THE PHARMACOLOGY OF CALCITONIN

BY

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DECLARATION

i) With the exception of the noradrenaline assays, the observations included in this thesis are the work of the author.

ii) During the course of this study the author has not been registered for the award of any other higher degree by any University or the Council for National Academic Awards. No part of this study has been previously submitted for the award of a higher degree.

iii) In partial fulfilment of the degree of Doctor of Philosophy the author has participated in seminars and conferences and has studied immunology and the electron microscopy to an advanced level.

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ABSTRACT

The mechanisms underlying the pharmacological effects of calcitonin (CT) have been investigated. The haemodynamic and antinociceptive effects of CT have been adopted as models for study as both are readily quantified, appear to involve modulation of neuronal activity and are of potential therapeutic use.

The antinociceptive effect of centrally administered CT in mice was found to be attenuated by pretreatment with p-chlorophenylalanine (but not by methysergide or α-methyl-p-tyrosine) and central administration of 5-hydroxytryptamine with CT overcame the effect of p-chlorophenylalanine. These observations form the basis of a model in which CT antinociception is attributed to modulation of the activity of central tryptaminergic neurones. However, CT did not alter the concentration of 5-hydroxytryptamine (or its major metabolite 5-hydroxyindoleacetic acid) in the mouse or rat brain. The hormone may therefore produce localised alterations in 5-hydroxytryptamine concentration or turnover.

As recent reports indicate that the CT gene is transcribed to produce calcitonin gene-related peptide (CGRP) rather than CT in neural tissue, the possible antinociceptive effects of salmon-CT, human-CT and CGRP have been compared. All three peptides produced antinociception but the effects of human-CT and CGRP were more transient and required higher doses. The possibility of action of CT on putative central CGRP receptors is discussed.

Salmon-CT i.v. has been found to produce a pressor response in rats made hypotensive by haemorrhage, but is without effect in normotensive rats or those made hypotensive by pithing. The pressor effect was greatly attenuated by chemical sympathectomy suggesting that the hormone potentiates sympathetic outflow. Centrally administered salmon-CT produced a pressor response in both normotensive and haemorrhaged rats, the latter effect was not greatly attenuated by chemical sympathectomy. It is concluded that the site and mechanism of action of centrally and peripherally administered salmon-CT differ in these models.

With regard to the cellular mechanism of action, CT was found to inhibit depolarisation stimulated uptake of ^{45}Ca by slices of rat midbrain, to increase the cyclic adenosine 3',5'-monophosphate content of mouse hypothalamus *in vivo*, and to stimulate adenylate cyclase activity in homogenates of mouse hypothalamus. These effects could clearly reflect effects of CT on cellular metabolism thus producing the altered neuronal activity assumed to underlie the antinociceptive and haemodynamic effects of the hormone.

ABBREVIATIONS

AchE	- Acetylcholinesterase
ACTH	- Adrenocorticotrophic hormone
ANOVA	- Analysis of variance
APUD	- Amine precursor uptake and decarboxylation
Asu	- L-amino suberic acid
ATP	- Adenosine triphosphate
AVP	- Arginine vasopressin
B_{max}	- Maximum binding
Brij 30	- Polyoxyethylene-4-lauryl ether
BSA	- Bovine serum albumin
cAMP	- Cyclic adenosine 3¦ 5'-monophosphate
cGMP	- Cyclic guanosine 3¦ 5'-monophosphate
CGRP	- Calcitonin gene-related peptide
CNS	- Central nervous system
c.p.m.	- Counts per minute
CSF	- Cerebrospinal fluid
CT	- Calcitonin (prefixed h-human, p-porcine, s-salmon, e-eel)
CTLI	- Calcitonin-like immunoreactivity
DADLE	- d-ala, d-leu-enkephalin
$d(CH_2)_5$Tyr(Me)AVP	- 1(β-mercapto-β,β-cyclopenta-ethylene propionic acid)
DHCC	- Dihydroxycholecalciferol
DNA	- Deoxyribonucleic acid
ED_{50}	- Concentration of agonist required to produce 50% of the maximum obtainable response
EDTA	- Ethylenediamine tetracetic acid
EGTA	- Ethylene glycol-bis(β-amino ethyl ether) N,N,N',N' tetra-acetic acid
GTP	- Guanosine triphosphate
Hepes	- N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
5-HIAA	- 5-Hydroxyindole acetic acid
5-HT	- 5-Hydroxytryptamine
5-HPT	- 5-Hydroxytryptophan

HPLC	- High pressure liquid chromatography
IC_{50}	- Concentration required to produce 50% of the maximal inhibitory effect
i.c.v.	- Intracerebroventricular
i.p.	- Intraperitoneal
i.v.	- Intravenous
K_d	- Dissociation Constant
K_{el}	- Elimination rate constant
MAP	- Mean arterial pressure
αMT	- α-Methyl, p-tyrosine
MW	- molecular weight
6-OHDA	- 6-Hydroxydopamine
OPT	- o-pthaldialdehyde
PCPA	- p-chlorophenylalanine
PTH	- parathyroid hormone
RNA	- Ribonucleic acid (prefixed m-messenger RNA)
s.c.	- Subcutaneous
$t_{\frac{1}{2}}$	- Half-life
Tris.	- 2-amino-2-(hydroxymethyl)propane-1,3-diol (tris)
V_d	- Volume of distribution

INTRODUCTION

I.1 THE DISCOVERY AND ISOLATION OF CALCITONIN

Until the discovery of calcitonin (CT) it was generally believed that parathyroid hormone (PTH) alone was responsible for regulation of plasma calcium (McLaen, 1957). However, in 1961 Copp, Davidson and Cheney perfused the parathyroid complex of dogs with hypercalcaemic blood and observed systemic hypocalcaemia which was more rapid than that produced by surgical removal of the parathyroid. These experiments led to the suggestion that a hypocalcaemic factor was released from thyroparathyroid tissue in response to hypercalcaemia. This factor termed "calcitonin", because of its role in regulation of calcium "tone", was assumed to be derived from the parathyroid glands (Copp, Cameron, Cheney, Davidson and Henze, 1962).

Hirsch, Gautier and Munson (1963) noted a greater reduction in plasma calcium of rats after parathyroidectomy by electrocautery than was observed after surgical removal of the parathyroid. This led to the conclusion that tissue damage in electrocautery caused a hypocalcaemic factor to be released from the adjacent thyroid. The thyroid origin of this hypocalcaemic factor was indicated by Hirsch et al (1963) who were able to extract a hypocalcaemic principal from the thyroid, and by Foster, Baghdiantz, Kumar, Slack, Soliman and MacIntyre (1964) who failed to produce systemic hypocalcaemia by perfusing the goat parathyroid with hypercalcaemic blood, but observed a rapid fall in systemic calcium concentration when the thyroid was also perfused. Copp and Henze (1964) however, maintained that calcitonin was of parathyroid origin leading to the development of the term "thyrocalcitonin" to describe the hormone secreted from the thyroid. Further convincing evidence of the thyroid origin of CT was obtained by Care (1965) who reported that direct addition of calcium to the thyroid arterial blood of pigs produced a rapid reduction in systemic plasma calcium, and by Care, Cooper, Duncan and Orimo (1968) who detected elevated levels of CT (determined by bioassay) in thyroid venous blood during perfusion with hypercalcaemic blood. Histological examination revealed that the thyroid glands of these pigs did not contain any parathyroid tissue. It has since been demonstrated that both substances are in fact the same and are derived from the parafollicular "C cells" (Foster et al, 1964; Pearse, 1968).

These cells, which derive phylogenetically from the neural crest and are of neuroectodermal origin, migrate during development to the ultimobranchial bodies of fish, amphibians and reptiles and predominantly to the thyroid of higher mammals including man. Immunofluorescence and immunochemical studies have since, conclusively shown CT to be localised in the parafollicular "C cells" of man (Woolf, Voelkel and Tashjian, 1974).

The relatively high concentrations of CT obtained from medullary thyroid carcinomas with malignant degeneration of the "C cells" has enabled the peptide to be isolated, sequenced and subsequently synthesised (Neher, Riniker, Rittel and Zuber, 1968; Rittel, Bruger, Kamber, Riniker and Sieber, 1968). To date, porcine human, bovine, ovine, salmon, chicken, eel and rat CT's have been isolated, although only porcine, human and salmon calcitonins are used therapeutically.

I.2 PERIPHERAL CALCIUM REGULATION AND THE ROLE OF CALCITONIN

The concentration of ionised calcium within cells is approximately 10^{-7}M, variations in this extremely low value are of critical importance to numerous biological functions, including muscle contraction and relaxation, endocrine and exocrine secretion and neuronal function. In the short term, rapid regulation of intracellular ionised calcium depends largely on the activity of intracellular organelles, however, in the long term the continuous influx of calcium must be counteracted by ejection through plasma membranes. Since both influx and efflux are concentration dependent, the efficiency of intracellular calcium regulation is ultimately dependent on the calcium concentration of extracellular fluid.

Calcium homeostasis is achieved by the coordinated effects of CT, PTH, and vitamin D (or more correctly its active metabolite, 1, 25-dihydroxycholecalciferol ; 1,25-DHCC). Briefly, 1,25-DHCC produced by the renal tubules when plasma calcium is low, acts on the small intestine to promote active absorption of calcium and plasma calcium is also elevated by actions on bone, and proximal tubules of the kidney. Similarly, PTH secreted from the parathyroid glands in response to hypocalcaemia increases calcium reabsorbtion by the renal tubules and indirectly facilitates intestinal absorbtion of calcium by promoting 1,25-DHCC production in the kidney.

The major effect of CT is considered to be inhibition of skeletal resorbtion as demonstrated by the decline in urinary hydroxyproline concentration (a sign of reduced skeletal resorbtion) after peripheral administration. In conditions of high bone turnover such as Paget's disease and growth, CT administration can lead to a reduction in plasma calcium. Raisz (1981) reported that there is little evidence to suggest that CT plays an essential role in the maintenance of calcium supply or plasma concentration in mammals, presumably because calcium efflux from adult bone forms a small proportion of calcium influx into plasma. However, in conditions such as growth, gestation and lactation where 1,25-DHCC levels are elevated (to meet increased demand for calcium and phosphates), CT levels are also increased preventing the destructive effect of 1,25

DHCC on bone. The protective effect of CT against calcium resorption was demonstrated by Swaminathan, Bates and Care (1972) who found that the plasma calcium concentration of young pigs was elevated following thyroidectomy and was returned to pre-surgery levels by CT infusion. Prevention of calcium resorption from bone cannot however be considered as the sole physiological effect of CT. The elevation of plasma calcium produced by feeding or gavage with calcium chloride (in young fasted rats) was found by Munson and Gray (1970) to be enhanced by thyroidectomy. Moreover, changes in serum calcium within the physiological range were found by Austin, Heath and Go (1970) to correlate with CT concentration in plasma. These results clearly support the suggestion of Gray and Munson (1969), that CT might protect against hypercalcaemia during the post prandial period in which calcium is rapidly absorbed from the gastrointestinal tract. As CT has been shown to modify renal clearance of calcium, and absorbtion of calcium from the gastrointestinal tract, the hormone can be considered to exert coordinated effects on kidney, gastrointestinal tract and bone which serve to prevent variations in plasma calcium and to protect bone from excessive calcium resorption.

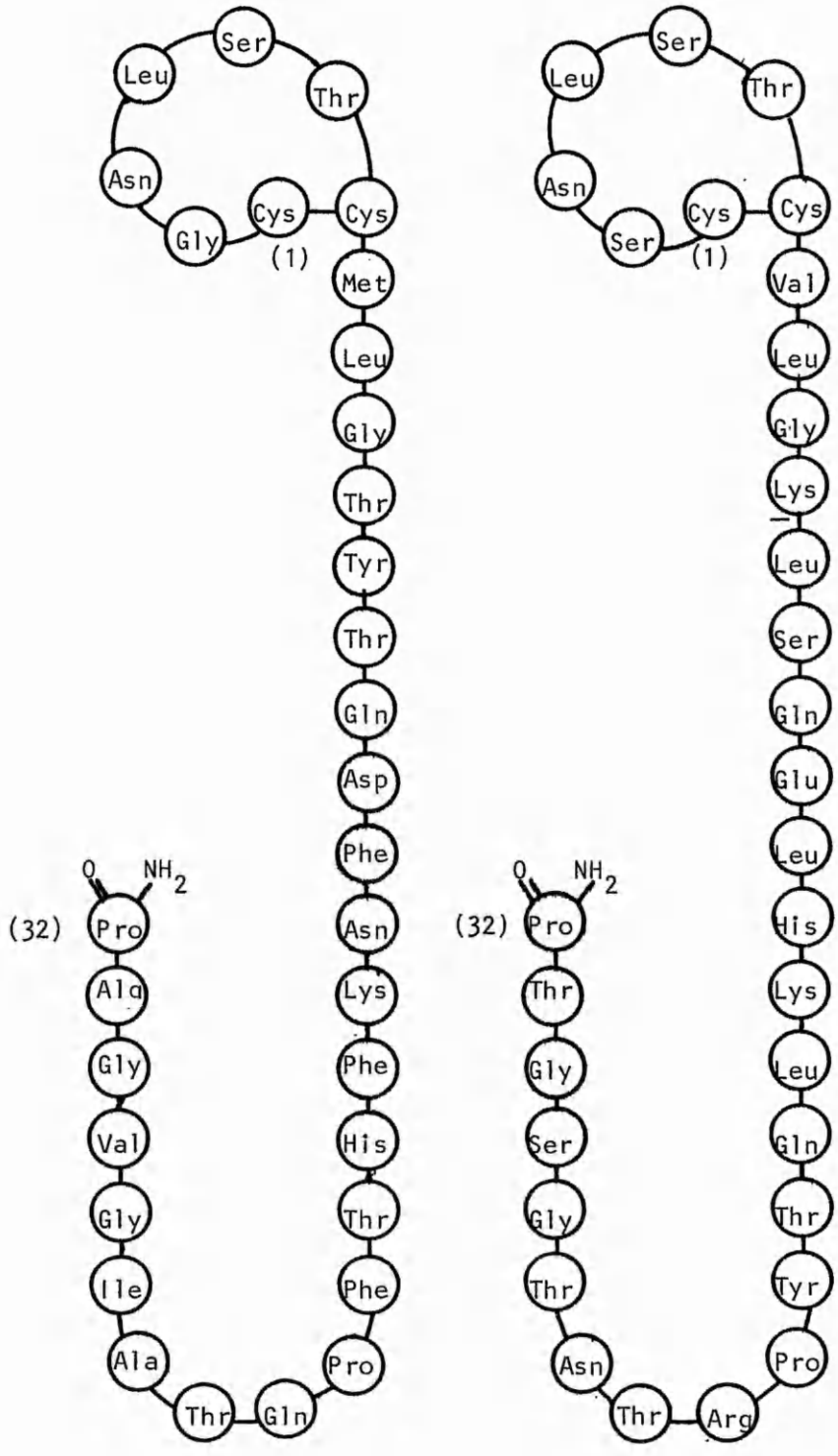
1.3 STRUCTURE AND EVOLUTION OF CALCITONIN

All forms of CT sequenced to date consist of a single chain polypeptide with a prolinamide residue at the carboxyl terminus and a 1-7 disulphide bridge at the amino terminus. In addition to the monomer an antiparallel dimer with disulphide bridges at the same positions between monomeric molecules has been demonstrated. In spite of considerable sequence variation between species, three major chemical groups of CT are generally recognised, teleost, artiodactyl and human, the sequences of salmon and human CT's are shown in figure 1.1 (see also 1.10). Only 9 residues have been found common to all sequenced CT molecules, these residues occur towards the two ends of the molecule suggesting their importance for biological activity (see 1.10).

Several groups have combined the techniques of high pressure liquid chromatography (HPLC) and radioimmunoassay (using antibodies raised against specific forms of CT) to investigate the occurrence of the various types of CT in different species. These studies have demonstrated that presence of human CT (or more correctly human CT-like immunoreactivity, CTLI), in the nervous systems of primitive chordates, including the sea squirt, Ciona intestinalis (Fritsch, Noorden and Pearse, 1979; Girgis, Galan, Arnett, Rogers, Bone, Ravazzola and MacIntyre, 1980) and in the brain of the hagfish, Myxine glutimosa (Girgis et al, 1980). MacIntyre and Craig, 1981, have also reported the unexpected observation of human CTLI in Escherica coli, Candida albicans and Aspergillus fumigutus. Finally, the ultimobranchial gland of amphibia has been found to contain human CTLI whereas that of birds and fish contains teleost CTLI (MacIntyre and Craig, 1981).

To explain the observations outlined above, MacIntyre and Craig, (1981) have proposed that human CT is an extremely ancient peptide which may have functioned as an intercellular messenger in unicellular and primitive multicellular organisms prior to the development of the skeletal system. Duplication of the human CT gene is thought to have occurred early in vertebrate evolution so that more recent forms of CT may have arisen from duplication of the gene for the parent peptide, human CT. In support of the duplicate gene theory MacIntyre's group have reported the observations of the co-existance

Figure I.1 Amino Acid Sequence of Human and Salmon Calcitonins



Human Calcitonin

Salmon Calcitonin

of human CT and teleost CT in mammals, reptiles, fish (Perez Cano, Girgis and MacIntyre, 1982) and birds (Perez Cano, Girgis, Galan Galan and MacIntyre, 1982). Those studies have now been extended by the demonstration of the co-existance of salmon and human CT-like peptides in the human thyroid, brain (Fischer, Tobler, Henke and Tschopp, 1983) and serum (Tobler, Tschopp, Dambacher and Fischer, 1984).

1.4 BIOSYNTHESIS OF CALCITONIN

Peptides destined for secretion are generally synthesised in the form of large precursor molecules (Zimmerman, Mumford and Steiner, (1980)) which undergo co-and post-translational modifications to yield the final secretory product. Jacobs, Potts, Bell and Habener (1979) and Goodman, Jacobs and Habener (1979) have found the major cell free translation product of cod, rat and human "C cell" derived mRNA to be a CT containing peptide with a molecular weight of 15000 termed procalcitonin, indicating that CT is no exception to the general scheme outlined above.

Jacobs, Goodman, Chin, Dee, Habener, Bell and Potts (1981a) extracted mRNA from carcinoma of the rat thyroid and determined the nucleotide sequence of large portions of the CT precursor by sequencing cloned DNA complementary to the sequence of the precursor coding mRNA. Nakanishi, Inoue, Kita, Nakamura, Chang, Cohen and Numa (1979) had previously used similar techniques to predict the sequence of pro-opiomelanocortin. The sequences of these precursor molecules are not compatible with the suggestion of Deftos, Burton, Bone, Catherwood Parthermore, Moore, Minick and Guillemin (1978) that CT is contained within the ACTH/endorphin precursor molecule. Subsequently, Jacobs, Lund, Potts, Bell and Habener (1981b) employed a cell free system to investigate the translation products of mRNA extracted from rat thyroid carcinoma. They found polyadenylated RNA to direct the synthesis of a precursor molecule (MW 15000) which was shown by immunoprecipitation with CT antiserum to contain a CT like sequence. Subsequent translation in the presence of microsomal membranes revealed that a leader peptide (MW 3000) was cleaved from procalcitonin, the ramaining sequence (MW 12000) was then glycosylated to form a 17000 MW molecule. The authors indicated that the only known sequence of the pro-calcitonin amenable to glycosylation is the Asn-Leu-Ser sequence at the 3-5 position of the CT molecule itself, and speculate that glycosylation of precursor molecules may play an important role in the regulation of post translational cleavages. Furthermore, they suggest that since secreted forms of CT have been found not to contain carbohydrate, the multiple forms

of immunoreactive CT found by various authors (Snider, Silva, Moore and Becker, 1977; Dermody, Rosen, Ananthaswamy, McCormick and Levy, 1981) might represent precursor forms with differing degrees of glycosylation.

Irrespective of the possible regulatory role of glycosylation it is apparent that post translational processing accounts for production of CT from pro-calcitonin, this processing is thought to involve cleavage of the amino terminal "leader sequence" (above) followed by removal of amino and carboxyl terminal cryptic sequences. Because of the possibility of biologically active sequences occurring within these cryptic sequences, MacIntyre et al (1982) synthesised the C-terminal peptide which flanks the CT sequence in human pro-calcitonin. This 21 residue peptide termed PDN-21 (now renamed Katacalcin) was found to have hypocalcaemic activity in the rat. Although katacalcin was found to be less potent than hCT, the effects of maximal doses of hCT and katacalcin were additive, possibly indicating distinct sites or mechanisms of action. Recently, radioimmunoassay and immunocytochemical techniques have been employed to demonstrate that katacalcin is localised in both normal and neoplastic medullary "C cells" (Ali-Rachedi, Varndell, Facer, Hillyard, Craig, MacIntyre and Polak, 1983), and circulates in concentrations approximately equal to hCT (MacIntyre, Hillyard, Murphy, Reynolds, Gainesdas and Craig, 1982). Moreover, simultaneous secretion of CT and katacalcin from human medullary thyroid carcinoma tissue has been observed (Iwasaki, Myers and Freake, 1983). Secretion of both peptides was found to be stimulated in a calcium dependent manner by depolarising solutions of potassium. The biological significance of this peptide has, however, yet to be established, particularly since MacIntyre, Hillyard, Reynolds, Gainesdas and Craig (1984) have recently reported the hypocalcaemic effect to be "highly variable at best".

In addition to post translational processing producing at least two biologically active peptides, variations in post transcriptional (pre-translational) processing of the procalcitonin gene has recently been reported. Rosenfeld and co-workers noted that cultures of rat medullary thyroid carcinoma cells were able to switch from high to low CT production and that this switch was associated with the

appearance of a new form of mRNA termed calcitonin gene related peptide mRNA (CGRP mRNA). To investigate the molecular basis of the phenomenom this group prepared circular DNA clones to both CT mRNA and CGRP mRNA and also generated a CT genome fragment. Using this approach Amara et al (1982) were able to demonstrate that both CT and CGRP mRNA coding sequences were present on the same gene, this CT gene was found to consist of four exons (regions of DNA which can potentially be transcribed to mRNA) one of which was not transcribed Tissue specific transcription of the three common coding exons with either the CT exon or the CGRP exon was found to produce two forms of mRNA with identical 5' sequences and non homologous 3' sequences, translation of these mRNA's produces either the CT precursor or the CGRP precursor.

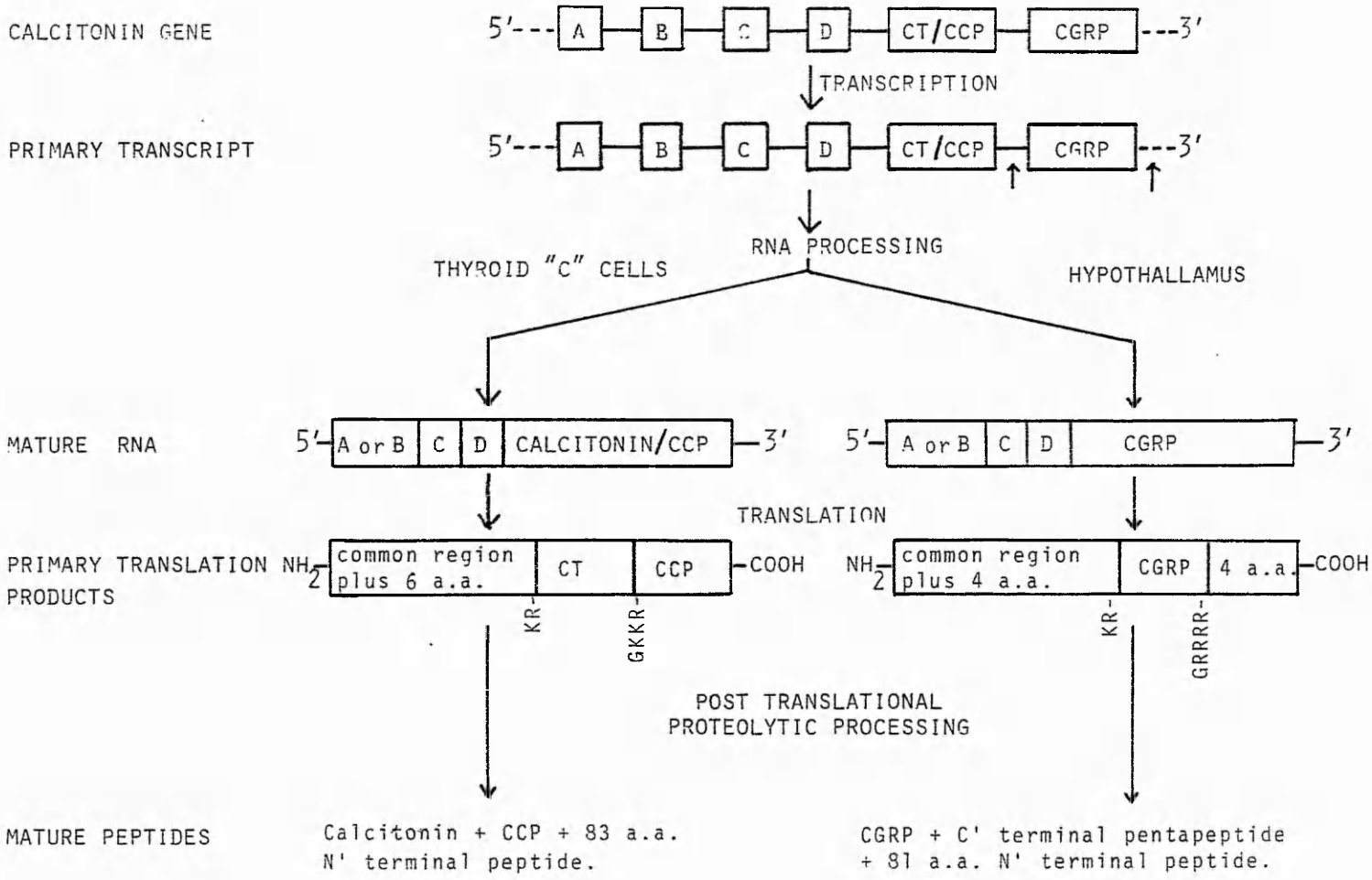
Although the splicing mechanism(s) determining production of CT mRNA or CGRP mRNA have not yet been established they could be of considerable physiological significance, particularly in light of the demonstration of tissue specific mRNA production. Amara et al (1982) found CGRP mRNA and CT mRNA to predominate in the hypothalamus and thyroid respectively. and speculated that the predicted peptide product of CGRP mRNA (CGRP) might act as a hypothalamic neuropeptide which could exert effects of its own. This suggestion is supported by the recent demonstration of depolarisation evoked release of CGRP from cultured rat trigeminal ganglion cells (Mason, Peterfreund, Sawchenko, Corrigan, Rivier and Vale, 1984). Rosenfeld, Mermod, Amara, Swanson, Sawchenko, Rivier, Vale and Evans (1983) raised anti CGRP antibodies against synthetic CGRP (sequence predicted from mRNA) and carried out immunofluorescence studies in the rat. The distribution of CGRP staining in the rat brain suggested to the authors that the peptide might play a role in sensory, integrative or motor components of ingestive behaviour and also in the processing of painful stimuli. It now appears that human CT is processed in a similar manner as Morris, Penico, Etienne, Tippins, Girgis and MacIntyre (1984) have described a human CGRP with 89% sequence homology (sequenced by fast atom bombardment) with the rat peptide. However, in contrast to rat CGRP the human peptide was detected peripherally in non neuronal tissue (in plasma and tumour tissue of patients with medullary thyroid carcinoma).

In summary, it appears that numerous mechanisms can contribute to the diversity of peptidergic systems. In the case of the CT gene tissue specific regulation of RNA processing leads to production of mRNA which is translated into precursors to either CT or CGRP. Subsequent post translational processing of precursor molecules (human pro-calcitonin) can produce more than one biologically active sequence (Figure I.2). The mechanism of regulation of RNA and precursor processing are not yet established (although polyadenylation and glycosylation are thought to be involved), and the possibility of tissue specific processing of the CT precursor has not yet been investigated.

The recombinant DNA and molecular biology techniques described by Rosenfeld et al (1983) have been applied to identification of previously unknown peptides. Subsequent synthesis of predicted peptides and preparation of antisera can rapidly lead to determination of the peptides distribution (Rosenfeld et al, 1983; Mulderry, Chater, Rodrigo, Allen, Rosenfeld and Polak, 1983; Sabate, Gibson, Morrison, Rosenfeld, Bloom and Polak, 1983; Ali-Rachedi et al, 1983). Since these techniques are relatively new to neuropeptide research (CGRP production is the first known example of tisse specific regulation of gene expression in the nervous and endocrine systems) it is relevant to consider potential drawbacks. Nakanishi et al (1979) have mentioned the possibility of deletion, addition or rearrangement of nuleotides during <u>in vitro</u> circular DNA synthesis from mRNA or in DNA cloning itself, in any case DNA cloning produces copies of a single mRNA molecule and the possible heterogeneity of precursor coding mRNA's cannot be discounted. Finally, the problem of antisera cross reacting with other unidentified molecules must always be considered, indeed, it should be noted that neither rat CGRP nor katacalcin have yet been sequenced or even extracted from biological tissues.

Figure 1.2 Alternative processing pathways in the expression of the calcitonin gene (from Rosenfeld et al, 1983)

Adenylation of the primary transcript at alternative sites (arrowed) leads to production of two alternative precursors. Post-translational processing of these precursors (cleavage at the signal sequence show, K-Lys, R-Arg. G-Gly) yields either CT (rat thyroid) or CGRP (rat neural tissue) in addition to the cryptic flanking peptides shown. The carboxyl terminal 16 amino acid flanking peptide of the rat CT precursor (CCP) is found in a position equivalent to that of the 21 amino acid peptide, katacalcin, in the human precursor.


CALCITONIN GENE
5'---
A
B
C
D
CT/CCP
CGRP
---3'
TRANSCRIPTION
PRIMARY TRANSCRIPT
RNA PROCESSING
THYROID "C" CELLS
HYPOTHALLAMUS
MATURE RNA
A or B
CALCITONIN/CCP
CGRP
TRANSLATION
PRIMARY TRANSLATION PRODUCTS
NH2
common region plus 6 a.a.
CT
CCP
COOH
common region plus 4 a.a.
4 a.a.
KR-
GKKR-
GRRRR-
POST TRANSLATIONAL PROTEOLYTIC PROCESSING
MATURE PEPTIDES
Calcitonin + CCP + 83 a.a. N' terminal peptide.
CGRP + C' terminal pentapeptide + 81 a.a. N' terminal peptide.

I.5 METABOLISM AND DISTRIBUTION OF PERIPHERALLY ADMINISTERED CALCITONIN

Several groups have used radioimmunoassay and bioassay to determine the rate of elimination of CT's from plasma of various species. In general, a rapid phase of elimination ($t_{\frac{1}{2}}$ approximately 2-20 minutes) followed by a slower phase ($t_{\frac{1}{2}}$ 30-300 minutes) has been reported (for review see Bennet and McMartin, 1978). Metabolism in plasma is unlikely to account for the rapid phase of elimination as several forms of CT have been found to be resistant to plasma metabolism in vitro (West, O'riordan and Care, 1969; Habener, Singer, Deftos and Potts, 1972a; Newsome, O'dor Parkes and Copp, 1973).

In the majority of estimations of the half-life of the slow phase of elimination, radioimmunoassay has been employed, raising the possibility that the slow elimination of immunoreactive metabolites was in fact determined. However, Habener, Singer, Neer, Deftos and Potts (1972b) found no evidence for circulating immunoreactive metabolites of $_{s}$CT or $_{p}$CT in the dog and concluded that the level of plasma CT fell slowly because of binding to plasma proteins. The rapid phase of elimination presumably reflects distribution of unbound plasma CT to tissues. DeLuise, Martin, Greenberg and Michelangeli (1972) have shown that levels of ^{125}I-CT's are rapidly reduced in rat plasma, after i.v. administration, by sequestering in kidney and liver. This group also found that human and porcine (but not salmon) CT's were considerably degraded by homogenates of rat liver, kidney, spleen and muscle. Although it is conceivable that homogenisation might release intracellular peptidases which would not normally metabolise CT's, the possibility of metabolism in the kidney is supported by the demonstration that isolated renal cells can degrade CT's (Hsu and Haymovits, 1974).

Although molecules of the size of CT would not normally be expected to cross the blood-brain barrier and whole body autoradiography studies (Hicks, Cooper and Waddel, 1971; Forslund, Slanina, Stridsberg and Appelgren, 1980) have failed to demonstrate redistribution of peripheral ^{125}I-CT to the CNS, evidence exists suggesting that this may in fact occur, and is discussed in chapter D.3.

1.6 DISTRIBUTION OF CALCITONIN AND CALCITONIN BINDING SITES

Several peptide hormone secreting cells have been shown to have the ability to take up and decarboxylate the amine precursors of neurotransmitters (Pearse, 1969). Such observations have led to the development of the APUD (amine precursor uptake and decarboxylation) concept. In essence, cells of the APUD series are thought to be derived from embryonic cells of the neuroectoderm, these cells retain characteristics of neuronal tissue and have been termed "neuroendocrine" (Pearse, 1977). As previously mentioned (1.1) CT secreting "C cells" of the thyroid or ultimobranchial glands are derived from the embryonic neural crest, a fact which presumably underlies their ability to take up and decarboxylate precursors of 5-Hydroxytryptamine and dopamine (Pearse, 1966 and 1977). In view of the origin of CT secreting cells it is perhaps not surprising that immunoreactive CT like molecules have been demonstrated in neural tissue of several invertebrates and primitive chordates (1.3). More recently reports have appeared in the literature describing the existance of CT like molecules and specific binding sites (putative receptors) for such molecules in the brain and pituitary of higher animals, including man.

Becker, Snider, Moore, Monaghan and Silva (1979) assayed CTLI and found that the thyroid contained approximately 20 times as much CTLI (67 ng/g net weight) as the next highest tissue, the jejunum which was followed by the thymus, urinary bladder and lung. Intermediate concentrations (1–3 ng/g net weight) were found in the rectum, testes, skeletal muscle, hypothalamus, pituitary, lymph nodes and stomach with the lowest concentration being found in the cerebellum and red blood cells. In addition to extrathyroidal CT, CT binding sites (putative receptors) have been demonstrated in tissues other than bone, these include kidney (Marx, Woodard and Aurbach, 1972) and brain (Fischer et al, 1981a). The distribution and characteristics of CTLI and CT binding sites within the CNS and pituitary are clearly relevant to the understanding of the central pharmacological actions of CT and are therefore described in more detail below.

1.6.a Calcitonin-like Immunoreactivity in Brain and Pituitary

Deftos et al (1978), using immunofluorescence staining (antisera raised against hCT) demonstrated uniform distribution of CTLI in the intermediate lobe of rat pituitary glands in addition to staining of discrete cells of the anterior lobe. Calcitonin-like immunoreactivity has now been demonstrated in the pituitaries of several species including pigs, sheep, trout, goldfish, rats (Deftos, Burton, Watkins and Catherwood, 1980) and man (Fischer et al, 1981a), although there is still considerable controversy on the exact distribution of CTLI.

Samaan and Leavens (1981) collected samples of blood from the pituitary vascular bed of patients with prolactin producing microadenomas during transnasal transsphenoidal surgery. Serum from this blood was found to possess greater CTLI (2.4 ± 0.9 ng.ml^{-1}) than peripheral blood (0.69 ± 0.19 ng.ml^{-1}). In contrast, peptides known to be secreted from the pituitary were found in at least 500 times greater concentration in the pituitary vascular bed. Furthermore, Cooper et al (1980) using antisera raised against hCT, were unable to demonstrate secretion of CTLI in thyroidectomised rats or from pituitaries <u>in vivo.</u> These observations indicate that secretion of CT-like molecules from the pituitary is unlikely to exert significant effects in the periphery, but the possibility of a paracrine role within the pituitary (Deftos et al 1978) cannot be discounted.

In addition to the pituitary CTLI has been demonstrated in the CNS or CSF of several species including snails (Schot, Boer, Swaab and Van Noorden, 1981) primitive chordates (Fritsch et al, 1979; Girgis et al, 1980), lizards (Galan Galan, Rogers, Girgis, Arnett, Ravazolla and MacIntyre, 1981a), pigeons (Galan Galan, Rogers, Girgis, and MacIntyre, 1981b), frogs (Yui, Yamada, Kayamori and Fujita, 1981), rats (Flynn, Margules and Cooper, 1981) and man (Becker et al, 1971; Becker, Silvas, Post, Ballenger, Carman, Snider and Moore, 1980) and has been localised to specific neurones in snails (Schot et al, 1981) and frogs (Yui et al, 1981).

The first demonstration of CTLI in the CNS of mammals was that of Becker et al (1979) mentioned previously. Since 1979 several groups have used radioimmunoassay to provide quantitative estimates of CTLI in the CNS and CSF of invertebrates. For example, Galen Galen et al, (1981b) found the hypothalamus, midbrain and brain stem of pigeons to contain 31.50 ± 9.65, 17.0 ± 5.0 and 2.85 ± 2.10 ng/ g wet weight respectively, wheras CTLI was undetectable in the cerebral cortex and cerebellum. Flynn et al (1981) using anti-hCT antibodies, estimated that the hypothalamus of Sprague-Dawley rats contained 0.21 ng immunoreactive material as compared to 1.16 ng in the anterior and 0.81 ng in the intermediate lobes of the pituitary, and noted that these three sources of CTLI contained less than one thousandth of that in the thyroid. Fischer et al (1981a) extracted immunoreactive CT from human autopsy specimens which was indistinguishable from hCT and its sulphoxide on HPLC. Concentrations of 0.7-0.9 pmol/g wet weight (≅ 3 ng/g wet weight) were reported in the posterior hypothalamus, pituitary stalk, anterior pituitary and posterior pituitary with intermediate concentratios being found in the anterior hypothalamus, substantia nigra and inferior colliculus. Detectable levels of CTLI at less than 0.1 pmol/g wet weight were found in the caudate nucleus, globus pallidus, amygdala, hippocampus cerebral cortex and cerebellum. Calcitonin-like immunoreactivity has also been found in human CSF extracted by lumbar puncture, with estimates varying from 11.1 ± 1.3 $pg.ml^{-1}$ (Pavlinac, Lenhard, Pathermore and Deftos, 1980) to 75 ± 8$pg.ml^{-1}$ (Fabbri et al, 1981). Several groups have used biochemical techniques to investigate the nature of pituitary and CNS CT-like molecules, this question remains controversial and is discussed in chapter D.1.c.

Finally, the question of whether CT-like peptides in the brain and pituitary are produced and secreted locally remains unanswered. Considerable evidence exists suggesting that peripheral CT is able to cross the blood-brain barrier (Stekolnikov and Abdukarimov, 1969; Fabbri, Santoro, Moreth, Cappa, Fraioli DiJulio, Galluzzi and LeManna, 1981). However, Pavlinac et al (1980) have failed to correlate plasma CT with CSF CTLI. Considering the small amounts of CTLI in the brain and pituitary compared to the thyroid and the lack of evidence for significant release of pituitary CTLI in to the general circulation

it seems unlikely that central or pituitary CT plays an important role in peripheral calcium metabolism. Alternatively, it has been suggested that CT-like peptides might play a neurotransmitter or paracrine role in the CNS (Austin and Heath, 1981).

I.6.b Calcitonin Binding Sites in Brain and Pituitary

Specific CT binding sites, or receptors, have been described in several preparations including plasma membranes of renal and bone cells (Marx et al, 1972a), breast cancer cells (Moseley, Findlay, Gorman, Michelangeli and Martin, 1983) and bronchial carcinoma cells (Hunt, Ellison, Underwood and Martin, 1977). Recently CT-specific binding sites have been described in the brain and pituitary of rats and man.

Koida, Nakamuta, Furakawa and Orlowski (1980) reported the existence of high affinity binding sites for ^{125}I-sCT in rat brain, Later, this group described the characteristics of ^{125}I-sCT binding to rat homogenates in more detail (Nakamuta, Furakawa and Koida, 1981). Specific binding (defined as that displaced by 1.0 nM sCT) was found to be saturable and to occur in a temperature and pH dependent manner, binding was inhibited by salmon,[amino suberic acid^{1,7}]-eel and porcine CT's (IC_{50}'s 2.0, 8.0 and 30.0 nM respectively) but not by a range of compounds including hCT,β-endorphin, substance P, 5-hydroxytryptamine, noradrenaline and atropine ($IC_{50} \geq 1$ M). The ability of CT's to displace ^{125}I-sCT correlates well with their affinity for binding to kidney particulate fraction (Marx et al, 1972a) and with their hypocalcaemic potency. Scatchard analysis of ^{125}I-sCT binding revealed two distinct binding sites, high affinity binding sites with a K_d of 0.65 nM and low affinity binding sites with a K_d of 12 nM. The greatest density of high affinity sites (B_{max}, 190 fmol/mg protein) was found in the hypothalamus followed by the midbrain, hippocampus and striatum, the highest density of low affinity binding sites (B_{max}, 200 fmol/mg protein) was found in the cerebellum. The affinity of sCT for binding sites in the CNS (measured by displacement of ^{125}I-sCT) is of the same order as that reported for receptor binding affinity of putative peptidergic neurotransmitters including met-enkephalin (K_d 1.8 and 5.8 nM in rat brain membranes, Simantov, Childers and Snyder, 1978) and substance P (K_d 3nM in rabbit CNS synaptic membranes, Nakata, Kusaka, Segewa, Jajima and Kitagawa, 1977).

Fischer et al (1981b) obtained broadly similar results when characterising the binding of ^{125}I-sCT to a crude membrane preparation of rat brain. The highest density of binding sites (3.18 fmol/mg

protein*) was found in the hypothalamus followed by the brain stem, midbrain, thalamus and striatum, areas with lowest density were the cortex, cerebellum spinal chord and hippocampus. They noted that binding was directed toward the carboxyl terminus (C' terminal fragments were more potent at displacing ^{125}I-sCT than were N' terminal fragments) and that the rate of dissociation was slow (67% of ^{125}I-sCT remained bound 6 hours after addition of excess sCT), but were unable to explain the tight association of ^{125}I-sCT with its binding sites. In the same year this group reported the characteristics of ^{125}I-sCT binding to homogenates of human brain (Fischer et al, 1981a). Again binding sites were most dense (1.07 fmol bound/mg protein*) in the hypothalamus followed by the pituitary stalk and median eminence. All other areas of the CNS bound less than 0.1 fmol/mg protein. Finally, Rizzo and Goltzman (1981) found binding of ^{125}I-sCT to homogenates of rat brain to be highly specific, saturable and dependent on both time and temperature, the authors suggest these results are consistent with the concept of binding to specific CNS receptors for the hormone.

In addition to investigations of the characteristics and distribution of binding sites in homogenates and preparations thereof several groups have used in vitro autoradiography to determine the distribution of central CT binding sites. The results obtained have been similar to those described above,for example Henke, Tobler and Fischer (1983) demonstrated extensive binding of ^{125}I-sCT to the anterior and dorsomedial areas of the rat hypothalamus with less binding in the ventromedial and lateral regions as well as the substantia nigra, arcuate and submaxillary nuclei. No binding was observed in the neocortex, cerebellum thalamus, basal ganglia or mamillary bodies. In addition to binding sites within the CNS Mauer, Marbach and Mousson (1982) were able to detect ^{125}I-sCT binding sites in the rat pituitary using autoradiography.

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*These values were determined in the presence of a fixed amount of ^{125}I-sCT and are therefore not equivalent to B_{max}.

Finally, Van Houten and co-workers used autoradiographical techniques to demonstrate that after peripheral (intracardiac) administration, ^{125}I-sCT binds selectively to circumventricular organs of the rat brain. These highly specialised regions are thought to serve as the main receptive system in brain for mediating the direct action of blood borne peptides on brain function (Van Houten, Goltzman and Posner, 1980; Van Houten, Rizzo, Goltzman and Posner, 1981).

In summary, the neuroectoderm origin of CT secreting thyroid "C cells" and the existence of immunoreactice CT like molecules in central neurons of primitive animals has led to the investigation and consequent demonstration of CTLI and CT specific binding sites within the CNS of mammals.

Although the source and physiological function of CT like molecules within the CNS and pituitary remains unclear it seems unlikely that release of CT from these regions into the general circulation would exert significant effects on peripheral calcium metabolism. Alternatively, it has been proposed that CT may perform a neurotransmitter or paracrine role within the CNS (Austin et al, 1981). Recently the authenticity of central CT has been disputed by Rosenfeld et al (1983) who were unable to detect CTLI or CT mRNA in rat pituitary or brain, this controversy is further explored in chapter D.2.

I.7 THE ROLE PLAYED BY NEURONAL CALCITONIN

I.7.a Calcitonin as a Neuropeptide

Peripheral CT secreting "C cells" are known to derive, phylogenetically, from the embryonic neuroectoderm layer. It is therefore not surprising that CT like molecules have now been demonstrated in central neurones which are also of neuroectoderm origin. Fritsch et al (1979) used immunofluorescence to demonstrate hCT-like immunoreactivity in neural complex of the sea squirt, Ciona intestinalis, and suggested that CT might be regarded as a new neurotransmitter. The location of CT-like immunoreactivity (CTLI) in neural tissue is supported by demonstrations of CTLI in frog CNS nerve terminals (Yui et al, 1981) and central nerve terminals of snail, rat and lizard CNS (Schot et al, 1981; Vanhoutten et al, 1981; Galan Galan et al, 1980). Several peripheral peptides are now known to play a role as central neuropeptides (e.g. angiotensin II, cholecystokinin, substance P, see Hokfelt et al, 1980). It is now recognised that such peptides may act as central neurotransmitters or neuromodulators, and may be stored and released in and from the same neurones as classical neurotransmitters, further supporting the similarity between the endocrine and neuronal systems Clearly a CT-like molecule may be considered as a central neuropeptide, but the possible neurotransmitter or neuromodulator role of CT is still open to speculation as discussed below.

I.7.b Calcitonin as a Central Neurotransmitter

To date, several authors (Fritsch et al, 1979; Fischer et al 1981b; Austin and Heath, 1981) have suggested that CT may act as a neurotransmitter. It is generally recognised that a number of criteria exist which may be fulfilled to substantiate claims of neurotransmitter status. Hence for classical neurotransmitters:-

i) the transmitter should be present in the nerve terminal
ii) precursors necessary for transmitter synthesis should be present or mechanisms should exist for concentration of the transmitter, in the nerve terminal
iii) depolarisation of the presynaptic nerve should cause the transmitter to be released

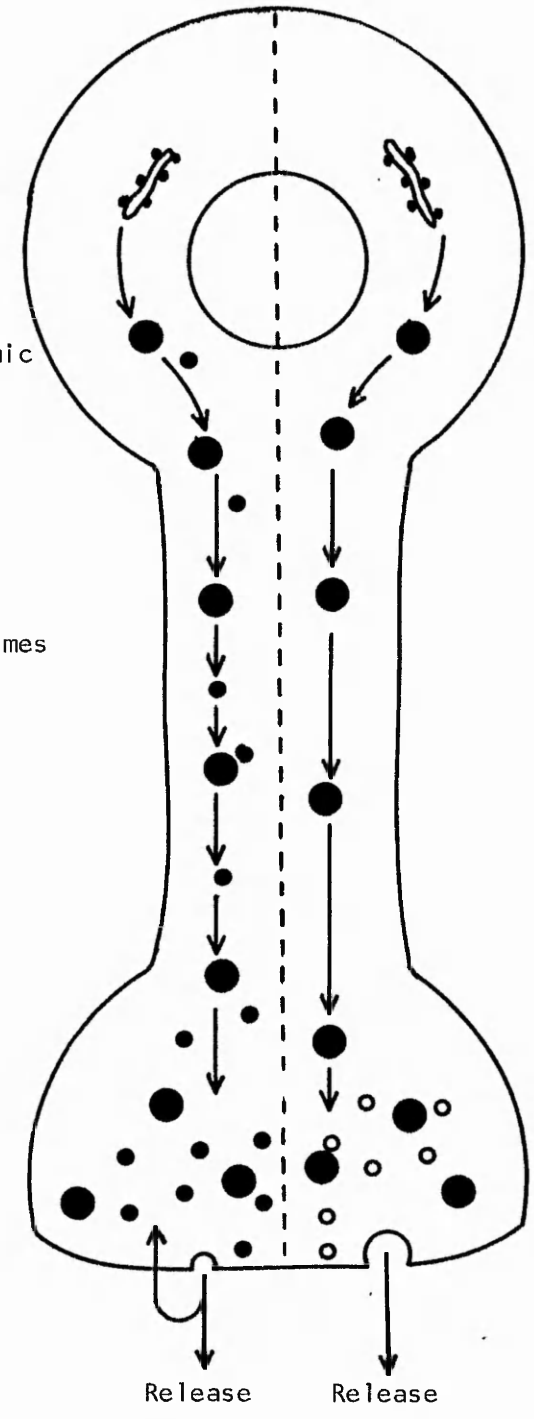
iv) application of the transmitter to the post synaptic membrane should produce the same effect as presynaptic depolarisation

v) post synaptic actions of the putative neurotransmitter should be identical to those of the endogenous chemical

vi) a system for rapid disposal or removal of the transmitter from the synaptic cleft should be present at the synapse.

Although CTLI has been demonstrated in nerve terminals (above) and iontophoretic and i.c.v. application of CT has been shown to produce modulation of neuronal activity (Miyahara and Oomura, 1981; Yamamoto et al, 1980), it is apparent that the available information on CT does not satisfy all of these criteria. It should be noted however, that there are no neuropeptides for which there is evidence to satisfy all of these criteria, some of which may not be applicable to peptide neurotransmitters. The major differences between transmission utilising classical neurotransmitters, such as noradrenaline, and peptide neurotransmitters are apparent in figure 1.3. A major difference is that peptides are thought to be produced (in the form of large precursors) only in the ribosomes of the cell soma (not in nerve terminals) and reuptake mechanisms do not seem to operate, hence, peptide molecules released from the nerve terminal must be replaced by axonal transport. This apparently inefficient and slow mechanism may be compensated by a long duration of action and the low concentration of peptide required to activate receptors. Thus, the long duration of the effect of CT on neuronal activity (Yamamoto et al, 1980) and the long receptor binding half-life of CT (Fischer et al, 1981) would be atypical for a classical neurotransmitter, but are still in accord with the proposed neurotransmitter role of CT. Alternatively, the hormone could serve a central neuromodulatory, neuroendocrine or paracrine role. The possibility of central neuromodulator action (modulating neuronal responsiveness to other neurotransmitters) is of particular interest as long duration of action is a characteristic of neuromodulatory peptides and may underlie their physiological and pharmacological effects. On the other hand, recent evidence (discussed below) suggests that CT is not synthesised centrally (Rosenfeld et al, 1983) and raises the possibility of an unidentified CT-like central neurotransmitter or neuromodulator candidate. It should be noted that central CT-like peptides could play more than one role in the CNS as is the case for

Figure 1.3 Diagramatic neurone demonstrating major features of neurones using classical neurotransmitter (left) and peptide transmitter (right). From Hökfelt et al (1980)



Production of

1) Synthesising enzymes

2) Storage vesicles (smooth endoplasmic reticulum)

Axonal transport of

1) Synthesising enzymes

2) Storage vesicles

Supply by

1) Axonal transport + storage

2) New synthesis

3) Reuptake

Production of

1) Peptide precursor

2) Storage vesicles

3) Conversion enzymes

Axonal transport of

1) Storage vesicles

Supply by

1) Axonal transport + storage

5-HT which, when released from nerve terminals in tight juxtaposition with postsynaptic receptors is considered to act as a neurotransmitter, but when released into the CSF is thought to act as a neurohormone (Moore, 1981).

I.8 PHYSIOLOGICAL AND PHARMACOLOGICAL EFFECTS OF CALCITONIN

I.8.a Effects of Calcitonin on Bone

Kinetic studies on rats indicated to Milhaud, Perault and Moukhtar (1965) that CT caused inhibition of bone resorption, a conclusion which was supported by studies on bone culture by Friedman and Raisz (1965). Inhibition of bone resorption is thought to occur as a result of reduced osteoclast activity which is manifested as a reduction in the number of nuclei and appearance of spaces between the surface of the cells and the bone (Hotrop, Raisz and Simmons, 1974). Inhibition of bone resorption leads to reduced excretion of urinary hydroxyproline (a useful index of the effect of CT therapy) in addition to decreasing circulating levels of calcium and phosphate (Austin and Heath, 1981). The major therapeutic use of CT is in the treatment of Paget's disease which is characterised by increased bone turnover and remodelling (Stevenson and Evans, 1981). In this condition CT reduces the elevated levels of plasma alkaline phosphatase and urinary hydroxyproline, the skin temperature over affected bone is reduced, neurological function is improved and new bone is observed replacing Pagetic tissue (Deftos and First, 1981). Alleviation of bone pain, which is also observed, may be associated with recovery of bone or with the reported analgesic effect of the hormone (see D.5.c).

I.8.b Effects of Calcitonin on Kidney

Calcitonin is thought to exert two distinct effects on the kidney Firstly it enhances renal production of 1,25 DHCC (Galente, Colston, MacAuley and MacIntyre, 1972). This effect is not observed in parathyroidectomised animals and may therefore be indirect (Lorenc, Tanaka, DeLuca and Jones, 1977). Secondly, CT has been shown to modify renal clearance of several ions, hence, sCT was shown by Maier (1977) to enhance renal clearance of sodium, chloride and potassium, and to reduce clearance of magnesium. Some confusion surrounds the effects of CT on renal excretion of calcium, as renal clearance has been reported to be increased, decreased or unaltered by CT. Reviewing this literature, Borle (1983) states that the doses shown by later groups to decrease

calcium clearance are more physiologically relevant and suggests that at physiological concentrations, CT enhances calcium reabsorption from the loop of Henle.

I.8.c Effects of Calcitonin on the Gastrointestinal Tract

Several clearcut pharmacological effects of CT on the gastrointestinal (G.I.) tract have been described. These include increased secretion of sodium, potassium, chloride and water by the small intestine (Gray, Bieberdorf and Fordtran, 1973), reduction in calcium absorption by the small intestine (Swaminathan, Kerr and Care, 1974), inhibition of the development of experimentally induced gastric ulceration (Orimi, Oyama and Ito, 1973). The relationship between CT and the G.I. tract has been reinforced by the demonstration that the G.I. hormones pancreozymin and gastrin are powerful secretagogues of CT (Care, 1970; Care, Bates, Swaminathan and Ganguli, 1971).

Morley, Levine and Silvis (1981a), measuring gastric acid secretion in rats, found centrally administered CT to be 1000 times more potent than the parenterally administered hormone, suggesting a central site of action. It seems unlikely that CT induced inhibition of gastric ulceration is caused solely by its effects on gastric acid secretion as Bates, Buckley and Strettle (1981c) found indomethacin induced gastric erosions in the rat to be markedly inhibited by sCT but resistant to maximally effective doses of drugs known to reduce gastric acidity or gastric secretion (atropine, cimetedine and an antacid preparation of magnesium and aluminium hydroxides).

Beuno, Fioramonti and Ferre (1983) compared the electrical activity of the small intestine in fed and fasted rats and found the fasted pattern to be characterised by regular "migrating myoelectric complex's" which were abolished by feeding. In fed animals i.c.v. CT (0.02-0.2 U) restored the fasted pattern of electrical activity within less than five minutes and with a duration of up to six hours. Similar effects were observed after i.v. administration of 100 times higher doses again suggesting a central site of action. The action of CT on intestinal motility occurred at doses which also inhibited gastric acid secretion, yet the effects were considered independent since pretreatment

with calcium gluconate blocked the effect of sCT on electrical activity without altering the effect on gastric acid secretion.

I.8.d Effects of Calcitonin on Food Intake

The postprandial rise in serum CT (Talmage, Doppelt and Cooper, 1975) suggested to Freed, Perlow and Wyatt (1979) that CT might be involved in the regulation of subsequent feeding behaviour. This group found sCT (12.5-50 $U.kg^{-1}$, s.c.) to produce a dose dependent reduction in rat feeding with a maximal effect between 4.5 and 8.3 hours after administration. The authors suggested that the anorectic effect was not due to illness or aversive effects of the peptide. Intracerebroventricular CT was effective at doses 25-50 times lower than those used peripherally suggesting that CT inhibits feeding by acting directly on the CNS and that endogenous CT might be involved in the regulation of feeding and appetite. Similar inhibition of feeding was observed by Cooper et al, (1980b) with i.c.v. doses of sCT (1.4 $U.kg^{-1}$) which had no effect on plasma calcium, pCT was found to be less effective and hCT was without effect. Levine and Morley (1981a) supported the earlier observations by demonstrating that centrally or peripherally administered CT reduced stress induced feeding in rats. Using this model, i.c.v. CT was as much as 1000 times more potent than the peripherally administered hormone (i.c.v. CT was effective at 0.002 U/rat) and the effect was thought not to be associated with elevation of blood glucose. This group also found CT (0.2 and 2 $U.ml^{-1}$) to inhibit the uptake of ^{45}Ca into explants of rats hypothalamus and suggested that the mechanism of CT induced suppression of feeding might be related to alteration of calcium flux in neuronal tissue. Morley and Levine, 1981b). In support of the role of CT in this hypothesis it should be noted that CTLI and CT binding sites have been demonstrated in the hypothalamus (Flynne et al, 1981; Koida et al, 1980) and that iontophoretic application of CT onto neurones of the lateral area of the hypothalamus has been found to inhibit activity in 64% of glucose sensitive and 15% of glucose insensitive neurones (Miyahara and Oomura, 1981).

I.8.e Anti-inflammatory Effects of Calcitonin

Calcitonin has been shown to exert anti-inflammatory effects in various species (Riesterer and Jaques, 1969; Abdullahi, DeBastiani, Nogarin and Velo, 1975; Strettle, Bates and Buckley, 1980) including man (Velo, De Bastani, Nogarin and Abdullahi, 1976). Although the mechanisms of the anti-inflammatory action are unclear, they could include modification of arachidonic acid metabolism (Cesarini, Colombo, Oligiati and Pecile, 1979) or calcium related changes in cellular or vascular permeability (Riesterer and Jaques, 1969). Cesarini et al (1979), using a cascade perfusion system, showed that CT inhibited the synthesis of prostaglandins and thromboxane, this effect which was attributed to cycloxygenase inhibition could clearly account for the anti-inflammatory effects of the hormone, and might be relevant to the mechanism of other effects such as inhibition of ulcer formation or inhibition of inflammation over Pagetic bone.

I.8.f Antinociceptive and Analgesic Effects of Calcitonin

Treatment of osteoarticular conditions (including Paget's disease) with CT has long been known to produce a marked alleviation of pain (Deftos and First, 1981) which may occur independently of the specific action on bone calcium metabolism (D.5.c). Observations of this nature led Pecile and co-workers to investigate the possibility that CT might exert an analgesic effect when administered centrally. This group determined the threshold voltage at which rabbits began to lick in response to electrical stimulation of the upper incisors (Pecile et al, 1975). The licking threshold was found to be increased after intracerebral administration of sCT (2 $\mu g.kg^{-1}$), the effect, which was maximal at 90 minutes, had a rapid onset and a duration of at least 2 hours. Since these initial observations the antinociceptive effects of centrally administered CT have been demonstrated by several groups including Yamamoto, Kumagai, Tachikawa and Hurayama, 1978 (using pCT in abdominal constriction tests, paw pressure tests and Haffner's test), Pecile, Olgiati and Sibilia, 1983 (using sCT hCT and pCT in the rat hot plate test), Bates, Buckley, Eglen and Strettle, 1981a (using sCT in the mouse acetic acid induced abdominal constriction tests).

Yamamoto et al, (1978) were able to produce significant antinociception with centrally administered doses of 10-60 $U.kg^{-1}$ of pCT in mice and 8-17 $U.kg^{-1}$ in rabbits whereas doses as high as 100 and 50 $U.kg^{-1}$ i.v. in mice and rabbits respectively produced no effects. Similarly, Bates et al, (1981a) obtained antinociceptive effects in the mouse abdominal constriction test with 0.04-50 $U.kg^{-1}$ sCT i.c.v. but were unable to achieve antinociception with s.c. doses as high as 1000 $U.kg^{-1}$. These facts alone indicate a central mechanism of action and therefore that antinociception occurs independently of the hypocalcaemic and anti-inflammatory actions of the hormone which are produced by peripheral administration.

Antinociception produced by central administration of CT presumably involves direct or indirect modulation of the activity of neurones involved in responses to painful stimuli, direct evidence for such an effect has been presented by the groups of Yamamoto and Pecile Pecile et al (1978) demonstrated that i.c.v. CT produced electroencephalograph (EEG) arousal in conscious rabbits, the maximum effect was found to coincide closely with the peak antinociceptive effect of pCT (90 minutes after administration using tooth licking threshold Pecile et al, 1975). In addition to EEG arousal and increase in licking threshold, Yamamoto, Tachikawa and Maeno (1980) demonstrated the effect of pCT on evoked potentials in the conscious rabbit. This group recorded potentials in the sensory cortex of immobilised rabbits produced by electrical stimulation of the tooth pulp or sciatic nerve, they demonstrated that morphine (1 and 2 $mg.kg^{-1}$) and i.c.v. pCT (8 and 17 $U.kg^{-1}$) produced similar inhibition of evoked potentials. The effects of morphine, but not pCT were antagonised by naloxone (0.5 $mg.kg^{-1}$). The authors suggest that a close relationship exists between the evoked potential components and pain sensation, it can therefore be concluded that morphine and pCT were able to modulate the action of common nociceptive pathways in the CNS by actions at different sites.

The electrophysiological data outlined above indicate that CT antinociception does not directly involve action at opiate receptors a conclusion which can be supported by evidence from antinociceptive tests. Braga, Ferri, Santagostino, Olgiati and Pecile (1978) demonstrated the relationship between morphine and pCT induced antinociception

on the lick threshold model in rabbits, they found that both drugs increased the threshold at which licking occurred and that on repeated administration tolerance developed to the effect of morphine but not to pCT. They further reported that morphine potentiated the effects of pCT and that unlike morphine the effect of pCT was not antagonised by naloxone (1 $mg.kg^{-1}$, i.p.). Yamamoto, Kumagai, Tachikawa and Maeno (1979), reported a similar lack of effect of levallorphan (a partial opiate antagonist) on the antinociceptive effect of centrally administered pCT in the mouse paw pressure test. The authors concluded that CT antinociception was independent of opiate systems and supported this by siting a personal communication from Miki stating that there was no cross tolerance between morphine and CT with regard to physical dependence. Bates, Buckley, Eglen and Strettle (1981b), investigated the modification of sCT antinociception by naloxone in the mouse abdominal constriction test using a wider range of doses of both sCT and naloxone. In this study naloxone was found to antagonise sCT antinociception but only at doses 10-1000 times higher than those required to antagonise the effect of morphine. CT has also been found to be inactive in several <u>in vitro</u> opiate bioassays including the superfused rat colon (Bates et al, 1982) and the guinea-pig myenteric plexus (Braga et al, 1978), Braga and co-workers have also shown that CT does not affect the binding of dihydromorphine to homogenates of guinea-pig brain.

Although CT appears not to exert its antinociceptive effect through opiate systems, there is evidence suggesting that the fundamental mechanisms may be similar. The dependence of opiate antinociception on central calcium metabolism has long been established, hence observations such as reversal of opiate antinociception by central administration of calcium ions or potentiation by calcium antagonists and chelators (EDTA) have led Schmidt and Way (1980), to suggest that the primary mode of action of opiates is modification of calcium metabolism. They propose that acute administration of opiates reduces calcium flux or binding at the nerve terminal, the consequent inhibition of transmitter release is then thought to produce analgesia. Satoh, Amane, Nakazawa and Takagi (1979), were able to attenuate the central antinociceptive effect of pCT (3U/animal, 14-17 g mice) with simultaneous administration of calcium ions (0.1 μmol/animal) at a

dose which was itself without effect. Bates, Buckley, Eglen and Strettle (1981c) using the acetic acid induced abdominal constriction test in mice found that i.c.v. administration of calcium ions caused hyperalgesia and that 1.7 $\mu mol.kg^{-1}$ (which alone was without significant effect) abolished the antinociceptive effect of sCT (2 $U.kg^{-1}$). Similarly, the calcium ionophore A23187 reduced the potency of sCT by approximately 100 fold when administered i.c.v. in a dose of 11.7 nmol kg^{-1} which was itself without effect (Bates, Buckley, Eglen and Strettle (1981d). Central administration of the highly selective calcium chelator EGTA or organic calcium antagonists (nifedepine and PY 108068) was found by Bates, Buckley, Eglen, McArdle, and Strettle (1982b), to produce dose dependent antinociception. Simultaneous administration of EGTA and a maximally effective dose of sCT did not produce additive effects indicating to the authors that a common mechanism was involved. In contrast, simultaneous administration of sCT and nifedepine gave greater antinociception than either drug administered alone indicating that the mechanisms of nifedepine antinociception are, at least in part, distinct from those of CT.

It can be concluded from the preceding paragraphs that although CT antinociception does not involve action at opiate receptors, there are considerable similarities between opiate and CT induced antinociception with regard to calcium metabolism (D.5.b).

Although the fundamental mechanism(s) of CT antinociception remain unclear it is apparent that central administration of CT results directly or indirectly in modulation of neuronal activity yet the neurotransmitter systems and sites of action involved have not been established. Yamamoto et al (1980), directly demonstrated the neuromodulatory action of CT on two specific nociceptive pathways:- 1) tooth pulp-spinal tractus nucleus of this trigeminal nerve-posterior group of nuclei of the thalamus-sensory cortex, and 2) sciatic nerve-spinal chord-ventral posterolateral nucleus-sensory cortex. However, even in these experiments the exact site of action of CT was not established since the effect could involve action at any number of sites from the spinal chord to the sensory cortex. Nakhla and Majumdar, (1978) have claimed that CT reduces plasma tryptophan and increases the concentration of 5-HT within the CNS, which, it was

suggested increased central acetylcholinesterase (AchE) activity. Bates, Buckley, Eglen, McArdle and Strettle (1982c) were unable to modify central AchE activity in mice with central or peripheral administration of sCT, however, the possibility of CT modifying central 5-HT has recently been supported by Dupuy, Peuchant, Vitiella, Jensen, Baghdiantz and Blanquet (1983), and remains of interest particularly in view of the established role of central 5-HT in regulation of responses to painful stimuli (D.4.c).

In spite of the lack of knowledge concerning precise mechanisms and sites of action of CT in the CNS, the marked antinociceptive effect of the hormone in several models has led to the initiation of clinical trials and investigations in humans. Fraioli, Fabbri, Gnessi, Moretti Santoro and Felici (1982a) injected sCT (1.5 $\mu g.kg^{-1} \simeq 4.3$ $U.kg^{-1}$) into the lumbar subarachnoid space of eight patients suffering from chronic intractable pain associated with terminal cancer. All patients reported a significant reduction in pain within 5 minutes of administration, and all but one patient reported complete absence of pain by 15 minutes. The duration of the analgesic effect of sCT ranged from 2-5 days whereas injection of saline produced no analgesia at any time. A sustained diuresis was the only side effect observed. Similarly, Fiore, Castorina, Malatino and Tamberino (1983) have observed analgesic effects after epidural administration of sCT ($\simeq$15 U) or hCT ($\simeq$9 U) and subarachnoid administration of hCT (15 $U.kg^{-1}$). These doses, which are lower than those used previously, produced more transient effects, although epidural administration of sCT (15 U $\simeq$ 3 μg) produced analgesia with a duration of as much as 6 hours. Although Shaw (1982), has warned of severe side effects (including convulsions and death) produced by intracisternal injection of sCT in dogs and baboons, Fraioli, Fabbri, Gnessi, Moretti, Santoro and Felici (1982b) indicate that these effects may be attributable to the preservatives and gelatin diluent in which CT is commercially provided in Italy. In the first investigation of the possible mechanisms(s) of action of the analgesic effect of sCT in humans Fabbri et al (1981), found that sCT (40 μg i.v.) did not modify the concentration of β-endorphin in the plasma or CSF. However, the marked increase in CTLI in the CSF (from 78 $\pm$ 8 to 220 $\pm$ 22 $pg.ml^{-1}$) after peripheral administration indicates that the peptide is able to cross the blood-brain barrier (or to induce secretion of an endogenous CT-like molecule into the CSF). The first demonstration of a

pathological condition involving central CT was given by Fabbri, Fraioli, Gnessi, Moretti, Bini, Gracu and Manfredi (1983). This group sampled plasma and CSF from a 17 year old male with a congenital absence of pain, a rare condition which is considered as a model of indifference to pain resulting from a central error in the processing of nociceptive messages. Elevated opioid activity was observed in the CSF but this was not thought to underlie the condition since it was reversed by naloxone. The authors suggested that the level of immunoreactive CT in the plasma (84.5 ± 4.3 pg.ml^{-1}) was normal, whereas the CSF level of 4630 ± 150 pg.ml^{-1} was 50-200 times higher than would normally be expected, and stated that confirmation of a link between the condition and central CT awaits the production of specific CT antagonists.

1.8.g Cardiovascular Effects of Calcitonin

The reduction in skeletal blood flow observed in patients receiving CT for treatment of Paget's disease (Wootton, Reeve, Spellacy and Tellez-Yudilevich, 1978) may contribute to the reduction in skeletal calcium turnover and to the CT-induced reduction in the elevated temperature of skin over Pagetic bone. Driessens and Vanhoutte (1981) investigated this phenomenom in vitro by measuring fluid pressure in dog tibia perfused with Krebs'-Ringer via the main nutrient artery, they observed that both pCT and sCT produced a dose dependent increase in pressure when added to the perfusate indicating that they caused constriction of bone blood vessels.

The vascular effects of calcitonin are not localised to bone nor is vasoconstriction a constant observation, Charbon and Pieper (1972) demonstrated that CT antagonised the vasodilator response to PTH in the hepatic and renal circulation but was without effect when administered alone. More recently Franchi, Chiarini, Matassi, Strazzulla Scordi, Righi and Nuzzagi (1983) have shown CT to improve cutaneous circulation in humans suffering from obstructive arterial diseases including Raynaud's syndrome.

In addition to the acute effects described above, chronic administration of sCT (but not pCT or hCT), at a dose of 5U.kg^{-1} over a period of 5 weeks, has been reported to attenuate the development

of the hypertension induced in rats by daily administration of deoxycorticosterone (DOCA) in combination with high NaCl intake (Aldred, Luna, Zeedyk and Bastian, 1976). The authors suggested that this effect might be related to the diuretic and natiuretic effects of the hormone.

Following work on similarities between opiate induced and CT induced antinociception, Bates and co-workers investigated the effects of CT in animal models known to respond to opiate agonists and antagonists (Bates, Buckley, Eglen, McArdle and Strettle, 1982a; Bates, Buckley, Eglen, McArdle, Strettle and Wood, 1983). During these investigations the effect of peripherally administered sCT, leu-enkephalin and naloxone on mean arterial pressure (MAP) of normotensive and hypotensive rats was determined. Salmon-CT and naloxone were found to be without effect on MAP of normotensive rats whereas leu-enkephalin produced transient dose dependent hypotension. In rats rendered hypotensive by haemorrhage, both naloxone (10 $mg.kg^{-1}$, i.v.) and sCT (0.1-10 $U.kg^{-1}$, i.v.) had pressor effects. These results reinforced earlier conclusions that sCT was not acting as a direct opiate receptor agonist but raised the possibility that the peptide might have opiate antagonist properties.

Reasons for the marked differences between the haemodynamic effects of peripherally administered sCT in normotensive, hypotensive and hypertensive rats remain to be determined, but presumably reflect differences between the experimental models used and between the effects of acute and chronic administation. The site and mechanism of the pressor response to sCT in rats rendered hypotensive by haemorrhage are not yet fully understood and investigations of this problem form a large part of the work presented in this thesis. Possible mechanisms of action include direct vasoconstriction as described by Driessens and Vanhoutte, (1981) or alternatively the pressor response could be achieved by modification of sympathetic tone, baroreceptor or chemoreceptor reflexes or secretion of adrenaline, vasopressin, ACTH etc. The possibility of central actions of CT after peripheral administration must also be considered as a route for effects on the cardiovascular system. Several peptides found within the CNS including endorphins, vasopressin, angiotensin, substance P, somatostatin, CGRP

etc.have been shown to have potent cardiovascular effects when administered centrally (Unger, Ganten, Lang and Rascher, 1981; Palkovits, 1981; Ganten, Unger, Simon, Schaz, Scholkens, Mann, Speck, Lang and Rascher, 1981; Fisher, Kikkawa, Rivier, Amara, Evans, Rosenfeld Vale and Brown, 1983) and may be involved in central mechanisms of blood pressure regulation (Unger et al, 1981).

Of particular relevance to this thesis is the work of Fisher and co-workers on CGRP, the alternative transcription product of the calcitonin gene found predominantly in the neural tissue of rats (Rosenfeld et al, 1983). This peptide was found to be localised in several areas of the brain including the nucleus tractus solitarius which is involved in relay of baroreceptor reflexes. When administered centrally to conscious rats CGRP caused elevation of the MAP and heart rate which was associated with a marked increase in plasma noradrenaline (Fisher et al, 1983). Similarities in structure between CT and CGRP discussed below (I.10) raise the possibility that CT might also act centrally in cardiovascular regulation.

I.8.h Effect of Calcitonin on Prolactin Secretion

Calcitonin-like immunoreactivity has been demonstrated in the pituitary, but it seems unlikely that the hormone is secreted from the pituitary to exert peripheral effects (I.6). An alternative possibility is that pituitary CT-like peptides regulate secretion of other pituitary hormones. The first investigation of this possibility was that of Reel, Pastushock, Vaitkus, Sokowski and Dermody (1977) who found that CT did not alter the release of thyrotropin or leuteinising hormone from pituitary cells in vitro. Subsequently CT-induced modification of prolactin release has been reported by the groups of Iwasaki and Pecile.

Iwasaki, Chihara, Iwasaki, Abe and Fujita (1979) reported that injection of 0.1-2.5 ng of [$Asu^{1,7}$]-eel CT (a stable analogue of eCT) into the cerebral ventricles of male rats produced a dose dependent rise in plasma prolactin with a duration of up to 40 minutes. Salmon, porcine and human CT's produced similar effects but were less potent. As i.v. administration of as much as 20 μg [$Asu^{1,7}$]-eCT had no effect on plasma prolactin levels the authors concluded that the hormone was

unlikely to act directly on the pituitary. Alternatively, they suggested that the effect might involve modulation of central tryptaminergic metabolism as Nakhla and Majumdar (1978) have reported CT induced elevation of central 5-hydroxytryptamine (5-HT) and i.c.v. 5-hydroxytryptophan (5-HTP, the immediate precursor of 5-HT) has been shown to increase prolactin secretion (Kamberi, Mical and Porter, 1973).

In direct contrast to the results described above, Oligati, Guidobono, Luisetto, Netti, Bianchi and Pecile (1981) reported that sCT reduced plasma prolactin in male rats after both i.v. ($10\ \mu g.kg^{-1}$) and i.c.v. ($\simeq 125\ ng.kg^{-1}$) injection. This effect was abolished by disruption of CNS control of the pituitary (median eminence lesion) and was not evident in haloperidol treated rats indicating to the authors that CT exerts its effects on prolactin secretion via the hypothalamic dopaminergic system. Although Pecile and co-workers were unable to explain the divergence between these results and those of Iwasaki et al (1979), their studies have been extended by the demonstration that i.c.v. sCT ($\simeq 125\ ng.kg^{-1}$) prevented the suckling stimulated rise in plasma prolactin of lactating rats (Olgiati, Netti, Guidobono and Pecile, 1982). Since prolactin, which circulates in high levels during lactation is thought to stimulate 1,25 DHCC production in the kidney, an inhibitory effect of CT on prolactin secretion could serve to prevent the sacrifice of skeletal calcium for milk production.

I.8.i Calcitonin and the Antistress Hypothesis

In reviewing the literature describing the physiological and pharmacological effects of CT, Dupuy (1983) has proposed that the hormone exerts a general antistress effect. In support of this hypothesis Dupuy notes that hypercalcaemia can be induced by secretion of catecholamines (Hsu and Cooper, 1975) and by the reduction in blood pH associated with intense exercise (Ruben and Bennet, 1981). Since both catecholamines and plasma calcium are established CT secretagogues (Bates, Bruce and Care, 1970) it is reasonable to assume that CT might be secreted in response to stresses, including exercise. Indeed, several stressful situations have been shown to increase circulating levels of CT, these include surgery (MacManus, Youdale and Braceland, 1975), heart infarction (Boer, Mulder, Fischer, Schopman, Hackeng and Silberbusch, 1981), premature birth (David, Salle, Putet and Gratmeyer,

1981) and trauma associated with bone fracture (Ekeland, Gautvik and MacIntyre, 1981). The reported effects of CT which might conceivably have protective or adaptive advantage in such stressful situations range from inhibition of stress induced ulcer formation (Bates and Barlet,1974), to production of analgesia (Pecile et al, 1975), protection against inflammation (Riesterer and Jaques, 1969), acceleration of wound healing (Lupulesku and Habowsky, 1978) and hyperglycaemia (Passariello, Giugliano, Scambato, Torella, d'Onofrio, 1981) in addition to established effects in calcium homeostasis.

Dupuy proposes that any situation producing disturbance of homeostasis provokes CT secretion which produces effects opposing the original disturbance. Evidence concerning the physiological role of CT in stress is, however, limited and this hypothesis cannot be considered proven. However, it is apparent that CT, like other peptides secreted in response to stress, may exert important but, as yet, undefined protective effects in such situation.

1.9 CALCITONIN AND SECOND MESSENGER SYSTEMS

In accord with the receptor theory of hormone action, it can be assumed that CT secreted from the thyroid glands is transported in the blood to target organs where it becomes bound to specific receptors in the cell membrane. The binding of hormone to receptor initiates a series of events which alter intracellular metabolism, eventually translating the extracellular event (binding of CT, the first messenger) to the ultimate cellular response. Although the mechanisms by which extracellular binding of CT produces intracellular effects are unclear they may involve second messenger systems.

In the late 1950's Sutherland and co-workers demonstrated that binding of glucagon to receptors on liver cells activated a membrane bound enzyme termed adenylate cyclase. This enzyme uses adenosine triphosphate (ATP) in the production of cyclic adenosine 3',5'-monophosphate (cAMP) which acts as an intracellular messenger (second messenger) activating protein kinases to phosphorylate various effector proteins (Sutherland and Rall, 1957; Sutherland, Robinson and Butcher, 1968). It is now clear that cAMP plays a critical role in the cellular effects of numerous hormones, neurotransmitters and agonists of their receptors, including noradrenaline and adrenaline (Klainer, Chi, Freidburg, Rall and Sutherland, 1962), dopamine (Clement-Cormier, Parrish, Petzold, Kebabian and Greengard, 1975), 5-HT (Nelson, Herbet, Adrien, Bockaert and Hamon, 1983), exogenous and endogenous opiates (Sharma, Klee and Nirenberg, 1975; Klee and Nirenberg, 1976), vasoactive intestinal polypeptide (Kerwin, Pay Bhoola and Pycock, 1980), vasopressin (Courtney and Raskind, 1983) and CT (Murad, Brewer and Vaughan, 1970). Cyclic nucleotides may therefore be the major second messengers involved in physiological and pharmacological effects of CT, alternatively, such effects could be achieved by a direct action or occur as a result of modified calcium metabolism.

The marked differences in the concentrations of intracellular and extracellular calcium ions (in most cells approximately 10^{-7} and 10^{-3} M respectively) and the calcium dependence of numerous biological processes makes this ion ideally suited to trigger changes in biological activity. In fact, it has been proposed that calcium ions

may themselves serve as second messengers (Rasmussen and Goodman, 1977) It now appears that the intracellular effects of calcium ions are relayed by complex formation with calcium binding proteins such as calmodulin, and that calcium dependent enzymes (such as protein kinases, calcium ATPase, adenylate cyclase and phosphodiesterase) are Ca-calmodulin dependent. Calcium binding is thought to activate calmodulin which in turn activates these effector proteins in a manner directly analagous to phosphorylation of effector proteins by cAMP activated protein kinases (figure I.4). The reported effects of CT on cyclic nucleotide and calcium metabolism are described below, although these effects are considered separately here the systems are clearly closely interrelated as discussed in section D.5.d.

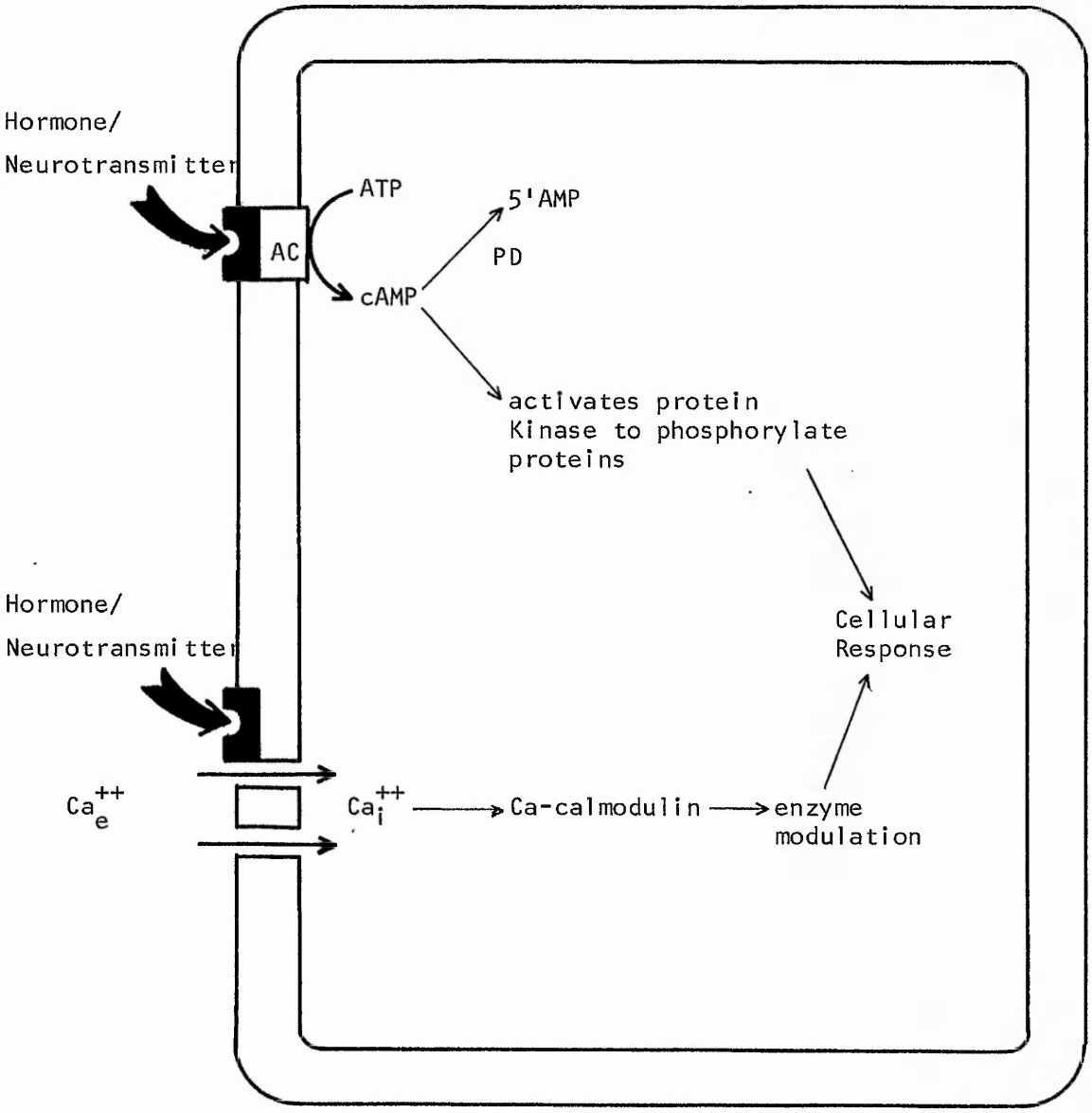
I.9.a Effects on Calcium

In addition to the established inhibitory effect of CT on efflux of calcium from bone (Friedman and Raisz, 1965) and intestinal calcium absorption (Swaminathan et al, 1974), CT has been shown to modify calcium transport in several other tissues including kidney (Kenny and Heiskell, 1965), liver (Yamaguchi, Takei and Yamamoto, 1976), myocardium (Chausmer, Weiss and Wallach, 1965) and hypothalamus (Levine and Morley, 1981; Koida, Yamamoto, Nakamuta, Matsuo, Okamoto and Mirimoto, 1982). In the majority of tissues studied CT has been found to increase calcium content although the hormone appears not to modify calcium content of salivary glands, small intestine, pancreas (Kenny and Heiskell, 1965), and brain (Eglen, 1983).

At the cellular level, CT has been shown to inhibit calcium efflux from bone (Harrell, Binderman and Rodan, 1973), kidney (Borle, 1969) and liver (Yamaguchi et al, 1976). Inhibition of calcium efflux could clearly account for the increased levels of intracellular calcium observed in most tissues (Borle, 1975). Kinetic analysis of ^{45}Ca uptake by hepatocytes revealed to Borle (1975) that CT enhanced the transport of calcium from the cytoplasm to a slowly exchanged calcium pool (assumed to be mitochondrial). Moreover, CT was found to enhance ^{45}Ca uptake by isolated mitochondria leading to the suggestion that CT induced inhibition of calcium efflux is achieved by intracellular calcium sequestering which reduces cytosolic calcium. This theory is discussed below (D.5.a).

Figure I.4 Diagram of cell using both calcium and cAMP as second messengers.

Binding of first messenger (hormone or neurotransmitter) to adenylate cyclase (AC) associated receptors produces physiological response by modulating production of cAMP which activates protein kinases to phosphorylate proteins. Similarly, calcium following the electrochemical gradient into cells (via receptor operated or voltage sensitive calcium channels) complexes with calmodulin to modulate the activity of Ca-calmodulin dependent enzymes. The possibility for interaction between the two systems is evident as Ca-calmodulin dependent enzymes include adenylate cyclase and phosphodiesterase (PD), and cAMP induced protein phosphorylation can alter the function of calcium channels and pumps.


Hormone/
Neurotransmitter
AC
ATP
5'AMP
PD
cAMP
activates protein
Kinase to phosphorylate
proteins
Hormone/
Neurotransmitter
Cellular
Response
Ca_e^{++}
Ca_i^{++}
Ca-calmodulin
enzyme
modulation

I.9.b Effects on Cyclic 3',5',-monophosphate

Calcitonin has been found to elevate levels of cAMP in a variety of preparations from bone, kidney (Murad et al, 1970; Marx et al, 1972a; Marx, Fedak and Aurbach, 1972b), parathyroid glands (Dufrense and Gitelman, 1972), bronchial carcinoma cells (Hunt et al, 1977) and rat glial cells (Loffler, Van Calker and Hamprecht, 1982). Adenylate cyclase activity of renal and skeletal membrane preparations was found by Marx et al (1972) to be stimulated by CT, an effect which could clearly account for the elevated levels of cAMP. Marx and Aurbach (1975) found CT stimulatable adenylate cyclase and ^{125}I-sCT binding sites to be distributed in the particulate (plasma membrane) fraction of kidney homogenates, and Goltzman (1980) observed a close correlation between binding affinity and potency at adenylate cyclase stimulation for sCT, hCT and a range of analogues. These results clearly indicate an association of peripheral CT receptors with membrane bound adenylate cyclase.

In contrast to the effect on renal membranes, Rizzo and Goltzman (1981) reported that sCT inhibited adenylate cyclase activity of rat brain homogenates and that sCT, pCT and hCT inhibited the activity of the enzyme in homogenates of hypothalamus in a dose dependent manner with potency proportional to the known biological potencies of the hormones. The reported inhibitory effect on CNS adenylate cyclase and stimulatory effect on glial cell cAMP accumulation are discussed below (R.6 and D.5.c).

1.10 STRUCTURE ACTIVITY RELATIONSHIPS

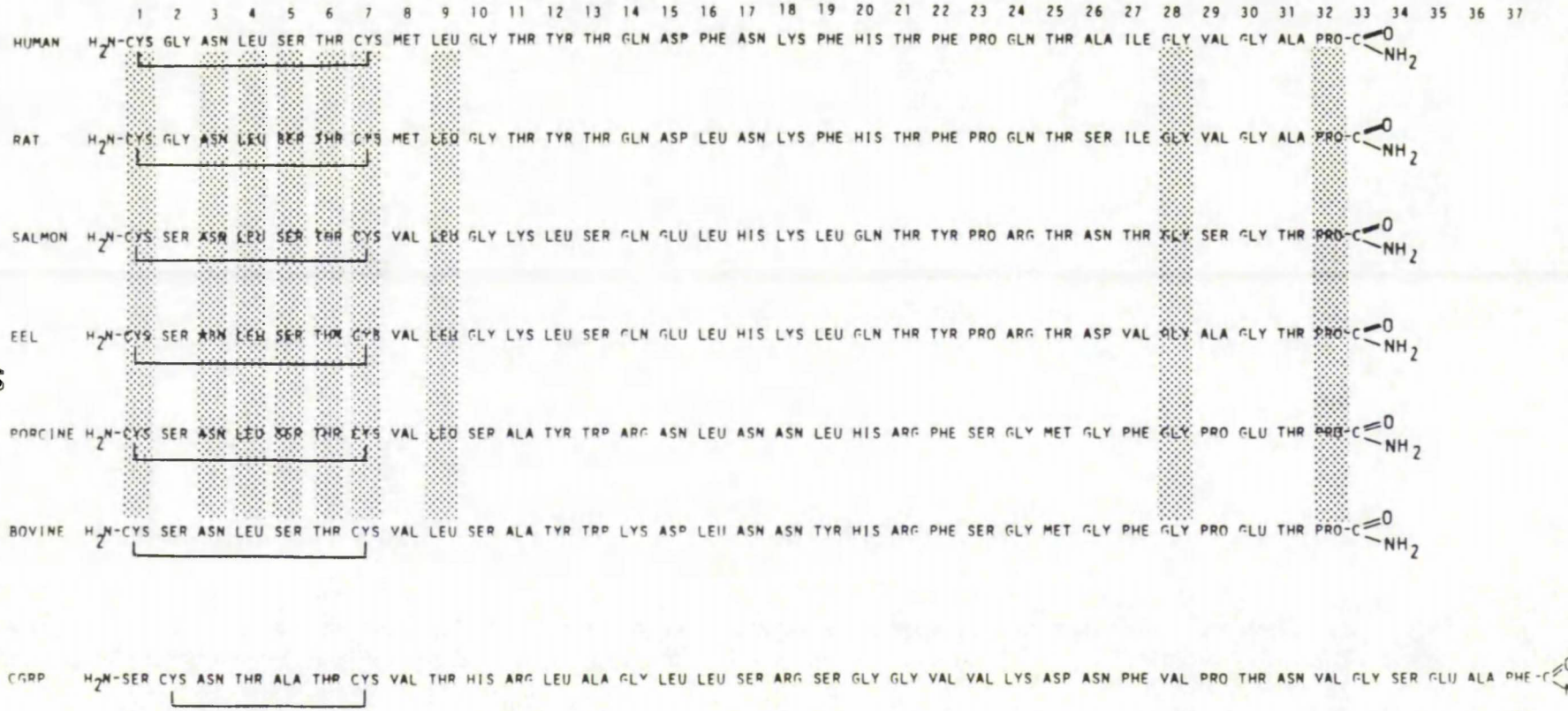
MacIntyre, Evans, Hobitz, Joplin and Stevenson (1980) noted that considerable variation exists between the structure of the CT's sequenced to date and that major changes are therefore possible without loss of biological activity. Indeed, the only residues common to all known calcitonins are the carboxy terminal prolinamide, glycine at position 28 and six of the seven bases in the N' terminal ring structure (figure 1.5). Sieber, Brugger, Kamber, Riniker, Rittel, Maier and Staehelin (1970) found that removal or deamination of the carboxy terminal prolinamide in pCT led to loss of hypocalcaemic activity, observations of this nature led MacIntyre to suggest that the whole CT molecule was necessary for biological activity. This assumption was disproved when Schwartz, Orlowski and Marcus (1981) demonstrated that des-ser^{2}-sCT was equipotent with sCT. Recently, Moe, Miller and Kaiser (1983) have focussed attention on the contribution of bases 8-22 to biological activity. Studies using circular dichroism have shown sCT and hCT to be 20-50% α-helical (dependent on conditions such as temperature and pH); and Moe et al (1983) have suggested that residues 8-22 might form an α-helical region. The CT molecule can therefore, be considered in terms of three domains, the N' terminal ring structure (1-17), the α-helical region (8-22) and a C' terminal random coil (23-32).

1.10.a Residues 1-7

All CT's sequenced to date have a seven base N' terminal ring structure joined by a disulphide bridge. Rittel, Maier, Brugger, Kamber, Rininker and Sieber (1976) found that biological activity was lost when the disulphide bridge was opened by methylations of the cysteines at 1 and 7. However, Morikawa, Munekata, Sakakibara, Noda and Otani (1976) found [Asn^{1,7}]-eel CT (in which the disulphide bond between cys^{1} and cys^{7} is replaced with an ethylene link between two molecules of L-amino suberic acid) to be as potent as eCT. These results suggest that the 1-7 link produces important conformational effects, but that the disulpide bond is not itself necessary for biological activity. The high degree of conservation of the N' terminal sequence indicates that this region plays an essential biological role yet

Figure 1.5 Amino acid sequence of human, rat, salmon, eel, porcine and bovine calcitonins and calcitonin gene-related peptide (CGRP).

Bases common to all sequenced calcitonin are indicated by shading.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
HUMAN H2N-CYS GLY ASN LEU SER THR CYS MET LEU GLY THR TYR THR GLN ASP PHE ASN LYS PHE HIS THR PHE PRO GLN THR ALA ILE GLY VAL GLY ALA PRO-C O NH2
RAT H2N-CYS GLY ASN LEU SER THR CYS MET LEU GLY THR TYR THR GLN ASP LEU ASN LYS PHE HIS THR PHE PRO GLN THR SER ILE GLY VAL GLY ALA PRO-C O NH2
SALMON H2N-CYS SER ASN LEU SER THR CYS VAL LEU GLY LYS LEU SER GLN GLU LEU HIS LYS LEU GLN THR TYR PRO ARG THR ASN THR GLY SER GLY THR PRO-C O NH2
EEL H2N-CYS SER ASN LEU SER THR CYS VAL LEU GLY LYS LEU SER GLN GLU LEU HIS LYS LEU GLN THR TYR PRO ARG THR ASP VAL GLY ALA GLY THR PRO-C O NH2
PORCINE H2N-CYS SER ASN LEU SER THR CYS VAL LEU SER ALA TYR TRP ARG ASN LEU ASN ASN LEU HIS ARG PHE SER GLY MET GLY PHE GLY PRO GLU THR PRO-C O NH2
BOVINE H2N-CYS SER ASN LEU SER THR CYS VAL LEU SER ALA TYR TRP LYS ASP LEU ASN ASN TYR HIS ARG PHE SER GLY MET GLY PHE GLY PRO GLU THR PRO-C O NH2
CGRP H2N-SER CYS ASN THR ALA THR CYS VAL THR HIS ARG LEU ALA GLY LEU LEU SER ARG SER GLY GLY VAL VAL LYS ASP ASN PHE VAL PRO THR ASN VAL GLY SER GLU ALA PHE-C O NH2

considerable modifications of this structure were shown by Rittle (1977) not to alter biological activity. A possible explanation of this paradox can be found by considering the previously described (1.4) experiments of Jacobs et al (1981b). This group found CT mRNA to direct the synthesis of a CT precursor which was glycosylated after removal of a 3000 MW leader sequence, the only known site on the precursor molecule amenable to glycosylation was the highly conserved Asn-Leu-Ser sequence at residues 3-5. It seems likely therefore, that the precise sequence of residues in the N'terminal ring structure is not essential for biological activity but that this region is highly conserved because of its important role in the processing of the precursor.

1.10.b Residues 8-22

In the CT's sequenced to date considerable variation is seen in the residues from positions 8-22 and structural modification, such as replacement of residues 15 and 21 of sCT with the lipophilic residues asparagine and phenylalanine (Maier,Riniker and Rittel, 1974), are possible without loss of biological activity. In contrast, Findlay, Michalengeli, Orlowski and Martin (1983) found that removal of the lipophilic residues at position 16 in sCT and pCT (Leu and Phe respectively) reduced biological activity to 20%.

Noting that circular dichroism studies of natural CT's indicate an α-helical content of 20-50%, Moe et al (1983) suggested that the region 8-22 might form a structurally important α-helix, this suggestion is supported by the secondary structure predictions of Merle, Lefevre and Milhaud (1979). Moe and co-workers also noted that axial projections of the predicted α-helical regions showed a tendency toward concentration of lipophilic residues on one side of the helix and that this tendency was most marked in more potent CT's (figure 1.6.a,b). To test the hypothesis that the structurally significant point of this region was the amphiphillic α-helix, Moe's group synthesised a model-CT (mCT) having residues 1-7 of hCT, 23-32 of sCT(1) and a central region composed of residues with a high potential for helix formation (e.g. leucine, glutamine and lysine) and a concentration of lipophilic residues on one side (figure 1.6.b). This peptide, had similar

Figure 1.6 a and b Axial projection of residues 8-23 of sCT, eCT, hCT, pCT, rat CGRP, and human CGRP.

The diagrams are based on the assumption that an α-helical conformation is adopted, and lipophillic residues are shaded.

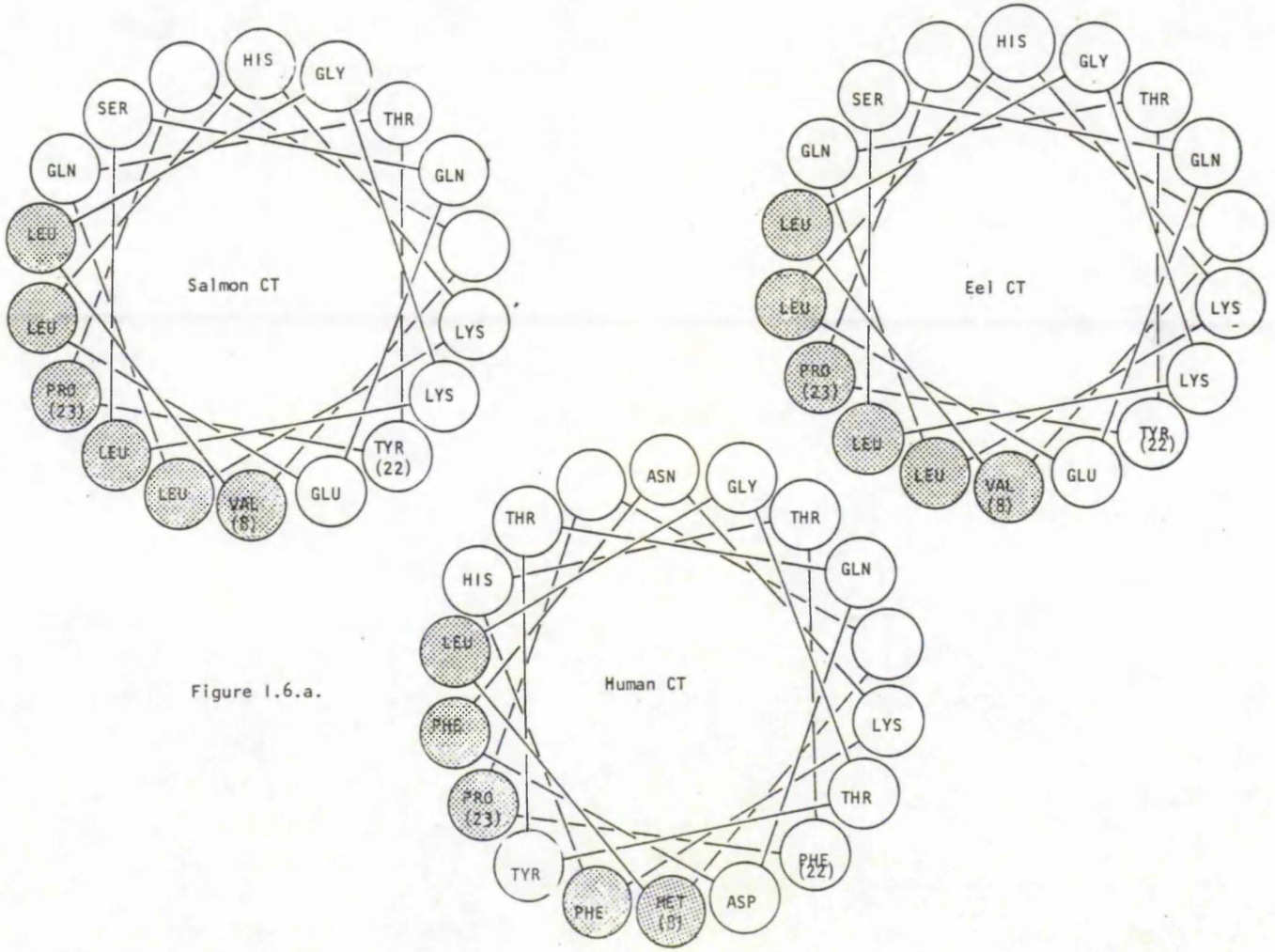

Salmon CT
HIS
GLY
SER
THR
GLN
GLN
LEU
LYS
LEU
LYS
PRO (23)
TYR (22)
LEU
LEU
VAL (8)
GLU
Eel CT
HIS
GLY
SER
THR
GLN
GLN
LEU
LYS
LEU
PRO (23)
LYS
TYR (22)
LEU
LEU
VAL (8)
GLU
Human CT
ASN
GLY
THR
THR
HIS
GLN
LEU
LYS
PHE
THR
PRO (23)
PHE (22)
TYR
PHE
MET (1)
ASP

Figure 1.6.a.

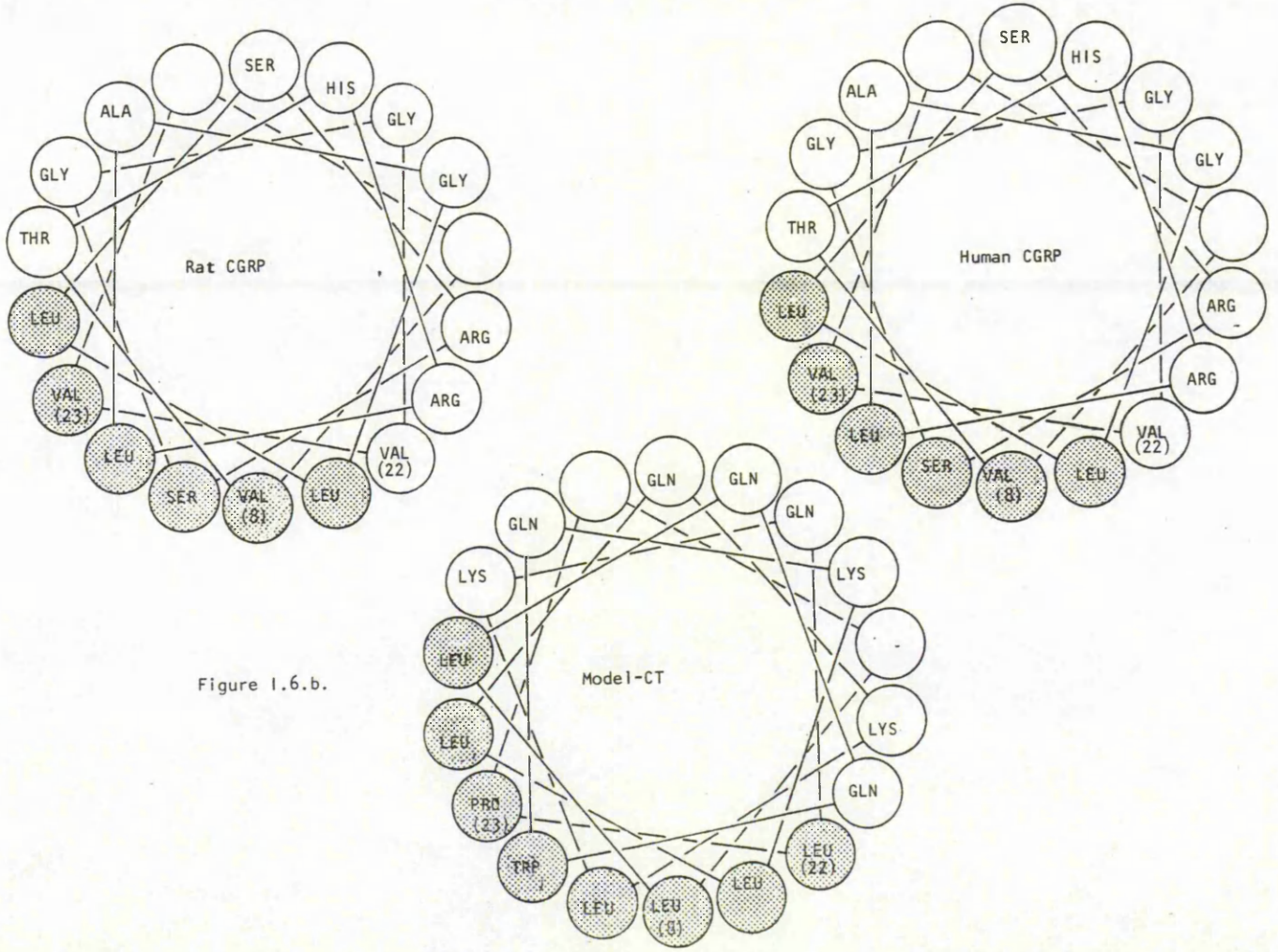

Rat CGRP
SER
HIS
GLY
GLY
ARG
ARG
VAL (22)
LEU
VAL (8)
SER
LEU
VAL (23)
LEU
THR
GLY
ALA
Human CGRP
SER
HIS
GLY
GLY
ARG
ARG
VAL (22)
LEU
VAL (8)
SER
LEU
VAL (23)
LEU
THR
GLY
ALA
Model-CT
GLN
GLN
GLN
LYS
LYS
LYS
GLN
LEU (22)
LEU
LEU (8)
LEU
TRP
PRO (23)
LEU
LEU
LEU

Figure 1.6.b.

hypocalcaemic and receptor binding potency to pCT. Epand, Epand, Orlowski, Schleuter, Boni and Hui (1983), later demonstrated that CT's interact *in vitro* with acidic phospholipids to form stable lipoprotein complexes and that this interaction is associated (in the case of sCT and pCT) with enhanced helical structure of the CT molecule. These results clearly indicate a structural role for residues 8-22, the amphiphillic α-helix could be involved in receptor binding, association with lipid membranes of target cells or in determining the spacing and orientation of C' terminal and N'terminal active sites.

The enhancement of the helical conformation by phospholipids may be significant *in vivo* as plasma membrane phospholipids could clearly promote the adoption of this structure. Interaction between the lipophilic face of the helix and the phospholipid plasma membrane might then enhance the stability of the hormone-receptor complex. The observations of Maier, Findlay and co-workers (above) are readily explained when the importance of the amphiphillic helix is known, since addition of lipophilic groups to the hydrophilic face of the helix (Maier et al, 1974) would not be expected to reduce activity whereas placing a hydrophilic residue in the lipophilic face (Findlay, 1983) would be expected to do so.

I.10.c Residues 23-32

Very little is known about the relationship between the structure of the C' terminal sequence and biological activity. This sequence, which is thought to have a random coil structure contains two amino acids common to all CT's (gly^{28} and pro^{32}), removal or deamination of the terminal proline residue has been found to abolish biological activity (Maier et al, 1974). In spite of considerable gaps in knowledge of the relationship between structure and activity of CT's and analogues, recognition of the major features necessary for biological function should be of assistance in predicting CT-like activity of other synthetic or endogenous molecules. The recently described peptide CGRP (Amara et al, 1983) is a case in point, although the primary structure of CGRP (a 37 base peptide with a 2-7 disulphide bridge and little sequence homology with any form of CT) is different from

that of known CT's, consideration of secondary structure suggests that similarities may exist. Firstly, it has been argued above that the precise sequence of residues in the N' terminal ring structure may not be important for biological activity. Although the six membered N' terminal ring structure of CGRP is different from the seven membered structure found in CT's it has considerable sequence homology with this region of des-ser^{2} sCT which has been shown to be equipotent with sCT (Schwartz, 1981). Secondly, like all known CT's the sequence 8-22 of CGRP contains regularly spaced lipophilic residues. Acidic phospholipids are known to enhance the α-helical content of some CT's, any such effect on CGRP could lead to the production of an amphiphillic α-helix in the 8-22 sequence of CGRP (figure I.6.b). In the C' terminal region CGRP, like all known CT's, possesses hydrophilic residues at positions 29 and 32 and a neutral hydrophobic residue at position 28, however, CGRP does not possess the two residues in this region common to all sequenced CT's (gly^{28} and pro^{32}). The lack of experimental evidence concerning relevance of C' terminal residues to biological action prevents prediction of the possible effects of those differences or of the residues 33-37 in CGRP. The relationship between CT and CGRP is explored more fully in chapter D.2.

1.11 AIMS OF THE PROJECT

It is evident from the preceding section that administration of CT to animals and man can produce a wide range of biological responses which may or may not be of physiological, pharmacological or therapeutic relevence. The aim of the experiments reported in this thesis was to investigate the pharmacological effects of CT and the mechanisms by which such effects might be produced. The pharmacological investigations have been concentrated on the anti-nociceptive and haemodynamic effects of CT which may be therapeutically relevant to man. As the effects observed in vivo appear to involve modulation of neuronal activity, biochemical studies have concentrated on the means by which CT might alter the activity of neuronal tissue.

MATERIALS AND METHODS

M.1 ANIMALS

The animals used in this project were either male or female CFLP mice of 20-30 g or were male or female Sprague Dawley rats of 150-350 g.

All animals were maintained in an ambient temperature of 21-23^{o}C with hours of darkness between 1800-0600. They were allowed access to food (Heygate and Son Ltd., breeding diet cubes containing 1.42% calcium and 1.09% phosphate) and water ad libitum.

Animals were either purchased from Hacking and Churchill Ltd., or bred from stock obtained from the same source.

M.2 CHEMICALS

Synthetic human and salmon calcitonins (hCT and sCT) were donated by the Armour Pharmaceutical Co. and by Ciba-Geigy Ltd. respectively, and naloxone was a gift from Endo Laboratories. Calcitonin gene related peptide (CGRP), and the vasopressin antagonist $d(CH_2)_5$Tyr(Me)AVP were purchased from the Merseyside Laboratories.

Leucine-enkephalin, d-ala-d-leu-enkephalin, vasopressin, Brij 30, 5-hydroxyindole acetic acid, 5-hydroxytryptamine, ∝-methyl-p-tyrosine, salicylic acid, o-pthaldialdehyde and parachlorophenylalanine were all purchased from Sigma Biochemicals Ltd. Radionucleotides ($^{45}Ca^{2+}$ and [^{3}H]-cyclic adenosine 3',5'-monophosphate) were obtained from Amersham International plc.

The remaining chemicals, which were of 'Analar' grade wherever possible, were purchased from major chemical suppliers (usually Sigma Biochemicals Ltd. or BDH Chemicals Ltd.).

M.3 SOLUTIONS

a) Physiological Salt Solutions:

i) Krebs' bicarbonate saline

NaCl	118	$mmol.l^{-1}$
KCl	4.7	$mmol.l^{-1}$
$MgSO_4$	1.0	$mmol.l^{-1}$
$CaCl_2$	2.5	$mmol.l^{-1}$
$NaHCO_3$	2.5	$mmol.l^{-1}$
KH_2PO_4	1.2	$mmol.l^{-1}$
Glucose	11	$mmol.l^{-1}$

at 37°C, pH 7.4, gassed with 5% CO_2 in O_2

ii) HEPES phosphate saline

NaCl	118	$mmol.l^{-1}$
KCl	5.9	$mmol.l^{-1}$
$MgCl_2$	1.2	$mmol.l^{-1}$
$CaCl_2$	2.5	$mmol.l^{-1}$
HEPES	11.7	$mmol.l^{-1}$

at 37°C, pH 7.4, gassed with O_2

iii) Phosphate buffer

100 ml, 1M KH_2PO_4 with 79 ml 1M NaOH was diluted to 200 ml with distilled water to produce 0.5 M phosphate buffer at pH 7.4. 50 mM phosphate buffer at pH 7.4 was prepared by diluting 50 ml, 0.2 m KH_2PO_4 and 39.5 ml, 0.2 M NaOH to 200 ml with distilled water.

iv) Tris/HCl buffer

0.2 M Tris (hydroxymethyl) aminomethane, 0.1 M HCl and distilled water were combined in a ratio of 25:42:37 to produce 0.05 M Tris/HCl buffer at pH 7.4.

b) Solutions for Drug Administration:

i) Vehicle for calcitonins

1	$mg.ml^{-1}$	bovine serum albumin (BSA)
0.01	$mol.l^{-1}$	acetic acid
0.9%		NaCl

adjusted to pH 7.4 with 1 M NaOH prior to use.

ii) Vehicle for i.c.v. injections

0.1	$mol.l^{-1}$	NaCl
0.05	$mol.l^{-1}$	Tris
1	$mg.ml^{-1}$	BSA

adjusted to pH 7.4 with 1 M HCl.

iii) p-chlorophenylalanine

0.9%	NaCl
0.5%	Brij (polyoxylaurylethylene ether)

injected as a suspension.

Remaining suspensions were administered in 0.9% NaCl unless otherwise stated.

M.4 INTRACEREBROVENTRICULAR INJECTION TECHNIQUE

M.4.a Mice

Drugs were administered into the lateral ventricles of conscious mice using the method of Haley and McCormick (1957) as modified by Cashin and Heading (1968).

Drugs were administered in a volume of 10 µl by means of an Agla ground glass syringe fitted with a 21 gauge needle which was sheathed to within 4 mm of the tip with fine capillary tubing. The syringe was housed in an Agla micrometer head attached to a racking assembly which was in turn mounted on a retort stand. Intracerebroventricular injection was achieved by rotating the racking screw to pass the needle vertically through the skull at a site 1.5 mm lateral to the bregma and 1.5 mm rostral to the coronal suture, the depth of penetration was limited by the sheathing of the needle.

The injection procedure took approximately 5 seconds, and although appearing initially stunned, the animals appeared to recover rapidly. The accuracy of the technique was assessed by injection of india. ink (10% solution in 0.9% NaCl) after which brains were removed and fixed in formol saline prior to examination under a dissecting microscope. In all brains examined the ink was found throughout the ventricular system and tissue damage appeared to be localised to the path of the needle.

M.4.b Rats

Urethane anæsthetised rats were placed in a Kopf 900 stereotaxic frame as described by DeGroot (1959). The stereotaxic frame has a vertical racking assembly onto which a micrometer syringe or drill may be attached. Using the drill in the racking assembly a 1 mm diameter hole was bored into the skull directly above the lateral ventricle. A 1 ml syringe with a 21 gauge needle was mounted in the micrometer head and then, using the racking assembly, the needle was lowered vertically through the hole into a lateral ventricle (DeGroot coordinates; horizontal +6.6; vertical + 1.5; lateral ± 1.3). Drug administration in a volume of 10 µl was then achieved by rotation of the micrometer screw.

At the end of each of 20 experiments, 10 μl of india ink (10% in 0.9% NaCl) was injected, the brains were removed and fixed in formol saline prior to examination under the dissecting microscope. In all but one case the ink was found to be distributed throught the ventricular system and tissue damage appeared to be localised to the needle tract.

M.5 THE ABDOMINAL CONSTRICTION TEST

Collier et al (1968) demonstrated that the injection of irritants into the peritoneal cavity of mice produced characteristic abdominal constrictions. This response consisted of a wave of muscular contractions passing caudally along the trunk of the animal accompanied by an inward twisting of the hind limbs. They further demonstrated that the rate of the abdominal constrictions could be reduced by agents known to produce analgesia and suggested the use of this model as a screen for such effects.

In this project acetic acid was used to produce peritoneal irritation, and the consequent abdominal constriction response was observed in animals placed individually on wooden boards of approximately 20 × 20 cm, held 20 cm above the bench, in an ambient temperature of 18-22^{o}C.

Preliminary experiments were carried out to determine a suitable dose of acetic acid and time for observation. A volume of 0.3ml 1% acetic acid in 0.9% NaCl injected i.p. was found to produce a relatively constant and reproducible, submaximal response of 3-4 constrictions per minute (c.p.m.) between 10 and 15 minutes after the injection. The dose and observation period was therefore chosen for use in all remaining experiments.

Modification of the abdominal constriction rate by various drugs was then assessed. Typically, drugs screened for potential antinociceptive effect were administered 10 minutes prior to the acetic acid injection, the only exceptions to this scheme were experiments using d-ala-d-leu-enkephalin (which was administered 5 minutes prior to the acetic acid) and experiments in which the time course of the antinociceptive effect of sCT, hCT and calcitonin gene-related peptide (CGRP) were investigated.

In each experiment a group of animals matched for size and sex received appropriate vehicle. Results can therefore be expressed either as c.p.m. or as percentage inhibition of the control constriction rate observed in the internal control group.

In order to eliminate observer bias all drug solutions were coded and experiments were performed "blind". The sex of the animals, time of day, date and ambient temperature were recorded to enable investigation of the possible effects of these variables on the control constriction rate.

M.6 ASSESSMENT OF LOCOMOTOR BEHAVIOUR

Modification of an animal's response to a noxious stimulus can be achieved either by alteration of its perception of the stimulus or by modification of its ability to respond to the stimulus. A drug which alters the constriction response to peritoneal irritation can, therefore, only be considered antinociceptive or hyperalgesic if it does not significantly impair motor function. The drugs used in the abdominal constriction test were, therefore, also tested, at a comparable time after administration, in the following tests:

M.6.a Measurement of Motor Discoordination

Motor discoordination was assessed by a rotating drum test based on that described by Dunham and Miya (1957). Animals were placed on a sandpaper covered drum of a Palmer Kymograph (diameter 15 cm rotating at 2 revolutions per minute) placed horizontally, 1 m above the floor. Unaffected animals were able to maintain their position on the drum by walking. Animals which fell from the drum were replaced immediately, the number of falls in a 2 minute period was counted and taken as an index of motor discoordination.

M.6.b Measurement of Investigative Behaviour

Investigative behaviour was assessed by the head dipping test of File and Wardill (1975). Immediately after investigation of motor discoordination animals were transferred to a 25 x 25 cm board containing 16 evenly spaced holes of 2.5 cm diameter. Control animals immediately began to investigate the environment by dipping their heads into the holes. The number of such dips in a 5 minute period was counted and taken as a measure of investigative behaviour.

M.7 DETERMINATION OF THE EFFECTS OF DRUGS AND PEPTIDES ON BLOOD PRESSURE AND HEART RATE

M.7.a Normotensive Rats

Rats were anaesthetised with urethane (1.4 $g.kg^{-1}$ in 0.9% NaCl) with half of the dose administered i.p. and the remainder s.c. The carotid artery was cannulated and connected to a Washington MDI chart recorder via a PT 400 pressure transducer for the determination of mean arterial pressure (MAP) and heart rate.

In animals which were to receive sCT and vehicle by i.v. injection the jugular vein was routinely cannulated and the trachea was cannulated in any animals exhibiting respiratory distress. Following surgery these animals were allowed a stabilisation period of at least 20 minutes, after which sCT (10 $U.kg^{-1}$) or vehicle (0.1 ml, 0.9% NaCl with 1 $mg.ml^{-1}$ BSA, followed by 0.2 ml 0.9% NaCl to flush cannulae) were administered i.v. All animals received one dose of sCT and one dose of vehicle in varied order and the MAP and heart rate were monitored for 60 minutes after each injection. Rectal temperature was maintained at $37 \pm 0.5^{o}C$ throughout by means of heating lamps.

Animals which were to receive sCT and vehicle by i.c.v. injection were treated as described above except that, the jugular vein was not cannulated, the animals were placed in a stereotaxic frame throughout the experiment, and stabilisation period was preceeded by a single sham i.c.v. injection (needle insertion without fluid ejection). Preliminary experiments had revealed that if this sham injection was omitted vehicle injection produced a gradual reduction in MAP. All animals again received a single injection of sCT (0.1, 1 or 10 $U.kg^{-1}$) and of i.c.v. vehicle in varied order, these injections were over a period of two minutes and in a volume of 10 µl (for details of i.c.v. injection technique see M.4.b).

M.7.b Rats Rendered Hypotensive by Haemorrhage

In determination of the effects of sCT, vehicle and naloxone on MAP and heart rate of haemorrhaged rats the preceding protocol was followed, except that the animals were rendered hypotensive (MAP reduced approximately 20% by removal of 1-3 ml arterial blood) after the initial stabilisation period, and were allowed a further 20 minute stabilisation before administration of the first dose of drug or vehicle. Where sCT or vehicle were administered centrally, the haemorrhage was preceded by the sham i.c.v. injection.

In order to investigate the mechanism of action of sCT in this preparation groups of 6-10 animals were pretreated with 6-hydroxydopamine (6-OHDA, 100 $mg.kg^{-1}$ i.p. in 0.1% ascorbic acid, 4,3 and 2 days prior to experimental use), bilateral vagotomy (5-10 minutes after removal of arterial blood) or the arginine vasopressin (AVP) antagonist $d(CH_2)_5Tyr(Me)AVP$ (0.1 $mg.kg^{-1}$ i.v. in 0.9% NaCl with 1 $mg.kg^{-1}$ BSA, five minutes prior to bleeding). The effect of 6-OHDA on the pressor response of haemorrhaged rats to i.c.v. sCT (1 and 10 $U.kg^{-1}$) was also assessed.

M.7.c Rats Rendered Hypotensive by Pithing

After cannulation of the carotid artery, jugular vein and trachea, a blunt ended pithing rod was passed through the orbit of the eye and then pushed via the foramen magnum down the spinal chord. As the rod passed down the spinal chord the characteristic extensions of the fore and hind limbs were used as an indication of the successful destruction of the CNS. The rats were then rapidly attached to a small animal ventilator (Scientific Research Instruments Ltd., 5056) and allowed at least 20 minutes for stabilisation. During this period a rectal temperature of $37 \pm 0.5^{o}C$ was obtained by means of heating lamps and this was maintained throughout the experiment.

The responsiveness of the MAP of the preparation to direct vasoconstriction was assessed by administration of vasopressin (1-100 $mU.kg^{-1}$ in 0.1 ml, 0.9% NaCl with 1 $mg.ml^{-1}$ BSA). The effect of sCT (10 $U.kg^{-1}$) in 0.1 ml, vehicle on MAP was then

monitored for 60 minutes after its administration and compared to the response observed after vehicle.

The pithed rat model was also used to investigate the duration of antagonism of pressor responses to vasopressin by $d(CH_2)_5Tyr(Me)AVP$. The maximal increase in MAP produced by vasopressin (5-100 $mU.kg^{-1}$ i.v.) was determined approximately 10, 60, 120 and 180 minutes after administration of $d(CH_2)_5Tyr(Me)AVP$ (0.1 $mg.kg^{-1}$ i.v. in 0.9% NaCl with 1 $mg.ml^{-1}$ BSA) and compared to the response observed in animals pretreated with vehicle alone.

M.8 MEASUREMENT OF ^{45}CALCIUM UPTAKE BY RAT BRAIN SLICES

A radiotracer method similar to that described by Cooke and Robinson (1971) was used to determine the effect of various ions, metabolic inhibitors and sCT on calcium uptake by slices of rat brain.

Rats were sacrificed by a blow on the head and exsanguinated, the brains were then rapidly removed and washed in chilled Krebs' solution (0-4°C). The cerebellum and cortex were removed from the brain on an iced Petri dish (following the guidelines of Glowinski and Iverson, 1966). The remaining tissue contains regions such as the hypothalamus and medulla which have been reported to contain a high density of CT binding sites (1.6). Longitudinal slices (0.5 mm) of this tissue (subsequently termed 'midbrain' for simplicity, were prepared on a McIlwain chopper and separated by shaking in 10 ml of Krebs' solution. Tissue slices (approximately 50 mg) were transferred to 2.5 ml of Krebs' solution at 37°C, and gassed with 5% O_2/95% CO_2 for 30 minutes to allow equilibration of the tissue with the incubation medium. A further 2.5 ml of gassed Krebs' solution at 37°C containing $\simeq$0.5 $\mu Ci.ml^{-1}$ $^{45}Ca^{2+}$ was then added.

The incubation was terminated after the appropriate time (2-90 minutes) by collection of the tissue on nylon mesh and washing with 1 ml of chilled Krebs' solution. The tissues were further washed for 30 minutes in 5 ml of iced Krebs' and finally rinsed on nylon with 1 ml of chilled Krebs' solution before blotting dry and placing in preweighed scintillation vials for weighing.

The tissues were solubilised using 0.5 ml of Soluene (BDH) at 50-60°C for 3 hours, and the solution was then neutralised with 1 ml of 0.5 M HCl prior to the addition of 10 ml scintillant (BDH cocktail T). The radioactivity in each of these samples and in 100 µl aliquots of the radiolabelled Krebs' solution was then determined by counting on a Packard scintillation counter (300C).

Several preliminary experiments were carried out in which times of preincubation, incubation and wash were varied. The effect of inclusion of lanthanum in the wash solution was also investigated using a HEPES

buffer throughout to overcome problems of precipitation encountered with the Krebs' solution.

After these preliminary investigations the 30 minute preincubation, 6 minute incubation and 30 minute wash were routinely used in investigation of the effect of various agents on calcium uptake. Drugs under investigation were added in 100 µl Krebs' solution 10 minutes prior to the initiation of the incubation, in experiments using modified Krebs' solutions the ionic composition of the solutions containing the radiolabel was varied to produce the desired incubation medium.

Since the concentrations of both calcium and $^{45}Ca^{2+}$ in the incubation medium are known, the apparent calcium uptake can be calculated from their ratio as follows:

$$\text{Apparent Ca uptake} = \frac{^{45}Ca^{2+}\text{(c.p.m.)/g.wet weight of tissue}}{\frac{^{45}Ca^{2+}\text{(c.p.m.)/ml medium}}{Ca^{2+}\text{mol/ml medium}}}$$

Results are expressed in µmol/g.wet weight

M.9 ASSAY OF CYCLIC ADENOSINE 3',5' MONOPHOSPHATE AND ADENYLATE CYCLASE ACTIVITY IN MOUSE BRAIN

M.9.a Cyclic Adenosine 3'5'-Monophosphate

Cyclic Adenosine 3',5'-Monophosphate (cAMP) was assayed by a protein binding method similar to that described by Brown, Albano, Ekins and Scherzi (1971) using reagents supplied in kit form by Amersham plc. Briefly, the method involves quantitative displacement of a known amount of radiolabelled cAMP from a highly specific binding protein by unlabelled cAMP in the sample. After separation of free and bound nucleotide the amount remaining bound is determined and used to calculate the concentration of nucleotide in the sample.

Groups of 8-10 mice were sacrificed by a blow on the head and immediately immersed in n-pentane, chilled on liquid nitrogen. After decapitation the heads were split longitudinally with an open razor and samples of hypothalamus and cortex were taken. Tissues were then homogenised in 10 ml Tris/EDTA buffer (0.05 M Tris/HCl plus 4 mM EDTA at pH 7.5), 1 ml samples of the homogenate were assayed for protein by the method of Lowry, Rosbrough, Farr and Randall (1951) and 50 µl samples, for cAMP measurement were placed in chilled Eppendorf microfuge tubes maintained on ice at 0-4°C throughout the assay. Standards of 0-16 pmol/50 µl cAMP in Tris/EDTA were also included at this stage. After addition of 50 µl [^{3}H] -cAMP (29 µCi/nmol) and 100 µl binding protein the samples were mixed with a vortex mixer and stored at 0-4°C for 2-3 hours. Separation of free and bound nucleotide was then achieved by addition of 100 µl of chilled charcoal suspension, vortex mixing, and centrifugation (3 minutes at 8000 g). 200 µl samples of the supernatant were then transferred to counting vials containing 4 ml scintillant (BDH cocktail T) and counted on a Packard 300C liquid scintillation counter. Tubes containing 150 µl Tris/EDTA treated as above, except for the the omission of the binding protein were included as blanks.

The radioactivity (c.p.m.) bound in the absence of cAMP was defined as C_o, and that bound in the presence of cAMP as C_x. A plot of C_o/C_x against cAMP concentration of the standards provided a standard curve which was linear in the range of 1-8 pmol cAMP/50 µl. All samples were assayed in duplicate and results are expressed in pmol/mg protein.

Preliminary experiments were performed to determine the relationship between homogenate concentration and cAMP concentration and the efficiency of extraction of cAMP added to tissue homogenates. After these experiments the assay was used in an investigation of the effect of sCT on cAMP concentrations in different regions of the mouse brain

M.9.b Adenylate Cyclase

Adenylate cyclase was assayed by determination of the rate of cAMP accumulation in tissue samples in the presence of a phosphodiesterase inhibitor using methods similar to those described by Albano, Maudsley, Brown and Barnes (1973). Regions of mouse brain obtained and dissected as described above were homogenised in 2.5 ml of aerated 50 mM Tris-HCl buffer at pH 7.4 with the addition of 10 mM NaCl and 10 mM KCl. 1 ml samples were removed and further diluted for protein assay using the method of Lowry et al (1951). 125 µl samples of the homogenate were transferred to Eppendorf microfuge tubes containing 375 µl aerated homogenisation medium with 3 mM $MgCl_2$, 2 mM ATP, 6 mM theophylline and test drug or vehicle in a shaking water bath at 30°C. Production of cAMP was terminated at fixed time intervals after the addition of homogenate (0-20 minutes) by boiling for 3 minutes. Denatured proteins were then precipitated by centrifugation (3 minutes at 8000 g) and 50 µl samples of supernatant were transferred to Eppendorf microfuge tubes, these samples and cAMP standards were stored frozen and then thawed prior to cAMP assay as described above.

Preliminary experiments were carried out to investigate the efficiency of extraction of cAMP concentration and period of incubation. The assay was then used to investigate the possible effects of various agents (GTP, NaF, and sCT) on the rate of cAMP production over a 10 minute incubation period. The rate of cAMP production expressed in pmol cAMP produced/mg protein/ minute is taken as a measure of adenylate cyclase activity.

M.10 ASSAY OF 5-HYDROXYTRYPTAMINE AND 5-HYDROXYINDOLE ACETIC ACID IN MOUSE BRAIN

The concentrations of 5-hydroxytryptamine (5-HT) and 5-hydroxy indole acetic acid (5-HIAA) in mouse brain regions were assayed by the spectrofluorimetric method of Maickel, Cox, Saillant and Miller (1968) as modified by Curzon and Green (1970). In addition, the rate of accumulation of 5-HIAA after treatment with probenecid 200 mg.kg i.p. in 0.3 ml 50 mM phosphate buffer) was determined and used as a measure of 5-HT turnover as described by Neff, Tozer and Brodie (1970).

After sacrifice by a blow on the head, the brains of mice were rapidly removed and dissected on an iced Petri dish into cortex, cerebellum and midbrain following the guidelines of Glowinski and Iverson (1966).

Tissues were weighed and then homogenised with an Ultraturrax homogeniser for 5 seconds in 3.5 ml acidified n-butanol (0.85 ml concentrated HCl added to 1 L n-butanol). After centrifugation for 5 minutes at 500 g 2.5 ml of the supernatant was transferred to a stoppered tube and shaken for 5 minutes with 5 ml n-heptane and 0.4 ml 0.1% cysteine in 0.1 M HCl. The aqueous phase containing 5-HT and the organic phase containing 5-HIAA were then separated by centrifugation for 5 minutes at 500 g and assayed as follows:

a) 5-Hydroxyindole acetic acid

5 ml of the organic phase obtained above was shaken with 0.8 ml 0.5 M phosphate buffer at pH 7.0 for 10 minutes. After centrifugation for 3 minutes at 500 g, 0.4 ml aliquots were transferred to glass tubes (12 x 100 mm), to which 0.04 ml of 1% cysteine solution, 0.8 ml of concentrated HCl, 0.02 ml of 0.1% OPT in methanol and 0.04 ml of 0.2% sodium periodate were added. The order of addition of reagents was strictly adhered to and all tubes were mixed by vortex, immediately after each addition. The tubes were allowed to stand for thirty minutes at room temperature and then placed in a boiling water bath for ten minutes and finally cooled with running water prior to measuring the fluorescence using a Perkin-Elmer 650-40 spectrofluorimeter (excitation 360 nm, emission 470 nm).

b) 5-Hydroxytryptamine

0.2 ml samples of the aqueous phase remaining after the extraction of 5-HIAA were transferred to tubes containing 1.2 ml of 0.004% OPT in 10 M HCl. The tubes were vortexed, boiled for 15 minutes and cooled prior to reading fluorescence at 360 nm excitation and 470 nm emission. Reagent blanks consisting of 0.2 ml, 0.1% cysteine in 0.1 M HCl reacted with the solution of OPT in HCl as above were also prepared.

Several brains were homogenised in n-butanol and pooled, 3.5 ml aliquots of this homogenate received either 5-HT (0.1 or 0.2 µg in 0.2 ml, phosphate buffer), 5-HIAA (0.08 µg in 0.4 ml, phosphate buffer) or an appropriate volume of buffer alone, these provided the internal standards which were included in all experiments.

In experiments where reagent standards (external standards) were also included these were taken through the assay procedures described above instead of extract. Concentrations of 5-HT and 5-HIAA were calculated by reference to the internal standards and expressed as µg/g wet weight of tissue.

Preliminary experiments were carried out to demonstrate emission spectra, extraction efficiency, specificity and dependence of fluorescence on homogenate concentration for both compounds. Having established the protocol, the effects of various treatments on levels of 5-HT and 5-HIAA and 5-HT turnover were investigated.

All cysteine and OPT solutions were prepared fresh on the day of use, 5-HT, 50 µg.ml and 5-HIAA, 20 $\mu g.ml^{-1}$ were stored in deionised water at $-20^{o}C$ and diluted in phosphate buffer for use. Deionised distilled water was used for preparation of all solutions and for the final rinse after washing of the glassware.

M.11 STATISTICS

Experimental design was arranged so that test results could be compared to those of a control group obtained simultaneously, such data was routinely analysed by means of paired Student's 't' tests ($P<0.05$ being accepted as a statistically significant difference). In experiments carried out over more than one day control results may have been grouped to provide a better estimate of the control response if no significant differences were observed between these control groups. Unless otherwise stated, values quoted are $\bar{x} \pm$ s.e., values of 'n' refer to the number of animals in the group.

More detailed analysis of results (analysis of variance and probit analysis) was achieved by linear regression procedures using a generalised linear interactive modelling package (GLIM II) on a DEC 20 computer (Appendix).

RESULTS

The results presented in this thesis were obtained from experiments in which the pharmacological, behavioural and biochemical effects of peptides and drugs were assessed. A wide range of methods have been employed drawing techniques from several biological disciplines, it has therefore been considered appropriate to provide discussion of the results obtained within each chapter of the results section. A more general discussion of the possible relationships between these results and their relevance to the understanding of the pharmacology of CT is included as a separate discussion section (D1-D5).

R.1 THE ABDOMINAL CONSTRICTION TEST

Introduction

The alleviation of pain associated with administration of CT to patients suffering from Paget's disease is well established (Deftos and First, 1981). It was this observation that prompted Pecile, et al (1975) to investigate the possible antinociceptive effect of the hormone. Since 1975, several reports have been published on the antinociceptive and analgesic effects of CT in various species (I.8.f). A large part of the work presented in this thesis involves investigation of the mechanisms by which antinociception is produced by central administration of CT. The acetic acid induced abdominal constriction test described by Collier et al, 1968 has been used throughout in conjunction with hole board and rotating drum tests for assessment of investigative and locomotor behaviour (File and Wardill, 1975; Dunham and Miya, 1957). Although the mechanisms involved in production of the abdominal constriction response by peritoneal irritants remain unclear, the method was considered most suitable for the present investigations for the following reasons:

i) Collier et al, 1968 have found the test to be an excellent indicator of analgesic potency in man by correlating the rank order of potency of known analgesics in man with their potency in inhibition of abdominal constrictions.

ii) Peritoneal irritants primarily activate deep nociceptors. This may be relevant to the antinociceptive effect of CT in Paget's disease in which deep pain occurs as distinct from the more superficial pain arising from surface tissues.

iii) The abdominal constriction rate can be reduced in a dose dependent manner by narcotic and non-narcotic analgesics (Collier et al, 1968).

iv) The test enables acquisition of quantitative data (rate of constriction) from large numbers of animals.

Abdominal constrictions can be produced by i.p. injection of numerous peritoneal irritants including acetic acid, acetylcholine, phenylquinone, arachidonic acid, bradykinin, histamine and potassium chloride. The first three members of this list appear to have been used most commonly in the screening of analgesics. Acetic acid was used as the peritoneal irritant throughout the experiments described below largely because a relatively constant and reproducible constriction response can be obtained within 10 minutes of administration.

A major disadvantage of this test is that increased permeability of blood vessels can accompany peritoneal irritation and abdominal constrictions, anti-inflammatory drugs may therefore inhibit abdominal constrictions by preventing the alteration in vessel permeability (Collier et al, 1968). False positive results can also be obtained with drugs which impair motor performance inhibiting the abdominal constrictions without producing antinociception. These problems can be minimised by screening potential antinociceptive drugs in a range of antinociceptive tests in parallel with tests for modification of motor performance. Hendershot and Forsaith (1958) reported that mice from one particular source gave an inconsistant and infrequent constriction response to phenylquinone leading to erratic and non reproducible results. Preliminary investigations were therefore carried out to establish the sensitivity of the test to the effects of known antinociceptive drugs and to sCT.

Results

Intraperitoneal injection of 0.3ml, 1% acetic acid in 0.9%NaCl produced the characteristic abdominal constriction response described above (M.5) the time course of which is shown in figure R.1. Abdominal constrictions were observed within 3 minutes of administration of irritant, the constriction rate was maximal after 7 minutes and declined gradually thereafter. Over the period 10-20 minutes after administration of irritant the constriction rate was relatively constant and reproducible, the 10th-15th minute after injection was, therefore, chosen as the earliest suitable period for counting of constrictions in all subsequent experiments.

Administration of acetic acid (0.01-5.0%, in 0.3 ml, 0.9% NaCl, i.p. ≃ 1-500 $mg.kg^{-1}$) produced a dose dependent abdominal constriction response (figure R.2) and the 1% solution was selected for use in all remaining experiments, allowing considerable range for modification by antinociceptive or hyperalgesic drugs.

Statistical analysis (analysis of variance on GLIM II) was carried out on data obtained over the period June 1982-June 1983 to determine whether circadian or circannual rhythms in pain sensitivity might modify the constriction response in the pooled control (vehicle treated) animals. This analysis revealed a pooled response of 3.5 ± 1.0 (mean ± s.d) constrictions per minute (c.p.m.), from 94 animals and no significant evidence for modification of this response with time of day or month of year was obtained (table 1). Similarly, no significant evidence was obtained for dependence of the response on sex of animals, ambient temperature or route of vehicle administration.

Inhibition of acetic acid induced abdominal constrictions was assessed 20 minutes after administration of a small range of established antinociceptive drugs which were used as positive controls. Acetyl salicylic acid, an antipyretic analgesic with peripheral and central actions (Atkinson and Collier, 1980), at s.c. doses of 0.25, 0.5 and 1.0 $mmol.kg^{-1}$, reduced the constriction rate 26.0 ± 10.0; 38.0 ± 11.0 and 43.0 ± 8.0% respectively (figure R.3). The ED_{50} of 0.2 $mmol.kg^{-1}$ is in relatively good agreement with that reported for the inhibition of acetylcholine induced abdominal constrictions by soluble aspirin (0.44 $mmol.kg^{-1}$, Bates et al, 1981a).

Similarly, i.c.v. administration of 1 and 10 μg of 5-HT reduced the constriction rate by 32.3 ± 10.0 and 56.7 ± 10.4% respectively, (figure R.4). The antinociceptive effect of centrally administered 5-HT has also been demonstrated in the rat 'hot plate' and 'tail flick' tests (Post, Fasmer, Holm and Ogren, 1984).

d-ala-d-leu enkephalin (DADLE), a metabolically protected form of leu-enkephalin with selective δ agonist activity (Ward and Takemori, 1982), produced marked dose dependent reduction in the rate of

abdominal constrictions when administered centrally (figure R.5). Doses of 5, 10 and 50 pmol/mouse, administered i.c.v. 15 minutes prior to the start of the abdominal constriction count reduced the constriction rate by 52.0 ± 6.5, 78.7 ± 6.8 and 96.7 ± 2.3% respectively. The ED_{50} of 4.3 pmol/mouse (95% confidence intervals 3.5-5.3 pmol/mouse ≃ 117-177 pmol.kg^{-1}) is in agreement with the results of Ward and Takemori (1982) who obtained an ED_{50} of 6 pmol/mouse for inhibition of acetic acid induced abdominal constrictions 10 minutes after administration. Vaught, Rothman and Westfall (1982) have suggested that certain types of δ receptor interaction can activate μ receptors this is supported by the fact that Ward and Takemori, (1982) demonstrated antagonism of DADLE (δ agonist) antinociception in the abdominal constriction test by β-funaltrexamine a highly selective μ antagonist. The demonstrated inhibition of abdominal constrictions by DADLE does not therefore conflict with the view of Tyers, (1980) that opiate antinociception in the abdominal constriction test is mediated by μ and/or κ receptors.

Intracerebroventricular sCT (figure R.6) at doses of 0.1, 1 and 10 U.kg^{-1} (≃ 6, 60 and 6000 pmol kg^{-1}) reduced the rate of abdominal constrictions by 23.6 ± 7.6, 38.1 ± 4.8 and 41.3 ± 7.8% respectively. Similar results were reported by Bates et al (1981a) who used the same test procedure.

The maximal doses of the drugs tested were without significant effect on locomotor or investigative behaviour (table 2). DADLE at 100 pmol/mouse completely abolished the abdominal constriction response but has been excluded from figure R.5 because investigative behaviour was significantly impaired (table 2).

Discussion

In this chapter results of preliminary experiments designed for determination of suitable procedures for antinociceptive testing have been presented. Counting of abdominal constrictions in mice from the 10th-15th minute after administration of 0.3 m., 1% acetic acid i.p. in mice was selected as a routine protocol for subsequent tests.

This procedure provides a relatively reproducible constriction rate with an adequate range for modification by antinociceptive or hyperalgesic drugs. In the majority of cases the protocol also provides time for animals to recover from the stress of injection of test substance prior to administration of peritoneal irritant. The observation of a relatively constant rate of abdominal constrictions as early as 10-15 minutes after injection of peritoneal irritant is advantageous since it enables the duration of the experiments and hence duration of stress to experimental animals to be minimised.

The abdominal constriction rate was found to be sensitive to modification by centrally acting narcotic and non-narcotic analgesic, none of which produced motor discoordination at the doses shown in the figures. These results indicate the value of the test for detection of drug induced antinociception.

Figure R.1 Time Course of the Acetic Acid Induced Abdominal Constriction Response. Values are $\bar{x} \pm$ s.e., n = 6

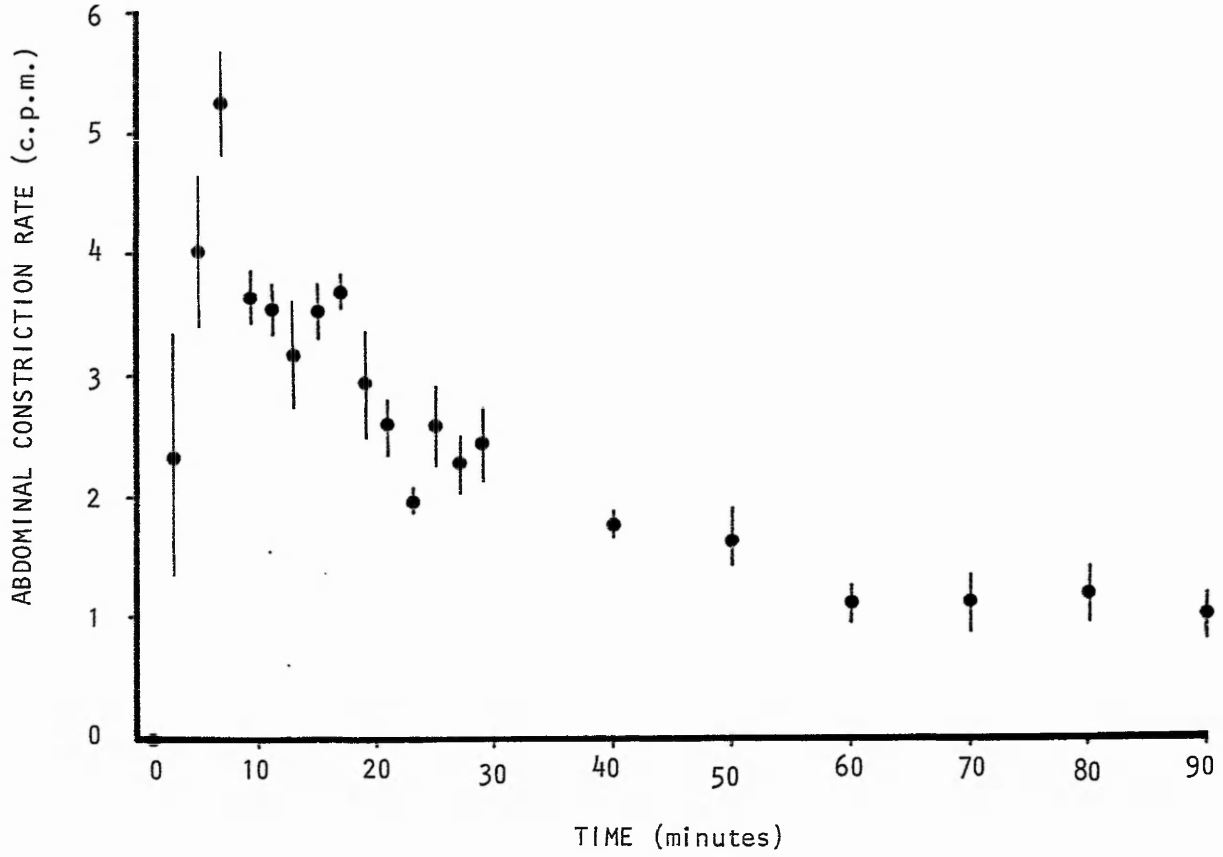

ABDOMINAL CONSTRICTION RATE (c.p.m.)
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
TIME (minutes)

Figure R.2 Relationship between Dose of Acetic Acid and Abdominal Constriction Rate.

Acetic acid was injected i.p. in 0.3 ml, 0.9% NaCl and abdominal constrictions were counted 10-15 minutes thereafter. Values are $\bar{x} \pm$ s.e., values of n are shown.

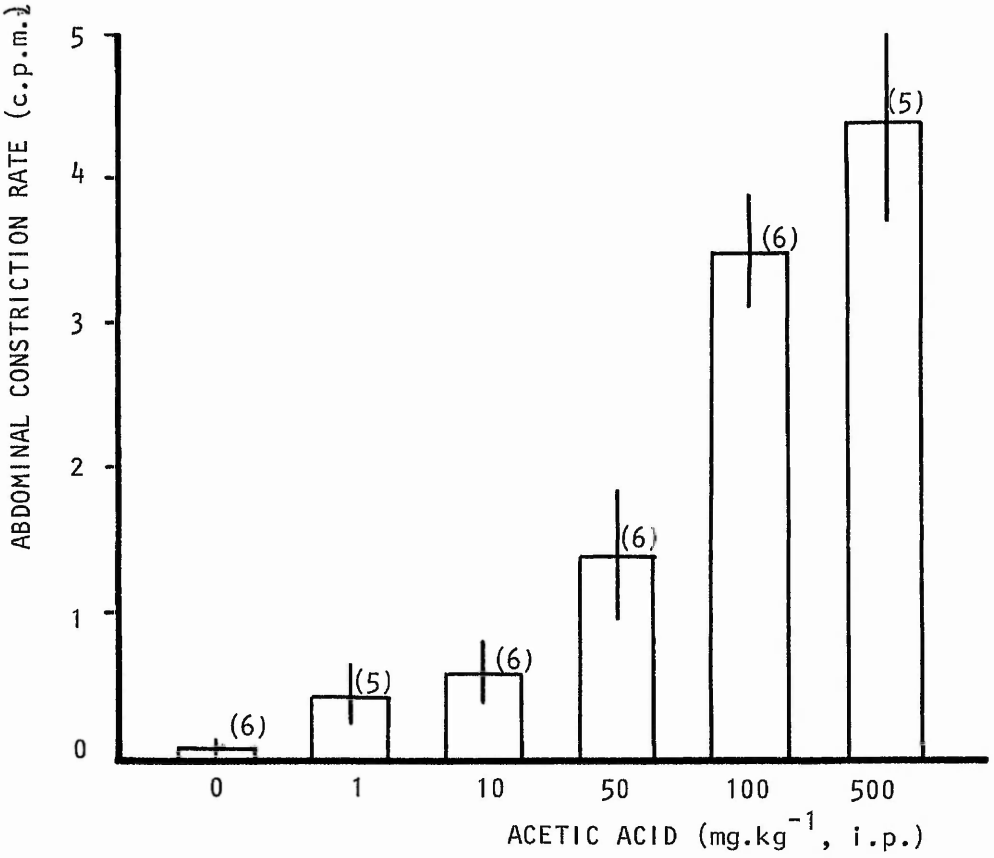

ABDOMINAL CONSTRICTION RATE (c.p.m.)
5
4
3
2
1
0
(6)
(5)
(6)
(6)
(6)
(5)
0
1
10
50
100
500
ACETIC ACID (mg.kg^{-1}, i.p.)

Table 1 Results of analysis of variance to determine possible effects of sex, time of day, ambient temperature and month on control abdominal contriction response

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	'F'	Significant (5%) 'F' value
Sex	59	1	59	1.37	3.95
Time	155	10	15	0.36	1.95
Temperature	101	5	20	0.47	2.34
Month	354	6	59	1.37	2.23
Residual	3054	71	43		
Total	3723	93			

Data was obtained by pooling control abdominal constriction rates (assessed 10-15 minutes after i.p. administration of 0.3 ml, 1% acetic acid) obtained over a period of one year (June 1982-June 1983). Analysis of variance (by linear regression) reveals no significant evidence for effect of variables tested in the control abdominal constriction rate.

Figure R.3 Effect of Acetyl Salicylic Acid on the Rate of Acetic Acid Induced Abdominal Constrictions.

Salicylic acid was administered s.c. in 0.1 ml, 0.9% NaCl 20 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e. values of n are shown.

* $P < 0.5$; ** $P < 0.01$

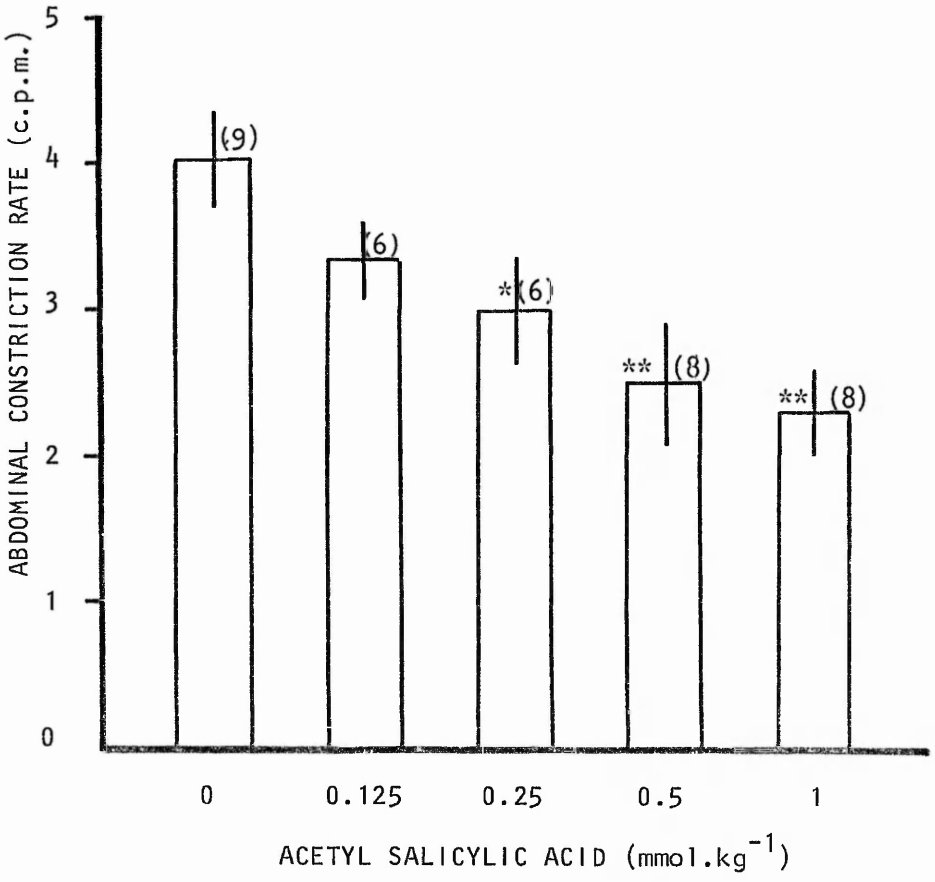

ABDOMINAL CONSTRICTION RATE (c.p.m.)
5
4
3
2
1
0
(9)
(6)
*(6)
** (8)
** (8)
0
0.125
0.25
0.5
1
ACETYL SALICYLIC ACID (mmol.kg^{-1})

Figure R.4 Effect of 5-HT on the Rate of Acetic Acid Induced Abdominal Constriction.

5-Hydroxytryptamine was administered i.c.v. in a volume of 10 µl 20 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e., n = 10.

* P < 0.05; ** P < 0.01

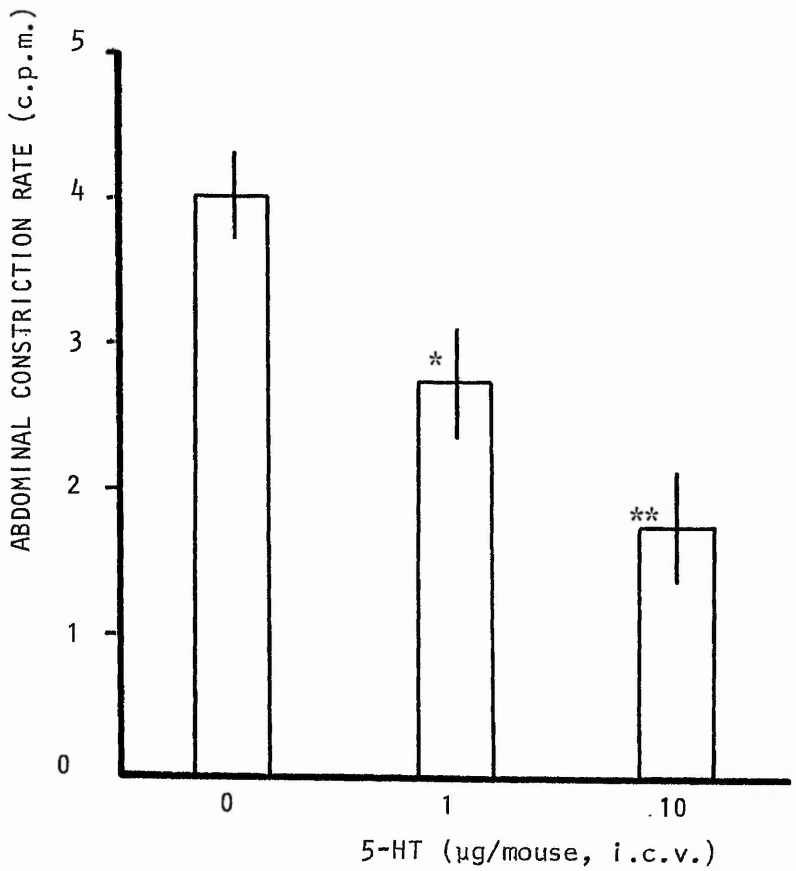

ABDOMINAL CONSTRICTION RATE (c.p.m.)
5
4
3
2
1
0
*
**
0
1
10
5-HT (µg/mouse, i.c.v.)

Figure R.5 Effect of DADLE on the Rate of Acetic Acid Induced Abdominal Constrictions.

d-ala-d-leu enkephalin was administered i.c.v. in a volume of 10 µl 15 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e. values of n are shown.

* $P < 0.1$

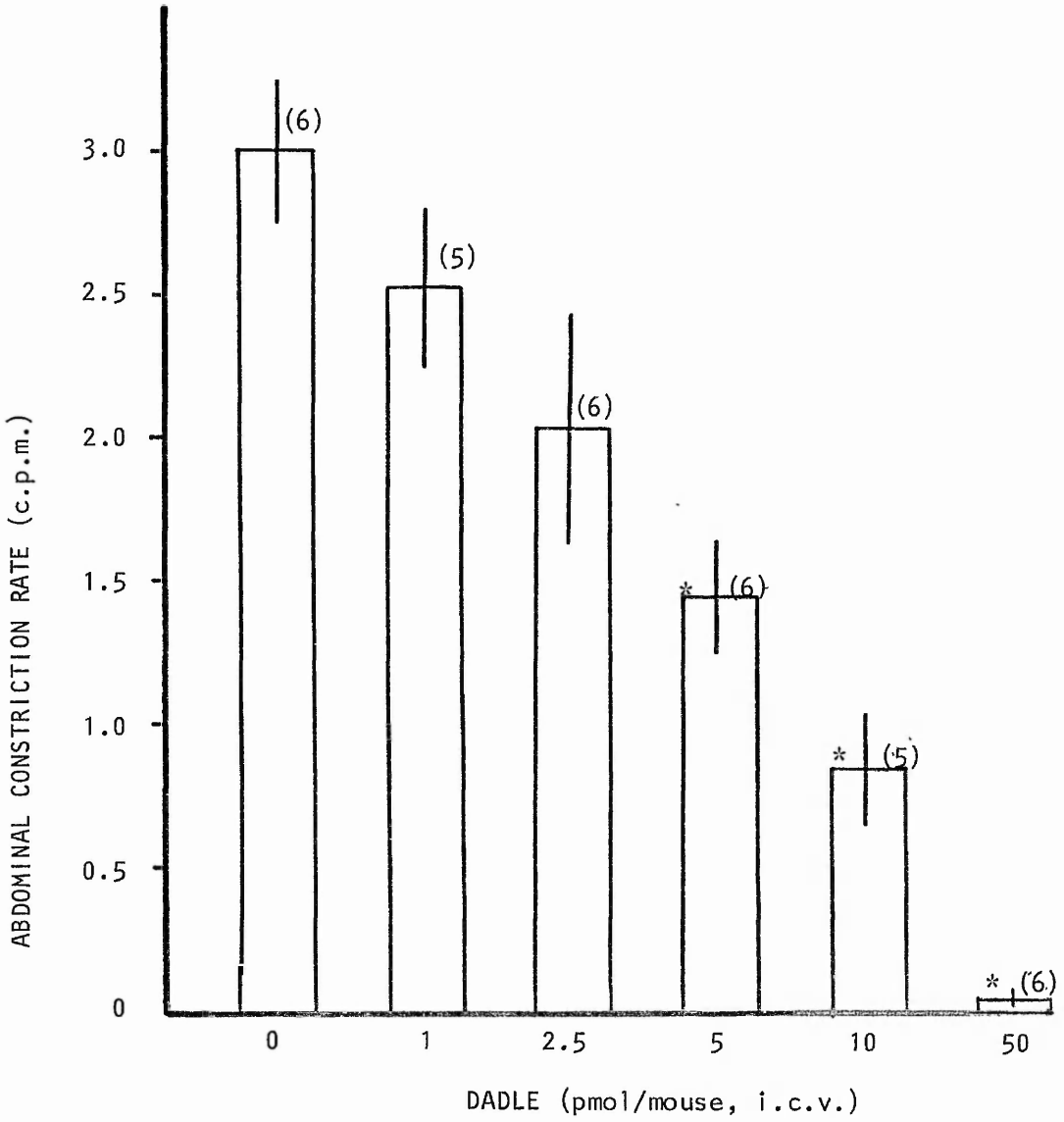

ABDOMINAL CONSTRICTION RATE (c.p.m.)
3.0
2.5
2.0
1.5
1.0
0.5
0
(6)
(5)
(6)
*
(6)
*
(5)
*
(6)
0
1
2.5
5
10
50
DADLE (pmol/mouse, i.c.v.)

Figure R.6 Effect of sCT on the Rate of Acetic Acid Induced Abdominal Constrictions.

Salmon CT was administered i.c.v. in a volume of 10 µl 20 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e. values of n are shown.

* $P < 0.05$

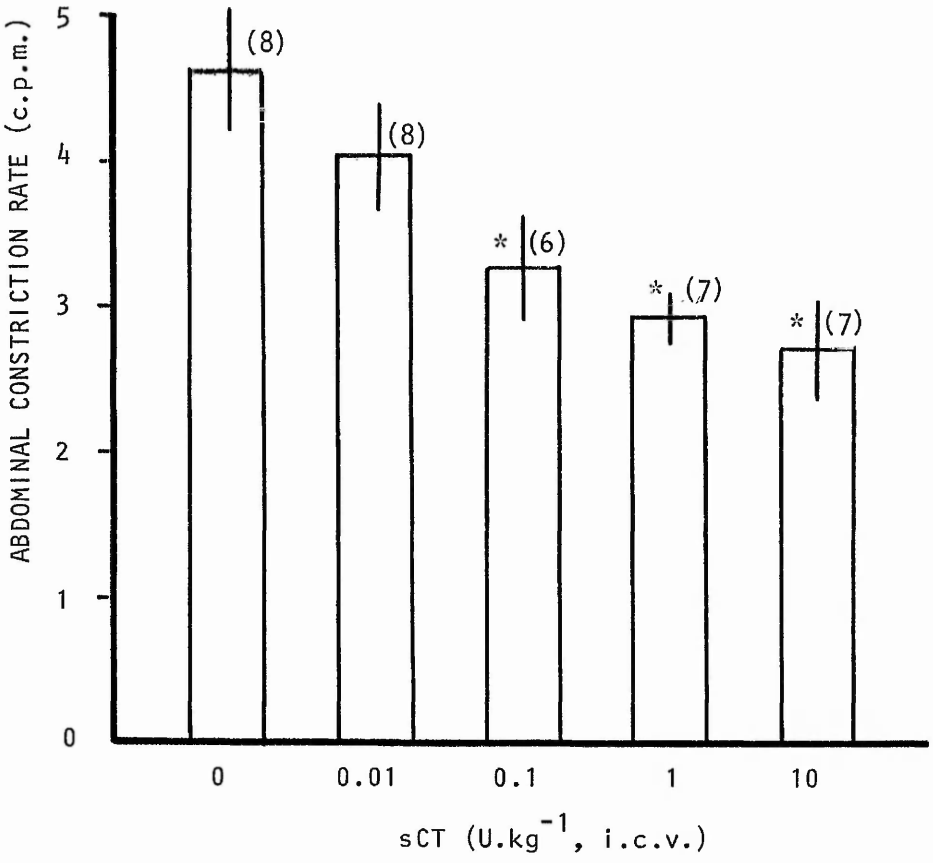

ABDOMINAL CONSTRICTION RATE (c.p.m.)
5
4
3
2
1
0
(8)
(8)
* (6)
* (7)
* (7)
0
0.01
0.1
1
10
sCT (U.kg^{-1}, i.c.v.)

Table 2 Effect of Various Antinociceptive Drugs on Mouse Locomotor Activity

	Motor Discoordination (falls/5 mins)	Investigative Behaviour (head dips/5 mins)
Vehicle, i.c.v.	1.0 ± 0.3 (14)	16.3 ± 1.9 (12)
5-HT, 1 µg/mouse i.c.v.	0.5 ± 0.2 (8)	21.7 ± 2.0 (8)
5-HT, 10 µg/mouse i.c.v.	1.6 ± 0.6 (7)	12.7 ± 2.1 (7)
DADLE, 50 pmol/mouse i.c.v.	2.2 ± 0.9 (6)	15.6 ± 0.7 (5)
DADLE, 100 pmol/mouse i.c.v.	2.2 ± 0.9 (6)	9.7 ± 2.7 (6)*
sCT, 2 $U.kg^{-1}$ i.c.v.	1.1 ± 0.7 (6)	18.4 ± 1.5 (7)
Vehicle, s.c.	1.2 ± 0.4 (6)	20.2 ± 2.0 (6)
Acetyl salicylic acid $0.5\ nmol.kg^{-1}$ s.c.	1.9 ± 0.3 (6)	16.0 ± 1.7 (6)
Acetyl salicylic acid $1.0\ nmol.kg^{-1}$ s.c.	1.8 ± 0.4 (6)	16.5 ± 1.8 (6)

Locomotor activity was assessed 20-30 minutes after administration of drugs or vehicle. Values are $\bar{x}$ ± s.e. (n), * $P < 0.05$

R.2 CALCITONIN ANTINOCICEPTION AND CENTRAL MONOAMINES

Introduction

The antinociceptive effects of several forms of CT have been demonstrated in various pharmacological screens (I.8.f). Although precise mechanisms and sites of action have yet to be elucidated it is apparent that the antinociception produced by opiates and CT is in many respects similar, hence :

i) both opiate and CT induced antinociception is thought to involve neuromodulatory action in the CNS;

ii) Yamamoto et al (1980) demonstrated that pCT and morphine inhibited potentials in the sensory cortex evoked by painful stimuli in a similar manner (I.8.f);

iii) the antinociceptive effects of both opiates and CT can be antagonised by central administration of calcium ions and ionophores, or potentiated by calcium antagonists and chelators (I.8.f).

However, several significant differences between CT and opiate induced antinociception have been reported, these include the lack of tolerance with repeated administration of CT (Braga et al, 1978) a tentative report of a lack of cross tolerance between morphine and CT (personal communication from Miki, cited by Yamamoto et al, 1979) and the lack of effect of opiate antagonists on CT antinociception at doses which antagonise morphine antinociception (Braga et al, 1978; Yamamoto et al, 1978; Bates et al, 1981b). Although CT clearly does not possess central opiate agonist activity the observed similarities may reflect action on common neuronal systems as demonstrated by Yamamoto et al (1980).

There is, at present, very little evidence available concerning the neuronal systems involved in CT antinociception. In contrast the literature detailing the relationships between opiate antinociception and central neurotransmitter systems is extensive, reflecting intensive

research carried out prior to characterisation of endogenous opiates (Hughes, Smith, Kosterlitz, Fothergill, Morgan and Morris, 1975). This literature abounds with contradictory experimental results which may reflect the action of opiates on several interacting neurotransmitter systems and the involvement of different systems in the response to varying painful stimuli (for review see Loh and Ross, 1979). However, it has been shown in vitro that opiates reduce the overflow of radiolabelled dopamine, noradrenaline and acetylcholine elicited by stimulation of slices of rodent brain either electrically or with depolarising concentrations of potassium (Celsen and Kuschinsky, 1974; Montel, Starke and Taube, 1975; Sharkawi and Shullman, 1969). Experiments in vivo have established that acute administration of morphine can increase the rate of turnover of noradrenaline, dopamine and 5-HT in the CNS of rats or mice in a naloxone reversible manner (Smith, Sheldon, Bedrorczyk and Villarreal, 1972; Goodlet and Sugrue, 1974) and that depletion of central catecholamines with α-methyl-p-tyrosine (α-MT) or central 5-HT with p-chlorophenylalanine (PCPA) can antagonise opiate induced antinociception. (Verri, Graeff and Corrado, 1967; Tennen, 1968).

In light of the demonstration by Yamamoto et al (1980) that opiates and CT can modulate common neuronal systems it is of interest to determine whether pharmacological modification of central monoamine systems (known to be involved in opiate antinociception) affects antinociception produced by sCT in the mouse abdominal constriction test.

Results

i) Effect of Methysergide on sCT Antinociception

Salmon CT at a dose of 2 $U.kg^{-1}$, i.c.v. produced a 45.4 ± 8.5% ($n = 7$) reduction in the abdominal constriction rate of mice pretreated with 0.1 ml, 0.9% NaCl, i.p. 60 minutes prior to counting of abdominal constrictions, whereas after methysergide (5 $mg.kg^{-1}$ in 0.9% NaCl) there was no statistically significant effect (figure R.7). Analysis of variance (ANOVA) revealed no evidence for an interaction between sCT and methysergide (see Appendix and figure R.7 subscript).

ii) Effect of PCPA on sCT antinociception

The abdominal constriction rate of 4.1 ± 0.2 cpm (n = 24) after pretreatment with PCPA (300 $mg.kg^{-1}$, s.c.) 72, 48 and 24 hours prior to test was not significantly greater than the rate observed after vehicle alone (3.98 ± 0.26 c.p.m., n = 23) nor were any overt behavioural effects of PCPA pretreatment noted. The antinociceptive effects of sCT were reduced by pretreatment with PCPA so that the effect of sCT was only significant at a dose of 10 $U.kg^{-1}$, whereas doses of 0.1, 1, 2, 10 and 50 $U.kg^{-1}$ produced antinociception after pretreatment with vehicle (figure R.8).

iii) Effect of PCPA on 5-HT Antinociception

Intracerebroventricular 5-HT (1, 5 and 10 µg/mouse) produced a similar dose dependent inhibition of the abdominal constriction rate after both PCPA (control 3.9 ± 0.4 c.p.m., n = 9) and vehicle (control 4.0 ± 0.4 c.p.m., n = 9), figure R.9.

iv) Effect of PCPA on Acetyl Salicylic Acid Antinociception

Acetyl salicylic acid at doses of 0.25-1.0 $mmol.kg^{-1}$, s.c., produced a dose dependent inhibition of the abdominal constriction rate after both PCPA (Control 3.8 ± 0.4 c.p.m., n = 10) and vehicle (control 3.5 ± 0.3 c.p.m., n = 9), figure R.10.

v) Effect of PCPA on d-ala-leu-enkephalin (DADLE) Antinociception

After vehicle pretreatment 5 pmol of DADLE, i.c.v., reduced the abdominal constriction rate by 44.0 ± 6.3% (n = 11). Whereas after PCPA pretreatment the inhibitory effect of DADLE did not attain statistical significance, figure R.11. Moreover, ANOVA revealed significant evidence for interaction (statistical) between PCPA and DADLE (see figure R.11 subscript).

vi) Effect of PCPA on the Antinociceptive Effects of Combinations of 5-HT and sCT

The abdominal constriction rate observed in animals receiving s.c. and i.c.v. vehicles alone was reduced in a dose dependent manner by 5-HT (figure R.12) as previously shown. After pretreatment with peripheral vehicle, sCT at a dose of 2 $U.kg^{-1}$ i.c.v. inhibited the constriction rate irrespective of the dose of 5-HT simultaneously administered 39.9 ± 9.5% with 0 µg; 40.0 ± 6.0% with 1 µg; 69.0 ± 9.2% with 10 µg 5-HT/mouse). Whereas the antinociceptive effect of sCT alone was antagonised by PCPA pretreatment, the effect of sCT in combination with 1 or 10 µg of 5-HT was unaffected by PCPA. Finally, the antinociceptive effect of the combination of 2. $U.kg^{-1}$ sCT and 10 µg 5-HT, i.c.v. was found to be greater than that of either treatment alone (figure R.13).

vii) Effect of α-methyl p-tyrosine (α-MT) on sCT Antinociception

Salmon-CT at doses of 0.1, 1 and 10 $U.kg^{-1}$, i.c.v. inhibited the constriction rate after pretreatment with either α-MT (Control 2.4 ± 0.2 c.p.m., n = 20) and vehicle (control 2.8 ± 0.2 c.p.m., n = 18), figure R.14.

viii) Effect of Pharmacological Modification on Monoamine Metabolism on Motor Activity

Locomotor activity and investigative behaviour were not significantly modified by any of the drugs used above(table 3). Reserpine (1 $mg.kg^{-1}$, s.c. in 0.1ml, 1% ascorbic acid, 8 hours prior to experimental use) which produces widespread depletion of central monoamines was not used in abdominal constriction tests because it was found to reduce investigative behaviour and motor coordination. Reserpine has therefore been included in table 3 as a positive control only.

Discussion

Several groups have found morphine antinociception to be antagonised by depletion of central monamines with reserpine (Verri et al, 1968; Fennessy and Lee, 1970). Fennessy and Lee (1970), for example, found that reserpine (1 $mg.kg^{-1}$) increased the ED_{50} of morphine in the phenylquinone writhing test between 2 and 24 hours after treatment. However, as reserpine at this dose was found to impair locomotor behaviour, alternative means of depletion of central monoamines were sought. PCPA in a dose regime that has been shown, by Koe and Weissman (1966), to inhibit tryptophan hydroxylase selectively, was found to reduce the concentration of 5-HT within the CNS (0.98 $\pm$ 0.05 g/g wet weight) by 47.7 $\pm$ 2.3%, n = 6, without significantly modifying the control abdominal constriction rate or locomotor behaviour. Similarly, α-MT which has been shown to inhibit dopa decarboxylase selectively (Spector et al, 1965) reduced CNS noradrenaline 56.0 $\pm$ 14.4%, from the control value of 0.48 $\pm$ 0.04μg/g wet weight (n = 4) and was without effect on both control constriction rate and locomotor activity, although the animals did appear somewhat sedated. The lack of effect of PCPA, α-MT and methysergide on control constriction rate indicates that adrenergic and tryptaminergic neurones are not essential to the response although the possibility of effects at higher doses cannot be discounted.

Pretreatment with α-MT produced no statistically significant antagonism of sCT antinociception, indicating that adrenergic mechanisms do not play a major role in this effect when acetic acid is used as the noxious stimulus. Similarly, Fennessy and Lee, 1970, have demonstrated that α-MT, at the dose employed above, does not modify morphine antinociception in the phenylquinone induced writhing test, and Clementi, Prato, Conforto and Scapagini (1984) were unable to modify sCT antinociception in the rat hot plate test by pretreatment with the α adrenoreceptor antagonist phentolamine.

Depletion of 5-HT with PCPA antagonised the antinociceptive effects of both sCT and DADLE but was without effect on salicylate or 5-HT antinociception. The latter two drugs can be considered as negative controls since neither cycloxygenase inhibition or interaction of 5-HT with its receptors would be expected to be antagonised by 5-HT depletion. As mentioned above (R.1) the antinociceptive effect of DADLE (a δ agonist) in the abdominal constriction test is considered by Ward and Takemori, (1982) to be mediated by μ receptors. Antagonism of DADLE antinociception by PCPA is, therefore, in agreement with reports of antagonism of morphine (the prototype μ agonist) antinociception with PCPA (Tennen, 1968, Fennesy and Lee, 1970). The dependence of sCT antinociception on tryptaminergic mechanisms is supported by the demonstration that the effect of PCPA is not observed when sCT and 5-HT (which presumably replaces depleted stores) are administered simultaneously.

Fennessy and Lee (1970) were able to antagonise morphine antinociception in the phenylquinone writhing test with PCPA but observed no such antagonism with methysergide. Similarly, no convincing evidence for methysergide induced antagonism of sCT antinociception was obtained above. Methysergide may block both post-synaptic 5-HT receptors and the presynaptic receptors which are necessary for feedback inhibition of 5-HT release. Interpretation of these results is therefore difficult because the net effect of facilitated 5-HT release and post-synaptic blockade is not known. To further confound the situation Clementi et al (1984) have reported that methysergide antagonises the antinociceptive effect of sCT in the rat hot-plate test. Although this report supports the overall conclusion that central tryptaminergic systems are involved in CT antinociception, it is clear that rational interpretation of these results awaits a more detailed knowledge of the effects of methysergide on the tryptaminergic systems involved in the models.

Yamamoto et al (1980) have directly demonstrated the neuromodulatory action of pCT and morphine on common nociceptive pathways. Similarly, the data above demonstrates the importance of tryptaminergic systems to the antinociceptive effect of both sCT and DADLE. The observed similarities between opiate and CT induced antinociception might therefore reflect, at least in part,

a neuromodulatory action on common tryptaminergic neurones. It is known that stimulation of tryptaminergic neurones in various Raphé nuclei known to be rich in 5-HT containing cell bodies can produce antinociception (Besson, Oliveras, Chaouch and Rivot, 1981). Since these neuronal systems are found in areas rich in CT-like immunoreactivity and CT binding sites, (I.6) CT antinociception could involve stimulation of these tryptaminergic neurones, and the consequent modification of 5-HT turnover could underlie the CT induced increase in 5-HT observed by Nakhla and Majumdar (1978) and Dupuy, Peuchant, Vitiello, Jensen, Baghdiantz and Blanquet (1983).

Figure R.7 Lack of Effect of Methysergide on sCT Antinociception

Salmon CT (2 $U.kg^{-1}$) was adminstered, to animals which had been pretreated with methysergide (5 $mg.kg^{-1}$ i.p. 60 minutes prior to sCT) or vehicle, 20 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$ (comparison of sCT treated animals to those receiving i.c.v. vehicle and appropriate pretreatment). ANOVA revealed significant modification of the constriction rate by sCT ($P < 0.05$) but no evidence was obtained for an effect of methysergide ($P > 0.1$), or of sCT-methysergide interaction ($P > 0.1$), on the constriction rate.

(See appendix for statistical methods).

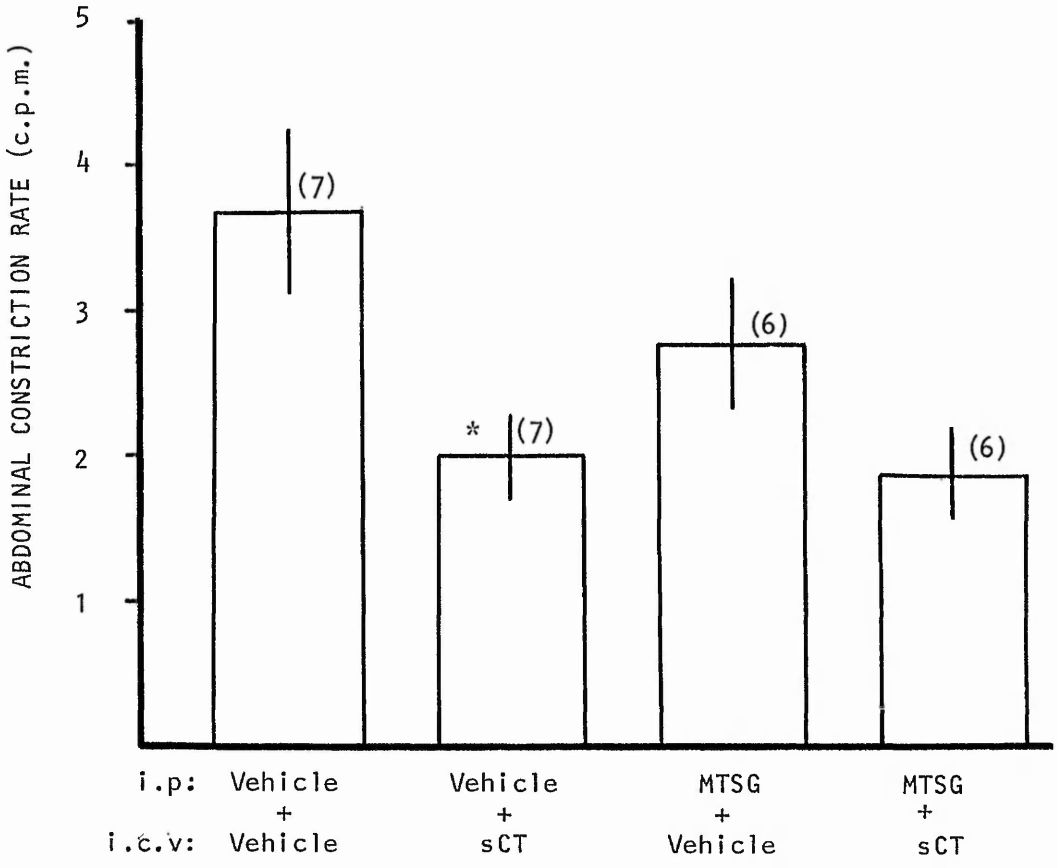

ABDOMINAL CONSTRICTION RATE (c.p.m.)
5
4
3
2
1
(7)
*
(7)
(6)
(6)
i.p: Vehicle + Vehicle
Vehicle + sCT
MTSG + Vehicle
MTSG + sCT
i.c.v:

Figure R.8 Antagonism of sCT Antinociception by PCPA Pretreatment.

Animals pretreated with PCPA (300 $mg.kg^{-1}$) or vehicle, 72, 48 and 24 hours prior to experimental use, received sCT or vehicle i.c.v. 20 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$; ** $P < 0.01$ (compared to control group with appropriate pretreatment).

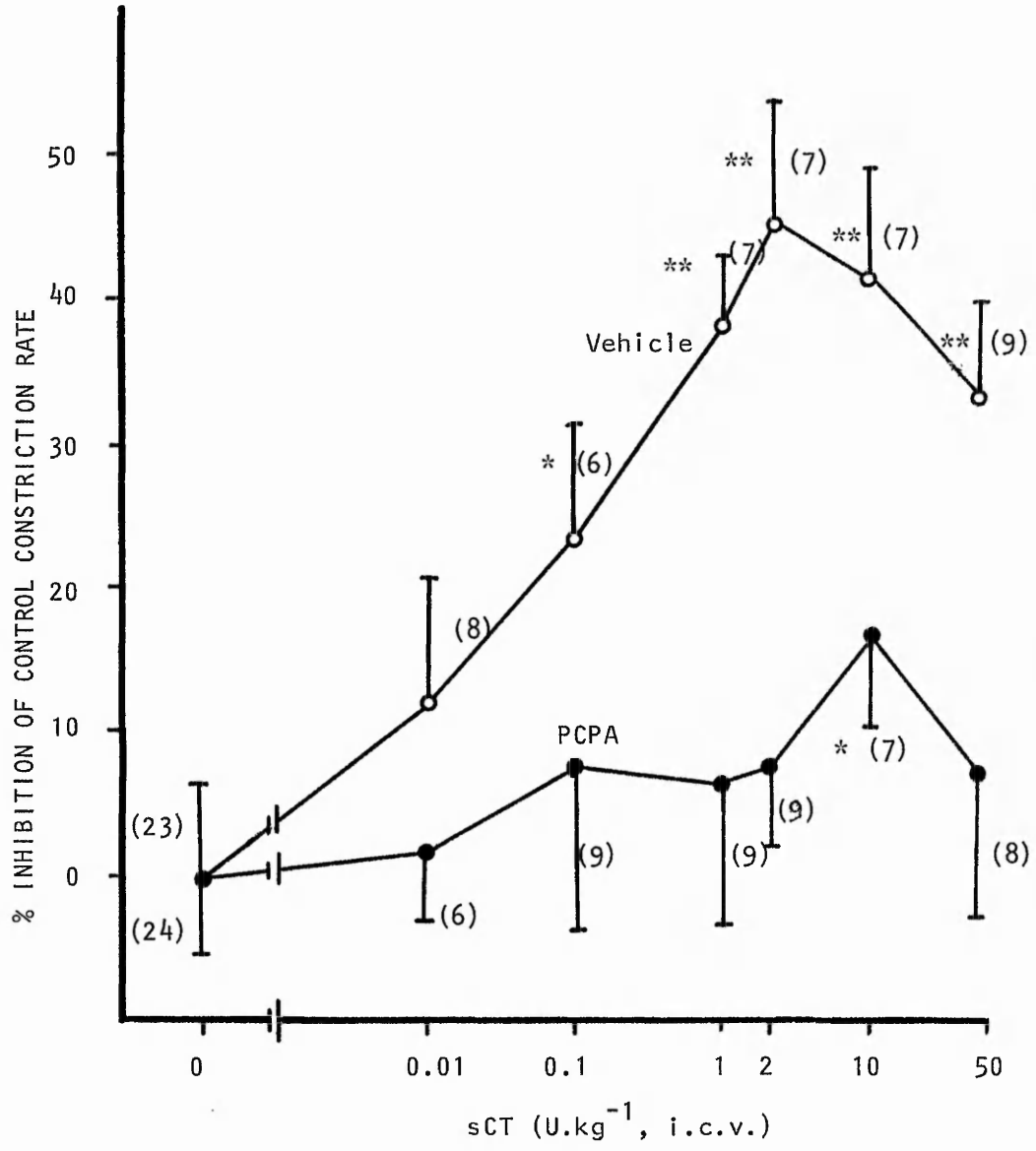

% INHIBITION OF CONTROL CONSTRICTION RATE
50
40
30
20
10
0
Vehicle
PCPA
**
(7)
**
(7)
**
(7)
**
(9)
*
(6)
(8)
(23)
(24)
(6)
(9)
(9)
(9)
*
(7)
(8)
0
0.01
0.1
1
2
10
50
sCT (U.kg^{-1}, i.c.v.)

Figure R.9 Lack of Effect of PCPA on 5-HT Antinociception.

Animals pretreated with PCPA or vehicle received 5-HT (or i.c.v. vehicle) i.c.v. 20 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e., values for n are shown.

* $P < 0.05$; ** $P < 0.01$ (compared to control group with appropriate pretreatment).

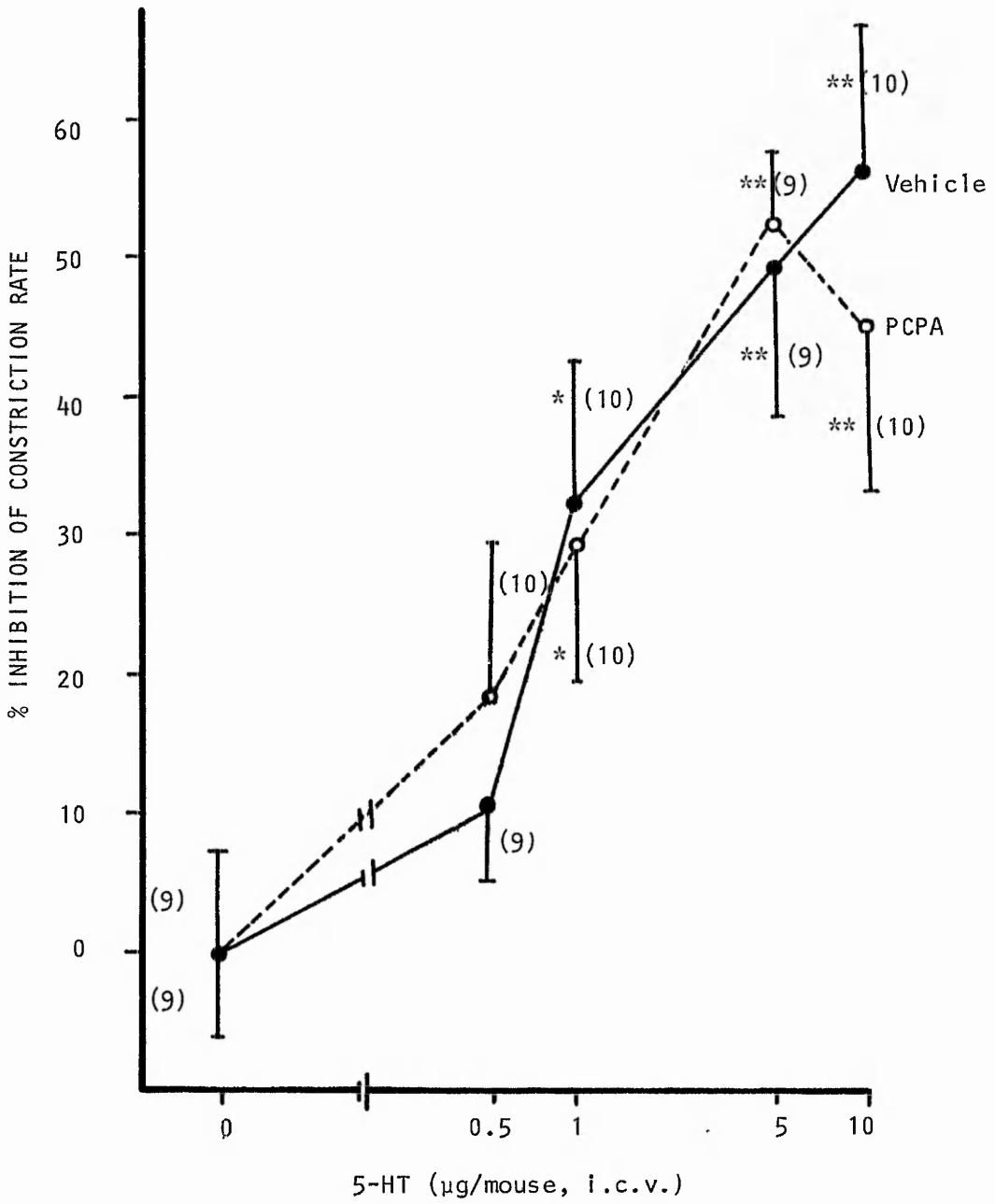

% INHIBITION OF CONSTRICTION RATE
60
50
40
30
20
10
0
(9)
(9)
(10)
(9)
* (10)
* (10)
** (10)
** (9)
** (9)
** (10)
Vehicle
PCPA
0
0.5
1
5
10
5-HT (μg/mouse, i.c.v.)

Figure R.10 Lack of Effect of PCPA on Acetyl Salicylic Acid Antinociception.

Animals pretreated with PCPA or vehicle received salicylate (or vehicle) s.c. 20 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$; ** $P < 0.01$ (compared to control group receiving appropriate pretreatment).

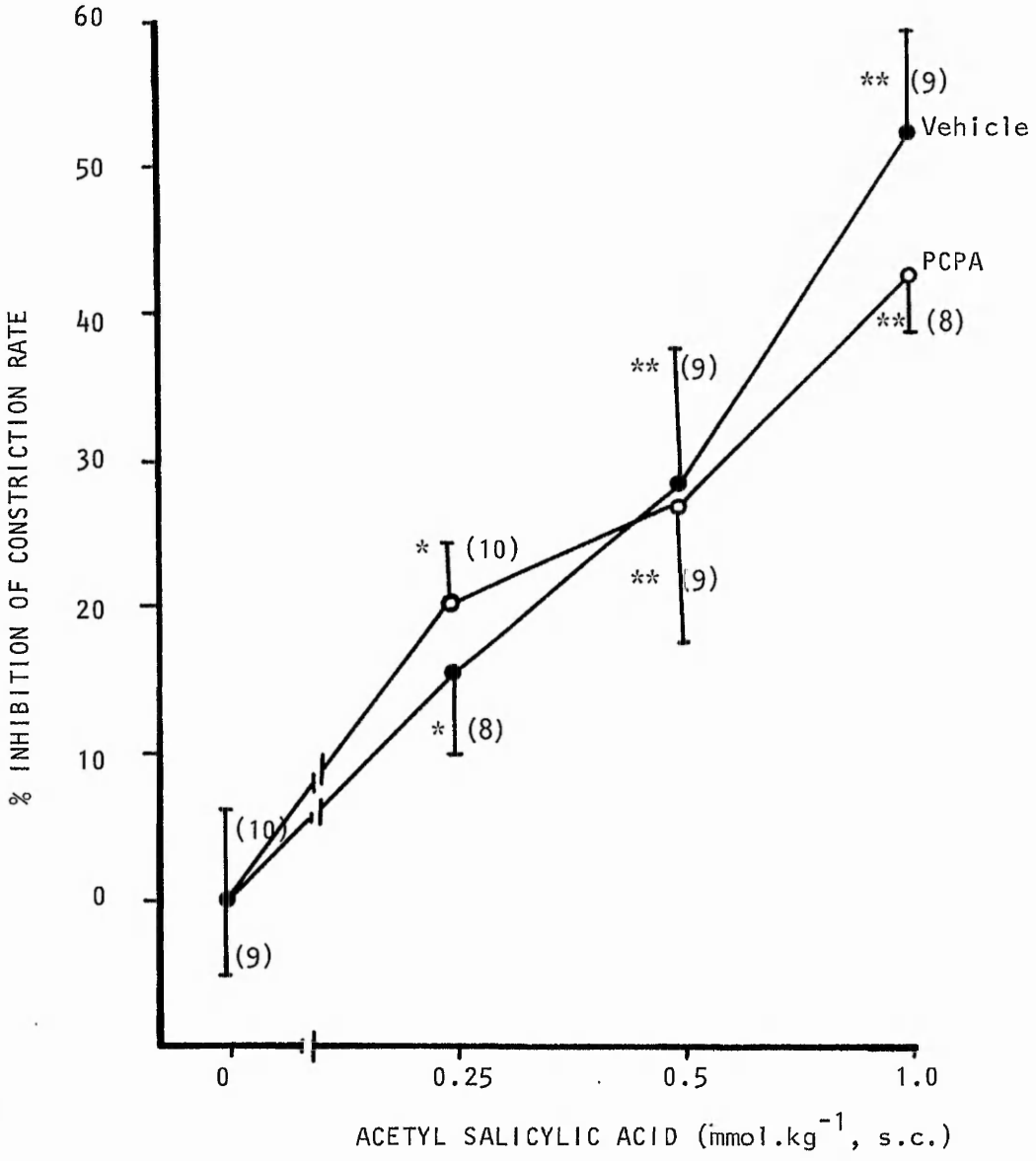

60
50
40
30
20
10
0
% INHIBITION OF CONSTRICTION RATE
** (9)
Vehicle
PCPA
** (8)
** (9)
* (10)
** (9)
* (8)
(10)
(9)
0
0.25
0.5
1.0
ACETYL SALICYLIC ACID (mmol.kg-1, s.c.)

Figure R.11 Antagonism of DADLE Antinociception by PCPA.

Animals pretreated with PCPA or vehicle received 5 pmol of DADLE (or i.c.v. vehicle) i.c.v. 15 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e., values of n are shown.

** $P < 0.01$ (compared to the control group receiving appropriate pretreatment). ANOVA revealed no significant evidence for an effect of PCPA on the constriction rate ($P > 0.1$) but evidence for the antinociceptive effect of DADLE ($P < 0.01$) and interaction between PCPA and DADLE ($P < 0.05$) was obtained.

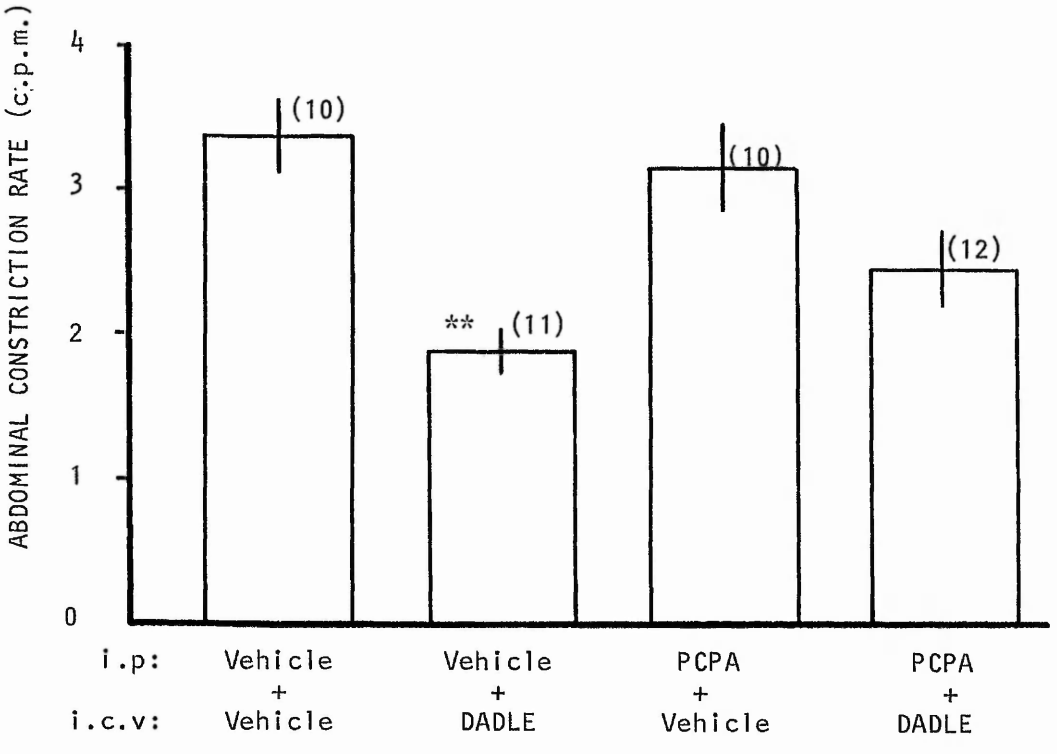

ABDOMINAL CONSTRICTION RATE (c.p.m.)
4
3
2
1
0
(10)
(10)
(12)
**
(11)
i.p:
i.c.v:
Vehicle + Vehicle
Vehicle + DADLE
PCPA + Vehicle
PCPA + DADLE

Figure R.12 Effect of PCPA on the Antinociceptive Effect of Combinations of 5-HT and sCT.

Animals pretreated with PCPA or vehicle received i.c.v. 5-HT, sCT or vehicle (or combination thereof) 20 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e., values of n are shown, inhibition of appropriate control constriction rate was significant at * $P < 0.05$ and ** $P < 0.01$.

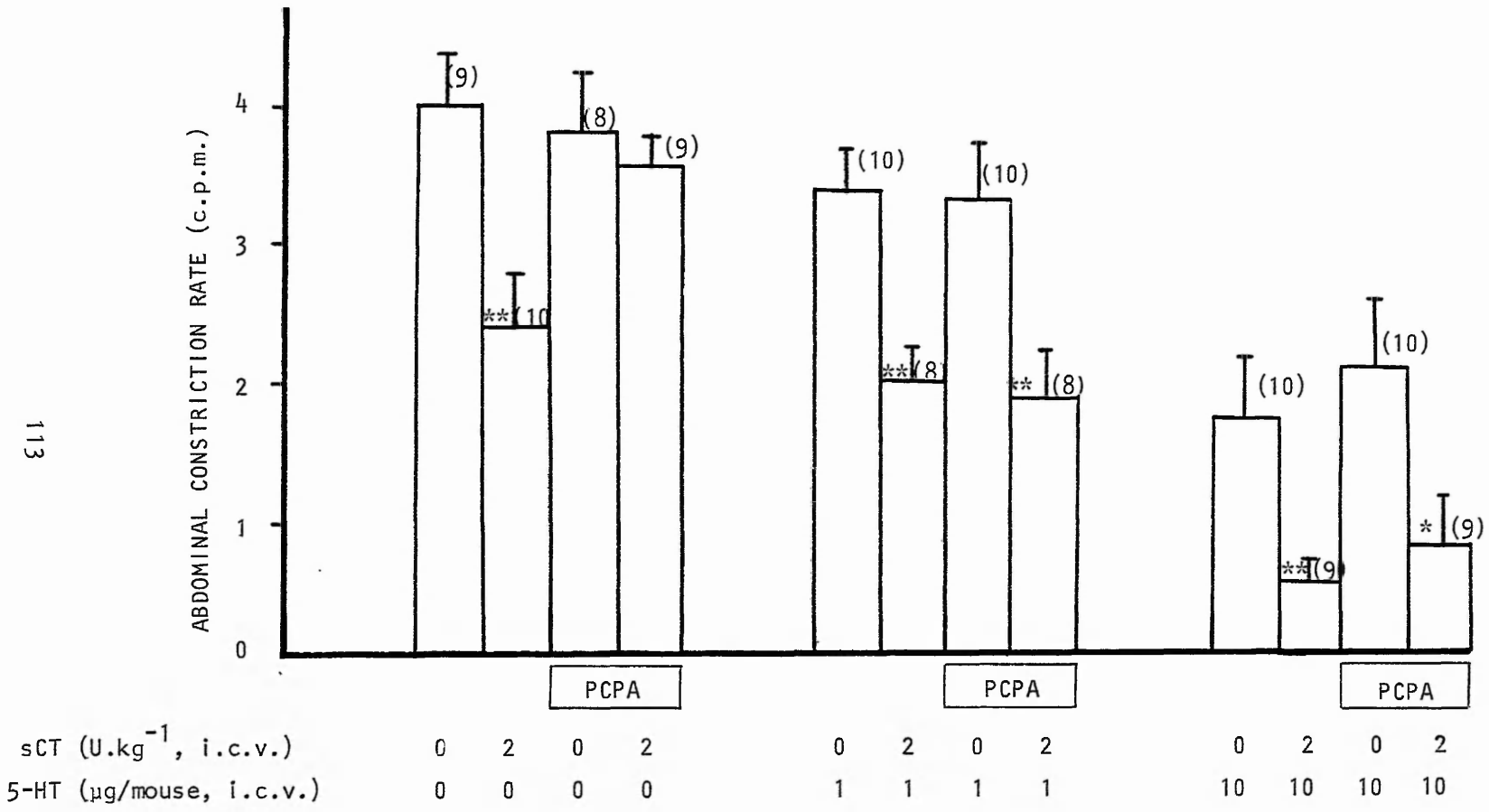

ABDOMINAL CONSTRICTION RATE (c.p.m.)
0
1
2
3
4
(9)
**(10)
(8)
(9)
(10)
(10)
**(8)
**(8)
(10)
**(9)
(10)
*(9)
PCPA
PCPA
PCPA
sCT (U.kg-1, i.c.v.) 0 2 0 2 0 2 0 2 0 2 0 2
5-HT (µg/mouse, i.c.v.) 0 0 0 0 1 1 1 1 10 10 10 10

Figure R.13 Antinociceptive Effect of sCT and 5-HT, Alone and in Combination.

The doses of sCT and 5-HT indicated were administered i.c.v. 20 minutes prior to counting the acetic acid induced abdominal constriction. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$; ** $P < 0.01$ (compared to group receiving vehicle alone).

Data shown above is extracted from Figure R.12.

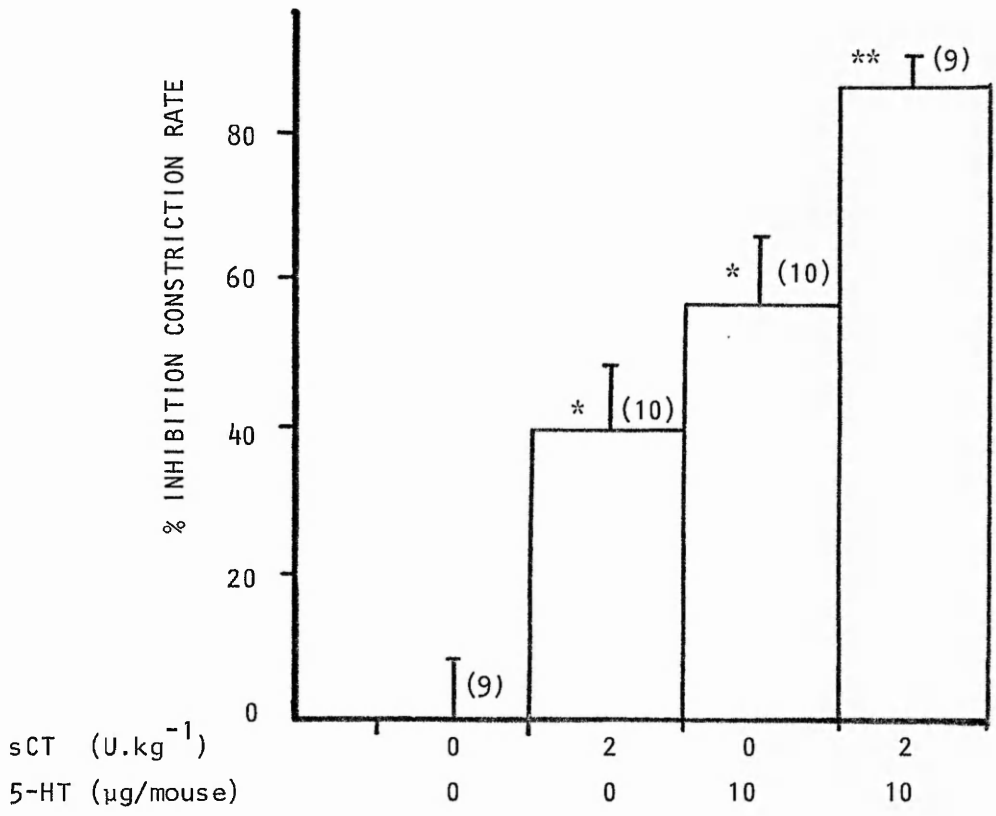

% INHIBITION CONSTRICTION RATE
0
20
40
60
80
(9)
*
(10)
*
(10)
**
(9)
sCT (U.kg-1)
5-HT (µg/mouse)
0
0
2
0
0
10
2
10

Figure R.14 Lack of Effect of α-methyl-p-tyrosine Pretreatment on sCT Antinociception.

Animals were pretreated with vehicle (0.45 ml, 50 mM phosphate buffer at pH 7.4) or α-MT (200 $mg.kg^{-1}$) i.p. 4 hours prior to experiment and received i.c.v. sCT or vehicle 20 minutes prior to counting the acetic acid induced abdominal constrictions. Values are $\bar{x} \pm$ s.e., values for n are shown.

* $P < 0.05$; ** $P < 0.01$ compared to control group receiving appropriate pretreatment.

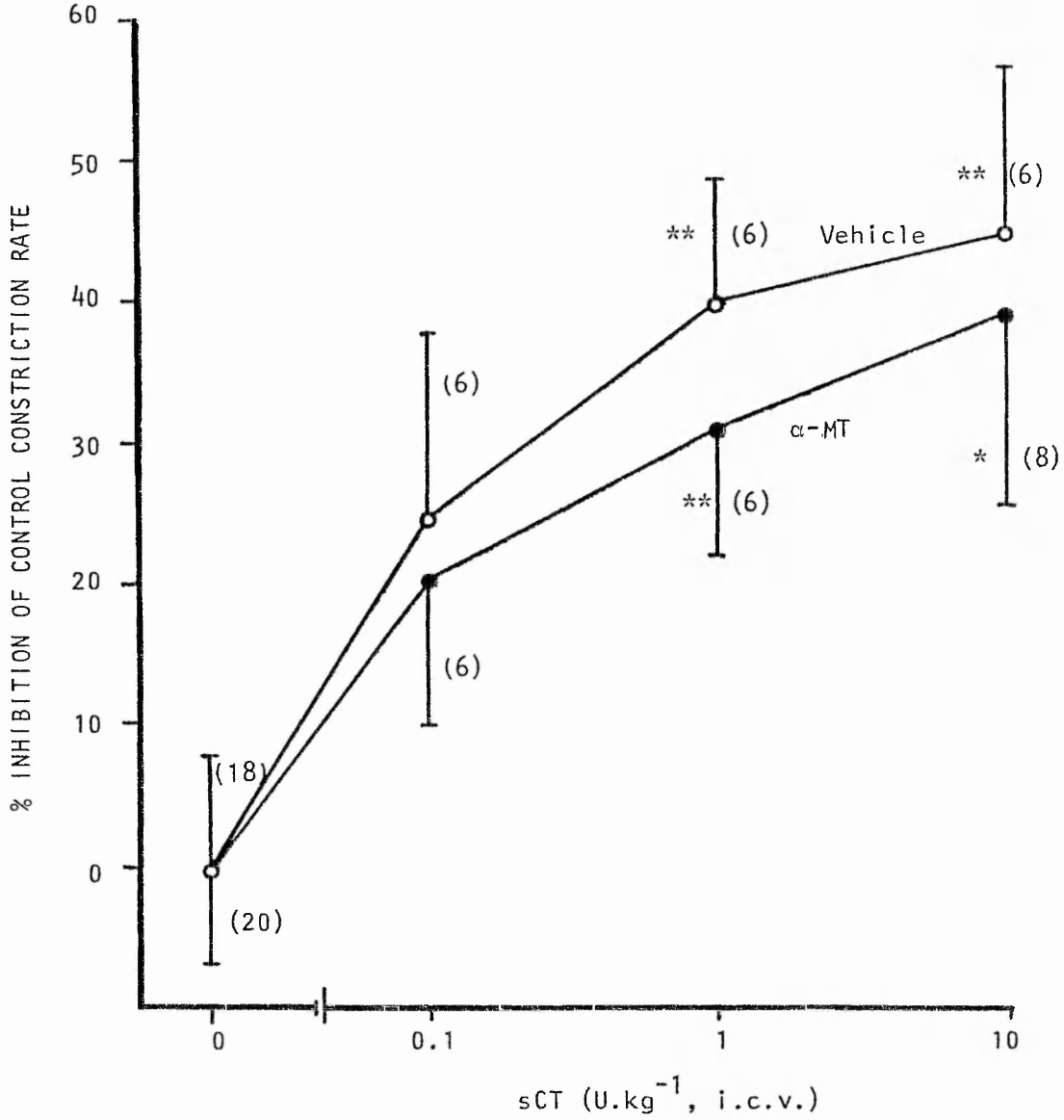

60
50
40
30
20
10
0
% INHIBITION OF CONTROL CONSTRICTION RATE
(18)
(20)
(6)
(6)
** (6)
** (6)
Vehicle
α-MT
** (6)
* (8)
0
0.1
1
10
sCT (U.kg-1, i.c.v.)

Table 3 Effect of Neurotransmitter Blockers and Depletors on Locomotor Activity

	Motor Discoordination (falls/5 mins)	Investigative Behaviour (head dips/5 mins)
Vehicle, s.c.	0.40 ± 0.20 (6)	18.5 ± 1.5 (6)
Methysergide, s.c.	0.30 ± 0.30 (7)	17.5 ± 1.5 (7)
Vehicle, i.p.	0.60 ± 0.15 (9)	18.0 ± 2.0 (9)
PCPA, i.p.	0.55 ± 0.25 (9)	19.0 ± 1.5 (9)
Vehicle, i.p.	1.05 ± 0.35 (8)	15.0 ± 3.5 (8)
α-MT, i.p.	2.50 ± 0.95 (7)	27.0 ± 6.0 (8)
Vehicle, s.c.	0.35 ± 0.20 (8)	19.0 ± 2.0 (8)
Reserpine, s.c.	1.50 ± 0.20 (7)*	11.5 ± 1.5 (8)*

Drug treatments were as follows; methysergide (5 $mg.kg^{-1}$) or vehicle (0.9% NaCl) administered s.c. in 0.1 ml, 1 hour prior to test; PCPA (300 $mg.kg^{-1}$) or vehicle (0.9% NaCl with 0.05% Brij) administered i.p. in 0.3 ml, 72, 48 and 24 hours prior to test; α-MT (200 $mg.kg^{-1}$) or vehicle (50 mM phosphate buffer at pH 7.4) administered i.p. in 0.1 ml 4 hours prior to test; Reserpine (1 $mg.kg^{-1}$) or vehicle (1% ascorbic acid) administered s.c. in 0.1 ml, 8 hours prior to test. Values are $\bar{x} \pm$ s.e. (n) * $P < 0.05$ compared to appropriate control (vehicle treated) group

R.3 COMPARISON OF THE ANTINOCICEPTIVE EFFECTS OF CENTRALLY ADMINISTERED CALCITONINS AND CALCITONIN GENE RELATED PEPTIDE

Introduction

Administration of various forms of CT into the cerebral ventricles of several species has been shown to result in antinociception (I.8.f). However, the existance of authentic CT in the CNS remains a controversial matter, as Fischer et al (1983) detected immunoreactive CT's in the rat CNS which were indistinguishable from authentic hCT and sCT, whereas Rosenfeld et al (1983) suggest CT-like immunoreactivity within the CNS reflects binding of antisera to molecules other than CT's (I.6.a). It seems possible that sCT antinociception involves interaction with systems associated with an, as yet, unidentified endogenous ligand. Rosenfeld et al (1983) suggest from the distribution of CGRP in the rat brain that the peptide may play a role in the processing of painful stimuli. This suggestion and the structural similarities between CT's and CGRP (I.10) raise the intriguing possibility of a common site of action. Alternatively, since production of CGRP has only been demonstrated in rats and humans, the central action of sCT in the mouse abdominal constriction test could involve interaction with receptors for an endogenous CT. It is therefore of interest to determine the possible antinociceptive effects of hCT (which differs from rat CT in only two amino acid residues) and CGRP in the mouse abdominal constriction test, and to compare the potency and duration of any such effects with that of sCT.

Results

i) Antinociceptive Effect of sCT, hCT and CGRP

Salmon CT at doses of 0.1-50 $U.kg^{-1}$ (0.006-30 $nmol.kg^{-1}$) administered i.c.v. 20 minutes prior to initiation of counting of abdominal constrictions, produced a dose dependent reduction in the acetic acid induced abdominal constriction rate of 4.0 ± 0.2 c.p.m. (n = 23) with an ED_{50} of 79 $m.U.kg^{-1}$ (4.7 $pmol. kg^{-1}$), this data which has been presented in figure R.6 has been replotted against dose in $nmol.kg^{-1}$

in figure 15a to enable direct comparison with the effects of other peptides. Similarly, i.c.v. injection of CGRP and hCT reduced the constriction rate (3.4 $\pm$ 0.2, n = 16 and 3.1 $\pm$ 0.2, n = 22) c.p.m. respectively) at doses of 1-10 nmol.kg^{-1}, with ED_{50}'s of 0.6 and 0.8 nmol.kg^{-1} respectively. All three peptides produced similar maximal inhibition of abdominal constrictions (approximately 40%) with a suggestion of reduction of the antinociceptive effect at the highest doses (figures 15a, 16a and 17a).

The time course of the observed antinociceptive effects was investigated by administration of peptides at various times prior to counting of acetic acid induced abdominal constrictions (constrictions were still counted 10-15 minutes after acetic acid administration). Preliminary experiments revealed the control constriction rate to vary considerably with timing of vehicle injection, it was therefore necessary to obtain an internal control response for each time. When comparing the response of vehicle treated groups obtained from the same experiment (a statistically valid comparison of an internally controlled experiment) the only constant observation was that the constriction rates at 10 minutes were significantly ($P < 0.05$) lower than those observed 20 minutes after administration of vehicle. Hence, the mean abdominal constriction rates in the sCT, hCT and CGRP experiments were 1.8, 2.6 and 1.4 c.p.m. respectively at 10 minutes, and 3.3, 3.0 and 3.0 c.p.m. respectively at 20 minutes.

The time courses of the antinociceptive effects of 0.6, 10 and 100 nmol, kg^{-1} (i.c.v.) of sCT, hCT and CGRP respectively, were determined by calculating % inhibition of the constriction rate observed in an internal control group (vehicle at the appropriate time) and are shown in figures 15b, 16b and 17b. Salmon CT was found to produce significant inhibition of constriction rate from 20-120 minutes after administration with no evidence for loss of effect with time. In contrast, the antinociceptive effects of hCT and CGRP attained statistical significance only at 20 minutes after administration.

Maximal doses of all three peptides were without effect in tests for modification of motor ability or investigative behaviour (table 4) and no overt behavioural responses were observed with any dose of peptide.

Discussion

In this chapter, the potential antinociceptive effects of sCT, hCT and CGRP have been compared in the mouse abdominal constriction test. The effect of sCT in this model is in good agreement with that reported by Eglen (1984) as previously noted (R.1). Similarly, intracerebral hCT has been shown, by Pecile et al (1983), to produce antinociception in the rat 'hot plate' test at doses of 5 and 10 $nmol.kg^{-1}$ which are comparable to the effective doses reported above. Although Rosenfeld et al (1983) have inferred from the distribution of CGRP in the rat brain that it might play a role in the processing of painful stimuli, the effect of the peptide in antinociceptive screens has not been reported (to the author's knowledge, August 1984). It has been noted previously that the abdominal constriction test is prone to false positive results particularly from anti-inflammatory drugs. The antinociceptive effects of sCT and hCT in the abdominal constriction test are supported by demonstrations of antinociceptive activity in several tests, since this is not so for CGRP the possibility of a false positive result cannot be discounted.

On the basis of ED_{50}'s (sCT 4.7, hCT 800, CGRP 600 $pmol.kg^{-1}$), it is possible to rank the potency of the three peptides:

$$sCT >> CGRP > hCT$$

The antinociceptive effect of sCT (0.6 $nmol.kg^{-1}$) was observed within 10 minutes of administration and was not altered significantly after 120 minutes, these observations compare well with those of Pecile et al (1975), who found antinociceptive effect of sCT at 12 $U.kg^{-1}$ in the rabbit licking threshold model to have a rapid onset and a duration of at least 2 hours, Pecile et al (1983), who found the antinociceptive effect of 2.5 $nmol.kg^{-1}$ sCT from 30-240 minutes after administration in the rat hot plate test, and Eglen (1984), who observed significant antinociception 20-60 minutes after administration of 0.6 $nmol.kg^{-1}$ sCT in the acetic acid induced abdominal constriction test in mice. The transient antinociceptive effect of hCT (10 $nmol.kg^{-1}$) observed in the abdominal constriction test contrasts with the

observations of Pecile et al (1983) who obtained significant antinociception with the equivalent dose of 10 µg/rat sCT at 30, 50 and 240 (but not at 120) minutes after administration in the rat hot plate test.

Comparison of the antinocicepitve potency of i.c.v. sCT (ED_{50} = 4.7 pmol.kg^{-1}) with that of other centrally acting peptides (ED_{50}'s of i.c.v. substance P, leucine-enkephalin, and neurotensin are approximately 25, 5000 and 1000 nmol.kg^{-1} respectively) reveals the marked potency of sCT. In hypocalcaemic assays the potency of sCT has been attributed to the slow rate of disappearance from the circulation (Habener et al, 1972b) which may, in turn, be dependent on the slow rate of metabolism (West et al, 1969) or the high receptor affinity and binding half-life of the peptide (Fischer et al, 1981), factors which could clearly affect the duration of the antinociceptive action of the three peptides studied. It is, therefore, unfortunate that binding studies have not yet been reported for CGRP or hCT.

Since, to date neither CGRP nor CGRP mRNA have been demonstrated in mouse CNS the antinociceptive effect of CGRP cannot be considered of physiological significance. However, it is tempting to speculate that molecules resembling either CGRP or hCT (e.g. rCT) play a physiological role in the central processing of painful stimuli and that the effect of such an endogenous ligand can be mimicked by administration of structurally similar endogenous ligands such as sCT. Such speculation can neither be supported nor refuted by the limited experimental data available at present, it is indeed unfortunate that the possibility of CGRP competition with CT receptor binding has not yet been reported.

Figure R.15 Dependence of sCT Antinociception on Dose (a) and Time (b).

In (a) sCT or vehicle were administered i.c.v. 20 minutes prior to counting the acetic acid induced abdominal constrictions. In (b) the abdominal constriction rate was determined at various times (shown) after administration of sCT (0.6 $nmol.kg^{-1}$) and is expressed as % inhibition of the rate observed in a control group receiving vehicle at the same time before acetic acid. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$; ** $P < 0.01$ (compared to internal control).

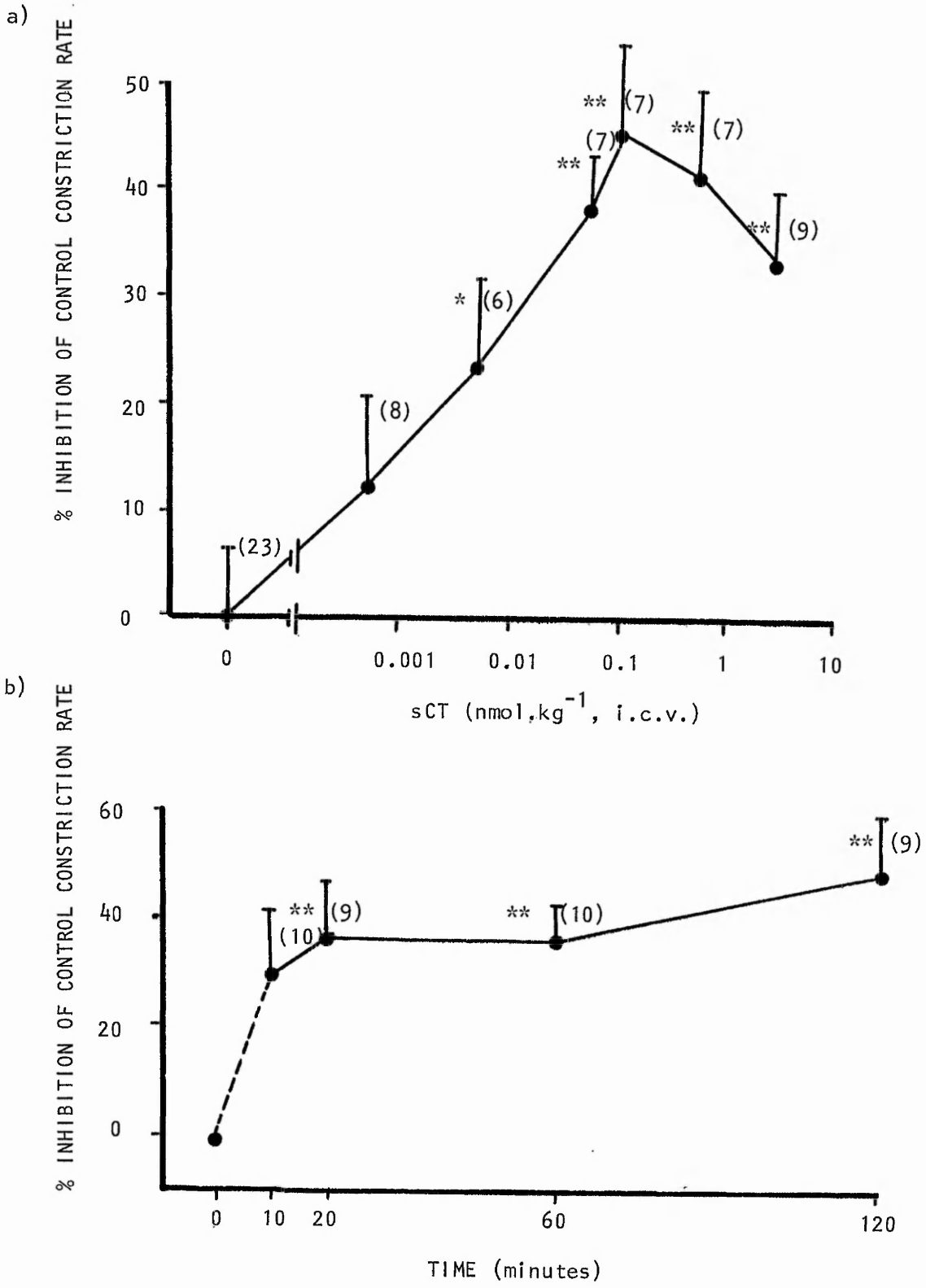

a)
% INHIBITION OF CONTROL CONSTRICTION RATE
50
40
30
20
10
0
(23)
(8)
* (6)
** (7)
** (7)
** (7)
** (9)
0
0.001
0.01
0.1
1
10
sCT (nmol.kg^{-1}, i.c.v.)
b)
% INHIBITION OF CONTROL CONSTRICTION RATE
60
40
20
0
** (10)
** (9)
** (10)
** (9)
0
10
20
60
120
TIME (minutes)

Figure R.16 Dependence of CGRP Antinociception on Dose and Time

In (a) CGRP or vehicle were administered 20 minutes prior to counting the acetic acid induced abdominal constrictions. In (b) the abdominal constriction rate was determined at various times (shown) after administration of CGRP (10 $nmol.kg^{-1}$) and is expressed as % inhibition of the rate observed in a control group receiving vehicle at the same time before acetic acid. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$; ** $P < 0.01$ (compared to internal control).

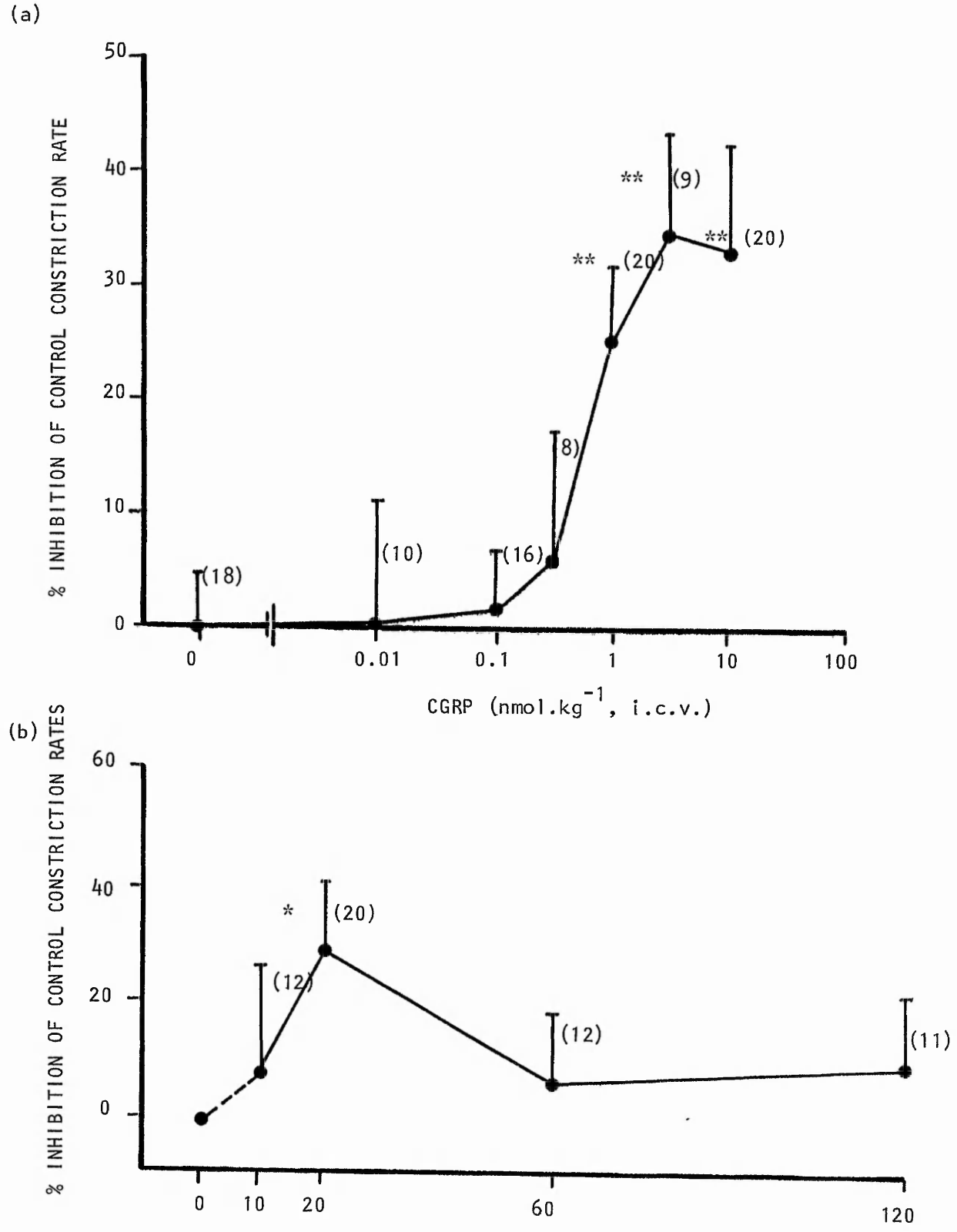

(a)
% INHIBITION OF CONTROL CONSTRICTION RATE
50
40
30
20
10
0
(18)
(10)
(16)
(8)
**
(9)
**
(20)
**
(20)
0
0.01
0.1
1
10
100
CGRP (nmol.kg^{-1}, i.c.v.)
(b)
% INHIBITION OF CONTROL CONSTRICTION RATES
60
40
20
0
*
(20)
(12)
(12)
(11)
0
10
20
60
120

Figure R.17 Dependence of hCT Antinociception on Dose and Time.

In (a) hCT or vehicle were administered 20 minutes prior to the acetic acid induced constrictions. In (b) the abdominal constriction rate was determined at various times (shown) after administration of hCT (10 $nmol.kg^{-1}$) and is expressed as % inhibition of the rate observed in a control group receiving vehicle at the same time before acetic acid. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$ (compared to internal control).

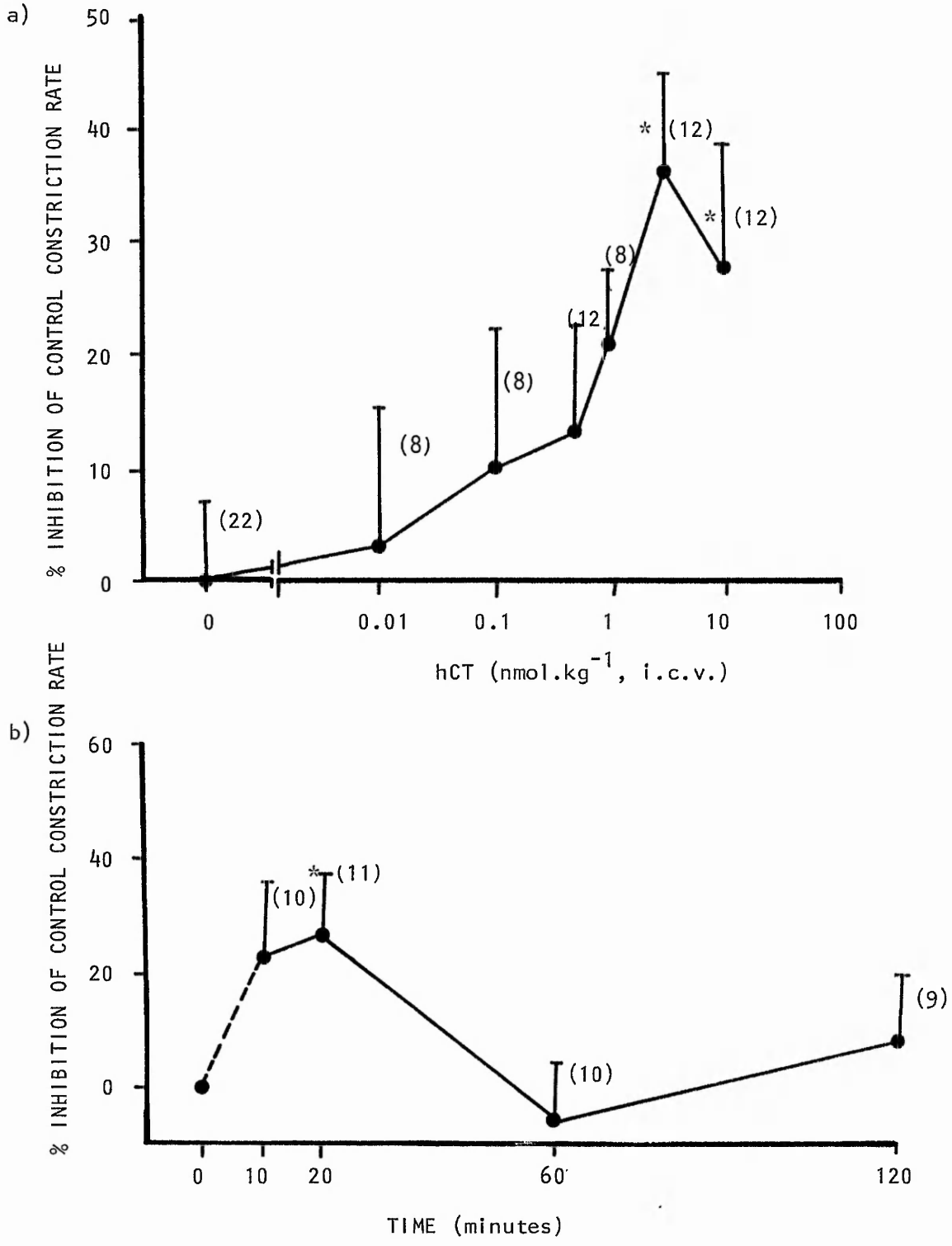

a)
% INHIBITION OF CONTROL CONSTRICTION RATE
50
40
30
20
10
0
(22)
(8)
(8)
(12)
(8)
* (12)
* (12)
0
0.01
0.1
1
10
100
hCT (nmol.kg^{-1}, i.c.v.)
b)
% INHIBITION OF CONTROL CONSTRICTION RATE
60
40
20
0
(10)
* (11)
(10)
(9)
0
10
20
60
120
TIME (minutes)

Table 4 Lack of Effect of Calcitonins and CGRP on Locomotor Activity

	Motor Discoordination (falls/5 mins)	Investigative Behaviour (head dips/5 mins)
Vehicle, i.c.v.	1.5 ± 0.6 (6)	15.0 ± 2.3 (6)
CGRP, i.c.v.	1.8 ± 0.7 (6)	17.6 ± 1.9 (6)
hCT, i.c.v.	1.3 ± 0.5 (6)	16.0 ± 2.0 (6)
Vehicle, i.c.v.	1.0 ± 0.3 (8)	16.3 ± 1.9 (8)
sCT, i.c.v.	1.1 ± 0.7 (8)	18.4 ± 1.5 (8)

Peptides (10 nmol.kg^{-1}, hCT or CGRP, or 0.12 nmol.kg^{-1} sCT) or i.c.v. vehicle were administered i.c.v. in a volume of 10 µl, 20 minutes prior to assessment of locomotor activity. Values are $\bar{x}$ ± s.e. (n), no significant modification of control values was observed at $P > 0.05$.

R.4 CARDIOVASCULAR EFFECTS OF CALCITONIN

R.4.a Peripheral Administration

Introduction

The involvement of peptide hormones such as vasopressin and angiotensin in cardiovascular regulation has been long established (Guyton, 1981). Although CT was shown to modify vascular tone as early as 1972 (Charbon and Pieper, 1972) and has been shown to inhibit the development of mineralocorticoid induced hypertension (Aldred, Luna, Zeedyk and Bastian, 1976), it was not until 1983 that the acute effects of CT on MAP were reported, (Bates et al, 1983).

Having demonstrated that central administration of sCT to mice produced antinociception which resembled that produced by opiates (I.8.f), Bates and co-workers investigated the possibility that sCT might produce opiate-like effects in biological models known to respond to opiate agonists and antagonists. These investigations revealed that in normotensive rats under urethane anæsthesia, leu-enkephalin, i.v., at doses of 20, 60 and 200 $nM.kg^{-1}$, produced transient dose dependent hypotension (Bates et al, 1983) whereas naloxone at a dose of 10 $mg.kg^{-1}$, i.v., and sCT, at doses of 0.1-10 $U.kg^{-1}$, i.v. did not alter MAP. In contrast, both naloxone and sCT produced a pressor response in rats rendered hypotensive by haemorrhage. The pressor effect of sCT was maximal 5 minutes after administration and the response to sCT (10 $U.kg^{-1}$) and naloxone (10 $mg.kg^{-1}$) had a duration of up to 60 minutes. Clearly sCT did not produce opiate agonists effects in this preparation, however the similarity between the response to naloxone and sCT raises the possibility of opiate antagonist action, alternatively sCT might act through mechanism(s) not directly related to opiate systems.

In addressing the problem of the mechanism of the pressor response to sCT in haemorrhaged rats pharmacological and surgical procedures have been used to selectively modify specific cardiovascular effector mechanisms in both normotensive and hypotensive rates. The results of these experiments are presented below.

Results

i) Cardiovascular Effects of sCT and Naloxone in rats Rendered Hypotensive by Haemorrhage

In haemorrhaged rats 10 mg.kg^{-1} of naloxone (i.v.) produced a gradual increase in MAP with the maximum observed increase in MAP of 16.8 $\pm$ 3.2 mmHg (n = 5) occurring 60 minutes after administration (figure R18). Intravenous administration of 10 U.kg^{-1} of sCT after haemorrhage produced a similar pressor response which was maximal 5 minutes after administration and remained significantly different to the vehicle response for 60 minutes (figure R19). In both of these experiments, and in all subsequent experiments on haemorrhaged rats, the change in MAP after vehicle did not differ significantly from zero at any time although the transient increase in MAP 5 minutes after vehicle was a consistant observation. Neither of the pressor responses described above were associated with changes in heart rate (table 5).

ii) Effect of sCT in Normotensive Rats and Effect of sCT and Naloxone on Leu-enkephalin Induced Hypotension

In normotensive rats i.v. administration of 10 U.kg^{-1} sCT produced no significant change in MAP (Figure R19). Administration of 2-600 nmol.kg^{-1} leu-enkephalin (i.v.) to normotensive rats produced transient hypotension, this response was abolished by pretreatment with 10 mg.kg^{-1} of naloxone (i.v.) but was not altered by pretreatment with 10 U.kg^{-1} of sCT (i.v.) figure R20.

iii) Effect of sCT and Vasopressin in Rats Rendered Hypotensive by Pithing

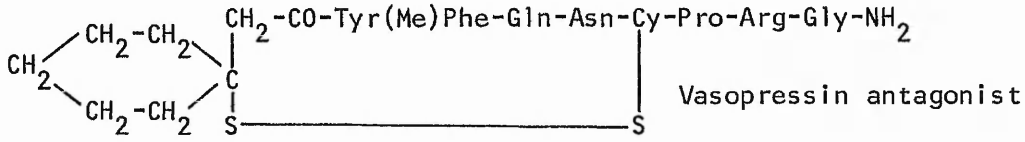
After destruction of the CNS by pithing rats had markedly reduced MAP's (figure R19) and were sensitive to the direct vasoconstrictor action of arginine vasopressin (AVP) at 10 and 100 U.kg^{-1}, i.v. The MAP of these animals was not altered by 10 U.kg^{-1} of sCT (i.v) although the heart rate was significantly reduced 5 and 15 minutes after sCT (figure R19 and table 5).

iv) Effect of sCT After Bilateral Vagotomy and Haemorrhage

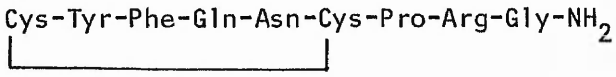
Administration of 10 $U.kg^{-1}$ of sCT, i.v., after bilateral vagotomy and haemorrhage produced a pressor response of similar magnitude (peak increase in MAP 9.7 ± 2.4 mmHg) and duration (60 minutes) to that observed after haemorrhage alone (figure R21 and R22). Again no significant alteration in heart rate was observed.

v) Effect of sCT after Vasopressin Antagonism and Haemorrhage

$d(CH_2)_5Tyr(Me)AVP$, a selective antagonist of the pressor response to AVP (Manning and Sawyer, 1982) was used to determine the importance of vasopressin in the pressor response to sCT. The structure of this peptide and of argine vasopression are shown below:



Vasopressin antagonist



Arginine vasopressin

Manning and Sayer (1982) report that $d(CH_2)_5Try(Me)AVP$ is a potent antagonist of the vasopressor response to arginine vasopressin with a pA_2 of 8.67. This antagonist exerts only weak antidiuretic activity (0.31 $U.mg^{-1}$). Preliminary experiments using pithed rats revealed that vasopressin produced a dose dependent pressor response which was abolished by pretreatment with the antagonist. There was no evidence for loss of effect of the antagonist over the three hour period studied (figure R23) indicating that 100 $\mu g.kg^{-1}$ i.v. (the dose and route used in subsequent experiments) of the vasopressin antagonist could maintain complete blockade of the vasopressor response to vasopressin for the duration of the subsequent experiments.

The pressor response to 10 $U.kg^{-1}$ sCT, i.v., was of similar magnitude (peak increase in MAP 8.3 ± 2.5 mmHg) and duration (60 minutes) after haemorrhage and vasopressin antagonist to that seen after haemorrhage alone (figure R24). No significant alteration in heart rate was produced by either the antagonist or sCT (table 6).

vi) Effect of sCT After Chemical Sympathectomy and Haemorrhage

Pretreatment of animals with 6-OHDA to produce chemical sympathectomy caused a significant reduction of MAP, from the initial MAP of 111.3 ± 4.0 mmHg (n = 8) observed after pretreatment with vehicle for 6-OHDA to the initial MAP of 92.0 ± mmHg (n = 10) observed after pretreatment with 6-OHDA. Pretreatment with 6-OHDA also greatly attenuated the pressor response to sCT after haemorrhage, so that i.v. administration of 10 $U.kg^{-1}$ of sCT produced a peak increase in MAP of only 4.5 ± 1.2 mmHg five minutes after administration, and the response only attained statistical significant at this time (figure R25). As in previous experiments, no significant changes in heart rate were observed after sCT.

Discussion

The response observed after administration of naloxone to normotensive and haemorrhaged rats is in good agreement with that described by Faden and Holaday. These workers have demonstrated that naloxone which is without effect in normotensive animals, produces a pressor response in those rendered hypotensive by haemorrhage (Faden and Holaday, 1979a), endotoxic shock (Holaday and Faden, 1978) or by severage of the spinal chord (Holaday and Faden, 1980), and suggest that naloxone acts by antagonism of the effects of endorphins released in response to stress. This theory is supported by the observations that peripheral administration of opiates produces hypotension in normotensive rats (Moore and Dowling, 1981) and exacerbates the hypotension produced by haemorrhage (Chance, 1981). The response to 10 $U.kg^{-1}$ of sCT (i.v.) shown above was similar to that of 10 $mg.kg^{-1}$ naloxone (i.v.) and was in general agreement with that reported by Bates et al (1983). Although both naloxone and sCT produced pressor responses after haemorrhage and were devoid of effect in normotensive

animals, the failure of sCT to antagonise leu-enkephalin induced hypotension indicates that sCT does not act as an opiate antagonist.

The lack of effect of sCT on heart rate indicates that the pressor response after haemorrhage might involve a direct or indirect effect on peripheral vasculature such as that demonstrated by Driessens and Vanhuotte, (1981). Destruction of the CNS by pithing caused a marked reduction in heart rate and MAP indicating effective destruction of central cardiovascular control centers. The lack of effect of sCT in pithed animals demonstrates the necessity of the CNS for production of the pressor response, the direct vasoconstrictor action of sCT on bone vasculature described by Driessens and Vanhoutte, (1981) does not therefore contribute significantly to the pressor response in haemorrhaged rats. Although sCT induced constriction of bone vasculature has not been demonstrated in the rat any such effect would not be expected to have marked effects on MAP because of the relatively poor supply of blood to bone.

The lack of effect of $d(CH_2)_5$Try(Me)AVP on the pressor response indicates that the response is not achieved by stimulation of vasopressin release from the posterior pituitary gland or by potentiation of the pressor effect of the elevated levels of vasopressin observed after haemorrhage. Similarly, the lack of effect of bilateral vagotomy indicates that inhibition of vagal baroreceptor afferents is not the mechanism by which sCT increases blood pressure.

The attenuation of the response to sCT after pretreatment with 6-OHDA demonstrates that peripheral adrenergic neurones are necessary for production of the response. After peripheral administration, 6-OHDA, an isomer of noradrenaline, becomes concentrated within adrenergic neurones by active uptake processes. In sufficiently high concentrations auto-oxidation of 6-OHDA occurs producing toxic peroxides, superoxides, hydroxyindoles and quinones which react non-specifically with neuronal structures to destroy the neurone (Kostrzewa and Jacobowitz, 1974), the selective destruction of sympathetic ganglia produced has been termed "chemical sympathectomy" (Theonen and Tranzer, 1968). Chemical sympathectomy produces a dose dependent depletion of noradrenaline, the extent and duration of

which varies from tissue to tissue. The dose regime employed (100 $mg.kg^{-1}$ 4, 3 and 2 days prior to experiment) has been shown by histological and biochemical means to selectively destroy adrenergic nerve terminals (Kostrzewa and Jacobowits, 1974) and was shown by Heath, Larson and Laakso (1980) to reduce thyroparathyroid noradrenaline concentration to 10% of normal. Both the CNS and the adrenal medulla are resistant to the effects of peripherally administered 6-OHDA, in fact it has been shown that the adrenal glands respond to diminished sympathetic function by a compensatory increase in catecholamine turnover (Kostrzewa and Jacobowits, 1974). Since 6-OHDA pretreatment does not reduce adrenal noradrenaline content it is apparent that release of noradrenaline from this source does not contribute greatly to the observed pressor response. Release of noradrenaline or adrenaline from the adrenal medula, or release of residual noradrenaline from other tissues, may however underlie the attenuated pressor response observed after sympathectomy. Alternatively, the response observed at 5 minutes might be associated with other cardiovascular effector systems such as modification of non vagal baroreceptor or chemoreceptor afferents, release of angiotensin or glucocorticoids etc.

Antagonism of the pressor response to sCT in hypotensive rats by pithing and by chemical sympathectomy indicates that the response involves facilitation of pressor sympathetic outflow from the CNS but leaves three important questions unanswered.

1) Where does sCT act to produce the pressor response?

Driessens and Vanhoutte, (1981) demonstrated that sCT did not modify the vasoconstrictor effect of noradrenaline or electrical stimulation. It therefore seems likely that sCT might either modulate transmission at sympathetic ganglia or might directly or indirectly affect central cardiovascular centres. The possibility of a central action of calcitonin is considered below.

2) Why is the response not observed in normotensive animals?

It has been demonstrated that the pressor response to intra-

cerebroventricular administration of peptides including substance P and enkephalins are drastically increased in spontaneously hypertensive rats (SHR) when compared to the parent strain of normotensive Wistar-Kyoto rats (Schaz, Stock, Simon, Schlor, Unger, Rockhold and Ganten, 1980; Unger et al, 1981). These observations and the demonstration of an impaired baroreceptor reflex in SHR rats (Schaz et al, 1980) led to the conclusion that the pressor action of these peptides was buffered by the baroreceptor reflex in Wistar-Kyoto rats and that the baroreceptor reflex is impaired in SHR rats. Clearly, similar baroreceptor buffering could explain the lack of effect of sCT in normotensive rats, the reduction of blood pressure after haemorrhage would reduce inhibitory feedback from the baroreceptors to the medulla and might, therefore, simply inhibit baroreceptor buffering of the sCT response. Alternatively, if sCT is unable to initiate sympathetic outflow from the CNS but merely potentiates existing neuronal activity (possibly by facilitation of ganglionic transmission) its effect would be most marked in conditions of elevated sympathetic activity such as haemorrhagic hypotension. The possibilities above could be readily investigated by determining the effects of sCT in normotensive rats with impaired baroreceptor reflexes (SHR or vagotomised rats), and the effects of sCT on the pressor response to sympathetic outflow produced by electrical stimulation of the spinal chord. Alternatively, it is known that hypoxia produced by insufficient capillary blood flow can increase capillary permeability in shock (Guyton, 1981) and that CT can reduce vascular leakage induced by histamine (Strettle, Bates and Buckley, 1980). Inhibition of capillary leakage is, however, unlikely to account for the observed pressor effect of sCT after haemorrhage as such leakage only occurs in the late stages of prolonged shock, and its inhibition would not therefore be expected to produce such a rapid change in MAP.

3) Why is facilitation of sympathetic activity not associated with tachycardia?

As the heart is innervated by sympathetic nerves from the cardioaccelerator centre of the medulla, it might be assumed that facilitation of sympathetic outflow would be associated with an increase in heart rate. However, in the results presented above,

neither sCT nor vehicle significantly altered heart rate of normotensive or haemorrhaged rats. Similarly, the results presented below demonstrate that the pressor response of rats to centrally administered sCT is not associated with modification of heart rate. Moreover, neither chemical sympathectomy nor removal of arterial blood produced the expected changes in heart rate and reflex bradychardia did not occur in response to the pressor effect of sCT. The pressor effect of centrally administered angiotensin is not evident in anaesthetised rats (Severs and Daniels-Severs, 1977), and urethane anaesthesia has been reported to elevate heart rate and to attenuate baroreceptor reflex control of heart rate (Fluckiger and Atkinson, 1984). Hence, although it is conceivable that sCT enhances sympathetic activity in vasopressor but not in cardioaccelératory fibres, it is premature to accept such a hypothesis without data from conscious animals.

R.4.b Central Administration

Introduction

Several neuropeptides are believed to be involved in central mechanisms of blood pressure regulation. Peptides including endorphins, angiotensin and substance P have been found in central cardiovascular regulatory centres (Palkovits, 1981) and produce haemodynamic responses which can be prevented by competitive antagonists after central administration (Shcaz et al, 1980; Phillips, Weyhenmeyer, Felix, Ganten and Hoffman, 1979). More recently CGRP, the major proposed product of CT gene expression within the CNS, has been found to be localised in areas of the brain involved in cardiovascular regulation including the parabrachial nucleus and the nucleus tractus solitarius which can be considered as the primary relay station for baroreceptor and chemoreceptor reflexes (Rosenfeld et al, 1983). Central administration of this peptide was found to produce marked pressor responses in conscious rats with an associated increase in both heart rate and plasma noradrenaline, the authors suggest that the response involves central stimulation of sympathetic outflow. In this section the possibility of centrally administered CT exerting haemodynamic effects has been investigated in both normotensive rats and in rats may hypotensive by haemorrhage.

Results

i) Effect of i.c.v. sCT on MAP of Normotensive and Hypotensive Rats

Intracerebroventricular administration of 0.1, 1 and 10 $U.kg^{-1}$ of sCT produced a dose dependent pressor response in both normotensive animals (figure R.26)and in those made hypotensive by haemorrhage (figure R.27), whereas vehicle produced no significant change in either group. The pressor effect of sCT was most pronounced after haemorrhage and MAP was significantly elevated from 5-60 minutes after administration of the higher doses. Neither vehicle nor sCT significantly altered the heart rate (table 5).

ii) Effect of 6-OHDA on the Response of Haemorrhaged Rats to i.c.v. sCT

Further groups of rats were pretreated with 6-OHDA or vehicle to determine the effect of chemical sympathectomy on the pressor response elicited by i.c.v. injection of 1 and 10 $U.kg^{-1}$ of sCT to rats made hypotensive by haemorrhage. In the vehicle pretreated group the MAP's before and after haemorrhage of 126.9 $\pm$ 6.6 and 101.2 $\pm$ 6.6 mm Hg respectively (n = 12) were again higher than those in the chemically sympathectomised animals which were 107.1 $\pm$ 3.9 and 86.8 $\pm$ 3.6 mm respectively (n = 10) although the reduction in MAP caused by removal of arterial blood did not differ singificantly. After pretreatment with the vehicle for 6-OHDA 1 and 10 $U.kg^{-1}$ of sCT (i.c.v.) produced a dose dependent pressor response (figure R.28) which, unlike the effect of i.v. sCT on MAP of haemorrhaged rats, was not significantly attenuated by chemical sympathectomy (figure R.29).

Discussion

The results presented above demonstrate that centrally administered sCT, at doses as low as 0.1 $U.kg^{-1}$ ($\simeq$ 2 pmol/rat), produces a pressor response with MAP being significantly elevated 5-60 minutes after administration of 10 $U.kg^{-1}$ ($\simeq$ 200 pmol/rat). The peak increase in MAP produced by i.c.v. administration of sCT at 10 $U.kg^{-1}$ to normotensive rats was 17.7 $\pm$ 1.7 mmHg. Consideration of the cardiovascular effects of other centrally administered peptides in normotensive rats reveals that the effects of sCT are of comparatively long duration and that the hormone

is comparatively potent. Hence, peak increases in MAP of approximately 15-30 mmHg have been observed after intraventricular administration of 36-360 nmol of leu- or met-enkephalin and after 50 μg (≃ 48 nmol) angiotensin, the duration of these effects did not exceed 20 minutes (Simon, Schaz, Ganten, Stock, Schlor and Ganten, 1978; Severs, Summy-Long, Daniels-Severs and Connor, 1971). Of particular relevance to this work, intraventricular CGRP was found to produce a peak increase in MAP of approximately 10 and 15 mmHg, with doses of 0.2 and 2.2 nmol. kg^{-1}, and the duration of these effects did not exceed 10 minutes (Fisher et al, 1983).

A comparison of the peak pressor responses to i.c.v. sCT (10 $U.kg^{-1}$) reveals that this effect, like that of i.v. sCT, is more marked after haemorrhage where the peak increase in MAP was 35.8 ± 3.9 mmHg (n = 6), than in normotensive animals where the peak increase in MAP was 17.7 ± 1.7 mmHg (n = 6). The possibility that this might merely reflect baroreceptor disinhibition produced by haemorrhage, could be investigated by determining the effect of i.c.v. (and i.v.) sCT in SHR rats and in rats subjected to bilateral vagotomy and/or sinoatrial denervation.

As the pressor response to i.c.v. sCT was of greater magnitude than the response to i.v. sCT (see figures R.19, R.26 and R.27) it can be assumed that centrally administered sCT acts directly on the CNS rather than by leakage into the peripheral circulation. However, the possibility of a direct central action of peripherally administered sCT must also be considered. Chemical sympathectomy, which antagonised the pressor response of haemorrhaged rats to i.v. sCT did not greatly modify the response to i.c.v. sCT, indicating that stimulation of sympathetic neuronal activity is not of major importance in the latter response. If peripherally administered sCT was acting directly on the same CNS structures as the centrally administered peptide, the response toi.v. sCT would be expected to be resistant to the effect of chemical sympathectomy. As this was not the case, these results indicate that both the site and mechanism of action of centrally and peripherally administered sCT may differ. Moreover, as Fisher

et al (1983) have suggested that the pressor effect of CGRP is achieved by stimulation of noradrenergic sympathetic outflow the mechanism of action of the cardiovascular effects of sCT and CGRP may differ.

Figure R.18 Effect of Naloxone (i.v.) on MAP of Haemorrhaged Rats.

The initial MAP of 127.1 ± 4.7 mm Hg was reduced to 103.2 ± 5.3 mm Hg by removal of arterial blood. Values are $\bar{x}$ ± s.e., n = 5

* $P < 0.05$ (compared to internal control (vehicle) response).

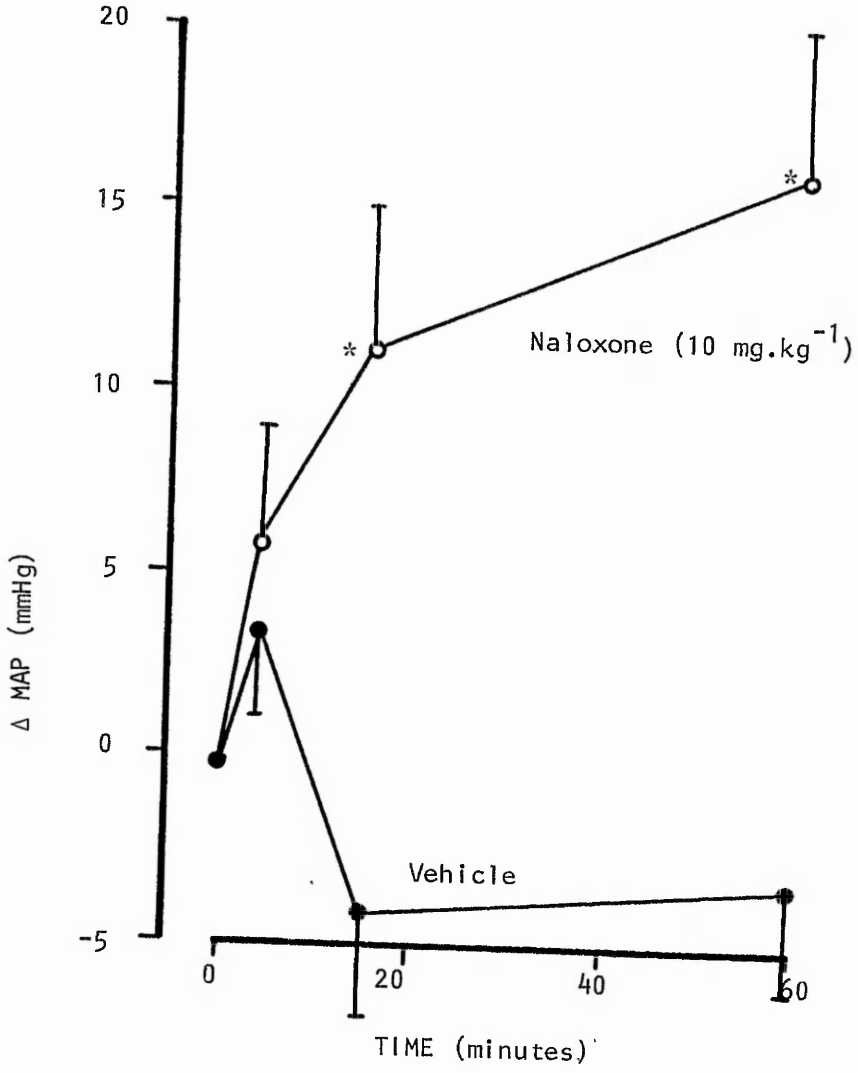

20
15
10
5
0
-5
Δ MAP (mmHg)
*
*
Naloxone (10 mg.kg^{-1})
Vehicle
0
20
40
60
TIME (minutes)

Figure R.19 Effect of sCT (i.v.) on MAP of Normotensive, Haemorrhaged and Pithed rats.

(a) The initial MAP of the normotensive rats was 103.2 ± 4.9 mm Hg (n = 5).

(b) The initial MAP of 108.1 ± 5.1 mm Hg (n = 6) was reduced to 82.7 ± 3.4 mm Hg by removal of blood.

(c) The initial MAP of 111.5 ± 5.0 mm Hg (n = 4) was reduced to 48.7 ± 5.0 mm Hg by pithing.

Values are $\bar{x}$ ± s.e., n = 4-6.

* $P < 0.05$; ** $P < 0.01$ (compared to internal control (vehicle) response).

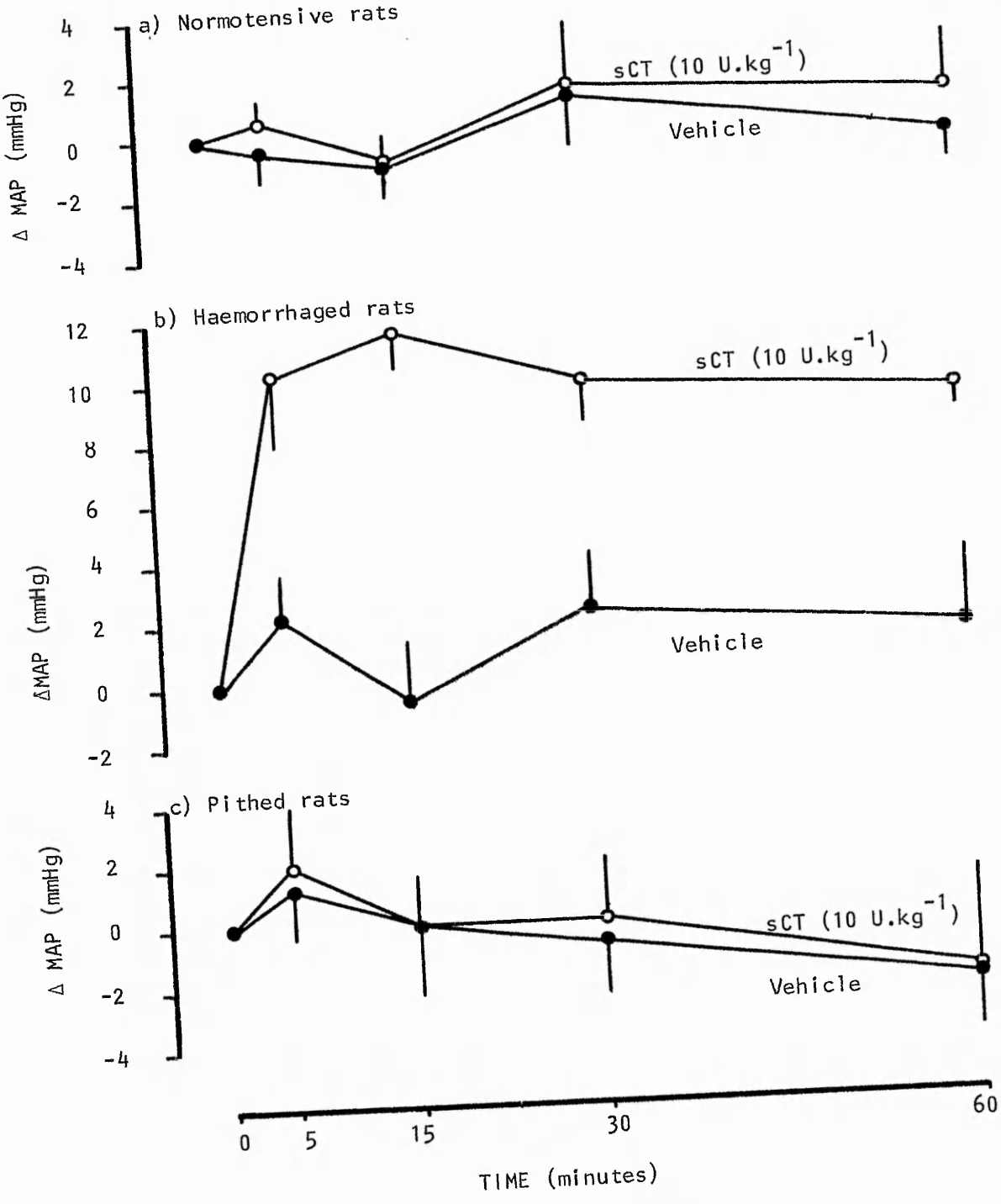

a) Normotensive rats
sCT (10 U.kg^{-1})
Vehicle
Δ MAP (mmHg)
4
2
0
-2
-4
b) Haemorrhaged rats
sCT (10 U.kg^{-1})
Vehicle
ΔMAP (mmHg)
12
10
8
6
4
2
0
-2
c) Pithed rats
sCT (10 U.kg^{-1})
Vehicle
Δ MAP (mmHg)
4
2
0
-2
-4
0
5
15
30
60
TIME (minutes)

Table 5 Effect of Calcitonin, Naloxone and Vehicle on Heart Rate of Normotensive, Pithed and Haemorrhaged Rats

Treatment	Drug, route	Heart Rate b.p.m.		Δ Heart Rate b.p.m.		
		Initial	t = 0	t = 5	t = 15	t = 60
NORMOTENSIVE	Vehicle, i.v.	412±16 (5)	401±14 (5)	+3±4 (5)	+6±7 (5)	+11±6 (5)
	sCT, i.v.			-2±5 (5)	+10±4 (5)	+13±9 (5)
PITHED	Vehicle, i.v.	450±24 (4)	405±13 (4)	+5±5 (4)	+7±5 (4)	0±22 (4)
	sCT, i.v.			-12±9 (4)*	-22±9 (4)*	-30±34 (4)
HAEMORRHAGED	Vehicle, i.v.	396±16 (6)	394±17 (6)	+2±7 (6)	+2±9 (6)	-6±11 (6)
	sCT, i.v.			12±7 (6)	+10±5 (6)	+10±4 (6)
HAEMORRHAGED	Vehicle, i.v.	399±12 (15)	379±13 (15)	+5±14 (6)	0±16 (6)	-2±10 (5)
	Naloxone, i.v.			-27±21 (5)	+1±17 (5)	+12±18 (5)
NORMOTENSIVE	Vehicle, i.c.v	—	445±8 (13)	+18±6 (6)	+5±5 (6)	+12±10 (6)
	sCT, i.c.v			+11±8 (7)	+17±10 (7)	+33±9 (7)
HAEMORRHAGED	Vehicle, i.c.v	420±23 (4)	395±31 (4)	+2±13 (4)	+2±13 (5)	+6±12 (5)
	sCT, i.c.v			+10±8 (5)	+18±11 (5)	+27±11 (4)

Animals received vehicle (0.9% NaCl with 1% BSA), naloxone (10 $mg.kg^{-1}$) or sCT (10 $U.kg^{-1}$) in a volume of 0.1 ml i.v., or received vehicle (100 mM NaCl, 1% BSA in 50 mM Tris at pH 7.4) or sCT (10 $U.kg^{-1}$) in a volume of 10 µl i.c.v. at t = 0. Heart rate was not significantly altered by haemorrhage or pithing ($P > 0.05$). Values are $\bar{x} \pm$ s.e. (n)
* $P \leq 0.05$ compared to internal control (vehicle) response.

Figure R.20 Hypotensive Response of Normotensive Rats to leu-enkephalin after Pretreatment with Naloxone, sCT or Vehicle.

Animals were pretreated with vehicle, naloxone or sCT (in 0.1 ml, i.v.) and the effect of leu-enkephalin on MAP was assessed 20-100 minutes thereafter. All animals received each dose of leu-enkephalin and the consequent reduction in MAP is expressed as a percentage of the reduction in MAP produced by the maximal dose of leu-enkephalin (6×10^{-7} mol.kg^{-1}). This maximum dose of leu-enkephalin produced a transient hypotensive response of -11.5 ± 1.9 mm Hg. Values are $\bar{x} \pm$ s.e., $n = 6$.

ANOVA revealed evidence for interaction between naloxone and leu-enkephalin ($P < 0.01$) but no evidence for interaction between sCT and leu-enkephalin ($P > 0.1$).

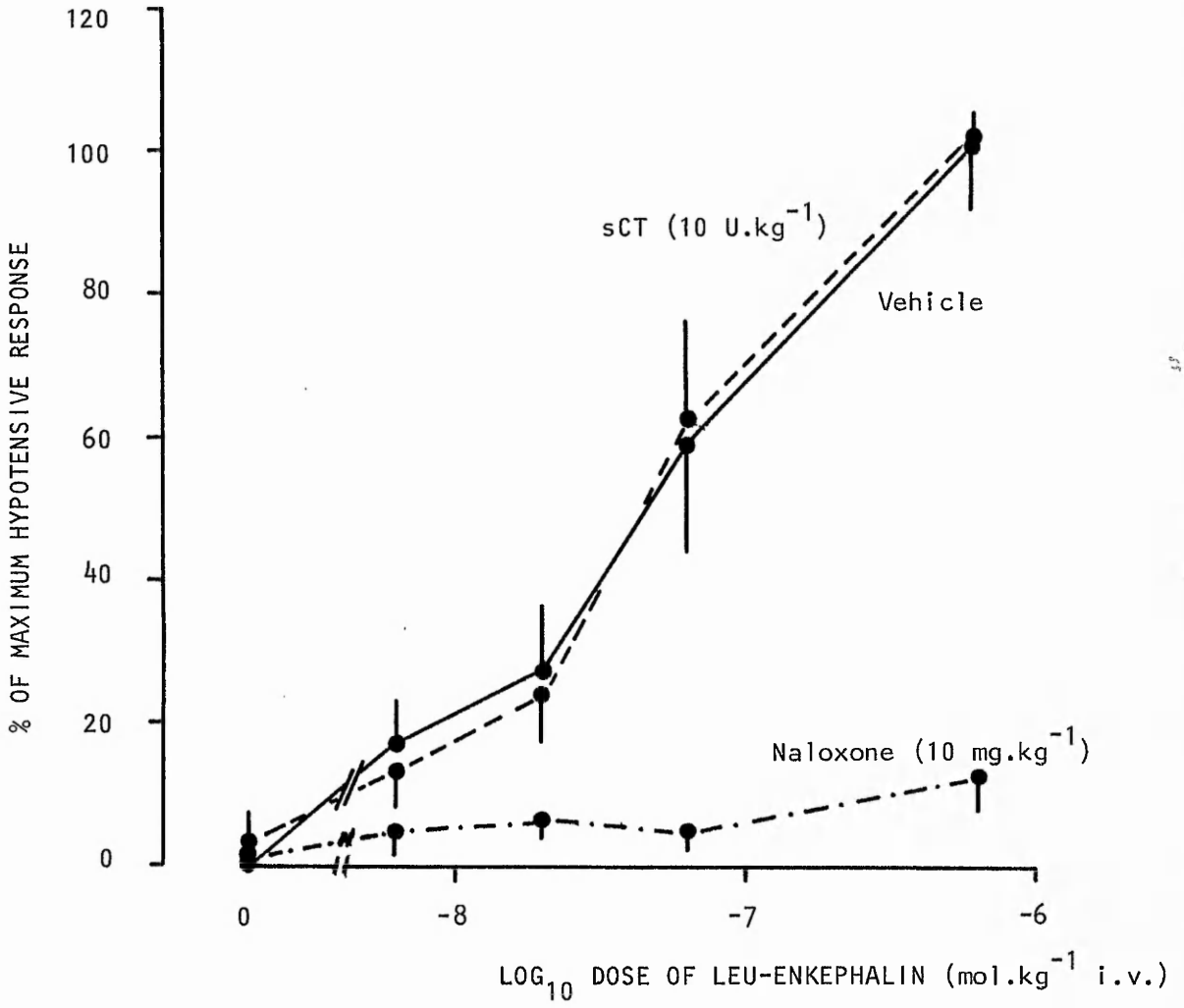

120
100
80
60
40
20
0
% OF MAXIMUM HYPOTENSIVE RESPONSE
sCT (10 U.kg^{-1})
Vehicle
Naloxone (10 mg.kg^{-1})
0
-8
-7
-6
LOG$_{10}$ DOSE OF LEU-ENKEPHALIN (mol.kg^{-1} i.v.)

Figure R.21 Pressor Effect of i.v. sCT in Pooled Pretreatment Control Group of Haemorrhaged Rats.

The initial MAP of 113.3 ± 2.5 mm Hg was reduced to 87.4 ± 5.1 mm Hg by removal of arterial blood. Values are $\bar{x}$ ± s.e., values of n are shown.

* $P < 0.05$ compared to internal control (vehicle) response.

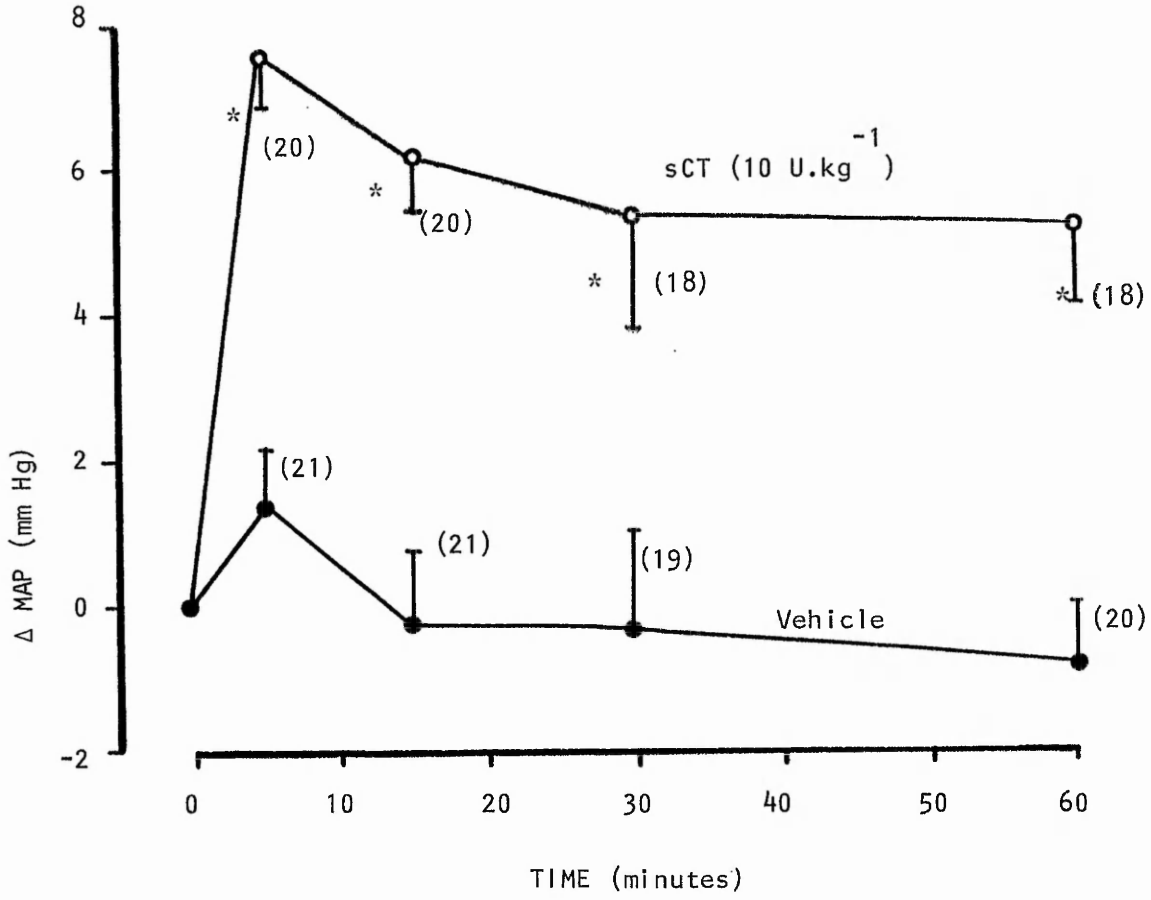

8
6
4
2
0
-2
Δ MAP (mm Hg)
*
(20)
*
(20)
*
(18)
*
(18)
sCT (10 U.kg-1)
(21)
(21)
(19)
Vehicle
(20)
0
10
20
30
40
50
60
TIME (minutes)

Figure R.22 Lack of Effect of Bilateral $V_a$gotomy on the Pressor Response of Haemorrhaged Rats to i.v. sCT.

Initial MAP of 122.4 $\pm$ 4.3 mm Hg was reduced to 95.9 $\pm$ 4.7 mm Hg by removal of arterial blood. Animals received bilateral vagotomy 5-10 minutes after haemorrhage. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.1$ (compared to internal control (vehicle) response).

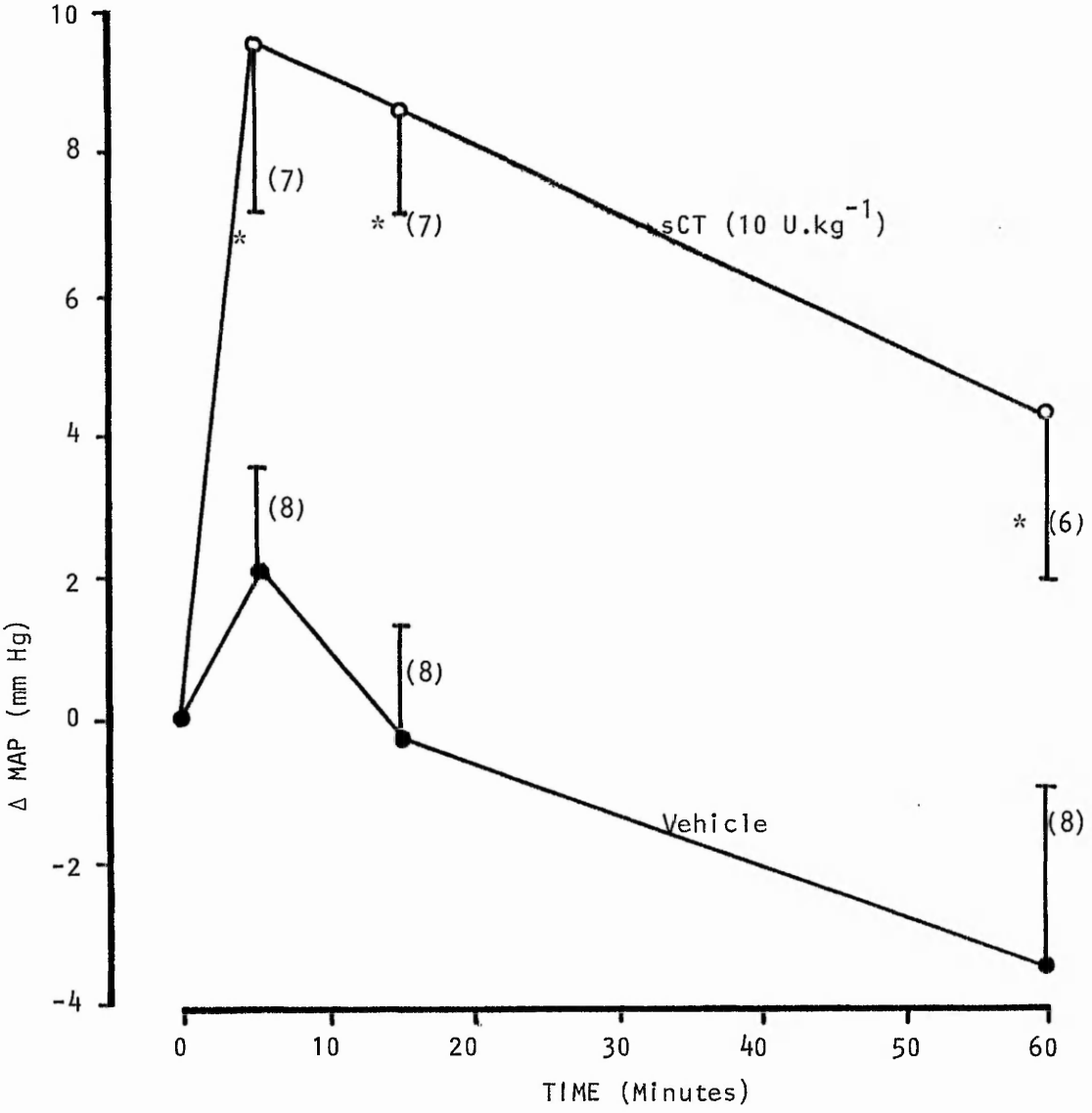

10
8
6
4
2
0
-2
-4
Δ MAP (mm Hg)
(7)
*
* (7)
sCT (10 U.kg^{-1})
(8)
* (6)
(8)
Vehicle
(8)
0
10
20
30
40
50
60
TIME (Minutes)

Figure R.23 Effect of Vasopressin Antagonist on the Pressor Response of Pithed Rats to Arginine Vasopressin.

Animals were pithed (MAP 52.0 ± 7.9 mm Hg, n = 6) and the effect of i.v. arginine vasopressin on MAP was determined approximately 10 (——) and 180 (- - - -) minutes after vehicle (●) or d(CH_2)Tyr(Me) AVP (○, 100 $g.kg^{-1}$). Values are means of four determinations.

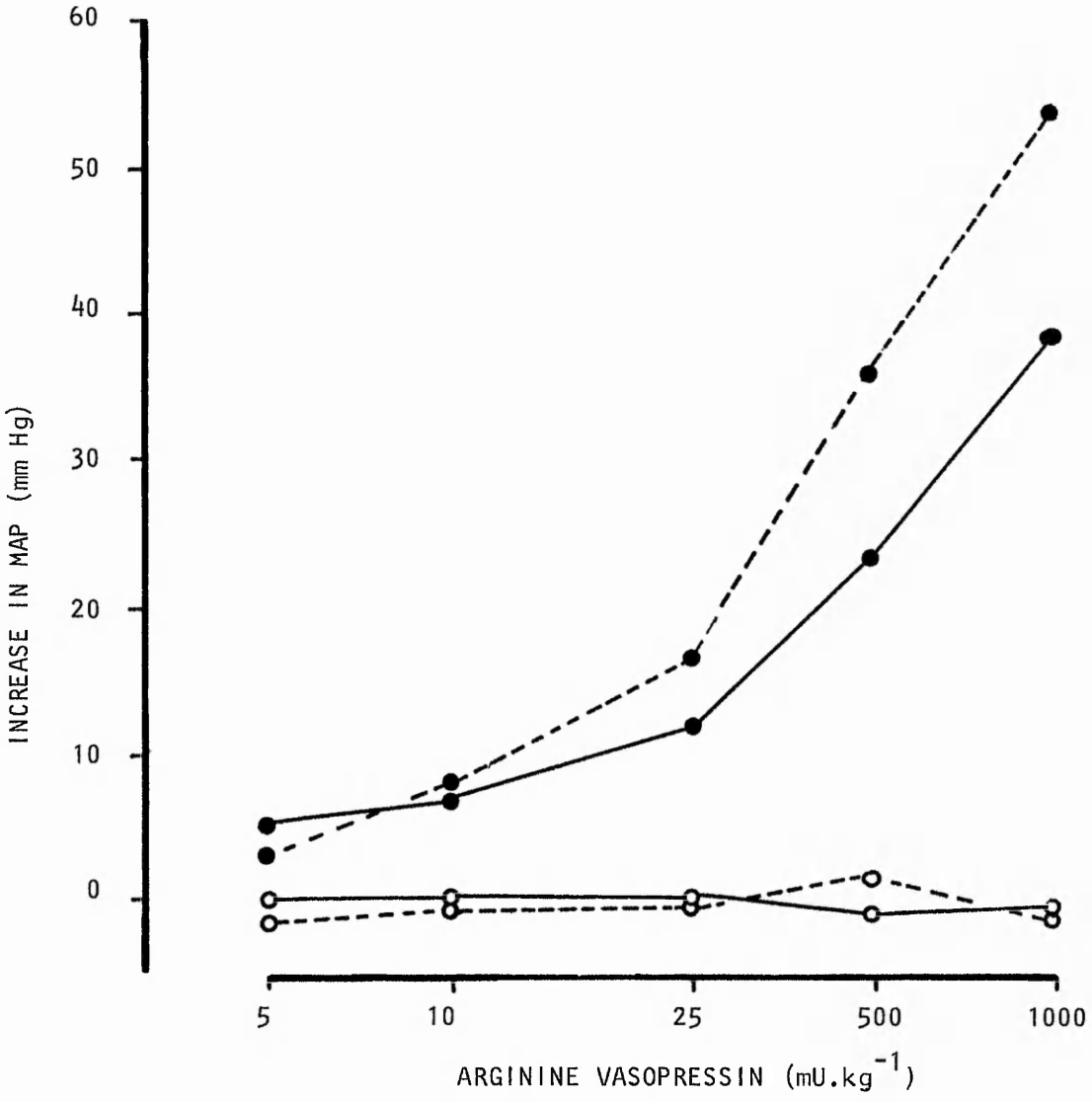

60
50
40
30
20
10
0
INCREASE IN MAP (mm Hg)
5
10
25
500
1000
ARGININE VASOPRESSIN (mU.kg^{-1})

Figure R.24 Lack of Effect of Vasopressin Antagonist on the Pressor Response of Haemorrhaged Rats to i.v. sCT.

The initial MAP of 104.5 ± 3.9 mm Hg was reduced to 83.7 ± 4.3 mm Hg by removal of arterial blood. $d(CH_2)_5Tyr(Me)AVP$ (100 $\mu g.kg^{-1}$) was administered i.v. 5 minutes prior to haemorrhage. Values are $\bar{x}$ ± s.e., values of n are shown.

* $P < 0.05$ compared to internal control (vehicle) response.

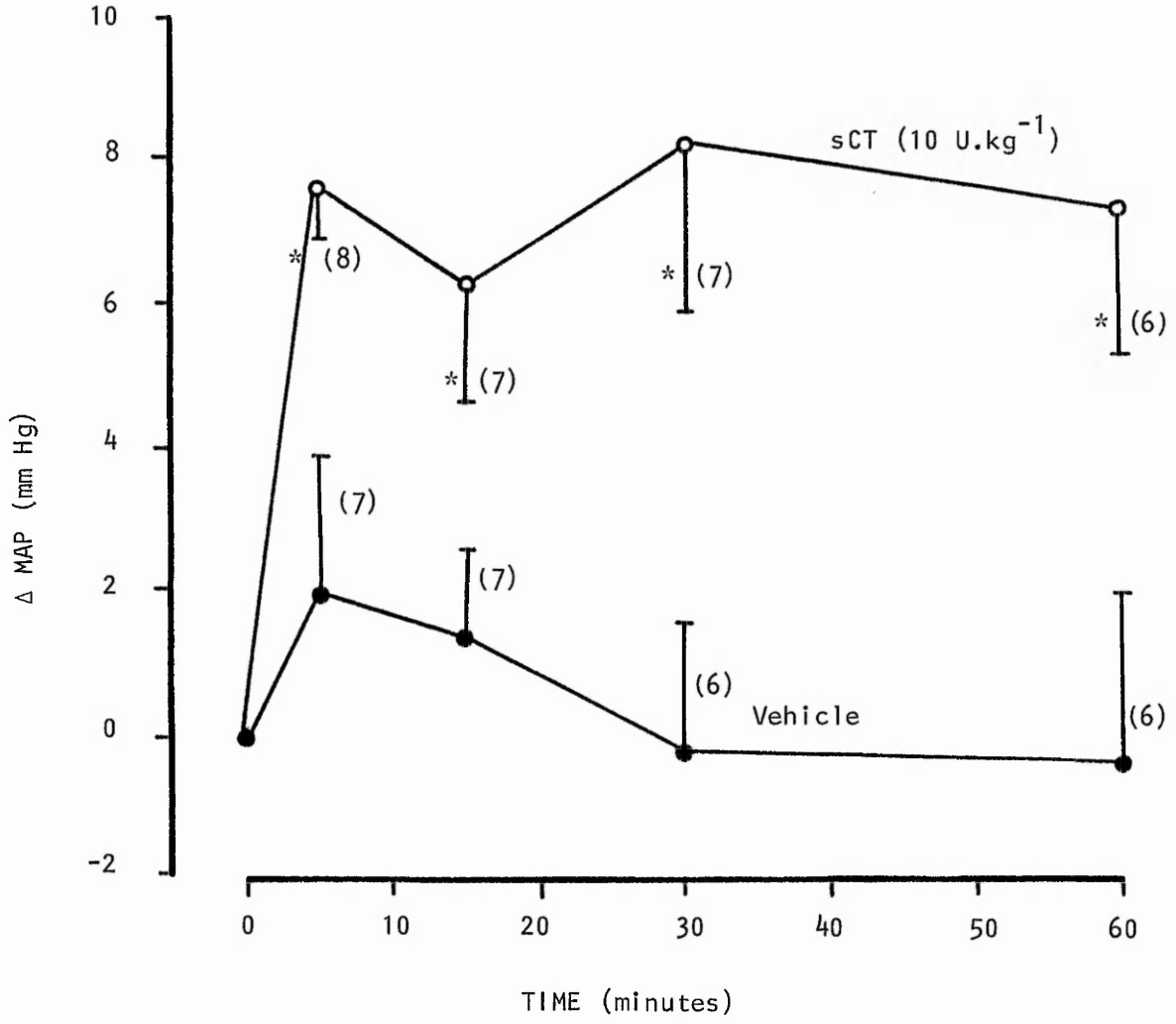

10
8
6
4
2
0
-2
Δ MAP (mm Hg)
sCT (10 U.kg^{-1})
* (8)
* (7)
* (7)
* (6)
(7)
(7)
(6)
Vehicle
(6)
0
10
20
30
40
50
60
TIME (minutes)

Figure R.25 Antagonism of the Pressor Response of Haemorrhaged Rats to i.v. sCT with 6-hydroxydopamine.

Animals pretreated with 6-OHDA (100 $mg.kg^{-1}$ i.p. in 0.1% ascorbic acid 4, 3 and 2 days prior to experimental use) had an initial MAP of 92.0 $\pm$ 3.2 mm Hg which was reduced to 75.4 $\pm$ 1.7 mm Hg by removal of arterial blood. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$ compared to internal control (vehicle) response .

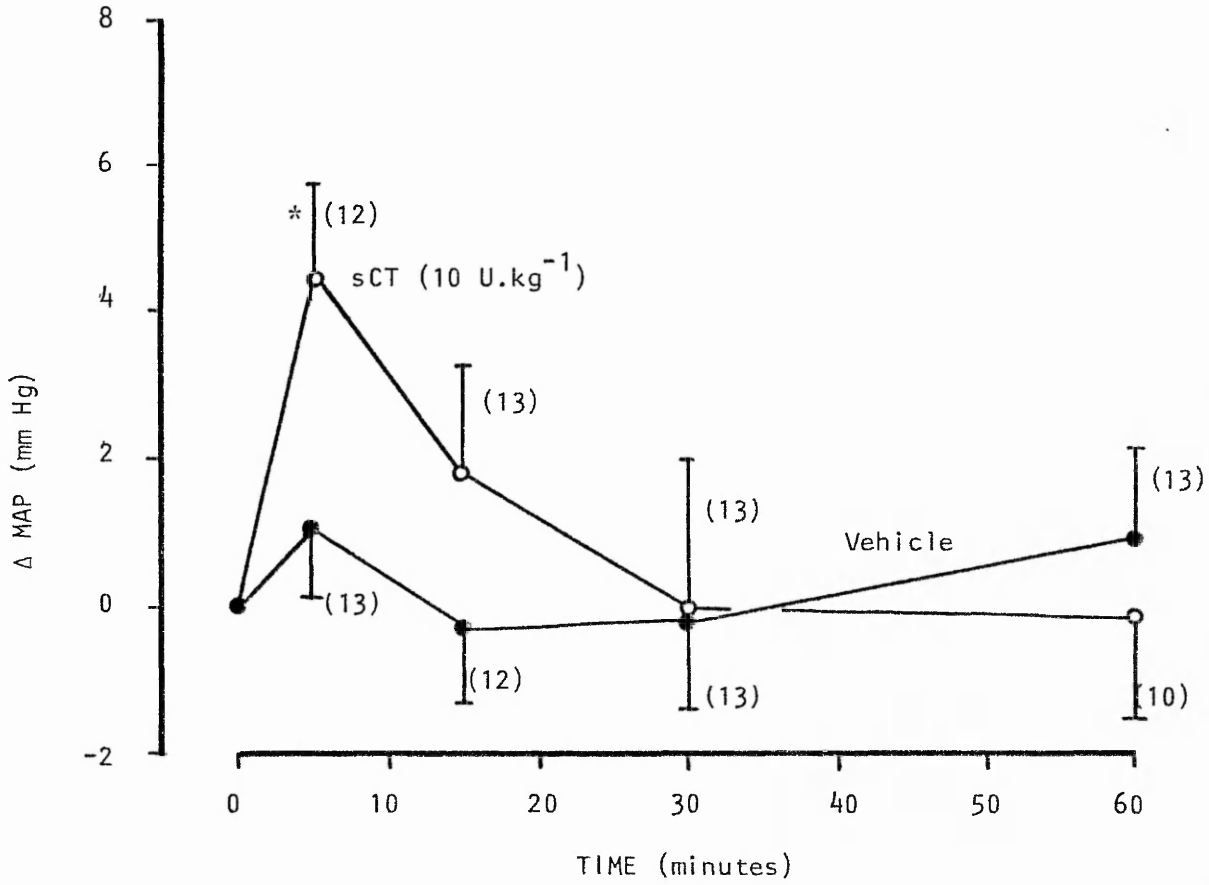

8
6
4
2
0
-2
Δ MAP (mm Hg)
*
(12)
sCT (10 U.kg^{-1})
(13)
(13)
(13)
Vehicle
(13)
(12)
(13)
(10)
0
10
20
30
40
50
60
TIME (minutes)

Table 6 Lack of Effect of Pretreatment and Administration of sCT and Vehicle on Heart Rate

Pretreatment	Drug,	Heart Rate (b.p.m.)		Δ Heart Rate (b.p.m.)		
		Initial	t = 0	t = 5	t = 15	t = 60
Grouped Control	Vehicle	407±10 (18)	373±12 (14)	+6±2 (13)	+7±4 (18)	+7±7 (18)
	sCT			+1±4 (17)	+7±3 (19)	+8±5 (18)
Bilateral Vagotomy	Vehicle	418±17 (6)	424±22 (5)	0±3 (7)	+3±2 (7)	+13±8 (6)
	sCT			+10±6 (5)	+8±4 (5)	+12±2 (5)
Chemical Sympathectomy	Vehicle	405±15 (11)	396±12 (10)	+4±4 (10)	+5±5 (13)	+22±8 (12)
	sCT			+11±5 (11)	+19±6 (11)	+22±8 (12)
Vasopressin Antagonist	Vehicle	415±22 (6)	385±22 (4)	-2±3 (6)	+7±9 (6)	+10±8 (6)
	sCT			+8±5 (6)	+12±5 (6)	+15±6 (6)

Groups of animals were pretreated as described in methods and rendered hypotensive by removal of arterial blood 20 minutes prior to administration of vehicle (0.9% NaCl with 1% BSA) or sCT (10 $U.kg^{-1}$) in a volume of 0.1 ml i.v. Values are $\bar{x} \pm$ s.e. (n). Initial heart rate did not differ between groups and haemorrhage did not significantly alter the heart rate. Similarly comparison of sCT and vehicle responses revealed no significant differences

Figure R.26 Pressor Effect of i.c.v. sCT in Normotensive Rats.

The initial MAP was 113.2 $\pm$ 4.3 mm Hg. Values are $\bar{x} \pm$ s.e., n = 5-6 except pooled vehicle where n = 16

** P < 0.01 compared to internal control (vehicle) response.

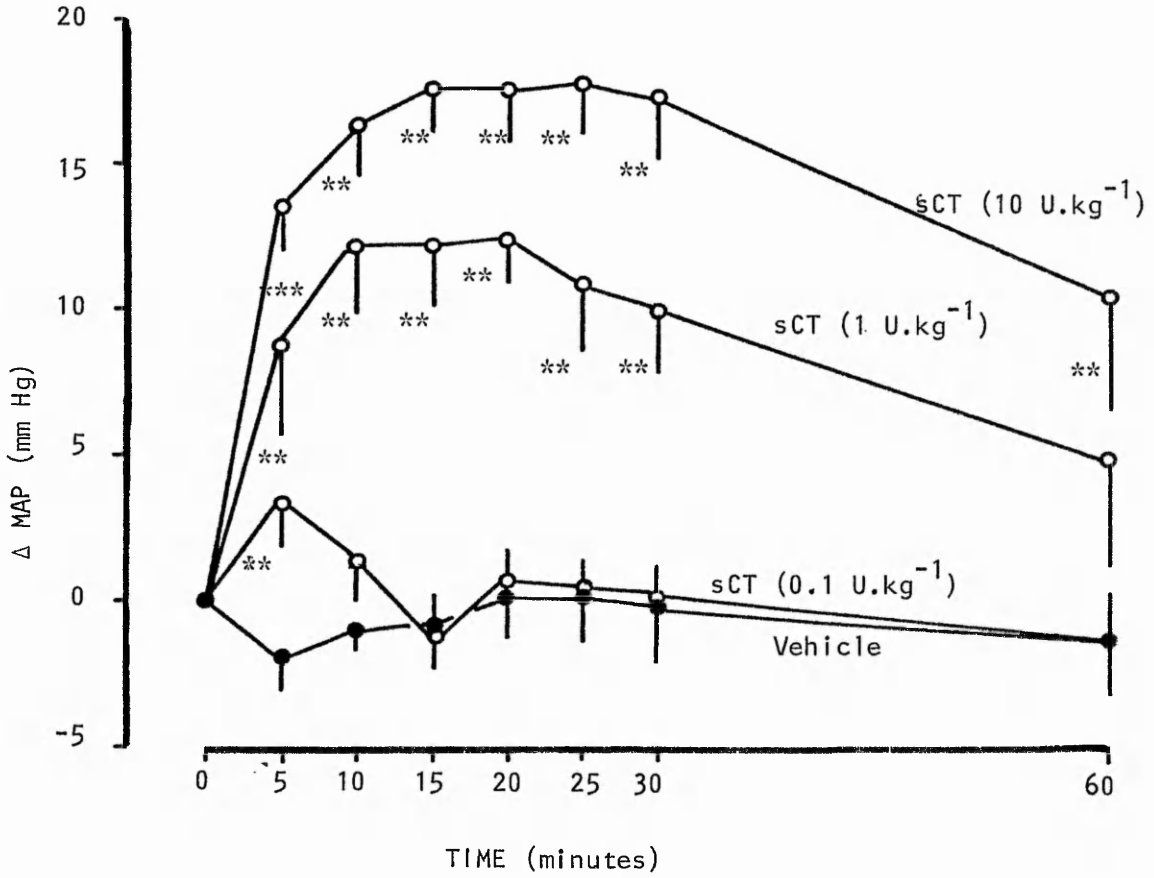

20
15
10
5
0
-5
Δ MAP (mm Hg)
sCT (10 U.kg^{-1})
sCT (1 U.kg^{-1})
sCT (0.1 U.kg^{-1})
Vehicle
0
5
10
15
20
25
30
60
TIME (minutes)

Figure R.27 Pressor Effect of i.c.v. sCT after Haemorrhage

The initial MAP of 121.2 ± 3.2 mm Hg (n = 14) was reduced to 90.9 ± 2.8 mm Hg by removal of arterial blood. Values are $\bar{x}$ ± s.e., n = 5-6 except pooled vehicle where n = 14.

* $P < 0.05$; ** $P < 0.01$ (compared to internal control (vehicle) response).

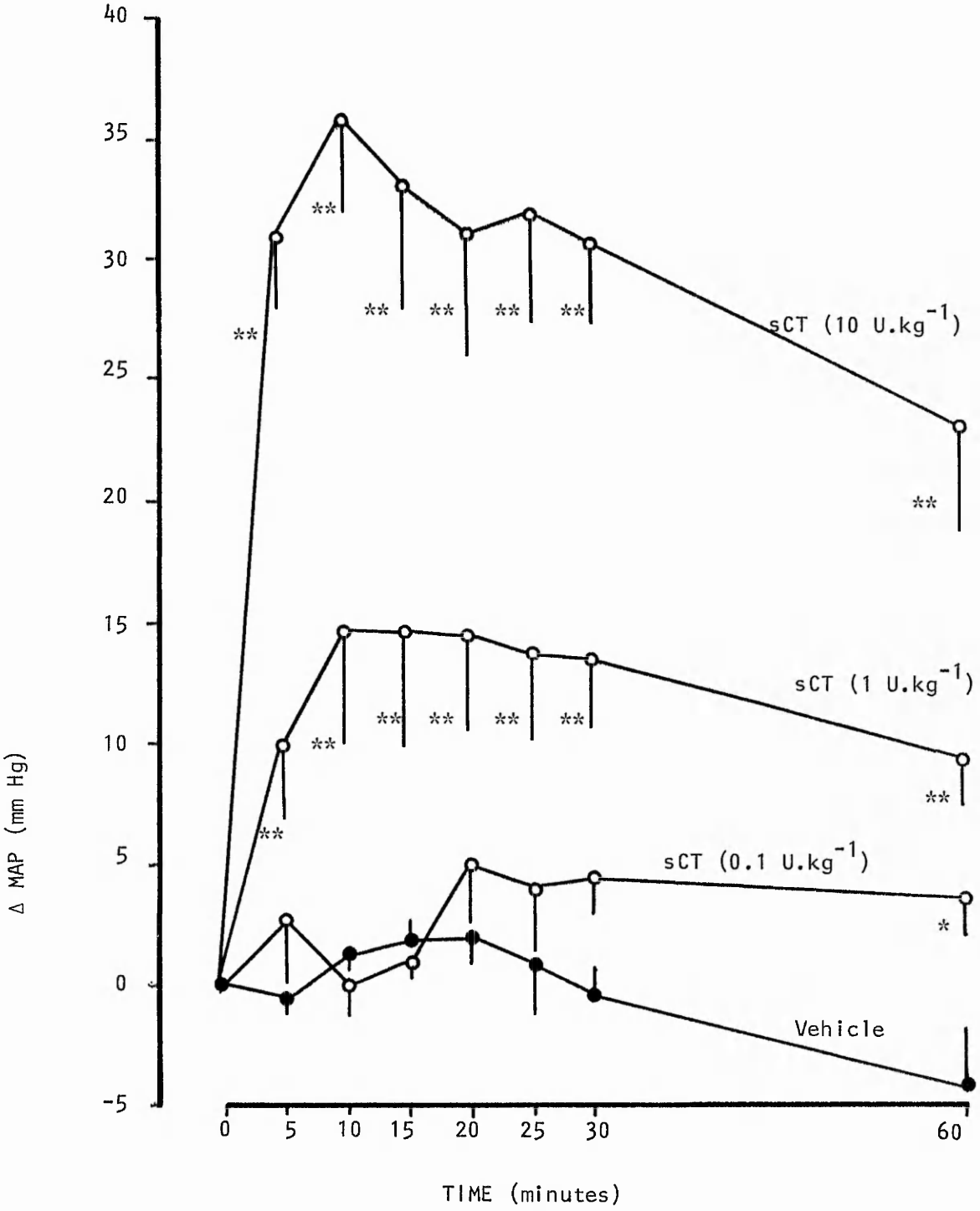

40
35
30
25
20
15
10
5
0
-5
Δ MAP (mm Hg)
sCT (10 U.kg-1)
sCT (1 U.kg-1)
sCT (0.1 U.kg-1)
Vehicle
**
*
0
5
10
15
20
25
30
60
TIME (minutes)

Figure R.28 Pressor Effect of i.c.v. sCT Haemorrhage and Pretreatment with Vehicle for 6-OHDA.

The initial MAP of 126.9 ± 6.6 mm Hg (n = 12) was reduced to 101.2 ± 6.6 mm Hg by removal of arterial blood. Values are $\bar{x}$ ± s.e., n = 5-6 except pooled vehicle where n = 12.

* $P < 0.05$, ** $P < 0.01$ (compared to internal control (vehicle) response).

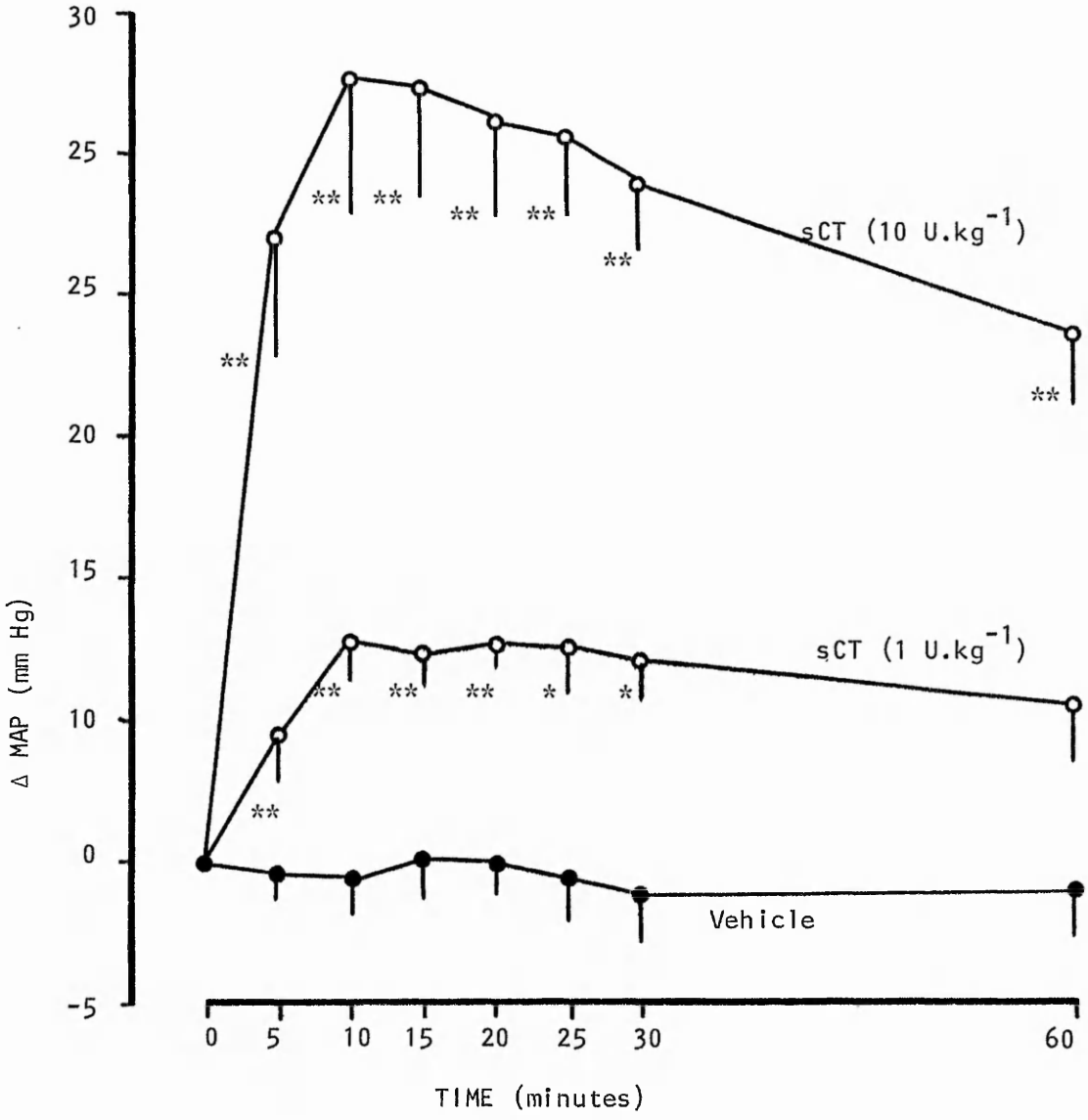
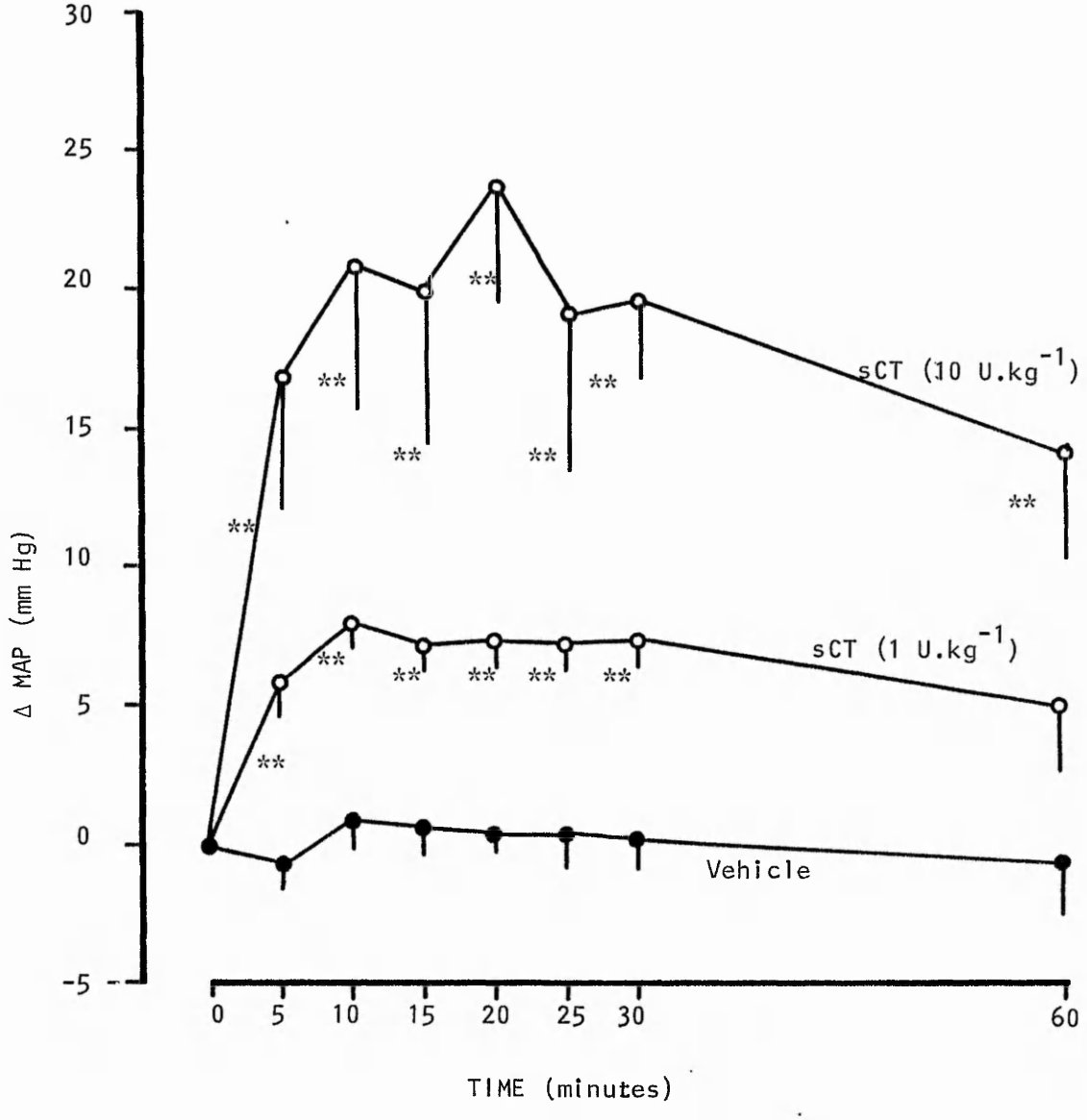

30
25
25
20
15
10
0
-5
Δ MAP (mm Hg)
sCT (10 U.kg-1)
sCT (1 U.kg-1)
Vehicle
**
*
0
5
10
15
20
25
30
60
TIME (minutes)

Figure R.29 Pressor Effect of i.c.v. sCT after Haemorrhage and 6-OHDA Pretreatment.

The initial MAP of 107.1 ± 3.9 mm Hg (n = 10) was reduced to 86.8 ± 3.6 mm Hg by removal of arterial blood. Values are $\bar{x}$ ± s.e., n = 5 except pooled vehicle where n = 10.

* $P < 0.05$; ** $P < 0.05$ (compared to internal control (vehicle) response).


30
25
20
15
10
5
0
-5
Δ MAP (mm Hg)
sCT (10 U.kg^{-1})
sCT (1 U.kg^{-1})
Vehicle
**
0
5
10
15
20
25
30
60
TIME (minutes)

R.5 ACCUMULATION OF ^{45}CALCIUM BY RAT BRAIN SLICES

Introduction

It is generally accepted that the major physiological action of calcitonin in humans is on bone, where it directly inhibits bone resorption (Friedman and Raisz, 1965). Calcitonin has also been shown to inhibit calcium efflux from other tissues including kidney (Borle, 1969) and liver (Yamaguchi et al, 1976). Such an effect could clearly explain the calcitonin induced increase in calcium content of kidney (Kenny and Heiskell, 1965), liver (Yamaguchi et al, 1976) and myocardium (Chausmer et al, 1965). Centrally CT has been found to inhibit calcium uptake by hypothalamic explants (Morley and Levine, 1981) and slices (Koida, et al, 1982). As numerous biological processes are known to be calcium, or calcium-calmodulin, dependent, it is apparent that a direct effect of CT on calcium distribution could be the mechanism by which the pharmacological effects of the hormone are brought about. The uptake of $^{45}Ca^{2+}$ by slices of rat brain has, therefore, been used as a model to study the possible effects of sCT on central calcium metabolism. An inherent problem in the use of radioisotopes to follow the transport of calcium in living tissues is the large proportion of calcium found in extracellular free and bound pools as compared to the small intracellular compartments which are often of greater interest. Preliminary experiments were therefore designed to establish a suitable experimental protocol for assessment of the uptake of radioisotopes into the intracellular compartment of the tissue. This experimental protocol was then used in determination of the effects of lanthanum, sodium azide, potasium and sCT on uptake of the radiosotope by brain slices.

Results

i) Effect of Duration of Wash

Preliminary experiments were performed in which brain slices were preincubated for 30 minutes, incubated in the presence of $^{45}Ca^{2+}$ for 6 minutes and then washed by transferring through a series of tubes

containing 5 ml of iced incubation medium without radiolabel. The radioactivity in the tissue at various times after termination of the incubation was then calculated from the activity of the separate washing solutions and from that remaining in the tissue slices. A plot of calculated calcium uptake (expressed as a % of that which would have been obtained without washing) against time (figure R.30) reveals that approximately 60% of the isotope was removed by a single wash of 1 minute duration. Pharmacokinetic analysis of this data (figure R30, inset) revealed that efflux of $^{45}Ca^{2+}$ behaved essentially as efflux from a two compartment system, following first order rate kinetics. Hence, $^{45}Ca^{2+}$ is removed rapidly, $t_{\frac{1}{2}}$ = 1.0 minutes, from a compartment with V_d of 55% of the tissue $^{45}Ca^{2+}$, and more slowly, $t_{\frac{1}{2}}$ = 15.7 minutes, from a second compartment with a V_d of 27% of tissue $^{45}Ca^{2+}$. As washout of the extracellular marker ^{3}H-inulin, from brain behaves as efflux from a rapidly eliminated single compartment ($t_{\frac{1}{2}}$ = 2.7 minutes, data not shown), it can be assumed that this compartment represents extracellular free calcium. Using the efflux data plotted above it has been calculated that the 30 minute wash in iced incubation medium, used in all subsequent experiments, would be expected to remove over 99% of the extracellular free radiolabel, and approximately 70% of the remainder.

ii) Effect of Lanthanum Wash

As the rapid phase of $^{45}Ca^{2+}$ efflux from the brain slices is thought to represent elimination of extracellular free calcium, it can be assumed that the slow elimination compartment represents all remaining sources of calcium, namely extracellular bound, and intracellular free and bound calcium. Van Breemen, Farinas, Gerba and McNaughton (1972) have used a wash in 2 mM lanthanum chloride to displace extracellular calcium from binding sites in rabbit aorta and this technique can therefore be used to determine the extent to which extracellular binding contributes to the observed apparent calcium uptake. Apparent calcium uptake was determined in brain slices preincubated for 30 minutes prior to incubation for 6 minutes in the presence of $^{45}Ca^{2+}$ and washing for a further 30 minutes in iced incubation medium containing 0 or 2 mm $LaCl_3$. This experiment was performed using a Hepes buffered incubation medium as addition of $LaCL_3$ to the modified Krebs' solution used elsewhere resulted in precipitation of lanthanum phosphates. The apparent calcium uptake

of 0.87 ± 0.04 mol.g^{-1} (n = 12) was reduced to 0.72 ± 0.06 mol.g^{-1} (n = 8) by inclusion of 2 mM $LaCl_3$ in the wash solution. These results indicate that approximately 18% of the estimated calcium uptake can be attributed to binding at extracellular sites, and that the majority of $^{45}Ca^{2+}$ uptake obtained in subsequent experiments (30 minute wash without $LaCl_3$) represents uptake of the radiolabel into the cells of the tissue slice.

iii) Effect of Preincubation Time

Borle (1981) has argued that steady state conditions are needed for the study of calcium transport in vitro, as the lack of equilibrium between tissue and incubation media can lead to artefactual results. In an investigation of the effect of preincubation time, apparent calcium uptake did not differ with preincubation periods of 10, 30 and 60 minutes (0.77 ± 0.04, 0.68 ± 0.01, 0.79 ± 0.07 µmol.g^{-1} respectively (n = 6). However, a relatively long period of 30 minutes was chosen for subsequent experiments as Varon and McIlwain (1961) have shown that incubation of brain tissues can result in tissue swelling, particularly during the first 10-15 minutes of incubation, which could be associated with artefactual ion fluxes, associated with anoxia and tissue swelling due to loss of intracranial pressure.

iv) Effect of Incubation Time

Apparent calcium uptake was determined after incubation for 6-60 minutes in either normal (5.9 mM K^+) or depolarising (56 mM K^+) incubation medium (figure R31). The uptake which was greater at all times in depolarising medium, increased in a hyperbolic fashion as previously demonstrated by Cooke and Robinson (1971) but did not appear to be maximal after an incubation of 60 minutes. This data provides further evidence that a suitable preincubation period has been chosen as Borle (1981) has found that incubation in non-steady conditions often results in non-hyperbolic uptake curves. The period of 6 minutes was chosen for all subsequent incubations as the use of a short incubation time has the advantage of minimising redistribution of radio label taken up.

v) Effect of Sodium Azide, Potassium and Lanthanum

As shown in figure R.32, the apparent calcium uptake by rat midbrain slices in standard incubation conditions was increased by 26 ± 2.0% in the presence of 56 mM K^+ and by 88 ± 15% in the presence of 3mM sodium azide. In a further experiment using a Hepes buffered incubation medium, 0.2 and 2.0 mM $LaCl_3$ was found to reduce the apparent calcium uptake by 24 and 72% respectively (figure R.33).

vi) Effect of sCT in Normal and in Depolarising Media

The apparent calcium uptake in normal (59 mM K^+) medium was not modified in the presence of 5 or 50 nM sCT. However, the stimulatory effect of depolarising incubation medium (apparent calcium uptake increased 26% by 56 mM potassium in the absence of sCT) was not seen in the presence of 5 and 50 nM sCT (70 and 700 $U.l^{-1}$) as shown in figure R.34.

Discussion

When $^{45}Ca^{2+}$ is used to follow the uptake of calcium by tissues the radioisotope in the incubation medium becomes redistributed with time until eventually the ratio of isotope to calcium is equal in all intra- and extracellular exchangeable pools. Initially uptake of radioisotope predominates, but later as the intracellular concentration of radiolabel increases, efflux of radioisotope increases, so that ultimately the amount of radiolabel in the tissue stabilises at a level where uptake and efflux are equal. It is therefore necessary to distinguish between apparent calcium uptake, which is dependent on redistribution of $^{45}Ca^{2+}$ taken up from the medium, and actual calcium uptake, which is not. The results presented above indicate that use of a 30 minute preincubation period followed by a 6 minute incubation and a 30 minute wash enables assessment of apparent calcium uptake, chiefly into intracellular compartments. However, as the wash was estimated to remove as much as 70% of the extracellular bound and intracellular free and bound radiolabel, and efflux of radiolabel may be significant even in a short incubation period, the apparent calcium uptake presumably underestimates actual calcium uptake.

Using an experimental protocol similar to that described above, Cooke and Robinson (1971) demonstrated that uptake of $^{45}Ca^{2+}$ by slices of rat cerebral hemispheres was increased 46% by incubation in 50 mM potassium and by 84% on incubation with 3 mM sodium azide. Moreover, measurement of resting membrane potentials (average -50 mV) indicated maintenance of intracellular sodium and potassium in control medium. This data which is in good agreement with that presented above, indicates that viable brain tissues accumulate calcium by passive diffusion following the electrochemical gradient and that $^{45}Ca^{2+}$ accumulation can be increased by depolarisation with potassium or by use of the metabolic inhibitor sodium azide (prevention of active extrusion by Ca-ATPase). The marked inhibitory effect of the inorganic calcium antagonist, lanthanum, further suggests that the major route of calcium uptake is via calcium channels. These control experiments using sodium azide, depolarising concentrations of potassium, and lanthanum therefore provide evidence of the viability of the tissues used.

Levine and Morley (1981) found that i.c.v. sCT supressed both spontaneous and $CaCl_2$ (1.25 nmol, i.c.v.) induced feeding in rats, and supported their suggestion that calcitonin might produce its anorectic effect by an action on hypothalamic calcium metabolism by demonstrating an inhibitory effect of sCT on uptake of $^{45}Ca^{2+}$ by hypothalamic explants. Doses of 0.2 and 2.0 $U.ml^{-1}$ (approximately 15 and 150 nM) were found to reduce $^{45}Ca^{2+}$ uptake by 25 and 30% respectively. These concentrations were considered by the authors to be equivalent to those which inhibited feeding <u>in vivo</u>. A more detailed study by Koida et al (1982) demonstrated that sCT at concentrations of 10-1000 nM reduced $^{45}Ca^{2+}$ uptake by small blocks of rat hypothalamus by approximately 10-15%. This effect was observed only in the hypothalamus and only after an incubation of 60 minutes. Unfortunately these authors do not comment on the fact that Levine and Morley (1981) observed an inhibitory effect with an incubation period of only 10 minutes.

The lack of effect of sCT on unstimulated apparent calcium uptake by rat brain slices is in contrast to the reported inhibitory effect in the hypothalamus. This discrepency may reflect differences in experimental protocol, particularly, the duration of incubation and tissue used may be critical (the tissue used in these experiments contained midbrain, brainstem, thalamus and subthalmus in addition to

the hypothalamus). However, the fact that sCT at doses of 5 and 50 nM prevented the potassium induced stimulation of apparent calcium uptake provides further evidence that sCT can modify calcium fluxes of CNS derived tissues in vitro; specifically the hormone may play a regulatory role in depolarisation elicited calcium flux.

Finally, it has been suggested by Koida et al (1982) that sCT induced inhibition of $^{45}Ca^{2+}$ uptake by the hypothalamus could be used as a model to elucidate the cellular events involved in the central effects of CT. It should be noted, however, that interpretation of the results of such experiments is difficult when the contribution of extracellular free and bound calcium to the uptake of radiosiotope is not known (as is the case in the experiments of Levine and Morley, 1981 and Koida et al, 1982), and that accumulation of $^{45}Ca^{2+}$ is dependent on rates of uptake and efflux, and the size of the exchangeable pool. Such considerations warrant the use of more detailed pharmacokinetic analysis of uptake and efflux curves than has been used to date. Moreover, Koida and co-workers have found that IC_{50} for displacement of ^{125}I-sCT from rat hypothalamus by sCT (1.08 nM) to be considerably lower than the minimum effective dose for inhibition of $^{45}Ca^{2+}$ uptake, and that C' terminal fragments of sCT which displace ^{125}I-sCT are without effect on $^{45}Ca^{2+}$ uptake. It is therefore conceivable that this effect is not receptor mediated.

Figure R.30 Effect of Duration of Wash on Apparent Calcium Uptake by Slices of Rat Midbrain.

Apparent calcium uptake was calculated from the radioactivity of the washing medium and that remaining in the tissue after 60 minutes. The inset shown the same data with the natural log of the calcium uptake plotted on the y axis enabling 'peeling' procedures for determination of pharmacokinetic parameters of calcium efflux. Values are $\bar{x} \pm$ s.e., n = 4.

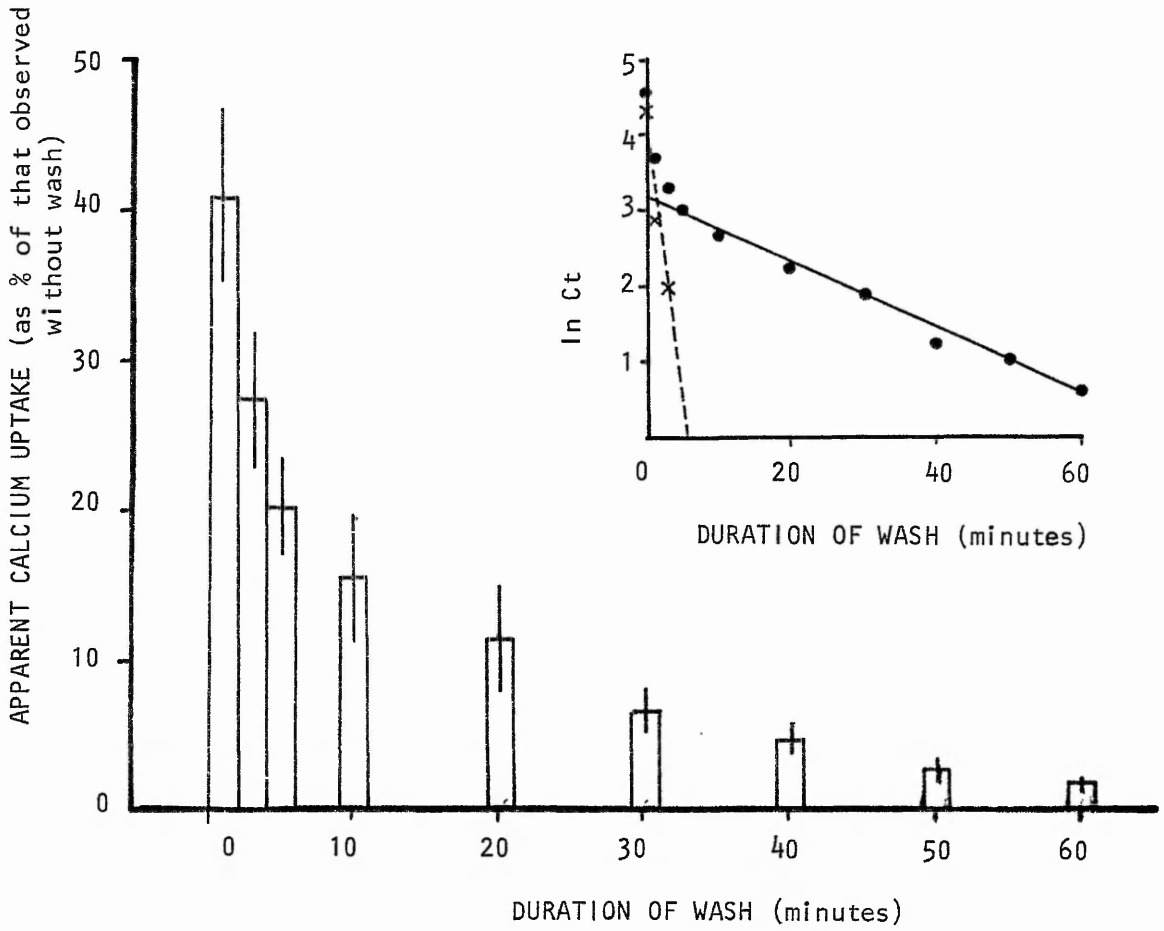

APPARENT CALCIUM UPTAKE (as % of that observed without wash)
50
40
30
20
10
0
0
10
20
30
40
50
60
DURATION OF WASH (minutes)
ln Ct
5
4
3
2
1
0
20
40
60
DURATION OF WASH (minutes)

Figure R.31 Effect of Duration of Incubation on Apparent Calcium Uptake.

Slices were preincubated for 30 minutes, prior to incubation in medium containing 56 mM (depolarising) or 5.9 mM (normal potassium), and $^{45}Ca^{2+}$ for the periods shown, and then washed for 30 minutes in iced Krebs. Values are $\bar{x} \pm$ s.e., n = 4.

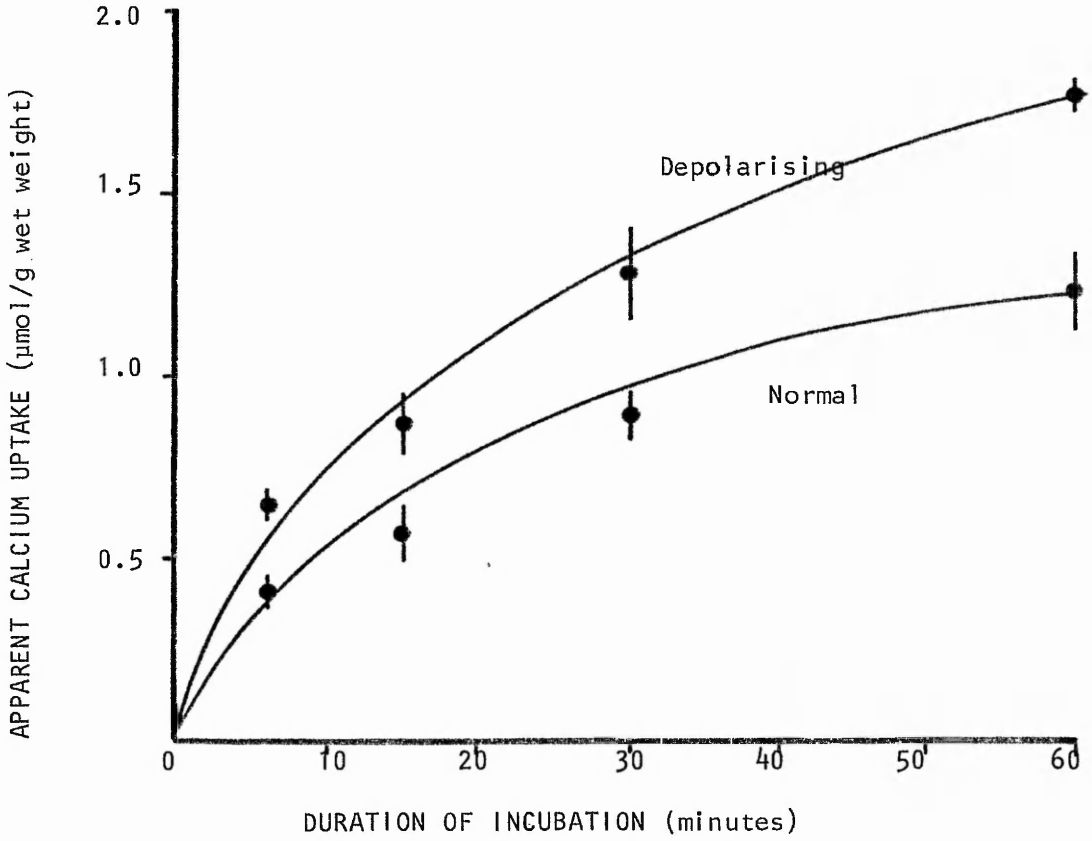

2.0
1.5
1.0
0.5
0
10
20
30
40
50
60
Depolarising
Normal
APPARENT CALCIUM UPTAKE (µmol/g wet weight)
DURATION OF INCUBATION (minutes)

Figure R.32 Effect of Potassium and Sodium Azide on Apparent Calcium Uptake.

The effect of 56 mM K^+ on apparent calcium uptake was assessed as described in R.30. Sodium azide was added (if appropriate) 10 minutes prior to incubation with $^{45}Ca^{2+}$. Values are $\bar{x} \pm$ s.e., values of n are shown.

** $P < 0.01$ (compared to control).

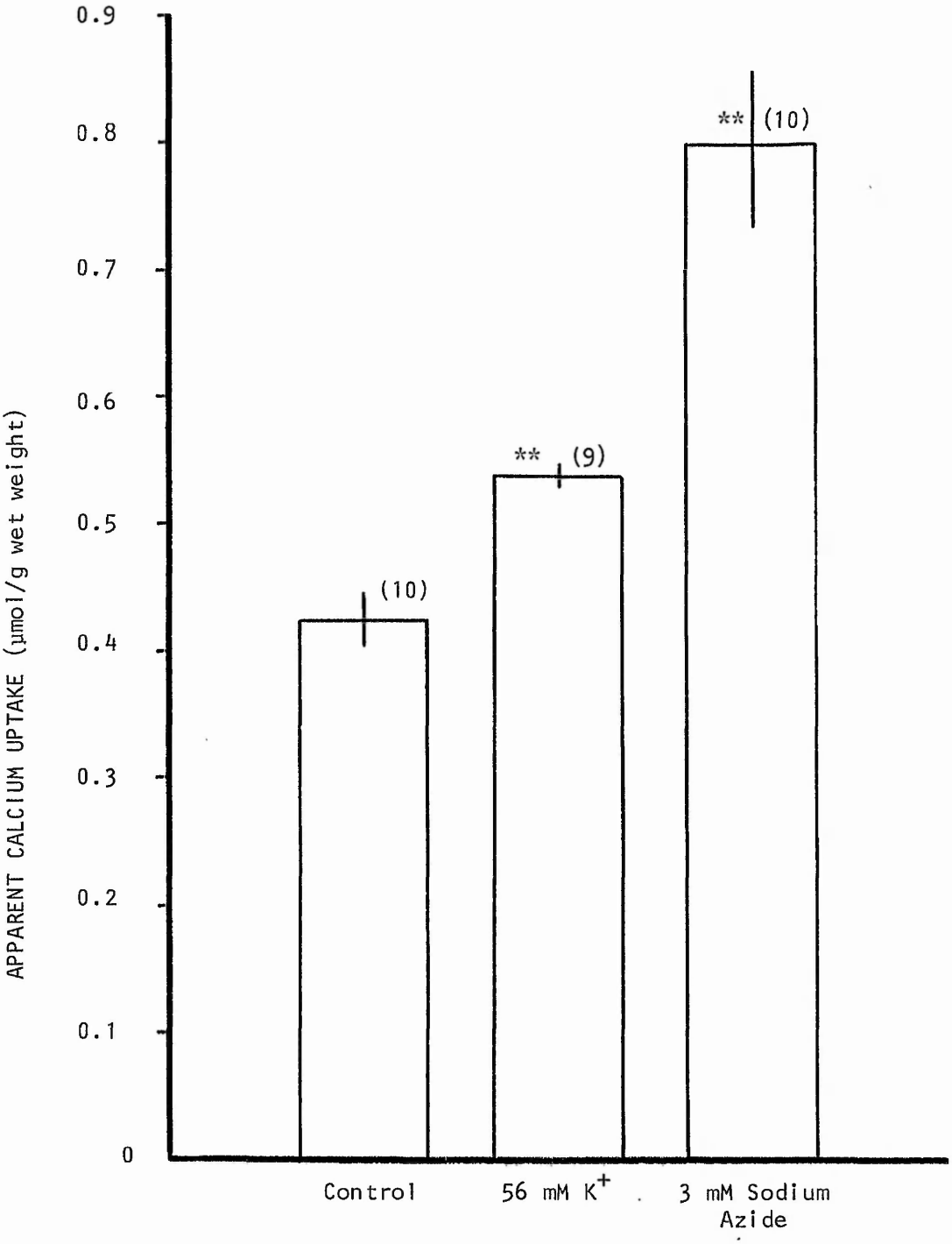

APPARENT CALCIUM UPTAKE (µmol/g wet weight)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(10)
** (9)
** (10)
Control
56 mM K+
3 mM Sodium Azide

Figure R.33 Effect of Lanthanum on Apparent Calcium Uptake.

This experiment was performed using Hepes buffered media (methods) $LaCl_3$ was added 10 minutes prior to $^{45}Ca^{2+}$. Values are $x \pm$ s.e., values of n are shown.

* $P < 0.05$; ** $P < 0.01$

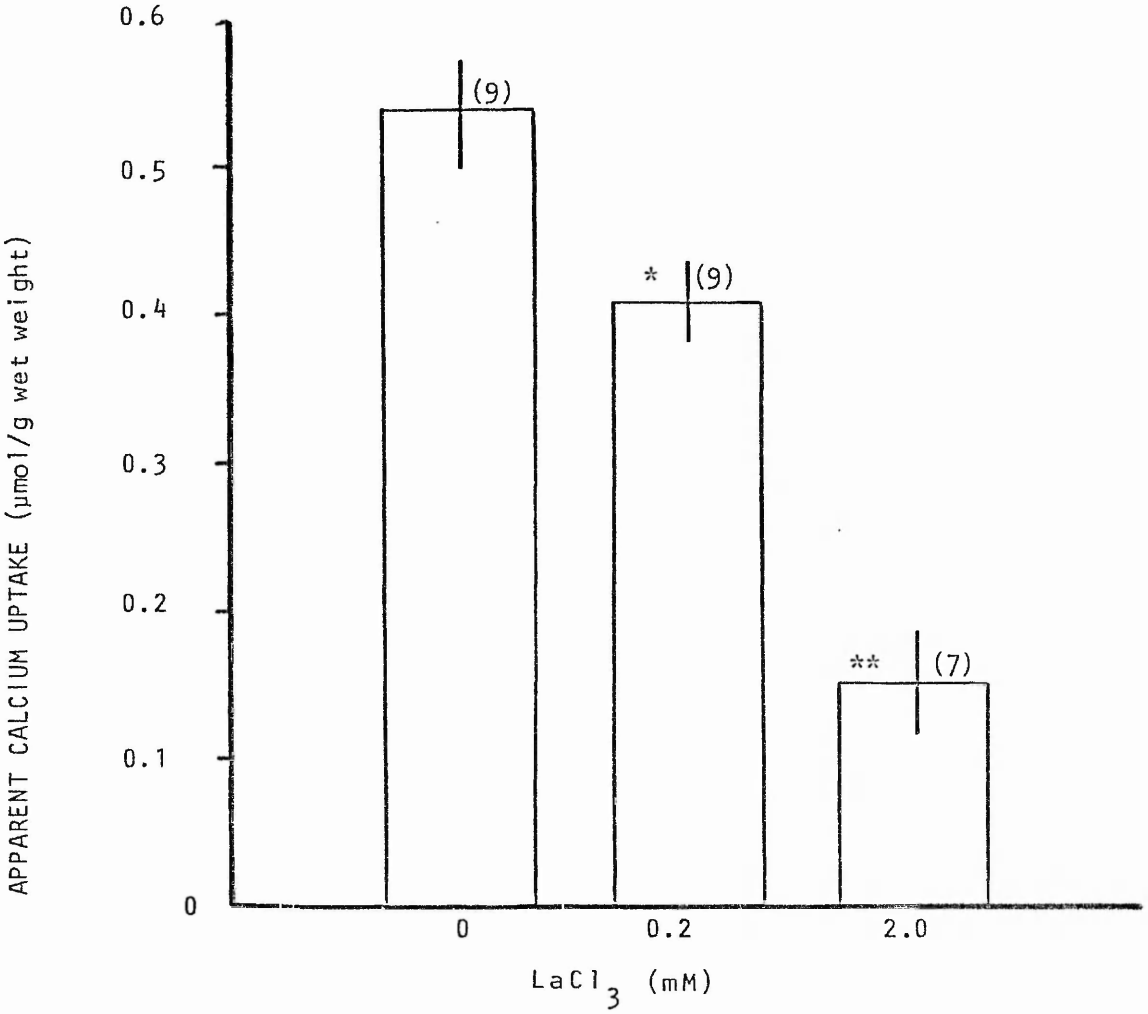
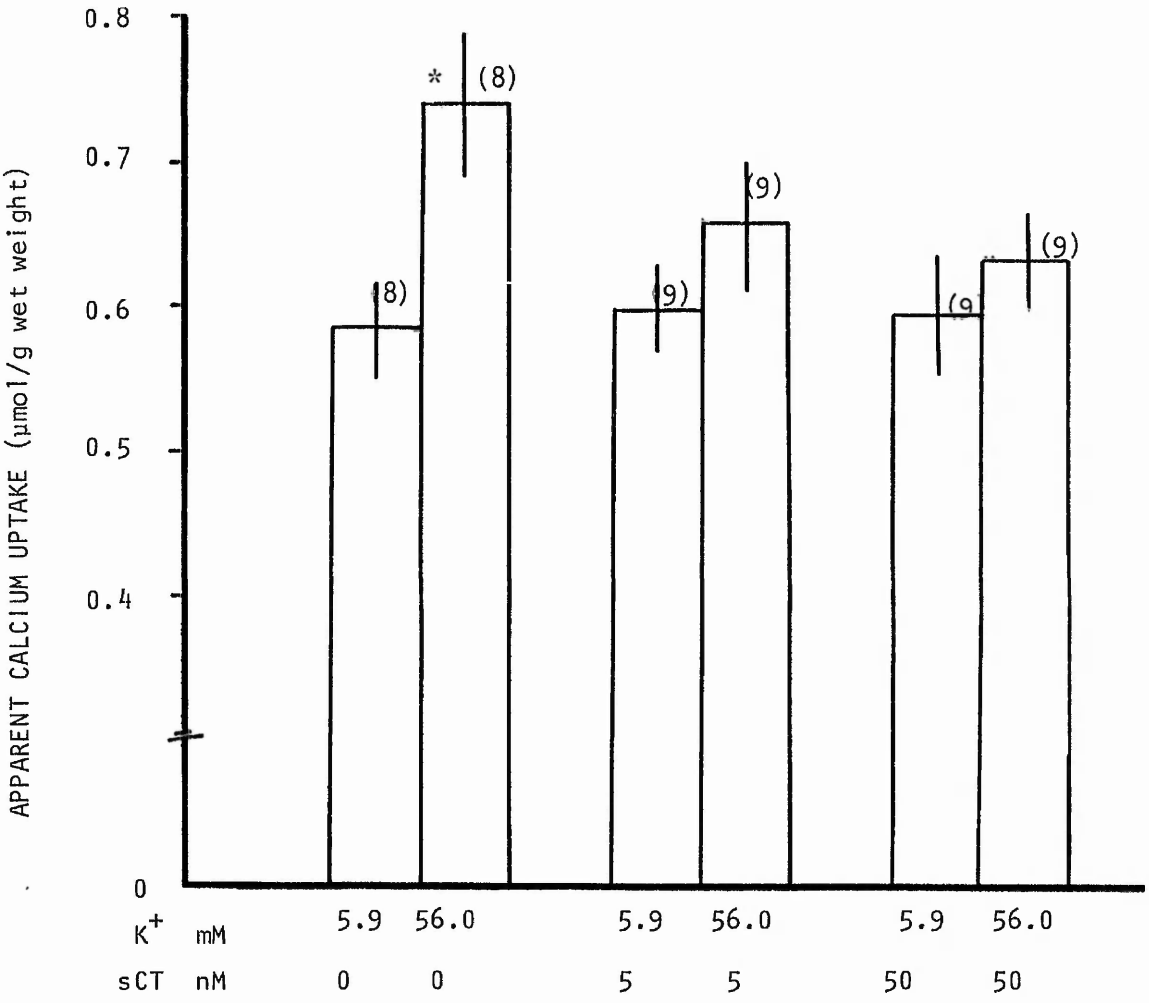

0.6
0.5
0.4
0.3
0.2
0.1
0
APPARENT CALCIUM UPTAKE (µmol/g wet weight)
(9)
* (9)
** (7)
0
0.2
2.0
LaCl3 (mM)

Figure R.34 Effect of sCT on Control and Potassium Stimulated Apparent Calcium Uptake.

Salmon CT was added to the incubation media 10 minutes prior to incubation in normal or depolarising solution. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$ compared to internal control (5.9 mM K^+).
5 nM sCT ≃ 70 $U.l^{-1}$


APPARENT CALCIUM UPTAKE (µmol/g wet weight)
0.8
0.7
0.6
0.5
0.4
0
*
(8)
(8)
(9)
(9)
(9)
(9)
K+ mM
5.9
56.0
5.9
56.0
5.9
56.0
sCT nM
0
0
5
5
50
50

R.6 CALCITONIN AND CENTRAL CYCLIC ADENOSINE 3', 5'-MONOPHOSPHATE METABOLISM

Introduction

It is now well established that numerous biologically active substances, including CT, can modify cyclic nucleotide metabolism in a wide range of tissues. Peripherally, CT has been found to elevate levels of cAMP in kidney, bone and mononuclear phagocytes and to increase adenylate cyclase activity in renal plasma membranes (I.9.b) Centrally, Loffler et al (1982) have reported CT to stimulate cAMP accumulation in cultured glial cells, yet paradoxically, adenylate cyclase activity of rat whole brain homogenates has been reported to be inhibited by CT (Rizzo and Goltzman, 1981). As modification of central cyclic nucleotide metabolism could clearly underlie the pharmacological effects of centrally administered CT, these previous experiments have now been extended by assessment of the effects of sCT on mouse brain cAMP and adenylate cyclase activity.

Results

i) Binding Assay Standard Curves

The protein binding assay used for cAMP is similar in principle to radioimmunoassay. In both cases a radiolabeled ligand is displaced from a high affinity binding site (on a naturally occurring protein for cAMP or on an antigen in radioimmunoassay) by unlabeled ligand. The proportion of radiolabel bound (determined as the specific activity in the presence of binding protein as a percentage of the activity in the same amount of labeled ligand in the absence of binding protein) is therefore dependent on the concentration of unlabeled ligand as shown in figure R.35 (inset). To simplify calculations a linear calibration curve can be obtained by calculating the activity in the absence of unlabeled ligand (C_o) divided by the activity in the presence of the standard (C_x) and plotting this value (C_o/C_x) against the concentration of the standards. The standard curves obtained for cAMP were linear over the range 1-16 pmol/sample.

ii) Relationship between Sample Concentration and cAMP Content

As shown above, when using the protein binding assay for cAMP a standard curve can be constructed which is linear over the range of 1-16 pmol per 50 µl sample of standard. It was, however, considered prudent to investigate the effect of tissue homogenate on the binding assay and to determine the relationship between sample concentration and cAMP content.

To investigate the possibility that the homogenate might interfere with the protein binding assay a standard curve was prepared from standards diluted in homogenate (one hypothalamus in 5 ml of 50 mM Tris/EDTA buffer at pH 7.4) and compared to a normal standard curve prepared by dilution of cAMP in buffer alone. The curve prepared in the presence of homogenate had a lower gradient than the normal standard curve, presumably reflecting non-specific binding of cAMP to the homogenate (figure R.36.b). However, as both standard curves appeared linear, non-specific binding is predicted to cause a constant underestimation (20-30%) of the actual cAMP content of samples in the range of homogenate concentrations used.

When a homogenate (one hypothalamus in 2.5 ml Tris/EDTA buffer) was serially diluted in buffer the assayed cAMP concentration of the samples was found to be directly dependent on the concentration of the samples (figure R.36.a) providing further evidence that any underestimation of cAMP concentration due to non-specific binding produces a constant error over the range of sample concentrations used.

iii) Effect of sCT on Mouse Brain Cyclic Nucleotide Levels

The cAMP concentrations of mouse hypothalami and cortices after i.c.v. administration of vehicle were 4.49 ± 0.16 and 4.83 ± 0.60 pmol.mg protein^{-1} ($n = 14$) respectively. The concentration of cAMP in the hypothalami was increased by i.c.v. administration of sCT at a dose of 50 U.kg^{-1} but was unaffected by 10 and 1 U.kg^{-1}. The cAMP concentration in the cortices was not significantly altered by i.c.v. sCT at doses of 1, 10 or 50 U.kg^{-1} (figure R.37).

iv) Adenylate Cyclase Activity of Mouse Hypothalamus Homogenates

Adenylate cyclase activity was assessed by the method of Albano et al (1973) in which the rate of cAMP accumulation is determined in a Tris-HCl buffered incubation medium containing ATP and a phosphodiesterase inhibitor (theophylline). Adenylate cyclase activity is expressed as pmol cAMP produced /mg protein/minute (pmol/mg/min). After incubation at 30°C adenylate cyclase activity was terminated by boiling for 3 minutes. Preliminary experiments were performed to find a suitable incubation period (during which cAMP accumulation is linearly dependent on time) and to assess the effects of boiling on the assay. Cyclic AMP accumulation in a homogenate of mouse hypothalamus was found to be directly dependent on time for the first 20 minutes of incubation (figure R.38b). The incubation period of 10 minutes was selected for all subsequent assays. When a series of cAMP standards were boiled prior to the protein binding assay the gradient of the standard curve was not significantly altered by boiling (figure R.38.a) indicating the suitability of this procedure for termination of the incubation.

v) Effect of Sodium Fluoride on Adenylate Cyclase

The adenylate cylcase activity of homogenates of mouse hypothalamus was 27.2 $\pm$ 1.4 pmol/mg/min (n = 6) with 10 μM, guanosine triphosphate (GTP), and 28.7 $\pm$ 3.5 pmol/mg/min (n = 6) without GTP, and was found to be increased by sodium fluoride at 5 and 15 mM in the presence and in the absence of GTP (figure R.39).

vi) Effect of sCT on Adenylate Cyclase

Adenylate cyclase activity of homogenates of mouse hypothalamus (control = 24.7 $\pm$ 1.6 pmol/mg/min, n = 9) was found to be increased in a dose dependent manner by sCT at 60 $\mu U.ml^{-1}$-60 $U.ml^{-1}$ (3.6 pM-3.6 μM) in the standard incubation medium containing 10 μM GTP (figure R.40). In a subsequent internally controlled experiment, sCT at concentration of 60 μU-2 $U.ml^{-1}$ again produced a dose dependent stimulation of basal adenylate cyclase activity in the presence of GTP, whereas in incubation medium lacking GTP only a modest stimulation of enzyme activity was observed attaining statistical significance

only at 2 $U.ml^{-1}$ (figure R.41). The basal adenylate cyclase activities in the presence and absence of GTP were the same as those given above for the effect of sodium fluoride on adenylate cyclase activity.

Discussion

The preliminary experiments described above indicate that the assays employed are suitable for the determination of cAMP and cGMP concentrations and adenylate cyclase activity in the brains of mice. The extraction efficiency was estimated at approximately 70-80% cAMP and adenylate cylcase values quoted are presumably, therefore, underestimated by approximately 20-30%. Procedures required for the correction of this error were not considered necessary as all experiments were internally controlled and were designed for estimation of hormone induced changes rather than for determination of absolute values.

The concentrations of cyclic nucleotides in the mouse brain are known to be greatly affected by post mortem changes (Steiner, Ferrendelli and Kipnis, 1972), which may partly account for the great variation in reported values. However, even when rapid methods of sacrifice and tissue fixation are employed considerable variations are seen in reported results. Hence, Steiner et al (1972) using liquid nitrogen to prevent post mortem changes obtained control values of 1.4 and 2.0 pmol.mg $protein^{-1}$ in the cerebral cortex and hypothalamus, whereas Schneider, Felt, Murphy and Goldman (1981), using microwave irradiation obtained control values of approximately 26 and 20 pmol. mg $protein^{-1}$ in these tissues. Notwithstanding the error introduced by extraction of cAMP, it is apparent that the control values of 4-5 pmol.mg $protein^{-1}$ reported above are within the reported range.

As with basal levels of cyclic nucleotides, the reported estimates of central adenylate cyclase activity vary greatly, presumably reflecting differences in experimental protocol. However, Kerwin et al (1980), using a similar assay method and incubation medium containing 10 µM GTP reported a value for the adenylate cyclase activity of rat hypothalamic homogenates (35.3 pmol/mg/min) which is in good agreement with the values obtained above (24-29 pmol/mg/min).

Central administration of sCT (50 $U.kg^{-1}$) was found to produce a modest but significant increase in the cAMP concentration of the hypothalamus, no such effect was obtained with 1 $U.kg^{-1}$ and although a similar tendency was observed in the cortex this did not attain statistical significance. The CT induced elevation of hypothalmaic cAMP could however, clearly be associated with the hormones central effects. Elevation of cAMP in vivo could theoretically be achieved by direct or indirect inhibition of cAMP metabolism by phosphodiesterase, or by direct or indirect stimulation of cAMP production by adenylate cyclase. Peripherally CT induced elevation of tissue cAMP can be attributed to its direct stimulatory effect on adenylate cyclase, and this possibility was therefore investigated centrally using homogenates of hypothalamus.

Salmon CT was found to produce a dose dependent stimulation of the adenylate cyclase activity of hypothalamic homogenates in the standard assay medium (containing 10 μM GTP) with significant stimulation at 0.12 nM-3.6 μM. These results are in relatively good agreement with those of Loreau, Lajotte, Wahbe and Ardaillou (1978) who stimulated adenylate cyclase of rat renal cortex membrane with approximately 0.01 nM-10 μM sCT, and those of Goltzman (1980) who stimulated rat and rabbit renal membrane adenylate cyclase with approximately 0.1- 10 μM sCT. Although Loffler et al (1982) found sCT to increase the accumulation of cAMP in cultures of rat glial cells at approximately 0.1-2 μM, sCT did not modify the accumulation in mouse glial cells. The stimulatory effect on mouse adenylate cyclase may therefore, not involve an action on glial cells.

The results of the experiments to determine the effect of GTP on the responses to sCT and NaF were in good agreement with those of Loreau et al (1978) who found that addition of 100 μM GTP increased the potency of sCT at stimulation of renal membrane adenylate cyclase by approximately three-fold (ED_{50} reduced from 1.97 to 0.67 nM), whereas addition of guanylimidophosphate did not modify the response to NaF. The results presented above indicate that, like peripheral CT receptor linked adenylate cyclase, the adenylate cyclase of hypothalamic homogenates can be stimulated by sCT in a GTP dependent

manner. It is not clear, however, whether the tendency toward stimulation of adenylate cyclase by sCT in the absence of added GTP can be attributed to a GTP independent effect or to the effect of tissue derived GTP.

The similarity between peripheral receptor linked adenylate cyclase and central adenylate cyclase with regard to GTP dependent stimulation by sCT provides evidence that the central effect is also receptor mediated and thus, may be physiologically or pharmacologically relevant. However, these results are in direct contrast with those of Rizzo and Goltzman (1981) who reported inhibition of rat hypothalamic homogenates' adenylate cyclases activity at doses of approximately 0.3-10 μM sCT. This discrepency may well reflect differences in experimental protocol, since Rizzo and Goltzman (1981) estimated the enzymes' activity at $4^{o}C$, and used a washed membrane fraction in an incubation medium without added guanyl nucleotide. The latter point is particularly surprising as the preparation was unlikely to contain tissue derived guanyl nucleotides and these authors had previously reported the stimulatory effect of sCT on renal membrane adenylate cyclase to be potentiated by guanyl-5'-ate imidophosphate, a GTP analogue which is resistant to hydrolysis to GDP. Moreover, the IC_{50} of the reported inhibitory effect (approximately 6 μM) was considerably higher than the concentration required to produce half-maximal inhibition of binding of ^{125}I-sCT to hypothalamic homogenates (1 nM). The authors suggest that this difference may reflect the different populations of CT binding sites reported in the CNS by Koida et al (1980) but as both binding and adenylate cyclase stimulation were determined on rat brain hypothalamic homogenates the receptors would have been from the same population. The reported inhibitory effect of high doses of sCT on central adenylate cyclase activity appears, therefore, not to be associated with receptor binding and may reflect a non specific or toxic effect.

Figure R.35 Cyclic Adenosine 3', 5'-monophosphate Protein Binding Assay Standard Curves.

50 µl samples of cAMP in Tris-HCl buffer were assayed as described in methods. The percentage $[^3H]$-cAMP remaining bound after incubation with cAMP standards is plotted against cAMP in the inset diagram. The same data, replotted as C_o/C_x against cAMP gives a linear standard curve. Values are means of two determinations.

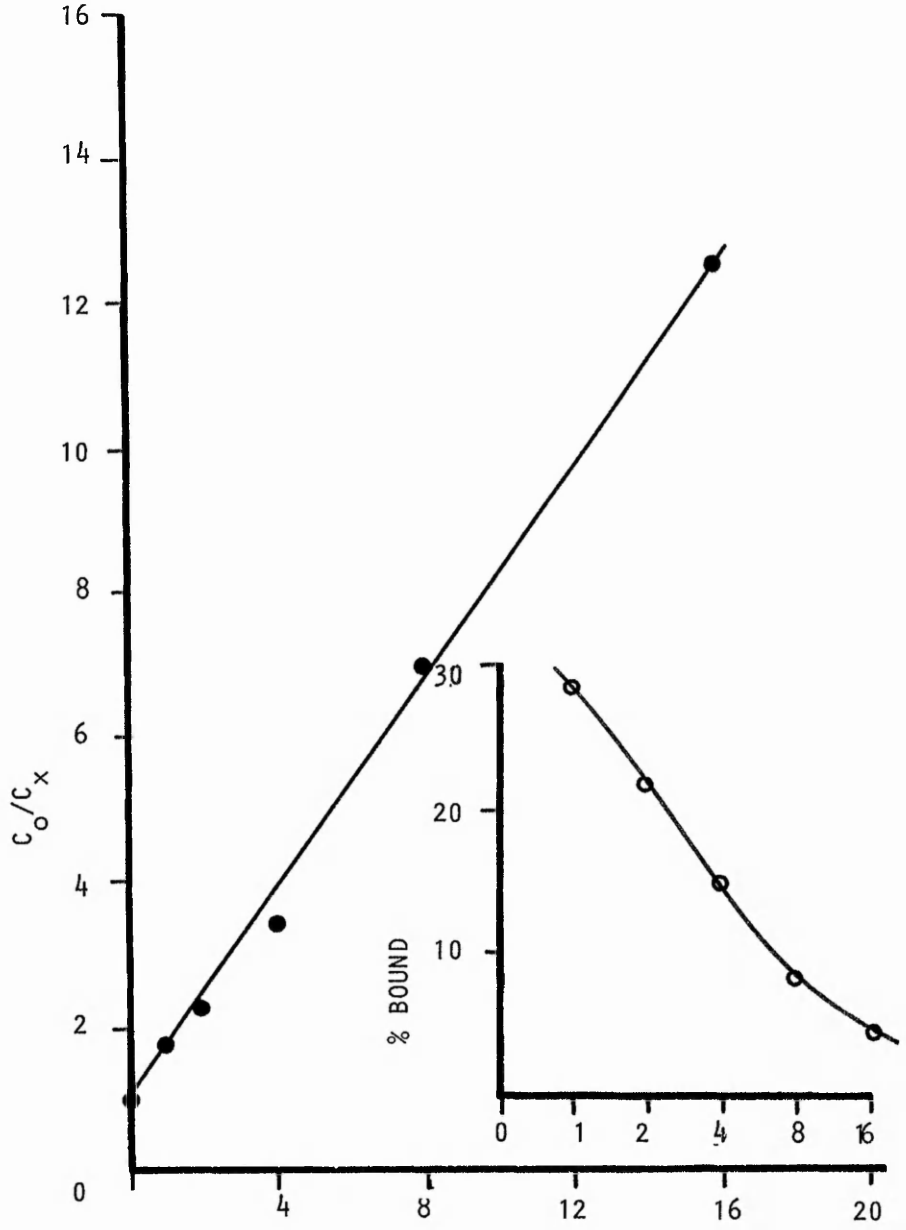

16
14
12
10
8
6
4
2
0
C_o/C_x
4
8
12
16
20
30
20
10
% BOUND
0
1
2
4
8
16
cAMP pmol/50 µl

Figure 36a Relationship Between Protein and cAMP Concentration

Hypothalamic homogenate was serially diluted prior to assay of cAMP and protein. Values are means of two determinations.

Figure R.36b Effect of Homogenate on cAMP Standard Curve.

cAMP standards were prepared in Tris-HCl or in hypothalamic homogenate (in Tris HCl). Values are the means of duplicate determinations.

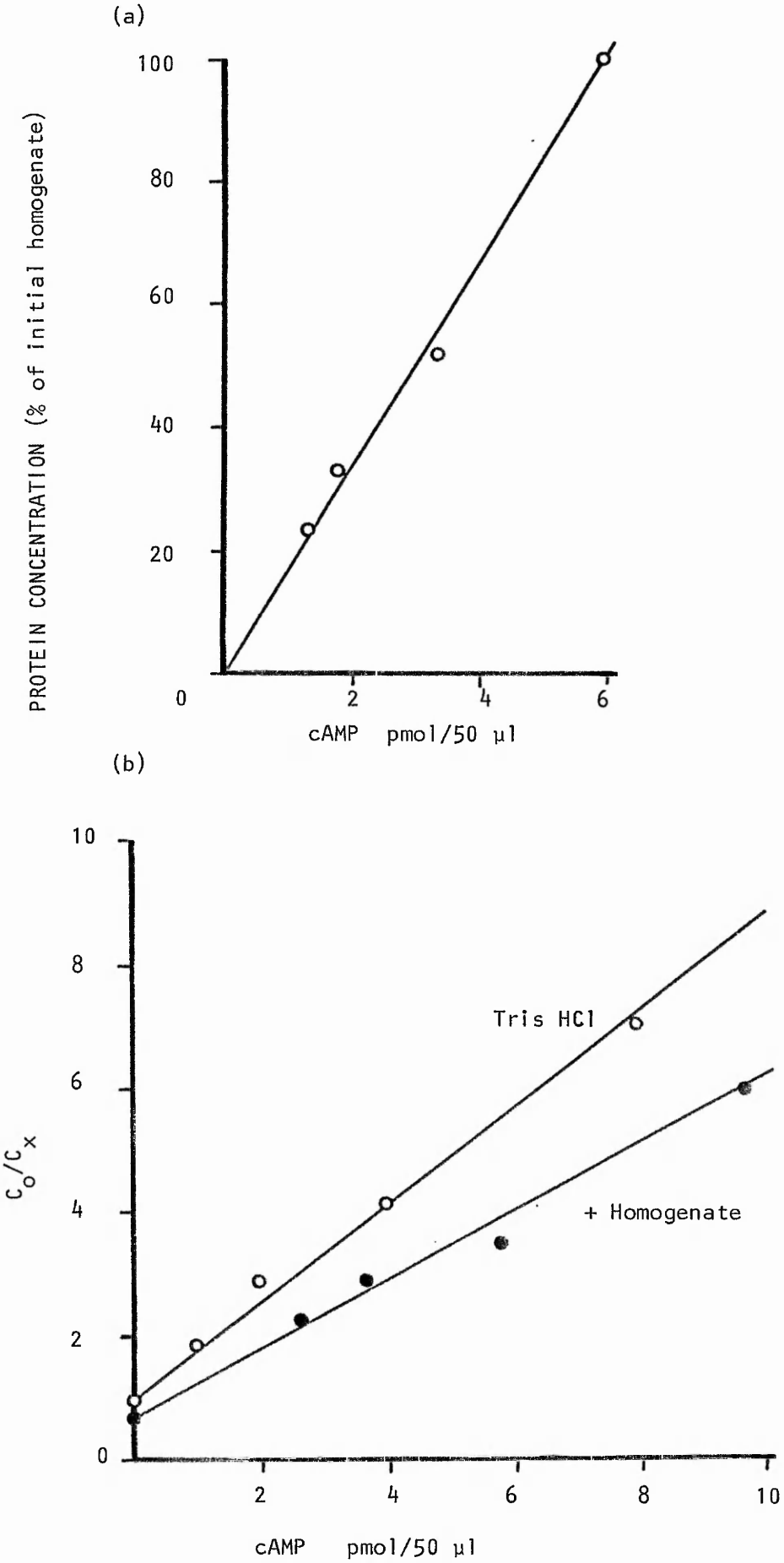

(a)
PROTEIN CONCENTRATION (% of initial homogenate)
100
80
60
40
20
0
2
4
6
cAMP pmol/50 µl
(b)
C_o/C_x
10
8
6
4
2
0
Tris HCl
+ Homogenate
2
4
6
8
10
cAMP pmol/50 µl

Figure R.37 Effect of i.c.v. sCT on cAMP Concentration of Mouse Brain.

Vehicle or sCT were administered 20 minutes prior to sacrifice, and cAMP was assayed as described in methods. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$ compared to animals receiving vehicle.

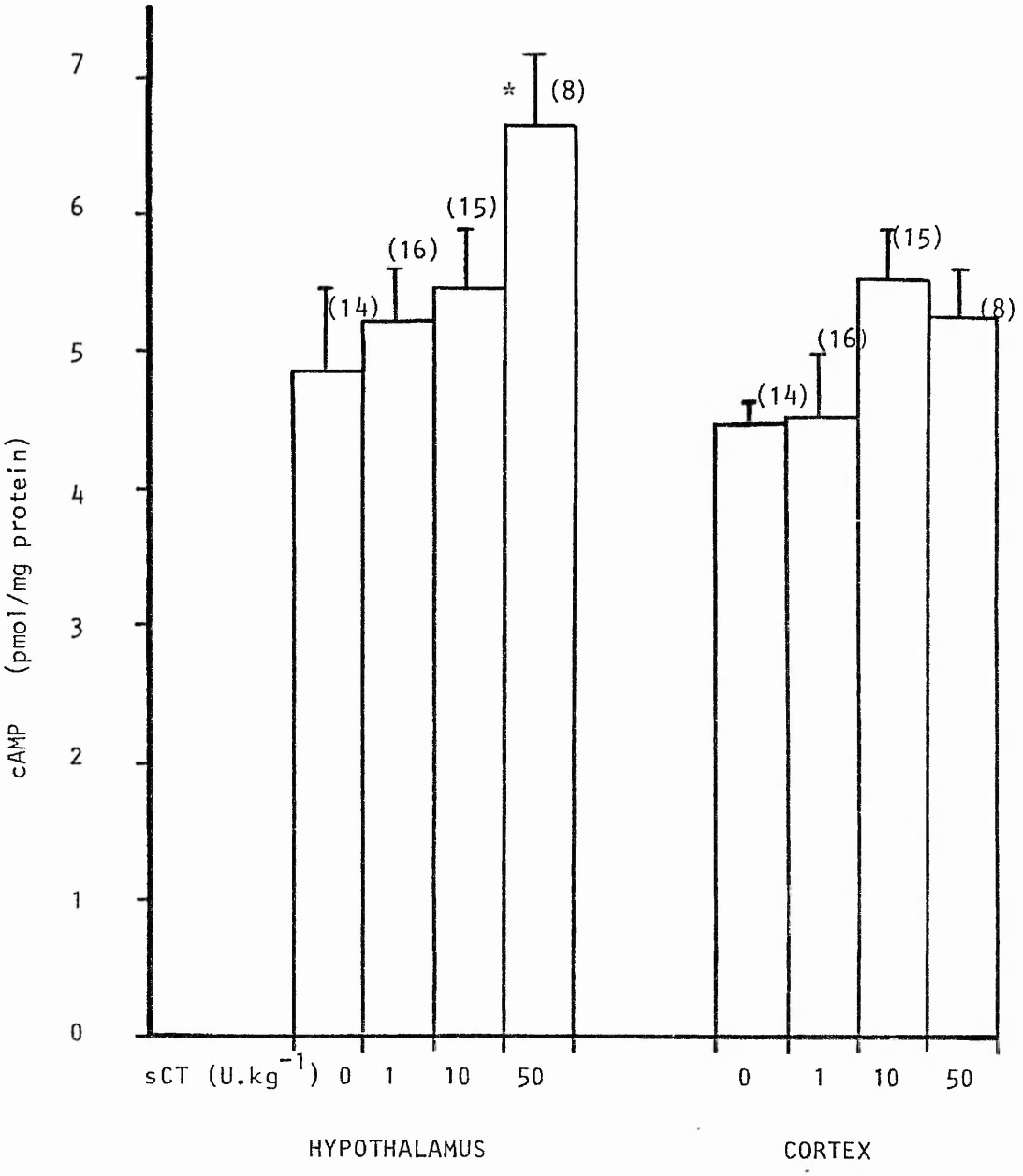
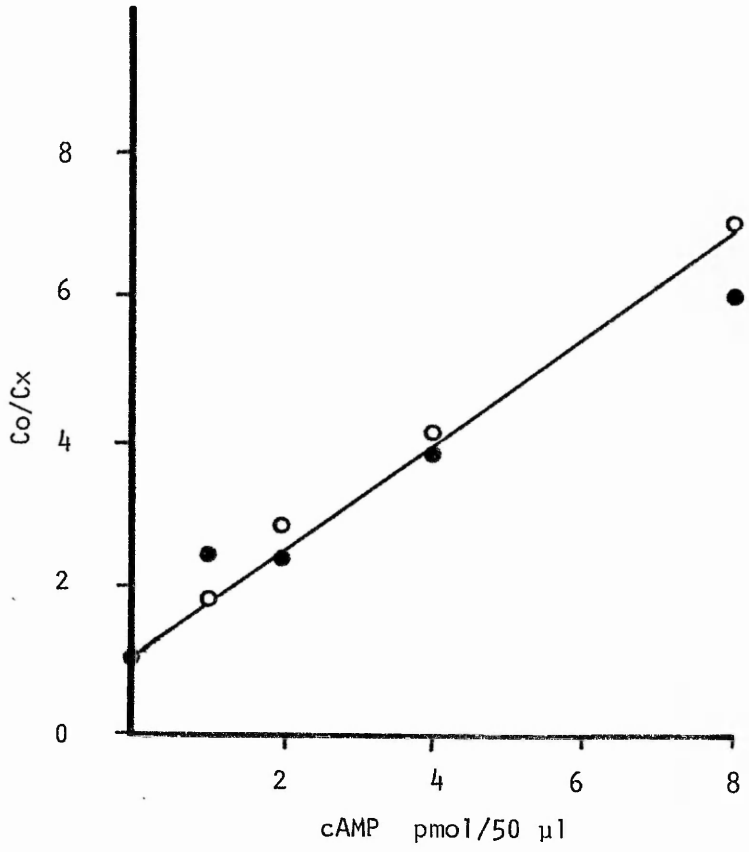

cAMP (pmol/mg protein)
0
1
2
3
4
5
6
7
*
(8)
(15)
(16)
(14)
(15)
(16)
(14)
(8)
sCT (U.kg^{-1}) 0 1 10 50
0 1 10 50
HYPOTHALAMUS
CORTEX

Figure R.38a Lack of Effect of Boiling on cAMP Standard Curve

Standards were either assayed directly (o) or were boiled for 3 minutes prior to assay (●). Values are means of three determinations.

Figure R.38b Accumulation of cAMP in Hypothalamic Homogenate.

Samples of homogenate were boiled to terminate enzyme activity at the times shown. Values are means of three determinations.


8
6
4
2
0
Co/Cx
2
4
6
8
cAMP pmol/50 µl

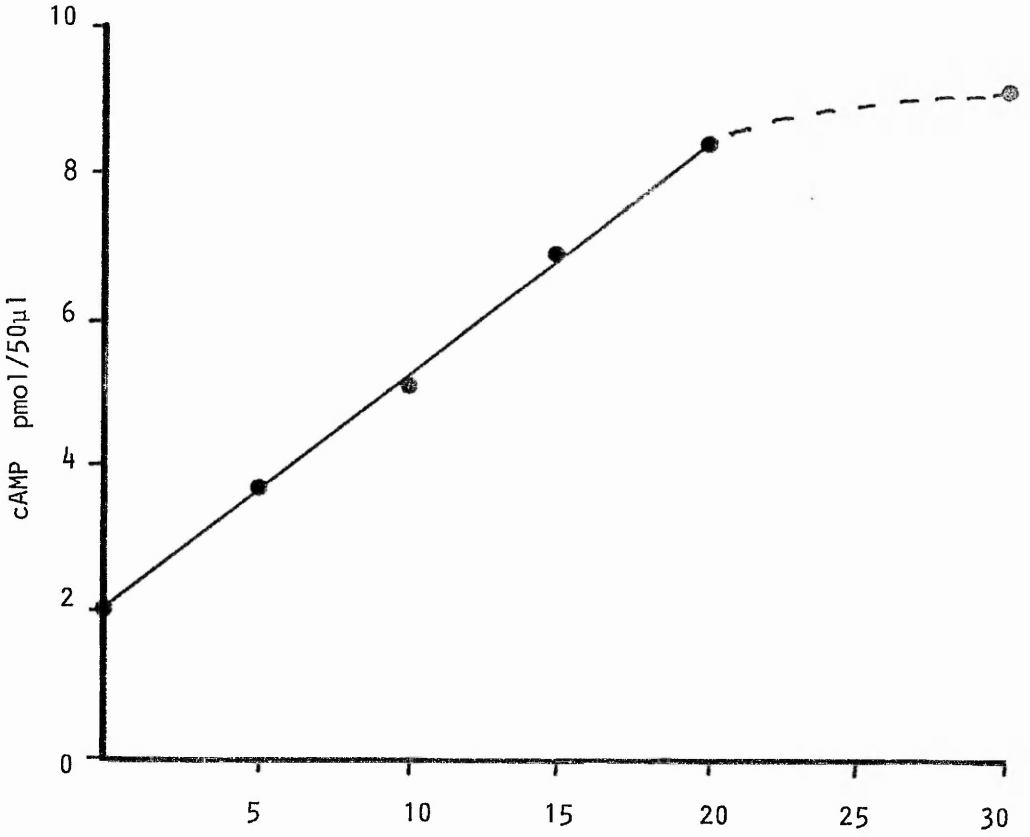

10
8
6
4
2
0
cAMP pmol/50µl
5
10
15
20
25
30
TIME (minutes)

Figure R.39 Effect of GTP on NaF Stimulation of Mouse Hypothalamic Adenylate Cyclase.

Adenylate cyclase activity was determined in the presence or absence of 10 μM GTP (basal adenylate cyclase activity was 28.7 ± 3.5 and 27.2 ± 1.4 pmol/mg/minute respectively). Values are $\bar{x}$ ± s.e., n = 6

* $P < 0.05$; ** $P < 0.01$ (compared to appropriate control group).

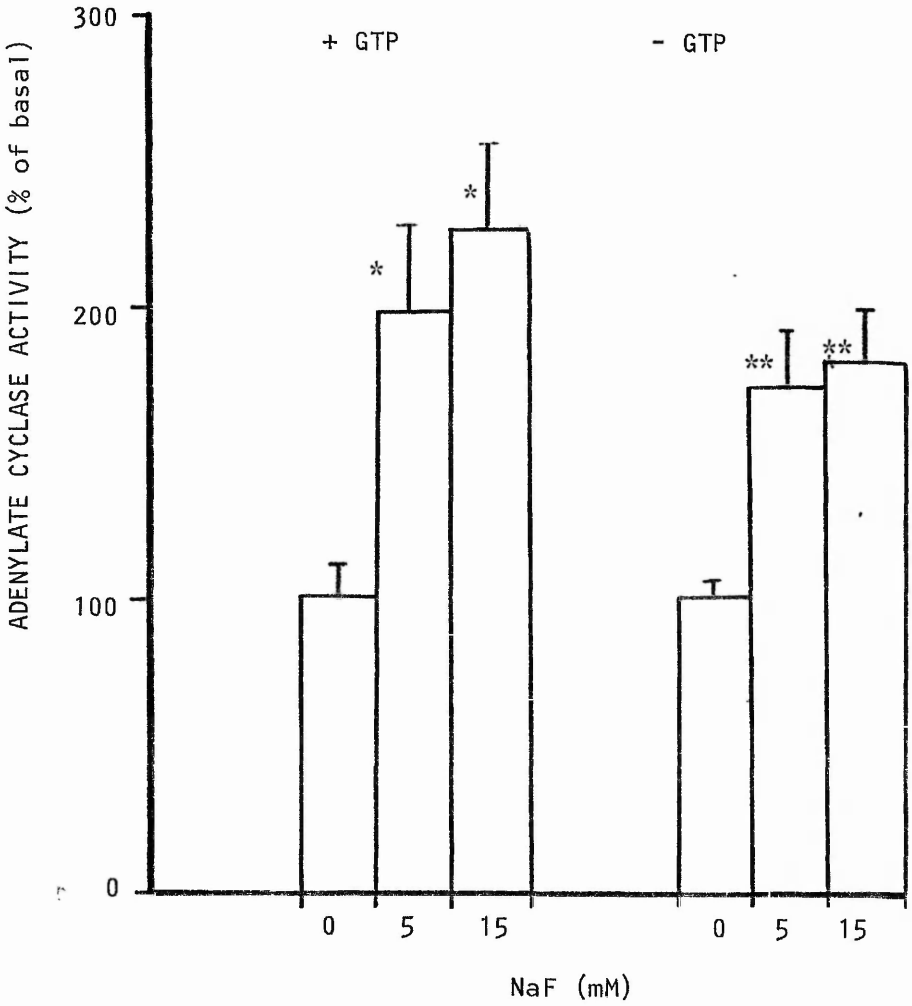

ADENYLATE CYCLASE ACTIVITY (% of basal)
300
200
100
0
+ GTP
- GTP
*
*
*
**
**
0
5
15
0
5
15
NaF (mM)

Figure R.40 Stimulation of Adenylate Cyclase in Mouse Hypothalamic Homogenates by sCT.

Enzyme activity determined from the cAMP produced by hypothalamic homogenate incubated for 10 minutes, at 30°C in a Tris-HCl buffered medium (pH 7.4) containing 2 mM ATP, 6 mM theophylline and 10 µM GTP. Incubation was terminated by boiling for 3 minutes. Significant stimulation was observed with 0.12 nM-3.6 µM sCT (≈2 mU-60 $U.ml^{-1}$). Control adenylate cyclase was 24.7 ± 1.6 pmol/mg/min (n = 9). Values are $\bar{x}$ ± s.e., values of n are shown.

* $P < 0.05$; ** $P < 0.01$

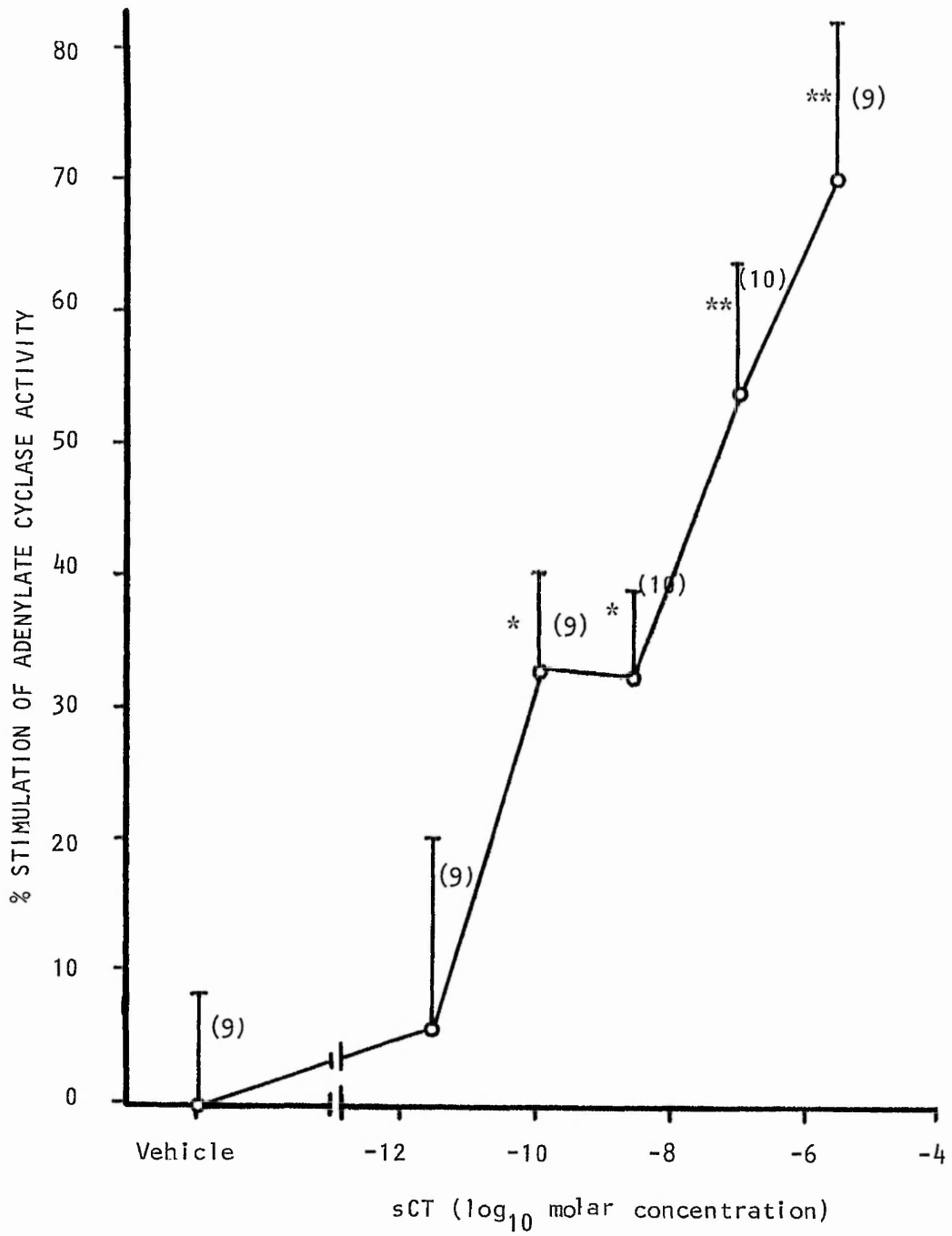
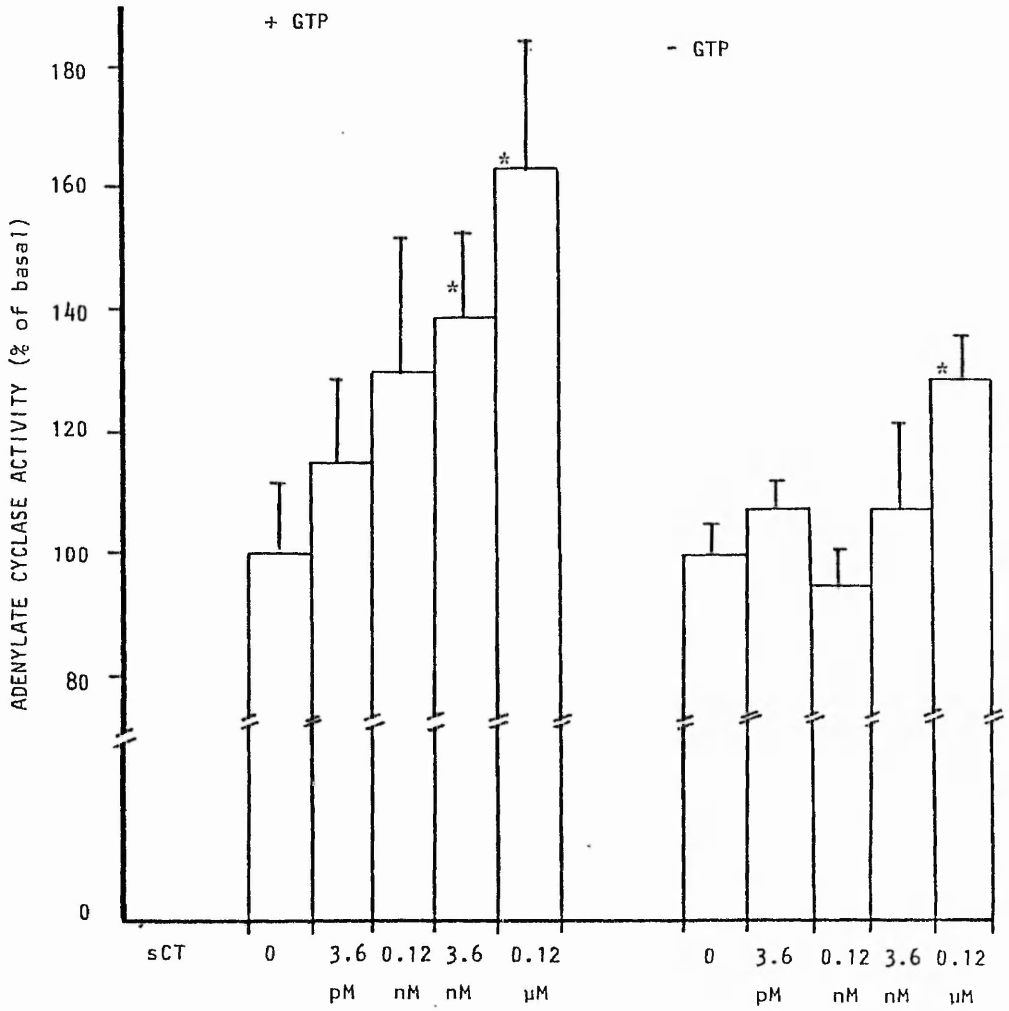

% STIMULATION OF ADENYLATE CYCLASE ACTIVITY
80
70
60
50
40
30
20
10
0
(9)
(9)
*
(9)
*
(10)
**
(10)
**
(9)
Vehicle
-12
-10
-8
-6
-4
sCT (log10 molar concentration)

Figure R.41 Effect of GTP on sCT Stimulation of Adenylate Cyclase of Mouse Hypothalamic Homogenate.

Adenylate cyclase activity was determined in the presence or absence of 10 μM GTP (basal activity was 28.7 ± 3.8 and 27.1 ± 1.4 pmol/mg/minute respectively). Values are $\bar{x}$ ± s.e., n = 6.

* $P < 0.05$ (compared to appropriate control).


+ GTP
- GTP
ADENYLATE CYCLASE ACTIVITY (% of basal)
180
160
140
120
100
80
0
*
*
*
sCT
0
3.6 pM
0.12 nM
3.6 nM
0.12 µM
0
3.6 pM
0.12 nM
3.6 nM
0.12 µM

R.7 EFFECT OF CALCITONIN ON CENTRAL TRYPTAMINERGIC METABOLISM

Introduction

It is apparent that the pharmacological effects of centrally administered CT may be produced by a direct or indirect action to modulate neuronal activity. Hence, Yamamoto et al (1980) have demonstrated that central administration of pCT (8 and 17 $U.kg^{-1}$) inhibited electrical potentials in the sensory cortex of immobilised rabbits evoked by electrical stimulation of the tooth pulp or sciatic nerve, and Miyahara and Oomura (1981) found that iontophoretic application of CT onto glucose sensitive neurones of the lateral hypothalamus caused a reduction in neuronal activity. However, very few published reports have addressed the problem of the identity of the neurotransmitter systems thought to be modulated by CT. A notable exception is the work of Nakhla and co-workers who have reported that a single intramuscular dose of pCT (20 $U.kg^{-1}$) increases both 5-HT concentration and acetylcholinesterase (AchE) activity in the rat brain (Nakhla and Majumdar, 1978) Clearly either or both of these effects could modulate neuronal activity and underlie the pharmacological effects of the hormone. Although the stimulatory effect of CT on rat brain 5-HT has recently been supported by Dupuy et al (1983), the effect of CT on brain AchE is in direct contrast to the results of Bates et al (1982b) who failed to observe any alteration in the enzyme activity after peripheral administration of sCT (20 $U.kg^{-1}$).

The results presented in this chapter were obtained from a series of experiments designed to extend previous studies of the effects of CT on central 5-HT. The assays used were based on those described by Curzon and Green (1970) in which 5-HT and 5-HIAA are extracted from crude brain homogenates and reacted with o-pthalaldehyde (OPT) to produce highly fluorescent products.

Results

i) Fluorescence Spectra, Standard Curves and Extraction Efficiencies

When a range of standard concentrations of 5-HT and 5-HIAA were assayed as described above (M.10) the relationship between fluorescence and indoleamine concentration was found to be linear over the range of 0.05-1.0 µg, for 5-HT and 0.025-1.0 µg for 5-HIAA. Furthermore, the

excitation and emission spectra obtained for 5-HT and 5-HIAA were essentially similar to those described by Maickel, Cox, Saillant and Miller (1968) for the 5-HT-OPT complex with excitation and emission maximal at approximately 360 and 470 nM respectively. Using a fluorimetric assay similar to that described above, Curzon and Green (1970) reported the efficiency of extraction of both 5-HT and 5-HIAA from acidified n-butanol to be 95-100%. However, as tissue indoleamines are extracted from an homogenate in n-butanol, rather than from the solvent alone, it was considered prudent to determine the possible effect of the tissue on the extraction efficiency. Known standards of 5-HT and 5-HIAA in phosphate buffer were either added to samples of acidified n-butanol or tissue homogenate in n-butanol prior to extraction and assay, or were assayed without extraction. After correction for sample dilution through the assay, and subtraction of appropriate blank values (phosphate buffer alone extracted from n-butanol or homogenate prior to assay or assayed without extraction) graphs of fluorescence against 5-HT or 5-HIAA concentration were plotted (figure R.42) and used for estimation of extraction efficiency. In agreement with the observation of Curzon and Green (1970), the efficiency of extraction of 5-HT and 5-HIAA from n-butanol did not differ significantly from 100%. However, in the presence of tissue homogenate, extraction efficiency was reduced to 69.4 $\pm$ 8.0% for 5-HT and 68.9 $\pm$ 14.5% for 5-HIAA. Although the reasons for this reduction in efficiency are unclear (possibilities include specific or non-specific binding of indoleamines to the tissue) it is apparent that the internal standardisation procedure adopted for subsequent assays should compensate for indoleamines lost in the extraction.

ii) Relationship between Homogenate Concentration and Fluorescence

When a sample of mouse brain was homogenised in 10 volumes of acidified n-butanol and serially diluted, prior to assay of 5-HT and 5-HIAA, the plots of fluorescence against concentration of homogenate were both found to be linear (figure R.43). In subsequent assays, tissue samples were homogenised in at least 10 volumes of solvent and a linear relationship between indoleamine concentration and fluorescence of these samples can therefore be assumed.

iii) Assay Specificity

As similar excitation and emission spectra have been obtained for 5-HT and 5-HIAA, fluorescence alone cannot be used to distinguish between these two indoleamines. The selectivity of the assays are therefore dependent on the efficiency of separation in the extraction. The selectivity of the assays used was assessed by addition of 5-HT to tissue homogenates prior to assay of 5-HIAA and by addition of 5-HIAA prior to assay of 5-HT. As shown in figure R.44, 5-HT was not found to interfere with the determination of 5-HIAA, whereas 5-HIAA was found to increase the fluorescence obtained in the 5-HT assay. In order to compensate for this interference, samples of homogenate plus 5-HT or 5-HIAA were assayed for 5-HIAA and 5-HT respectively. In 6 separate experiments, 1 µg 5-HIAA was found to produce fluorescence equivalent to that produced by 0.19 ± 0.02 µg 5-HT in the 5-HT assay. Where possible, the values obtained for 5-HIAA interference in the 5-HT assay and the 5-HIAA content of the samples were used to correct 5-HT determinations for 5-HIAA fluorescence.

iv) Effect of Pargyline, Probenecid and p-chlorphenylalanine (PCPA) on Mouse Brain 5-HT and 5-HIAA

In a series of internally controlled experiments, the effects of pargyline, probenecid and PCPA on mouse whole brain 5-HT and 5-HIAA concentration were determined. The rationale for the use of these drugs in control experiments is evident from figure R.45 and the results of these experiments are shown in figures R.46 and R.47. The control values (vehicle pretreated) obtained for 5-HT concentration were 1.08 ± 0.17, 0.75 ± 0.06 and 0.98 ± 0.05 µg/g wet weight in three separate experiments (n = 6-9) and for 5-HIAA concentration were 0.43 ± 0.03 and 0.61 ± 0.07 µg wet weight in two separate experiments (n = 6-9), these values are in good agreement with values reported in the scientific literature. Hence, Curzon and Green (1970) found the concentration of 5-HT and 5-HIAA in the rat whole brain to be 0.83 and 0.35 ug/g wet weight respectively. Thirty minutes after inhibition of monoamine oxidase with 75 $mg.kg^{-1}$ of paragyline (i.p.) mouse whole brain 5-HT was found to be elevated by 62.0 ± 5.0% and 5-HIAA was reduced by 17.1 ± 4.8%. In contrast, blockade of 5-HIAA efflux from

the CNS with 200 mg.kg^{-1} of probenecid (i.p.) elevated whole brain 5-HIAA by 28.6 ± 13.1% without significantly altering the concentration of 5-HT. These results are essentially similar to those of Neff et al (1967) who used the rate of 5-HIAA accumulation after probenecid, and Tozer, Neff and Brodie (1966) who used the rate of decline of 5-HT after pargyline, to estimate the rate of 5-HT turnover in the rat brain. Inhibition of tryptophan hydroxylase with PCPA (300 mg.kg^{-1}, 72, 48 and 24 hours prior to experimental use) reduced the concentration of both 5-HIAA and 5-HT by 74.6 ± 6.2 and 47.7 ± 2.3% respectively, the necessity for repeat administration was demonstrated by the modest reduction in 5-HT (23.1 ± 6.6%, n = 8) produced by a single injection of PCPA (300 mg.kg^{-1} 24 hours prior to experimental use). Koe and Wiessman (1966) have reported that this dose of PCPA reduced rat brain 5-HT to 7 ± 1% of control values indicating the marked sensitivity of rats to this treatment.

v) Rate of Turnover of Mouse Brain 5-HT

As mentioned above, the rate of central 5-HT turnover has been estimated from the rate of accumulation of 5-HIAA after blockade of acid transport from the CNS with probenecid (Neff et al, 1967) and from the rate of decline of central 5-HIAA after monoamine oxidase inhibition with pargyline (Tozer et al, 1966). Preliminary experiments (figure R.48) revealed that mouse whole brain 5-HIAA increased linearly for 90 minutes after administration of probenecid, the 5-HT turnover rate (estimated from the gradient of this graph) of 0.27 ± 0.04 µg/g/hr is in good agreement with the values of 0.4 and 0.192 µg/g/hr obtained by Neff et al (1967) and Goodlet and Sugrue (1974) respectively, using similar methods of assessment in rats. The lack of effect of probenecid on central 5-HIAA after pargyline pretreatment has also been demonstrated in rats by Neff et al (1967).

vi) Effect of sCT on Mouse and Rat Brain 5-HT and 5-HIAA

As shown in table 7, central and peripheral administration of sCT at doses of 20 U.kg^{-1} i.p. and 1-10 U.kg^{-1} i.c.v. failed to modify the concentration of either 5-HT or 5-HIAA in either midbrain or

cerebellum plus cortex regions of mouse brain, determined at various times after administration of sCT. Furthermore, 20 $U.kg^{-1}$ i.p. failed to modify the concentration of these indoleamines in either region of rat brain (table 8). These results are in direct contrast to those of Nakhla and Majumdar (1978) who found that rat brain 5-HT was elevated by 56%, 30 minutes after intramuscular administration of pCT at a dose of 20 $U.kg^{-1}$, and those of Dupuy et al (1983) who found rat whole brain 5-HT to be increased by approximately 45%, 45 minutes after peripheral administration of sCT (20 $U.kg^{-1}$).

Discussion

The results of the preliminary experiments described in sections i-iv above, provide evidence that the experimental procedures adopted are suitable for a simple and specific spectrofluorimetric assay of brain 5-HT and 5-HIAA. The reliability of these assays are further supported by the fact that the determined values, which do not vary greatly ("within experiment" standard errors are typically below 10%), are in good agreement with previously reported values, and can be modified in a predictable manner by various pharmacological procedures. However, in direct contrast to the work of Nakhla and Majumdar (1978) and Dupuy et al (1983) sCT at 20 $U.kg^{-1}$, i.p., was found not to modify the concentration of 5-HT in rat brain 5-HT. Moreover, central administration of sCT to mice, at doses known to produce antinociception (1 and 10 $U.kg^{-1}$) and at times when antinociception might be expected (20, 45 and 90 minutes after administration), failed to modify central concentrations of 5-HT or 5-HIAA. These results suggest that the pharmacological effects of CT are unlikely to be achieved by gross elevation of central 5-HT levels.

As reported effects of CT on rat brain AchE activity and 5-HT concentration are contradicted by the work of Bates et al (1982c) and by the results presented above, it seems pertinent to discuss the work of Nakhla and Majumdar and of Dupuy and co-workers in some detail. In 1978, Nakhla and Majumdar reported that a single i.m. injection of pCT (20 $U.kg^{-1}$) to rats of 150-200 g caused a reduction in plasma calcium and free tryptophan concentrations, and increased cerebral AchE activity and whole brain 5-HT. Nakhla and Majumdar proposed that CT increased central AchE activity by promoting the uptake of tryptophan

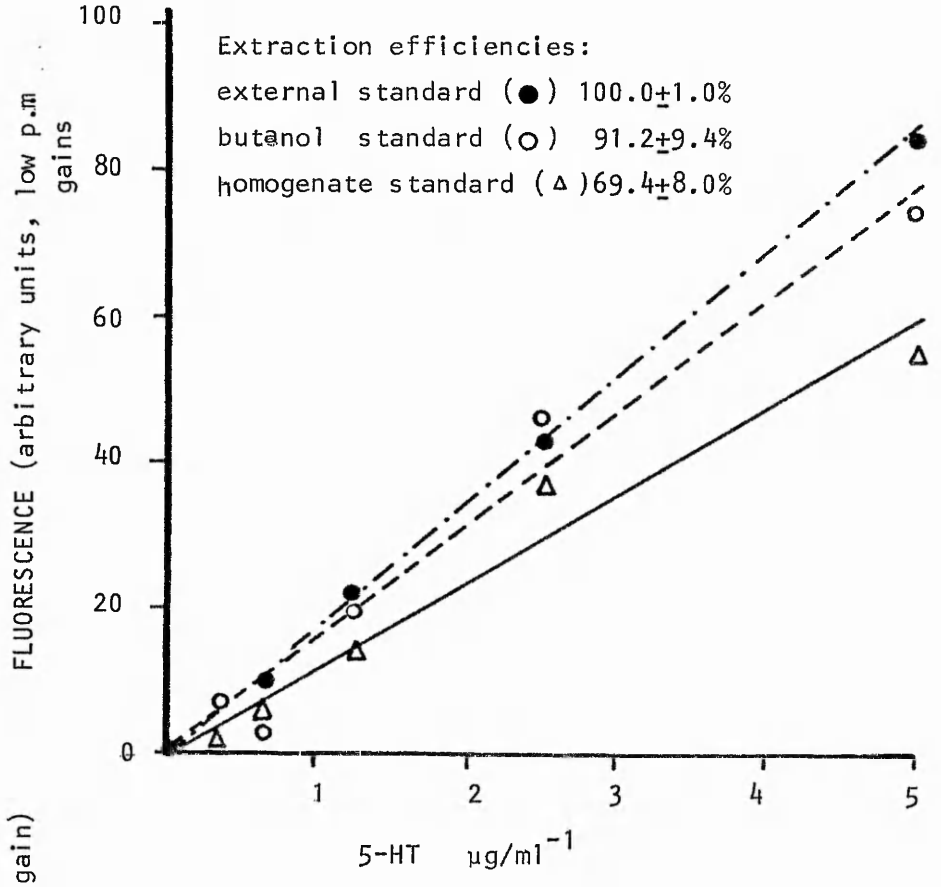
into the CNS and that this caused an increase in control 5-HT which in turn increased central AchE activity. The authors suggested that this hypothesis is supported by the demonstration that PCPA pretreatment prevented the reduction in plasma tryptophan and the increase in cerebral AchE activity (Nakhla, 1980). Further support for this hypothesis was provided by Dupuy et al (1983) who found that a single dose of sCT (20 $U.kg^{-1}$) elevated central 5-HT in rats at 45, but not at 90, minutes after administration. However, mechanisms underlying the effect of central 5-HT on AchE activity have not been proposed by either group, indeed, Bates et al (1982c) have failed to modify central AchE activity of rats with i.p. sCT, and in mice were unable to modify central AchE activity with a range of treatments including peripheral administration of tryptophan (300 $mg.kg^{-1}$ s.c. and 300 or 600 $mg.kg^{-1}$ p.o.) PCPA (300 $mg.kg^{-1}$ i.p.), sCT (20 $U.kg^{-1}$ s.c.) and central administration of sCT (50 $U.kg^{-1}$ i.c.v.) and 5-HT (0.67 $mg.kg^{-1}$ i.c.v.). In addition to the controversy surrounding the possible effects of CT on AchE activity, the reported reduction in plasma free tryptophan after pCT (Nakhla et al, 1978) is in direct contrast to the increase observed by Dupuy et al (1983) using sCT. A possible explanation for this latter discrepency is that Nakhla and Majumdar (1978) collected blood samples in heparinised tubes, whereas Dupuy et al (1983) avoided the use of heparin which they stated can modify the binding of tryptophan to serum albumin. It is, of course, possible that similar differences in experimental protocol (route of administration, type of CT used, age, sex and species of experimental animal, treatment of tissues after removal etc) might, at least in part, explain differences in reported effects on AchE activity and 5-HT levels. However, a serious criticism of the work of both Nakhla and Majumdar and of Dupuy and co-workers is that their experiments were generally not sufficiently controlled. Hence, central 5-HT concentrations after CT administration have been compared to the values obtained prior to administration, and the possibility of artifactual effects due to handling stress and diurnal variation in 5-HT levels can therefore not be rejected. The importance of suitable internal controls is demonstrated by referring to the effect of sCT (20 $U.kg^{-1}$ i.p.) on rat brain 5-HT (table 8). Although the concentration of midbrain 5-HT was significantly greater 45 minutes after sCT administration that at 20 or 90 minutes, ($P < 0.05$) this effect cannot be attributed to the hormone, as the control value was also elevated at 45

minutes. Furthermore, neither group have commented on the fact that their control values for central 5-HT concentrations (2.5 and - 3.0 µg/g reported by Dupuy et al, 1983, and Nakhla and Majumdar, 1978, respectively) are 2-4 times higher than the values reported elsewhere.

Figure R.42 Graphs to Show Efficiency of Extraction of 5-HT (a) and 5-HIAA (b)

The fluorescence shown is that produced when indoleamines were either not extracted (●) or were extracted from butanol (o) or from homogenates (Δ). All values are corrected by subtraction of appropriate blank and for dilution during assay. Gradients were determined by linear regression and were used for determination of extraction efficiencies. Values are means of three determinations.

a)


Extraction efficiencies:
external standard (●) 100.0±1.0%
butanol standard (○) 91.2±9.4%
homogenate standard (△)69.4±8.0%
FLUORESCENCE (arbitrary units, low p.m gains
100
80
60
40
20
0
1
2
3
4
5
5-HT µg/ml^{-1}

b)

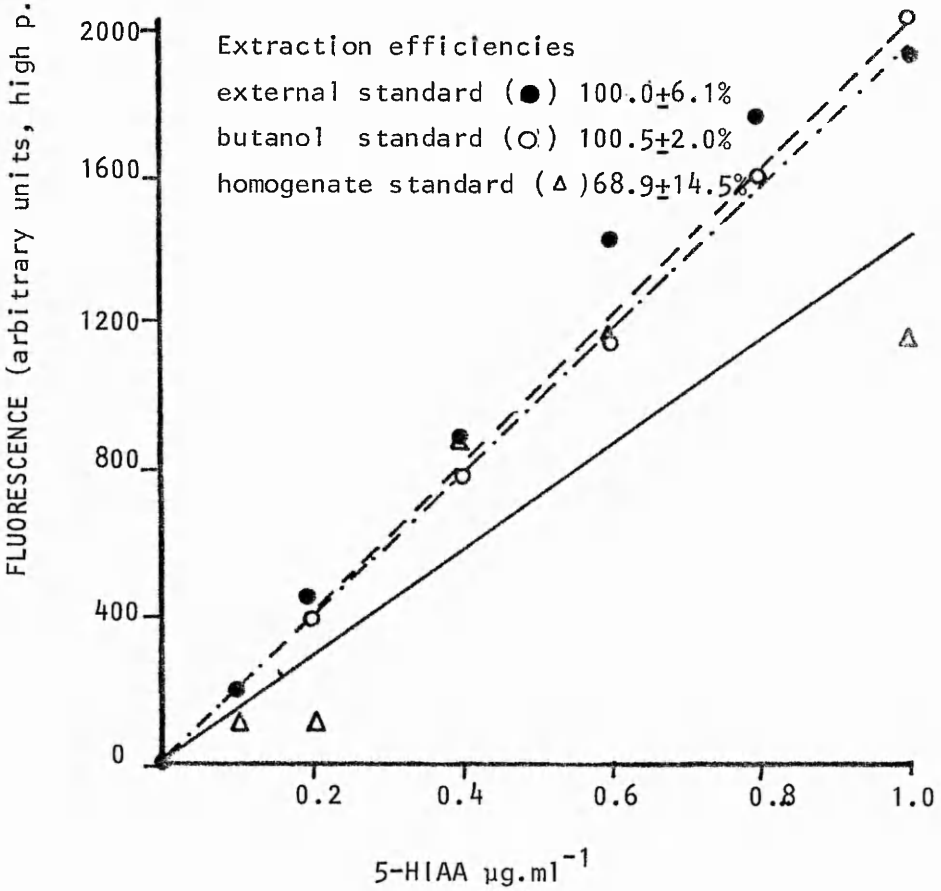

Extraction efficiencies
external standard (●) 100.0±6.1%
butanol standard (○) 100.5±2.0%
homogenate standard (△)68.9±14.5%
FLUORESCENCE (arbitrary units, high p.m. gain)
2000
1600
1200
800
400
0
0.2
0.4
0.6
0.8
1.0
5-HIAA µg.ml^{-1}

Figure R.43 Relationship Between Homogenate Concentration and Fluorescence of Extracted 5-HT and 5-HIAA.

The original homogenate (CNS tissue in 10 volumes of acidified n-butanol) was assayed directly or diluted further prior to assay. Values are means of three determinations. Photomultiplier gain was set to normal for 5-HIAA and high for 5-HT.

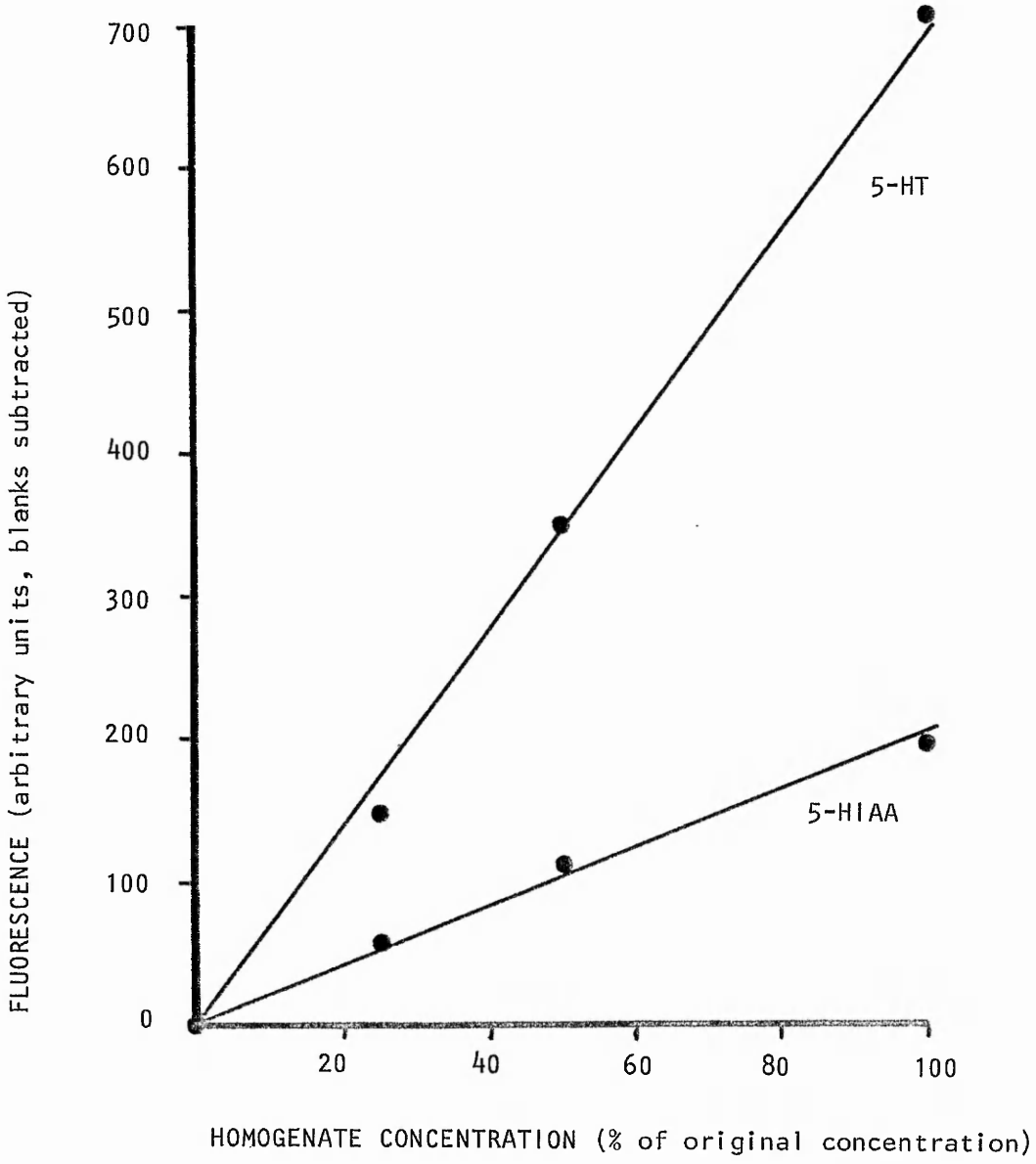

700
600
500
400
300
200
100
0
FLUORESCENCE (arbitrary units, blanks subtracted)
5-HT
5-HIAA
20
40
60
80
100
HOMOGENATE CONCENTRATION (% of original concentration)

Figure R.44 Specificity of 5-HT and 5-HIAA Assays.

The 5-HT and 5-HIAA content of samples of mouse brain homogenate with added indoleamines (shown) was determined. In subsequent assays 1 µg 5-HIAA was found to produce fluorescence equivalent to that of 0.19 ± 0.02 µg 5-HT in the 5-HT assay, whereas 5-HT did not consistantly produce fluorescence in the 5-HIAA assay. Values are means of three determinations.

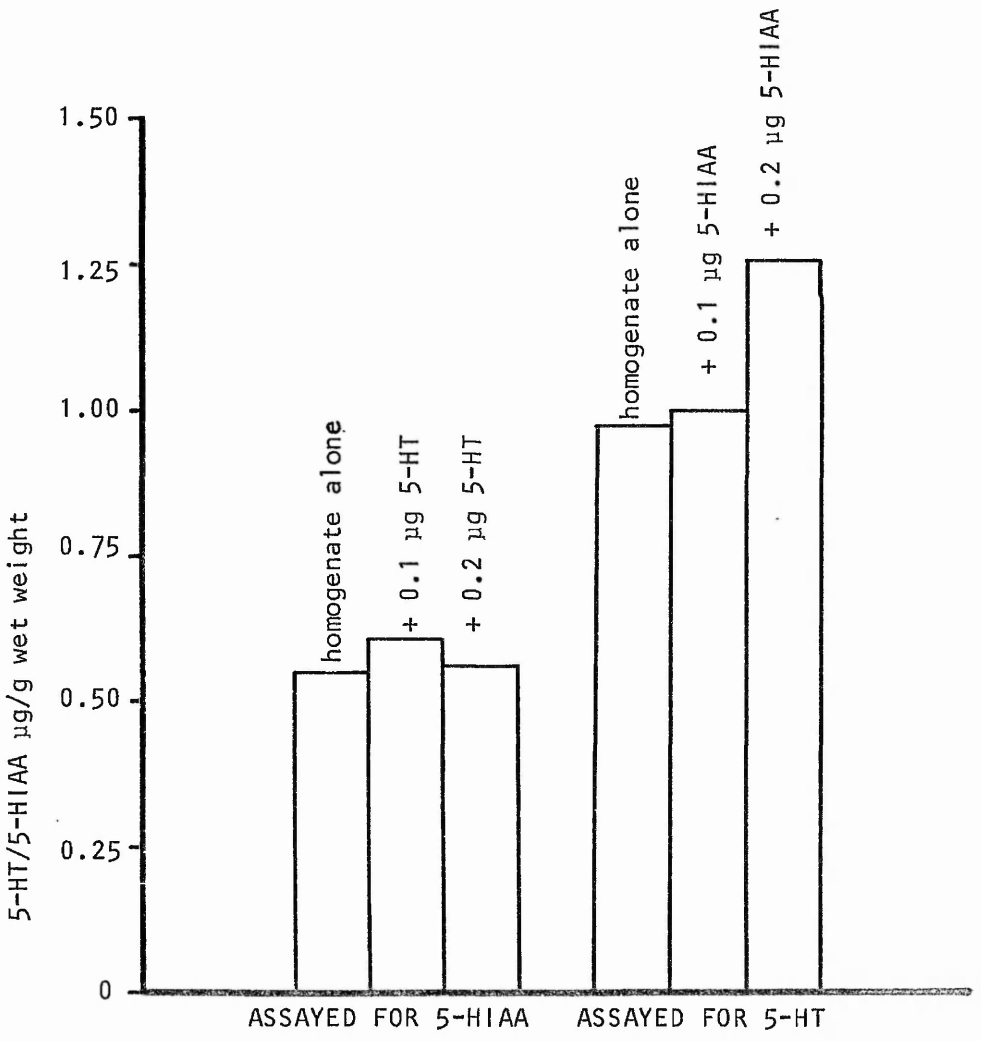

1.50
1.25
1.00
0.75
0.50
0.25
0
5-HT/5-HIAA µg/g wet weight
homogenate alone
+ 0.1 µg 5-HT
+ 0.2 µg 5-HT
homogenate alone
+ 0.1 µg 5-HIAA
+ 0.2 µg 5-HIAA
ASSAYED FOR 5-HIAA
ASSAYED FOR 5-HT

Figure R.45 Diagram to Illustrate Main Features of Central 5-HT Turnover (Adapted from Neff et al, 1967).

Inhibition of tryptophan hydroxylase with PCPA leads to reduction in central 5-HT and 5-HIAA concentrations. Inhibition of monoamine oxidase with pargyline increases central 5-HT and reduces central 5-HIAA concentration. Inhibition of transport of 5-HIAA across the blood-brain barrier with probenecid causes accumulation of 5-HIAA in the CNS.

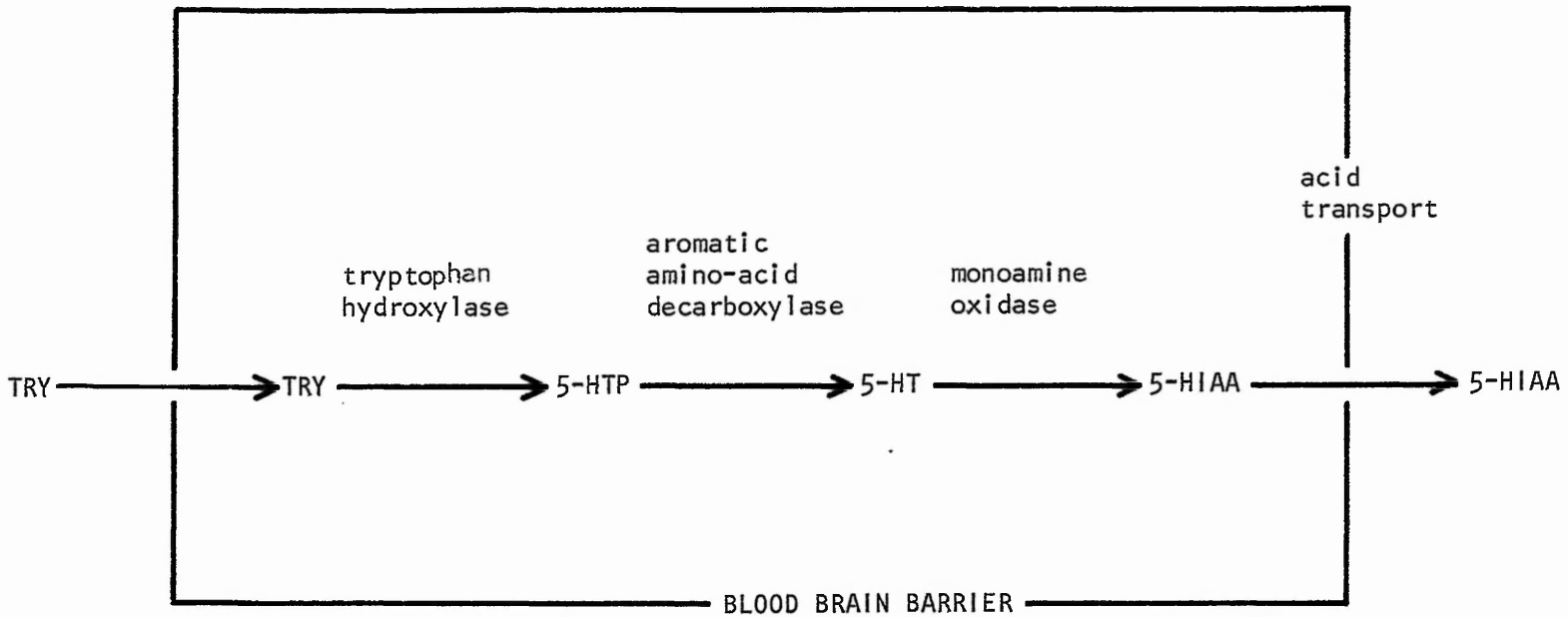

acid
transport
tryptophan
hydroxylase
aromatic
amino-acid
decarboxylase
monoamine
oxidase
TRY
TRY
5-HTP
5-HT
5-HIAA
5-HIAA
BLOOD BRAIN BARRIER

Figure R.46 Effect of Pargyline, Probenecid and PCPA on Mouse Whole Brain 5-HT.

Animals were sacrificed 30 minutes after pretreatment with vehicle, pargyline (75 $mg.kg^{-1}$) or probenecid (300 $mg.kg^{-1}$), or were pretreated with PCPA (300 $mg.kg^{-1}$) or vehicle (0.9% NaCl with 0.5% Brij 30) either 24 hours (PCPA x 1) or 72, 48 and 24 hours (PCPA x 3) prior to sacrifice. Values are $\bar{x} \pm$ s.e., values of n are shown.

** $P < 0.01$ compared to appropriate control (vehicle) group.

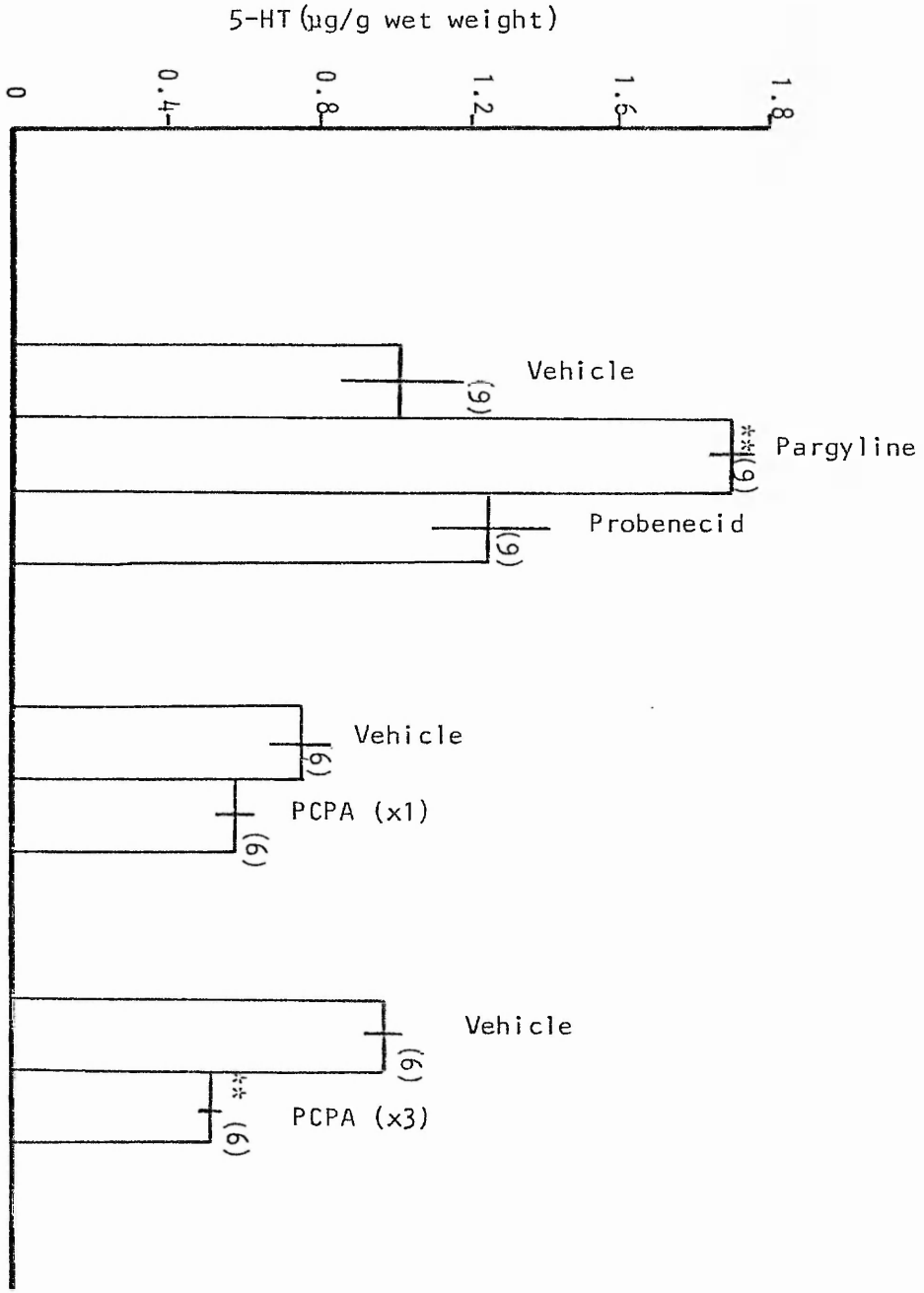

5-HT (µg/g wet weight)
0
0.4
0.8
1.2
1.6
1.8
Vehicle
(9)
**(9)
Pargyline
Probenecid
(9)
Vehicle
(6)
PCPA (x1)
(6)
Vehicle
(6)
** (6)
PCPA (x3)

Figure R.47 Effect of Pargyline, Probenecid and PCPA on Mouse Whole Brain, 5-HIAA.

Animals were sacrificed 30 minutes after pretreatment with vehicle, pargyline (75 $mg.kg^{-1}$) or probenecid (300 $mg.kg^{-1}$) in a volume of 0.1 ml, i.p., or were pretreated with vehicle (0.9% NaCl with 0.5% Brij 30) or PCPA (300 $mg.kg^{-1}$) 72, 48 and 24 hours prior to sacrifice. Values are $\bar{x} \pm$ s.e., values of n are shown.

* $P < 0.05$; ** $P < 0.01$ (compared to appropriate control (vehicle) group.

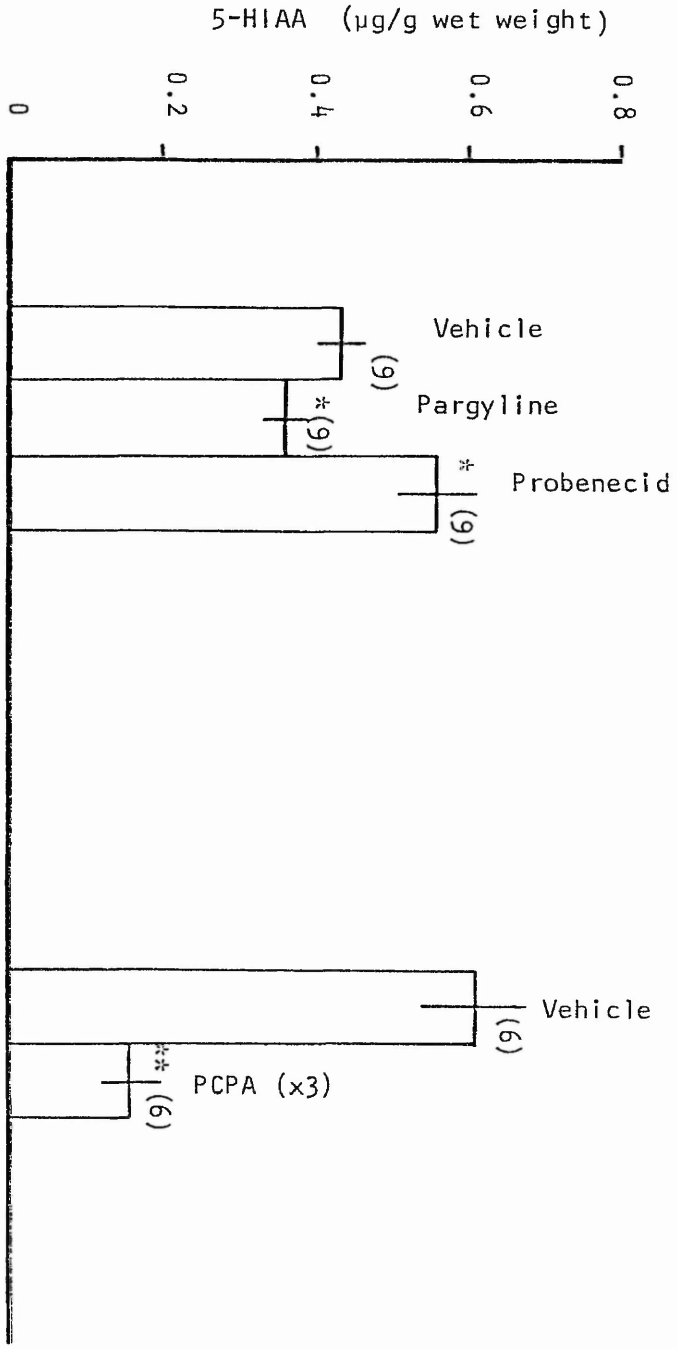

5-HIAA (µg/g wet weight)
0
0.2
0.4
0.6
0.8
Vehicle
(9)
Pargyline
*
(9)
*
Probenecid
(9)
Vehicle
(6)
**
PCPA (x3)
(6)

Figure R.48 Effect of Pargyline Pretreatment on Probenecid Induced Accumulation of 5-HIAA in Mouse Brain.

Animals received pargyline (75 $mg.kg^{-1}$) or vehicle i.p. 30 minutes prior to administration of probenecid (300 $mg.kg^{-1}$; i.p.). 5-HIAA accumulated at the rate of 0.27 $\pm$ 0.04 µg/g/hour and 0.00 $\pm$ 0.02 µg/g/hour after vehicle and pargyline respectively. Values are $\bar{x} \pm$ s.e., n = 6-8

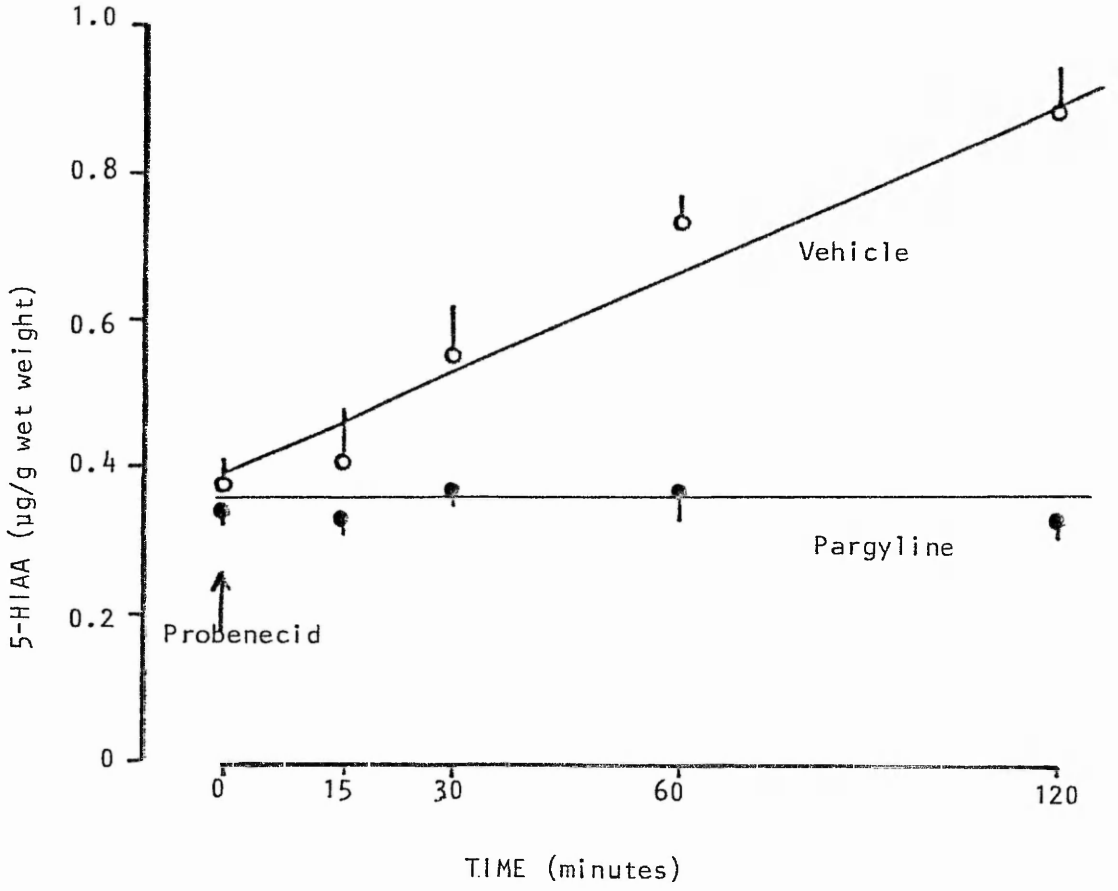

1.0
0.8
0.6
0.4
0.2
0
5-HIAA (µg/g wet weight)
Vehicle
Pargyline
Probenecid
0
15
30
60
120
TIME (minutes)

Table 7 Lack of Effect of sCT on Mouse Brain 5-HT and 5-HIAA

	"Midbrain"		Cortex and Cerebellum	
	5-HT	5-HIAA	5-HT	5-HIAA
Vehicle, i.p. 20 mins	1.025±0.056 (6)	0.666±0.034 (6)	0.510±0.044 (5)	0.318±0.025 (6)
sCT 20 $U.kg^{-1}$ i.p 20 mins	1.147±0.111 (6)	0.696±0.039 (6)	0.474±0.018 (6)	0.312±0.014 (6)
Vehicle, i,p. 45 mins	1.102±0.053 (5)	0.811±0.069 (5)	0.377±0.062 (4)	0.548±0.158 (4)
sCT 20 $U.kg^{-1}$ i.p 45 mins	1.251±0.131 (5)	0.845±0.115 (5)	0.353±0.077 (4)	0.586±0.155 (4)
Vehicle, i.c.v 20 mins	0.705±0.030 (6)	-	0.447±0.039 (6)	-
sCT 1 $U.kg^{-1}$ i.c.v., 20 mins	0.731±0.046 (6)	-	0.448±0.031 (6)	-
sCT 10 $U.kg^{-1}$ i.c.v., 20 mins	0.712±0.030 (6)	-	0.490±0.010 (6)	-
Vehicle, i.c.v 20 mins	1.488±0.072 (6)	0.390±0.043 (6)	0.364±0.029 (6)	0.119±0.008 (6)
sCT, 10 $U.kg^{-1}$ i.c.v., 20 mins	1.408±0.101 (6)	0.391±0.023 (6)	0.401±0.012 (6)	0.135±0.010 (6)
Vehicle, i.c.v 45 mins	1.436±0.103 (6)	0.419±0.026 (6)	0.381±0.015 (6)	0.131±0.006 (6)
sCT, 10 $U.kg^{-1}$ i.c.v., 45 mins	1.555±0.055 (6)	0.413±0.020 (6)	0.366±0.016 (6)	0.146±0.007 (6)
Vehicle, i.c.v. 90 mins	1.585±0.089 (5)	0.414±0.022 (6)	0.398±0.032 (6)	0.127±0.006 (6)
sCT, 10 $U.kg^{-1}$ i.c.v., 90 mins	1.523±0.020 (6)	0.427±0.013 (6)	0.384±0.017 (6)	0.144±0.006 (6)

Indoleamine concentrations (expressed in µg/g wet weight) after sCT did not differ significantly ($P > 0.05$) from those of appropriate control (vehicle) groups. Where 5-HIAA was not determined, 5-HT is not corrected for 5-HIAA flourescence. Values are $\bar{x} \pm$ s.e. (n).

Table 8 Lack of Effect of sCT on Rat Brain 5-HT and 5-HIAA

	"Midbrain"		Cortex and Cerebellum	
	5-HT	5-HIAA	5-HT	5-HIAA
Vehicle, i.p. 20 mins	1.617±0.122 (6)	1.024±0.074 (6)	0.441±0.036 (5)	0.290±0.018 (5)
sCT, 20 $U.kg^{-1}$ i.p 20 mins	1471±0.106 (5)	1.055±0.098 (6)	0.422±0.013 (6)	0.288±0.010 (6)
Vehicle, i.p. 45 mins	1.908±0.131 (5)	1.023±0.039 (5)	0.484±0.097 (6)	0.284±0.012 (6)
sCT, 20 $U.kg^{-1}$ i.p 45 mins	1.893±0.082 (5)	1.048±0.044 (6)	0.463±0.017 (6)	0.303±0.020 (6)
Vehicle, i.p. 90 mins	1.543±0.093 (6)	1.006±0.059 (6)	0.476±0.023 (6)	0.323±0.010 (6)
sCT, 20 $U.kg^{-1}$ i.p 90 mins	1.593±0.131 (6)	1.214±0.115 (6)	0.518±0.053 (6)	0.330±0.011 (6)

Indoleamine concentration (expressed in µg/g wet weight) after sCT did not differ significantly ($P > 0.05$) from those appropriate control (vehicle) groups. Values are $\bar{x} \pm$ s.e. (n).

DISCUSSION

D.1 SUMMARY OF RESULTS AND SCOPE OF DISCUSSION

Centrally mediated effects of CT can be assumed to involve direct or indirect modification of neuronal activity. Similarly, evidence has been presented indicating that the pressor response of haemorrhaged rats to peripherally administered (i.v.) sCT involves modulation of the activity of peripheral sympathetic nerves. The work presented in this thesis therefore falls into three distinct categories:

i) Determination of the pharmacological effects of drugs and peptides using in vivo models suitable for the quantification of the responses to CT induced alteration of neuronal activity. The models used in the majority of experiments were, the mouse acetic acid induced abdominal constriction test, in which sCT, hCT and CGRP were found to produce antinociception after central administration, and the normotensive and hypotensive urethane anaesthetised rat in which sCT was found to produce profound pressor responses, particularly after central administration, or in rats rendered hypotensive by haemorrhage.

ii) The use of pharmacological and surgical procedures to determine the identity of the neuronal systems affected by sCT. The antinociceptive effect of centrally administered sCT in the mouse acetic acid abdominal constriction test was found to be dependent on the integrity of central tryptaminergic systems but apparently not dependent on central noradrenergic systems. The pressor effect of peripherally administered sCT in haemorrhaged rats was found to be dependent on the integrity of both the CNS and the peripheral sympathetic nervous system, whereas the latter appears not to be necessary for the pressor effect of centrally administered sCT.

iii) Determination of the biochemical effects of sCT in order to elucidate mechanisms by which CT might alter neuronal activity. In contrast to previous reports CT was found not to alter central 5-HT (or 5-HIAA) concentrations indicating that gross alterations in the concentration of this indoleamine do not produce (or occur as a result of) the central neuronal action of CT. Investigation of possible second messenger systems revealed that sCT can elevate the concentration of cAMP and the activity of adenylate cyclase in the mouse hypothalamus

and can inhibit potassium stimulated uptake of $^{45}Ca^{2+}$ by slices of rat midbrain.

In the following discussion it is the author's intention to relate these observations to each other, to the published results of other workers, and to broader concepts of relevance to the understanding of the pharmacological effects of CT. It is also intended to propose models and mechanisms to account for the observed effects and to discuss the physiological, pharmacological and therapeutic relevance of the effects of CT reported herein.

D.2 THE RELATIONSHIP BETWEEN CALCITONIN AND CALCITONIN GENE-RELATED PEPTIDE

Several groups have employed biochemical techniques to investigate the nature of CT-like immunoreactive material demonstrated in the CNS and pituitary. For example, Catherwood and Deftos (1980) extracted immunoreactive material from porcine pituitary which was recognised by antisera raised against pCT, but not by that raised against hCT and was found to be of similar size to pCT when subjected to gel filtration chromatography. Similarly, Fischer et al (1981) found the predominant forms of immunoreactive CT extracted from human pituitary to co-elute with synthetic monomeric hCT and its sulphoxide on reverse phase HPLC. More recently this group (Fischer et al, 1983) reported approximately equimolar concentrations of immunoreactive hCT and its carboxy terminal flanking peptide (CCAP) in extracts of human brain (again indistinguishable from the authentic peptides by HPLC). These results provide evidence that authentic CT's exist in the CNS and that the precursor is processed (and presumably produced) centrally.

In contrast, the opposite conclusion is indicated by the results of Rosenfeld and co-workers. Amara et al (1982) have demonstrated that the CT genome, which is transcribed to CT mRNA in the thyroid, is transcribed to CGRP mRNA in the neural tissues of rats. Furthermore, Rosenfeld et al (1983) demonstrated CGRP immunoreactivity in the rat CNS but failed to demonstrate central CTLI with antisera which recognised thyroid CT. Cooper et al (1980a) had previously reported that only a small proportion (two out of fifteen) of antisera capable of recognising authentic CT's detected CTLI in the brain or pituitary, clearly indicating that the immunoreactive molecules demonstrated in the brain and pituitary are not identical to those found peripherally but that these immunoreactive molecules are similar enough to cross react with some antisera.

Structural similarities between CT and CGRP (which may prove to be more striking when the tertiary structure of CGRP is determined, see I.10) raise the possibility that CGRP may be the endogenous molecule recognised by antisera raised against CT's. A logical extension of this argument is that the CT binding sites within the

CNS might prove to be receptors for CGRP and that receptor mediated central effects of CT's might be related to the physiological role of endogenous CGRP. With the present lack of experimental data concerning the secondary structure of CGRP, the affinity of CGRP for central CT binding sites and the distribution of central CGRP binding sites, these possibilities can only be explored by comparison of a) the affinity of antisera recognising central CTLI for synthetic CT's and CGRP's; b) the distribution of central CGRP-like and CT-like immunoreactivities; and c) the pharmacological profiles of centrally administered CT and cGRP. These comparisons, which are limited by a paucity of experimental data are explored below.

The majority of demonstrations of central CTLI were reported prior to the availability of CGRP and possible cross reactivity of antisera with CGRP has not therefore been assessed. One notable exception is the report of Fischer et al (1983) in which separate antisera recognising human CTLI and salmon CTLI in extracts of human brain did not cross react with rat CGRP in amounts of up to 1.3 nmol (50% binding of sCT and hCT was observed with 6 and 9 fmol respectively) indicating that these antisera were not recognising rat CGRP in the human brain extracts (cross reactivity with human CGRP was not determined).

Rosenfeld et al (1983) reported that the distribution of CGRP like immunoreactivity was different to that of any known neuropeptide. Although the distributions of central CGRP like immunoreactivity and CTLI are broadly similar (both are concentrated in the hypothalamus, brain stem and anterior lobe of the piuitary gland and sparse in the cortex and cerebellum), detailed comparison of distributions is impossible because central CTLI has not been thoroughly mapped.

At present (August 1984) the only reported pharmacological effects of centrally administered CGRP are the production of antinociception in mice (chapter R.3), elevation of MAP in rats (Fischer et al, 1983) and inhibition of gastric acid secretion in rats (Lenz, Mortrud, Rivier, Vale and Brown, 1984). Comparisons of the effects of i.c.v. sCT and CGRP on the rate of acetic acid induced abdominal constrictions in mice (results presented in the present work) reveals

that both peptides produce antinociception but that the potency ($ED_{50} \simeq 600$ pmol.kg^{-1}) and duration of action of CGRP were considerably less than those of sCT ($ED_{50} \simeq 4.7$ pmol.kg^{-1}). The antinociceptive effect of CGRP was found to resemble that of hCT in both potency ($ED_{50} \simeq 800$ pmol.kg^{-1}) and duration.

Comparison of the effects of centrally administered sCT and CGRP on rat blood pressure (chapter R.4.b. and Fisher et al, 1983) reveals that sCT at doses of 0.1-10 U.kg^{-1} (0.06-0.6 nmol.kg^{-1}) produces a pressor response in normotensive rats with MAP elevated 5-60 minutes after administration of the higher doses, whereas, the pressor response produced by i.c.v. administration of CGRP, at doses of 1 and 10 nmol.kg^{-1}, had a duration of no more than ten minutes. It would clearly be advantageous to compare the haemodynamic effects of CT's and CGRP in internally controlled experiments using the same animal model (e.g. all animals conscious or all animals anaesthetised).

Comparison of the inhibitory effects of CT and CGRP on gastric acid secretion is hampered because the effects of CGRP have, as yet, only been reported in the form of a brief abstract. However, it is apparent that both peptides can inhibit gastric acid secretion in rats after central or peripheral administration (I.8.c. and Lenz et al, 1984),and that both peptides are highly potent (Lenz et al, 1984, reported inhibition of gastric acid secretion after administration of 0.22-2200 pmol CGRP, and Bueno et el, 1983, observed reduced gastric acid secretion after i.c.v. administration of approximately 1.2 and 12 pmol sCT). Lenz et al (1984) were unable to antagonise the effect of i.c.v. CGRP by peripheral administration of CGRP antibodies, indicating that CGRP, like CT (Morley et al, 1981a), can act centrally to inhibit gastric acid secretion.

The observations described above are compatible with the suggestions that CGRP may play a central role in the processing of painful stimuli, cardiovascular regulation (Rosenfeld et al, 1983) and regulation of gastric acid secretion, and that CT's might act as agonists at central CGRP receptors. However the pressor effect of centrally administered sCT in haemorrhaged rats was not attenuated by chemical sympathectomy (chapter R.6.), and the pressor effect of

centrally administered CGRP was reported to be associated with increased levels of plasma adrenaline and noradrenaline (Fisher et al, 1983). Accepting the conclusion of Fisher and co-workers, that centrally administered CGRP elevates blood pressure by increasing noradrenergic sympathetic outflow, these results indicate that the pressor responses to centrally administered sCT and CGRP are achieved by different mechanisms and presumably at distinct sites of action. An altenative interpretation of the observations of Fisher et al (1983) is that the elevated levels of plasma adrenaline and noradrenaline do not occur as a result of enhanced noradrenergic sympathetic activity (these catecholamines could be secreted from the adrenal medulla), or that the enhanced sympathetic activity does not contribute to the observed response. These alternative interpretations could readily be resolved by comparison of the effects of chemical sympathectomy on the pressor responses to centrally administered sCT and CGRP in the same model.

It is apparent from the preceding paragraphs, that the nature of CTLI and the possibility of CT action on central CGRP receptors remain unresolved, and may remain so until central CT-like materials are extracted and sequenced and central CGRP receptors are characterised. When considering the relationship between central CTLI and CGRP it should be noted that duplication of the CT gene is thought to have occurred. Assuming similar organisation of the two CT genes, the information coding for CGRP will also be duplicated, explaining the observation of two separate forms of rCGRP in the rat brain (Rosenfeld et al, 1983) and two separate forms of CTLI in the human brain (Fischer et al, 1983). Moreover, the recent report that human CGRP differs from the rat peptide, in that it is found in thyroid tissues (Morris et al, 1984), may be significant. Clearly it is unwise at the present time to be dogmatic about the distribution of the multiple peptide products of the CT/CGRP genomes which may be expressed to varying degrees in different tissues and species.

D.3 SITE OF ACTION OF PERIPHERALLY ADMINISTERED CALCITONIN

Peripherally, the actions of CT are thought to be mediated by binding to highly specific, high affinity binding sites such as those demonstrated in bone and kidney (Marx et al, 1972a) and in pulmonary carcinoma cells (Hunt et al, 1977). The blood-brain barrier is normally thought to prevent large peptide hormones in the periphery from exerting direct effects on the CNS. However, considerable evidence exists suggesting that this may not be the case for CT. This evidence which is of direct relevance to the haemodynamic effects of peripherally administered sCT described in chapter R.4.a, is discussed below.

i) Pharmacological actions of CT which are generally thought to be centrally mediated are often observed after peripheral administration of the hormone. For example, Levine and Morley (1981a) found i.c.v. CT to be 1000 times more potent than the parenterally administered hormone at inhibition of gastric acid secretion in the rat. Similarly, Bueno et al (1983) found that i.v. CT was 100 times less effective than i.c.v. CT at abolishing the fasted pattern of electrical activity of the rat intestine. Doepfner (1983) stated that penetration of the blood brain barrier by peptides was approximately 0.1% indicating that peripherally administered CT's could exert direct central effects.

ii) Stekolnikov and Abdukarimov (1969) employed spectrophotometric and chromatographic techniques to demonstrate the appearance of CT within the CSF after peripheral administration to the rabbit. Similarly, peripheral administration of sCT (40 μg) has been found to increase radioimmunoassayable CT in the CSF of man from 75.8 to 220.2 pg.ml^{-1} (Fabbri et al, 1981), although the possibility of plasma contamination of CSF samples cannot be excluded. In contrast, whole body autoradiography of mice (Hicks et al, 1971) and rats (Forslund et al, 1980) has failed to demonstrate redistribution of peripherally administered CT to the CNS.

iii) Peripherally administered CT may exert effects on areas of the CNS not protected by the blood-brain barrier. Specific binding sites (considered by the authors to be receptors) for blood-borne CT have been demonstrated in the circumventricular organs which are not protected by the blood-brain barrier (Vanhouten et al, 1981). These highly specialised organs are thought to serve as the main receptive system for mediating the direct effects of blood-borne peptides on the brain.

Irrespective of the ability or lack of ability of the hormone to cross the blood-brain barrier, it is apparent that peripherally administered CT could exert direct central effects. As the majority of the results presented above concern the antinociceptive and haemodynamic effects of CT, it is pertinent to consider the possibility of such actions occurring centrally after peripheral administration. In the abdominal constriction test i.c.v. sCT has been shown to produce antinociception at doses of 0.1-50 $U.kg^{-1}$ whereas s.c. doses of 0.001-1000 $U.kg^{-1}$ produced no such effect (Bates et al, 1981a). Pecile et al (1983) have reported that sCT at 20 µg/rat s.c. produced significant antinociception (in the rat hot plate test) on the second and third dose of a chronic treatment schedule (two injections per day giving a daily dose of approximately 600 $U.kg^{-1}$). It therefore seems possible that tests for the antinociceptive effects of CT's administered acutely by peripheral injection merely provide insufficient time for the redistribution of the hormone to the CNS.

The possibility of a central effect of peripherally administered CT is of particular relevance to interpretation of the haemodynamic effects of sCT reported in chapter R.4.b. As the pressor response of haemorrhaged rats to peripherally administered sCT (10 $U.kg^{-1}$, i.v.) was of similar magnitude and duration to that produced by 1 $U.kg^{-1}$ i.c.v. it might be concluded that approximately 10% of the peripheral dose is redistributed to a central site of action. This hypothesis would not however, be in accord with the fact that the peripheral doses required to affect gastric acid secretion and intestinal electrical activity are 100-1000 times greater than the corresponding central doses. Moreover, as the pressor response to i.v. sCT was markedly attenuated by chemical sympathectomy, whereas the response

to i.c.v. sCT was not, separate mechanisms and sites of action are indicated. Hence, although the sites at which peripherally administered sCT produces its haemodynamic effects are not known, it presumably does not involve a direct action on the CNS. As no evidence was obtained for a direct vasoconstrictor action in pithed rats, and the hormone has been shown not to potentiate the vasoconstrictor effect of exogenous noradrenaline (Driessens and Vanhoutte, 1981), it appears that sCT may act peripherally to modulate the activity of noradrenergic sympathetic neurones.

D.4 SITE OF ACTION OF CENTRALLY ADMINISTERED CALCITONIN

In the previous chapter it has been argued that although mechanisms exist by which peripherally administered CT may act directly upon the CNS, the haemodynamic effects of i.v. sCT reported in chapter R.4.a appear to involve a peripheral site of action. Although the possibility is not generally considered, it is also conceivable that centrally administered CT's could act peripherally, either by crossing the blood-brain barrier or by leakage into the periphery at the site of injection. However, Bates et al (1981a) failed to demonstrate antinociception in the mouse abdominal constriction test with peripheral administration (s.c.) of 0.01-1000 $U.kg^{-1}$ sCT, whereas central administration of 0.1-50 $U.kg^{-1}$ has been found to produce significant antinociception in the same test (R.2). These results clearly indicate that the antinociceptive effect of centrally administered sCT, which has been studied in this thesis, involves a direct action on the CNS. Similarly, the pressor response of haemorrhaged and normotensive rats to centrally administered sCT is either attenuated or absent after peripheral administration, indicating again that the centrally administered peptide produces the observed effects by a direct action on the CNS.

As the regions of the CNS accessible to centrally administered CT's have not been reported, it is only possible to assume that centrally administered CT's act on the specific binding sites demonstrated in several regions of the CNS. The distribution and characterisation of such binding sites is therefore of crucial importance to the understanding of the central role and effects of CT and related peptides and is discussed below.

D.4.a The Distribution of Central Calcitonin Binding Sites, and their Status as Receptors

As stated previously, the peripheral actions of CT are thought to be mediated by binding to highly specific high affinity binding sites. Specific binding of CT to peripheral tissues has been shown to be dependent on both time and temperature and to be highly selective (Goltzman, 1980), and renal CT binding sites have been

shown to be associated with cell membranes and membrane bound adenylate cyclase (Marx and Aurbach, 1975). Convincing evidence that such binding sites represent functional receptors associated with adenylate cyclase is that the relative potencies of salmon, porcine and human CT's are similar whether assessed by competetive binding analysis (40:1:0.05, Marx et al, 1972a), adenylate cyclase activation (20:1;0.05) or in vivo bioassay (20:1:0.05, Potts, Niall and Keutman, 1970). Evidence of this nature has led to the general acceptance that peripherally, CT specific binding sites represent CT receptors.

Similar evidence has accumulated indicating that central CT specific binding sites might also represent receptors. Hence, ^{125}I-CT binding to rat brain homogenates has been shown to be dependent on time, temperature and pH and to be inhibited by salmon, eel and porcine CT's with IC_{50}'s proportional to their hypocalcaemic activity (Nakamuta et al, 1981, see I.6), and CT has been shown to modify central cyclic nucleotide metabolism both in vitro and in vivo (R.6). Evidence of this nature led Rizzo and Goltzman (1981) to suggest that central CT binding sites represent specific CNS receptors for the hormone. In light of the controversy surrounding the existence of authentic CT in the CNS (above) the alternative suggestion made by Nakamuta et al (1981), and more recently by Rosenfeld et al (1983), that central CT binding sites represent receptors for an unidentified CT-like molecule in the CNS cannot be discounted. Although the endogenous ligand for these receptors may not be authentic CT, the fact that authentic CT's bind to such receptors and that such binding may result in the behavioural effects of centrally administered CT's is not disputed.

Evidence for the role of CNS binding sites in the pharmacological effects of centrally administered CT can be derived from calculations based on the estimated density of such binding sites within the CNS and the pharmacologically effective doses of CT. Koida et al (1980) reported the maximum amount of specific binding (B_{max}) of ^{125}I-sCT to rat whole brain homogenates to be 56 fmol.mg^{-1}. Assuming the rat brain to contain approximately 180 mg of protein and that 50% of ^{125}I-sCT binding is non-specific, these receptors can be estimated to be fully occupied by addition of approximately 10 pmol of sCT per rat if the sCT is distributed rapidly and evenly throughout the CNS. The

equivalent dose of $1U.kg^{-1}$ is close to the dose reported in chapter R.2 to produce the maximum antinociceptive effect in mice ($2U.kg^{-1}$), and has been found to produce haemodynamic and antiulcerogenic effects as well as inhibition of gastric acid secretion and modification of prolactin secretion. Although this calculation can clearly be criticised on the grounds that *in vivo* binding characteristics, rates of displacement of CT from central receptors, and the distribution of centrally administered CT's are not known, the results are compatable with the suggestion that the pharmacological effects of centrally administered CT's are mediated by binding to the central high affinity binding sites.

In conclusion, considerable evidence exists to suggest that some of the central CT binding sites represent receptors for an endogenous CT-like molecule. Irrespective of the nature of this endogenous molecule (see D.2) it is known that authentic CT's bind to these receptors with high affinity and it can be assumed that the pharmacological effects of centrally administered CT's occur as a result of binding to the receptors. In order to further understand the central pharmacological actions of CT it is necessary to consider the anatomical distribution of central CT binding sites, the mechanism by which receptor binding modulates target cell activity and the pharmacological classification of these target cells.

D.4.b Correlation Between the Central Pharmacological Effects of Calcitonin and the Anatomical Distribution of Central Calcitonin Binding Sites

In rat and human brains the greatest density of CT specific binding sites is found in regions such as the hypothalamus and brain stem and the lowest density occurs in the cortex and cerebellum (Nakamuta et al, 1981; Fischer et al, 1981a). In the human brain, at least, the density of CT binding sites appears to correlate well with the distribution of CTLI, with the notable exception of the pituitary which was found to contain a high concentration of CTLI but very few CT binding sites. More recently the technique of *in vitro* autoradiography has been used to provide a more detailed knowledge of the distribution of central CT binding sites. As peptidergic

neurones are thought not to possess reuptake systems for peptides, the sites labelled in this manner presumably represent receptors rather than neuronal peptide stores. The distribution of central CT binding sites reported by Henke et al (1983) and Oligati et al (1983) is in general agreement with the distribution of binding sites in homogenates, i.e. regions of highest density include the hypothalamus and brain stem and little or no binding is observed in the cortex and cerebellum. More specifically, in the hypothalamus, dense labelling was observed in the anterior and dorsomedial areas whereas binding was sparse in the ventromedial and lateral parts. In hypothalamic control of feeding and gastric acid secretion, it is assumed that the ventromedial nucleus balances the stimulatory effect of the lateral hypothalmic area. An inhibitory effect of CT on the lateral hypothalamic area could therefore produce the inhibition of feeding and gastric acid secretion observed after central administration. Indeed, iontophoretically applied CT has been shown to inhibit the activity of glucose sensetive neurones of the lateral hypothalamus. Similarly, dense labelling was observed in the mediobasal hypothalamus, which is thought to be involved in the control of prolactin secretion, and in the anterior part of the hypothalamus which receives afferent inputs associated with the suckling stimulus of prolactin secretion (Oligiati et al 1983). These binding sites could clearly be relevant to the reported effects of CT on basal and suckling stimulated prolactin release. (1.7.h).

Oligiati et al (1983) considered the most striking feature of CT binding to rat brain to be the dense clustering of binding sites in regions of the brain stem and spinal chord that regulate pain perception. These areas included the dorsal horns of the spinal chord at laminae IV and V, the nucleus of the spinal tract of the trigeminal nerve, various Raphé nuclei and the periaqueductal grey matter which receive nociceptive input from laminae IV and V of the spinal chord. Assuming redistribution from the site of central administration to such binding sites CT might produce analgesia by inhibition of nociceptive inputs to the brain or by inhibition of ascending or descending pain pathways. Of particular relevance to the mechanism of the antinociceptive effect of sCT investigated in this thesis, it should be noted that the ascending pathways described

above, and the Raphé nuclei are rich in the cell bodies of tryptaminergic neurones (below). Interaction with these neuronal systems might also be relevant to the cardiovascular effects of centrally administered sCT reported in chapter R.4 as the Raphé nuclei with the highest density of ^{125}I-sCT binding sites (Raphé obscurus, pallidus and magnus) are located in the medulla close to the vasomotor and cardioaccelerator centres, and tryptaminergic neurones from the medullary Raphé nuclei innervate the dorsal horns of the lateral sympathetic column. The developing understanding of the correlation between the pharmacological effects of centrally administered CT and the functional regions of the CNS in which CT binding sites are found provides further evidence that the effects of centrally administered CT may reflect physiological roles of the endogenous peptide.

D.4.c Pharmacological Identity of Neuronal Systems Modulated by Calcitonin

Although iontophoretic or i.c.v. application of CT has been shown to modulate neuronal activity, the neurotransmitters used by such neurones remain largely a matter of speculation. The first study of direct relevance to the neurotransmitter systems modulated by CT was that of Nakhla and Majumder (1978) who reported that peripheral administration of CT to rats elevated central AchE activity. This report does not, however, appear to have found support in subsequent investigations.

The possibility of CT induced modulation of central dopaminergic activity was investigated by Nicoletti, Ferrara, Bernadini, Clementi, Prato, Drago and Scapagnini (1982). This group reported that centrally administered sCT and hCT (0.25 and 1 µg respectively) produced antidopaminergic activity in rats (potentiation of haloperidol induced catalepsy and inhibition of apomorphine induced hypermobility). The models used are thought to reflect striatonigral dopaminergic activity, yet concentrations of dopamine and its major precursor dihydroxyphenyl acetic acid (DOPAC) were found to be unchanged in the corpus striatum . As CT was found to decrease the activity of the gamma amino butyric acid (GABA) synthesising enzyme, glutamate decarboxylase, in the substantia nigra it was suggested that CT modifies nigrostriatal GABA-ergic activity which indirectly modulates striatonigral dopaminergic activity.

The possibility of CT induced modulation of central noradrenergic activity has not yet been investigated in depth, although the antinociceptive effect of CT, at least, appears to be unrelated to central adrenergic systems. Hence, results presented above demonstrate that the antinociceptive effect of i.c.v. sCT in the mouse abdominal constriction test is not significantly modified by pretreatment with the dopa decarboxylase inhibitor α-MT (at a dose which reduced the concentration of noradrenaline within the CNS by 56%). Furthermore, the antinociceptive effect of centrally administered sCT (in the rat hot plate test) has been reported to be unaffected by blockade of central α-adrenergic receptors with phentolamine (Clementi et al, 1984).

In addition to the proposed effect of CT on central dopaminergic activity, considerable evidence has now accumulated to suggest a link between central CT and 5-HT:

i) Peripheral CT secreting cells 'C cells' derive from the embryonic neuroectoderm layer and retain the ability for uptake and decarboxylation of precursors to 5-HT and dopamine in accord with the APUD concept of Pearse (1969, 1977). It therefore seems reasonable to assume that central neurons containing CTLI might also possess this ability. As co-storage and co-release of neuropeptides and classical neurotransmitters appears as a developing pattern for CNS organisation (e.g. co-storage of substance P and 5-HT, Chan-Palay, 1981) the possibility of co-storage and co-release of 5-HT and CT-like peptides exists.

ii) CT administration has been reported to increase the concentration of central 5-HT (Nakhla and Majumdar, 1978; Dupuy et al, 1983). Although these results are not supported by those presented above (R.6) reasons for this discrepency are not known. The previous reports of CT induced elevation of central 5-HT levels cannot therefore be discounted, moreover it is conceivable that CT might modulate 5-HT turnover without altering absolute concentration.

iii) The side effects of peripherally administered CT in humans have been successfully treated by the 5-HT antagonist pizotifen (Crisp, 1981).

iv) Centrally administered CT produces a wide range of biological responses in which central tryptaminergic mechanisms may play a regulatory role. Hence, central administration of CT and, direct or indirect, stimulation of central 5-HT receptors has been found to inhibit feeding (see I.8.d and Blundell, 1977). Central administration of 5-HT is known to stimulate prolactin release (Kamberi, Mical and Porter, 1973) and central tryptaminergic neurones are thought to be involved in the suckling induced rise in plasma prolactin (Kordon, Blake, Terkel and Sawyer, 1974), indicating that CT induced modification of prolactin secretion (I.8.h) could be related to effects on central tryptaminergic neurones. Drugs such as PCPA, which inhibit central tryptaminergic activity have been reported to reduce arterial pressure (Chalmers, 1975), raising the possibility that the pressor response to centrally administered sCT (R.4.b) might be associated with an increase in central tryptaminergic activity. Finally, several groups have reported the antinociceptive effect of enhanced central tryptaminergic activity produced by central administration of 5-HT (R.2 and Post, Fasmer, Holm and Ogren, 1984; Yaksh and Wilson, 1979), blockade of monoamine oxidase or reuptake (Yaksh and Wilson, 1979), or by electrical stimulation of various Raphé nuclei (Besson et al, 1981). The antinociceptive effect of centrally administered CT might, therefore, be related to an enhancement of central tryptaminergic neurone activity.

v) In vitro autoradiography has revealed that CT binding sites are very dense in the dorsal horns of laminae IV and V of the spinal chord, in the medullary Raphé nuclei and in the periaqueductal grey matter. A major group of descending tryptaminergic neurones with their cell bodies in the medullary Raphé nuclei surrounding the preiaqueductal grey matter innervate the spinal chord and the dorsal and ventral horns of the lateral sympathetic column. It is therefore possible that at least some of the CT binding sites in these regions reflect receptors on tryptaminergic neurones.

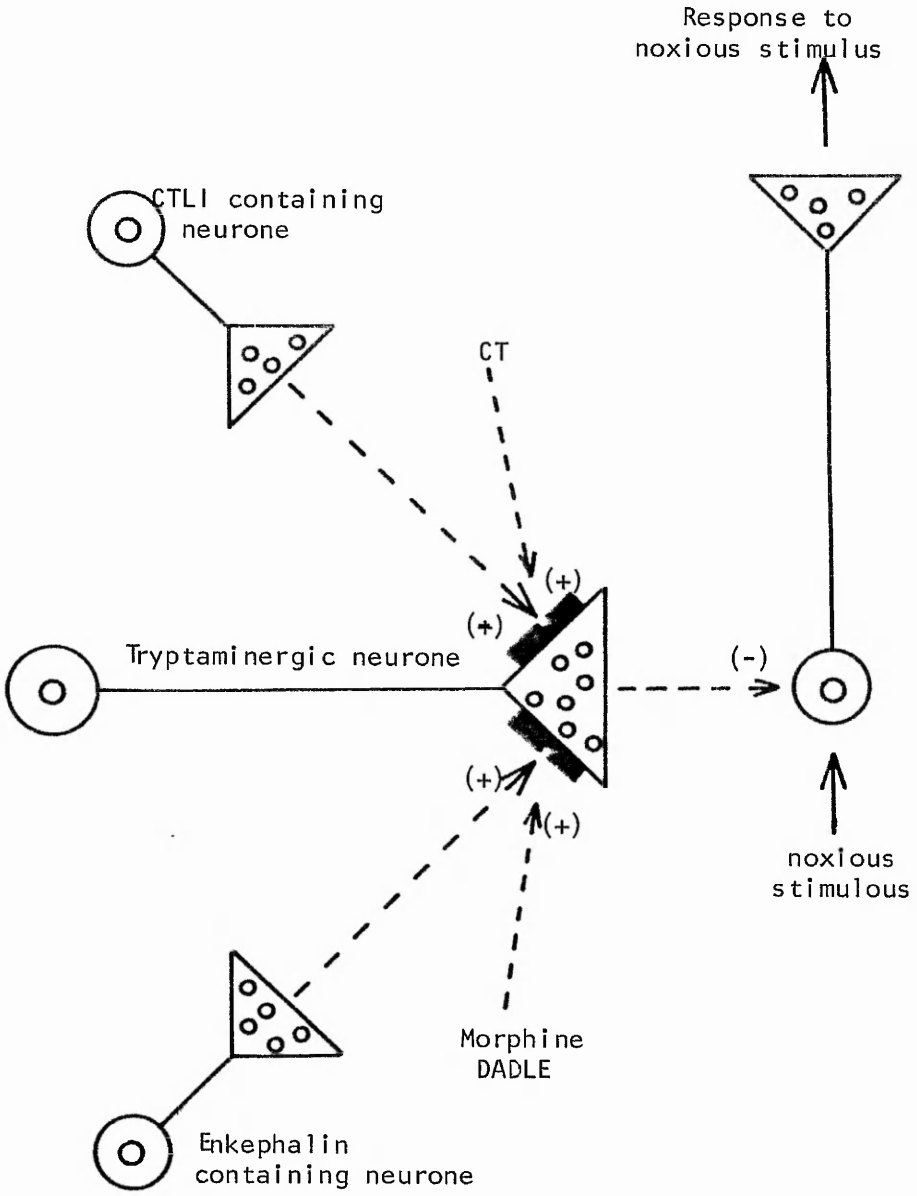
vi) Results presented above demonstrate that the antinociceptive effect of centrally administered sCT in the mouse abdominal constriction test is dependent on the integrity of central tryptaminergic systems (CT antinociception was antagonised by PCPA pretreatment and returned after central administration of 5-HT). Although methysergide produced no significant modification of CT antinociception in this test, Clementi et al (1984) have recently reported that methysergide antagonised the antinociceptive effect of centrally administered CT in the rat hot plate test. The reasons for this discrepency are unknown (see R.2) but the report of Clementi and coworkers indicates that the dependence of CT antinociception on central tryptaminergic systems is not peculiar to mice or to the abdominal constriction test.

Taken together these results provide strong evidence for a link between central CT and tryptaminergic neuronal systems. Specifically, tryptaminergic neurones of the Raphé nuclei, brain stem or spinal chord are implicated as the site at which the antinociceptive effects of CT are produced. A simple model to account for such observations is shown in Figure D.1. This model is not intended to precisely describe the anatomical arrangement of central neuronal systems involved in CT induced and opiate induced antinociception, nor is it intended to imply that this is the only possible model compatible with the results presented in this thesis. The model is however in accord with the observations reported in chapter R.2, that:

i) 5-HT administered centrally produces antinociception which is not antagonised by the pretreatment with PCPA whereas the antinociceptive effects of centrally administered sCT and DADLE are attenuated by PCPA pretreatment, and

ii) the antinociceptive effect of the maximal effective dose of sCT ($2U.kg^{-1}$) was enhanced by simultaneous administration of 5-HT suggesting that maximal activity of CT at its central binding sites may produce insufficient release of 5-HT for tryptaminergic neurones to maximally stimulate 5-HT receptors in the production of antinociception.

Figure D.1 Simplistic Model to Account for Dependence of CT Antinociception on Central Tryptaminergic System.


Response to
noxious stimulus
CTLI containing
neurone
CT
(+)
(+)
Tryptaminergic neurone
(-)
(+)
(+)
noxious
stimulous
Morphine
DADLE
Enkephalin
containing neurone

Although this model can be used to account for several of the results obtained in the experiments reported in this thesis, it is presumably an oversimplification of the situation found in vivo, and several points clearly warrant further discussion.

Firstly, it should be noted that the distance between the site of release of the CT-like peptide and the receptors for the peptide could be a few μm or several mm. Classically, neurotransmitters are thought to act at receptors on post-synaptic membranes which are in tight juxtaposition with the synaptic membrane of the pre-synaptic neurone. However, neuropeptides playing a paracrine, neuroendocrine or neuromodulatory role may act at receptors more distant from the site of release.

Secondly, an enkephalinergic neurone is shown innervating the tryptaminergic pathway as such an arrangement could underlie the observation that both CT and morphine produce similar inhibition of the neuronal potantials evoked by painful stimuli (Yamamoto et al, 1980). However it is not the authors' intention to imply that this is always the case or that receptors for enkephalins and CT-like peptides are necessarily located on presynaptic nerve terminals.

Thirdly, it should be stressed that CT-like peptides and endogenous opiates may act as inhibitory transmitters and CT has been shown to inhibit the activity of glucose sensitive neurones in the lateral hypothalamus (Miyahara and Oomura, 1981). It therefore seems conceivable that these peptides produce antinociception by inhibiting the activity of interneurones which themselves inhibit 5-HT release from tryptaminergic neurones.

Finally, it should be noted that release of 5-HT from the tryptaminergic neurone is shown inhibiting the activity of a neurone in the primary pain pathway. This pathway has been included in the model to account for the observation that centrally administered calcium ions and ionophores produce hyperalgesia and can antagonise the antinociceptive effects of CT's and opiates (seeD.5.b). If it is assumed that central administration of calcium ions enhances neuronal activity indiscriminantly, it must also be assumed that the enhanced activity in pathways increasing

the response to painful stimuli is sufficient to mask the effect of increased activity in pathways tending to reduce the response to such stimuli. Accepting such a model, it is evident that antagonism of the antinociceptive effects of CT's and opiates by central administration of calcium ions and ionophores, and potentiation of such effects by administration of calcium chelators and antagonists does not necessarily indicate modulation of central calcium metabolism to be the primary mechanism of action of these peptides (see D.5.b).

D.5 MECHANISMS OF THE EFFECTS OF CALCITONIN ON THE CNS AT THE CELLULAR AND SUBCELLULAR LEVELS

Physiologically significant hormonal effects are mediated by binding of the hormone to receptors on or in the target cell, and second messenger systems may serve to translate the extracelluar event (hormone binding) to the ultimate cellular response (1.9). As the role of CT in calcium homeostasis is established, it is not surprising that extensive research effort has focused on modification of cellular calcium metabolism as a mechanism for the hormones effects on cellular activity. Considerable biochemical and behavioural evidence has accumulated to suggest that alteration of cellular calcium metabolism is the mechanism by which CT (and opiates) produces its physiological and pharmacological effects. This evidence, and evidence which indicates that CT may act by alternative means, such as alteration of cyclic nucleotide metabolism, is discussed below.

D.5.a Effects of Calcitonin on Cellular and Subcellular Calcium Metabolism

Calcitonin has been shown to inhibit the efflux of calcium from bone (Friedman and Raisz, 1965), kidney (Kenny and Heiskell, 1965), liver (Yamaguchi et al, 1975) and myocardium (Chausmer et al, 1965), an effect which could clearly account for the increased levels of calcium observed in most tissues (Borle, 1975). Kinetic analysis of the uptake of $^{45}CA^{2+}$ by kidney cells indicated to Borle (1975) that CT increased the cellular accumulation by increasing the size of the slowly exchangable calcium pool (assumed by Borle to be mitochondrial). Borle (1983) proposed that the fundamental mechanism of action of CT is stimulation of mitochondrial calcium uptake (cytosolic calcium is thought to be maintained by uptake into mitochondria, endoplasmic reticulum and plasma membranes), and that it is the reduction in cytosol calcium concentration that leads to an inhibition of efflux and consequent increase in cellular calcium. In neural tissue Eglen (1983) failed to show that CT induced an increase in the total calcium concentration, any inhibitory effect on efflux in neural tissue must therefore be associated with a reduced uptake. The net effect, reduction in calcium turnover in neural tissue could clearly account for the observed reduction in $^{45}Ca^{2+}$ uptake.

Borle's hypothesis can, however, be firmly criticised, as it is dependent on the observation that CT stimulates $^{45}Ca^{2+}$ transport into isolated mitochondria. Although it is suggested that extracellular CT becomes internalised in vivo to exert this effect (Borle, 1975), Marx and Aurbach (1975) were able to separate the ^{125}I-sCT binding fraction of renal homogenates from the mitochondrial fraction by means of sucrose density gradient centrifugation. The direct effect of CT on mitochondria can not, therefore be receptor mediated and is unlikely to be of physiological or pharmacological significance.

Although the reported direct effect of CT on mitochondrial calcium uptake may not be physiologically or pharmacologically relevant, Borle's kinetic analysis of $^{45}Ca^{2+}$ uptake by kidney cells provides a valuable indication of the mechanism by which CT might modify cellular calcium metabolism. As noted above, Borle (1975) found that CT increased cellular accumulation of $^{45}Ca^{2+}$ and increased calcium sequestering in a slowly exchangeable pool, the consequent reduction in cytosolic calcium concentration was thought to account for the observed inhibition of cellular efflux. In the CNS Eglen (1983) failed to modify the calcium concentration of regions of mouse brain by central administration of sCT (0.5-20 $U.kg^{-1}$, i.c.v.). Therefore any CT induced inhibition of calcium efflux from central tissues must presumably be associated with inhibition of uptake. Indeed, CT has been found to inhibit the uptake of $^{45}Ca^{2+}$ by unstimulated hypothalamic tissue (Levine and Morley, 1981a; Koida et al, 1982) and potassium stimulated slices of rat midbrain (R.5). Interpretation of the results reported for hypothalamic calcium uptake is difficult as the authors do not appear to have adopted procedures suitable for determining whether the radiolabel is taken up into intracellular or extracellular compartments. However, preliminary experiments reported in chapter R.5, have provided a suitable protocol for the assessment of the effects of CT on uptake of $^{45}Ca^{2+}$ in central tissues, in which the majority of radiolabel is taken into the intracellular compartment. In these experiments sCT was without effect on basal $^{45}Ca^{2+}$ uptake, but prevented potassium stimulated uptake. If inhibition of depolarisation induced $^{45}Ca^{2+}$ uptake occurs as a result of reduced neuronal excitability, these results may reflect a neuromodulatory role of the peptide. In tissue slice experiments it is impossible to determine whether CT acts

directly to modify calcium distribution, or whether alteration of calcium flux occurs as a result of direct action on a distinct second messenger system. Second messenger systems which might indirectly account for the cellular effects of CT (including effects on calcium metabolism) are discussed below.

D.5.b Evidence for the Dependence of Central Opiate and Calcitonin Induced Antinociception on Central Calcium Metabolism

Many studies have demonstrated the important role of calcium in the central actions of opiate analgesics. It has been shown that central administration of calcium can produce hyperalgesia whereas central administration of calcium antagonists or the calcium chelating agent EGTA (ethylene glycol-bis(β-amino ethyl ether) tetracetic acid) can produce antinociception (Schmidt and Way, 1980). Moreover, central administration of subeffective doses of calcium can antagonise the antinociceptive effect of morphine (Harris, Loh and Way, 1975), and central administration of subeffective doses of EGTA or calcium antagonists can potentiate morphine antinociception (Harris et al, 1975; Iwamato, Harris, Loh and Way, 1978). In addition to these behavioural studies acute narcotic administration has been found to reduce the total calcium content of central tissues (Cardenas and Ross, 1975) and to reduce both the rate of calcium uptake and the total calcium content of synaptosomes prepared from rat or mouse brains (Cardenas and Ross, 1975). As neurotransmitter release is known to be calcium dependent, Schmidt and Way (1980) have proposed that the reduction in calcium available to the nerve terminal, after acute opiate administration, inhibits neurotransmitter release (see R.2) and that this inhibitory effect produces antinociception by an undefined mechanism.

Similar evidence exists to support the dependence of CT antinociception on central calcium metabolism. Hence, Satoh et al (1979) and Bates et al (1981c) have shown central CT antinociception to be antagonised by i.c.v. doses of calcium which were themselves without effect. This antagonism presumably involves an increase in cellular calcium, as Bates et al (1981d) found CT antinociception to be antagonised by the calcium ionophore A25187 (again at a dose which was not hyperalgesic). Moreover, Bates et al (1982b) have shown that CT antinociception can be potentiated by central administration of EGTA and the organic calcium antagonists, nifedepine and PY 108 068 (at doses which do not themselves produce antinociception).

Calcitonin is thought not to act on central opiate receptors, but considerable similarities exist between opiate and CT induced antinociception, which may reflect a common mechanism of action. Hence, the proposal of Schmidt and Way (1980) - that the primary mechanism of opiate action is a reduction in neuronal calcium uptake may be extended to account for the mechanism of action of CT. It should be noted however, that the evidence in support of this mechanism of action for opiates is considerably stronger than that for CT (e.g. central administration of CT appears not to reduce calcium content or uptake or to inhibit neurotransmitter efflux) and that the observations described above do not prove modification of calcium metabolism to be the primary mechanism of action of either opiates or CT. Furthermore, this hypothesis can be firmly criticised on two grounds:

i) It is assumed that reduced neuronal activity produces analgesia, although this may be the net effect of increased neuronal activity, evidence outlined above (D.4.c) indicates that an increase in the activity of some groups of neurones produces antinociception. The antinociceptive effect of enhanced tryptaminergic activity in the CNS is of direct relevance to the experiments described in chapter R.2, and is discussed above. It can, therefore, be assumed that central administration of calcium or calcium ionophores either selectively enhances the activity of neuronal systems, producing nociception, or that the net effect of calcium and calcium ionophores on neuronal systems with divergent effects is enhanced nociception.

ii) Radioligand binding to putative opiate and CT receptors of CNS membrane preparations has been shown to be inhibited by calcium ions, an effect which could, at least in part, account for antagonism of the peptide induced effects (Pert and Snyder, 1973 Fischer et al 1981b).

In conclusion, the antinociceptive effects of centrally administered CT and opiates are undeniably dependent on central calcium metabolism, it is not clear however, whether this merely reflects the calcium dependence of the neurones modulated by these peptides or the possibility that these peptides alter neuronal activity by direct modification of neuronal calcium metabolism.

D.5.c Effects of Calcitonin on Central Cyclic Adenosine 3',5'-monophosphate Metabolism

Peripheral CT receptors of kidney and bone have been shown to be associated with membrane bound adenylate cyclase indicating that modulation of cAMP production might be the mechanism by which receptor binding ultimately leads to modulation of the activity of the target cells. As adenylate cyclase activation is observed in membrane preparations, this effect cannot occur as a result of modified calcium metabolism (effects of CT on cellular calcium metabolism of intact cells or tissues could, however, be achieved as a result of adenylate cyclase activation). In CNS tissue CT has been shown to alter cyclic nucleotide metabolism both in vivo (CT induced increase in hypothalamic cAMP reported above) and in vitro (increase in cAMP accumulation of rat glial cells reported by Loffler et al, 1982 , and modification of adenylate cyclase activity reported by Rizzo and Goltzman, 1981 and above).

The stimulatory effect of sCT on hypothalamic adenylate cyclase reported in chapter R.6 is in direct contrast to the inhibitory effect reported by Rizzo and Goltzman (1981). However, the studies of Rizzo and Goltzman were performed in the absence of added GTP, and the concentrations of sCT found to inhibit adenylate cyclase in hypothalamic homogenates were at least 1000 times greater than the concentrations required to inhibit binding of ^{125}I-sCT to the same preparation ($ED_{50} \simeq 1$ nM, $IC_{50} \simeq 6$ µM). These results therefore contrast to the situation in the periphery as the ED_{50}'s reported for adenylate cyclase stimulation are generally in good agreement with the IC_{50}'s for displacement of ^{125}I-sCT from peripheral receptors (e.g. Loreau et al, 1978, reported the IC_{50} and ED_{50} for CT binding and stimulation of adenylate cyclase in the rat renal cortex were approximately 0.5 and 1.6 nM respectively). A possible interpretation of these results is that the inhibitory effect of CT on central adenylate cyclase is not receptor mediated and is presumably, therefore, not physiologically or pharmacologically relevant.

In direct contrast to the results of Rizzo and Goltzman (1981), the characteristics of the stimulatory effect of sCT on hypothalamic adenylate cyclase demonstrated in chapter R.6 are compatible with the

suggestion that the enzyme stimulation occurs as a result of receptor binding. Hence, the effect appears to be dependent on the presence of GTP (as is the case for the renal membrane, CT receptor linked, adenylate cyclase), and occurs at doses known to inhibit ^{125}I-sCT binding to hypothalamic homogenates. The value reported by Rizzo and Goltzman to reduce ^{125}I-sCT binding to hypothalamic homogenates by 50% was 1 nM, this concentration lies within the range of doses found to stimulate hypothalamic adenylate cyclase activity. Clearly further characterisation of the central sCT dependent adenylate cyclase enzyme is required to establish whether this effect is receptor mediated (for example guanyl nucleotide dependence could be reassessed in washed membrane preparations to avoid contamination with endogenous GTP and the possible correlation between the distribution of central CT binding sites and central CT dependent adenylate cyclase could be investigated). If the enzyme proves to be receptor linked, this preparation, associated with binding studies, could provide a valuable model for investigation of the relationship between structure and activity at central CT binding sites.

D.5.d Interactions and Alternatives

In the preceding sections CT induced alterations of cellular cAMP and calcium metabolism have been considered as possible mechanisms by which CT could modify neuronal activity. It should, however, be recognised that CT might modulate the activity of target cells by exerting effects on other second messenger systems. In this respect, the possibility that CT might affect cyclic guanosine 3',5'-monophosphate or phosphatidyl inositol (PI) metabolism clearly warrant investigation.

The possibility of CT induced modification of PI metabolism is of particular interest because of the link between PI hydrolysis and mobilisation of intracellular calcium (for review see Downes, 1983). In the PI response receptor binding is thought to activate phospholipase C to hydrolyse phosphoinositides of the plasma membrane forming inositol phosphates and diacylglycerol. Phosphoinositides which are highly polar, are thought to influence the properties of calcium binding sites and/or calcium channels within the plasma membrane, and their hydrolysis may lead to mobilisation of calcium. Alternatively, inositol phosphates (particularly the triphosphates) may act by mobilisation of calcium from intracelluar stores (Burgess and Putney,

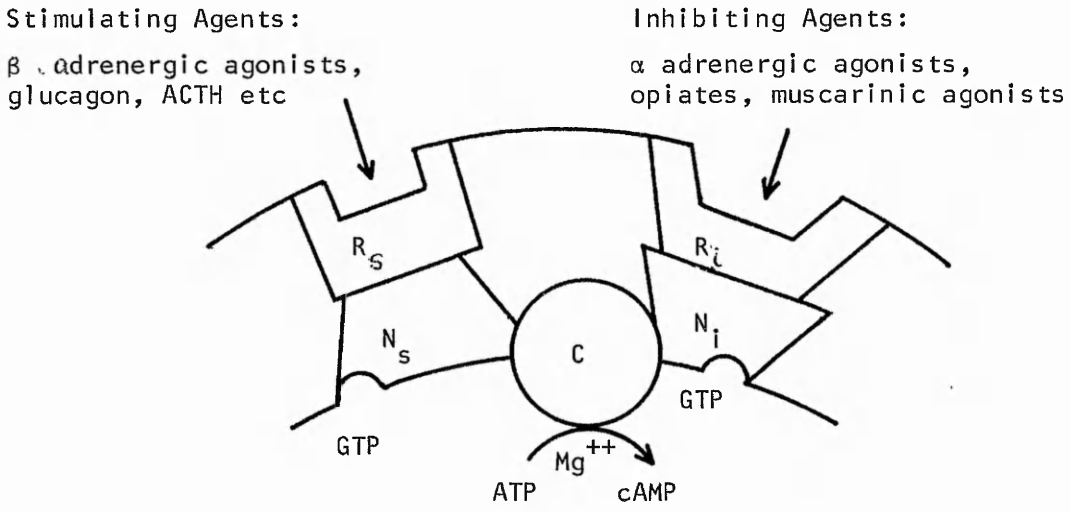


Figure D.2 Schematic representation of dual regulation of adenylate cyclase system by stimulatory and inhibitory hormones and neurotransmitters, from Rodbell, 1980.

Binding of GTP to the nucleotide regulatory component (N) on the inner face of the cell membrane either stimulates (N_s) or inhibits (N_i) the action of the adenylate cyclase catalytic unit (C). Under normal conditions the receptor unit (R) and the nucleotide regulatory unit exist as the complex R-N, in this situation R inhibits the binding of GTP to N. Hormone or neurotransmitter binding to R triggers the release of this inhibitory effect leading to GTP binding and consequent stimulation (GTP binding to N_s) or inhibition (GTP binding to N_i) of adenylate cyclase activity. Calcitonin is assumed to bind to an R_s type receptor whereas NaF acts directly on the catalytic unit.

1984, reported that inositol triphosphates caused mobilisation of $^{45}Ca^{2+}$ from the endoplasmic reticulum of hepatocytes). When considering the possibility of action of CT and PI metabolism, it should be noted that direct interactions between CT's and phospholipids have been reported (see I.10). Hence Epand et al (1983) have reported that CT's bind to phopholipids, including PI, and that such binding enhances adoption of the α-helical conformation in the CT molecule. If this observation is of physiological significance, it is conceivable that CT's might modify the rate of PI metabolism by reducing the amount of substrate available to phospholipase C rather than direct modulation of the enzymes activity.

A further possibility which warrants discussion, is that CT might exert direct effects on more than one second messenger system (figure D.2). This possibility is exemplified by the cellular actions of adrenergic agonists. Hence, agonist binding to β_1 and β_2 adrenoreceptors activates adenylate cyclase to increase intracellular cAMP concentration; activation of α_2 adrenoreceptors inhibits adenylate cyclase and α_1 activation increases intracellular levels of free calcium by provoking the hydrolysis of inositol phospholipids in the cell membrane (Downes, 1983). As Koida et al (1980) have demonstrated two distinct populations of CT binding sites in the rat brain, it would be interesting to determine whether these binding sites are associated with distinct second messenger systems.

Because of the paucity of experimental evidence it is only possible to speculate on the possible interrelationships between second messenger systems involved in the cellular effects of CT. The close relationship between PI metabolism and calcium metabolism has been mentioned above. Similarly, cyclic nucleotide metabolism is closely linked to cellular calcium metabolism. Hence, a direct effect on cellular calcium transport, or a general effect on calcium distribution within the CNS, could modulate neuronal activity by involvement in calcium or Ca-calmodulin dependent processes (depolarisation-secretion coupling, neurotransmitter release and synthesis, cyclic nucleotide metabolism, protein phosphorylation, acetylcholinesterase and tryptophan hydroxylase activity etc. for review see Carvalho, 1982). Alternatively, a direct effect on cyclic nucleotide

metabolism could modulate neuronal activity and exert secondary effects on calcium metabolism (possibly by phosphorylation of calcium channels and pumps or by altering the distribution of calmodulin between cytosol and cell membrane).

In the preceding discussion it has been assumed that the effects of CT on central cellular activity are mediated by receptor binding and consequent modulation of the activity of second messenger systems. The possibility that CT might indirectly modify central neuronal activity by exerting more widespread effects on central calcium metabolism is of interest, but does not appear to be supported by experimental evidence (e.g. Eglen, 1983, found that central and peripheral administration of sCT did not alter the concentration of calcium in regions of mouse brain, and Stekolnikov and Adukarimov, 1969, reported that CT administration did not modify the concentration of calcium in the CSF) and has therefore not received detailed attention in this thesis.

Finally, the possibility that CT might modulate Ca-calmodulin dependent cellular activity by a direct action must be considered as recent studies by Gnessi, Camilloni, Fabbri, Politi, DeLuca, DiStazion, Moretti and Fraioli (1984) have indicated that CT might interact directly with calmodulin. This group prepared a fluorescent conjugate of dansyl chloride and calmodulin and observed that the intensity of fluorescence, which was calcium dependent, could be modified by CT's. Calcitonin was also found to inhibit calmodulin dependent phosphodiesterase in a dose dependent manner. A possible explanation of these observations is that CT binds to calmodulin and modifies the ability of calmodulin to bind calcium. Reid (1984) has indicated that many of the drugs which bind to calmodulin (such as several major tranquilisers) do so by interaction with a specific amphipillic α-helical region in the calmodulin molecule. β-endorphin, for example, is thought to contain an amphiphillic α-helical region which promotes binding to a similar region in the calmodulin molecule. It is tempting to speculate that the proposed amphiphillic α-helical region at residues 8-22 in CT's (and possibly CGRP's) could promote binding of these peptides to calmodulin and consequent alteration of Ca-calmodulin dependent cellular activity. This possibility remains to be investigated, as

does the physiological or pharmacological relevance of interactions between CT's and calmodulin.

D.6 SIGNIFICANCE OF THE HAEMODYNAMIC AND ANTINOCICEPTIVE EFFECTS OF CALCITONIN

D.6.a Physiological Significance

Central administration of CT produces a wide variety of pharmacological effects. The doses required for pharmacological activity are usually extremely low (typically 0.1 — 10 $U.kg^{-1}$ ≃ 6-600 $pmol.kg^{-1}$ ≃ 6.5-650 ng/rat), in good agreement with the amounts estimated to produce 100% occupancy of the CNS receptors (D.4.a). The central pharmacological effect of the hormone may, therefore, reflect a physiological role of the endogenous peptide. As the pressor effect of sCT (which is most marked after haemorrhage) and the antinociceptive effect of the hormone provide support for the antistress hypothesis of Dupuy (1983), the pharmacological effects of centrally administered CT may prove to reflect such a physiological role for the endogenous peptide. It would, however, be premature to accept this hypothesis as the nature and effects of the endogenous CT-like molecule remain unknown.

D.6.b Pharmacological Significance

Since the discovery of the endogenous opiates (Hughes et al, 1975), considerable effort has been spent in elucidating the identity, mechanism and action and site of action of central neuropeptides. The ultimate aim of this research being the provision of drugs or methods for therapeutically useful modification of central peptidergic activity. A fundamental problem, particularly with the behavioural aspects of such research, is that neuropeptides are often rapidly metabolised by peptidases, consequently transient effects are produced which are often not readily quantified. Salmon CT is therefore an atypical peptide, in as much as the hormone has a long duration of action (analgesic effects of sCT were observed as much as 5 days after administration of sCT, Fraioli et al, 1982 a and b) which may be related to stabilisation of the receptor hormone complex, and the hormone produces several quantifiable effects. The reports of Amara et al (1982) and Rosenfeld et al (1983) indicating that authentic CT does not exist in the mammalian CNS have added impetus to CT research as the genome of CT and related peptides provides a model for the study of peptide gene translation and precursor

processing. Moreover, these results indicate the necessity for reassessment of experiments on the hormones central effects. If authentic-CT does not exist centrally the observed effects of centrally administered CT presumably reflect agonist (or antagonist) action at receptors for, as yet, undefined central peptides. Salmon-CT might, therefore be considered as a powerful pharmacological tool for investigation of the effects of this peptide.

D.6.c Therapeutic Relevance

It has been stated that the antinociceptive and haemodynamic effects of CT were selected as models for study because these effects are readily quantified and may prove to be therapeutically useful. In fact this therapeutic potential may already be partly realised. In the treatment of Pagetic bone disease with CT, analgesia is often reported prior to any other signs of improvement such as reduction of serum alkaline phosphatase or urinary hydroxyproline. Continued treatment often results in a return of urinary hydroxyproline and serum alkaline phosphatase to pretreatment values (indicating a return to elevated rates of bone turnover) without loss of the analgesic effect of the hormone. These observations clearly indicate that the analgesic effect of CT in Paget's disease cannot be entirely attributed to its effects on bone turnover, and may reflect a peripheral antinociceptive effect of the hormone, similar to that observed after repeated peripheral administration of large doses of sCT to rats (Pecile, 1983). A second effect of CT therapy often observed prior to biochemical signs of improvement is the reduction in temperature and redness over Pagetic bone indicating the occurrence of local haemodynamic effects. As CT has been shown to exert a direct constrictor effect on skeletal vasculature (Driessens and Vanhoutte, 1981) the reduction in blood flow to bone might contribute to both the reduction in calcium turnover and to the observed reduction in swelling, heat and redness. These results provide a strong indication that the haemodynamic and antinociceptive effects of the hormone might contribute to its therapeutic efficacy in the treatment of Pagetic bone disease.

A second area of therapeutic use which has attracted much interest recently is the potent and durable analgesic effect observed after subarachnoid administration of CT's to humans. Because of the risks

inherent in central injections, it is apparent that this therapy can only be considered in the most severe cases such as chronic intractable pain of terminal carcinoma. A more promising means of administration is epidural injection as sCT has recently been found to produce long lasting analgesia when administered by this route (Fiore et al, 1983). No extensive reports have yet been published on the side effects of centrally administered CT's in humans (although Shaw, 1982, has warned that central injection of sCT can produce convulsions and death in dogs), it would indeed be interesting to know whether the hormone produces any change in blood pressure.

Faden and Holaday (1979) have suggested that naloxone could be used to increase MAP in the treatment of hypovolaemic and endotoxic shock. A major problem with such therapy is that the analgesic effect of endogenous and exogenous opiates might be antagonised. In direct contrast to the view of Faden and Holaday, Guyton (1981) states that morphine, by inhibition of neurogenic elements contributing to hypotension in shock, can be therapeutically useful in the treatment of shock. Clearly CT, which produces analgesia and elevates arterial pressure, might prove useful in the treatment of traumatic shock associated with both pain and hypotension.

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APPENDIX

STATISTICAL ANALYSIS BY GENERALISED INTERACTIVE MODELLING (GLIM) PROCEDURES

The statistical analyses reported in this thesis were performed, in part, by means of a generalised interactive modelling procedure (GLIM II package on a DEC 20 computer). This procedure is based on the assumption that a variable under investigation has a structure, and that the variable is in some way dependent on more basic variables or factors (e.g. biological response is dependent on dose of drug administered). The GLIM package provides a means of constructing a model in which the observed variable is described in terms of its linear dependence on these factors (transformations of data are often required to meet the assumption of linear dependence). As the relationship between the dependent variable and the predictor variable (factor) is assumed to be linear, the appropriate mathematical description of this relationship can readily be obtained by linear regression analysis which provides an estimate of the "goodness of fit" of the model (sum of squares of residuals). This procedure therefore, has much in common with analysis of variance by linear regression, and construction of linear models in an interactive session on GLIM provides data which can be presented in the form of an ANOVA table or in the form of a mathematical model.

The GLIM procedure has been used in preference to more traditional statistical procedures for the following reasons;

i) The modelling session is interactive so that large numbers of factors can be included or excluded when constructing the model.

ii) The GLIM package has a powerful "calculate" function, simplifying routine calculations performed on raw data.

iii) The GLIM package offers a convenient means of applying mathematical transformations to data (e.g. Probit transformation of dose response curve data for determination of ED_{50}'s), and assessing whether such transformations significantly improve the fit of the model.

An example of the use of the GLIM procedure for analysis of variance is shown below:

Analysis of the Effect of Methysergide on sCT Antinociception (see R.2)

```
*       $UNITS 26
        $DATA CON ANT SCT
        $DINPUT  30
```

(data is read from fortran file 30, this data is coded as follows:
CON - constrictions per 5 minutes;
MTS - methysergide, 1 - vehicle, 2 - 5 $mg.kg^{-1}$;
ICV - i.c.v. dose of sCT, 1 - vehicle, 2 - 2 $U.kg^{-1}$

```
        $FACTOR MTS 2 ICV 2
        $FIT : +ICV : +MTS : +MTS.ICV

             Deviance        d.f.
1            1089            25
2             811            24
3             769            23
4             746            22

        $DIS E L
             Estimate        s.e.    parameter
1             18.29          2.200   %GM
2           - 8.286          3.112   ICV (2)
3           - 4.452          3.239   MTS (2)
4             3.786          4.580   ICV (2).MTS (2)

        LINEAR PREDICTOR ICV MTS ICV.MTS
```

* User commands are prefixed $, computer output is shown indented but not prefixed

The model constructed to describe the constriction rate in terms of the dose of methysergide and sCT is:

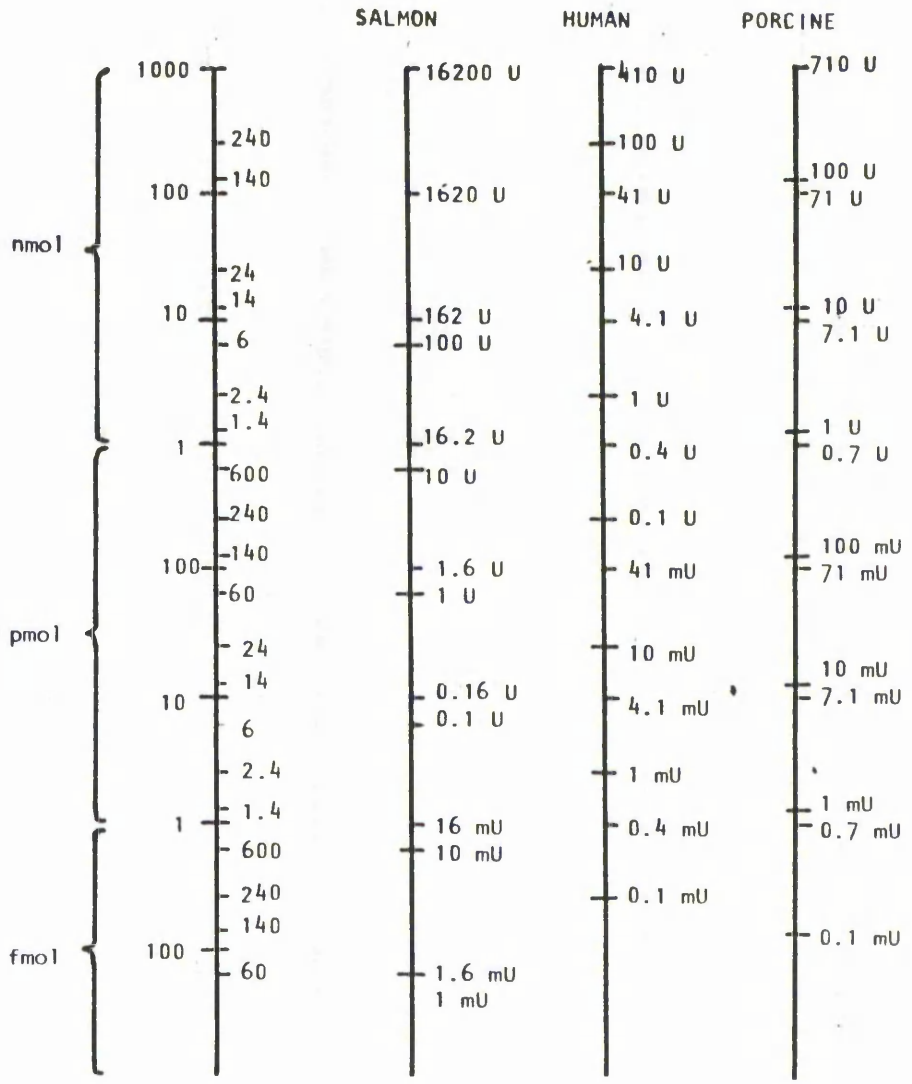
$$\text{Constriction rate} \quad 18.29 + (-8.286 \times \text{ICV}(2)) + (-4.452 \times \text{MTS}(2) + (\text{ICV}(2) \times \text{MTS}(2) \times 3.786)$$

(Standard errors have been omitted for clarity). A more familiar presentation of this data is:

Source of variation	s.s	d.f	m.s.	F
i.c.v sCT	278	1	278	8.1 (1,25)
Methysergide	42	1	42	1.2 (1,24)
Interaction	23	1	23	0.7 (1,23)
Residual	746	22	34	
Total	1089	25		

The F values reveal that the presence or absence of sCT is a significant source of variation ($P < 0.01$), but that methysergide, which is itself not a significant source of variation ($P > 0.1$), does not interact significantly with sCT ($P > 0.1$). This analysis therefore reveals no significant evidence for modification of sCT antinociception by methysergide (see R.2).

CHART SHOWING COMPARATIVE POTENCY'S OF sCT, hCT and pCT.



Conversions have been based on the following potency estimates ; hCT, 120 U.mg^{-1}; pCT, 200 U.mg^{-1}; sCT, 4500 U.mg^{-1}, (values obtained from Diem, K.and Lentner, C. 1972, Documenta Geigy, and from potency of synthetic sCT supplied by the Armour Pharmaceutical Co.). The potency of synthetic and extracted calcitonins may differ and may vary from batch to batch, values given must therefore be treated as rough approximates.

One MRC unit is defined as the calcium lowering activity of one MRC standard ampoule. Ten MRC mU lowers plasma calcium approximately 10% in starved 150g rats bled 1 hour after i.v. injection.

Br. J. Pharmac. 76, 270P, (1982).

LACK OF EFFECT OF CALCITONIN ON THE RESPONSE OF THE RAT COLON TO LEU- AND MET- ENKEPHALIN, OR ACETYLCHOLINE, IN VITRO

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Calcitonin, administered by central injection, possesses analgesic properties (Braga et al, 1978; Bates et al, 1981a) not observed when the hormone is administered peripherally. Calcitonin-induced analgesia in the mouse shares several features in common with opiate analgesia, including antagonism by calcium ions and naloxone. However, the dose of naloxone required to antagonise calcitonin-analgesia in the mouse is 10-100 fold greater than those required to antagonise opiate analgesia (Bates et al, 1981b), which suggests that calcitonin may not interact directly at the opiate receptor.

We have studied the effect of calcitonin on the rat colon, a tissue which contains excitatory opiate receptors of the μ and δ types (Boura and Olley, 1981, Gillan and Pollock, 1980). In this tissue, opiates produce a contractile response, possibly by activation of serotoninergic neurones (Huidoboro-Toro et al, 1981).

The middle third of the colon was removed from CFY rats (♂,♀, 200-300g) and suspended from an isotonic transducer under 1g tension. The tissue was superfused with warmed (37°C), gassed (5% CO_2/95% O_2) Krebs solution (Boura et al, 1981) at a rate of 4ml min^{-1}. Agonists and antagonists were injected into the superfusion fluid. Each piece of tissue was tested with acetylcholine (10^{-8}–10^{-3}M) prior to the application of the opioid peptides leu and met- enkephalin or salmon calcitonin (4700 i.u. mg^{-1}; mw = 3600). Agonist potency was determined from the EC_{50}, and the potency of the antagonists was determined by estimation of the pA_2 value.

The preparation contracted in response to acetylcholine (EC_{50} = 5 x 10^{-7}M) and the response was antagonised by atropine (pA_2 = 7.1). Both leu and met- enkephalin elicited a contractile response (leu- enkephalin EC_{50} = 3 x 10^{-8}M; met- enkephalin EC_{50} = 6 x 10^{-8}M), with a threshold concentration of 10^{-8}M and a maximum response at 10^{-6}M. The maximum response to both peptides was only 20-50% of the maximum response to acetylcholine. The response of the rat colon to the opioid peptides was antagonised by naloxone with a pA_2 value of 7.1 for leu- enkephalin and 7.0 for met- enkephalin. These results for naloxone and the maximum enkephalin response are similar to those of Boura and Olley, 1981.

Salmon calcitonin (2.5 x 10^{-12} – 2.5 x 10^{-4}M; n = 4) did not stimulate the rat colon; nor did it affect the responses to either of the opioid peptides. Similar observations have been reported by Braga et al (1978) using the transmurally stimulated guinea-pig ileum as the opiate bioassay preparation.

We conclude that calcitonin does not interact with the μ or δ opiate receptors in this preparation. Since opiate-induced analgesia in the abdominal constriction test is mediated via the μ receptor, we also conclude that the central analgesic effect of calcitonin is not mediated by direct interaction with opiate receptors of the μ type.

The salmon calcitonin was generously donated by Armour Pharmaceuticals Corporation Eastbourne, U.K.

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Br. J. Pharmac. 76, 271P, (1982).

INHIBITION OF ABDOMINAL CONSTRICTIONS BY CALCIUM ANTAGONISTS AND THEIR INTERACTION WITH CALCITONIN AND DIVALENT CATIONS

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The analgesia induced by the opiates (Chapman and Way, 1980) and calcitonin (Bates et al, 1981) may result from changes in the distribution of calcium ions within the cells of the brain. In addition, the central injection of lanthanum ion, a calcium flux inhibitor, also results in analgesia which is reversed by naloxone. However, the possible analgesic properties of the 'slow' calcium channel antagonists have not been studied. In this investigation, the analgesic properties of the calcium antagonists nifedipine and PY 108-068 have been studied.

Groups of 10 CFLP mice (♀,♂, 30g) were given intracerebroventricular (i.c.v.) injections of nifedipine, PY 108-068, salmon calcitonin (SCT), $CaCl_2$ or $MgCl_2$, dissolved in 10µl of tris-saline pH 7.4 containing 50% dimethyl sulphoxide. 10 minutes later, mice were given an i.p. injection of acetic acid (0.3ml of 1% w/v) and the frequency of abdominal constrictions was counted between the 10-14th minute following the injection of acid. In the control mice, which received an i.c.v. injection of vehicle, the rate of abdominal constrictions was 3.02 ± 0.1 min^{-1} ($\bar{x}$ ± se). Some groups of animals did not receive acetic acid, but were used to assess locomotor activity and investigative behaviour, using standard rotating drum and hole board tests respectively.

The i.c.v. injection of 0.33, 3.3 or 6.6 µmoles kg^{-1} nifedipine caused 5 ± 5, 26 ± 4* and 50 ± 2%* ($\bar{x}$ ± se, *$p<0.005$) inhibition of the frequency of abdominal constrictions. At these doses, there was not impairment of locomotor or investigative behaviour.

The simultaneous i.c.v. injection of $CaCl_2$ (1.7 µmoles kg^{-1}), together with 3.3 µmoles nifedipine, reversed the effect of the calcium antagonist. This dose of $CaCl_2$ is inactive by itself. In contrast, the simultaneous i.c.v. injection of $MgCl_2$ (3.3 µmoles kg^{-1}) together with nifedipine (3.3 µmoles kg^{-1}) did not reverse the effect of nifedipine.

Intracerebroventricular injection of SCT (0.1 or 2 i.u. kg^{-1}) inhibited the frequency of abdominal constrictions by 22 ± 4* and 49 ± 4%* ($\bar{x}$ ± se;*$p<0.005$). The simultaneous i.c.v. injection of SCT (0.1 i.u. kg^{-1} and nifedipine (0.33 µmoles kg^{-1}) produced a summation of the effects of these agents. Similar results were obtained when SCT (2 i.u. kg^{-1}) and nifedipine (3.3 µmoles kg^{-1}) were simultaneously injected i.c.v.

The i.c.v. injection of 0.33, 3.3 or 6.6 µmoles kg^{-1} PY 108-068 caused 34 ± 3*, 43 ± 6* and 57 ± 4%* ($\bar{x}$ ± se;*$p<0.005$) inhibition of abdominal constrictions. At these doses, there was no impairment of locomotive or investigative behaviour.

In summary, nifedipine and PY 108-068 given by i.c.v. injection, significantly reduce the frequency of abdominal constrictions induced by acetic acid. The effect of nifedipine was additive with that of calcitonin and, like the effect of calcitonin, could be reversed by i.c.v. injection of calcium ion but not by i.c.v. injection of magnesium ion.

The drugs used were generously donated by the following: salmon calcitonin (Armour Pharmaceutical Corp.), nifedipine (Bayer UK Ltd.) and PY 108-068 (Sandoz Ltd.).

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SALMON CALCITONIN AND CENTRAL ACETYLCHOLINESTERASE ACTIVITY

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Nakhla and Majumdar (1978) reported that intramuscular administration of porcine calcitonin decreases plasma tryptophan and increases central 5-hydroxytryptamine (5-HT) in rats; this rise is thought to mediate an increase in central acetylcholinesterase (AchE) activity. Either of these changes might be associated with the anti-nociceptive action of calcitonin (Pecile et al, 1975; Bates et al, 1981).

To test this hypothesis, groups of 10 CFLP mice (♂&♀,30g) or Wistar rats (♂&♀, 250-300g) were given drugs or appropriate vehicle. One hour later (15 min in the case of eserine) the brains were removed. Cortex, cerebellum and the remaining tissue were homogenised separately in 10ml, 0.1M phosphate buffer at the appropriate pH. The protein concentration of the homogenates was determined according to the method of Lowry et al (1951) and AchE activity was determined according to Ellman et al (1961) at pH 7.4°C and 21°C for mouse brain or pH 8 and 37°C for rat brain. The results are shown in Table 1.

Table 1 AchE activity of brain homogenates

Treatment	Cerebellum	Cortex	Remainder
Tryptophan 300mg.kg^{-1} s.c.	1.32 ± 0.05	6.50 ± 0.50	5.18 ± 0.46
(Control Mice)	1.36 ± 0.23	6.07 ± 0.22	5.84 ± 0.29
5-HT 0.67mg.kg^{-1} i.c.v.	1.57 ± 0.09	6.11 ± 0.23	4.91 ± 0.19
(Control Mice)	1.75 ± 0.09	6.54 ± 0.21	5.37 ± 0.19
p-chlorophenylalanine 300mg.kg^{-1} p.o.	1.24 ± 0.07	5.17 ± 0.17	4.53 ± 0.14
(Control Mice)	1.39 ± 0.10	5.20 ± 0.29	4.45 ± 0.27
Salmon calcitonin 20 IU.kg^{-1} s.c.	1.02 ± 0.12	4.15 ± 0.21	3.90 ± 0.05
(Control Mice)	1.05 ± 0.08	4.37 ± 0.27	3.91 ± 0.18
Salmon calcitonin 50 IU.kg^{-1} i.c.v.	1.45 ± 0.14	6.44 ± 0.30	5.48 ± 0.18
(Control Mice)	1.76 ± 0.19	6.45 ± 0.32	5.18 ± 0.20
Salmon calcitonin 20 IU.kg^{-1} s.c.	4.41 ± 0.21	7.53 ± 0.34	7.07 ± 0.32
(Control Rats)	4.33 ± 0.27	7.18 ± 0.37	7.23 ± 0.43
Eserine 2mg.kg^{-1} s.c.	1.77 ± 0.20	3.30 ± 0.25**	3.16 ± 0.24*
(Control Mice)	2.06 ± 0.24	5.65 ± 0.25	4.48 ± 0.41

Results expressed in μ.mol.hr^{-1} (mg protein)$^{-1}$, $\bar{x}$ ± s.e., n = 7-10, *P<0.02, **P<0.001.

No significant change in AchE activity was observed after any of these treatments except for eserine, which was used as a control for the assay system.

In conclusion, we are unable to provide evidence either that agents which modify 5-HT metabolism influence AchE activity in the brain or that the central anti-nociceptive action of salmon calcitonin is likely to be associated with changes in AchE activity.

We thank Armour Pharmaceutical Corporation for the donation of salmon calcitonin.

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CALCIUM ANTAGONISTS: DIFFERENTIAL EFFECTS ON GUINEA-PIG AND RAT INTESTINAL SMOOTH MUSCLE

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The effects of the calcium slow channel blocking agents (calcium antagonists) on cardiac and vascular smooth muscle have been extensively studied (e.g. Naylor and Poole-Wilson, 1981). There are, however, relatively few reports on the effects of these agents on the smooth muscle of the alimentary tract of species other than the guinea pig. We have compared the effects of the calcium antagonists. verapamil, nifedipine and PY 108-068 upon the response of smooth muscle from the alimentary tract of rats and guinea pigs to stimulation by acetylcholine.

Segments of colon (middle third) and ileum were isolated from rats (Sprague-Dawley, 250g, ♂,♀) or guinea pigs (Duncan-Hartley, 350g, ♂,♀) and suspended under 1g tension in Krebs solution at 37°C, gassed with 5% CO_2 in oxygen. The calcium ion concentration of the Krebs solution was 2.5 $mmol.l^{-1}$. In experiments using lanthanum, the tissue was immersed in Hepes buffered solution containing the same concentration of calcium ion and gassed with oxygen.

Cumululative dose-response curves to acetylcholine were obtained in the presence or absence of the calcium antagonists in normal or calcium deficient Krebs. The antagonists were added 1 or 60 minutes prior to the addition of acetylcholine.

The responses of the guinea pig ileum and colon to acetylcholine were antagonized noncompetitively by all four agents. The responses of both tissues were completely abolished by $10^{-7}M$ of the organic antagonists and $2mmol.l^{-1}$ lanthanum. These results are similar to those of Ticku and Triggle (1976).

In contrast, the rat ileum and colon were relatively insensitive to the organic antagonists which failed to significantly reduce the response to acetylcholine at concentrations up to $10^{-6}M$ (n = 4). Pre-incubation of the rat tissue with the calcium antagonists for periods up to 1 hour did not increase their potency (n = 4) nor was the effect of nifedipine increased by pre-incubation of the tissue in calcium deficient Krebs solution (n = 4), containing the minimum concentration of calcium ions ($0.5mmol.l^{-1}$) required to maintain a maximum response to acetylcholine However, the maximum responses of the rat ileum and colon were reduced (63 ± 3% and 83 ± 7% respectively; $\bar{x}$ ± se, n = 4) by $2mmol.l^{-1}$ lanthanum ion. Pre-incubation of the tissue in calcium free solution abolished the response to acetylcholine.

We conclude that in contrast to similar tissue from the guinea pig, the responses to acetylcholine of the rat ileum and colon are very insensitive to the organic calcium antagonists verapamil, nifedipine and PY 108-068.

The drugs used were generously donated by the following: verapamil (Abbott Laboratories Ltd.), nifedipine (Bayer UK Ltd.) and PY 108-068 (Sandoz Ltd.).

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CALCITONIN IN HAEMORRHAGIC SHOCK

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The hypotension observed after haemorrhagic or endotoxic shock, has been shown to be exacerbated by morphine (Chance et al, 1982) and reversed by naloxone (Faden & Holaday, 1979). We have studied the effects of calcitonin, a centrally acting antinociceptive agent (Bates et al, 1981), on the blood pressure in anaesthetised, normotensive, haemorrhaged and pithed rats.

Sprague-Dawley rats (150-350g) were anaesthetised with i.p. urethane (1.4g kg^{-1}). The jugular vein and carotid artery were cannulated for drug administration and blood pressure recording respectively. Vehicle (0.154M NaCl, with 1mg ml^{-1} BSA) or drugs (leucine enkephalin (LE), naloxone and salmon calcitonin (sCT)), were administered on a latin-square system.

Separate groups of animals were bled by withdrawing arterial blood, until the mean arterial pressure (MAP) was reduced by 20%. After a 20 minute stabilisation period, vehicle or drugs (naloxone or sCT) were administered. The MAP was monitored for 60 minutes thereafter. The change in MAP was determined from the post-haemorrhage value, and statistical analysis was undertaken by Student's t test.

In normotensive animals, LE produced a significant, ($p < 0.05$) transient hypotension. Pretreatment with naloxone (10mg kg^{-1}), administered, i.p., 30 minutes prior to LE, completely abolished the responses to LE. sCT (0.01-100 i.u. kg^{-1}) was without effect on the blood pressure of normotensive animals.

Table 1 Change in MAP, after drug treatment in haemorrhaged animals (mean ± SE MEAN, *$p < 0.05$

Treatment	Change in MAP (mm Hg) - Post-Haemmorrhage 5 Minutes	15 Minutes	60 Minutes
Vehicle	+4.3 ± 3.3	-3.8 ± 2.9	-3.5 ± 3.6
sCT (0.1 i.u. kg^{-1})	+2.6 ± 3.3	+1.8 ± 2.4	+6.4 ± 0.87*
sCT (1 i.u. kg^{-1})	+7.0 ± 3.2*	+6.6 ± 3.0*	+7.0 ± 2.1*
sCT (10 i.u. kg^{-1})	+16 ± 4.8*	+15.8 ± 4.2*	+11.4 ± 6.2
Naloxone	+5.7 ± 2.9	+11.0 ± 3.8*	+17.2 ± 2.8*

As shown in Table 1 Naloxone produced a rise in MAP, which was significantly different at 15 minutes, and was sustained for 60 minutes. sCT (0.1-10 i.u. kg^{-1}) produced a dose-dependent increase in MAP, at 5 minutes which was sustained for 60 minutes. In pithed animals, sCT (10 i.u. kg^{-1}) produced no observable effect, although these animals were responsive to a direct-acting vasoconstrictor such as vasopressin (1.5-300mU kg^{-1}).

We have shown that sCT exerts a pressor effect in rats rendered hypotensive by haemorrhage. There was no effect observed with sCT in normotensive animals. The absence of an effect in the pithed animal indicates that the pressor action of sCT in haemorrhage is probably not by direct peripheral vasoconstriction.

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CALCITONIN ANTINOCICEPTION AND SEROTONERGIC TRANSMISSION

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Morphine antinociception in mice can be antagonised by inhibition of serotonin synthesis with p-chlorophenylalanine (pCPA) (Fennessy and Lee, 1970), and potentiated by intracerebral (i.c.) administration of serotonin (Sewell and Spencer, 1975). Much evidence of this nature exists linking opiate antinociception with central serotonergic systems. We have investigated the possible involvement of serotonin in the central antinociceptive action of salmon calcitonin (SCT) in the abdominal constriction test (Bates et al, 1981).

Groups of mice (CFLP, ♂, ♀, 25-30g) were treated with vehicle or pCPA (300 mg.Kg^{-1} S.C. in 0.9% NaCl with 0.05% polyoxyethylene-4-lauryl ether, pH 7.4) 72, 48 and 24 hours prior to test. On the day of test the mice were given SCT (0.01 - 50 IU, Kg^{-1}) or serotonin (0.5 - 10 µg/mouse) in 10µl of vehicle (50mM Tris, 100mM NaCl in 1% bovine serum albumin at pH 7.4) using the i.c. injection technique of Haley and McCormick (1957). Ten minutes later the mice received, i.p., 0.3 ml of a solution containing 1% acetic acid in 0.9% NaCl and the abdominal constriction rate was counted between the 10th and 14th minutes after injection.

The statistical validity of the results was assessed using analysis of variance and Student's 't' test. In all cases groups of mice (6-10) under test were compaired to an internal control group receiving the appropriate vehicle.

The abdominal constriction rate after treatment with vehicle (i.p. and i.c.) was 3.98 ± 0.26 ($\bar{x}$ ± s.e., n = 23; pooled controls) constrictions.min^{-1}. Pretreatment with pCPA had no significant effect on the abdominal constriction rate (4.12 ± 0.22, n = 24), whereas, SCT (0.1, 1, 2, 10, 50 IU.Kg^{-1}) produced a dose dependent reduction of the abdominal constriction rate (23.6 ± 7.6%, 38.1 ± 4.8%, 45.4 ± 8.5%, 41.3 ± 7.8%, 33.0 ± 6.7% respectively, n = 6-9, $P < 0.025$). After pretreatment with pCPA, SCT (i.c.) produced no significant reduction in abdominal constriction rate at 0.1, 1, 2 or 50 IU.Kg^{-1}, although at 10 IU.Kg^{-1} SCT, the constriction rate was reduced by 19.9 ± 6.1%, n = 7, $P < 0.05$. Intracerebral serotonin (1, 5, 10 µg/mouse) caused a dose dependent reduction in abdominal constriction rate (32.3 ± 10.0%, 49.2 ± 10.3%, 56.7 ± 10.4% respectively, n = 9-10, $P < 0.025$) which was not antagonised by pretreatment with pCPA. Furthermore, simultaneous i.c. administration of serotonin (10 µg/mouse) and SCT (2 IU.Kg^{-1}) caused a reduction in abdominal constriction rate (86.6 ± 4.0%, n = 9) which was significantly ($P < 0.02$) greater than the effect observed with either serotonin (56.7 ± 10.4, n = 10) or SCT (39.8 ± 9.5%, n = 10) alone. In addition the antagonism of SCT antinociception by pCPA was not apparent when serotonin (1 or 10 µg/mouse) was injected simultaneously with the SCT (2 IU.Kg^{-1}).

These results suggest that central serotonergic systems may be involved in the antinociceptive action of SCT.

The salmon calcitonin was generously donated by Armour Pharmaceutical Corporation.

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POSSIBLE MECHANISMS OF ACTION OF CALCITONIN IN HAEMORRHAGIC HYPOTENSION

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It has been reported that i.v. salmon calcitonin (SCT) produces a pressor response when administered to rats rendered hypotensive by haemorrhage (Bates et al, 1983). This effect was not due to peripheral vasoconstriction and was not observed in normotensive animals. We have investigated the mechanism(s) of this response by determining the effect of SCT after bilateral vagotomy, chemical sympathectomy (Kostrzewa et al,1974), and vasopressin antagonism (Kruszyski et al, 1980, Htzinikolaou et al, 1981).

Sprague-Dawley rats (♂,♀, 150-350g) were anaesthetised with urethane, cannulated for drug administration and blood pressure recording and bled to reduce the mean arterial pressure (MAP) as previously described (Bates et al,1983). After a 20 minute stabilisation period SCT (10 i.u. Kg^{-1} in 0.1ml, 0.15M NaCl with 1mg ml^{-1} (BSA) or vehicle were administered i.v. The change in MAP was monitored for a further 60 minutes.

Groups of 6-10 animals were pretreated with 6-Hydroxydopamine (6-OHDA,100mg Kg^{-1} i.p. in 1mg ml^{-1} ascorbic acid, 4,3 and 2 days prior to experimental use), bilateral vagotomy (5-10 minutes after bleeding) or the vasopressin antagonist [1-β-mercapto-β,β-cyclopentamethylene propionic acid), 2-(O-methyl) tyrosine, arginine vasopressin ($d(CH_2)_5$Tyr(Me)AVP, 0.1mg kg^{-1}, i.v. in 0.15M NaCl with 1mg ml^{-1} BSA, 5 minutes prior to bleeding). In each case control groups received appropriate pretreatment vehicle. There were no differences between these 3 groups which have therefore been combined as the pretreatment control.

Table 1. (Change in MAP After Drug Treatment ($\bar{x} \pm$ s.e., mmHg)

Pretreatment	Drug	5 minutes	15 minutes	60 minutes
Combined controls(n,18-21)	vehicle	+1.4±0.8	-0.3±1.1	-0.8±0.9
	SCT	+7.6±0.7 *	+6.2±0.8 *	+5.3±1.2 *
Bilateral vagotomy(n,6-8)	vehicle	+2.2±1.4	-0.2±1.8	-3.4±2.5
	SCT	+9.6±2.4 *	+8.7±1.6 *	+4.5±2.6 *
6-OHDA (n,10-12)	vehicle	+1.1±0.9	-0.3±1.0	+0.9±1.3
	SCT	+4.5±1.2 *	+1.8±1.5	-0.1±1.4
$d(CH_2)_5$Tyr-(Me)AVP(n,6-8)	vehicle	+2.0±2.0	+1.4±1.2	-0.3±2.4
	SCT	+7.7±0.8 *	+6.3±1.7 *	+7.4±2.1 *

*P<0.05, 2 tailed Students 't' test.

The pressor response to SCT was unaffected by bilateral vagotomy or $d(CH_2)_5$Tyr(Me) AVP (at a dose which, in pithed rats, has been shown to abolish for 3 hrs. the pressor response of 1-100 mU Kg^{-1} vasopressin). However after pretreatment with 6-OHDA the pressor response only reached statistical significance at 5 minutes. These results suggest that pressor response to SCT in rats rendered hypotensive by haemorrhagic shock may involve an increase in sympathetic tone, rather than modification of vagal afferents or the secretion of vasopressin.

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COMPARISON OF THE ANTINOCICEPTIVE EFFECTS OF CENTRALLY ADMINISTERED CALCITONINS AND CALCITONIN GENE-RELATED PEPTIDE

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Rosenfeld et al (1983) have demonstrated that alternative processing of the RNA transcribed from the calcitonin gene produces tissue specific forms of mRNA. Thyroid mRNA encodes a precursor to the hormone calcitonin whereas neural mRNA encodes a novel peptide termed calcitonin gene-related peptide (CGRP). The distribution of CGRP suggests that peptide may be involved in processing of painful stimuli. Several forms of calcitonin produce antinociception when administered centrally (Pecile et al, 1975; Bates et al, 1983). In this communication we compare the antinociceptive effects of centrally administered CGRP, human calcitonin (hCT) and salmon calcitonin (sCT).

Groups of mice (CFLP, ♂, ♀, 25–35g) received sCT (10 $U.kg^{-1}$ = 0.6 $nmol.kg^{-1}$). hCT (0.01–10 $nmol.kg^{-1}$) or CGRP (0.01–10 $nmol.kg^{-1}$) in 10µl of vehicle (50 mM Tris, 100 mM NaCl in 1% BSA, pH 7.4) by the intracerebroventricular (i.c.v.) injection technique of Haley and McCormick (1957). Ten minutes later the animals received, i.p., 0.3ml of a solution containing 1% acetic acid in 0.9% NaCl. The rate of consequent abdominal constrictions (constrictions per minute, c.p.m.) was determined between the 10th and 14th minute after injection. The possibility of peptides modifying locomotor or investigative behaviour was also assessed by the rotating drum and hole board tests.

The statistical validity of the results was assessed using Student's 't' tests, groups of mice under test being compared to an internal control group receiving vehicle alone.

The control abdominal constriction rate (3.12±0.22 c.p.m., n=22) was significantly reduced by i.c.v. hCT at 5 and 10 $nmol.kg^{-1}$ (36.6±8.5, 27.5±11.4% inhibition of internal control respectively, n=11–12, $P<0.05$). CGRP at 1, 5 and 10 $nmol.kg^{-1}$ i.c.v. reduced the control constriction rate (3.45±0.15, n=25) by 25.1±7.0, 34.9±9.0 and 33.3±9.4% respectively (n=19–20, $P<0.01$). Doses below those shown were without effect and maximal doses of peptides did not significantly modify locomotor or investigative behaviour.

In a further experiment significant inhibition of abdominal constriction was observed 20, 60 and 120 min. after sCT (10 $U.kg^{-1}$ ≈0.6 $nmol.kg^{-1}$, i.c.v) whereas antinociceptive effects of CGRP and hCT (10$nmol.kg^{-1}$) were only evident 20 minutes after administration.

We have previously reported a minimum antinociceptive dose of 0.1 $U.kg^{-1}$ (≈6 $pmol.kg^{-1}$) sCT in this model. The marked potency and duration of action of sCT as compared to hCT has been reported for the hypocalcaemic action of the hormones and may be associated with its higher receptor affinity and binding half-life.

In summary, we have demonstrated that central administration of relatively high doses of both hCT and CGRP can produce transient antinociception in mice, these results support the suggestion of Rosenfeld et al, 1983, that CGRP might play a role in central processing of painful stimuli.

The sCT and hCT were donated by the Armour Pharmaceutical Corporation and Ciba-Geigy respectively, CGRP was purchased from Merseyside Laboratories.

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CALCITONIN IN HAEMORHAGIC HYPOTENSION.

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The hypotension observed in haemorrhagic shock has been shown to be exacerbated by morphine and reversed by naloxone. Bates et al (1983) have reported that i.v. administration of salmon calcitonin (sCT) a peptide hormone with central antinociceptive activity produces a pressor response in rats rendered hypotensive by haemorrhage, but is without effect in normotensive animals or those made hypotensive by pithing. Administration of sCT (10 $U.kg^{-1}$, i.v. in 0.1ml, 0.9% NaCl with 1mg.ml BSA) to urethane anaesthetised rats 20 minutes after removal of arterial blood to reduce MAP (mean arterial pressure) by 20% produced a pressor response (change in MAP significantly greater after sCT than after vehicle, $P<0.05$) with a duration of at least 60 minutes. A similar response was observed when animals received bilateral vagotomy or vasopressin antagonist ($d(CH_2)_5$Tyr-(Me)AVP, 0.1$mg.kg^{-1}$ i.v.), whereas chemical sypathectomy (6-OHDA, 100$mg.kg^{-1}$, 4,3, and 2 days prior to experiment) markedly attenuated the increase in MAP. We conclude that the pressor response to sCT in rats rendered hypotensive by haemorrhage may involve an increase in sympathetic tone rather than modification of vagal afferents or the secretion of vasopressin.

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PRESSOR EFFECT OF CENTRALLY ADMINISTERED CALCITONIN.

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We have recently reported that salmon calcitonin (sCT) produces a pressor response after peripheral administration to rats rendered hypotensive by haemorrhage, but is without effect in normotensive rats (Bates et al., 1983).Similarly calcitonin gene-related peptide, the product of calcitonin gene transcription in the rat CNS, has been found to increase mean arterial pressure (MAP) after intracerebroventricular (i.c.v) administration (Fisher et al., 1983). We have therefore determined the effect of i.c.v. sCT on MAP of urethane anaesthetised normotensive rats and those rendered hypotensive by removal of arterial blood.

Change in MAP (mmHg) produced by i.c.v. sCT in normotensive rats (A), and rats rendered hypotensive by haemorrhage (B).

	Dose $U.kg^{-1}$ (n)	5 min.	15 min.	30 min.	60 min.
A	0 † (16)	−2.0±1.0	−0.9±1.1	−0.2±1.9	−1.4±1.8
	0.1 (5)	3.4±1.2 *	1.4±1.0	0.2±0.9	−1.4±1.7
	1.0 (5)	8.8±3.0 *	12.2±2.0 *	9.8±2.0 *	4.8±3.8 *
	10 (6)	13.5±1.7 *	17.5±1.5 *	17.2±2.1 *	10.3±4.2 *
B	0 † (14)	−0.6±0.3	1.9±1.1	−0.3±1.0	−4.1±1.1
	0.1 (4)	2.7±2.7	1.0±0.6	4.5±1.4 *	3.7±1.4 *
	1.0 (5)	9.8±3.1 *	14.6±4.7 *	13.4±2.9 *	9.2±1.9 *
	10 (6)	31.0±3.2 *	33.0±4.9 *	30.6±4.3 *	23.0±4.2 *

Values are $\bar{x}$±s.e; n=number of animals; †pooled vehicle; *significantly greater than vehicle ($P<0.01$), Students 't' test.

In summary, i.c.v. administration of sCT produced a dose dependent pressor response in both normotensive animals and those rendered hypotensive by haemorrhage. This effect which was most marked after removal of arterial blood, had a duration of at least 60 minutes (1 and 10 $U.kg^{-1}$). These results indicate that the previously reported pressor effect of sCT after haemorrhage may involve actions on the CNS and that CT, or structurally related peptides, might play a role in central mechanisms of cardiovascular regulation.

Bates et al (1983) Brit.J. Pharmac. 79, 255P.
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PRESSOR EFFECT OF CENTRALLY ADMINISTERED SALMON CALCITONIN (sCT).

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We have previously demonstrated that sCT produces a dose dependent pressor response when administered peripherally to rats made hypotensive by haemorrhage, but is without effect in normotensive rats and those made hypotensive by pithing (Bates et al., 1983). An increase in sympathetic tone has been implicated as the mechanism underlying the pressor response of haemorrhaged rats to i.v. sCT (Bates et al., 1984), and of normotensive rats to intracerebroventricular (i.c.v) calcitonin gene-related peptide (Fisher et al., 1983). We have therefore determined the effect of i.c.v. sCT on mean arterial pressure (MAP) of normotensive rats and those rendered hypotensive by haemorrhage, and assessed the effect of chemical sympathectomy on the response observed in haemorrhaged animals.

Groups of Sprague-Dawley rats which were untreated or were pretreated with either 6-hydroxydopamine (6-OHDA, 100 $mg.kg^{-1}$ i.p. in 1 $mg.ml^{-1}$ ascorbic acid, 4,3, and 2 days prior to experimental use) or with appropriate vehicle, were cannulated for blood pressure recording under urethane anasthesia as previously described (Bates et al., 1983). The animals were then placed in a stereotaxic frame to facillitate i.c.v. injection and were given a single sham i.c.v. injection (needle entry without fluid ejection) followed by a 30 minutes stabilisation period. Where appropriate, arterial blood was removed to reduce MAP by approximately 20%, 10 minutes after the sham injection. After stabilisation, sCT (0.1, 1 or 10 $U.kg^{-1}$) or vehicle (50 mM Tris, 100 mM NaCl, in 1% bovine serum albumin, pH 7.4) was administered (i.c.v) in a volume of 10 µl and the change in MAP was monitored for 60 minutes thereafter. All animals received a single dose of sCT and of vehicle in varied order, statistical analysis was by comparison (Student's 't' test) of sCT induced effects with those of the vehicle treated internal controls.

In normotensive rats i.c.v. sCT produced a dose dependent pressor response with peak increases in MAP of 12.4 ± 1.4 and 17.7 ± 1.7 mm Hg for 1 and 10 $U.kg^{-1}$ respectively, ($n=4-6$, $P < 0.05$), whereas vehicle produced no significant change in MAP. A greater pressor response was produced by i.c.v. administration of sCT to rats rendered hypotensive by haemorrhage (peak increases in MAP; 14.6 ± 4.6 and 35.8 ± 3.9 mm Hg for 1 and 10 $U.kg^{-1}$. Furthermore, the pressor response of hypotensive animals to i.c.v. sCT (1 and 10 $U.kg^{-1}$) was not significantly altered by chemical sympathectomy. In contrast, the pressor effect of i.v. sCT (10 $U.kg^{-1}$) has been shown to be greatly attenuated by chemical sympathectomy (Bates et al., 1984).

These results provide further evidence that calcitonin or structurally related peptides might play a role in central cardiovascular regulatory systems, and indicate that the mechanisms underlying the haemodynamic effects of centrally and peripherally administered sCT may differ.

The sCT was kindly donated by the Armour Pharmaceutical Corporation.

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