THE PHARMACOLOGY OF CALCITONIN

BY

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DECLARATIONS

(i) The observations included in this thesis are entirely 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 for the degree of Doctor of Philosophy the author has participated in seminars and conferences, and studied immunology and biochemistry to an advanced level.

PUBLICATIONS

1. R.F.L. Bates, G.A. Buckley, R.M. Eglen and R.J. Strettle (1981) Comparison of the Analgesic Effects of Subcutaneous and Intracerebroventricular Injection of Calcitonin on Acetic Acid Induced Abdominal Constrictions in the Mouse. Br. J. Pharmacol.72 575P.

2. Ibid. (1981) Interaction of Calcium Ions and Salmon Calcitonin in the Production of Analgesia in the Mouse. Br.J. Pharmacol. 73 302-303P.

3. Ibid. (1981) The interaction of Naloxone and Calcitonin in the Production of Analgesia in the Mouse. Br. J. Pharmacol. 74 278P.

4. Ibid. (1981) Hyperalgesia Induced by Chronic Subcutaneous Injection of Calcitonin. Br. J. Pharmacol. 74 279P.

5. Ibid. (1981) Antagonism of Calcitonin Induced Analgesia by Ionophore A23187. Br. J. Pharmacol. 74 857P.

6. Ibid. (1982) Desensitisation to Calcitonin Induced Analgesia by Withdrawal of Chronic Calcitonin Treatment. Br. J. Pharmacol. 75 119P.

7. R.M. Eglen, R.F.L. Bates and G. A. Buckley (1982) Analgesia Induced by Central Injection of Calcitonin.

Proceedings of VIIIth International Society for Neurochemistry. in press.

8. Ibid (1982) Withdrawal Hyperalgesia Following Chronic Subcutaneous Injection of Calcitonin. Ibid.

9. R.F.L. Bates, G.A. Buckley, R.M. Eglen, C.A. McArdle and R.J. Strettle (1982) Lack of Effect of Calcitonin on the Response of the Rat Colon to Leu and Met. Enkephalin or Acetylcholine in vitro. Br. J. Pharmacol. 76 270P.

10. Ibid (1982) Inhibition of Abdominal Constrictions by Calcium Antagonists and Their Interaction with Calcitonin and Divalent Cations. Br. J. Pharmacol. 76 271.

11. Ibid (1982) Salmon Calcitonin and Central Acetylcholinesterase Activity. Br. J. Pharmacol. 77 518P.

12. R.F.L. Bates, G.A. Buckley, R.M. Eglen, C.A. McArdle and S.J. Wilkinson (1982) Calcium Antagonists - Differential Effects on Guinea-Pig and Rat Intestinal Smooth Muscle. Br. J. Pharmacol. 77 567P.

13. R.F.L. Bates, G.A. Buckley, R.M. Eglen, C.A. McArdle, R.J. Strettle and D.A.R. Wood (1983) Calcitonin in Haemorrhagic Shock. Br. J. Pharmacol. in press.

ABSTRACT

The antinociceptive properties of calcitonin have been studied. Antinociception was only observed after intracerebroventricular (i.c.v.) injection. There was no effect observed after i.v., i.p. or s.c. administration. The concentration of plasma calcium was reduced following administration of the hormone by all of these routes. It was concluded that antinociception induced by calcitonin was independent of its action on the concentration of plasma calcium.

The i.c.v. injection of calcium (but not magnesium) ions or the ionophore A23187, simultaneously with calcitonin antagonised the antinociceptive effects of the hormone. The i.c.v. injection of EGTA or the calcium antagonists, nifedipine and PY 108 068, simultaneously with calcitonin, potentiated the antinociceptive effects of calcitonin. Similar results have been reported for the opiates. However, the opiate antagonist, naloxone, was relatively ineffective in reversing the antinociceptive effects of the hormone.

Withdrawal of animals from chronic pretreatment with calcitonin administered by s.c. injection, produced a long lasting hyperalgesia. In these animals, the antinociceptive effects of calcitonin, but not aspirin, were reduced.

In contrast to that of the opiates, calcitonin was not effective in altering the total calcium concentration of the brain, after i.c.v. or s.c. injection. However, in a similar fashion to the opiates, calcitonin reduced the uptake of ^{45}calcium by stimulated and non-stimulated brain slices, in vitro.

Calcitonin was ineffective when applied to the superfused rat colon, in vitro. This tissue produced a contractile response to the opiates. Calcitonin, unlike the opiates, did not reduce the blood pressure of the anaesthetised normotensive rat. However, it did resemble the action of naloxone, in producing a pressor effect in rats rendered hypotensive by haemorrhage.

In contrast to previous workers, calcitonin was found to be ineffective in increasing the activity of cortical acetylcholinesterase.

It is concluded that antinociception induced by calcitonin resembles that of the opiates, in that an alteration in calcium flux is involved. However, the hormone is unlikely to act via the opiate receptor to produce this response.

INTRODUCTION

1.1 THE ROLE OF CALCIUM AND ITS REGULATION

Calcium subserves a number of functions in the living organism, a fact which is reflected in the complexity of its regulation. A wide variety of cell function is subject to small fluctuations in ionized calcium concentrations within localized areas of the cell. The maintenance of precise extracellular calcium concentrations is therefore of vital importance.

Calcium homeostasis is achieved by the coordinate control of three agents: parathyroid hormone (PTH), vitamin D (or more specifically its active metabolites), and calcitonin. A reduction in the plasma calcium concentration produces an increase in the rate of secretion of PTH, which, in turn, increases bone resorption and restores the calcium concentration. Any increase in plasma calcium ion concentration inhibits the secretion of PTH, but stimulates the secretion of calcitonin. Calcitonin acts to restore the plasma calcium concentration. This relationship is shown in Figure 1.

The role of Vitamin D, or more correctly, its active metabolite, 1,25-dihydroxy vitamin D, is to promote the absorption of calcium and phosphate from the bone tissue. Vitamin D and PTH have a number of complementary functions, since the action of PTH on the gut and kidney is much reduced in the absence of Vitamin D.

Figure 1

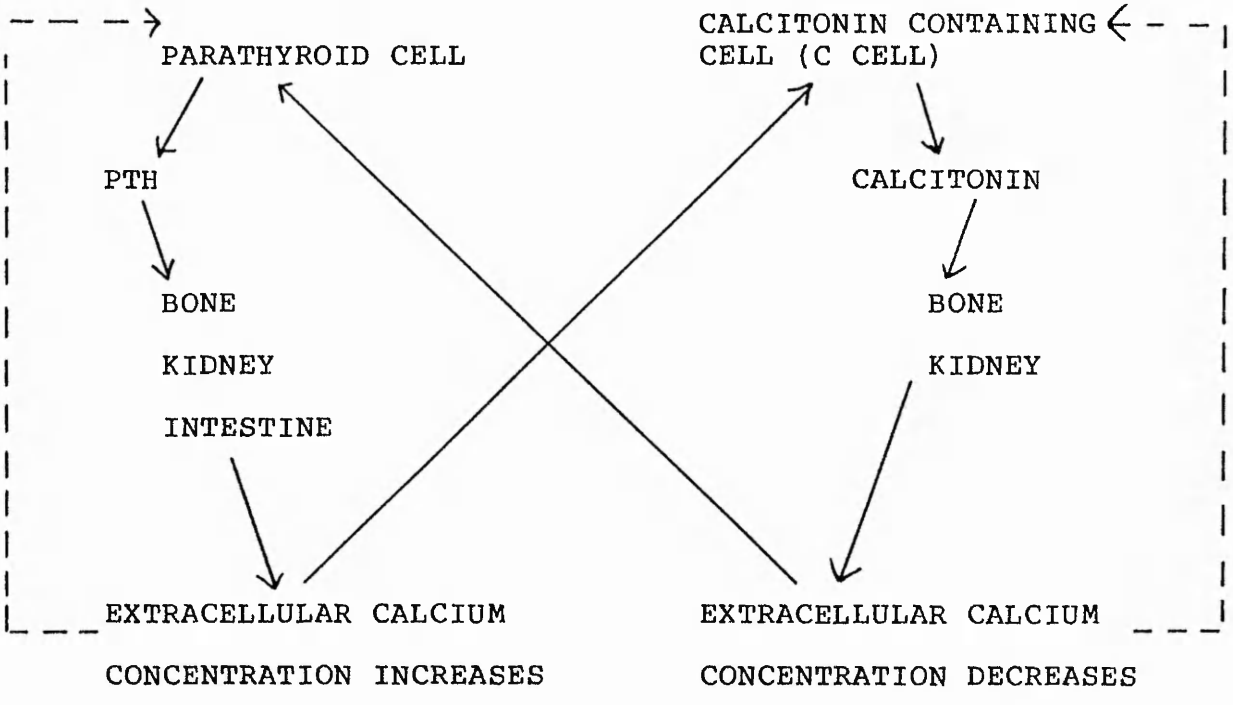


Figure 1 Hormonal control of calcium metabolism

——→ Stimulation - - - -→Inhibtion

1.2 THE ROLE OF CALCIUM IN ANTINOCICEPTION

This thesis is devoted to the antinociceptive properties of calcitonin. The relationsip between calcium and calcitonin is intimate and it is therefore relevant to discuss the relationsip between calcium ions and the production of antinociception.

Analgesia is a state of diminished perception of pain whilst antinociception reflects a decreased perception of noxious stimuli (Collier, 1964).

This question of terminology will be discussed more fully in a later section, but in the ensuing discussion the term antinociception will be used. Antinociception may be induced by administration of a wide range of substances including drugs of the opiate class, such as morphine, and by drugs of the antipyretic class, such as aspirin. Opiate drugs (and the opiate peptides such as endorphin or enkephalin) inhibit the perception of noxious stimuli by acting on the central nervous system (CNS). It is thought that a fundamental event in the production of antinociception is an alteration in the distribution of calcium ions within the central nervous system (Chapman and Way, 1980). Calcium has a number of actions in the CNS, where it may serve as a membrane stabiliser, influence the propagation of the nervous impulse and membrane permeability. Calcium is also important in the release of neurotransmitter and for many post-synaptic actions. It is therefore apparent that modulation of calcium within the CNS will influence a number of CNS functions.

The simultaneous injection of calcium ions with opiates, such as morphine, results in antagonism of their antinociceptive actions (Harris, Loh and Way, 1975). This is also seen in a number of opiate actions, unrelated to antinociception, such as relaxation of intestinal smooth muscle (Opmeer and van Ree, 1980). Calcium is, however, not the only divalent cation to do this since manganese and magnesium are also capable of antagonising opiate-induced antinociception (Harris et al, 1975). The antinociceptive properties of the opiates can be potentiated by agents which inhibit calcium flux. These agents include lanthanum (Iwamoto, Harris, Loh and Way, 1978) and the calcium chelator, ethylene diamine tetra acetic acid (EDTA), Harris et al, (1976). These agents are also capable of inducing antinociception when administered themselves. The response to lanthanium is inhibited by naloxone (Iwamoto et al, 1978). The intracerebroventricular (i.c.v.) injection of calcium ions can induce an increased perception of noxious stimuli (Chapman and Way, 1980).

The concentration of calcium in all regions of the brain is reduced by treatment with opiate drugs (Cardenas and Ross, 1975). This effect is antagonised by the opiate antagonists, naloxone and levallorphan (Cardenas and Ross, 1975). Similar observations have also been made at the subcellular level, using synaptosomes (Cardenas and Ross, 1976). It has been suggested that the reduction in available calcium at the nerve terminal, which occurs after acute opiate administration alters the release of neurotransmitter (Chapman and Way, 1980). There is, in

fact, surprisngly little evidence to support this hypothesis, although Shikimi Kaneto and Hano (1967) have shown that morphine at a high concentration ($1mmol.l^{-1}$) inhibits the potassium evoked release of acetylcholine from brain slice preparations.

In contrast to the acute administration of opiates, the chronic administration of opiates results in an increase in the total concentration of calcium within the CNS. This has also been seen in synaptosomes (Ross, 1978). The binding of calcium to brain tissue is increased following acute administration of opiates and is reduced after chronic treatment with opiates (Cardenas et al, 1976).

Consideration of data such as that presented above led Chapman and Way (1980) to propose the following:

(i) acute opiate administration decreases calcium flux and/or the binding at the nerve terminal, which causes a reduction in neurotransmitter release and eventually, by an as yet undefined mechanism, antinociception;

(ii) a homeostatic response then occurs, which restores the neuronal calcium concentration to control levels and eventually produces an elevation in the calcium concentration;

(iii) adaptation and tolerance result because subsequent administration of opiates is required in increasing concentrations to produce the same antinociceptive response;

(iv) when opiate administration is discontinued, the elevated concentration of calcium produces an increase in neurotransmitter release, causing hyperalgesia and other symptoms of withdrawal.

1.3 CALCITONIN

1.3.1 ISOLATION AND DISCOVERY

In 1962 Copp, Cameron, Cheney, Davidson and Henze demonstrated that perfusion of the thyroid/parathyroid complex in dogs with hypercalcaemic blood, caused a reduction in the plasma calcium concentration which was more rapid than that caused by parathyroidectomy. In addition, when hypercalcaemic blood from the thyroparathyroid veins of a donor dog was injected into a recipient dog, a decrease in the plasma calcium concentration still occurred. It was concluded that a humoral factor was released from the parathyroid gland during perfusion with hypercalcaemic blood. However, the presence of an active principle in the thyroid gland was suggested by Hirsch, Gautier and Munson (1963). It was shown that the fall in plasma calcium concentration observed after parathyroidectomy (by electrocautery) was greater than that observed after surgical parathyroidectomy. It was concluded that the electrocautery of adjacent thyroid tissue had caused the release of hypocalcaemic factor. The origin of this factor was later confirmed by extraction of a factor with hypocalcaemic activity from the thyroid gland (Hirsch et al, 1963). Copp et al (1962), however, maintained that his new hypocalcaemic hormone was derived from the parathryoid gland. It was called calcitonin, in order to distinguish it from the hypocalcaemic factor derived from the thyroid gland, i.e. thyrocalcitonin.

It was eventually shown that both substances were derived from cells named c-cells. In man, such cells are

almost exclusively present in the thyroid gland, but in other species (such as those used by Copp) they may exist in the parathyroid glands and other tissues of the cervical region as well as the thyroid gland.

The c-cells migrate from the last ultimobranchial pouch, in the embryo, which in lower animals forms a discrete body. In mammals, the ultimobranchial cells fuse to join the thyroid gland. The ultimate phylogenetic origin of the c-cells is the neural crest. This fact is important, when considering the potential presence of calcitonin within the brain.

1.3.2 CALCITONIN IN EXTRATHYROIDAL TISSUE

Although calcitonin was found originally in the c-cells of the thyroid/parathyroid gland complex, there is considerable evidence for its presence in other tissues (Becker, Snider and Moore, 1979). The majority of studies have used the technique of immunoreactive staining and it is, therefore, more correct to refer to the presence of calcitonin like-immunoreactivity (CLI) rather than calcitonin per se. The use of such staining techniques may also show the presence of fragments, precursors and molecules which in part or whole resemble the calcitonin molecule. The antisera used should therefore be highly specific.

CLI can be detected in the plasma and urine of thyroidectomised patients (Becker et al, 1979) which suggests that there occurs at least one of the following processes:

(i) extrathyroidal synthesis in c-cells present in tissues other than the thyroid gland;

(ii) receptor bound hormone originating from an intact thyroid gland prior to its removal;

(iii) incomplete suppresion of the calcitonin genome in a number of cells throughout the body.

The relative distribution of CLI has been assessed by Becker et al (1979) who ranked the distribution of the CLI with respect to the ratio of the tissue concentration to the cardiac blood concentration. The tissue having the highest ratio is the thyroid gland which is about 200 times more than the next highest tissue, the jejunum. There then follows

the thymus, urinary bladder and the lung. Intermediate ratios are found in the rectum, testis, skeletal muscle, hypothalamus, pituitary lymph nodes and stomach. The lowest ratios were found in the red blood cells and the cerebellum.

1.3.3 CALCITONIN-LIKE IMMUNOREACTIVITY IN THE PITUITARY GLAND

The initial reports related to the association of calcitonin with the CNS were concerned with the pituitary gland since medullary carcinoma of the thyroid gland, in addition to the elevated calcitonin concentrations, also secrete several other peptide hormones, including ACTH (which is secreted from the pituitary gland). There is frequently coproduction of ACTH and calcitonin from peptide secreting tumors (Rosenberg, Hahn, Orth, Deftos and Tanaka, 1978). These considerations led Deftos, Burton, Bone, Catherwood, Parthmore, Moore, Minick and Guillemin (1978) to study the normal pituitary gland for the presence of CLI. CLI was located in the anterior lobe and the intermediate lobe, but not in the posterior lobe. Later work by other groups confirmed this observation.

Mendelsohn, D'Agostino, Eggleston and Baylin (1979) reported that the hormone was found in cells which also contained ACTH. This observation has not been confirmed by other workers although Woolfe, De Leelis, O'Brian and Tashjian (1979) reported that CLI was present in the anterior lobe with a similar distribution to ACTH. Gagel, Voelkel, Woolfe and De Leelis (1979) showed that CLI was present in the anterior lobe only, and the distribution of CLI staining was similar, but not identical, to that of ACTH. Watkins,Moore, Burton, Bone, Catherwood and Deftos (1980) showed that the cells containing CLI did not have the same distribution as the corticotrophs i.e. cells containing

ACTH.

These differences i.e. whether CLI is present in the pituitary intermediate lobe, and whether CLI distribution is similar to that of ACTH, have yet to be resolved. These discrepancies may arise from the use of different species and/or antisera raised to recognise different portions of the calcitonin molecule.

Using specific antisera for specific species Deftos, Burton, Watkins and Catherwood (1980) have found that the CLI was present in the anterior pituitary lobe of the pig, cow, sheep, trout and goldfish. In these species the presence of CLI in the intermediate lobe was not a consistent observation. Watkins et al (1980) have shown the presence of CLI in the rat pituitary. Cooper, Peng, Obie and Garner (1980) have also reported the presence of CLI in the pituitary vascular bed of the human. However, Zillotto, Luisetto, Cecchino, Gastoldo, Francia, Padovan and Heynan (1980) could find no correlation between pituitary hyperfunction and calcitonin concentration in man although high calcitonin concentrations were found in a third of the patients examined. No explanation was offered for this discrepancy.

Samaan and Leavens (1981) have found that CLI was present in the pituitary vascular bed of man, at concentrations 2-3 times higher than that found in the peripheral blood. However, the serum concentration of FSH, ACTH, TSH were 1000 fold that found in the peripheral blood. No change was found in patients with elevated ACTH concentrations.

In contrast to the above studies, Weber and Voight (1979) have observed that with immunostaining for calcitonin in rat pituitary slices, there was no specific calcitonin activity. These authors considered that the positive CLI staining observed by other authors was due to cross reactivity by calcitonin antisera with the disulphide bridge found in the 16K precursor molecule fragment (a precursor molecule for ACTH). However, they also presented data which showed that calcitonin did not affect the binding of specific 16K antisera. Finally in order to study whether the calcitonin molecule was contained in the precursor, which would render it inaccessible to antisera, tryptic digests of the 16K and 30K precursor molecules were incubated with calcitonin antisera. Again, no CLI within these fragments was detected. These findings firstly contradict the suggestion that ACTH and calcitonin are contained within a common precursor as suggested by Deftos et al (1978) and secondly the suggestion of Cooper et al (1980) that the CLI observed within the pituitary was not calcitonin per se but a peptide (possibly a precursor) that was sufficiently related in structure to cross-react with calcitonin antisera.

The work of this group remains to be fully answered, although the balance of evidence would support the following: calcitonin, or a molecule nearly related to calcitonin is present in the anterior lobe of the pituitary gland in all species studied. Secondly, the presence of calcitonin in the vascular pituitary bed is greater than that of

the normal plasma concentration, which suggests that the hormone is secreted from the gland. Finaly, there is little evidence to suggest that calcitonin and ACTH are contained within the same cells or are co-secreted.

1.3.4 CALCITONIN-LIKE IMMUNOREACTIVITY IN THE CENTRAL NERVOUS SYSTEM

Fritsch, van Noorden and Pearse (1980) have reported that CLI was present in the neural ganglia of Ciona intestinalis, a primitive protochordate. The molecule found resembled human calcitonin in its immunological staining properties. This work was confirmed by Girgis, Galan Galan, Arnett, Rogers, Bone, Ravazzola and MacIntyre (1980). These authors found that CLI was present in the nervous systems of several protochordates and a cyclostome, Myxine. In all these species, the molecule detected resembled the human form of calcitonin in its immunological characteristics. Since this work, CLI has been detected in the CNS of a variety of animals including the frog (Yui, Yamada, Kayamori and Fujita, 1981), lizard (Galan Galan, Rogers, Girgis, Arnett and MacIntyre, 1980), pigeon (Galan Galan, Roger, Girgis and MacIntyre, 1981), rat (Flynn, Margules and Cooper, 1981) and man (Becker, Snider and Moore, 1980).

The following table (Table 1) shows this data and includes the concentrations present but the wide variation in the methods of assay and unit used makes comparison difficult. There appears to be a considerable variation between species, but it may be concluded that the area with the highest concentration of CLI is the hypothalamus and pituitary gland, whilst the areas with lowest concentrations of CLI are the brainstem and cortex.

The precise location of the CLI is unclear, although Fritsch et al (1980) have shown that CLI is present in

Table 1 Concentration of calcitonin, or calcitonin-like immunoreactivity, in the central nervous systems of a number of species.

AUTHOR	METHOD	TISSUE	SPECIES	CONCENTRATION	UNITS
Flynn et al (1980)	RIA	Hypothalamus	Rat	0.21	ng/hypo.
	RIA	Pituitary	Rat	1.16	ng/pit
Cooper et al (1980)	RIA	Pituitary	Rat	0.046	ng.mg ww^{-1}
	RIA	Thyroid	Rat	106	ng.mg ww^{-1}
Samaan et al (1981)	CLI	Pituitary Vascular Bed	Man	2.4	ng ml
Galan	RIA	Hypothalamus	Pigeon	31.5	ng gww^{-1}
Galan et al (1981)	RIA	Mid Brain	Pigeon	17	ng gww^{-1}
	RIA	Brain Stem	Pigeon	2.85	ng gww^{-1}
Girgis et al (1980)	RIA	Whole Brain	Myxine	1300	pg CT equivalent
	RIA	Whole Brain	Amphioxious	160	pg CT equivalent
	RIA	Whole Brain	Ascidiella	320	pg CT equivalent
	RIA	Whole Brain	Styella	33	pg CT equivalent
	RIA	Whole Brain	Ciona	22-50000	pg CT equivalent

Table 1 Continued

AUTHOR	METHOD		SPECIES	CONCENTRATION	UNITS
Yui et al (1981)	RIA	Whole Brain	Frog	1.83	pg mg tissue
Becker et al (1979)	CLI	Hypothalamus	Man	0.015	ng mg fat free dry weight
	CLI	Pituitary	Man	0.013	ng mg fat free dry weight
	CLI	Cortex	Man	0.0064	ng mg fat free dry weight
	CLI	Cerebellum	Man	0.0017	ng mg fat free dry weight

specific neurones of Ciona intestinalis. Yui et al (1981) have shown that CLI in the CNS of the frog is present in two distinct neuronal populations; those neurones present in the parvicellular area and those in the nucleus infundibularis. In this latter area these cells are bipolar and project toward the third ventricle and the CSF.

The authors were unable to state whether the neurones were secretory in nature. This study is important since it is the only study of a vertebrate brain to localise CLI activity to specific neuronal systems. If such a study is applicable to higher animals then it implies that calcitonin may be released into the CSF with the possibility that it may modulate neuronal function.

CLI has been found in the CSF of man, although the source of this CLI is unknown. Stekol'nikov and Abdurkarimov (1969) have reported that a small proportion of calcitonin given by peripheral injection enters the CSF. This has also been reported by Carmen and Wyatt (1979) to be the case in rabbits and in monkeys.

1.3.5 CALCITONIN BINDING SITES IN THE CNS

Since calcitonin exerts its effects in peripheral tissues by binding to specific receptor sites, it is reasonable to suggest that the same occurs within the CNS, with which either calcitonin or material with CLI (of central or peripheral origin) will combine. Van Houten, Goltzman and Posner (1980) first reported the existence of specific binding sites for calcitonin in the circumventricular areas of the rat brain. The technique used was that of in vivo autoradiography. The greatest density of binding was observed in the organum vasclosum lamina terminalis area which contains high concentrations of CLI. In the median eminence binding was restricted in the zona externa as bilaterally overlapping foci in the medial and lateral palisade zones. An earlier paper by Foslund, Stridsberg, Stanina and Appelgren (1980) used whole body autoradiography after subcutaneous injection of I^{125} calcitonin, and found no evidence for binding to the CNS. However, calcitonin binding to the CNS was not specifically looked for.

Koida, Nakamuta, Furukawa and Orlowski (1980) measured the binding of I^{125} calcitonin to rat brain homogenates. All of the areas studied exhibited binding, even areas such as the cortex and cerebellum (which contain low concentrations of CLI). The specific binding was determined by means of displacng the bound labelled hormone with 'cold' hormone. The highest density of binding was found in the hypothalamus and thalamus and the lowest in the cerebellum and cortex. This distribution was similar to the

distribution of CLI in the rat brain. The analysis of binding of the hormone to whole brain homogenates showed two distinct binding sites. The low affinity site was shown to have a k_D and V_{max} of 10 $nmol.l^{-1}$ and 10 $fmol.mg\ protein^{-1}$ whilst the high affinity site had a k_D and V_{max} of 0.57 $nmol.l^{-1}$ and 56 $fmol.mg^{-1}$ protein respectively.

This group of workers further studied the kinetics of binding I^{125} calcitonin to various brain regions (Nakamuta, Furukawa and Koida, 1981). The hypothalamus and midbrain showed the binding of highest affinity and the greatest number of binding sites. The lowest values were found in the cerebellum. The binding to the high affinity binding sites was similar to those reported for other peptides in the brain. The binding sites were selective for calcitonin since ACTH, β-MSH and enkephalin did not dsplace the bound hormone. A variety of neurotransmitter blockers and neurotransmitters were also ineffective in influencing calcitonin binding.

Fischer, Sagar and Martin (1981) have also found binding sites for salmon calcitonin using membrane fractions isolated from whole rat brain. The binding was a function of temperature and, after binding, the hormone was not degraded. The binding was highest in the hypothalamus, confirming work by other authors. Human calcitonin and a sulphone analogue did not influence binding. The analogue, salmon calcitonin (10-32), a fragment without hypocalcaemic activity, was at least as effective as salmon calcitonin (1-32) in the affinity of its binding. The authors concluded

that the amino acids 1-7, which form the ring structure, were unimportant for binding to the tissue. It was also shown by these workers that the hormone remained bound for as long as 6 hours after administration. This has also been shown by Goltzman (1980) for the binding of calcitonin in the periphery (which may explain the long duration of action of the hormone in the periphery after its removal from the circulation). The binding of the hormone to the brain was unaffected by a number of neuropeptides, as well as by naloxone and morphine. Of particlar relevance to this project is the fact that the binding of calcitonin was strongly inhibited by 1 to 8 $mmol.l^{-1}$ $CaCl_2$. The role of calcium ions in the production of antinociception has been discussed previously.

The work of Rizzo and Goltzman (1981) has shown that the binding of I^{125} calcitonin to rat brain was consistent with the concept that such binding is to receptors i.e. the binding is highly specific, saturable and the kinetics are time and temperature dependent. These authors also found that the greatest amount of binding was in the area of the hypothalamus.

To set the work of these authors in context with work undertaken to study calcitonin receptors outside the brain, one may compare this work with that of Goltzman (1980) who studied the binding of I^{125} salmon calcitonin to skeletal and renal tissue membranes.

In both the brain and the periphery, the binding was time and temperature dependent, and equilibrium conditions

were established after 1 hour. However, the rate of degradation was different in the brain, compared to the periphery. At 37°C, there was 80% degradation at 60 minutes in the renal membranes, whilst there was only 15% degradation at the same time in the brain. (Fischer et al (1980) have also reported that the bound calcitonin in the brain is only degraded after 6 hours in the brain.) In addition, the amount of hormone bound to renal membranes (at 4°C for 60 minutes) was 20 fmol.mg protein^{-1}, when incubated with 8 x 10^{-11} mol.l^{-1} calcitonin. Under identical conditions, the amount of hormone bound to the brain was 40 fmol.mg protein^{-1} i.e. double that bound to the renal membranes.

One may conclude from this data the following: the amount of hormone bound and the amount of degradation differ in the brain with regard to the periphery. However, it must be stated that an endogeous ligand present in the brain may be similar in its binding characteristics to the calcitonin found in the periphery. The characteristics of the binding of salmon calcitonin to the brain are consistent with those of neuromodulators, such as enkephalin or substance P (Koida et al, 1980).

In summary, calcitonin or CLI is present in relatively high concentrations in the CNS of a number of animals throughout the evolutionary scale. The ancestral form of the hormone appears to be human. There is some evidence to suggest that within the mammalian brain, the calcitonin present in the CNS may be different to the calcitonin found

within the thyroid gland (see section on biosynthesis).

A common finding is that the hypothalamus and areas associated with the ventricular system, contain areas of high concentrations of CLI, and are also areas of high affinity of binding. These facts agree well with the known effects of calcitonin on the CNS; effects which appear to be related to an action on the hypothalamus areas.

1.3.6 CALCITONIN BIOSYNTHESIS

Peptides destined for secretion are generally synthesised in the form of a large precursor. Such precursors are then processed by a variety of mechanisms and this accounts for the development of whole peptide families, similar in structure, yet different in function.

Until very recently the synthesis of calcitonin was thought to occur solely by the following means (Jacob, 1981):

Calcitonin is formed from a precursor molecule (m.wt. 15000) known as procalcitonin. The calcitonin sequence is present near the carboxy terminal, flanked by basic amino acids. (Basic amino acid pairs such as ARG - ARG or ARG - LYS are common enzyme cleavage points in the processing of large precursor molecules.) The initial event is the co-translational cleavage of procalcitonin and cleavage of the leader sequence to form calcitonin. Carboxypeptidase activity then trims the basic amino acids from the carboxy terminal. Enzymatic conversion of the PRO - GLY amino acid sequence to the carboxyl terminal prolinamide found in the mature form then occurs. The function of a carbohydrate moiety attached to the precursor is unknown, but it may lend specificity to post-translational cleavages (Jacob, 1981).

There are also a number of calcitonin related mRNA molecules which can give rise to products other than procalcitonin which have been denoted as calcitonin gene related product (CGRP) mRNAs (Amara, Jonas, Rosenfeld, Ong and Evans, 1982). These mRNAs can give rise to translational products which do not contain calcitonin.

Which mRNA is processed depends on the tissue. As shown in Figure 2, in the thyroid gland, the calcitonin mRNA is translated, as described above. In the hypothalamus the variant CGRP mRNA predominates. However, both are derived from the same gene. The sequence of this peptide has only predicted from the mRNA - it has yet to be isolated from the hypothalamus. This substance may represent proposed endogenous calcitonin-like ligand in the brain. It is also possible that, in studies using CLI and RIA techniques to search for the presence of calcitonin in the brain, the positive results obtained may indicate not calcitonin but the above peptide. It would be interesting to study the effects of this substance on CNS function.

The situation has been further complicated by the discovery by MacIntyre, Hillyard, Murphy, Reynolds, Gaines, Das and Craig (1982) of a peptide flanking calcitonin the precursor molecule, procalcitonin, which has biological activity. This substance, denoted PDN - 21, has a similar potency towards lowering the plasma calcium concentration as calcitonin. This substance has the structure (H-Asp-Met-Ser-Ser-Asp-Leu-Glu-Arg-Asp-His-Arg-Pro-His-Val-Ser-Met-Pro-Gln-Asn-Ala-Asn-OH). It is unknown whether such a substance is present in the CNS.

The discovery of CLI in the pituitary gland led Deftos et al (1979) to speculate as to whether the substance in this area was derived from Pro-Opio Melano Cortin (POMC) which gives rise to endorphin, ACTH and MSH. Nakahanishi, Inove, Kita, Nakamura, Chang, Cohen and Numa (1980) proposed

that there was a slight sequence homology between calcitonin and POMC in that there were 7 amino acids which comprised a 'calcitonin-like skeletal structure'. This was thought by Deftos et al (1979) to account for its occurrence with ACTH (Watkins et al, 1980). However, Weber et al (1979) have shown that calcitonin bears no immunological resemblence with POMC or its fragments.

CALCITONIN GENE	58 Noncoding ---	5' Noncoding ---	Common Coding ---	Common Coding ---	Calcitonin --- CGRP --- 3'
		Exon A	Exon B	Exon C	Exon D
PRIMARY TRANSCRIPTION	5' --- A ----------	B ----------	C ----------	D ----------	Calcitonin --- CGRP --- 3'

RNA PROCESSING

	THYROID C CELLS	HYPOTHALAMUS
MATURE mRNA	5' --- A or B --- C --- D --- Calcitonin --- 3'	5' --- A or B --- C --- D --- CGRP --- 3'

mRNA TRANSLATION

PRIMARY TRANSLATION PRODUCT	NH_2 -- Common Region + 6 a.a. Calcitonin --- COOH	NH_2 -- Common Region + 4 a.a. CGRP --- COOH
MATURE PEPTIDES	CALCITONIN + 83 AMINO ACID N' TERMINAL PEPTIDE	CGRP + 81 AMINO ACID N' TERMINAL BUFFER
	PERIPHERY	CNS

Figure 2 Calcitonin Biosynthesis in Periphery and CNS (after Amara et al, 1982).

1.3.7 CALCITONIN STRUCTURE AND EVOLUTION

Calcitonin exhibits several species differences with regard to its strucutre and amino acid sequence (MacIntyre, 1978). The calcitonin isolated from the human, rat, cow, pig, sheep, eel and salmon are all different. All possess a similar structure consisting of a straight chain peptide of 32 amino acids with 7 amino acids forming a disulphide ring at the amide terminal and a prolinamide at the carboxy terminal. Non-mammalian calcitonins are more resistant to degradation in plasma than mammalian calcitonins and also possess a higher affinity for receptors in the periphery. These two facts account for their greater potency in reducing the concentration of plasma calcium (Habener, Neer, Deftos and Potts, 1972). The entire molecule is required for activity and consequently in vivo degradation produces inactive fragments (Austin, 1981). Porcine, human and salmon calcitonin differ in their site of degradation and metabolism. Porcine calcitonin is mainly degraded in the liver, whilst salmon and human calcitonin are metabolised in the kidney (De Luise, Martin and Melick, 1970).

Immunological cross-reactivity experiments have demonstrated 3 main animal groups of the hormone:

(i) pacific salmon, rainbow trout, cod, tuna, eel, domestic fowl, pigeon and quail

(ii) sheep, pig and cow;

(iii) rat and human.

The ancestral form of the hormone is considered to be the human form (Girgis et al, 1980) and the genetic divergence

occurred early in its evolution. A further divergence occurs between the mammals and the non-mammals and again between the rat, human and artiodactyls (see Figure 3). It has also been shown (Perez-Cano, Girgis, Galan Galan and MacIntyre, 1982) that there may be simultaneous expression of two different calcitonin genes in the same species. The pigeon possesses both salmon and human calcitonin immunoreactivity in the thyroid gland. Normally only one gene is expressed whilst the others remain dormant. In the pigeon both genes are expressed, the human calcitonin to a lesser degree.

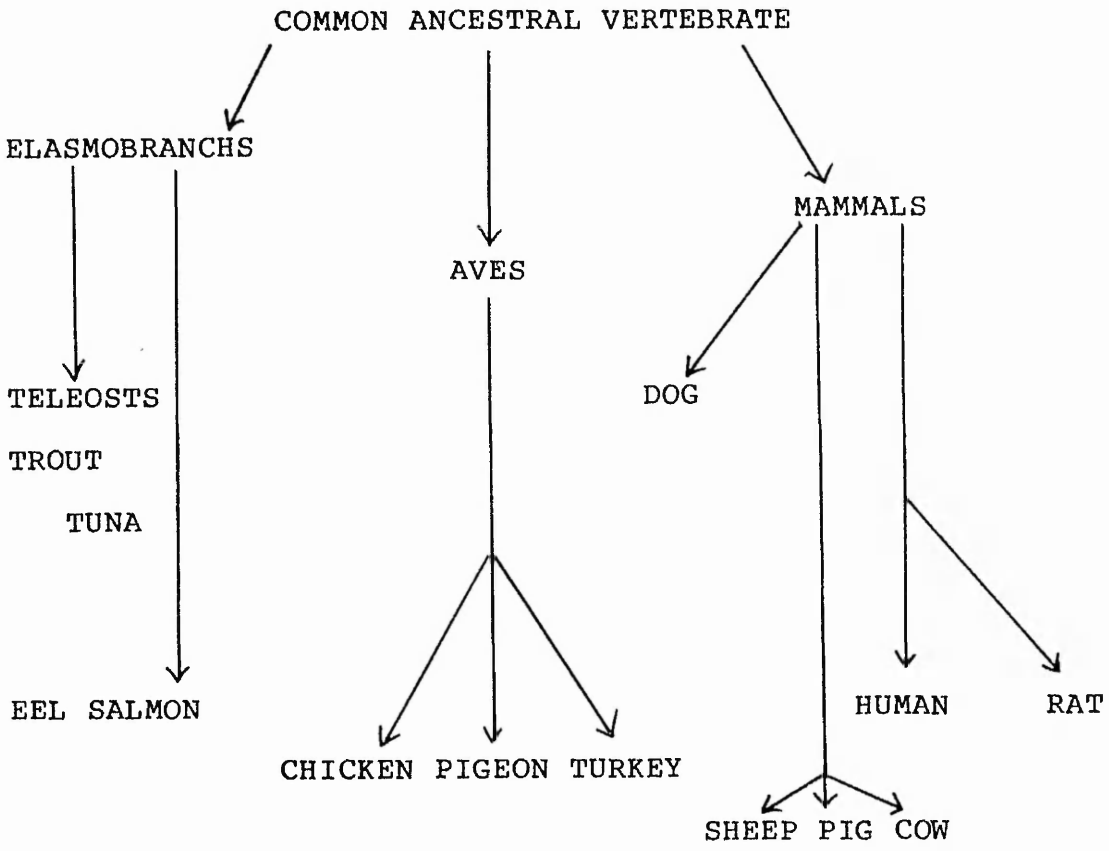


Figure 3 Evolution of Calcitonin

1.3.8 PHYSIOLOGY OF CALCITONIN

1.3.8.1 ROLE IN THE REGULATION OF CALCIUM IN MAN

Calcitonin in normal adults plays little part in the regulation of plasma calcium concentration (MacIntyre, 1981). However, in times of relative calcium deficiency (where bone turnover is high) such as pregnancy, lactation and growth, calcitonin acts to prevent resorption of the skeleton (MacIntyre, 1981). It does this by directly inhibiting oesteoclast activity and in the long term reducing their number. Normal women are relatively deficient in plasma calcitonin compared with men, although the concentration of calcitonin does increase sharply during pregnancy and lactation (MacIntyre, 1981). High concentrations of the hormone are also seen in the plasma of the foetus and the neonate. In these conditions the plasma concentration of 1,25 dihydroxy vitamin D is raised in order to increase the plasma calcium concentration. The function, in the presence of calcitonin is not fulfilled at the expense of the skeleton.

1.3.8.2 ROLE IN THE REGULATION OF CALCIUM IN OTHER SPECIES

The demands of a light skeleton and egg laying provide additional stresses on the calcium metabolism in birds. Birds have, therefore, a highly efficient homeostatic control of plasma calcium concentration, which includes intestinal absorption of dietary calcium. Most calcium is stored in the shell gland. The efficiency of plasma calcium regulation in birds probably explains why no hypocalcaemic

effect is seen following calcitonin injection. The same is also true for the reptiles, although the lack of effect of the hormone in these animals may also be due to their low rate of bone turnover. Bony fish also have a rapid efficient homeostatic control of calcium, with calcium being stored partly in the bone but also in the gills. Calcitonin, released from the ultimobranchial glands, seems to protect the skeleton from excessive resorption.

In summary, calcitonin does not exert a marked effect on the plasma calcium concentration of most species either because of a low rate of bone turnover or because of rapid calcium homeostatic mechanisms.

Deftos (1981) has suggested that calcitonin has other roles other than a purely endocrine role. This involves a local regulatory role, i.e. a paracrine and possibly a neurocrine function in the CNS. Calcitonin may, therefore, modulate communication between cells, in both the unicellular organisms (where it may act as an intracellular regulator) and in oligocellular organisms (where it could exert a paracrine role). As animals became more complex, the hormone evolved to subserve a specialised endocrine role.

This view of calcitonin may be considered in terms of the evolution of hormones in general. As a means of cellular communication, hormones evolved early (insulin, for example, is present in fungi and in E.coli; protozoa contain ACTH and B-endorphin). Roth (1982) has proposed that the general function of hormones, to mediate cellular

communication, is the same in both unicellular and multicellular organisms. The difference is that in unicellular organisms there is no distinction between endocrine and exocrine function, and the hormones subserve a purely paracrine function.

Bern (1980) proposes that in primitive animals calcitonin secretion may have been regulated by changes in the external calcium concentration, i.e. at this level, calcitonin secretory cells (which may possibly be neurosecretory cells) would possess a sensory function. Calcitonin-like immunoreactivity is present in the neural cells of a number of primitive organisms (Fritsch et al, 1980), and its secretion may be regulated by changes in external calcium. It is also possible that the putative sensory function of calcitonin neurones may be maintained in higher animals. In the vertebrates, dendritic processes from both hypothalamus and caudal secretory neurones have been observed projecting into the ventricular system and the central canal respectively (Yui et al, 1981). It was suggested (Bern, 1980) that such processes may represent a retention of earlier, direct control mechanisms which were at one time located externally. This implies that such neurones may be sensitive to changes in the calcium concentration of the CSF.

This may explain why calcitonin is effective on a number of CNS functions at concentrations which would not affect the plasma calcium concentration. Calcitonin, secreted from specific neurones in small quantities, could exert effects

on the brain, possibly by an alteration in the calcium concentration of the CSF.

Girgis et al (1981) have suggested that, in mammals, calcitonin may have 2 functions: a classical endocrine role to regulate the plasma calcium concentration and secondly to regulate the calcium concentration of the CSF, and consequently CNS function. Though this is pure speculation, it is not without precedent since, in teleosts, prolactin-containing cells are sensitive to a reduction in the environmental osmotic pressure. In mammals this function is thought to be maintained in addition to its traditional role in lactation.

1.3.9 ACTION OF CALCITONIN AT THE CELLULAR LEVEL

The actions of calcitonin, like many peptide hormones, at the cellular level can be broadly divided into 2 categories - its effects on the intracellular calcium concentration and secondly, its effects on intracellular cAMP concentration

1.3.9.1 CALCIUM

Many workers have shown that calcitonin reduces the transport of calcium in bone, kidney (Kenny and Heiskell, 1965), liver (Yamaguchi and Yamamoto, 1976) and myocardium (Chausmer, Weiss and Wallach, 1965). In the majority of studies the intracellular concentration is elevated. This could occur because of one, or all, of the following processes occurring:

(i) a promotion of uptake,

(ii) a decrease in the exchangeable pool of calcium,

(iii) an inhibition in efflux.

One should note that calcitonin does not elevate the calcium concentration in all tissues, e.g. there is no effect on the salivary glands and the pancreas. In contrast to other workers, Chausmer et al (1965) and Kenny and Heiskell (1965) have failed to show any effect on the calcium concentration of bone cells and the small intestine respectively. Their use of a crude extract of calcitonin may account for this.

The majority of authors consider that calcitonin increases the tissue calcium content by an inhibition of calcium efflux. Only the work of Wase et al (1967) has suggested that calcitonin actually promotes uptake.

Yamaguchi et al (1976) have shown that calcitonin inhibits the efflux of calcium from liver cells both in vivo and in vitro. However, Borle (1975a) has suggested that the situation may be more complex than simply considering the uptake or efflux of calcium. He proposes that it is the regulation of cytoplasmic activity which is dependent upon the exchange of calcium between the mitochondria and the cytoplasm. In liver cells, in vitro, Borle (1975b) has shown that calcitonin stimulates the transport of calcium from the cytoplasm to the mitochondria, which reduces the available pool of exchangeable calcium. A secondary effect of this process is that calcium efflux is inhibited.

An important aspect of Borle's work is that, from a theoretical standpoint, the conditions under which the uptake experiments are undertaken are crucial. Firstly, the uptake of ^{45}calcium measured is not equivalent to the calcium uptake. Secondly, the effects of hormones are different when examined under equilibrium conditions, compared to non-equilibrium conditions. This was illustrated by Borle (1975a), investigating the effect of calcitonin on calcium uptake into kidney cells. Simple uptake data indicated a stimulation of calcium uptake. However, rigorous kinetic analysis revealed that calcitonin depressed both calcium influx and efflux - the increased uptake was caused solely by an enlargement of the exchangeable pool. These conditions may account for the discrepancies seen in the effects of calcitonin on calcium since not all were performed under equilibrium.

1.3.9.2 3' 5' cAMP

The activity of cyclic 3' 5' adenosine monophosphate (cAMP) as a second messenger is related to the concentration of intracellular calcium. Since calcitonin exerts an effect on the intracellular calcium concentration, it is likely to also influence intracellular cAMP activity.

Calcitonin has been shown to elevate the concentration of cAMP in a variety of tissues including kidney (Murad, Brewer and Vaughan, 1970), bone (Murad et al, 1970) and, surprisingly, mononuclear phagocytes (Minker, Newbrey and Walling, 1977). The actions of calcitonin on kidney and bone may be mimicked by elevating the intracellular cAMP concentration. (This effect of calcitonin on cAMP is in contrast to the action of PTH and prostaglandin E_2; agents which do not increase bone resorption and do not alter cAMP levels.) The work of Borle (1975b) is, in this respect, an exception. Initially, he demonstrated that cAMP stimulated the efflux of calcium from mitochondria. However, calcitonin reversed this effect of cAMP even though the hormone also elevated the intracellular concentration of cAMP. He explained this by postulating that the increase in intracellular cAMP caused by calcitonin was due to a decrease in the cytoplasmic calcium, which depressed the adenyl cyclase activity. This is still not a complete explanation since the decrease in adenyl cyclase activity will raise the intracellular cAMP concentration and which would, in turn, cause an efflux in calcium from mitochondria. Borle (1976) later retracted this part of his

work that claimed that cAMP affected the accmulation of calcium by mitochondria.

The action of calcitonin on CNS calcium and cAMP concentration have been little studied. In contrast to its actions in the periphery, calcitonin has been shown to inhibit the uptake of calcium into hypothalamic explants (Levine and Morley, 1981). This would reduce the intracellular calcium concentration - the opposite of its action in the periphery. However, a reduction in the calcium content of the brain may yet occur because of the following reasons:

(i) the central effects of calcitonin are effects which are thought to be elicited by a decrease in neuronal calcium;

(ii) related to this is the fact that the central effects of calcitonin are the opposite of the effects observed when calcium ions are injected into the CNS. The central injection of calcium ions, for example, are capable of inducing feeding (Levine and Morley, 1980) and also hyperalgesia (Chapman and Way, 1980). As will be discussed, the central injection of calcitonin can induce an inhibition of feeding and antinociception.

(iii) As a corollary to the ability of calcium ions to antagonise calcitonin-induced antinociception (Satoh et al, 1979), Fischer et al (1980) have shown that calcium ions can inhibit the binding of calcitonin to preparations from rat brain.

(iv) Carmen and Wyatt (1979) has shown that s.c.

injections of calcitonin cause an increase in the concentration of calcium in the CSF. This may account for the ability of the hormone to limit manic depressive illness - a condition which is associated with a decrease in the calcium concentration of the CSF.

To date, there have been 2 studies concerning the effect of calcitonin on the activity of adenylate cyclase in the brain. Rizzo and Golzman (1981) measured the effect of salmon calcitonin on the adenylate cyclase activity of homogenates from whole rat brain or from homogenates from the hypothalamus. The activity of the enzyme was inhibited by salmon calcitonin at concentrations between 10^{-6} to 10^{-4} $mol.l^{-1}$. Porcine and human calcitonin were less effective than salmon calcitonin in this respect. However, this action of the hormone is in direct contrast to its action in the periphery. The concentrations of the hormone required to inhibit adenyl cyclase were very high and were far in excess of the doses required for binding.

Loffler, van Caulker and Hamprecht (1982) investigated the action of human calcitonin and PTH on the cAMP concentration in primary cultures of rat and mouse glial cells. Calcitonin elevated the concentrations of cAMP in rat glial cells only; no effect was observed in the mouse. In the rat a threshold effect was observed at 10^{-8} $mol.l^{-1}$, and a plateau was still not reached at 2×10^{-6} $mol.l^{-1}$. The rise was complete by 5 to 10 minutes and sustained for at least 30 minutes. The rapid onset and long duration of action correlates well with the rapid onset and long

duration of action observed both for the binding of the hormone to neural tissue and for its known activity on the CNS.

1.3.10 PHARMACOLOGY OF CALCITONIN

The primary effect of calcitonin is to reduce plasma calcium concentration by means of its action on the major target organs of the bone and kidney (MacIntyre, 1981). In addition, the hormone also has a number of effects which may not be related to its hypocalcaemic action. These include effects on the CNS and on the periphery. The latter are discussed below.

1.3.10.1 EFFECTS ON BONE

Pharmacological doses of calcitonin almost completely inhibit bone resorption, both in vitro and in vivo. The primary use of calcitonin as a pharmacological agent is in the treatment of Paget's disease. This condition is characterised by a state of excessive bone resorption, usually accompanied by an increase in the plasma concentration of alkaline phosphatase and urinary hydroxyproline (Deftos,1981). The major therapeutic sign of the effectiveness of calcitonin is the alleviation of bone pain which occurs in 80% of patients. There is an improvement in neurological function and a decrease in skin temperature over the diseased bone. Urinary alkaline phosphatase declines and is a major index of successful calcitonin therapy. Histologically new bone is formed and abnormal tissue replaced (Austin, 1980).

1.3.10.2 EFFECTS ON KIDNEY

Calcitonin has two distinct effects on the kidney. Firstly,

it enhances the excretion of not only phosphate but secondly, sodium, potassium, calcium and magnesium (Ardaillou, 1975). Calcitonin also enhances the renal production of 1,25 dihydroxy vitamin D. This effect on vitamin D metabolism may be indirect since it is dependent upon the presence of intact thyroid glands.

1.3.10.3 EFFECTS ON THE GI TRACT

Calcitonin increases small bowel secretion of electrolytes and water and inhibits gastric emptying and gastric acid secretion (Austin, 1981). The action of calcitonin on gastric acid secretion was originally inferred from a number of lines of evidence. There is a marked coincidence between primary hyperparathyroidism and peptic ulceration. In addition, in normal patients there is also an increase in the acidity of the gastric juice after hypocalcaemia. Finally, several gastrointestinal hormones are powerful secretagogues for calcitonin including gastrin and pancreozymin.

Calcitonin inhibits basal secretion and pentagastrin stimulated secretion of gastric juice in a number of species (Hotz, Goebell, Muneand and Ziegler, 1974). A decrease in the rate of gastric emptying and also an inhibition in pepsin output had been observed (Bobalik, Klesszylki, Aldred and Bastian, 1974). The majority of these studies have employed pharmacological concentrations of the hormone and there is no consistent relationsip between the concentration of plasma calcium and the inhibition in gastric secretion.

Calcitonin is, therefore, thought to act directly.

Calcitonin has also been studied as a potential antiulcer agent because of its ability to reduce gastric secretion. The hormone has been shown to be effective when applied to a number of models of ulceration (Doepfner and Ohnhais, 1972; Bates and Barlet, 1974) and in a number of species. The exact mechanism of action is unclear although it may differ from 'classical' antiulcer agents (Strettle, 1981).

1.3.10.4 EFFECTS ON THE ANTI-INFLAMMATORY RESPONSE

Calcitonin, when applied to a number of models has a potent anti-inflammatory response (Abdullahi, Ammagoi-Martelli, Bramm, Franco and Velo, 1975). Again the mechanism of this is unclear, although it may be due to either a reduction in the plasma calcium concentration or due to an inhibition in prostaglandin synthesis.

This effect of calcitonin will be described more fully in the discussion. Strettle et al (1981) concluded that the action of calcitonin was independent of its effect on plasma calcium concentration and also that its action was dissimilar to that of other anti-inflammatory agents.

1.3.10.5 EFFECTS ON THE CARDIOVASCULAR SYSTEM

A number of workers have shown that calcitonin in large concentrations can affect the cardiovascular system. Barabanova and Yegorov (1980) has shown that calcitonin can affect conduction of electrical impulses between heart cells

of the S-A node of the guinea-pig and the rabbit. Calcitonin can also affect the responsivity of systemic blood vessels (Driessens and Van Houtte, 1981) to PTH. The significance of these findings is unclear. The action of calcitonin on the blood pressure of normotensive and haemorrhaged animals has been studied in this project.

1.3.11 EFFECT OF CALCITONIN ON THE CNS

Calcitonin, as discussed, may exert a neuromodulatory influence on the CNS. Calcitonin has been reported to have 4 effects on CNS function:

(i) antinociception
(ii) inhibition of food intake
(iii) control of prolactin secretion
(iv) inhibition of gastric acid secretion.

Carmen and Wyatt (1979) have also claimed that the hormone is of value in the treatment of manic depressive illness. Though this is not a physiological function, it is included in this section since it suggests some mechanism by which calcitonin may affect the CNS.

1.3.11.1 ANTINOCICEPTIION

The alleviation of pain as a consequence of treatment of Paget's disease with calcitonin is well documented (Deftos, 1981). It was this observation that led Pecile, Ferri, Braga and Olgiati (1978) to study the effect of calcitonin, administered by i.c.v. injection on the nociceptive response to electrical stimulation of the tooth pulp in rabbits. A rise in the threshold of electrical stimulation required to elicit a licking response was taken as an antinociceptive response. Antinociception was observed after the administration of 2 i.u. kg^{-1} porcine calcitonin. The effect was rapid in onset and reached a maximum after 90 minutes. The injection of 16 i.u. kg^{-1} produced a greater effect, of longer duration. In addition, at both of these

doses the concentration of plasma calcium was reduced, which indicated to the authors a degree of leakage of the hormone from the ventricles to the circulation. This leakage was considered to represent leakage of the hormone from the wound site caused by implantation of the cannulae.

Braga, Ferri, Santagostini, Olgiati and Pecile (1978) investigated the relationship between calcitonin-induced antinociception and opiate- induced antinociception. The same antinociceptive test as described above was used and the hormone was injected chronically, i.e. by repeated, daily i.c.v. injection. Repeated injections of morphine over a period of 5 days produced tolerance. In contrast, no such tolerance was seen with the administration of calcitonin. In addition, calcitonin-induced antinociception was not antagonised by the opiate antagonist, naloxone. The simultaneous i.c.v. injection of calcitonin and morphine produced a potentiation of the antinociceptive effects. The authors concluded that the mechanism of antinociception induced by calcitonin was different from that of the opiates. In vitro work by Braga et al (1978) underlined the fact that calcitonin did not interact with opiate mechanisms in the production of antinociception. Calcitonin did not affect the binding of dihyromorphine to homogenates of guinea-pig brain, nor was it active in an opiate bioassay, i.e. the guinea-pig myenteric plexus. However, the question of whether calcitonin exerted its effect by a central or peripheral mechanism was still unresolved.

Yamamoto, Kumagai, Tachikawa and Marayawa (1978)

studied the effects of porcine calcitonin in a number of antinociceptive tests. These included the acetic acid-induced abdominal constriction test and the paw pressure test. Calcitonin was administered by either i.c.v., i.v. or s.c. injection into mice and rabbits. (This paper has not been published in English and all but the summary is in Japanese.) The antinociceptive effects were seen only when the hormone was administered centrally and they were of long duration (over 90 minutes). In a further paper by this group (Yamamoto, Kumagai, Tachikawa and Maeno1978) the effect of porcine calcitonin was studied in the paw pressure test in mice. Antinociception persisted for up to 30 minutes following i.c.v. injection of calcitonin, although the maximum effect was obtained at 10 minutes. The antinociceptive responses to calcitonin were not antagonised by the (partial) opiate antagonist, levallorphan. The authors concluded that calcitonin exerted its antinociceptive action independently of the opiate system. In the discussion of this paper, the authors cited a personal communication from Miki, stating that there was no cross tolerance between calcitonin, morphine or phenobarbital with regard to physical dependence. This study has not been expanded upon to date.

The data presented above suggest that the mechanism of calcitonin-induced antinociception is independent of the opiate system. Satoh, Amano, Nakagawa and Takagi (1979) have reported that the fundamental mechanisms of calcitonin-induced antinociception and that of opiate-induced

antinociception may, however, be similar, i.e. the modulation of calcium flux. To support this they administered calcitonin, by i.c.v. injection, and assessed the antinociceptive properties by the paw pressure test. The EC 50 was shown to be 2.25 i.u. kg^{-1} and a maximum effect was reached 5 minutes after injection. The simultaneous injection of calcium ions and calcitonin reduced the antinociceptive properties of the hormone with regard to both the maximum and the duration of the response. The authors also suggested that, although both morphine and calcitonin-induced antinociception was attenuated by calcium ions, the site of action of these agents was different. The injection of porcine calcitonin into the reticularis gigantocellularis (an area sensitive to the effects of morphine) did not result in antinociception.

Evidence of a neurophysiological nature also suggests that calcitonin acts differently to the opiates. Yamamoto, Tachikawa and Maeno (1981) studied the effects of morphine and calcitonin on the production of evoked potentials by painful stimuli. These potentials, measured in the sensory cortex, were evoked by (i) stimulation of the tooth pulp, or (ii) the sciatic nerve. Potentials evoked by the former consisted of 2 components and the potentials evoked by the latter consisted of 3 components. Morphine attenuated the second component in the potentials evoked by the tooth pulp stimulation and the third component produced by sciatic stimulation. These results were considered to bear a close relationship to potentials evoked by pain sensation.

Calcitonin, by i.c.v. injection alone, also attenuated these potentials in a similar manner. However, in contrast to morphine, the effect of calcitonin was not antagonised by naloxone. There was no effect of calcitonin after peripheral administration. These results indicated to the authors that calcitonin can inhibit specific antinociceptive pathways in a similar manner to morphine, i.e. (i) tooth pulp - spinal tractus nucleus of the trigeminal nerve - posterior group of the nucleus of the thalamus - sensory cortex, and (ii) sciatic nerve - spinal chord - ventral postero-lateral nucleus - sensory cortex. The authors concluded that, although calcitonin may act on the same pathways as the opiates, because the effects on the evoked potentials were similar, the receptors involved were different.

Fabri, Santoro, Moretti, Cappa, Fraioli, Di Julio, Galuzzi and La Manna (1981) measured the concentration of calcitonin and B endorphin in the plasma and CSF of human patients after peripheral injection of calcitonin. The hypothesis was that the antinociceptive effect of calcitonin on bone pain in Paget's disease might be related to stimulation of the release of B endorphin. There was no change in the concentration of B endorphin in blood plasma or CSF, although calcitonin levels rose in both the plasma and CSF.

Fraioli, Fabri, Gresssi, Moretti, Santoro and Felici (1982) have studied the effect of calcitonin (2 - 3 i.u. kg^{-1}) injected into the subarachnoid space, in patients

suffering from chronic intractable pain, arising from terminal cancer. Analgesia was assessed subjectively and within 5 minutes of injection all patients reported an alleviation of the pain. Complete absence of pain was obtained for at least 48 hours thereafter. (Injections of placebo were ineffective.) However, Shaw (1982) has published a letter which warns of potential dangers of this technique, since he found severe toxic side effects and some fatalities after administration of the hormone into the subarachnoid space in dogs and baboons. Fraioli et al (1982) have published a reply to this, in another letter, stating that they observed no side effects and also that they used a pure calcitonin preparation. The implication was that the toxic effects reported by Shaw (1982) may have arisen from use of the commercially available calcitonin, 'Calcimar', which contains a preservative in a gelatin diluent. The question of toxicity after i.c.v. injection of calcitonin has still not been resolved, although no such effects have been seen by this author when the pure salmon calcitonin was injected.

1.3.11.2 INHIBITION OF FOOD INTAKE

Calcitonin has been shown to inhibit the intake of food after either central or peripheral administration. Freed, Perlow and Wyatt (1979) have shown that when calcitonin (0.2 - 1 i.u. kg^{-1}) was administered by i.c.v. injection to rats, there was an inhibition of feeding. This effect was also seen after s.c. injection although the doses required were

25 - 50 fold greater than those required for i.c.v. injection. The effect obtained after s.c. injection may represent a small proportion of the hormone crossing the blood brain barrier, and acting on the brain. Also, since the dose required to exert an effect after s.c. injection was so large, then it was considered that the post prandial rise in the plasma calcitonin concentration would exert a central anorectic effect. These authors have also found that the full integrity of the molecule was needed for full anorectic activity, since the i.c.v. injection of tryptic digests of the hormone were inactive. It was suggested by these workers that calcitonin acted as a long term regulator of feeding since the maximum effect of the hormone was seen only when it was administered several hours prior to feeding. Another interpretation could be that calcitonin released prior to the onset of feeding, may be central in origin. A portion of this calcitonin may then enter the circulation, which would explain the anticipatory rise in plasma calcitonin concentration observed by other authors. Finally, Freed et al (1979) have shown that the dose of calcitonin required to exert a central anorectic effect was much smaller than the doses of other anorectic peptides such as ACTH, angiotensin II and enkephalin.

Cooper, Obie, Margules, Flynn and Walker (1980) have confirmed the work of Freed et al (1979) since they found that the i.c.v. injection of calcitonin (30 i.u. kg^{-1}) resulted in a 50% reduction in food consumption. In contrast, no effect was observed after s.c. injection,

although the concentration of plasma calcium was reduced. The i.c.v. injection of 1.4 i.u. kg^{-1} reduced the food consumption by 90%. However, only a 25% inhibition in food intake was achieved by the i.c.v. injection of the same dose of porcine calcitonin. The i.c.v. injection of human calcitonin was ineffective. The authors concluded that the post-prandial rise in calcitonin observed was not responsible for the anorectic effect since the mammalian forms of the hormone were relatively ineffective.

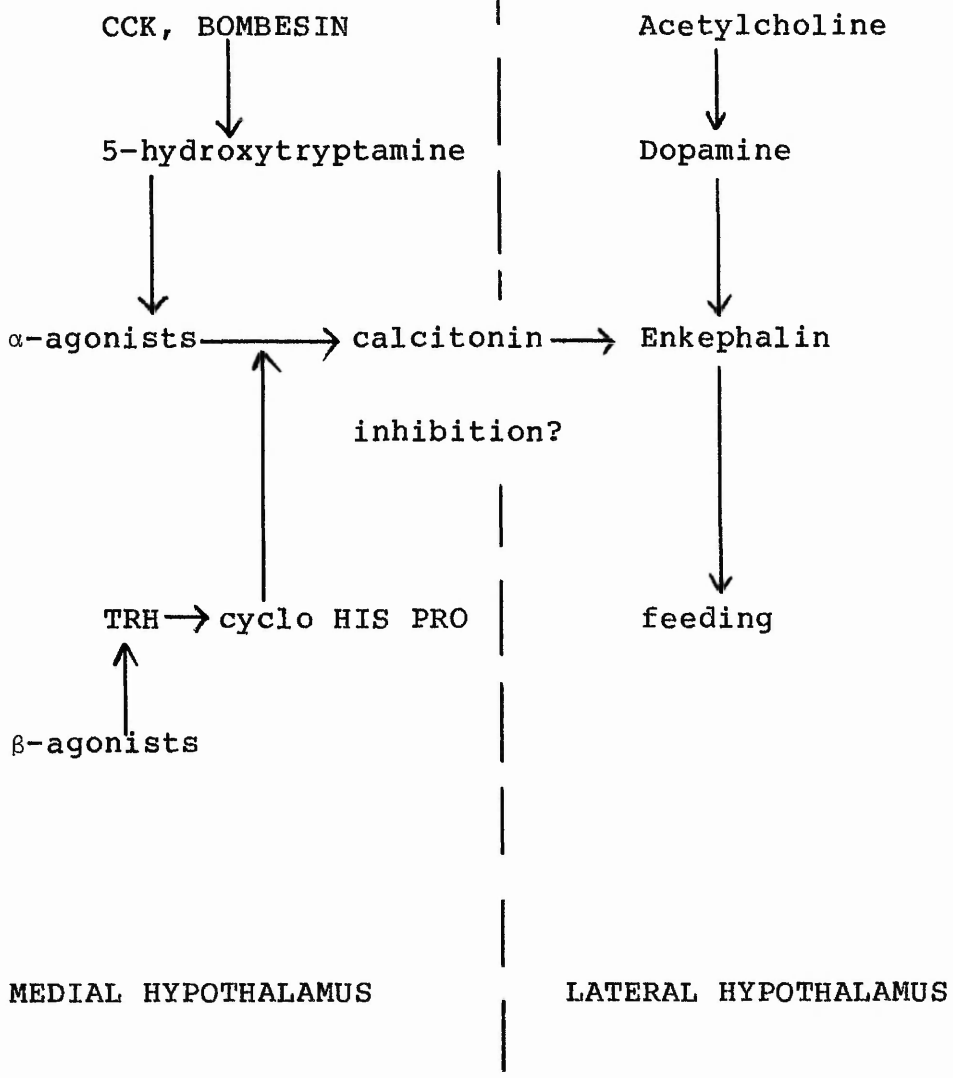
Morley and Levine (1981) have studied the anorectic effect of calcitonin in a number of feeding models. Calcitonin reduced stress-induced eating both by peripheral injection (1 - 40 i.u. kg^{-1}) and by i.c.v. injection (0.002 - 2 i.u. kg^{-1}). In agreement with other workers, the administration of the hormone by the central route was much more effective than that given peripherally. Since there was no disruption in general behaviour (e.g. sedation) it was unlikely that the hormone inhibited feeding by altering feeding behaviour. 2 i.u. kg^{-1} calcitonin by i.c.v. injection completely abolished feeding for 8 hours. There was no concomitant reduction in the plasma glucose concentration, which occurred with the use of other anorectic peptides such as bombesin and CCK - 8. The i.c.v. injection of calcitonin also reversed the feeding induced by the i.c.v. injection of calcium ions.

Calcitonin inhibited feeding induced by the injection of the GABA agonist, muscimol (Morley, Levine and Kneip, 1981). In this paper the authors proposed a model of

regulation of feeding in the hypothalamus in which calcitonin was designated an inhibitory role. A rise in the concentration of the hormone in the medial hypothalamus would inhibit the tonically active dopaminergic - enkaphaliergic feeding mechanism in the lateral hypothalamus (see Figure 4).

Calcitonin (as measured by CLI) is found in high concentrations in the hypothalamus (Becker et al, 1979). Miyahara and Oomura (1981) have shown that calcitonin applied directly to neurones in the lateral hypothalamus inhibits the firing rate of those neurones (64%) sensitive to glucose. Only 15% of those neurones not sensitive to glucose were inhibited by calcitonin.

Figure 4


CCK, BOMBESIN
Acetylcholine
5-hydroxytryptamine
Dopamine
α-agonists
calcitonin
Enkephalin
inhibition?
TRH
cyclo HIS PRO
feeding
β-agonists
MEDIAL HYPOTHALAMUS
LATERAL HYPOTHALAMUS

1.3.11.3 CONTROL OF PROLACTIN SECRETION

Calcitonin has been shown to exert an equivocal effect on the secretion of prolactin. However, the relationsip between calcitonin and prolactin is of some importance, because of their respective roles in pregnancy and lactation.

Iwasaki, Chihara, Iwasaki, Abe and Fujita (1979) have reported that administration of $ASN^{1,7}$ eel calcitonin (2.5 µg peptide per rat by i.c.v. injection) produced an increase in the rate of secretion of prolactin in rats. This effect was not antagonised by prior administration of naloxone and was not observed when calcitonin was added (10^{-8} -10^{-6} $mol.l^{-1}$) to pituitary cell cultures. No effect was seen, in vivo, when eel calcitonin was administered peripherally.

The opposite results were reported by Pecile et al (1981). In both rats and in human patients, it was shown that peripherally administered calcitonin reduced plasma prolactin concentration, for as long as 4 hours after administration. When calcitonin was given by i.c.v. injection to rats, a similar effect was observed. In addition, prolactin secretion induced by either morphine or by stress was blocked by the i.c.v. injection of calcitonin. This effect was unrelated to the hypocalcaemic effect of the hormone. Calcitonin injected by i.c.v. injection to rats with lesions between the CNS and the anterior pituitary gland did not affect the release of prolactin at low concentrations (25 ng per rat); there was, however, an effect at higher doses (250 ng per rat). The authors considered that the inhibitory effect of calcitonin was of

central origin, i.e. of hypothalamic origin. In addition, the inhibitory action of calcitonin was antagonised by the dopamine antagonist, haloperidol. Calcitonin was active at a concentration much lower (7 pmoles per rat) than any other known peptide neuromodulator of prolactin secretion including neurotensin, substance P, endorphin and TRH.

The role of calcitonin in lactation was further studied by this group (Olgiati, Netti, Guidobone and Pecile, 1981). Calcitonin significantly reduced the secretion of prolactin in lactating rats, but not in ovariectomised, oestrogen treated rats. The concentrations of calcitonin needed were greater than those required in stress or morphine-induced prolactin secretion. Prolactin stimulates kidney production of vitamin D metabolites and may exert an effect on the efflux of calcium from the bone. The group, therefore, proposed that calcitonin has an important neuroendocrine role in reducing prolactin release, and, therefore, protection of the skeleton.

1.3.11.4 INHIBITION OF GASTRIC ACID SECRETION

Morley and Levine (1981) have reported that calcitonin by i.c.v. injection is a potent inhibitor of gastric acid secretion measured in rats. This effect was 1000 fold more potent by central injection than by peripheral injection. The i.c.v. injection of the hormone inhibited the secretion of gastric acid induced by insulin and also by TRH. The authors considered this to be related to a central effect of the hormone and may be related to the role of the hormone in

satiety, by inhibiting post-prandial gastric acid secretion. No other studies have been reported on this effect.

1.3.11.5 CALCITONIN AND MANIC DEPRESSIVE ILLNESS

A reduction in the concentration of calcium in the CSF has been found to accompany an elevation in mood and the motor activity of depressed patients (Carmen and Wyatt, 1979). Similar changes also occur in acute psychotic agitation. Calcitonin has been used to treat psychoses with s.c. injection causing a reduction in the CSF calcium concentration (Carmen and Wyatt, 1979). There was also a decrease in the frequency of agitated episodes. The effect of the hormone on the calcium concentration of the CSF are in direct contrast to the findings of Stekolinikov et al, (1969), but were consistent with the alleviation of the symptoms.

Calcitonin also reduced the duration of hyperexia seen in the psychotic patients, either by altering muscle function or by a central effect on the temperature control centre in the hypothalamus. This effect has not been confirmed by other workers such as Strettle (1981) who could find no effect of the hormone on yeast-induced pyrexia in rats.

Calcitonin delayed the onset of sleep by a biphasic action: an initial reduction in the calcium ion concentration of the CSF (which may increase sleep latency) and a secondary homeostatic compensation to elevate the CSF calcium concentration back to greater than or normal levels. Over compensation would induce depression, which was

frequently seen in the morning after calcitonin administration.

1.3.11.6 POSSIBLE MECHANISMS OF ACTION OF CALCITONIN ON THE CNS

Since the relationship between calcitonin and the calcium ion concentration is both complex and intimate, it is reasonable to suggest that the fundamental mechanism of the action of the hormone in the brain is to modulate the calcium flux in the CNS. This has been discussed previously. A secondary effect of this would be an alteration in the concentration and turnover of neurotransmitters. However, to date there is very little work undertaken to show the effects of the hormone on brain neurochemistry. Carmen and Wyatt (1979) have reported that the concentration of the following monoamines in the CSF did not change as a result of administration of calcitonin (s.c.): dopamine, 3,4 dihydroxyphenylacetic acid; homovanillic acid; noradrenaline; vanilmandelic acid and 3-methoxy-4-hydroxyphenylglycol; tyramine; parahydroxyphenyl acetic acid; octapaine; parahydroxymandelic acid.

The work of Nakhla and Majumdar (1978) and later papers (detailed in the results section) report that peripherally injected calcitonin increased the activity of acetyl-cholinesterase in the cortex but not in the cerebellum. This was thought to be due to a mobilisation of tryptophan from the blood to the brain where it was converted to 5 hydroxytryptamine. This substance mediated the increase in

acetylcholinesterase activity. This interesting hypothesis has been studied in this project and will be discussed in detail later.

1.3.11.7 THE STATUS OF CALCITONIN AS A NEUROTRANSMITTER

There is at present much interest in the role of peptides as neurotransmitters in the CNS. There are a large number of such substances found within the brain, although not all have been shown to possess physiological function. The potential role of calcitonin or CLI as a neurotransmitter has been speculated upon by a number of authors (Fritsch et al, 1978; Fischer et al, 1980). However, in order to substantiate this there are a number of recognised criteria which must be satisfied. These were originally formulated for the 'classical' neurotransmitters, such as acetylcholine or noradrenaline. The biosynthesis of peptides and their secretion and disposal are thought to be more complex than those of the more traditional neurotransmitters (Hokfelt, Johansson, Ljundahl, Lundberg and Schultzberg,1980). Neuropeptides are frequently derived from large precursor molecules, which may be differentially processed at various sites to produce whole families of related peptides of quite different functions. The brain peptides have discrete regional distributions and are often synthesised in the same neurones as the 'classical' neurotransmitters. The release of neuropeptides is probably similar to that of other neurotransmitters, i.e. by a depolarising stimuli, which is dependent upon the entry of calcium. Peptides facilitate or

inhibit the actions of neurotransmitters at synapses and vice versa. Multiple subclasses or receptors exist for the peptides. The disposal of neuropeptides released from central or peripheral terminals may involve a variety of mechanisms including degradation by peptidases, reuptake and diffusion.

The above considerations may apply to calcitonin: it is derived possibly from a large precursor, it has a discrete regional distribution and may have subclasses of receptor sites. Finally, there is probably peptidase degradation. However, there is no evidence to date to indicate its existence in the neurone with other neurotransmitters and no evidence to indicate that it is released by depolarising stimuli.

Criteria used to assess neurotransmitter function will be described in relation to calcitonin below:

(i) the transmitter substance must be identified as being present in the terminal of the nerve. Calcitonin has been shown to be present in neuronal terminals in the frog (Yui et al, 1981) and rat brain (Van Houten et al, 1981);

(ii) the substances needed to synthesise the transmitter in sufficient quantity should be present in the neurone. There is no evidence to suggest this for calcitonin;

(iii) when the presynaptic nerve ending is depolarised, the substance should be released in a quantity adequate to produce a response. There are, to date, no studies to indicate this with respect to calcitonin;

(iv) when the substance is applied directly to the post-

synaptic membrane it should produce the same effect as the substance released from the presynaptic terminal by depolarisation. Calcitonin has been shown, in one study, to inhibit neuronal activity when applied iontophoreticaly (Murayama et al, 1981);

(v) the post-synaptic actions of the applied chemical must be identical to those of the endogenous chemical. Similar considerations as those above apply here as well;

(vi) an adequate system for the disposal or removal of the chemical from the post-synaptic site must exist. Calcitonin has been shown to remain bound to brain membranes for as long as 6 hours (Fischer et al, 1981), which is a long time for a neurotransmitter;

(vii) pharmacological agents that interfere with the action of the transmitter should interact with the putative transmitter substance when applied to the site of action. There are, to date, no specific calcitonin antagonists, a serious drawback in the study of the pharmacology of the hormone.

It should be noted that few, if any, neuropeptides satisfy all of these criteria. Peptides are thought to act more as modulatory agents of neuronal function. The proposed actions of the hormone to modulate calcium flux within the CNS would place it in a prominent position to influence neuronal function.

1.4 AIMS OF THE PROJECT

The previous sections have illustrated the relationship between calcitonin and the CNS. The major aim of this project is to characterise the antinociceptive actions of calcitonin. Calcitonin, as described, has the ability to influence the calcium flux of cells in the periphery and possibly in the CNS. In previous sections it was reviewed how the opiates produce their antinociceptive effects, by influencing calcium fluxes within the CNS. It is an aim of this project to investigate the relationship between calcium and calcitonin, with respect to the production of antinociception. This aim involves the use of calcium ions, the calcium chelator, EGTA, the calcium ionophore, A23187, and the dihydropyridine calcium slow channel blocking agents, nifedipine and PY 108068, in conjunction with calcitonin in order to study the antinociceptive response. In addition, the action of calcitonin wll be compared and contrasted with the opiates on the total calcium concentration of the brain and the uptake of calcium by brain slices.

In the introduction it was described how the action of calcitonin on the CNS may be independent of an action on the opiate system. An aim of this thesis is to explore this aspect, firstly by studying the effect of the opiate antagonist, naloxone, on calcitonin-induced antinociception, and secondly, applying the hormone to two systems, in which activation of the opiate receptor does not lead to antinociception, i.e. the contraction of the rat

colon, and the changes in blood pressure of either the normotensive or haemorrhaged rat.

Finally, the hypothesis by Nakhla and Majumdar (1978) that calcitonin increases cortical acetylcholinesterase activity has been studied. The aim, to duplicate and extend these studies, is included since it is a mechanism by which calcitonin may exert a profound effect on the CNS, and which may include antinociception.

METHODS

2.1 MATERIALS

Animals

The animals used in this project were:

a) male and female, CLFP mice (Hacking and Churchill Ltd), weight range 20 to 30 g.

b) male and female, Sprague Dawley rats (Hacking and Churchill Ltd), weight range 200 to 300 g.

All animals were maintained at 23°C with hours of darkness between 1800 to 0600 hours. They were allowed access to food and water ad libitum. The diet consisted of 'modified rat/mouse diet cubes, ration no. 42' (Heygate and Sons Ltd).

Physiological Solutions

(i) Krebs Bicarbonate Saline

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

Gassed with 5% CO_2 in oxygen. pH 7.4 (at 37°C).

(ii) HEPES Phosphate Saline

NaCl	118	$mmol.l^{-1}$
KCl	5.9	
$MgCl_2$	1.2	

$CaCl_2$	2.5
HEPES	11.7

Gassed with oxygen. pH 7.4 (at 37°C).

(iii) Phosphate Buffer

19 mls 0.2 $mol.l^{-1}$ monobasic sodium phosphate + 81 mls 0.2 $mol.l^{-1}$ dibasic sodium phosphate. Dilute to 200 mls. pH 7.0.

(iv) Salmon Calcitonin Vehicle

1 $mg.ml^{-1}$ bovine serum albumin (BSA)

0.01 $mol.l^{-1}$ acetic acid

0.154 $mmol.l^{-1}$ NaCl

pH 7.4.

(v) Vehicle for I.C.V. Injection

a) salmon calcitonin

0.100 $mol.l^{-1}$ NaCl

0.05 $mol.l^{-1}$ Tris

1 $mg.ml^{-1}$ BSA

pH 7.4

b) A23187 and calcium antagonists

0.200 $mol.l^{-1}$ NaCl

0.10 $mol.l^{-1}$ Tris

Diluted 1 in 2 with dimethyl sulphoxide

Adjusted to pH 7.4, with NaOH.

(vi) Aspirin Vehicle

1% gum acacia

0.154 $mol.l^{-1}$ NaCl

Adjusted to pH 7.4, with NaOH.

2.2 I.C.V. INJECTION TECHNIQUE

Injections were made into the cerebral ventricles of conscious mice by the method of Haley and McCormick (1957), using modifications suggested by Cashin and Heading (1968). The site of injection was 1.5 mm lateral to the bregma and 1.5 mm rostral to the coronal suture. An aglar syringe and micrometer head was fitted with a 21 gauge needle. The needle was sheathed to within 4 mm of the centre of the bevel to limit the penetration depth. The volume injected was 10 µl. To facilitate operation of the apparatus with one hand, the syringe was fitted in an X block fitted to a racking screw, which itself was fitted to a retort stand. Penetration was achieved by rotating the racking screw, which lowered the syringe. In practised hands the procedure was complete within 5 seconds and the animals, though dazed for about 2 minutes, were fully recovered within 5 minutes.

The site of injection was assessed by using indian ink (1 in 5 dilution in 0.154 $mol.l^{-1}$ NaCl). The brain was carefully removed and prepared for histological examination (see appendix). In 95% of the brains examined, ink was present throughout the ventricular system, and tissue damage was restricted to the needle tract.

2.3 ASSESSMENT OF ANTINOCICEPTION

The method used was that of Collier, Dineen, Johnson and Schneider (1968) and it has been called by these authors the abdominal constriction test. 0.3 mls of acetic acid, in 0.154 $mol.l^{-1}$ NaCl, was administered by i.p. injection. The response to this peritoneal irritant consists of a wave of muscle constriction passing caudally along the trunk of the animal, accompanied by the inward twisting of the hind limbs. Initially a range of acetic acid concentrations was used, in order to establish a concentration which gave a rate of three - four constrictions per minute; in further experiments, a concentration of 1% acetic acid was used.

Secondly, to choose a period when the response was at a maximum and was constant, the time course of the response to the i.p. injection of 0.3 mls 1% acetic acid was followed for a period of a hundred minutes. In further experiments, the number of abdominal constrictions was counted between the tenth to fourteenth minute inclusive, following the injection of acid.

The abdominal constrictions were counted, with the animals placed individually on boards, placed nine inches above the bench. These boards were washed between each experiment. The temperature of the room (usually between 19 and 21^{o}C) and the time at which the experiment was undertaken were recorded.

Substances to be screened for potential antinociceptive activity, and their appropriate vehicle, were injected five minutes prior to the injection of acetic acid. The only

exception to this scheme was leucine enkephalin, which was injected five minutes after the injection of acid. This scheme was adopted because of the lability of this substance.

The results are either expressed as absolute number of constrictions per minute, or as the percentage change (increase or decrease) in the frequency of abdominal constrictions per minute, from control values (i.e. the frequency of abdominal constrictions per minute of the vehicle injected animals).

In each series of experiments, a group of control animals were included, to allow for any potential day to day variation.

2.4 MEASUREMENT OF MOTOR DISCOORDINATION

The method used was adapted from that of Dunham and Miya (1957). Animals were placed on a slowly rotating sandpaper-covered drum (15 cm diameter, 4.7 $cm.min^{-1}$). The apparatus was placed three feet from the floor. Unaffected animals were able to maintain position at the top of the drum by continuously walking. The number of times each animal fell from the drum, over a two minute period, was recorded. The animals were immediately replaced back on the drum, once they had fallen.

2.5 MEASUREMENT OF INVESTIGATIVE BEHAVIOUR

Animals, immediately after measurement of motor coordination, were placed in the middle of a board containing sixteen equally spaced holes (File and Wardill, 1975) and the board was placed nine inches above the bench. The number of 'head dips' in to these holes were counted over a five minute period. A 'head dip' was defined as when the head entered the hole to a depth to the base of the ears. After the animal had been tested, the board was washed clean.

2.6 MEASUREMENT OF PLASMA CALCIUM CONCENTRATION

Blood samples were withdrawn from conscious animals (previous experiments had shown that the use of anaesthetics had effects on the plasma calcium concentration) by cardiac puncture. Generally, 1 ml of blood was withdrawn, using a 21 gauge needle, into a heparinised syringe, and transferred to polythene heparinised tubes. The samples were centrifuged at 1000 g for five minutes, and the plasma aspirated and placed in chilled plastic tubes (Eppendorf). The samples were either frozen or the plasma calcium concentration was immediately determined.

Plasma calcium concentration was measured using a Corning 940 Calcium Analyser, which, with an aliquot volume of 100 µl, gave a coefficient of variance of 1.1%. This was well wthin the recommended manufacturer's value of 1.5%. One problem arose with the use of this fluorometric titration procedure, in that it was found that the concentration of haemoglobin in haemolysed samples gave erroneouly low readings. This has also been shown by Griebel, Knoblock and Koch (1979) and consequently, any samples which had haemolysed were discarded. The values from the machine were read in $mmol.l^{-1}$ and each value determined was the mean of three analyses.

2.7 BRAIN DISSECTION PROCEDURE

The animals were killed, decapitated and the skull was opened by cutting along the saggital suture. The brain was removed by scooping out the tissue with a microspatula, after first severing the pituitary stalk and ol factory nerves. The tissue was immediately transferred to 10 mls ice cold NaCl (0.154 $mol.l^{-1}$). This washed the brain free from any fragments of bone and congealed blood. The brain was then removed from this solution and lightly blotted on ashless filter paper (Whatman). The tissue was then placed in an ice cold glass dish. Five specified regions were dissected: cerebellum, brain stem, cortex, hypothalamus and midbrain. The brain stem corresponds to the medulla oblongata and pons; the midbrain corresponds to the striatum, thalamus and the subthalamus; the cortex corresponds to the telencephalon, without the striatum and includes the white and grey matter of the cerebral cortex.

The dissection was performed after the method of Glowinski and Iversen (1966). The rhombencephalon was separated from the rest of the brain by a transverse cut, and dissected into the cerebellum and the brain stem. A transverse cut at the optic chiasma, passing through the anterior chiasma, was made which delimited the anterior portion of the hypothalamus. The remainder of the hypothalamus was dissected by taking the anterior commisure as a horizontal reference point and the line between the mammilliary bodies and posterior hypothalamus as the caudal limit. The remainder of the cerebral hemispheres were

peeled away from the striatum and corpus callosum, and combined with the frontal cortex removed earlier. This forms the cortex complex. The remaining tissue was denoted as the midbrain. The reproducibility of the dissection may be judged by the statistical distribution of the weights of the regions are isolated (see Table 2).

Table 2

Weights of Mouse Brain Regions

Region	Weight (mg)	% Error
Cerebellum	62.4 ± 1.45	2
Brain Stem	63.9 ± 4.64	7
Hypothalamus	10.2 ± 0.54	5
Cortex	107.5 ± 4.71	4
Mid Brain	177.0 ± 7.1	4

Table 2 Analysis of mouse brain regions dissected according to the procedure outlined in methods. Values are mean ± s.e.m.; n = 40 - 60.

2.8 MEASUREMENT OF TOTAL BRAIN CALCIUM CONCENTRATION

Preliminary Experiments

There are a number of methods available in the literature (Ross, Medina and Cardenas, 1974; Ames and Nesbitt, 1958; McDonald, 1978) to measure total tissue calcium concentration. These include wet ashing, dry ashing and solubilisation. Wet ashing is a procedure where the tissue is heated to boiling in concentrated inorganic acids until the residue of inorganic salts remains. Preliminary experiments using this technique resulted in contamination from the glazed crucibles used, in addition to difficulties in redissolving the residue for analysis. Dry ashing, a procedure where the sample is heated to a white ash in a muffle - furnace, was also tested and found to be unsatisfactory because of problems of spluttering of the sample as well as contamination problems. The third method, that of solubilisation, was also tried using concentrated NaOH and heating to 70^{o}C. This procedure has the advantage that the protein concentration can be determined on the same sample and the potential hazards of concentrated boiling inorganic acids are avoided. The disadvantage is that the amount of tissue which can be used with this technique is small, with a possible loss in accuracy in the calcium analysis.

To avoid the problems outlined above the method used was that of Lolley (1963), in which trichloroacetic acid (TCA) was used to extract the calcium from the tissue.

The brains of freshly killed mice were removed and

dissected as previously described. Tissue samples were transferred to preweighed plastic tubes (Eppendorf), and weighed. The calcium concentration could thus be expressed in terms of mg. wet weight of tissue ($mg.w.w^{-1}$). 500 µl of 10% TCA (w/v) was added to the samples of all regions except the hypothalamus to which 250 µl of TCA was added. The tubes were then weighed again, in order to calculate the volume within which the tissue sample was homogenised. The tubes were then left for five hours, centrifuged for ten minutes at 1000 g, 100 µl aliquots of the supernatants were then used to determine the calcium concentration.

Calcium analysis was undertaken using a Corning 940 Analyser. The procedure was similar to that described for the analysis of the plasma samples. The various experimental details used to assess the accuracy of the technique are detailed in the Results section.

2.9 MEASUREMENT OF RADIOLABELLED ^{45}CALCIUM UPTAKE BY BRAIN SLICES

Mouse brains were removed, as detailed previously, and sliced longitudinally, using a McIlwain tissue chopper. The slices were 0.4 mm thick. The slices were placed in 10 mls ice cold HEPES buffer solution and vortexed for ten seconds to separate the slices. Individual slices were then transferred to 2.5 mls of warm (37^{o}C), gassed (100% oxygen) Hepes buffer solution and preincubated for fifteen minutes in the presence of drug or vehicle. 2.5 mls of warm (37^{o}C), gassed (100% oxygen) buffer containing approximately 0.25 $\mu Ci.ml^{-1}$ $^{45}Ca^{2+}$ was then added for six minutes. In experiments where potassium stimulated uptake was measured, 2.5 mls of solution containing the labelled calcium was added such that the final potassium concentration was 56 $mmol.l^{-1}$.

Following incubation in the labelled solution, the slices were rapidly strained over a nylon mesh, washed with ice cold buffer and placed in 5 mls ice cold buffer for fifteen minutes. The slices were then blotted after straining, and weighed in vials. 0.5 mls of tissue solubiliser (Soluene) was added and the vials were left for 24 hours at 21^{o}C, or three hours at 70^{o}C. Once the slices had dissolved, 0.5 mls 4 N HCl and 10 mls of scintillant were also added. Vials were counted using standard conditions for one minute each. Six vials, containing 5 µl of labelled incubating solution were also counted to act as 'spiked' samples. (This enabled cpm to be converted to dpm.)

Apparent calcium uptake was calculated as follows:

$$\text{APPARENT CALCIUM UPTAKE} = \frac{\text{DPM } ^{45}\text{CALCIUM g.w.w}^{-1}.\text{CALCIUM CONC ml}^{-1}\ \text{g.w.w}^{-1}\ \text{MEDIA}}{\text{DPM } ^{45}\text{CALCIUM ML}^{-1}\ \text{MEDIA}}$$

2.10 SUPERFUSION OF THE RAT COLON

Male and female rats were stunned and the middle third of the colon was removed, washed and suspended under 1g tension in the superfusion apparatus. The superfusion fluid was warmed ($37^{o}C$), gassed krebs (5% CO_2/O_2) and was passed over the tissue at a rate of 4 $ml.min^{-1}$. Agonists or antagonists were either injected into the superfusion fluid, via a rubber manifold, or were placed in the fluid reservoir for continuous administration. The tissue was allowed at least 15 minutes to equilibrate before use, and each piece of tissue was tested initially with 10^{-4} $mol.l^{-1}$ acetylcholine. This technique has been described by Boura and Olley (1981).

2.11 MEASUREMENT OF BLOOD PRESSURE IN NORMOTENSIVE AND HAEMORRHAGED RATS

Rats were anaesthetised by urethane (1.4 $g.kg^{-1}$), with 50% of the dose administered s.c. and the remainder i.p. The jugular vein and carotid artery were then cannulated. In animals which exhibited respiratory distress, the trachea was also cannulated

Substances to be administered were either given by i.v. injection or by i.p. injection. Blood pressure was recorded using a Washington P400 transducer, connected to the carotid artery cannula. After surgery, the animals were allowed 20 minutes to gain a stable blood pressure.

In the normotensive animals vehicle, leucine enkephalin or calcitonin were administered on a latin square basis. (Animals pretreated with naloxone (10 $mg.kg^{-1}$) were administered with this agent 30 minutes, i.p., prior to testing with leucine enkephalin or vehicle.)

In experiments usng haemorrhaged animals, the procedure was as follows: the animals were haemorrhaged by withdrawing arterial blood until the blood pressure had declined by 20%. A period of 20 minutes was then allowed to enable the animals to stabilise. At the end of this period drugs or vehicle were then injected i.v. and the change in mean arterial pressure was monitored over the following 60 minutes.

2.12 MEASUREMENT OF BRAIN AECTYLCHOLINESTERASE ACTIVITY

Mouse brains were removed as described previously and dissected into three regions: the cortex, cerebellum and the remainder. Each region was homogenised for thirty seconds in 10 mls of 0.1 $mol.l^{-1}$ phosphate buffer. One ml of this solution was then analysed for activity of acetylcholinesterase (Achase) activity, by the method of Ellman, Courtney, Andres and Featherstone (1961). The activity is expressed per mg protein, estimated using the method of Lowry, Rosenbrough, Farr and Randall (1951).

The reaction mixture contains 2.5 $mmol.l^{-1}$ acetylthiocholine as the enzyme substrate and dithionitro benzene (DTNB) as the indicator. This mixture produces the yellow anion, even in the absence of Achase, and a blank run was always undertaken with phosphate buffer in place of the tissue homogenate. These readings were subtracted from the subsequent tissue readings. 0.5 ml of brain homogenate solution was added to 0.5 ml of reaction mixture, and the change in absorbance, read at 612 nm, was measured over a period of one minute. A chart recorder was connected to the absorption spectrophotometer and calibrated to give the changes in enzyme activity in $\mu moles.ml^{-1}$ homogenate.

The protein concentration was determined using three solutions. Solution A (containing 2% sodium carbonate in 0.1 N NaOH); solution B (containing 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate) and solution C (which was a 1 in 50 dilution of solution B by solution A). One ml of brain homogenate was added to 5 mls of Folin - Ciocalteau solution (diluted 1

in 2 with phosphate buffer) was then added, mixed and left for at least forty-five minutes for the colour to develop. Samples were read at 750 nm against a and a blank range of standard protein solutions (using BSA) was also prepared, to act as a standard curve.

Enzyme activity was then expressed as:

$\mu moles.hour^{-1}.mg\ protein^{-1}$.

RESULTS

3.1 I.C.V. INJECTION

The ability to accurately place substances in the cerebral ventricles is important, since this route of administration is extensively used in this project.

The technique was assessed for its accuracy by the injection of ink and subsequent histological examination. The results of injection of ink have already been discussed in the Methods section. This section describes the effects of injecting drugs into the ventricular system. These agents were injected in order to duplicate the method of Haley and McCormick (1957).

Results

(i) Acetylcholine

Immediately following injection, all animals became hyper-excitable and were sensitive to touch or sound. The animals became depressed and remained hunched five minutes after injection. They remained in this condition for as long as thirty minutes after injection. In some cases turning behaviour was observed, with the animals turning ipsilaterally to the site of injection. The injections of higher concentrations of acetylcholine (5 µg) resulted in more severe effects similar to those described above.

(ii) Noradrenaline

Initially after injection of noradrenaine (10 µg) the animals became depressed. The ears were flattened against the head, the animals were stationary and some exhibited symptoms of tachyapnea. The 'Straube-Tail' reaction was observed in one animal.

(iii) 5-hydroxytryptamine

Immediately after injection of 50 µg the animals went into a series of seizures, followed by tremor and tachyapnea. About five minutes after the injection the animals were fully recovered.

(iv) Atropine

The i.c.v. injection of atropine (20 µg) resulted in tonic seizures, extreme sensitivity to sound and touch. Thirty minutes after injection fatalities had occurred in 4 out of 5 animals tested.

Discussion

The results obtained after the i.c.v. injection of the drugs are similar to those obtained by other workers (Haley and McCormick, 1957; Feldberg and Sherwood, 1953). (It should be noted that the effects described above may also be obtained even if the drugs were not placed in the ventricles). The evidence, however, taken together with the histological examination, suggests that the technique is reproducible and may be confidently used to assess the effects of drugs on the CNS administered by i.c.v. injection.

It is considered that the advantages of the technique, i.e. rapidity of screening large numbers of animals and the absence of any side effects from anaesthetics, outweigh the disadvantage of the lesser precision of the technique when compared to the i.c.v. injection techniques using precise stereotaxic coordinates. The technique has been used extensively by authors studying the central actions of a range of potential antinociceptive peptides (e.g. Chipkin, Morris, English, Rosamund and Stewart, 1981).

3.2 ASSESSMENT OF ANTINOCICEPTION

General Considerations

In man, the perception of pain is strongly influenced by a psychic component, i.e. the mental state of the patient strongly influences the nature and degree of pain perceived. Drugs intended for use as analgesic agents are screened in animal models. This poses problems, since the psychic components of pain are difficult to build into an animal model. Therefore, such a model only measures the antinociceptive properties of the substance under test. Collier (1964) has stated that the term pain cannot be used in spinal animals and, 'even in intact animals, this term has an affective connotation of unknown validity'. A more appropriate term is nociception; nociception relates to a response which is dependent on sensory endings ('nociceptors'), and motor, central and sensory neurones and muscles (Collier, 1964). Any agent which raises the minimal stimulus needed to elicit a nociceptive response in animals is denoted antinociceptive in its action.

This project is a study into the mechanism by which calcitonin exerts an antinociceptive effect and this term will be used throughout, in preference to the term analgesia.

All routine antinociceptive tests in animals rely on a degree of motor activity to demonstrate that noxious stimulus has exerted an effect. Therefore, the absence of motor response cannot be taken as evidence of an antinociceptive action, unless the possibility of motor

discoordination is excluded (Collier, 1964). It is for this reason that antinociceptive testing is frequently accompanied by tests of motor coordination.

Choice of antinociceptive test

Calcitonin, when used therapeutically in Paget's disease, dramatically alleviates pain arising from the joints of periosteum (Martin and Woodhouse, 1976). This pain may be classified as deep pain, in contrast to the less severe pain arising from superficial tissues. In addition to the analgesic properties of calcitonin in human patients, it is also active in a number of animal antinociceptive tests. The hormone is, therefore, both analgesic and antinociceptive in its action. The test to be used, therefore, must be a procedure in which deep nociceptors are primarily activated.

A second consideration is that the test must be sensitive to both classes of analgesic agents, i.e. the narcotic and the antipyrexic classes. Thirdly, the test must be quantitative in nature, to allow comparison between various treatments to be rigorously determined. Finally, to enable valid statistical comparison to be made, the test must allow the rapid precise screening of large numbers of animals.

The test which satisfies these criteria best is the abdominal constriction test (Collier, Dineen, Johnson and Schneider, 1966).

Following the i.p. injection of a noxious agent, the rat or mouse exhibits a response denoted as the abdominal constriction response. (The features of this response have

been described in the Method.) The precise nature of the response has yet to be defined. The i.p. injection of substances as diverse as acetylcholine, acetic acid and phenylquinone can elicit the response (Collier et al, 1968). The latency of the onset of the response, when acetylcholine, bradykinin and 4% w/v sodium chloride are injected, is short probably because these substaces act directly on the nociceptors. In contrast, the latency period prior to the onset of the response is long, when acetic acid or phenylquinone are injected. This fact provides a latency period to allow complete recovery from an i.c.v. injection prior to antinociceptive testing.

This test has shown that most substances known to be analgesic in man, have proved effective and show identical orders of potency. In this respect, this test is more predictive of analgesic potency of substances than other antinociceptive tests, such as the tail - clip test, for example (Collier, 1964).

There are two main disadvantages to the use of this test. Firstly, increased capilliary permeability which accompanies peritoneal irritation, means an antinociceptive effect may not easily be distinguished from an anti-inflammatory response. This problem is important when investigating calcitonin, which also possesses potent anti-inflammatory actions (Strettle, Bates and Buckley, 1980). Secondly, the test has been shown to produce 'false positives', i.e. substances which are not antinociceptive in any other tests, but which inhibit abdominal constrictor

activity (Collier et al, 1968). These problems are partly overcome by assessing the actions of putative antinociceptive agents in a range of tests, and partly by running in parallel, tests for motor coordination and investigative activity. Calcitonin has been shown to be active in a range of antinociceptive tests, includng tooth - pulp stimulation (Pecile et al, 1978), tail - pinch test (Yamamoto et al, 1978) and the abdominal constriction test (Yamamoto et al, 1978).

This section, in view of the above discussion, is devoted to the characterisation of the abdominal constriction test, with a view to establishing conditions for use in testing calcitonin for antinociceptive activity.

Results

The i.p. injection of acetic acid (0.1% to 5%; w/v) in a vehicle (0.3 mls) produced a dose dependent increase in the frequency of abdominal constrictions (Figure 5). The injection of 10% acetic acid proved lethal to the animals and is not included in the dose response curve.

The time course of the response to 1%* showed that the abdominal constrictions began five minutes after injection, and reached a plateau between ten and twenty-five minutes following injection. The response had almost ceased by a hundred minutes (Figure 6).

The administration of substances by i.c.v. injection reqired the use of either a tris - saline vehicle (as in the case of calcitonin, calcium ions, magnesium ions and EGTA) or a dimethylsulphoxide vehicle (for use with A23187,

*acetic acid

nifedipine or PY 108068). The comparison between animals given an i.c.v. injection of either vehicle, showed no significant difference between the two (see Table 3).

The experiments using this technique were undertaken over a period of one year, and also at various times of the day. The sensitivity to pain has been shown to follow both a circadian and circannual rhythm (Frederickson, Burgis and Burgis, 1977). No such rhythms can be observed in the sensitivity to peritoneal irritation by acetic acid (see Figures 7,8).

The sensitivity, and use of the technique, was assessed by the use of two positive control substances, aspirin (an antipyretic analgesic) and leucine enkephalin (a narcotic analgesic). In both cases, the test was sensitive to the antinociceptive properties of these agents (aspirin EC50 was 1.8×10^{-4} mol.kg^{-1}, Figure 9; leucine enkephalin EC50 was 3×10^{-6} mol.kg^{-1}, Figure 10).

Discussion

These results show that the abdominal constriction response may be elicited by acetic acid, and that for future experiments the i.p. injection of 0.3 mls of 1% acetic acid was used as the standard noxious challenge. This produces 3 to 4 constrictions minute^{-1}, a figure which allows a wide range of constrictions for either antinociceptive or hyperalgesic responses.

The time course of the response shows that the use of the period between the 10th and 14th minute after injection of acetic acid is on the plateau of the response.

Consequently, this was the period chosen for further antinociceptive testing procedures.

The lack of rhythm in the sensitivity to peritoneal irritation implies that antinociceptive testing is not subject to possible errors occurring as a result of the time of day or year.

Finally, the EC50 values for aspirin or leucine enkephalin illustrate that the test is responsive to both peripherally or centrally acting analgesic agents. The EC50 value for aspirin was similar to that reported by Collier et al (1964) as was the EC50 value for leucine enkephalin. Chapman, Hu and Way (1980) have reported an EC50 value of 2 x 10^{-6} $mol.kg^{-1}$ for leucine enkephalin.

Table 3

Number of Abdominal Constrictions per minute in animals injected with different vehicles.

Vehicle	Frequency of Abdominal Constrictions (min^{-1})
Tris - saline	3.42 ± 0.21
Dimethylsulphoxide - tris - saline	3.24 ± 0.19

Table 3 Control number of abdominal constrictions in animals treated with 2 different vehicles. Values are mean ± s.e.m.; n = 50-100.

vehicle was injected i.c.v., and the noxious stimulus was 0.3 ml 1% acetic acid injected i.p.

Figure 5 Effect of acetic acid on the frequency of abdominal constrictions, expressed as a percentage of maximal response. Values are mean ± s.e.m.; n = 6-10 *$p < 0.05$.

The maximal response was the number of abdominal constrictions obtained with 2% acetic acid.

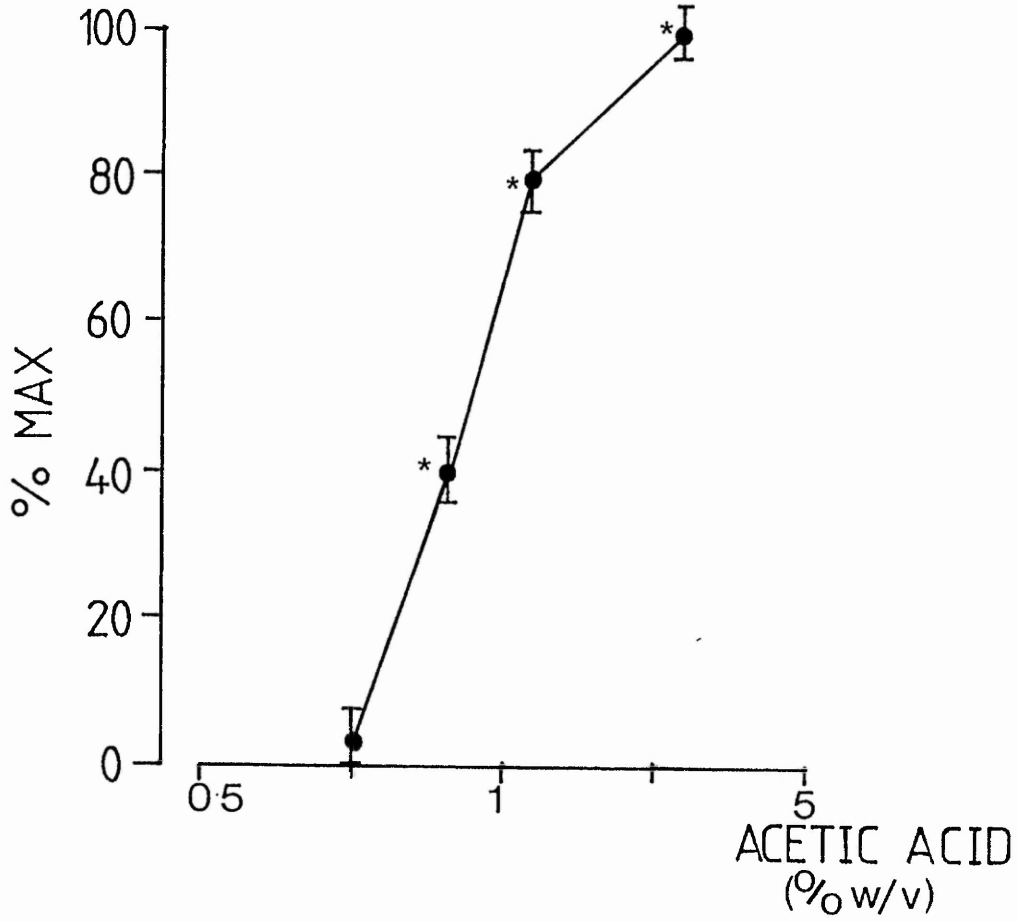


Figure 5

Figure 6 Time course of abdominal constriction response to acetic acid (1%). Values are mean ± s.e.m.; n = 10.

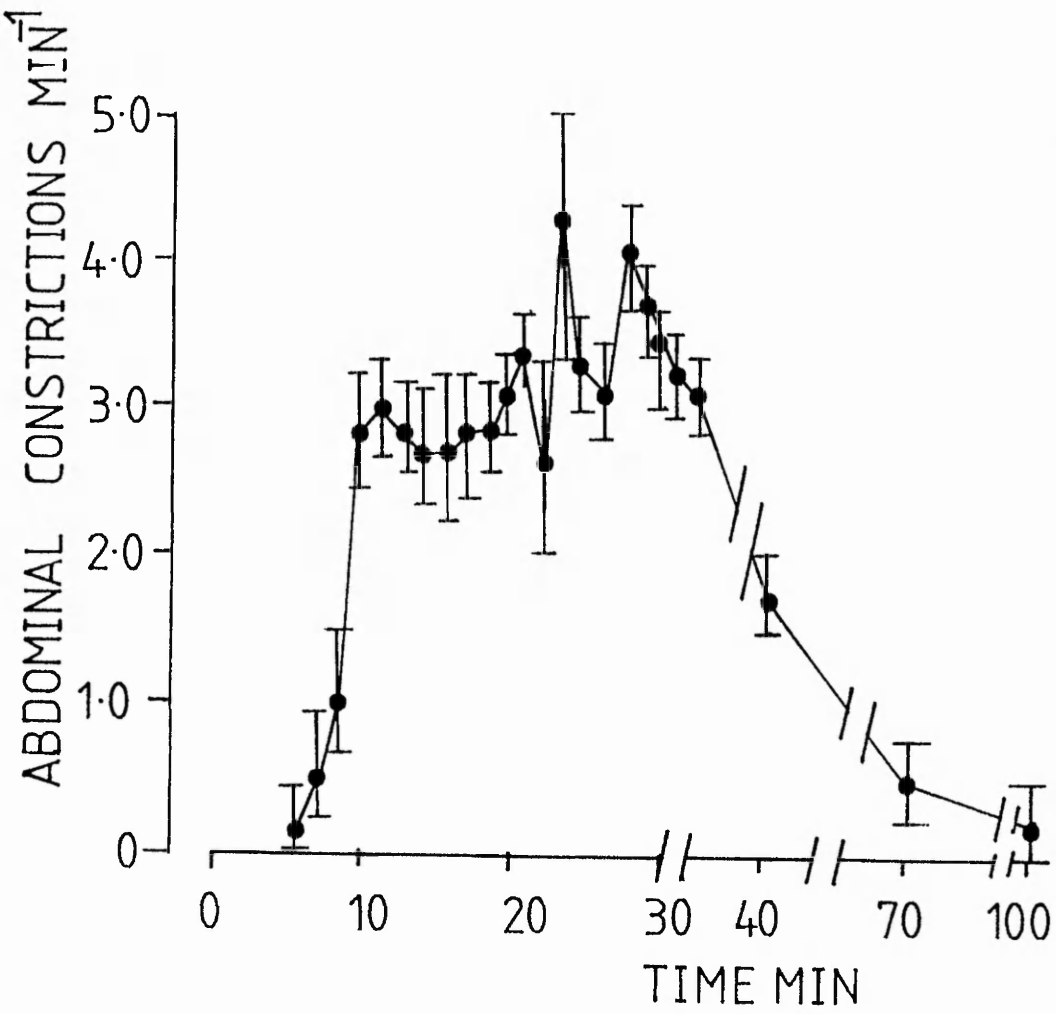

ABDOMINAL CONSTRICTIONS MIN⁻¹
5·0
4·0
3·0
2·0
1·0
0
0
10
20
30
40
70
100
TIME MIN

Figure 6

Figure 7 Abdominal constriction response in animals injected with vehicle as a function of time of day. Values are mean ± s.e.m.; n = 6-30.

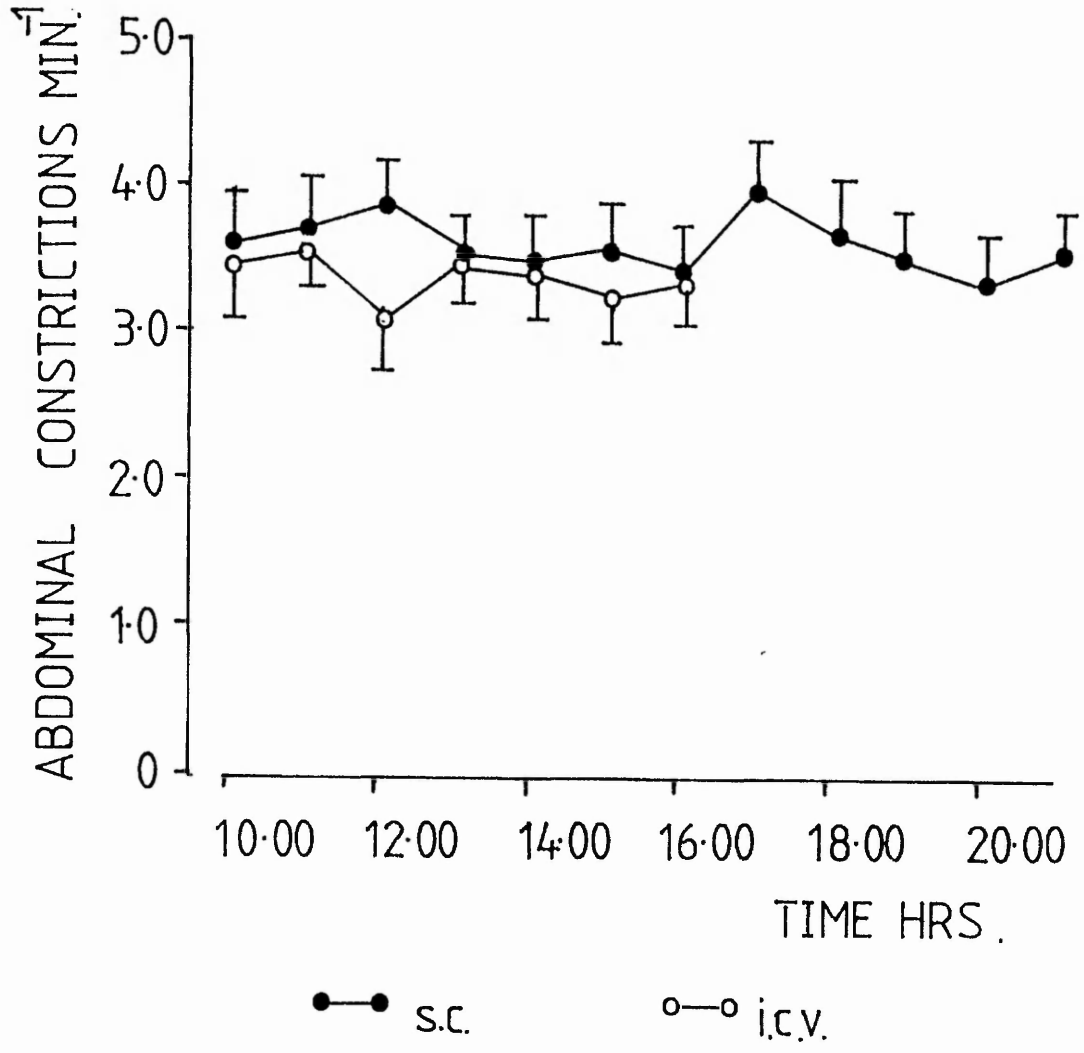

ABDOMINAL CONSTRICTIONS MIN.⁻¹
5·0
4·0
3·0
2·0
1·0
0
10·00
12·00
14·00
16·00
18·00
20·00
TIME HRS.
s.c.
i.c.v.

Figure 7

Figure 8 The abdominal constriction response in animals injected with vehicle, plotted as a function of time of year. Values are mean ± s.e.m.; n = 10-20.

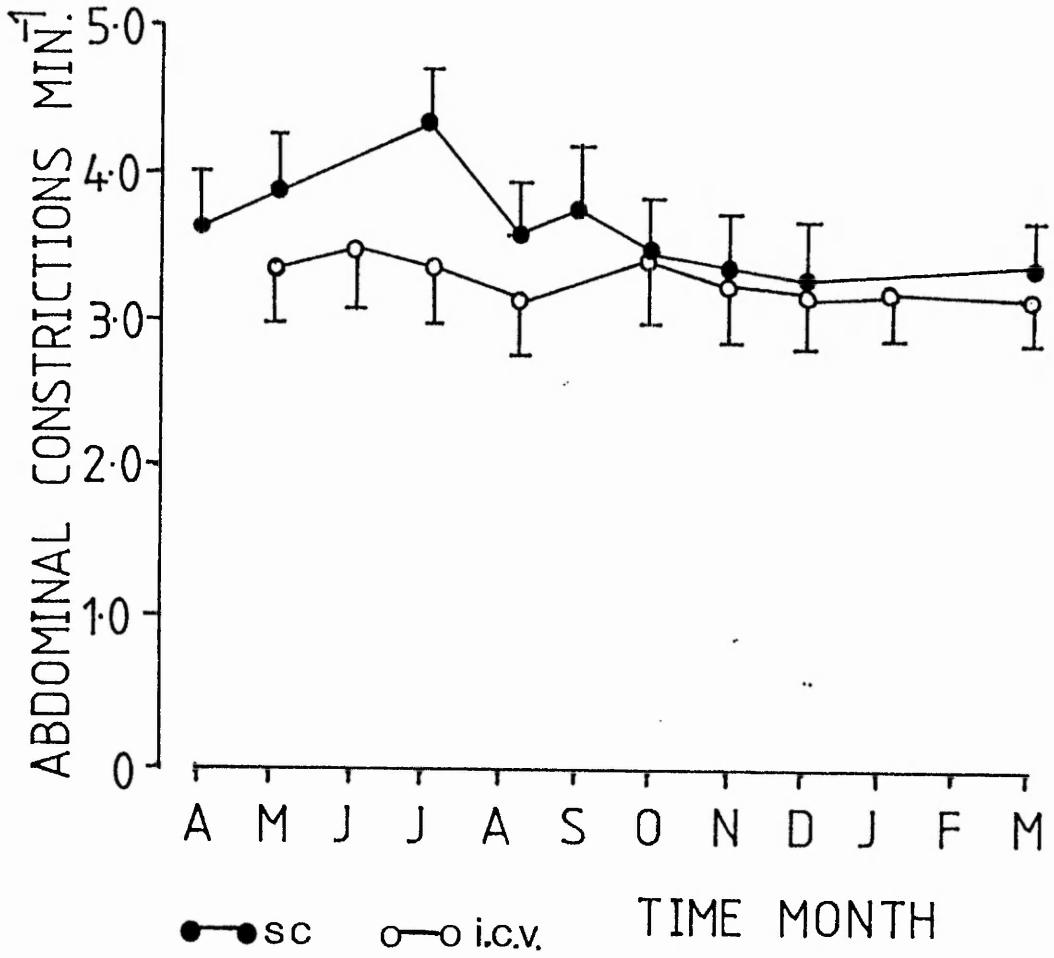

ABDOMINAL CONSTRICTIONS MIN.⁻¹
5·0
4·0
3·0
2·0
1·0
0
A M J J A S O N D J F M
TIME MONTH
sc
i.c.v.

Figure 8

Figure 9 Effect of aspirin on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *p < 0.05.

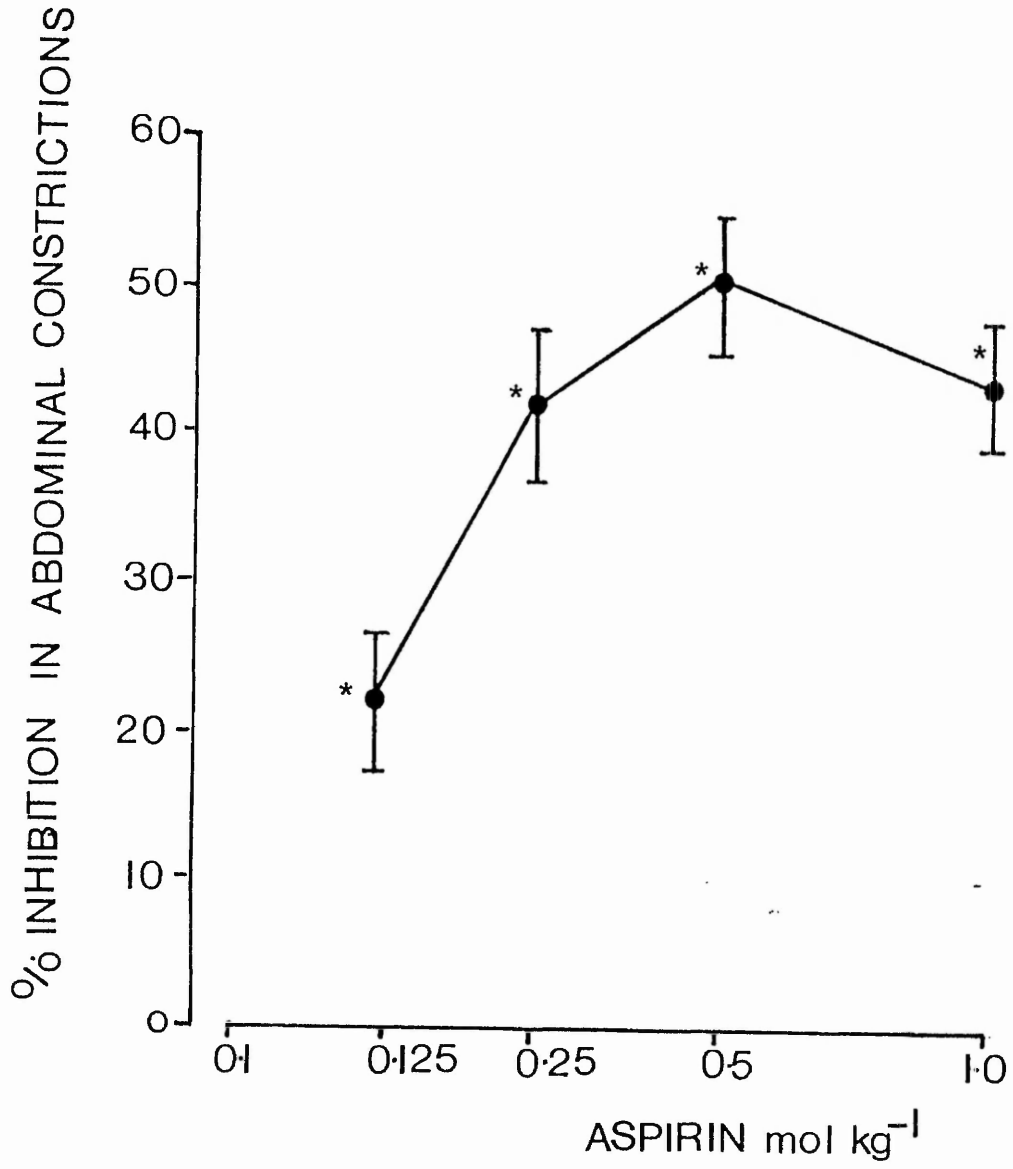

% INHIBITION IN ABDOMINAL CONSTRICTIONS
60
50
40
30
20
10
0
0·1
0·125
0·25
0·5
1·0
ASPIRIN mol kg⁻¹
*
*
*
*

Figure 9

Figure 10 Effect of leucine enkephalin on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10
*p < 0.05.

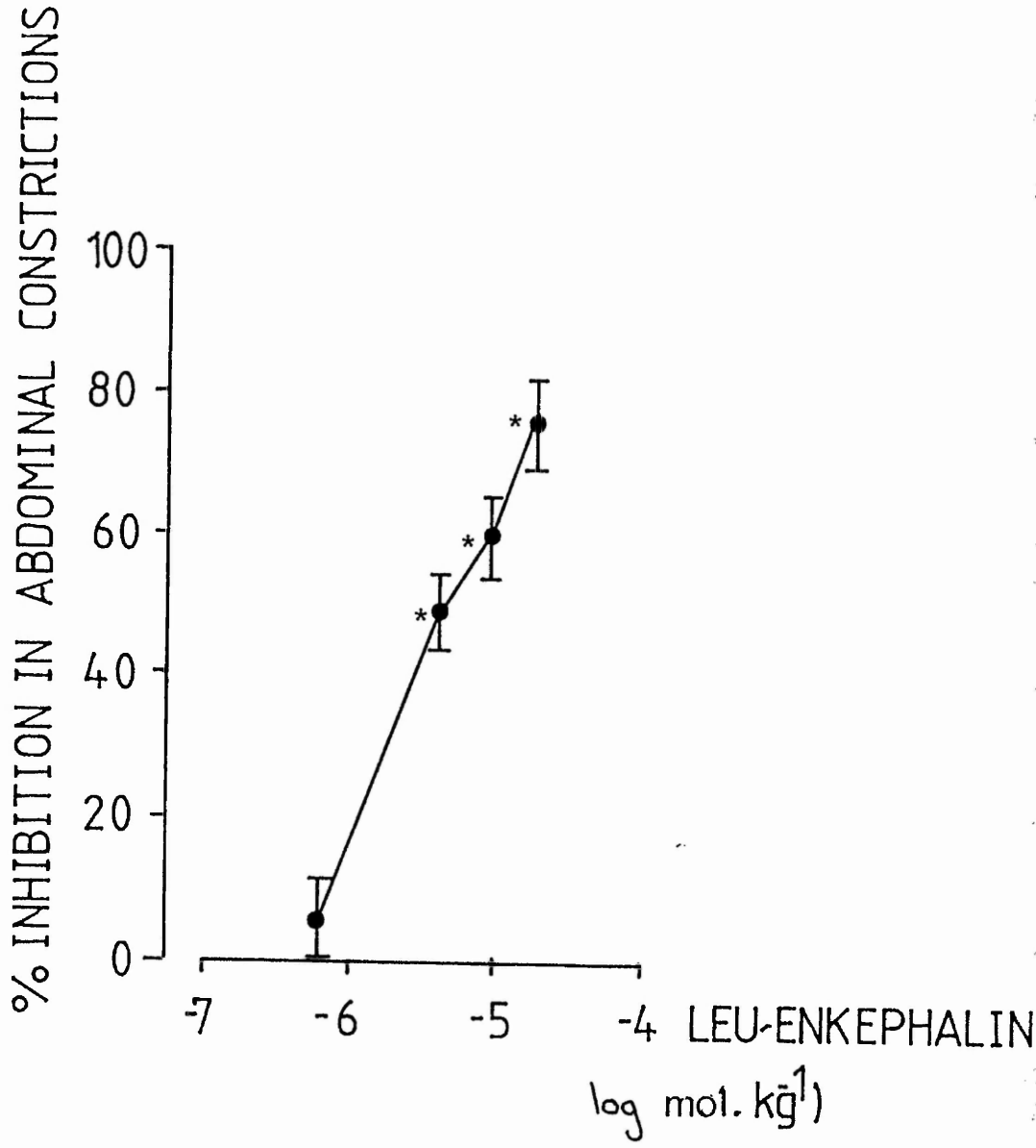

% INHIBITION IN ABDOMINAL CONSTRICTIONS
100
80
60
40
20
0
-7
-6
-5
-4
LEU-ENKEPHALIN
log mol. kg⁻¹)
*
*
*

Figure 10

3.3 ACUTE ADMINISTRATION OF CALCITONIN

Introduction

This section describes the effects of salmon calcitonin upon the frequency of abdominal constrictions. The anti-nociceptive properties of calcitonin have been studied by a number of workers, as cited in the introduction, and in all studies the hormone has been administered by i.c.v. injection. The possibility remains, therefore, that this property of the hormone may be an effect secondary to a peripheral action, possibly caused by the hormone leaking out from the brain into the circulation (Chapman and Way, 1980). Consequently, the effects of calcitonin administered by i.c.v. (0.04 - 50 i.u. kg^{-1}), subcutaneous (1 - 50 i.u. kg^{-1}), intravenous (10 i.u. kg^{-1}) and intraperitoneal (10 i.u. kg^{-1}) injection have been studied. In a separate series of experiments, calcitonin was injected, as before, and plasma calcium concentration was measured. To reduce the possibility of a 'false positive' result the hormone was injected by i.c.v. injection, and the animals were screened for any impairment in investigative and motor coordination. Finally, the peptide pentagastrin was administered by i.c.v. injection, in order to assess whether the activity of calcitonin in the abdominal constriction test was also due to a non-specific peptide effect. The concentrations of pentagastrin used were equimolar and a 100 fold greater than the concentration of calcitonin used (1.8 and 180 nmoles. kg^{-1} respectively).

Results

Salmon calcitonin, when it was administered by subcutaneous injection (10 - 50 i.u. kg^{-1}, Figure 11), intravenous injection (10 i.u. kg^{-1}, Figure 12) and intraperitoneal injection (10 i.u. kg^{-1}, Figure 12), was without effect on the frequency of abdominal constrictions. In all cases the concentration of plasma calcium was significantly reduced.

Salmon calcitonin doses of 0.04, 0.2, 2, 10 and 50 i.u. kg^{-1} by i.c.v. injection caused 14 ± 6.4, 37 ± 9.3, 38 ± 15.7, 50 ± 8.0 and 31 ± 8.2 (mean ± s.e., Figure 13) % inhibition in the frequency of abdominal constrictions respectively. 10 i.u. kg^{-1} calcitonin, by i.c.v. injection, produced a significant ($p < 0.05$) reduction in the plasma calcium concentration (0.18 $mmol.l^{-1}$). The blood was collected at fifteen minutes after the injection of calcitonin to correspond to the time at which antinociceptive testing was undertaken.

The inhibitory effect of calcitonin (10 i.u. kg^{-1}) on the frequency of abdominal constrictions, were shown to persist for up to sixty minutes following the i.c.v. injection of 10 i.u. kg^{-1} (Figure 14).

The effect of calcitonin on the frequency of abdominal constrictions was unlikely to be due to a non-specific peptide effect, since the i.c.v. injection of equimolar concentrations of pentagastrin, and 100 fold greater, was without effect on the frequency of abdominal constrictions.

The i.c.v. injection of calcitonin (10 i.u. kg^{-1}) was without effect on the investigative behaviour or motor coordination (see Table 4).

Figure 11 Lack of effect of salmon calcitonin (sCT), injected s.c., on the rate of abdominal constrictions. Values are mean ± s.e.m.; n = 10.

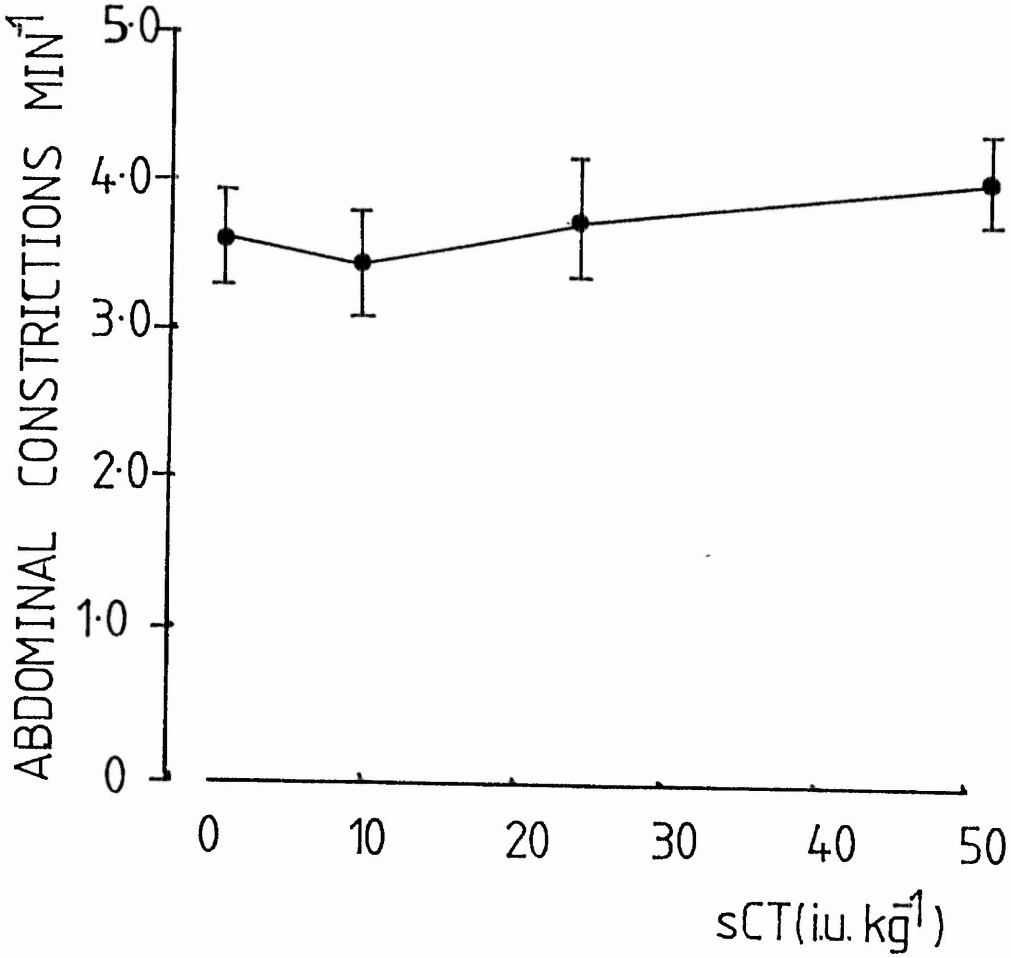

ABDOMINAL CONSTRICTIONS MIN^{-1}
5·0
4·0
3·0
2·0
1·0
0
0
10
20
30
40
50
sCT(i.u. kg^{-1})

Figure 11

Figure 12 Effect of salmon calcitonin (sCT) on the frequency of abdominal constrictions, compared to the appropriate vehicle injected animals, when administered by the s.c., i.p., i.v. or i.c.v. route. Values are mean ± s.e.m.; n = 6-10 *$p < 0.05$.

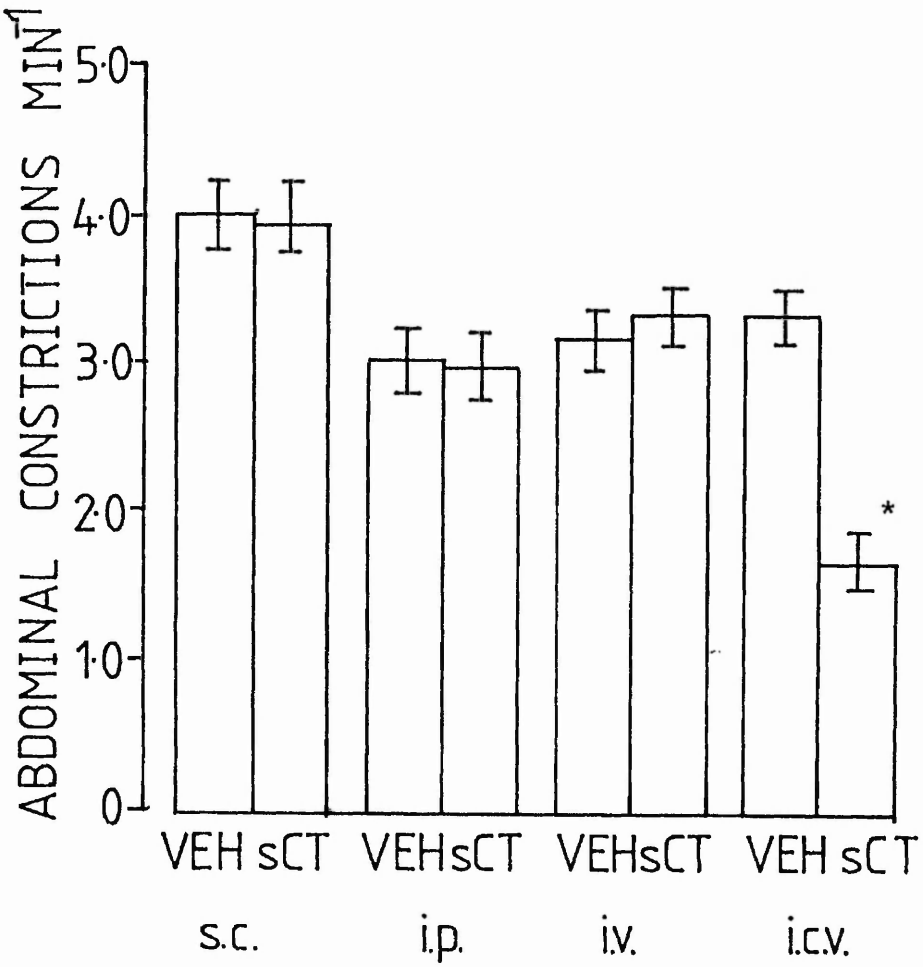

ABDOMINAL CONSTRICTIONS MIN⁻¹
5·0
4·0
3·0
2·0
1·0
0
VEH sCT VEH sCT VEH sCT VEH sCT
s.c.
i.p.
i.v.
i.c.v.
*

Figure 12

Figure 13 Inhibitory effect of salmon calcitonin (sCT), injected i.c.v., on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *p < 0.05.

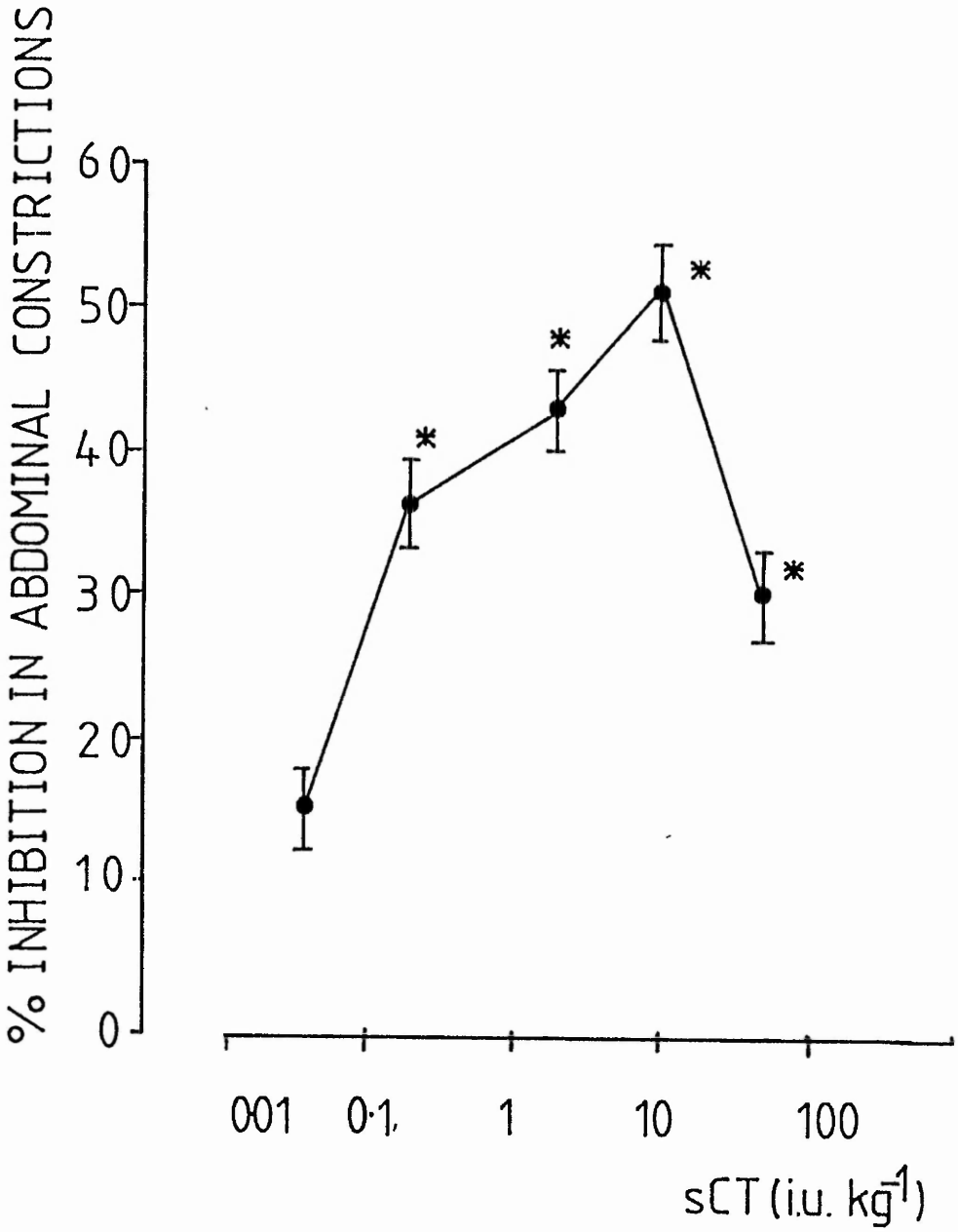

% INHIBITION IN ABDOMINAL CONSTRICTIONS
60
50
40
30
20
10
0
*
*
*
*
001
0·1
1
10
100
sCT (i.u. kg⁻¹)

Figure 13

Figure 14 Time course of the antinociceptive effect of i.c.v salmon calcitonin (sCT 10 i.u. kg^{-1}). Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

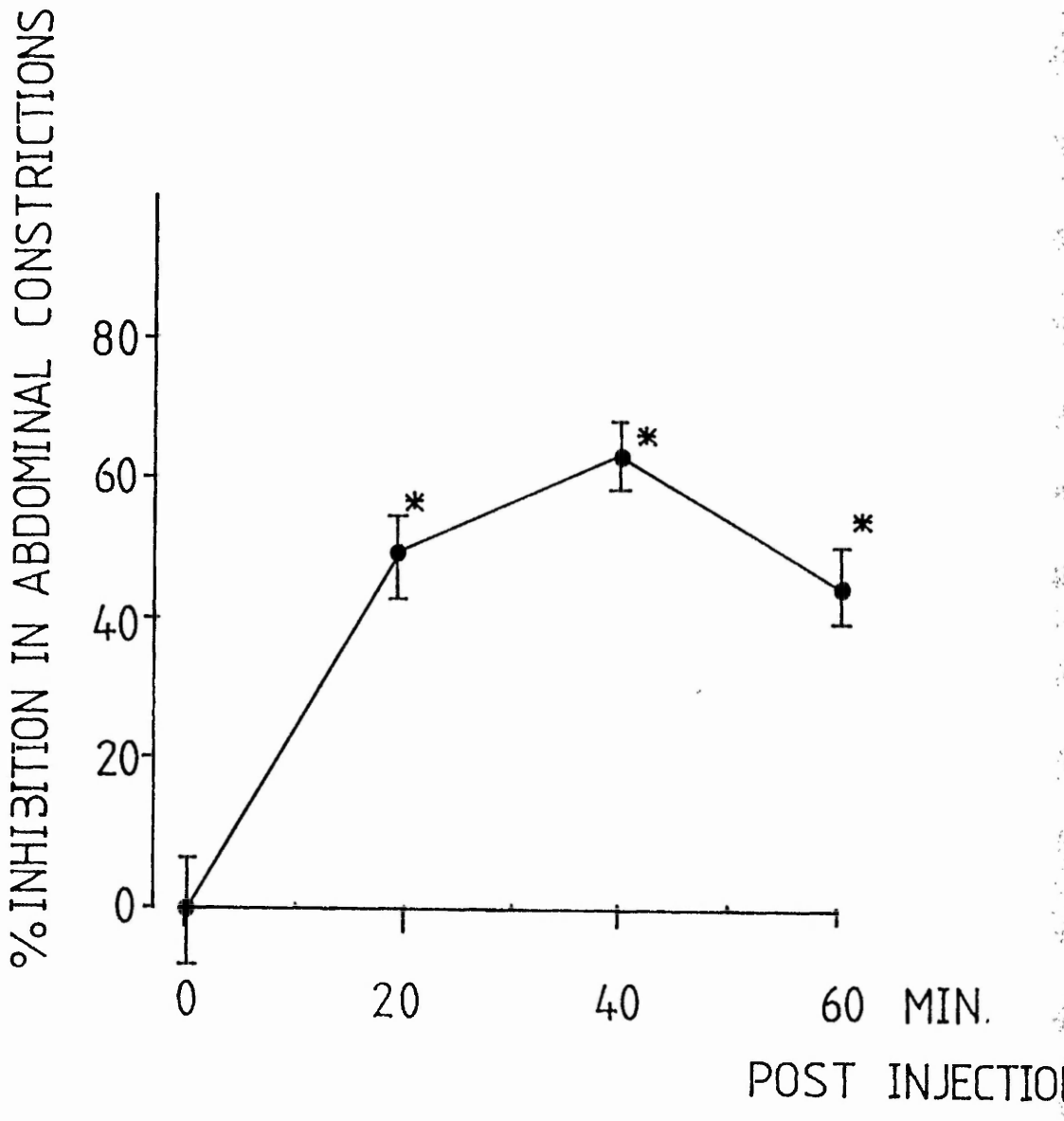

% INHIBITION IN ABDOMINAL CONSTRICTIONS
80
60
40
20
0
*
*
*
0
20
40
60 MIN.
POST INJECTIO

Figure 14

Table 4 Results of Investigative Behaviour and Motor Discoordination Tests

Treatment	Investigative Behaviour (head dips.min^{-1})	Motor Discoordination (no.times animal fell from drum min^{-1})
Calcitonin		
(10 i.u. kg^{-1})	8.04 ± 0.25	0.18 ± 0.001
Vehicle	7.8 ± 0.15	0.12 ± 0.01
$CaCl_2$		
(3.3 µmol.kg^{-1})	9.2 ± 0.09	0.15 ± 0.009
(6.6 µmol.kg^{-1})	3.2 ± 0.12 *	0.56 ± 0.002 *
(13.2 µmol.kg^{-1})	1.3 ± 0.15 *	1.3 ± 0.13 *
Vehicle	8.5 ± 0.32	0.15 ± 0.008
A23187		
(11.7 µmol.kg^{-1})	8.9 ± 0.18	0.20 ± 0.012
(117 µmol.kg^{-1})	8.6 ± 0.20	0.31 ± 0.20
(1170 µmol.kg^{-1})	3.8 ± 0.14 *	1.5 ± 0.18 *
Vehicle	9.0 ± 0.12	0.38 ± 0.02
Nifedipine		
(6.6 µmol.kg^{-1})	8.5 ± 0.45	0.42 ± 0.09
PY 108 068		
(6.6 µmol.kg^{-1})	7.8 ± 0.19	0.38 ± 0.02
Vehicle	9.0 ± 0.32	0.32 ± 0.12

All treatments above were administered by i.c.v. injection

Table 4 Continued

Treatment	Investigative Behaviour (head dips.min^{-1})	Motor Discoordination (no.times animal fell from drum.min^{-1})
4 s.c. injections of calcitonin withdrawn from treatment for 48 hours	8.6 ± 0.38	0.13 ± 0.009
Vehicle	8.9 ± 0.42	0.15 ± 0.010

Table 4 Results of investigative behaviour and motor discoordination tests. Units are head dips.min^{-1} (investigative behaviour) and number of falls off drum.min^{-1} (motor discoordination). Values are mean ± s.e.m.; n = 6-10 *$p < 0.05$.

Discussion

These results demonstrate that calcitonin posseses anti-nociceptive activity only when it is administered by i.c.v. injection, and not when it is administered by subcutaneous, intraperitoneal or intravenous injection. This property of the hormone is, therefore, possibly due to an action on the CNS, or more specifically on areas of the brain accessible to diffusion of calcitonin from the ventricular system. This action is in agreement with other workers such as Pecile et al (1975) and Braga et al (1978), in that the action was observed after central injection and persists for at least one hour. The hypocalcaemic activity of calcitonin, observed after i.c.v. administration of calcitonin, would appear to be unrelated to the antinociceptive actions of the hormone, as was first suggested by Chapman and Way (1980).

The ability of calcitonin, after i.c.v. administration, to reduce plasma calcium concentration may reflect either that the hormone entered the systemic circlation from damage caused at the site of injection or it may be due to the blood brain barrier allowing the passage of the hormone from the cerebrospinal fluid to the bloodstream. Since calcitonin was inactive when it was administered by peripheral injection, it may be that the blood brain barrier only allows the passage of the hormone in one direction, i.e. from the cerebrospinal fluid to the blood and not vice versa. However, it has been reported that calcitonin may enter the cerebrospinal fluid after it has been given by peripheral injection (Carmen and Wyatt, 1979).

In addition, the idea proposed by Chapman and Way (1980) that the antinociceptive properties of calcitonin are dependent upon a decrease in the plasma calcium concentration is not confirmed by the results presented here. Although calcitonin, either by i.c.v. or subcutaneous administration, results in a significant reduction in the plasma calcium concentration, the results show that the hormone is effective only by i.c.v. injection. It is interesting to note that the peripheral administration of the antipyretic analgesic, acetylsalycyclic acid, has been shown to produce a reduction in the plasma calcium concentration.

The possibility that the properties of antinociception induced by calcitonin are related to changes in the calcium flux within the brain, rather than in the peripheral nervous system, is studied in the following section.

3.4 EFFECT OF DIVALENT CATIONS

The antinociceptive properties of the opiates (Chapman and Way, 1980), dibutryl cGMP (Vocci, Welch and Dewey, 1980) and acetylcholine (Widman, Rosun and Dewey, 1978) have been shown to be strongly influenced by divalent cations. Chapman and Way (1980) have proposed that the initial event consists of a lowering of neuronal calcium. The consequent reduction in neurotransmitter release produces antinociception. (This has been more fully described in the Introduction.)

This section is devoted to this facet of the antinociceptive process, and the effects of $CaCl_2$, $MgCl_2$, on calcitonin-induced antinociception have been studied. In addition, the effect of EGTA, a calcium chelating agent has also been studied. This substance was used in preference to ethylenediaminetetraacetic acid (EDTA), since its affinity for calcium is over 250,000 times more specific than the corresponding magnesium chelate, at pH 7.4 and 37°C (Wallick, Allen and Schwartz, 1973). By comparison, the affinity of EDTA for calcium is only about 100 times more than the affinity for magnesium under the same conditions.

Chapman and Way (1980) have reported that the i.c.v. injection of calcium ions produces an increased sensitivity to pain, whilst the converse is true for the calcium chelators. These results are consistent with the concept that a reduction in neuronal calcium produces antinociception. The effects of these substances have been assessed when they have been administered alone or in conjunction with calcitonin.

Results

Effects of Calcium Ions

The i.c.v. injection of $CaCl_2$ (0.003 - 1.7 $\mu mol.kg^{-1}$) was without significant effect in the frequency of abdominal constrictions (Figure 15). The i.c.v. injection of 3.3 $\mu mol.kg^{-1}$ $CaCl_2$ resulted in a significant increase in the number of abdominal constrictions ($p < 0.05$), Figure 15. The i.c.v. injection of higher concentrations of calcium ions (6.6 - 13.2 $\mu mol.kg^{-1}$) produced behavioural disturbances and impairment in motor coordination (see Table 4).

Effect of Calcium Ions on Antinociception-Induced by Calcitonin

The simultaneous i.c.v. injection of calcitonin, 2 i.u. kg^{-1}, and $CaCl_2$ resulted in antagonism of the antinociceptive effects of the hormone. This was a dose-dependent effect of the calcium and calcitonin was no longer significantly antinociceptive when injected simultaneously with 0.33 and 1.7 $\mu mol.kg^{-1}$ $CaCl_2$ (Figure 16). These concentrations of calcium were without effect when administered alone.

Effect of Magnesium Ions

The i.c.v. injection of $MgCl_2$ (3.3 $\mu mol.kg^{-1}$) was without effect on the frequency of abdominal constrictions. This concentration was without effect on the antinociceptive properties of calcitonin (2 i.u. kg^{-1}) when injected simultaneously (Figure 17).

Effects of EGTA and Interaction with Calcium Ions and Calcitonin

The i.c.v. injection of EGTA (3.3 $\mu mol.kg^{-1}$) resulted in a significant reduction in the frequency of abdominal constrictions ($p < 0.05$). The i.c.v. injection of an equimolar concentration of $CaCl_2$ simultaneously with the EGTA reversed the effect of EGTA (Figure 18).

The i.c.v. injection of calcitonin (10 i.u. kg^{-1}) decreased the frequency of abdominal constrictions from 3.24 ± 0.2 to 2.36 ± 0.21 constrictions min^{-1} (mean ± s.e.). When calcitonin was injected simultaneously with EGTA (10 i.u. kg^{-1} and 3.3 $\mu mol.kg^{-1}$ respectively), no additional decrease was observed (Figure 18). However, when 2 i.u. kg^{-1} calcitonin was injected together with EGTA (3.3 $\mu mol.kg^{-1}$) a greater effect was observed, when compared to the effects of these agents injected alone at these concentrations.

Discussion

These results illustrate the ability of calcium ions to modulate the antinociceptive properties of both EGTA and calcitonin. They also show that agents capable of altering the brain calcium concentration can modulate the sensitivity to peritoneal irritation themselves. The hyperalgesia induced by calcium ions has also been shown by Chapman and Way (1980) using identical concentrations of calcium ions. In addition, the antinociceptive effects of EGTA have also been shown by these authors. These results disagree with the work of these authors wth regard to the effects of magnesium ions. The ability of this ion to induce hyperalgesia and to

angatonise opiate-induced antinociception has been shown by Harris et al (1975). These results were obtained using another antinociceptive test which may account, in part, for this discrepancy.

The ability of calcium ions to antagonise antinociception induced by calcitonin has also been shown by Satoh, Amano, Takahro and Takagi (1979). These authors showed that calcium ions reduced both the potency and duration of the antinociception induced by porcine calcitonin. This paper, whilst in good agreement with the results presented in this section, may be strongly criticised on the grounds that a hightly impure preparation of calcitonin was used. (98 i.u. mg^{-1}:- highly purified porcine calcitonin is usually of the order of 300 i.u. mg^{-1}.) This implies that to achieve the doses used in the paper (15 i.u. kg^{-1}), in the volume injected (10 µl),a very viscous solution would have been used centrally.

However, it would appear that calcitonin resembles opiate analgesia in that antinociception induced by the hormone can be antagonised by calcium ions. The inability of magnesium ions to antagonise calcitonin antinociception, in contrast to its action on opiate antinociception, may be a reflection of the specificity of the hormone for calcium. The relationship between calcitonin and EGTA is also similar to that observed between opiates and EGTA. The results shown here, where the maximum effective antinociceptive concentration of calcitonin (10 i.u. kg^{-1}) is not potentiated by EGTA, suggests that the two processes

involved are related. However, such potentiation does occur when a submaxmal concentration of calcitonin (2 i.u. kg^{-1}) is used in conjunction with EGTA.

Figure 15 Effect of $CaCl_2$ on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 * $p < 0.05$.

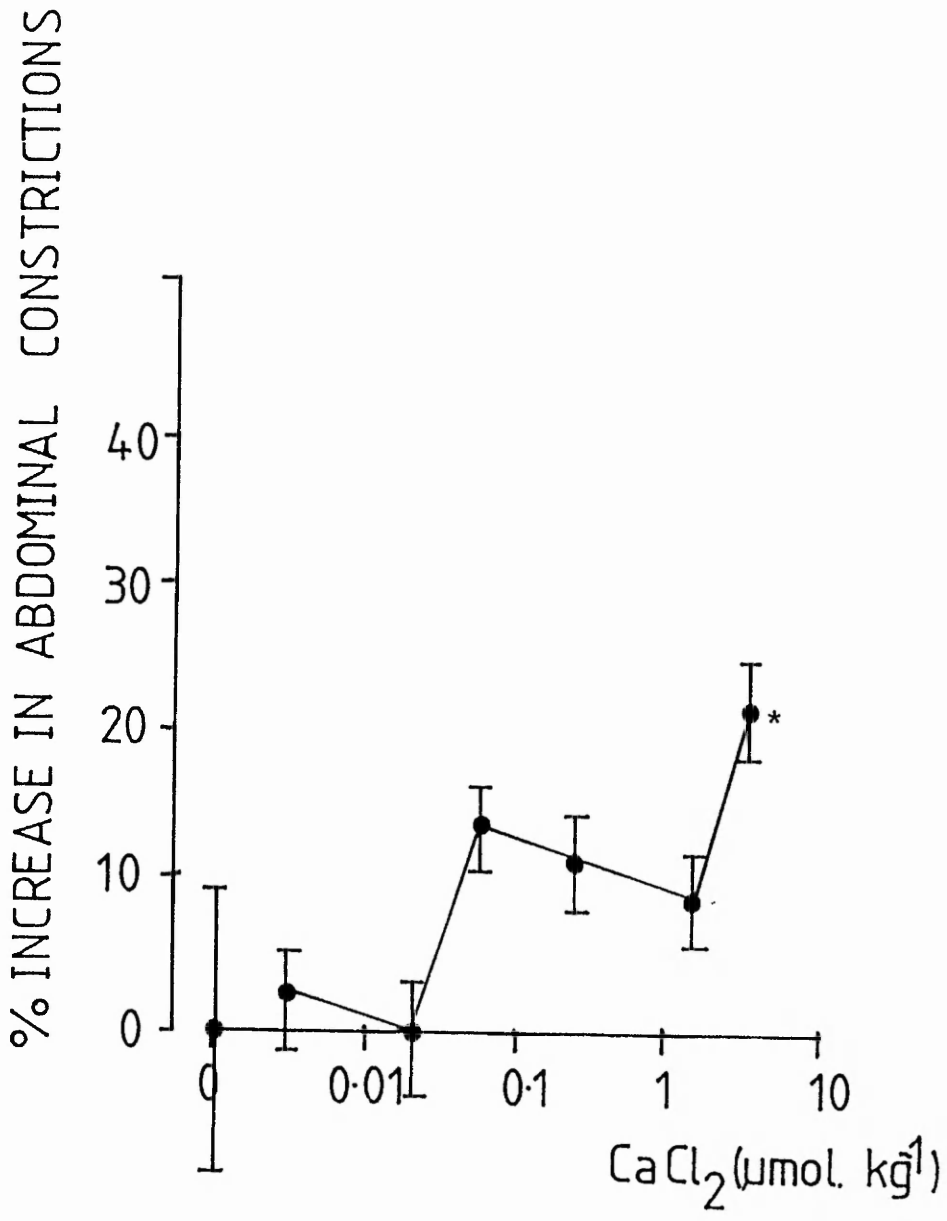

% INCREASE IN ABDOMINAL CONSTRICTIONS
40
30
20
10
0
0
0·01
0·1
1
10
*
CaCl2 (μmol. kg-1)

Figure 15

Figure 16 Reversal of the antinociceptive response to salmon calcitonin (sCT, 2 i.u. kg^{-1}) by $CaCl_2$. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

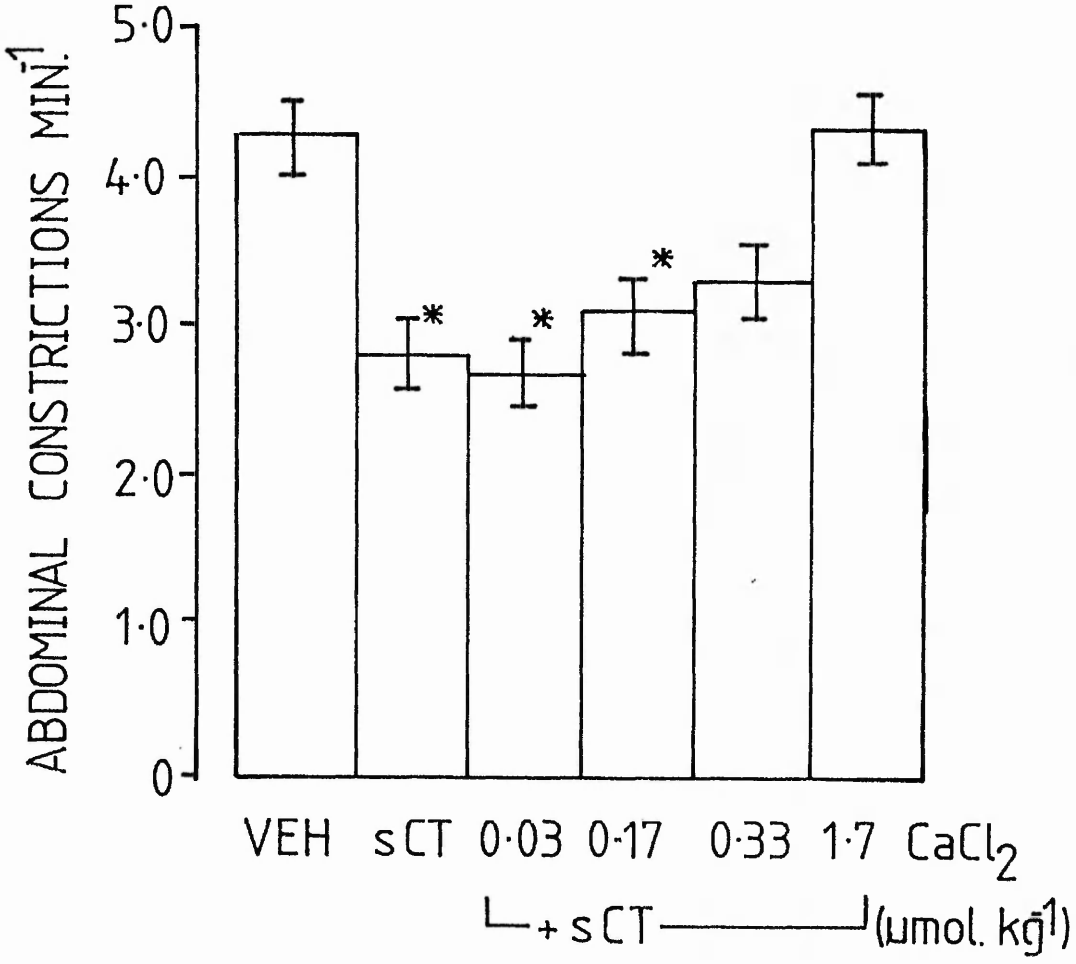

ABDOMINAL CONSTRICTIONS MIN.-1
5·0
4·0
3·0
2·0
1·0
0
*
*
*
VEH
sCT
0·03
0·17
0·33
1·7
CaCl2
+ sCT
(μmol. kg-1)

Figure 16

Figure 17 Effect of $MgCl_2$ (3.3 $\mu mol.kg^{-1}$), salmon calcitonin (2 i.u. kg^{-1}), either alone or in combination, on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 * $p < 0.05$.

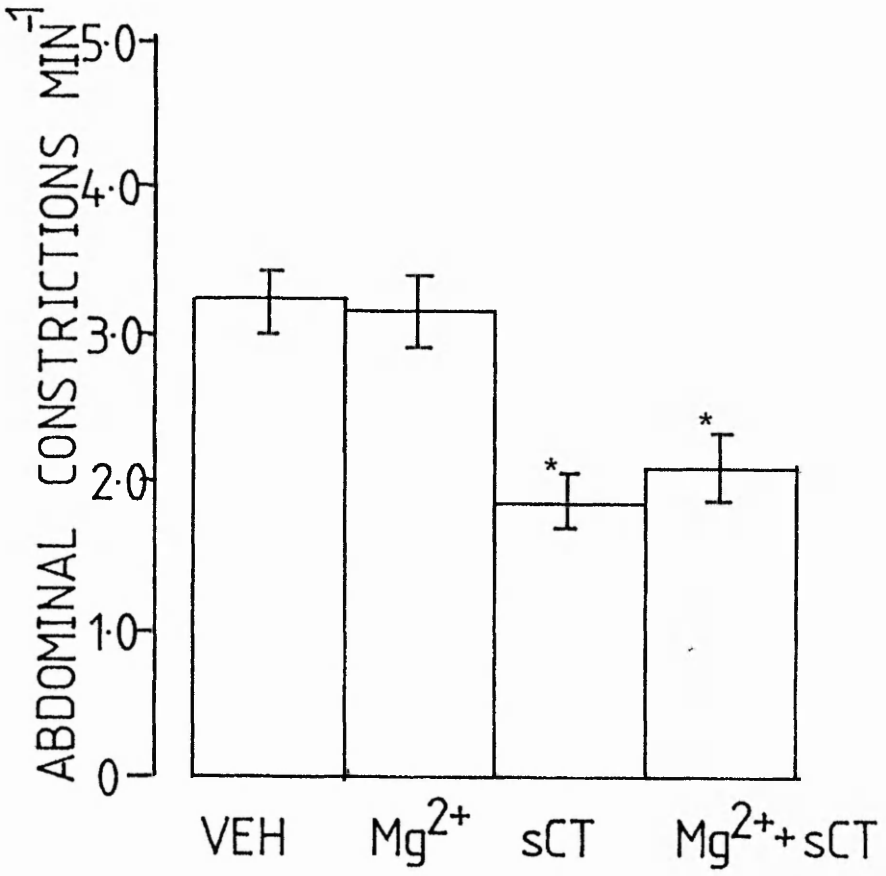

ABDOMINAL CONSTRICTIONS MIN^{-1}
5·0
4·0
3·0
2·0
1·0
0
*
*
VEH
Mg^{2+}
sCT
Mg^{2+}+sCT

Figure 17

Figure 18 Effect of salmon calcitonin (10 i.u. kg^{-1}), EGTA (3.3 $\mu mol.kg^{-1}$), alone or in combination, and a combination of EGTA (3.3 $\mu mol.kg^{-1}$) and $CaCl_2$ (3.3 $\mu mol.kg^{-1}$) on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

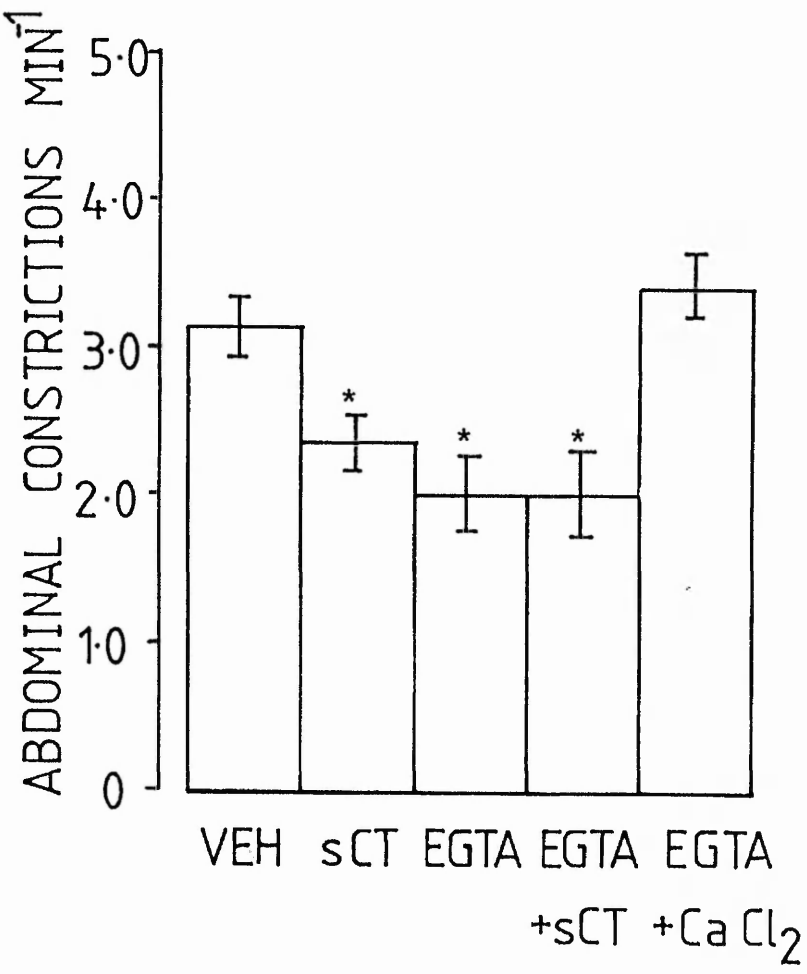

ABDOMINAL CONSTRICTIONS MIN-1
5·0
4·0
3·0
2·0
1·0
0
*
*
*
VEH
sCT
EGTA
EGTA
+sCT
EGTA
+Ca Cl2

Figure 18

3.5 EFFECT OF THE CALCIUM IONOPHORE, A23187

The class of calcium ionophores, of which A23187 is at present the most selective (Reed and Lardy, 1980), permit study of the effects of raising the intracellular calcium concentration. The effect of these agents on opiate-induced antinociception has been studied using primarily the ionophore X537A, a compound which permits the entry of a range of divalent cations into the cell, including magnesium, barium and strontium. A23187, in contrast, is highly selective for calcium ions with a slight affinity for magnesium ions (Reed and Lardy, 1980). Calcium ionophores have been shown to increase the ability of calcium ions, at previously inactive concentrations, to antagonise antinociception induced by opiates (Harris et al, 1975) and dibutryl cGMP (Vocci et al, 1980).

This section concerns the effect of A23187 on the antinociception induced by calcitonin. The compound has also been used in conjunction with calcium and magnesium ions, in order to study the selectivity of A23187 with regard to effect of calcium ion movement.

Results

The i.c.v. injection of A23187 (1.17 -11.7 nmol.kg^{-1}) was without effect on the frequency of abdominal constrictions. A slight, but significant ($p < 0.05$) increase was observed after the i.c.v. injection of 117 nmoles of A23187 (Figure 19). Higher concentrations of A23187 (1170 nmol.kg^{-1}) resulted in a state of depression of motor activity and a reduction in motor coordination. This was reflected in the

reduction in abdominal constrictions (Table 4).

As shown in Figure 20, the i.c.v. injection of $CaCl_2$ (3.3 $\mu mol.kg^{-1}$) resulted in a significant increase in the frequency of abdominal constrictions. The response to simultaneous injection of maximal concentrations of A23187 (117 $nmol.kg^{-1}$) and $CaCl_2$ (3.3 $\mu mol.kg^{-1}$) was not significantly different from the effects of these agents when administered alone. The i.c.v. injection of $MgCl_2$ (3.3 $\mu mol.kg^{-1}$) was without effect on the frequency of abdominal constrictions, and the simultaneous injection of $MgCl_2$ (3.3 $\mu mol.kg^{-1}$) and A23187 (117 $nmol.kg^{-1}$) did not result in any change in the frequency of abdominal constrictions when compared to the effects of these agents alone (Figure 21).

The antinociceptive properties in calcitonin (0.04 - 10 i.u. kg^{-1}) were antagonised by the simultaneous injection of A23187 (11.7 and 117 $nmol.kg^{-1}$). The potency of calcitonin was reduced by about 100 fold by the simultaneous i.c.v. injection of 11.7 $nmol.kg^{-1}$ A23187 (Figure 22). This concentration of A23187 was inactive when administered alone, and was without effect on investigative and motor behaviour (see Table 4).

Discussion

These results demonstrate, further, the importance of the calcium ion in antinociception. When A23187 is present in sufficient concentrations (117 $nmol.kg^{-1}$), an increased sensitivity to peritoneal irritation occurs. Therefore, the ionophore has facilitated the cellular entry of calcium ions (or possibly elevated the concentration of calcium ions in

the C.S.F.). The lack of effect of magnesium either alone or in combination with A23187 implies firstly, that it is the calcium ion that is important and secondly, demonstrates the selectivity of the ionophore for calcium ions.

The results also show that the antinociception induced by calcitonin is related to a reduction in the concentration of calcium within the CNS. This may be at the cellular level, but may be an action at the blood brain barrier. The ability of calcium ionophores to antagonise antinociception induced by calcitonin is similar to the ability of these agents to antagonise the antinociceptive properties of the opiates. These results do not, however, give any indication concerning the cellular site of action of the hormone to produce its effects because calcium and ionophores have widespread sites of action.

Figure 19 Effect of A23187 on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

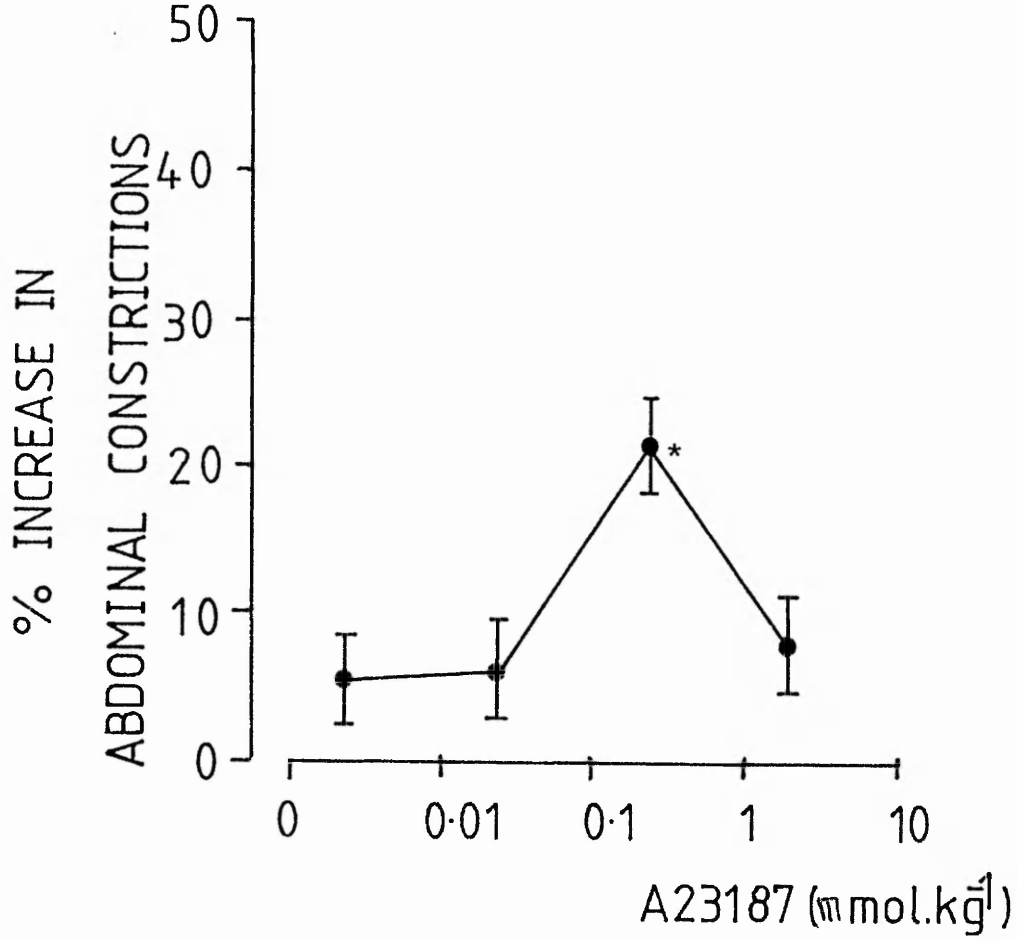

% INCREASE IN
ABDOMINAL CONSTRICTIONS
50
40
30
20
10
0
*
0
0·01
0·1
1
10
A23187 (mmol.kg⁻¹)

Figure 19

Figure 20 Effect of A23187 (117 $nmol.kg^{-1}$), $CaCl_2$ (3.3 $\mu mol.kg^{-1}$), alone or in combination, on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 * $p < 0.05$.

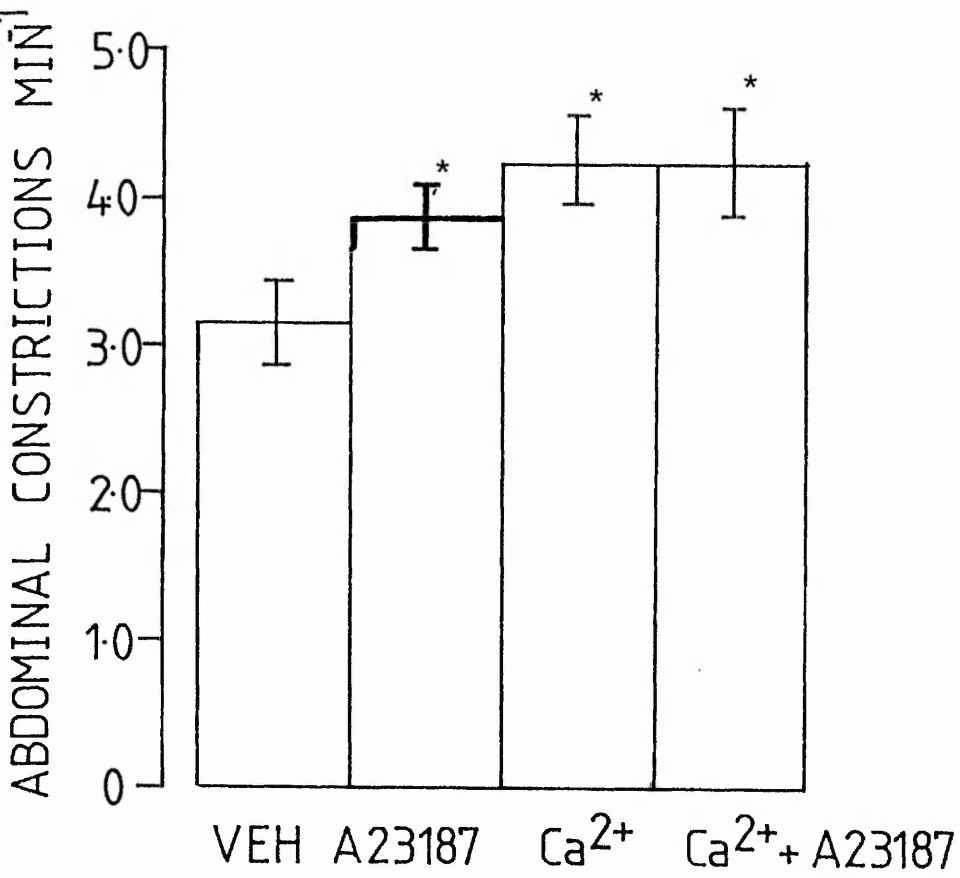

ABDOMINAL CONSTRICTIONS MIN⁻¹
5·0
4·0
3·0
2·0
1·0
0
*
*
*
VEH
A23187
Ca2+
Ca2+ + A23187

Figure 20

Figure 21 Effect of A23187 (117 nmol.kg^{-1}), $MgCl_2$ (3.3 µmol.kg^{-1}), alone or in combination, on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *p < 0.05.

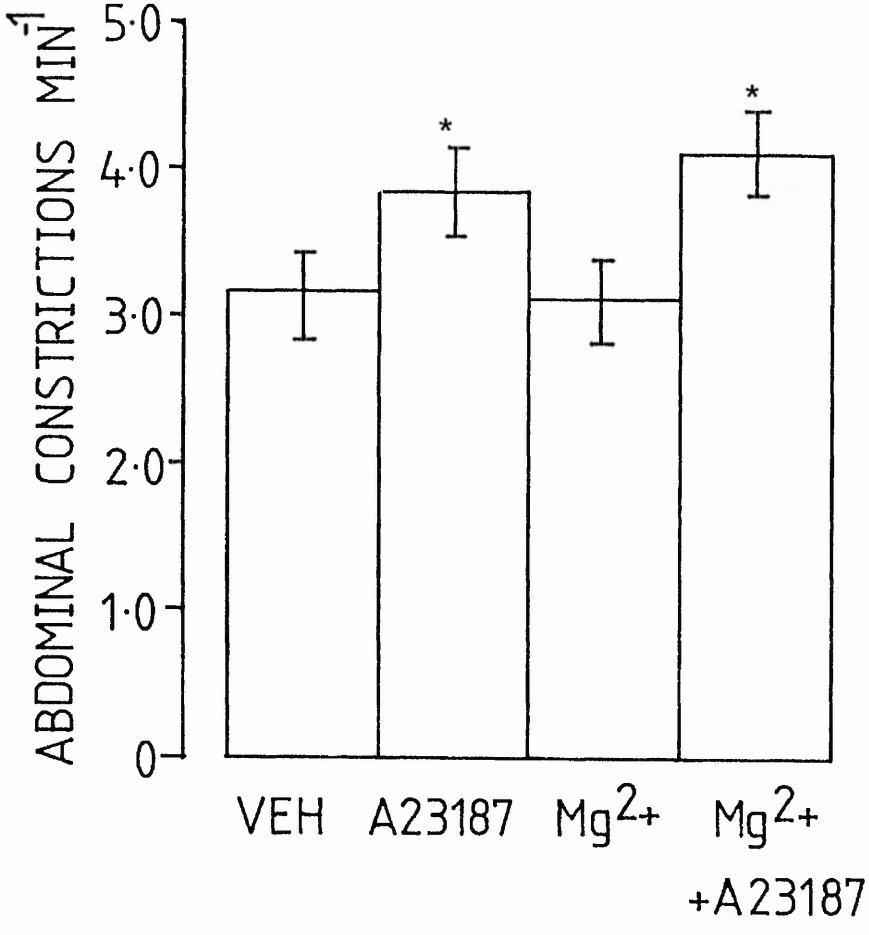

ABDOMINAL CONSTRICTIONS MIN-1
5·0
4·0
3·0
2·0
1·0
0
*
*
VEH
A23187
Mg2+
Mg2+ +A23187

Figure 21

Figure 22 Effect of A23187 (117 nmol.kg^{-1}) on the antinociceptive response to salmon calcitonin. Values are mean ± s.e.m.; n = 10 *p < 0.05.

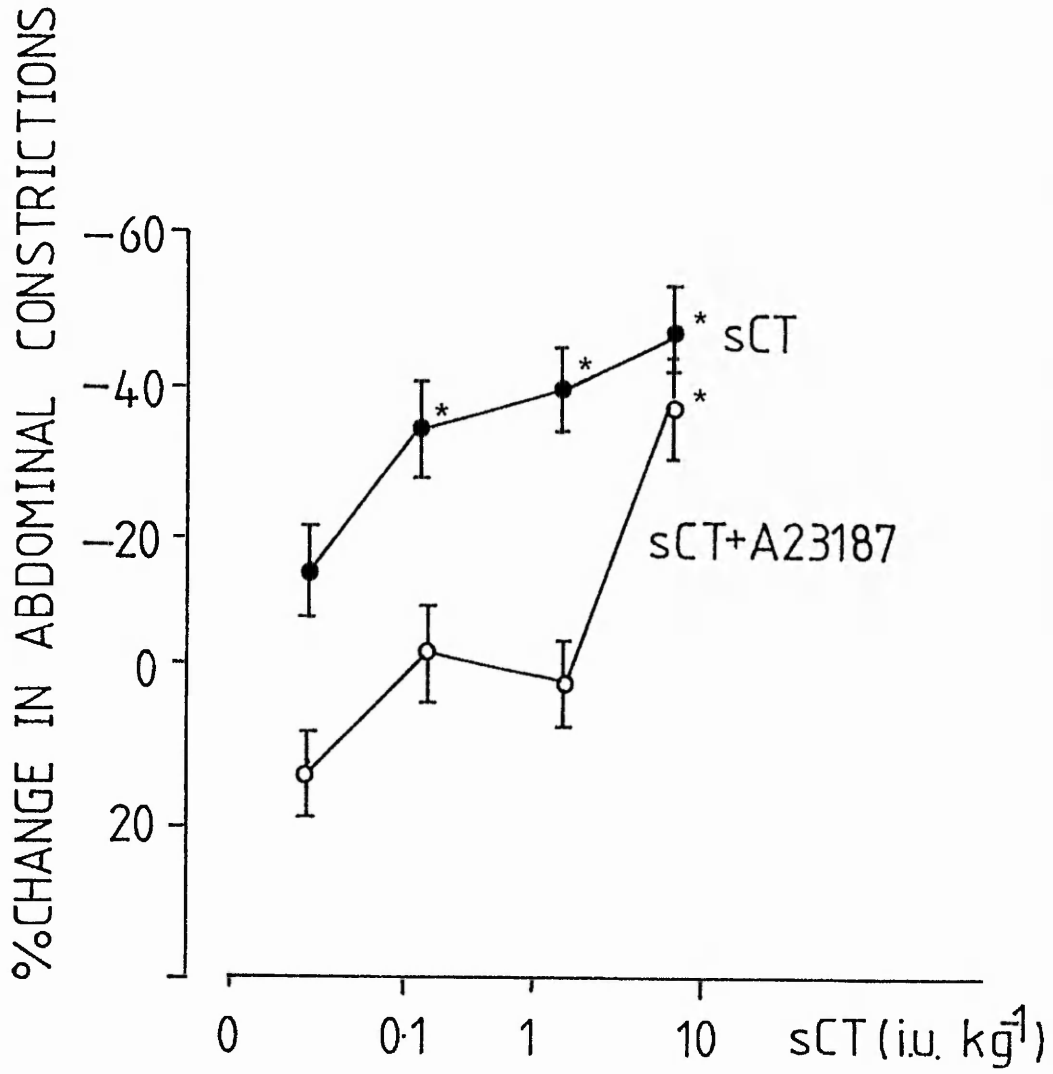

%CHANGE IN ABDOMINAL CONSTRICTIONS
-60
-40
-20
0
20
sCT
sCT+A23187
*
0
0·1
1
10
sCT (i.u. kg⁻¹)

Figure 22

3.6 EFFECT OF THE CALCIUM ANTAGONISTS

In agreement with the concept of a reduction in neuronal calcium eliciting antinociception, it has been shown that the i.c.v. injection of lanthanum ions, an agent which displaces calcium from calcium channels, results in antinociception (Iwamoto, Harris, Loh and Way, 1978). This effect is antagonised by the opiate antagonist, naloxone, (Iwamoto et al, 1978) and by calcium ions (Harris et al, 1976). Chapman and Way (1980) have proposed that this implies that the fundamental processes involved in both lanthanum and opiate antinociception are similar.

In the past decade, a class of drugs have been developed which, at least in cardiac muscle, preferentially block the entry of calcium via the voltage-sensitive calcium channel (Nayler and Poole-Wilson, 1981). These agents have since been shown to influence the entry of calcium into a number of tissues includng neural tissues.

This section is devoted to the effects of two calcium antagonists, nifedipine and a structurally similar compound, PY 108068, on nociception and their interaction with calcitonin. The rationale for the experiments parallels the work undertaken by other workers using lanthanum and its effect on antinociception induced by the opiates.

Results

The i.c.v. injection of nifedipine (0.33 - 6.6 $\mu mol.kg^{-1}$) resulted in a significant inhibition in the frequency of abdominal constrictions (Figure 23). The same was true when PY 108068 (0.33 - 6.6 $\mu mol.kg^{-1}$, Figure 23) was injected.

Both of these agents were ineffective at these concentrations when administered by subcutaneous injection. These agents, when administered by i.c.v. injection (0.33 - 6.6 $\mu mol.kg^{-1}$) were without effect on motor coordination and investigative behaviour (see Table 4).

The simultaneous injection of $CaCl_2$ (1.7 $\mu mol.kg^{-1}$) and nifedipine (3.3 $\mu mol.kg^{-1}$) antagonised the inhibitory effect of nifedipine on the frequency of abdominal constrictions. In contrast, the simultaneous injection of $MgCl_2$ (3.3 $\mu mol.kg^{-1}$) with nifedipine (3.3 $\mu mol.kg^{-1}$) had no effect on the inhibitory effect of nifedipine on the abdominal constriction frequency (Figure 24).

The i.c.v. injection of calcitonin (0.1 or 2 i.u. kg^{-1}) produced a 22 ± 8% and 50 ± 5% (mean ± s.e.) reduction in the frequency of abdominal constrictions. The simultaneous injection of calcitonin (0.1 i.u. kg^{-1}) and nifedipine (0.33 $\mu mol.kg^{-1}$) - both threshold concentrations - produced a summation of the effects observed when these agents are administered alone. This was also observed when near maximal concentrations of calcitonin and nifedipine (2 i.u. kg^{-1} and 3.3 $\mu mol.kg^{-1}$ respectively) were injected simultaneously (Figure 25).

Discussion

The calcium 'slow channel' blocking agents, nitedipine and PY 108068, are effective as antinociceptive agents only when administered by central injection. The same is also true for lanthanum (Harris et al, 1976). It is frequently assumed that the slow channel blocking agents selectively

block 'voltage-sensitive' channels. One can, therefore, imply that the antinociceptive properties of these agents are related to a reduction in cellular calcium produced by blockade of the voltage-sensitive calcium channel. Lanthanum, however, presumably induces antinociception by displacing calcium from the calcium channel. The addition, observed in the maximal effects of calcitonin and nifedipine, may indicate that two distinct mechanisms are operating. There may be different effects on different types of calcium channels.

Finally, the ability of calcium, but not magnesium, in antagonising the antinociceptive effects of these agents indicates the specific action of these agents in blocking calcium entry.

Figure 23 Effect of nifedipine and PY 108 068 on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *p < 0.05.

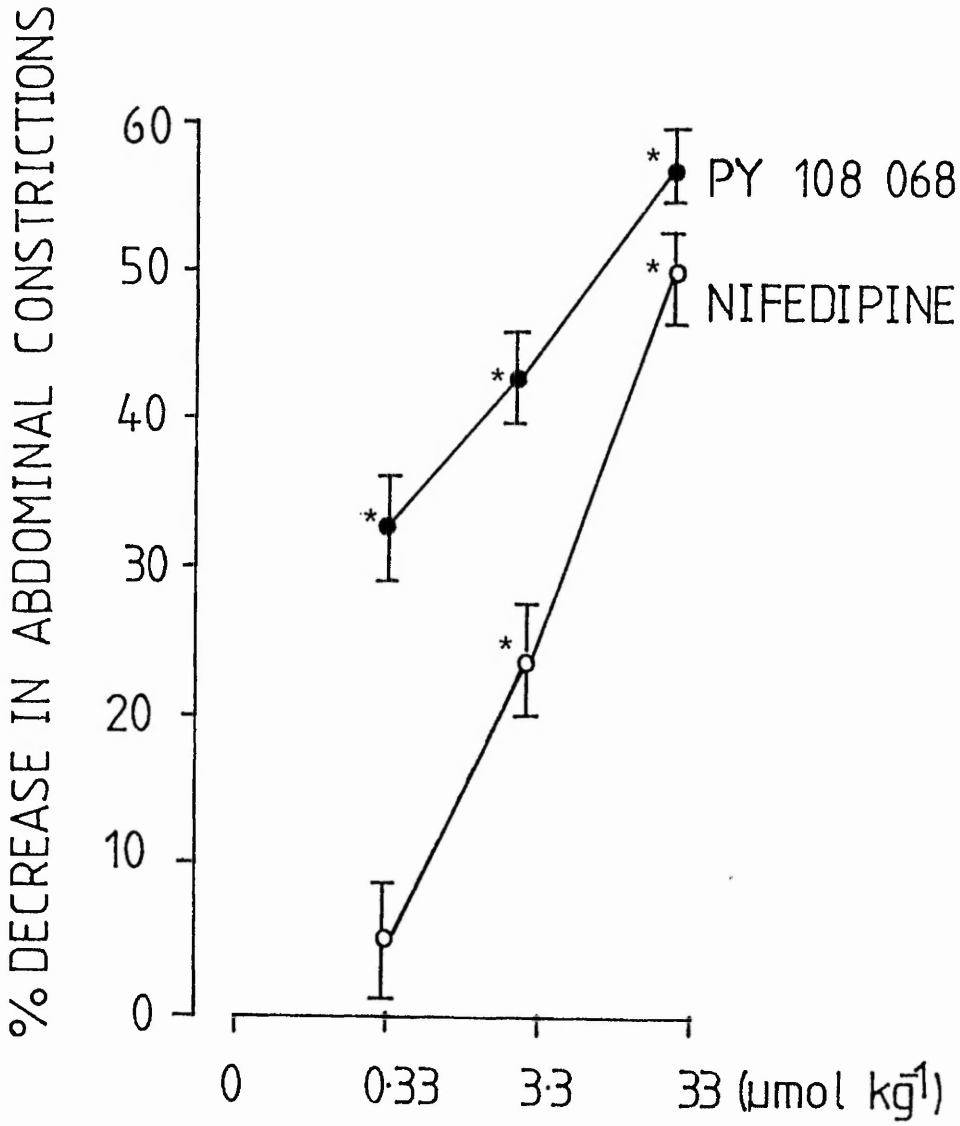

% DECREASE IN ABDOMINAL CONSTRICTIONS
60
50
40
30
20
10
0
PY 108 068
NIFEDIPINE
*
0
0·33
3·3
33 (μmol kg⁻¹)

Figure 23

Figure 24 Effect of nifedipine (3.3 $\mu mol.kg^{-1}$), $CaCl_2$ (1.7 $\mu mol.kg^{-1}$) amd $MgCl_2$ (3.3 $\mu mol.kg^{-1}$), either alone or in combination, on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

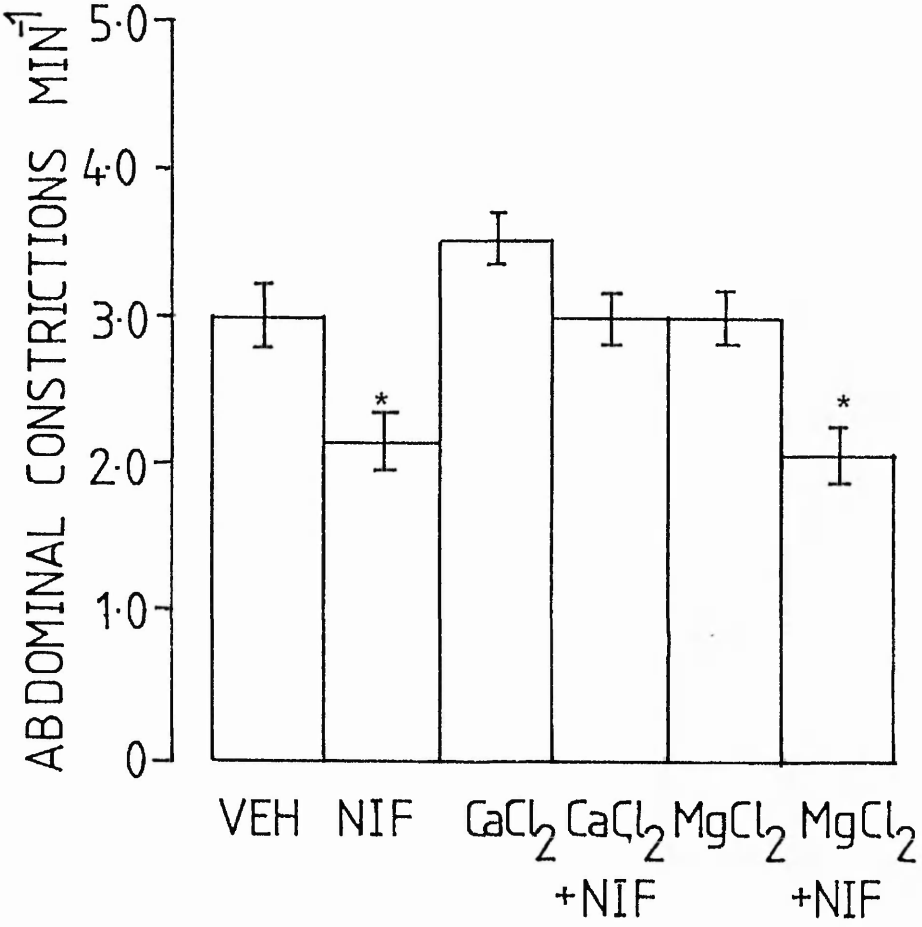

ABDOMINAL CONSTRICTIONS MIN⁻¹
5·0
4·0
3·0
2·0
1·0
0
*
*
VEH
NIF
CaCl2
CaCl2 +NIF
MgCl2
MgCl2 +NIF

Figure 24

Figure 25 Effect of nifedipine (3.3 and 0.33 $\mu mol.kg^{-1}$) and salmon calcitonin (2 and 0.1 $i.u.kg^{-1}$), either alone or in combination, on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

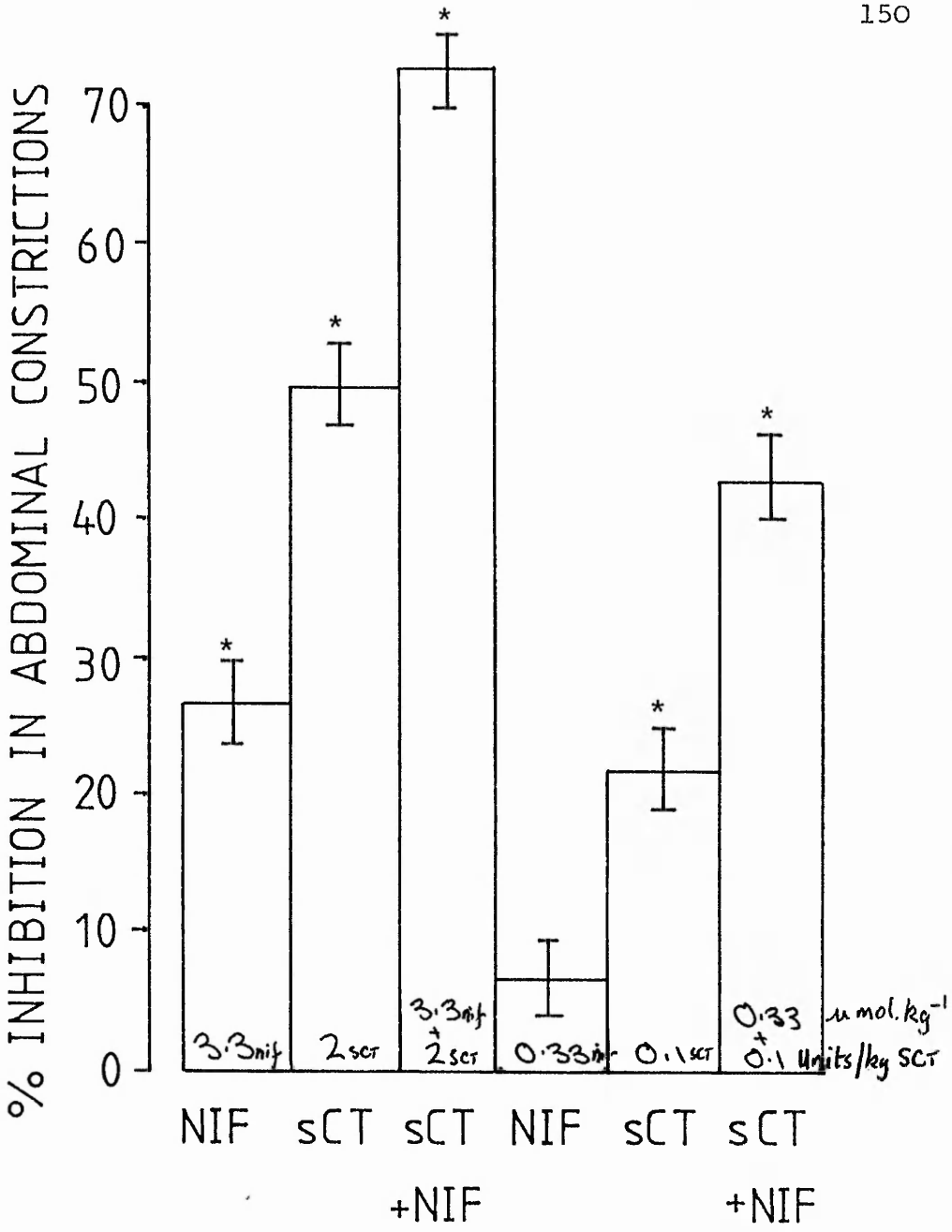


Figure 25

Doses of nifedipine in μmol kg^{-1}
" " sCT in Units/kg

3.7 EFFECT OF NALOXONE

The data presented so far has illustrated the similiarities between antinociception induced by the opiates and by calcitonin, with regard to their interaction with calcium ions. This section describes the interaction between calcitonin and the opiate antagonist, naloxone. Braga et al (1978) have reported that naloxone does not antagonise the antinociceptive properties of calcitonin. The dose of naloxone used was 1 $mg.kg^{-1}$ and this did not influence the antinociceptive properties of 12 i.u. kg^{-1} calcitonin.

In the following experiments the effects of naloxone, administered either alone or in conjunction with calcitonin, have been studied, using a range of concentrations of each agent.

Results

The i.p. injection of naloxone (0.01 - 10 $mg.kg^{-1}$) produced a significant ($P < 0.05$) increase in the frequency of abdominal constrictions, at the concentrations of 1 and 10 $mg.kg^{-1}$ (Figure 26). Naloxone, at a concentration of 0.01 $mg.kg^{-1}$ was without effect on the antinociceptive actions of calcitonin (Figure 27). However, naloxone at concentrations of 0.1 and 1 $mg.kg^{-1}$ antagonised the antinociceptive properties of calcitonin at all concentrations up to and including 2 i.u. kg^{-1} (Figure 28). The antinociceptive properties of 10 i.u. kg^{-1} calcitonin were only antagonis ed by the administration of 10 $mg.kg^{-1}$ (Figure 29) naloxone.

Discussion

These results show that at high enough doses naloxone possesses hyperalgesic properties. This has been shown by other workers and has been ascribed by Jacobs, Tremblay and Colombel (1974) to be due to antagonism of a prevailing enkephalin tone.

The results are in agreement with those of Braga et al (1978) in that the antinociception induced by calcitonin is independent of the opiate receptor, since the concentration required to antagonise the antinociceptive effects of calcitonin are about 10 - 1000 times those required to antagonise morphine analgesia (Collier et al, 1968). The concentration required to antagonise morphine analgesia, induced after peripheral injection, is 0.01 $mg.kg^{-1}$. The results also show that naloxone will antagonise calcitonin antinociception only at concentrations where either the effect of naloxone is non-specific (and other effects are seen such as anorexia and depression) or at concentrations where naloxone is hyperalgesic itself.

Nonetheless, the action of naloxone on antinociception induced by calcitonin apears to be competitive in nature, since the maximal effective concentration of calcitonin (10 i.u. kg^{-1}) can be antagonised by 10 $mg.kg^{-1}$ naloxone.

In later sections the interaction between calcitonin and the opiate system will be further investigated using an in vitro opiate bioasay and also a non-analgesic opiate system, the role of opiates in the regulation of blood pressure.

Figure 26 Effect of naloxone on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

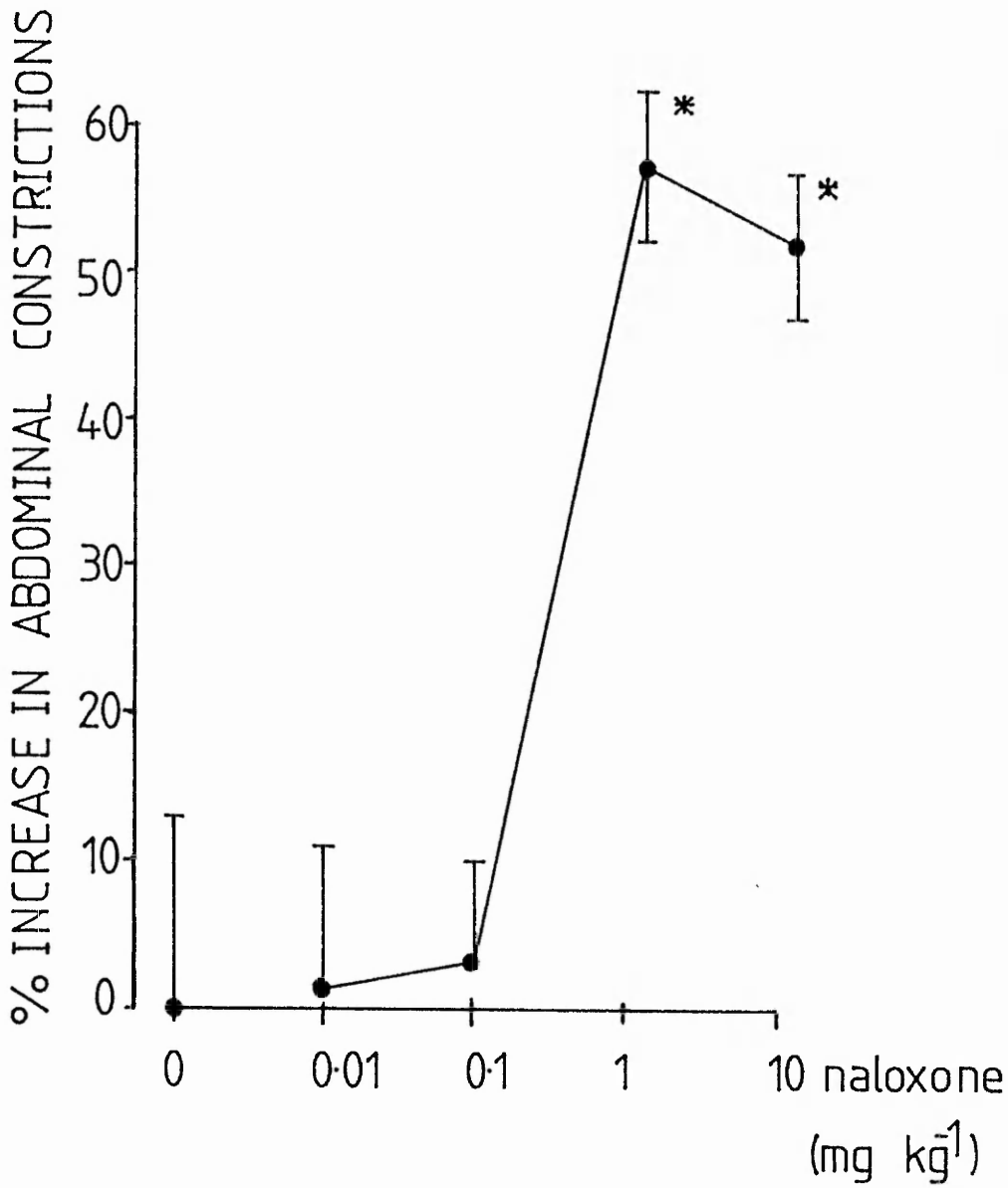

% INCREASE IN ABDOMINAL CONSTRICTIONS
60
50
40
30
20
10
0
*
*
0
0·01
0·1
1
10 naloxone
(mg kg⁻¹)

Figure 26

Figure 27 Effect of salmon calcitonin (2 i.u.kg^{-1}) and naloxone (0.01 - 1 mg.kg^{-1}) on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

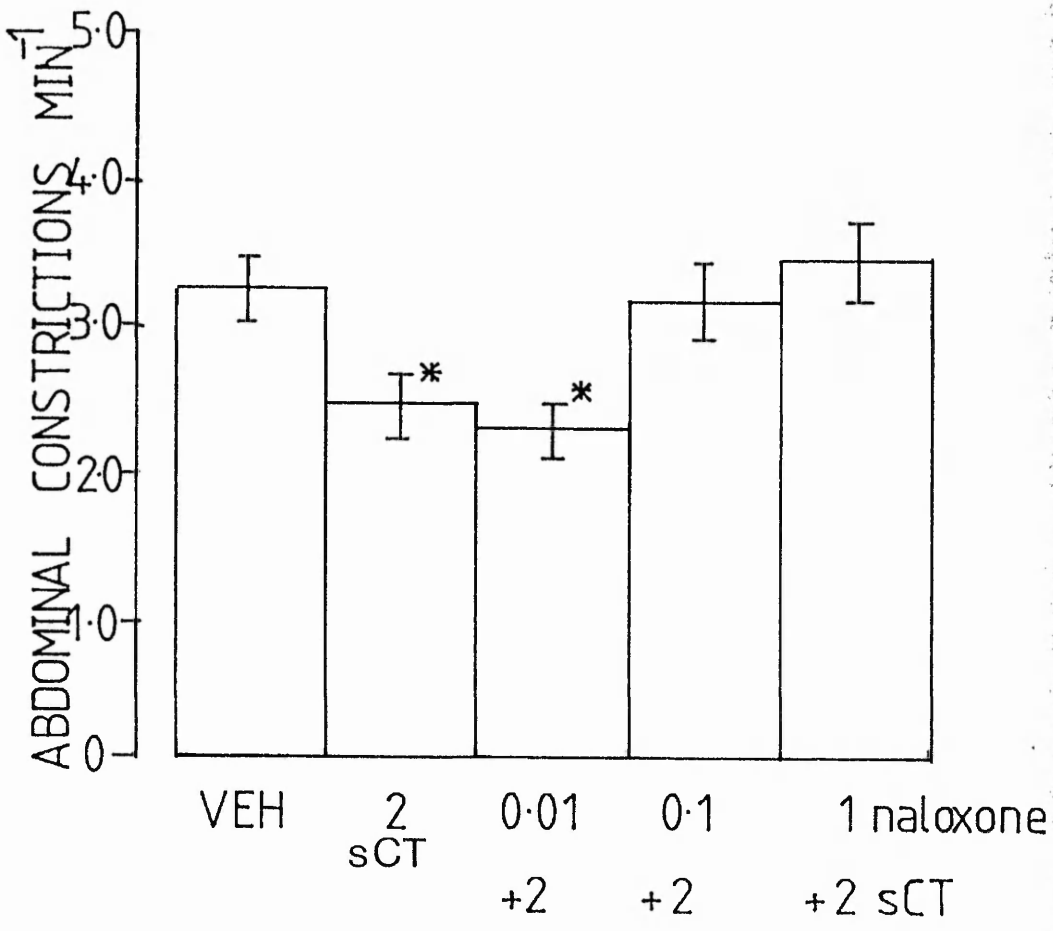

ABDOMINAL CONSTRICTIONS MIN⁻¹
5·0
4·0
3·0
2·0
1·0
0
*
*
VEH
2 sCT
0·01 +2
0·1 +2
1 naloxone +2 sCT

Figure 27

Figure 28 Effect of naloxone (1 $mg.kg^{-1}$) on the antinociceptive response to salmon calcitonin. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

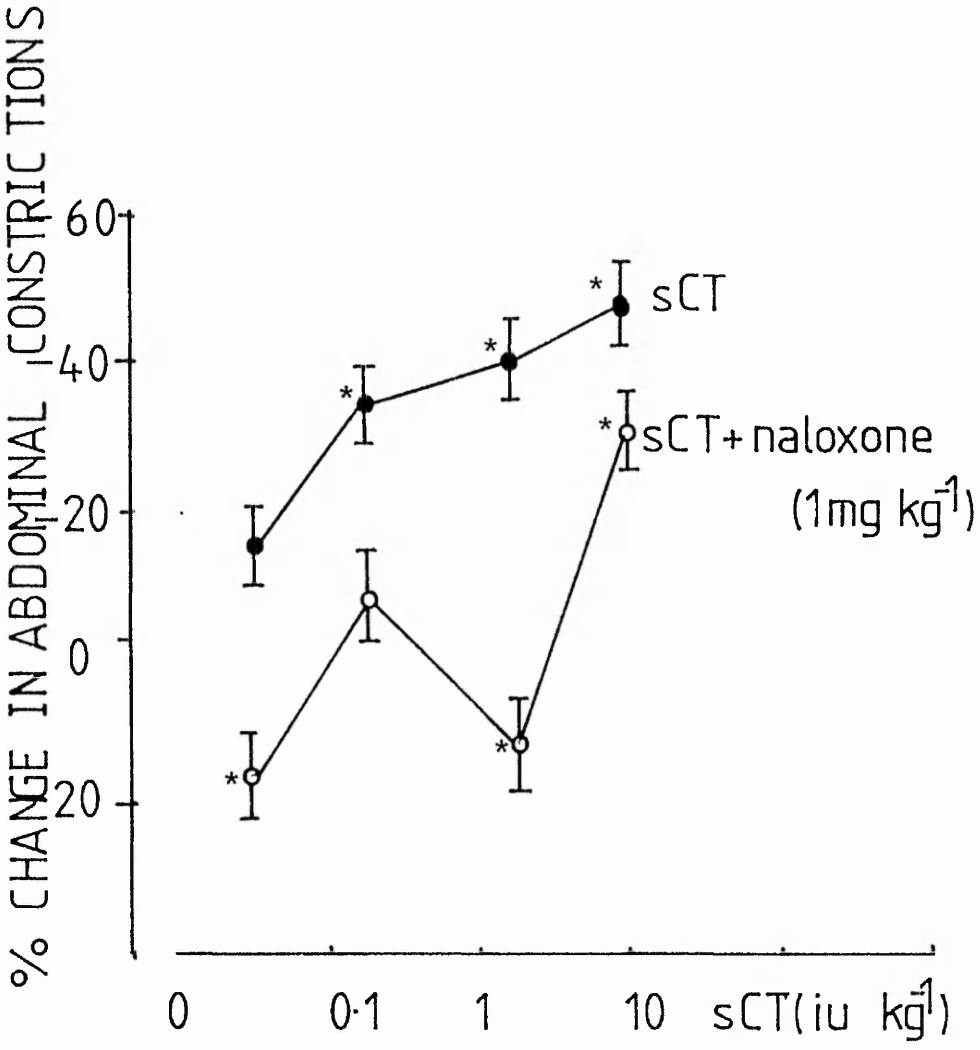

% CHANGE IN ABDOMINAL CONSTRICTIONS
60
40
20
0
20
sCT
sCT+ naloxone
(1mg kg⁻¹)
0
0·1
1
10
sCT(iu kg⁻¹)

Figure 28

Figure 29 Effect of salmon calcitonin (10 i.u. kg^{-1}) and naloxone (10 $mg.kg^{-1}$) on the frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

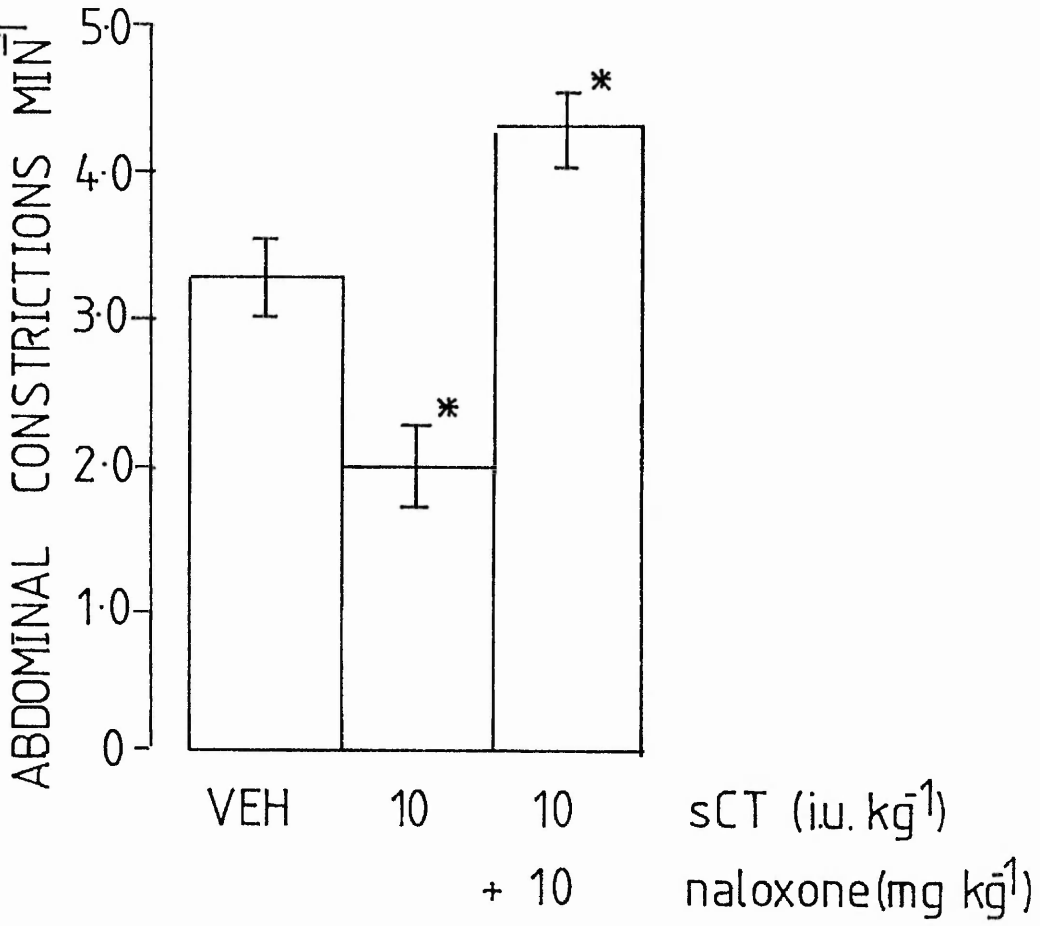

ABDOMINAL CONSTRICTIONS MIN⁻¹
5·0
4·0
3·0
2·0
1·0
0
*
*
VEH
10
10
+ 10
sCT (i.u. kg⁻¹)
naloxone (mg kg⁻¹)

Figure 29

3.8 CHRONIC ADMINISTRATION OF CALCITONIN

The effect of chronic administration of calcitonin on its antinociceptive properties have been little studied, and only then with regard to the i.c.v. route of administration (Pecile et al, 1978). These workers showed that, in contrast to chronic administration of opiates, tolerance does not develop to the effects of calcitonin, even after 5 daily injections of 10 i.u. kg^{-1}. This section describes the results of chronic administration of calcitonin, given by subcutaneous injection. The sensitivity to peritoneal irritation is assessed after a varying number of injections (1 to 8), and at various times after the final injection. In the majority of studies calcitonin was administered on alternate days, for the specified period, or number of injections.

The second part of this section describes the action of calcitonin (0.04 - 10 i.u. kg^{-1}), aspirin (0.125 - 0.5 $mmol.kg^{-1}$), $CaCl_2$ (3.3 $\mu mol.kg^{-1}$), $MgCl_2$ (3.3 $\mu mol.kg^{-1}$) and EGTA (3.3 $\mu mol.kg^{-1}$) administered 48 hours after the 4th s.c. injection of calcitonin. These experiments are designed to study the modulation of the effects of these substances produced by withdrawal of the animals from the calcitonin pretreatment.

Results

(i) Effect of Number of Calcitonin Injections

Animals, pretreated with 1, 2 or 3 s.c. injections of calcitonin (10 i.u. kg^{-1}), and examined at 12, 24, 48 or 96 hours after the final injection, exhibited no changes in the

frequency of abdominal constrictions. However, animals pretreated with 4 injections of calcitonin, and examined at 6, 12, 24, 48 or 96 hours after the final injection, exhibited an progressive increase in the frequency of abdominal constrictions (Figure , 31). This was significantly ($P < 0.05$) greater than the respective vehicle injected animals at 12, 24 and 48 hours. The maximal increase was observed at 48 hours.

The increase in the frequency of abdominal constrictions observed after the 4th injection was dependent upon the dose of calcitonin (0.1 - 10 i.u. kg^{-1}) used during the pretreatment (Figure 32).

Animals examined only 10 minutes after either the 5th or 8th injection of calcitonin (10 i.u.kg^{-1}) were, in contrast, not significantly more sensitive to peritoneal irritation.

(ii) Effect of Pretreatment on Plasma Calcium Concentration

Animals, which had received 4 s.c. injections (10 i.u.kg^{-1}) exhibited a significant reduction ($P < 0.05$) in plasma calcium concentration one hour after the final injection. This was restored to control values 12 and 48 hours after the final injection (Figure 33). (In such animals, examined 24 and 48 hours after the final injection, there was no impairment in motor coordination or in investigative behaviour, see Table 4.)

(iii) Effect of Calcitonin Pretreatment on Calcitonin-Induced Antinociception

The i.c.v. injection of calcitonin (0.04 - 10 i.u. kg^{-1}), in

animals withdrawn for 48 hours from the 4th and final s.c. injection of calcitonin (10 i.u. kg^{-1}), resulted in antinociception which was about 100 fold less effective when compared to animals not pretreated (Figure 34). (EC50 = 0.36 i.u. kg^{-1} in non-treated animals; 66 i.u. kg^{-1} in pretreated animals). In contrast, aspirin (0.125 - 0.5 $mmol.kg^{-1}$) was unaffected with regard to its anti-nociceptive properties (EC50 = 0.44 $mmol.kg^{-1}$ in untreated animals; 0.25 $mmol.kg^{-1}$ in pretreated animals, Figure 345.

(iv) Effect of Calcitonin Pretreatment on the Responses to $CaCl_2$, $MgCl_2$ and EGTA

Animals pretreated with 4 s.c. injections of calcitonin (10 i.u. kg^{-1}) and tested 48 hours after the final injection, were injected i.c.v. with either $CaCl_2$, $MgCl_2$ or EGTA, and their effects compared to their effects in non-treated animals. The response to $CaCl_2$ (3.3 $\mu mol.kg^{-1}$) was not significantly different to its effect in non-treated animals in that the frequency of abdominal constrictions was not increased further. The i.c.v. injection of $MgCl_2$ (3.3 $\mu mol.kg^{-1}$) was without effect on the frequency of abdominal constrictions again when compared to animals injected with vehicle. The i.c.v. injection of EGTA (3.3 $\mu mol.kg^{-1}$) was less effective in reducing the frequency of abdominal constrictions when compared to its effect in non-treated animals (Figure 36).

Discussion

The increased sensitivity to peritoneal irritation observed after chronic calcitonin pretreatment differs from the

withdrawal hyperalgesia observed when animals are withdrawn from chronic morphine pretreatment, in that no gross behavioural disturbances are seen. There are no signs of the 'wet-dog shake' syndrome or of jumping behaviour (Collier, 1964). That the increased sensitivity to peritoneal irritation is due to calcitonin withdrawal specifically is reflected in the fact that a further s.c. injection of calcitonin abolishes this increase in sensitivity at 10 minutes after the injection.

There is circumstantial evidence to suggest that the phenomenon, though induced by withdrawal from chronic peripheral pretreatment, is a central effect possibly mediated by activating neuronal systems, either inside or outside the blood brain barrier. Firstly, the time course of the response development is of long duration (6 - 48 hours) and is not accompanied by a prolonged effect on plasma calcium concentration. Secondly, the antinociceptive effects of calcitonin (after i.c.v. injection) are antagonised by injecting into animals withdrawn from calcitonin pretreatment. However, the antinociceptive effects of aspirin are unaffected. Since calcitonin acts centrally to induce antinociception whilst aspirin acts at the periphery, this would suggest that the withdrawal hyperalgesia is due to the activation of neuronal systems.

However, such conclusions present problems in that one is postulating a potent effect of repeated peripheral administration of calcitonin on the CNS - or more specifically on the areas susceptible to centrally

administered calcitonin. Other workers (e.g. Levine and Morley, 1981; Iwasaki, Chirara, Iwasaks, Abe and Fujita, 1979) have shown that calcitonin can affect CNS function. The difference, in these cases, is that the effect of centrally injected calcitonin is similar to the effect of the peripherally administered calcitonin, albeit at much lower concentrations.

In the case of the chronically administered calcitonin, the development of 'tolerance' does not occur with repeated central injection. It is important to define 'tolerance' in this context. In animals withdrawn from chronic calcitonin pretreatment, the animals are less sensitive to the effects of centrally administered calcitonin. However, the animals are not tolerant in the usual sense of the term since it is only the withdrawal hyperalgesia which causes this desensitisation. One explanation may be that the chronic administration of calcitonin under the regime used by Pecile et al (1978) was only maintained for 5 days. The development of 'tolerance' after chronic peripheral administration requires at least 10 days. Alternatively, the mechanism in the development of hyperalgesia may be unrelated to the central effect of calcitonin. The prolonged peripheral administration of calcitonin may mobilise the other agents involved in the regulation of body calcium concentration, i.e. PTH and/or vitamin D may be mobilised as a compensatory mechanism in response to the continual lowering of plasma calcium. It will be shown (see Discussion) that both of these substances can influence

CNS calcium metabolism, and the existence of a vitamin D binding protein (Bainbridge and Owen Parkes, 1981) in the CNS may suggest a central action for the active metabolite of this vitamin. In fact, the lipophilic structure of vitamin D and its active metabolites would make this substance a suitable candidate to cross the blood brain barrier and influence CNS activity.

The exact mechanism by which the increase in sensitivity to peritoneal irritation occurs may also involve calcium. This derives from experiments in which calcium, magnesium or EGTA were injected by i.c.v. injection in animals withdrawn from chronic calcitonin pretreatment. Chapman and Way (1980) have suggested that during chronic opiate pretreatment the neuronal calcium increases as a homeostatic response to the acute depletion after every opiate injection. Consequently, sudden withdrawal of opiates renders the animals hyperalgesic, because of the elevated neuronal calcium. In animals withdrawn from chronic calcitonin pretreatment, the i.c.v. injection of calcium at a concentration (which in non-treated animals produces an increased sensitivity to noxious stimuli) does not produce an additional increase in the sensitivity to peritoneal irritation. This may be a reflection of the fact that the cells of the CNS (not necessarily the neurones) already have an increased intracellular calcium concentration. The converse may be true when EGTA is administered into pretreated animals. The already elevated cellular calcium concentration offsets the reduction in

intracellular calcium caused by EGTA and the antinociception is less evident. It is interesting to note that animals rendered tolerant to morphine are also tolerant to EGTA (Chapman and Way, 1980). In contrast to the results obtained here, animals rendered tolerant to morphine exhibit increased sensitivity to noxious stimuli when calcium ions are injected by i.c.v. injection. The i.c.v. injection of magnesium ions is without effect in either treated or non-treated animals, which again underlines the specificity to the calcium ions in this process.

Figure 30 Effect of the number of salmon calcitonin injections (s.c. 10 i.u. kg^{-1}) on the frequency of abdominal constrictions. The animals were assessed 48 hours after the final injection. Values are mean ± s.e.m.; $n = 10$ $*p < 0.05$.

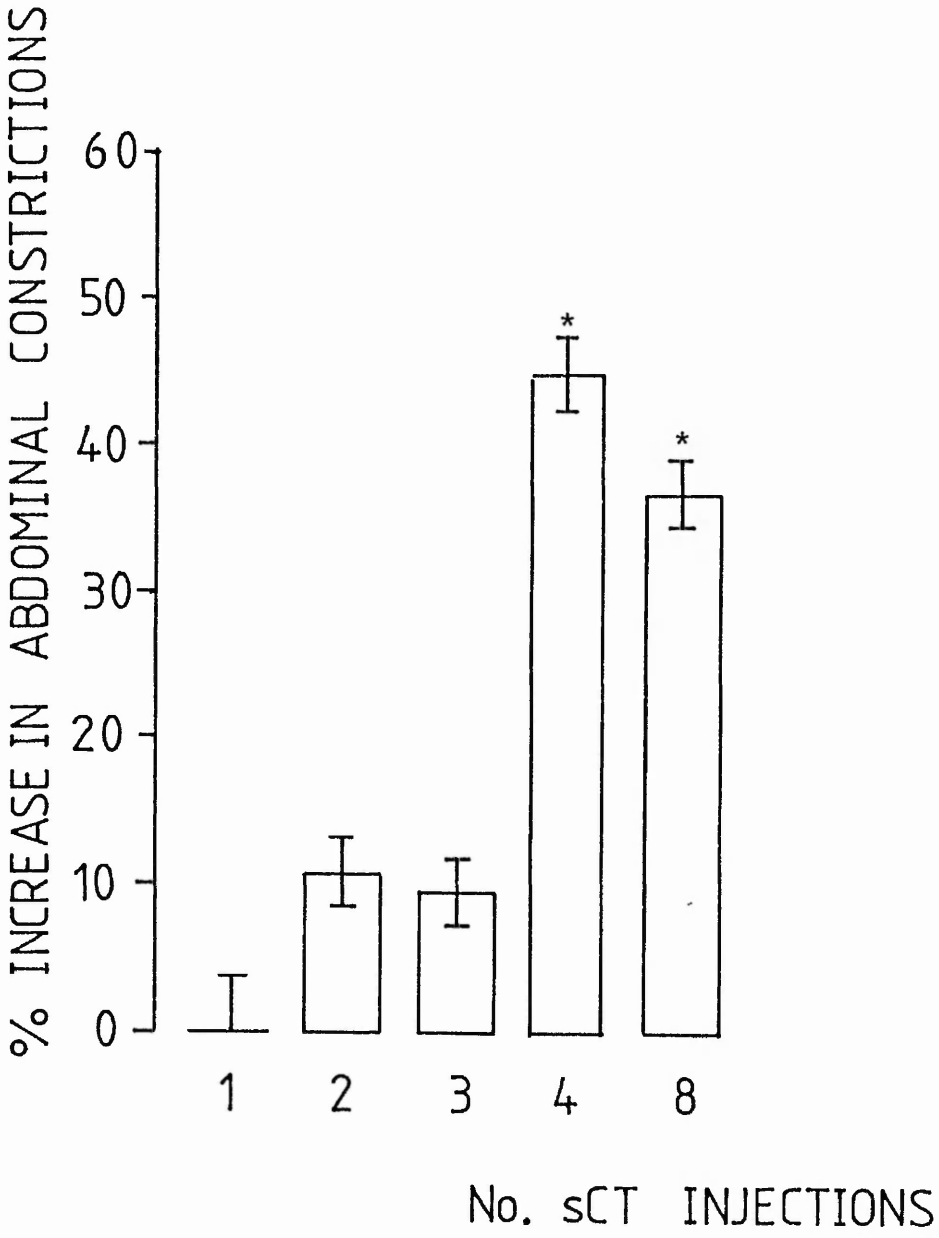

% INCREASE IN ABDOMINAL CONSTRICTIONS
60
50
40
30
20
10
0
*
*
1
2
3
4
8
No. sCT INJECTIONS

Figure 30

Figure 31 Time course of the hyperalgesic response to 3 and 4 injections of salmon calcitonin (s.c. 10 i.u. kg^{-1}). Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

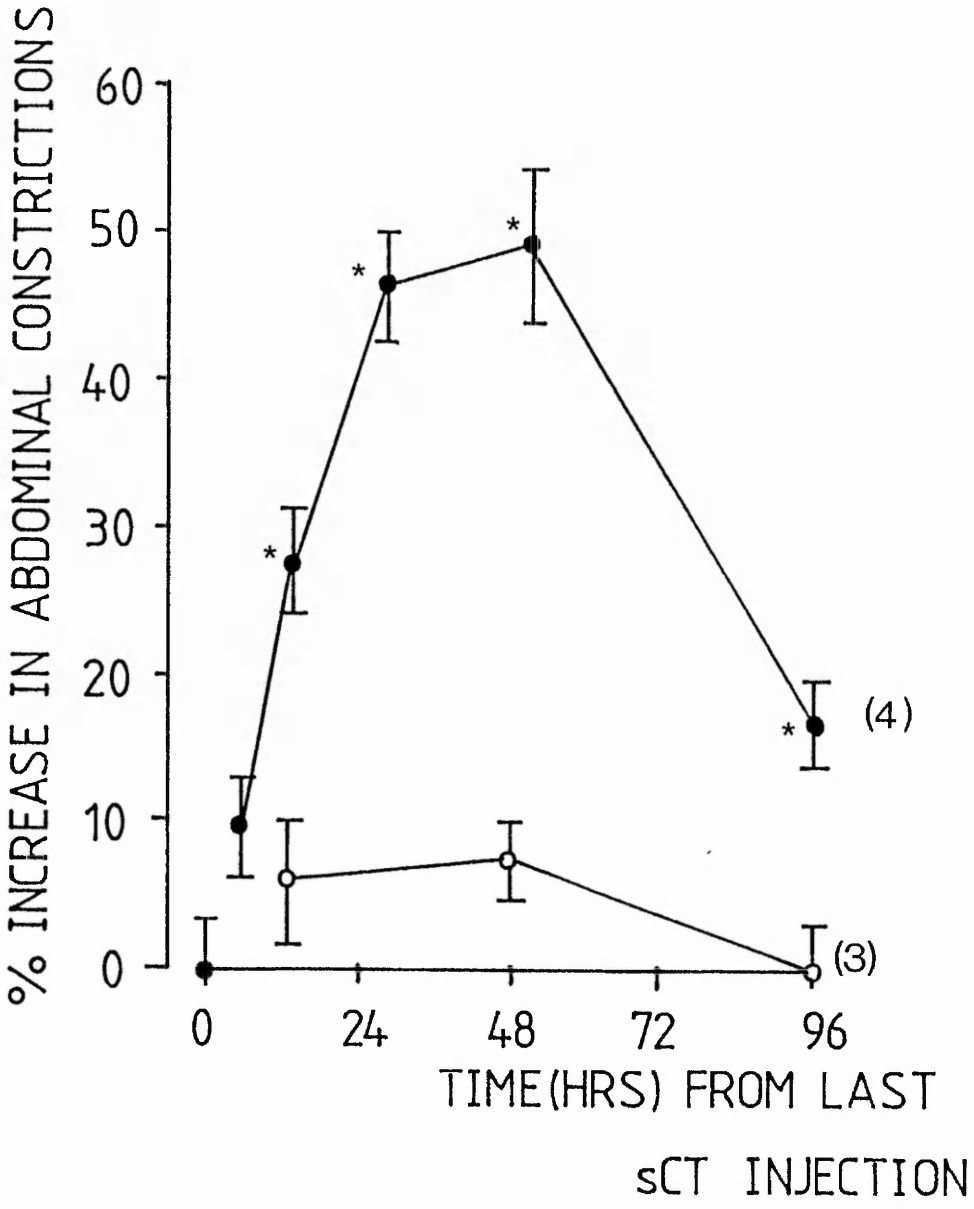

% INCREASE IN ABDOMINAL CONSTRICTIONS
60
50
40
30
20
10
0
0
24
48
72
96
TIME(HRS) FROM LAST
sCT INJECTION
(4)
(3)
*
*
*
*

Figure 31

Figure 32 Effect of salmon calctonin pretreatment (4 s.c. injections, animals examined 48 hours after final injection) and frequency of abdominal constrictions. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

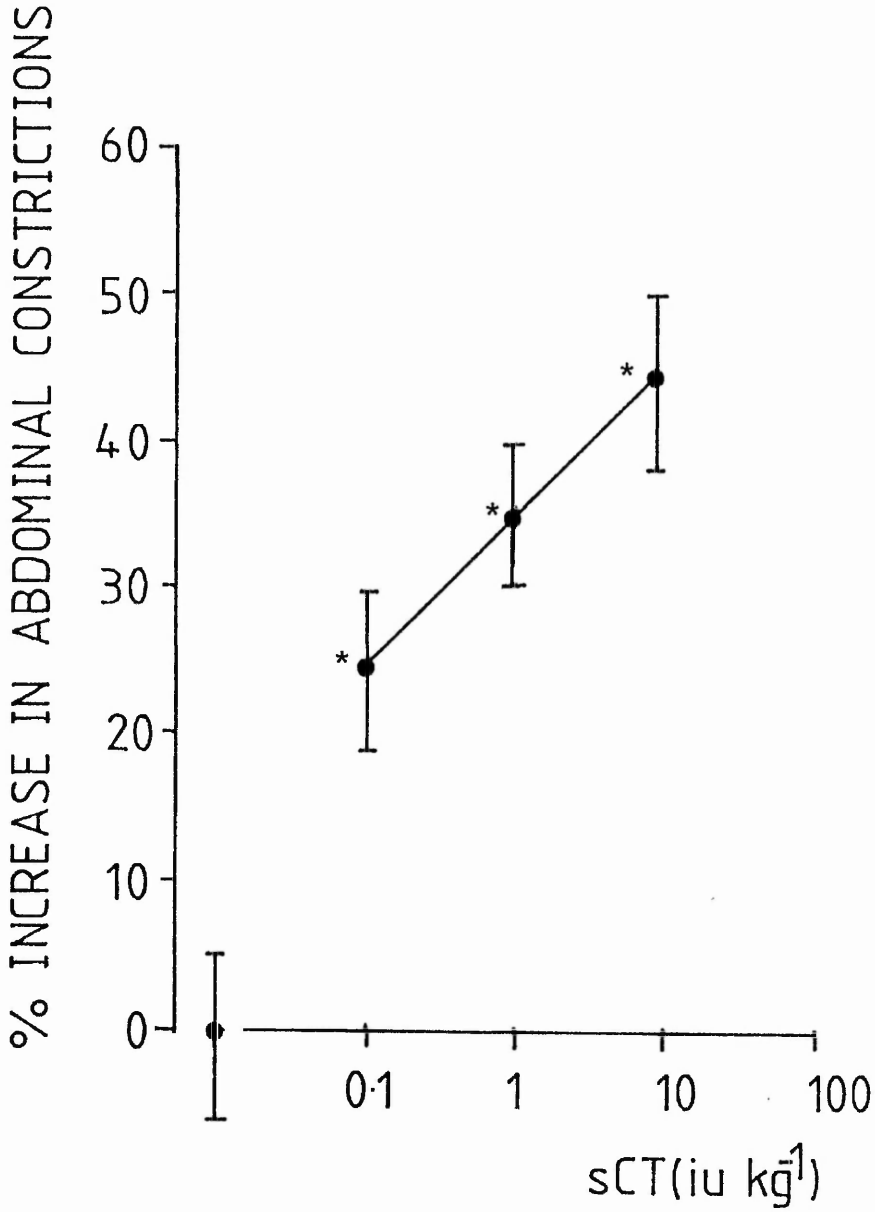

% INCREASE IN ABDOMINAL CONSTRICTIONS
60
50
40
30
20
10
0
0·1
1
10
100
sCT(iu kg⁻¹)
*
*
*

Figure 32

Figure 33 Effect of salmon calcitonin pretreatment (4 s.c. injections) on the concentration of plasma calcium. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

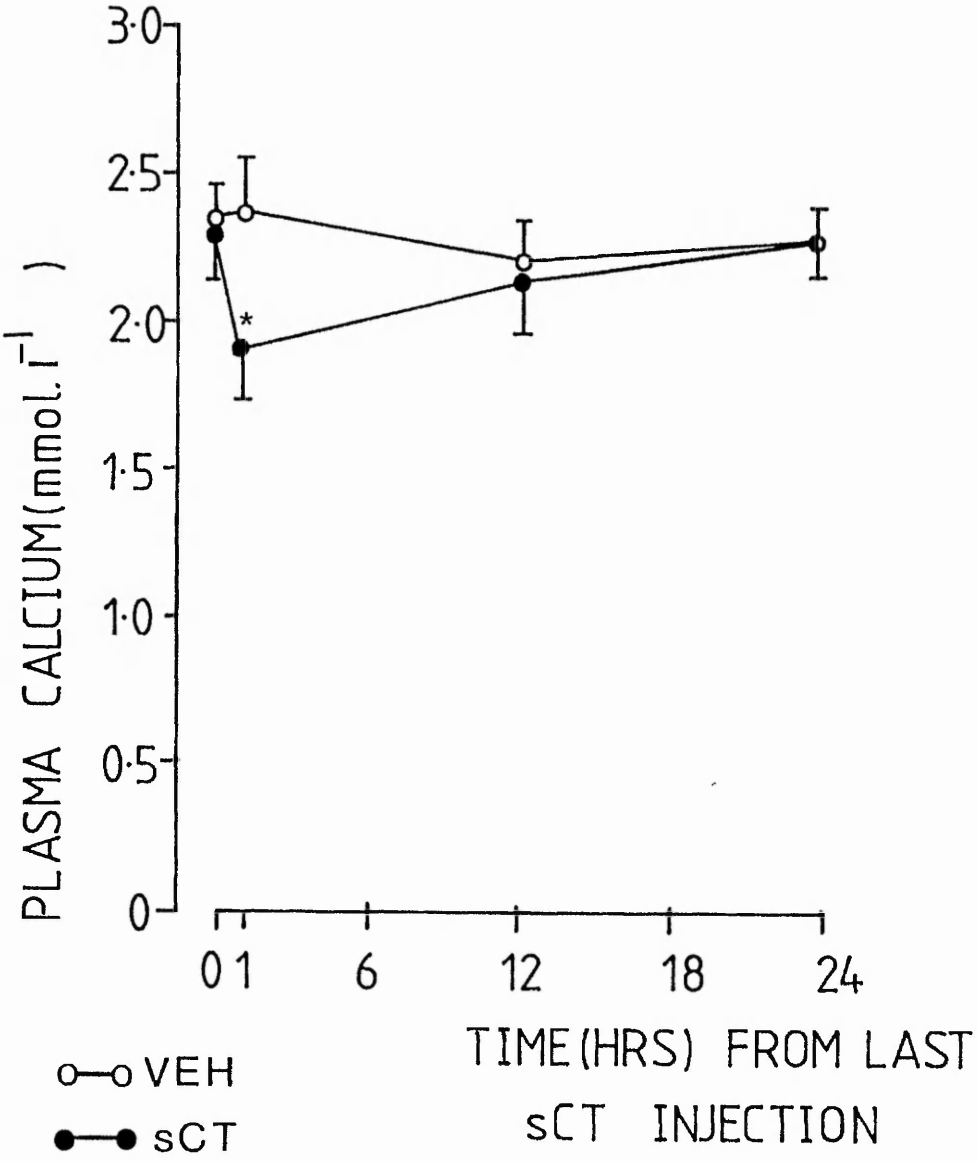

3·0
2·5
2·0
1·5
1·0
0·5
0
PLASMA CALCIUM(mmol.l⁻¹)
*
0 1
6
12
18
24
TIME(HRS) FROM LAST
sCT INJECTION
VEH
sCT

Figure 33

Figure 34 Effect of salmon calcitonin pretreatment (4 s.c. injections, animals examined 48 hours after final injection) on the antinociceptive response to salmon calcitonin. Values are mean ± s.e.m.; n = 10 *$p < 0.05$.

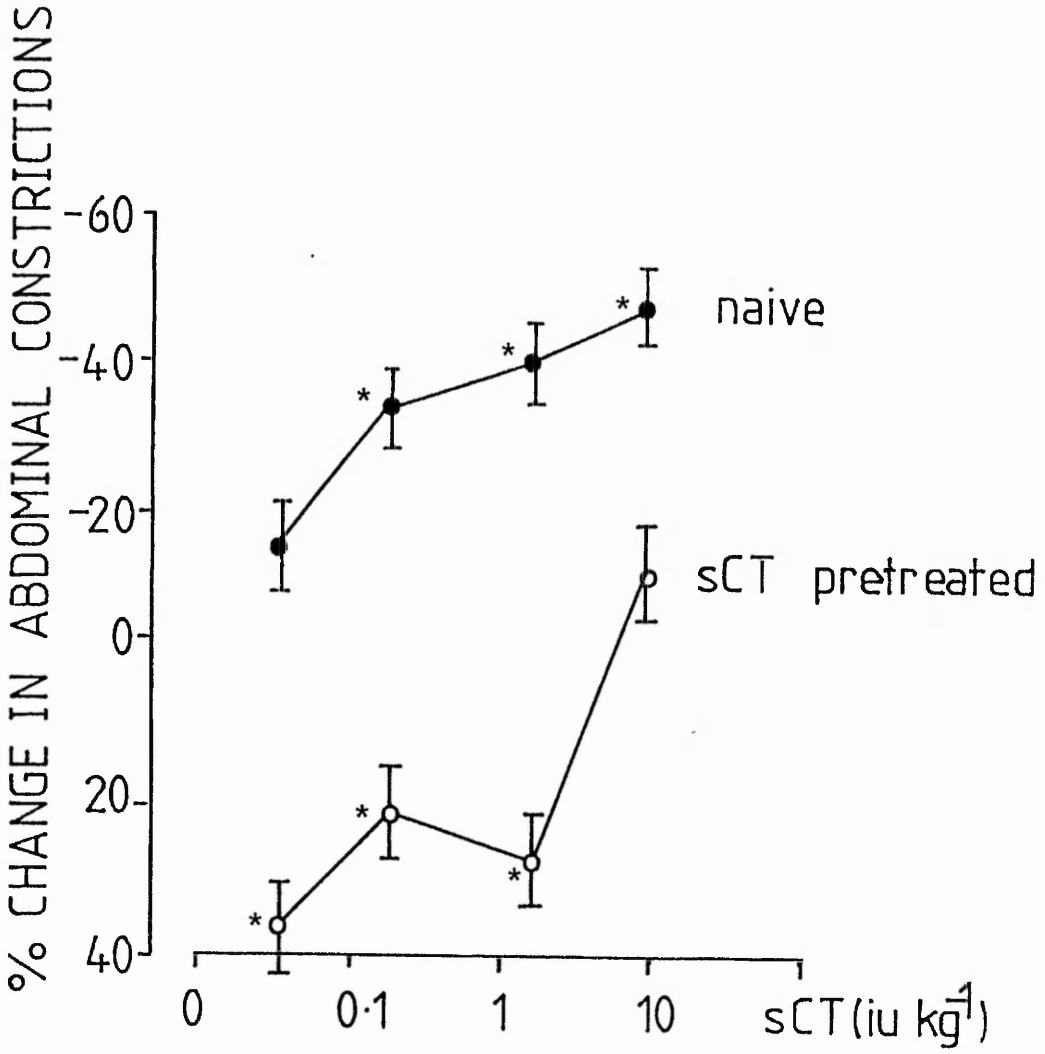

% CHANGE IN ABDOMINAL CONSTRICTIONS
-60
-40
-20
0
20
40
naive
sCT pretreated
0
0·1
1
10
sCT (iu kg⁻¹)

Figure 34

Figure 35 Effect of salmon calcitonin pretreatment (4 s.c. injections, animals examined 48 hours after final injection) on the antinociceptive response to aspirin. Values are mean ± s.e.m.; n = 10 *p < 0.05.

significantly different from vehicle injected animals.

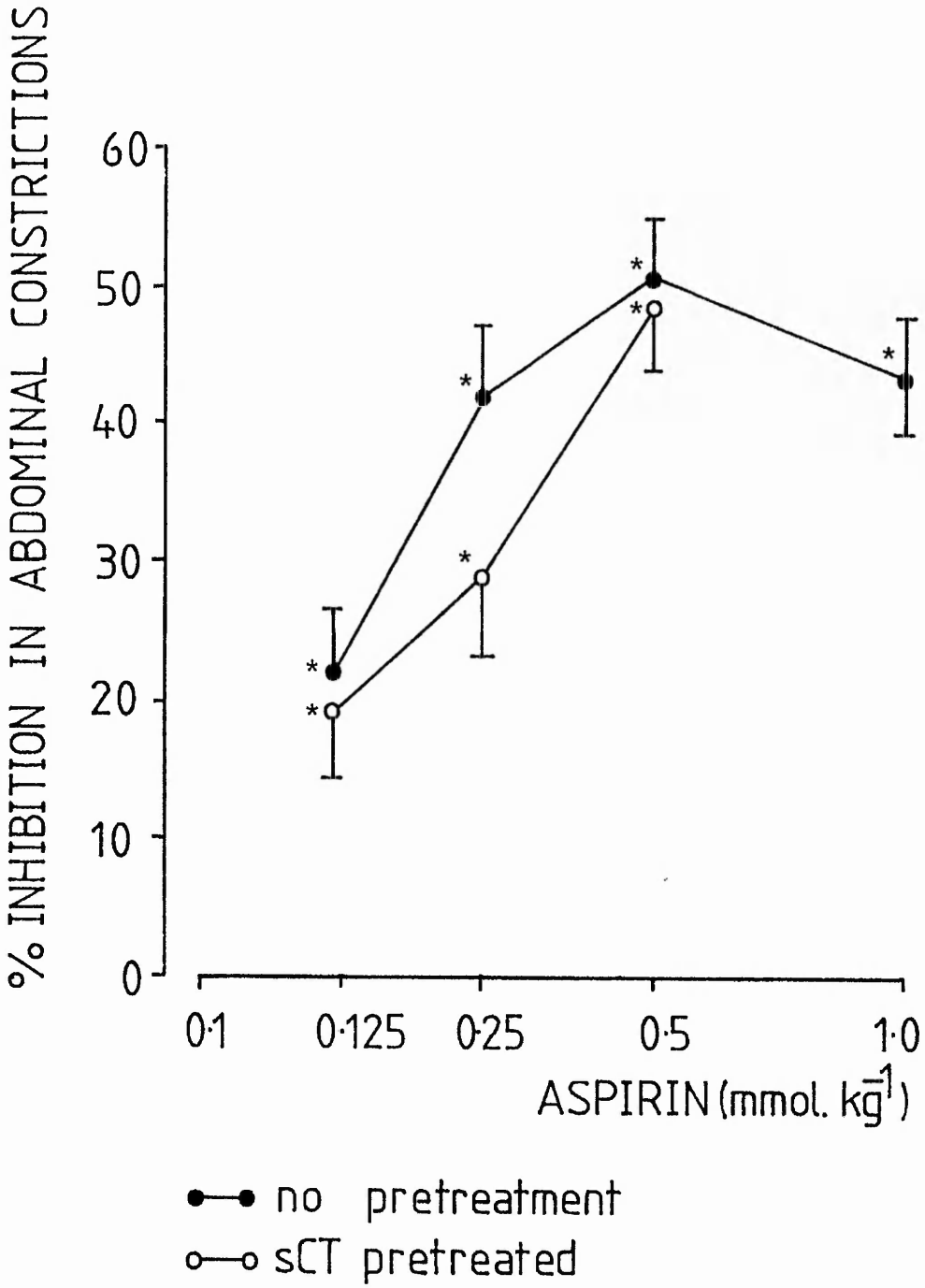

% INHIBITION IN ABDOMINAL CONSTRICTIONS
60
50
40
30
20
10
0
0·1
0·125
0·25
0·5
1·0
ASPIRIN (mmol. kg⁻¹)
no pretreatment
sCT pretreated

Figure 35

Figure 36 Effect of salmon calcitonin pretreatment (4 s.c. injections, animals examined 48 hours after final injection) on the response to $CaCl_2$ (3.3 $\mu mol.kg^{-1}$), $MgCl_2$ (3.3 $\mu mol.kg^{-1}$) and EGTA (3.3 $\mu mol.kg^{-1}$). Values are mean ± s.e.m.; n = 10 *$p < 0.05$

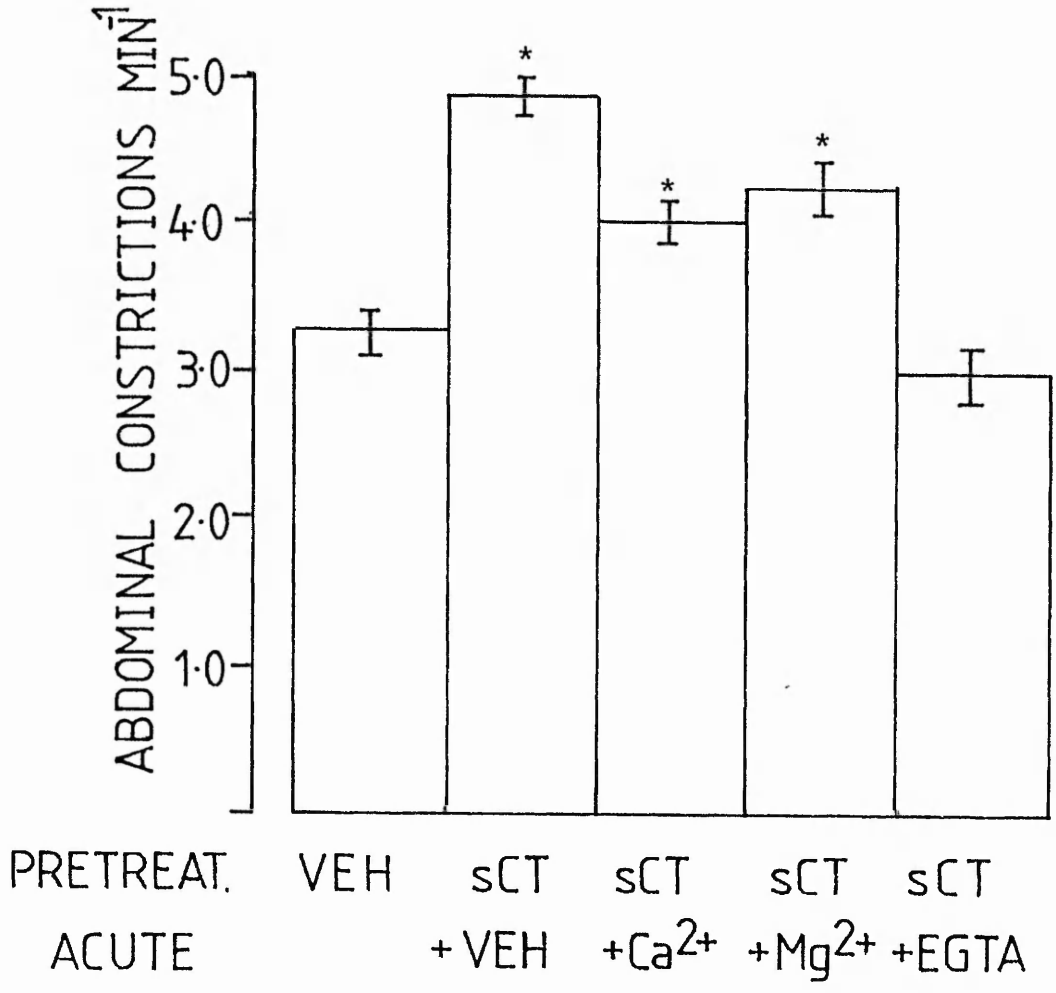

ABDOMINAL CONSTRICTIONS MIN⁻¹
5·0
4·0
3·0
2·0
1·0
*
*
*
PRETREAT.
ACUTE
VEH
sCT
+VEH
sCT
+Ca2+
sCT
+Mg2+
sCT
+EGTA

Figure 36

3.9 EFFECT OF CALCITONIN AND CALCIUM ANTAGONISTS ON THE TOTAL TISSUE CALCIUM CONTENT OF THE BRAIN

The action of the opiates on the total brain tissue calcium concentration has been extensively studied (Ross, Medina and Cardenas, 1974; Cardenas and Ross, 1975; Yamamoto, Harris, Loh and Way, 1878). These workers have shown that the acute administration of morphine results in a reduction in the total tissue calcium concentration in all regions. This effect is also reversible by opiate antagonists (Ross, Lynn and Cardenas, 1976). The reduction in calcium concentration is observed using synaptosomes and also preparations of synaptosomal membranes (Ross, Lynn and Jones, 1976; Ross, 1977). However, this effect is not restricted to the opiates since reserpine (Ross et al, 1976) and pentobarbital (Yamamoto et al, 1978) have been shown to reduce total brain tissue calcium concentration.

Conversely, animal models of addiction have been shown to possess elevated total brain tissue calcium concentration (Ross et al, 1976). Again this is evident when using synaptosomes from chronically addicted animals (Ross, 1977).

This section describes the effect of acute administration of calcitonin and the calcium 'slow channel blocking' agents, nifedipine, PY 108-068 and lanthanum on the total tissue calcium concentration of the CNS. The technique in this thesis, though known for some time, differs from the more usual method of extraction of calcium by concentrated nitric and perchloric acids (e.g. Ross, 1977) in that calcium is extracted using trichloroacetic acid

after the method of Lolley (1963).

The technique also uses an automated titration procedure to determine the calcium concentration. In the majority of studies the calcium concentration is determined using atomic absorption techniques. Therefore, an appreciable part of this section is devoted to determining the characteristics of the technique. In addition, reserpine has been used a positive control as an agent which has been shown by Ross et al (1974) to alter the calcium concentration of the brain.

Results

The sensitivity of the calcium analyser (CORNING 940) was found to be sufficient to assay calcium in the range of concentrations expected from the tissue samples. The relationship between the calculated values and the observed values was linear throughout the whole concentration range with no deviation at the lowest concentration (Figure 37).

Tissue samples were analysed using both fluorometric titration and atomic absorption techniques, in order to compare the sensitivity of the assays. These results are shown in Table 5, and statistical analysis shows no significant difference between the two techniques.

A recovery experiment was also performed, in order to check the extraction procedure. Samples of tissue to which a known quantity of calcium was added, showed a recovery of 110 ± 6.4 % (mean ± s.e.) which is considered to be within acceptable limits.

Intracerebroventricular injection of $CaCl_2$

(3.3 mmol.kg^{-1}) resulted in an elevation of cortical calcium concentration of 0.25 µmol.gww^{-1}. This compares with a calculated value of 0.20 µmol.gww^{-1}, assuming that the injected solution remains in the lateral ventricles.

The reproducibility of the analysis of (i) replicate analysis of aliquots from the same sample and (ii) analysis of aliquots from different samples are shown in Table 6. The standard error in each case was less than 10%, and again is considered to be within acceptable limits.

The s.c. injection of reserpine (8 mg.kg^{-1}) was used as a positive control. Analysis of tissue samples taken 2 hours after injection showed no significant change in the calcium concentration. However, at 18 hours after injection, the calcium concentration had declined in all regions (Table 7). These results parallel the changes observed in the behaviour of the animals; at 2 hours the animals were relatively unaffected by the reserpine, whereas at 18 hours after administration the animals were heavily sedated. The core temperature of the animals had decreased by 8 ± 2.1^{o}C (mean ± s.e.; n = 6).

The i.c.v. injection of calcitonin (0.5 - 20 i.u. kg^{-1}) was without effect on the calcium concentration, in any of the regions studied. (Samples were taken at 15 minutes after the i.c.v., injection which corresponds to the time at which the antinociceptive testing was undertaken.) The s.c. injection of calcitonin (10 i.u. kg^{-1}) was also without effect on the calcium concentration (Table 7).

The i.c.v. injection of nifedipine (3.3 µmol.kg),

PY 108-068 (3.3 $\mu mol.kg^{-1}$) or lanthanum chloride (3.3 $\mu mol.kg^{-1}$) produced no consistent change in the brain calcium concentration, although all these agents at these concentrations are antinociceptive when administered by i.c.v. injection. All three agents, produced no significant change in the calcium concentration in the brain stem but a significant increase in the mid brain. $LaCl_3$ and PY 108-068 produced an increase in the cerebellum, whilst nifedipine produced a decrease. $LaCl_3$ and nifedipine produced a decrease in the hypothalamus, whilst PY 108-068 produced an increase. Finally, PY 108-068 was without effect in the cortex, whilst $LaCl_3$ and nifedipine produced an increase.

Discussion

These experiments were designed to show that the assay was comparable to the existing literature. One can conclude that the assay is reproducible, gave good recovery of calcium and was sensitive to a range of calcium concentrations. The method was also comparable to the usual method of calcium analysis, i.e. atomic absorption.

The use of reserpine as a positive control agreed with other workers (Ross et al, 1974) in that a reduction in calcium concentration occurred in all regions of the brain. However, the results were at variance with other workers in that 18 hours was required to produce a fall in the calcium concentration (Ross et al, 1974, observed a fall after 30 minutes).

The action of calcitonin seemed to differ from

the opiates in that it did not affect the calcium concentration of the brain, even at concentrations which are supramaximal antinociceptive doses (20 $i.u.kg^{-1}$). As noted in the Introduction, there are few studies concerning the effect of calcitonin on the calcium concentration of the CNS. However, Stekol'nikov and Abduraminov (1969) reported that calcitonin, administered i.p. or i.v. (no dose was mentioned), produced a rise in the calcium concentration of the CSF. Carman and Wyatt (1979) have reported converse results in that i.m. injection of calcitonin (2 $i.u.\ kg^{-1}$) produced a reduction in the CSF calcium concentration. In both studies the changes seen were small but significant.

Experiments which measure the total tissue calcium concentration of the brain would not detect any change in the calcium concentration of the CSF, since any change would be 'masked' by changes in the surrounding tissue. It is, in fact, an important criticism of this experiment that the technique allows no conclusions to be made concerning any potential specific changes in the calcium concentration of either the CSF or the brain tissue. All that can be concluded is that the action of the hormone differs from the action of the opioids. This conclusion may be reflected in the evidence accumulated in other work (e.g. Braga et al, 1978), that the hormone acts in a fashion independent of the opiate system.

The results obtained using the calcium antagonists in this experiment are confusing and allow no definite conclusions to be drawn. More concentrations of these

agents would need to be administered before any definite trends may be seen.

Figure 37 Comparison of observed with calculated values for analysis of standard $CaCl_2$ solutions using CORNING 940 calcium analyser.

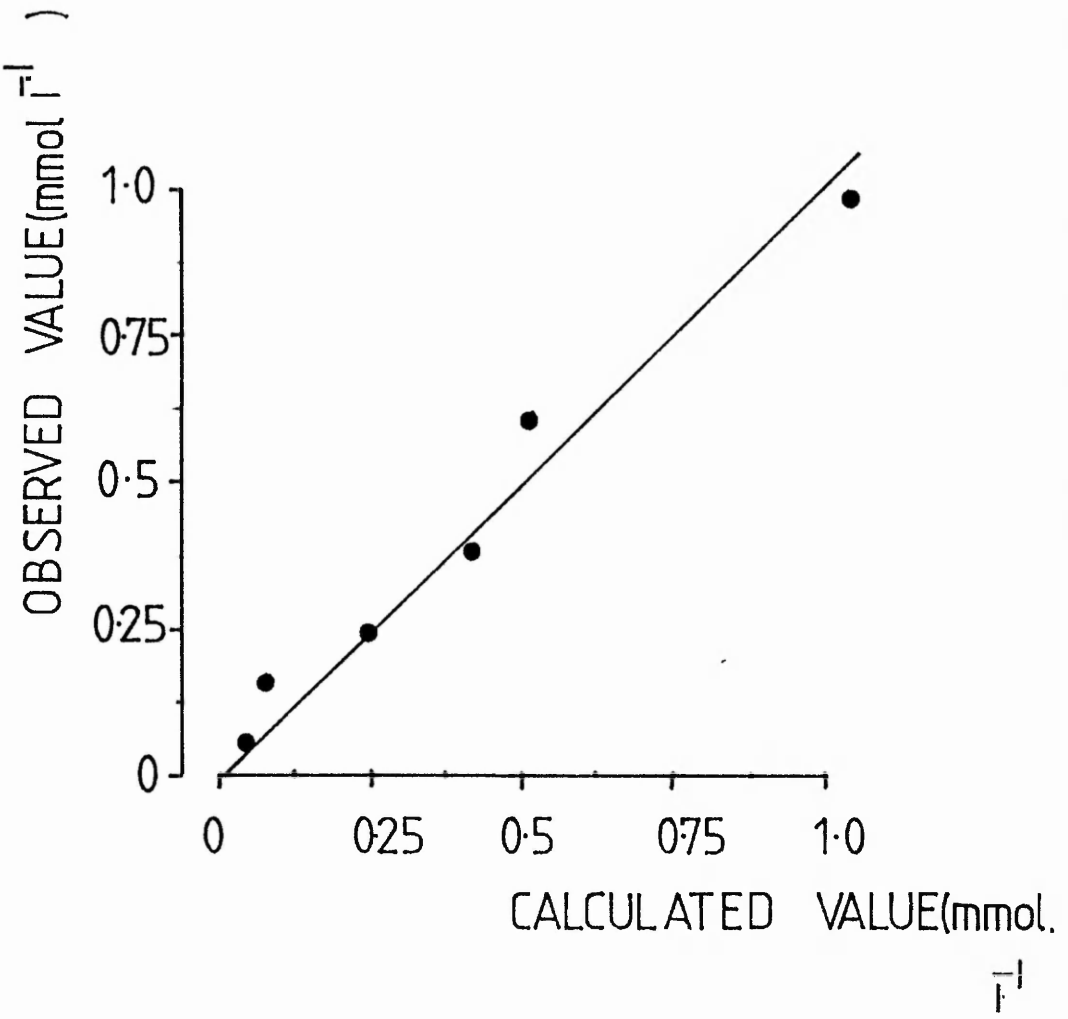

OBSERVED VALUE(mmol l⁻¹)
1·0
0·75
0·5
0·25
0
0
0·25
0·5
0·75
1·0
CALCULATED VALUE(mmol. l⁻¹)

Figure 37

Table 5 Total Brain Calcium Concentration of Mouse Brain Regions as assessed by Fluorometric Titration and by Atomic Absorption Techniques

Region	Calcium Concentration ($\mu mol.g.w.w.^{-1}$)	
	Fluorometric Titration	Atomic Absorption
Cerebellum	1.40 ± 0.14	1.38 ± 0.23
Brain Stem	1.28 ± 0.04	1.34 ± 0.10
Cortex	1.12 ± 0.02	1.15 ± 0.05
Mid Brain	1.17 ± 0.08	1.20 ± 0.10

Table 5 Comparison of analysis of the total tissue calcium concentration as assessed by either fluorometric titration or by atomic absorption techniques, from the same sample. Values are mean ± s.e.m.; n = 8-10.

Table 6 Replicate Analysis of Total Calcium Concentration of Mouse Brain

(i) Replicate analysis from same sample

Region	Calcium Concentration ($\mu mol.g.w.w^{-1}$)
Cerebellum	1.39 ± 0.06
Brain Stem	1.27 ± 0.05
Hypothalamus	1.26 ± 0.10
Cortex	1.22 ± 0.07
Mid Brain	1.16 ± 0.03

(ii) Replicate analysis from different samples

Region	Calcium Concentration ($\mu mol.g.w.w^{-1}$)
Cerebellum	1.28 ± 0.10
Brain Stem	1.18 ± 0.08
Hypothalamus	1.26 ± 0.05
Cortex	1.32 ± 0.10
Mid Brain	1.29 ± 0.03

Table 6 Replicate analysis from same sample and from different samples. Values are mean ± s.e.m. n = 5.

Table 7 Effect of Calcitonin and Reserpine on the Total Calcium Concentration of Mouse Brain

Route of Injection and Treatment	Region	Calcium Concentration ($\mu mol.g.w.w.^{-1}$) Test	(Vehicle)
Calcitonin	Cerebellum	1.48 ± 0.09	(1.58 ± 0.05)
(0.5 i.u. kg^{-1}	Brain Stem	1.85 ± 0.08	(1.68 ± 0.05)
i.c.v.)	Hypothalamus	1.62 ± 0.10	(1.52 ± 0.13)
	Cortex	1.38 ± 0.04	(1.34 ± 0.06)
	Mid Brain	1.52 ± 0.05	(1.41 ± 0.03)
(10 i.u. kg^{-1}	Cerebellum	1.53 ± 0.04	(1.62 ± 0.05)
i.c.v.)	Brain Stem	1.56 ± 0.05	(1.53 ± 0.05)
	Hypothalamus	1.53 ± 0.08	(1.67 ± 0.06)
	Cortex	1.35 ± 0.04	(1.33 ± 0.02)
	Mid Brain	1.45 ± 0.04	(1.46 ± 0.03)
(20 i.u. kg^{-1}	Cerebellum	1.64 ± 0.07	(1.55 ± 0.05)
i.c.v.)	Brain Stem	1.60 ± 0.04	(1.68 ± 0.05)
	Hypothalamus	1.50 ± 0.07	(1.64 ± 0.10)
	Cortex	1.40 ± 0.03	(1.43 ± 0.05)
	Mid Brain	1.48 ± 0.05	(1.48 ± 0.05
(10 i.u. kg^{-1}	Cerebellum	1.39 ± 0.06	(1.35 ± 0.14)
s.c.)	Brain Stem	1.27 ± 0.05	(1.27 ± 0.04)
	Hypothalamus	1.26 ± 0.06	(1.31 ± 0.02)
	Cortex	1.22 ± 0.07	(1.34 ± 0.08)
	Mid Brain	1.19 ± 0.08	(1.16 ± 0.06)

Table 7 Continued

Route of Injection and Treatment	Region	Calcium Concentration ($\mu mol.g.w.w.^{-1}$) Test (Vehicle)
Reserpine	Cerebellum	1.34 ± 0.12*(1.74 ± 0.04)
(8 $mg.kg^{-1}$	Cortex	1.24 ± 0.02*(1.57 ± 0.05)
s.c. 20 hrs	Remainder	1.18 ± 0.05*(1.42 ± 0.08)
post injection)		

Table 7 Effect of calcitonin and reserpine on the total calcium concentration of the mouse brain. Animals treated with vehicle are shown in parenthesis. Values are mean ± s.e.m.; n = 6-10 *$p < 0.05$ from vehicle treated animals tested concurrently.

3.10 EFFECT OF CALCITONIN, NALOXONE, SODIUM AZIDE AND LANTHANUM ON THE UPTAKE OF ^{45}CALCIUM IN BRAIN SLICES

This section is similar to the previous section in that calcitonin is applied to a system which has been shown to be affected by the opiates. In both slices and synaptosomes, the opiates have been shown to inhibit the uptake of calcium in either stimulated or non-stimulated tissues (Kakunaga et al, 1966; Guerro-Munoz, Cerrate, Guerrero and Way, 1979). This has been postulated by Chapman and Way (1980) to be the mechanism by which the opiates reduce the calcium concentration in the neuronal terminal.

In this section, the effect of calcitonin has been investigated using both stimulated and non-stimulated tissues. Levine and Morley (1981) have shown that calcitonin inhibits the uptake of calcium into explants of hypothalami, although no experiments were undertaken using potassium stimulated preparations.

In addition, a number of experiments have been undertaken to characterise the system used, i.e. the dependence of ^{45}calcium uptake on time, concentration, temperature and region of the brain. In addition, the method was further studied by varying the preincubation time and washing time. The problem of adequate preincubation time with either the Krebs solution or the drug to be used and the consequent effect on uptake have already been discussed, in connection with the work of Borle (1974a,b). Borle's work stresses the importance of undertaking experiments under steady state conditions.

In this work, two positive controls will be used: lanthanum, an agent which displaces calcium binding to the cells, and sodium azide, an agent which inhibits the efflux of calcium by inhibiting the sodium-calcium exchange pump (Cooke and Robinson, 1971).

Results

The reproducibility of the slicing procedure was demonstrated by statistical analysis of all the slices used. The weight was 20.8 ± 0.76 (mean ± s.e.; n = 299). The 4% error is considered to be within acceptable limits.

(i) Effect of Preincubation Time and Washout Time

The uptake of calcium was found to be unaffected by preincubating the tissue for a period of more than one minute. All further experiments were undertaken using a preincubation of fifteen minutes (Figure 38).

It was also found that after a washout time of five minutes, the uptake of calcium was constant. In further experiments, a washout time of fifteen minutes was used (Figure 39).

(ii) Time Course of the Uptake Process

The time course of calcium uptake exhibited a rapid initial phase which had declined after forty minutes (Figure 40).

(iii) Effect of External Calcium Concentration on Uptake

The uptake of calcium was dependent upon the external calcium concentration of the media. A rapid increase was seen in the range of 0 - 10 $mmol.l^{-1}$ and reached a plateau when the external calcium concentration was raised to 15 $mmol.l^{-1}$ (Figure 41).

(iv) Effect of Temperature on Uptake

The uptake of calcium was dependent on the incubation temperature and was linearly related between the temperature of 20 and 50°C. However, there was a higher uptake observed at 6°C than at 20°C (Figure 42).

(v) Effect of Brain Region on Uptake

Calcium uptake was not significantly different in the 4 regions studied, i.e. the cortex, midbrain, brain stem and cerebellum (Figure 43).

(vi) Effect of Drugs on Uptake

Sodium Azide (10^{-3} $mol.l^{-1}$) significantly ($p < 0.05$) increased the accumulation of ^{45}calcium in both the non-stimulated and stimulated slices. Lanthanum (2×10^{-3} $mol.l^{-1}$) significantly reduced the accumulation of ^{45}calcium in both the non-stimulated and stimulated slices (Figure 44).

Calcitonin (0.04 - 2 i.u. ml^{-1}) signiticantly reduced the uptake of ^{45}calcium in both the stimulated (Figure 45) and non-stimulated (Figure 46) slices. Naloxone (10^{-7} $mol.l^{-1}$) significantly reduced the uptake of ^{45}calcium in the stimulated slices only. Naloxone (10^{-7} $mol.l^{-1}$) did not affect the inhibitory action of calcitonin on ^{45}calcium uptake in the non-stimulated slices. However, the effects of naloxone (10^{-7} $mol.l^{-1}$) and calcitonin (2 i.u. ml^{-1}) on ^{45}calcium uptake were additive, when compared to the effects of these agents when used alone (Figure 47) in stimulated slices.

Discussion

The use of a preincubation period of fifteen minutes, and a washout time of fifteen minutes, appears to be sufficient to allow, steady state conditions to develop, and the vast majority of ^{45}calcium, present in the extracellular space, to be removed, respectively (Cooke and Robinson, 1971).

The uptake of calcium, under resting conditions, is a passive process, governed by the kinetics of diffusion. The efflux of calcium is an exchange process and the rate of efflux is directly related to the intracellular calcium concentration. The time course of calcium uptake, reported here, is consistent with this idea. The rapid, initial phase is terminated after twenty minutes, when presumably the intracellular calcium concentration reaches a critical value, which then stimulates efflux.

Similarly, the relationship between calcium uptake and extracellular calcium concentration also reflects this model. The linear relationsip between uptake and extracellular calcium concentration, which would be predicted from diffusion kinetics, does not persist at extracellular calcium concentrations greater than 10 mmol.l^{-1}. At such concentrations, the intracellular calcium concentration is such that the efflux is stimulated and calcium uptake is now governed by both influx and efflux mechanisms.

The relationship between uptake and temperature under diffusion kinetics is linear. This is seen in these experiments between the temperatures of 10 to 50^{o}C. The higher uptake observed at 4^{o}C is of interest in that it may

be due to the fact that efflux may be completely inhibited at this temperature, whilst a small amount of uptake may still occur.

As described in the Methods, the slices used in these experiments are taken from whole brain tissue. It is important, therefore, to show that the uptake of calcium does not significantly vary from one brain region to another. This does not occur in at least the 4 regions studied.

The literature concerning the use of potassium as a stimulatory agent is extensive, and the concentrations used vary from 20 to 200 $mmol.l^{-1}$. The experiments described in this section used a potassium concentration of 56 $mmol.l^{-1}$ - a concentration which is most commonly used. It may be argued that the stimulation (20 - 30%) reported in these experiments using this concentration of potassium are low in comparison to reports in the literature (e.g. Kakunaga, 1966). However, comparison of the work described here, with that of Cooke and Robinson (1971), who used an identical experimental technique, shows similar degrees of potassium stimulation

The use of lanthanum and sodium azide as positive controls show that the technique is comparable to the literature, since the effects of these agents are similar to those of other workers (e.g. Cooke and Robinson, 1971).

The inhibitory effect of calcitonin on the uptake of calcium is similar to the effect reported by Levine and Morley (1981). The results presented here also show that calcitonin can inhibit the uptake of calcium in potassium

stimulated slices.

However, several problems arise as a result of this work. The evidence from central administration of the hormone and from several in vitro experiments would tend to indicate that the action of the hormone on the CNS is a specific effect. This idea is hard to reconcile with such a large effect of calcitonin on brain slice calcium uptake. If the effect of calcitonin is as great as it appears from this slice work, then one would expect a marked change in the total calcium concentration of the brain - which was not observed in the previous section. In addition, the large effects of calcitonin on calcium uptake, if true in vivo, would result in large non-specific disturbances in CNS function, which also does not occur. Obviously, further work will need to be done in order to resolve this question.

These experiments, the physiological significance of which is open to question, also give no indication as to the site of action of the hormone either wth respect to the locus of action or to the type of cell involved, i.e. is its action on glia or on neurones. Finally, the effect of naloxone in these experiments is also open to question. Naloxone, in these experiments inhibited the uptake of calcium, a finding which is at variance to the literature (e.g. Guerrero-Munoz et al, 1979), which consistently show that naloxone alone is without effect on calcium uptake.

Figure 38 Effect of time of preincubation on apparent calcium uptake by brain slices. Values are mean ± s.e.m; n = 6.

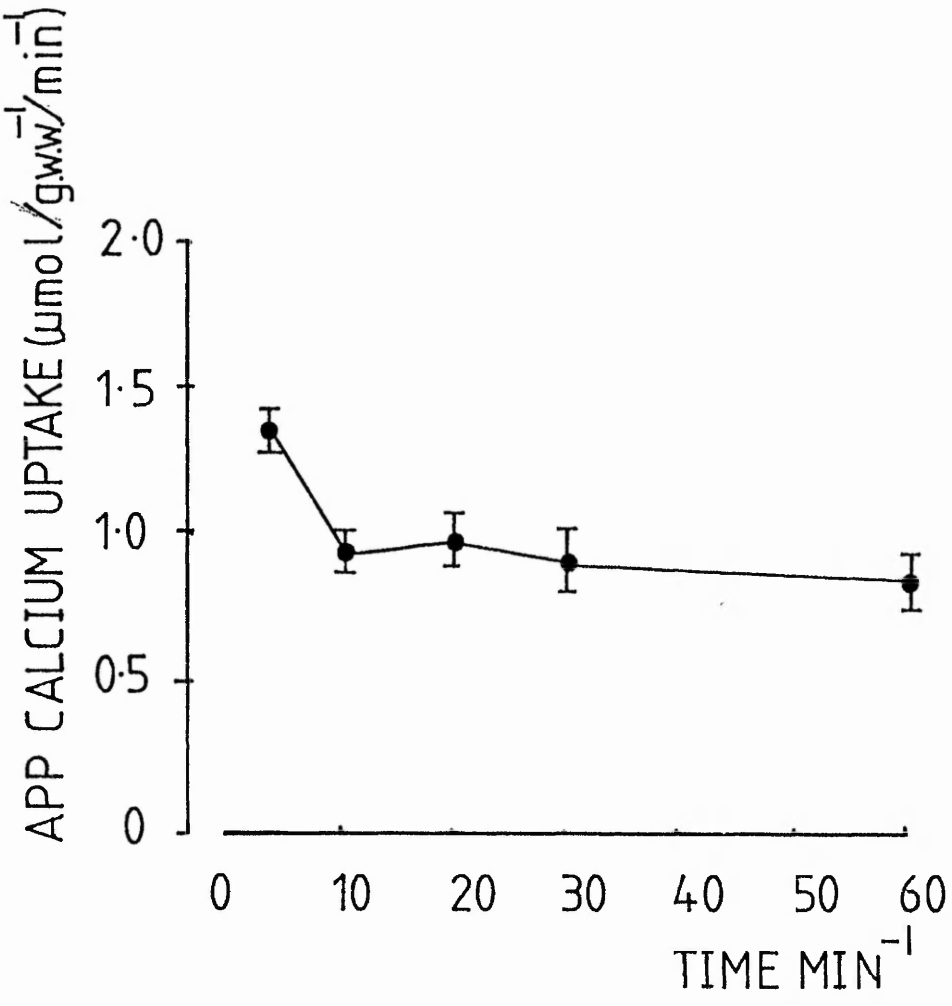

APP CALCIUM UPTAKE (umol/g.w.w/min)
2·0
1·5
1·0
0·5
0
0
10
20
30
40
50
60
TIME MIN-1

Figure 38

Figure 39 Effect of time of washout on apparent calcium uptake by brain slices. Values are mean ± s.e.m.; n = 6.

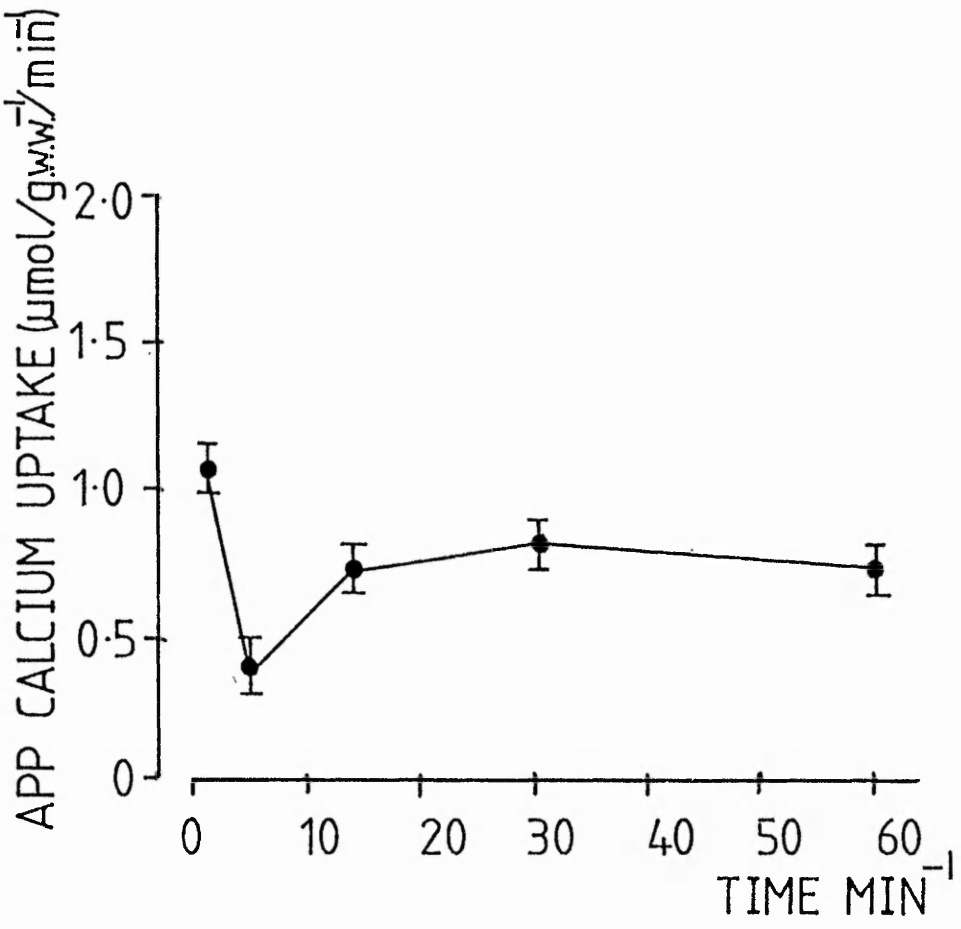

APP CALCIUM UPTAKE (μmol/g.w.w.$^{-1}$/min^{-1})
2·0
1·5
1·0
0·5
0
0
10
20
30
40
50
60
TIME MIN^{-1}

Figure 39

Figure 40 Time course of apparent calcium uptake by brain slices. Values are mean ± s.e.m.; n = 6.

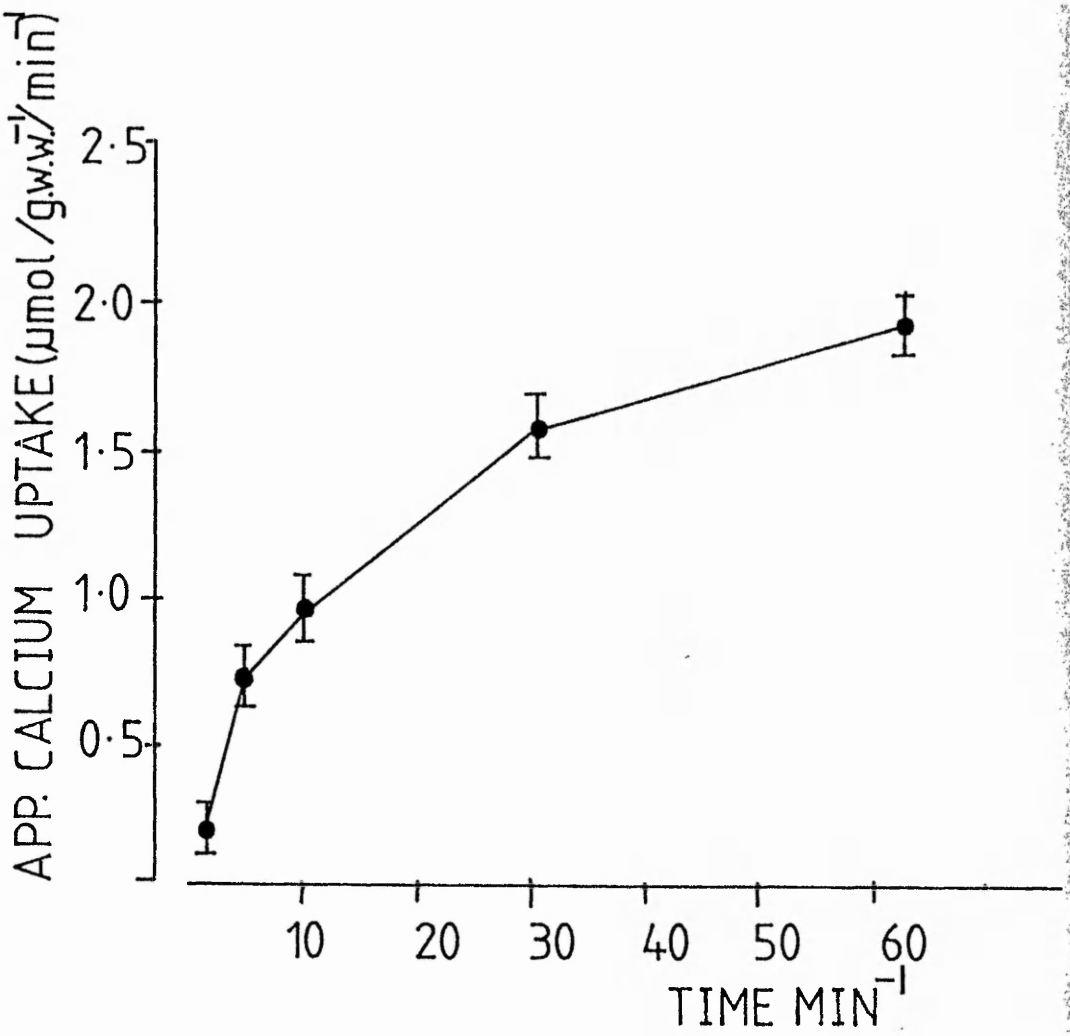

APP. CALCIUM UPTAKE (μmol/g.w.wt⁻¹/min⁻¹)
2·5
2·0
1·5
1·0
0·5
10
20
30
40
50
60
TIME MIN⁻¹

Figure 40

Figure 41 Relationship between external calcium concentration and apparent calcium uptake by brain slices. Values are mean ± s.e.m.; n = 6.

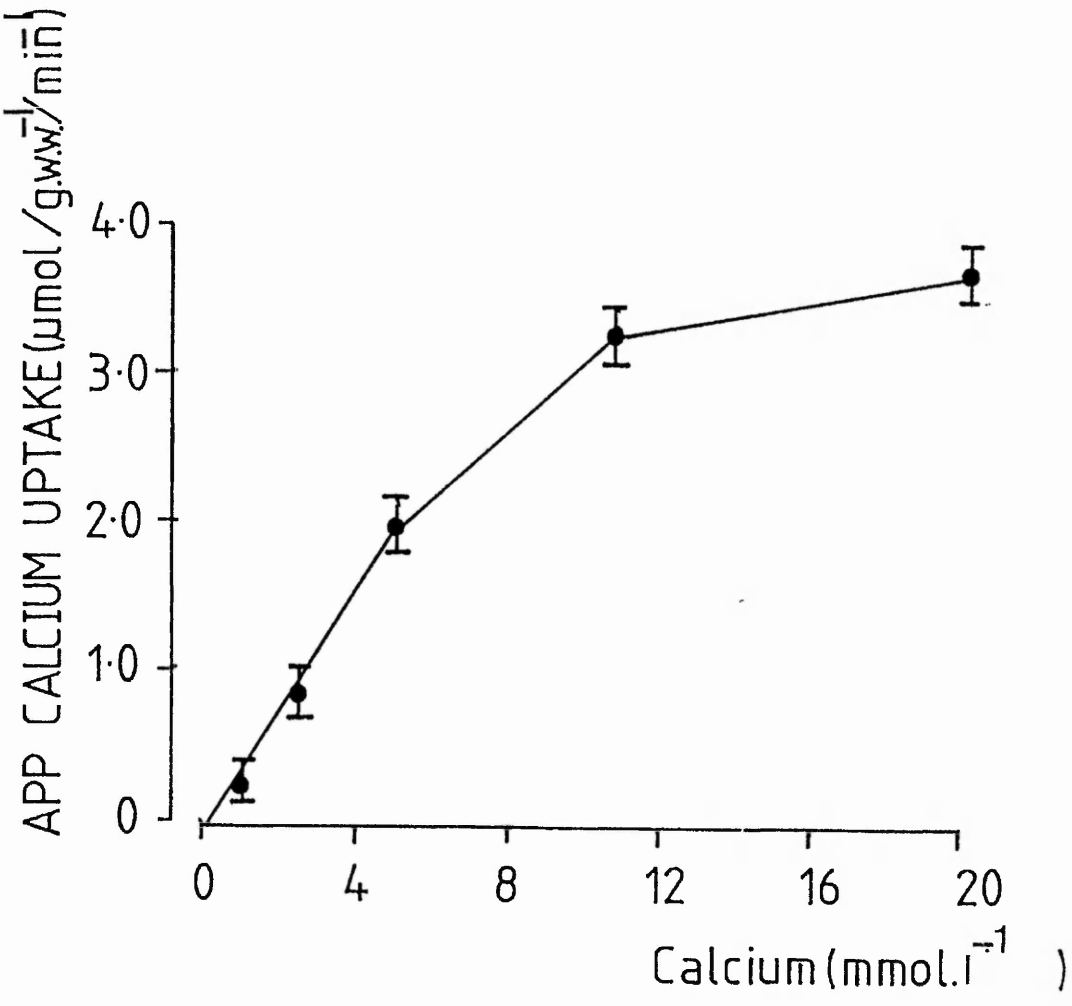

APP CALCIUM UPTAKE (μmol/g.w.w.$^{-1}$/min^{-1})
4·0
3·0
2·0
1·0
0
0
4
8
12
16
20
Calcium (mmol.l^{-1})

Figure 41

Figure 42 Relationship between temperature and apparent calcium uptake by brain slices. Values are mean ± s.e.m.; n = 6

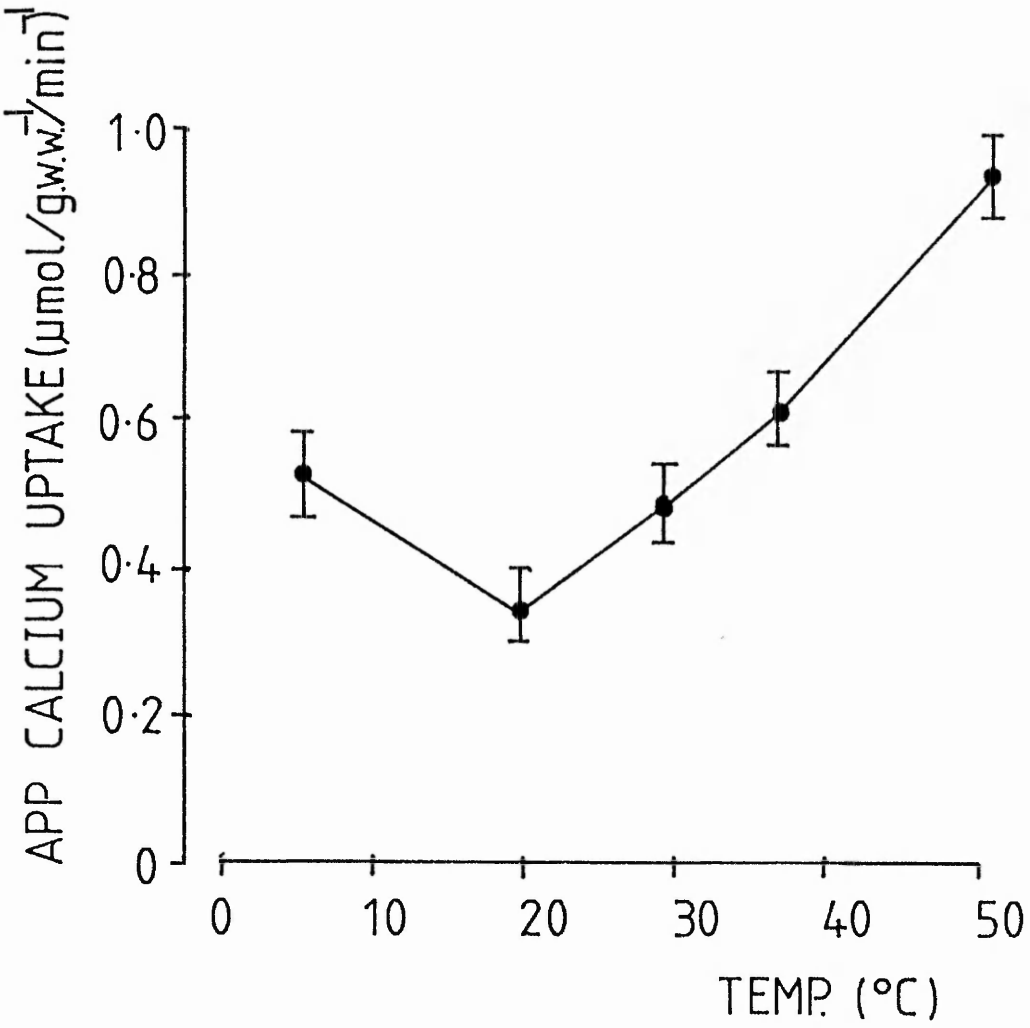

APP CALCIUM UPTAKE (µmol/g.w.w.$^{-1}$/min^{-1})
1·0
0·8
0·6
0·4
0·2
0
0
10
20
30
40
50
TEMP (°C)

Figure 42

Figure 43 Apparent calcium uptake by various regions of the brain (B.S. = brain stem; CERE = cerebellum; M.B. = mid brain; CTX = cortex). Values are mean ± s.e.m.; n = 6.

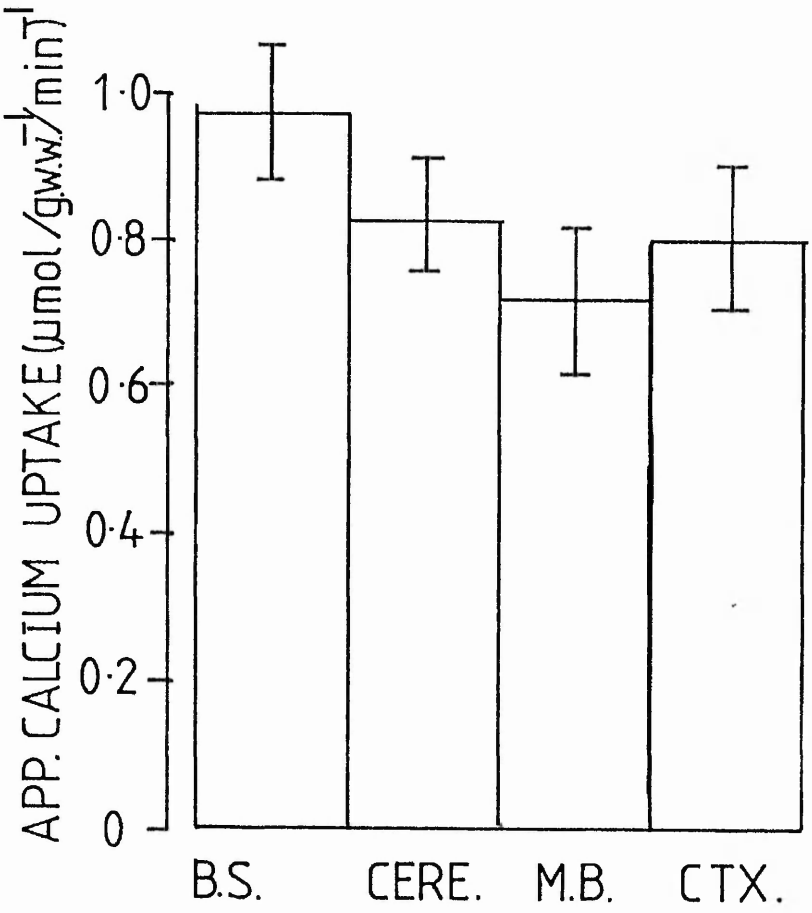


Figure 43

Figure 44 Effect of $LaCl_2$ (2 $mmol.l^{-1}$), Na azide (1 x 10^{-3} $mol.l^{-1}$) on apparent calcium uptake in stimulated and non-stimulated brain slices. Values are mean ± s.e.m.; n = 5 *$p < 0.05$.

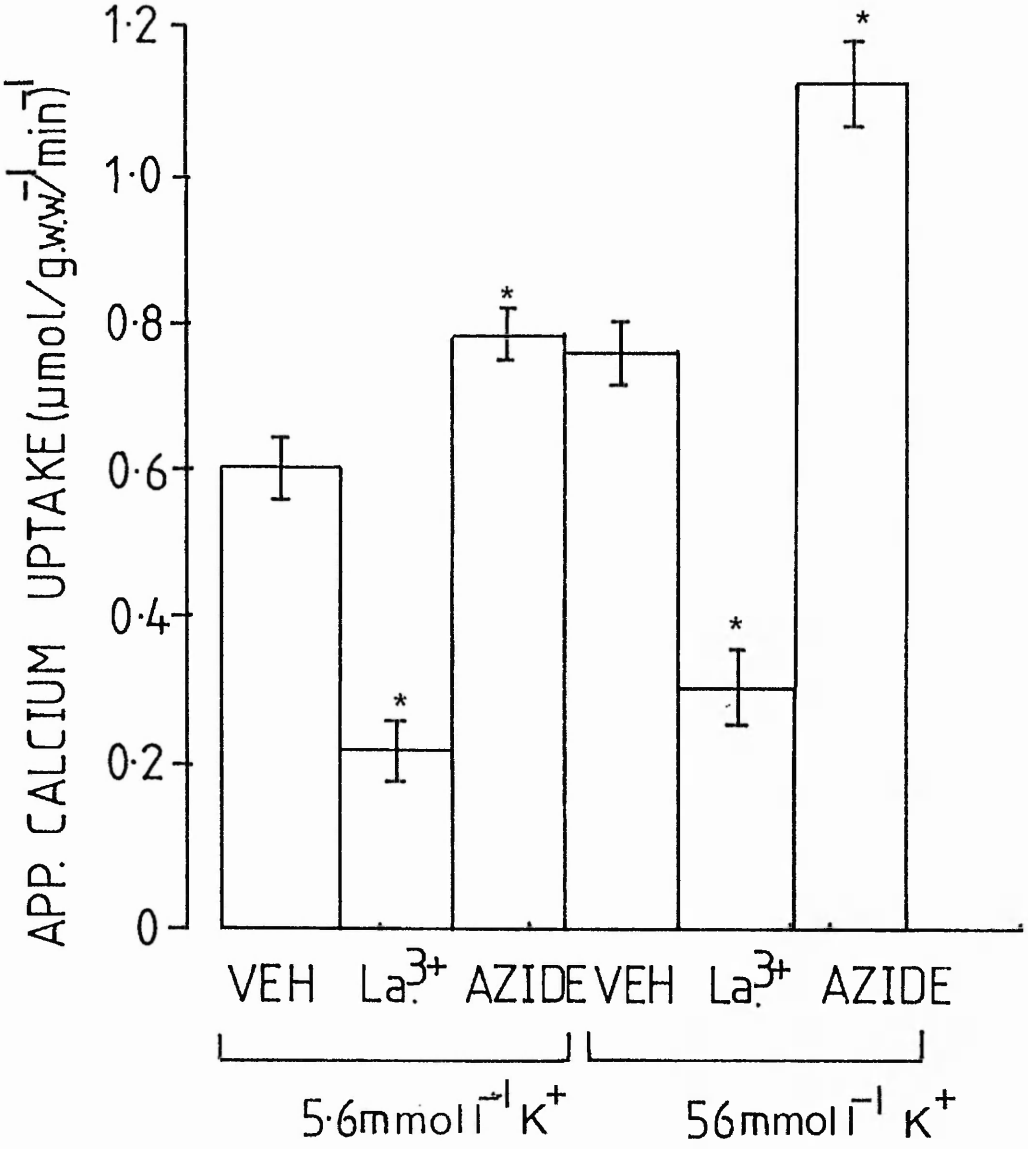

APP. CALCIUM UPTAKE (μmol/g.w.w.⁻¹/min⁻¹)
1·2
1·0
0·8
0·6
0·4
0·2
0
*
VEH
La3+
AZIDE
VEH
La3+
AZIDE
5·6mmol l⁻¹ K+
56mmol l⁻¹ K+

Figure 44

Figure 45 Effect of salmon calcitonin on apparent calcium uptake in stimulated brain slices. Values are mean ± s.e.m.; n = 5 *p < 0.05.

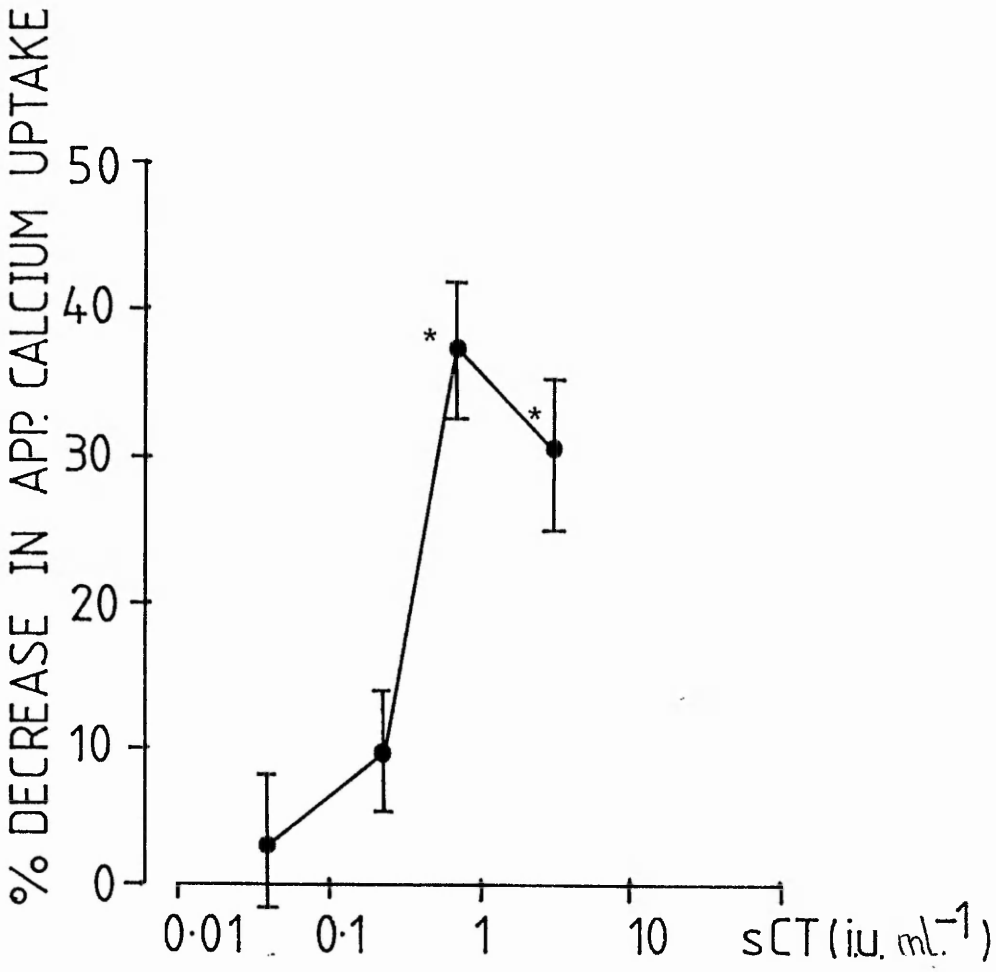


Figure 45

control uptake level was 0.58 µmol/g ww^{-1}/min^{-1}.

Figure 46 Effect of salmon calcitonin on apparent calcium uptake in non-stimulated brain slices. Values are mean ± s.e.m.; n = 5 *p < 0.05.

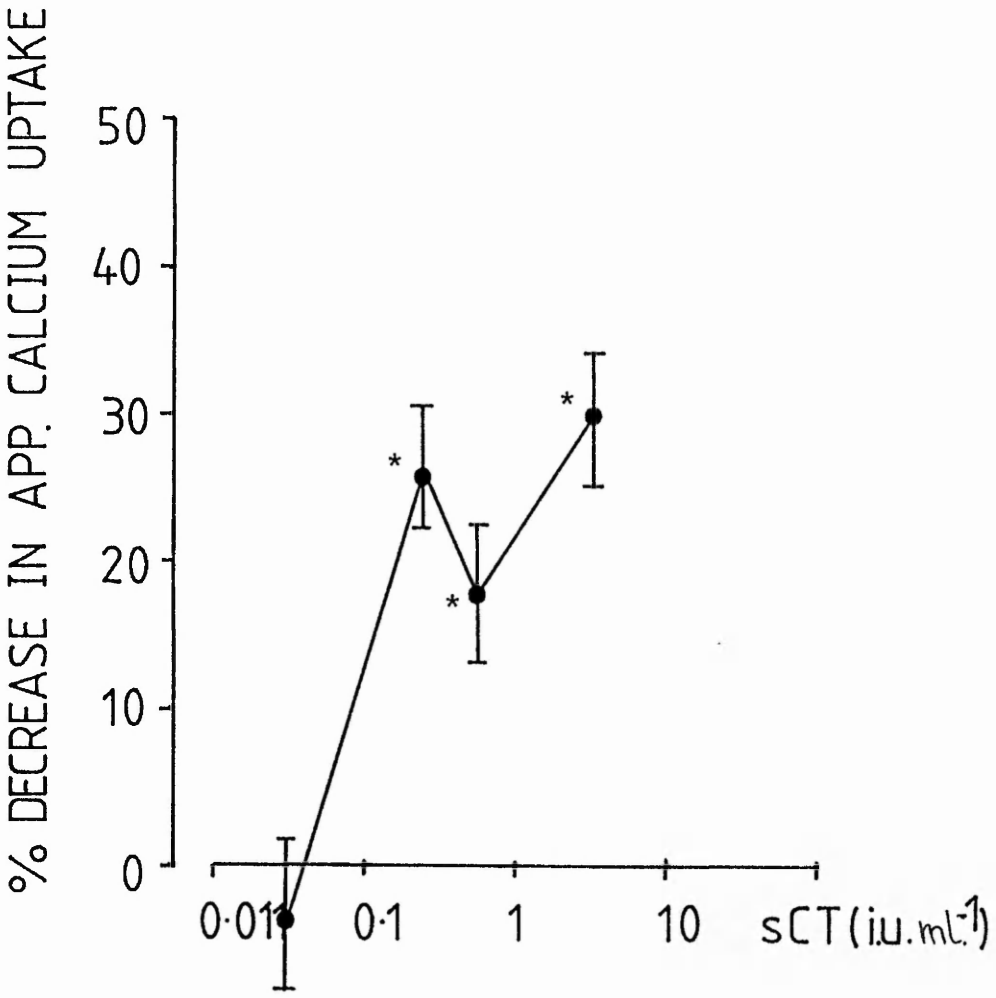

% DECREASE IN APP. CALCIUM UPTAKE
50
40
30
20
10
0
0·01
0·1
1
10
sCT (i.u.ml⁻¹)
*
*
*

Figure 46

Figure 47 Effect of salmon calcitonin (2 i.u. ml^{-1}), naloxone (1 x 10^{-7} $mol.l^{-1}$) on apparent calcium uptake in non-stimulated and stimulated brain slices. Values are mean ± s.e.m.; n = 5 *$p < 0.05$.

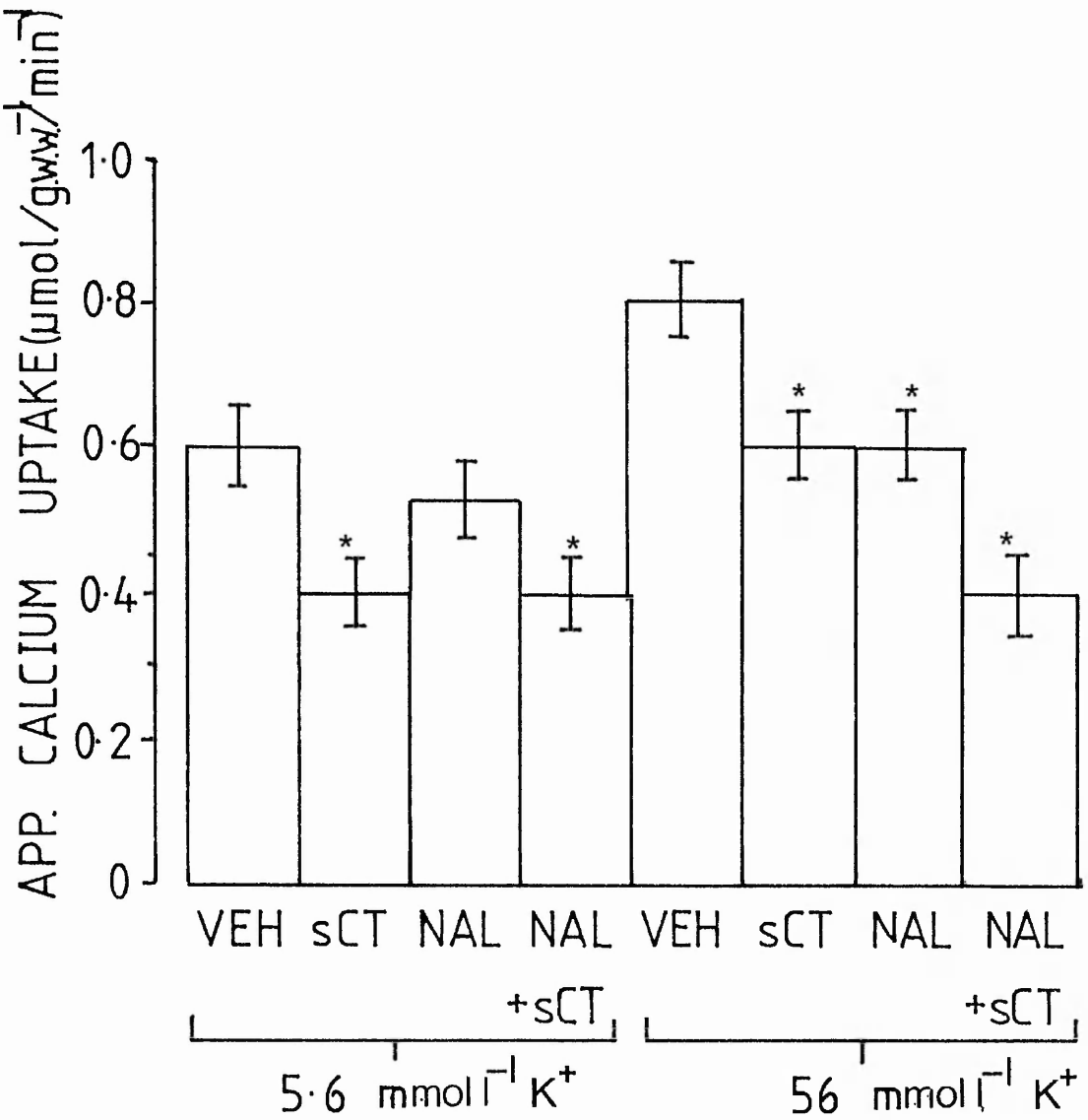

APP. CALCIUM UPTAKE(μmol/gww/min)
1·0
0·8
0·6
0·4
0·2
0
VEH
sCT
NAL
NAL
+sCT
VEH
sCT
NAL
NAL
+sCT
5·6 mmol l⁻¹ K⁺
56 mmol l⁻¹ K⁺

Figure 47

3.11 SUPERFUSION OF THE RAT COLON

To date, there are a number of in vitro opiate bioassays and different assays activate different combinations of opiate receptors. Braga et al (1978) have studied the effect of calcitonin on the guinea-pig myenteric plexus-longitudinal muscle preparation (Pert and Snyder, 1973). Calcitonin (0.4 or 4 i.u. ml^{-1}) was inactive in this preparation, whilst morphine produced a marked inhibitory effect on contractions induced by electrical field stimulation

This section describes the effect of calcitonin in a relatively novel opiate bioassay, that of the superfused rat colon. This tissue produces a contractile response, when opiates are applied (Gillan and Pollock, 1980; Nijkamp and van Ree, 1980; Huidobro-Toro and Way, 1981; Boura and Oliey, 1981). This excitation is thought to be mediated by activation of serotonergic neurones (Huidobro-Toro and Way, 1981), although Blanquet, Bouvier and Gonnella (1982) have proposed that the opiates act by removing an inhibitory effect of non-adrenergic, non-cholinergic neurones. The subclasses of opiate receptor involved in the response to the opiates are thought to be of the delta type, predominantly, although mu receptors can be demonstrated.

In view of the novelty of the preparation, a major portion of this section will be devoted to an investigation into the mechanism by which the opiates induce the response. The second part of the preparation is devoted to studying the potential activity of calcitonin in this assay.

Results

Preliminary Experiments

A number of preliminary experiments were undertaken in which the tissue was placed in an organ bath, using a similar technique to that of Gillan and Pollock (1980). This technique meant that the tissue remained in contact with the opiates for long periods. It was observed that the tissue rapidly desensitised to the opiates. The addition of 10^{-7} $mol.l^{-1}$ leucine enkephalin, or methionine enkephalin resulted in a series of rhythmic contractions (2.3 ± 0.2 min^{-1} mean ± s.e. mean, n = 6). These contractions were abolished by the addition of naloxone (10^{-6} $mol.l^{-1}$). After 2 - 3 minutes the amplitude of these contractions diminished and the second addition of enkephalin (10^{-7} $mol.l^{-1}$), even after extensive washing out of previous enkephalin doses, did not elicit any further contraction (Figure 48).

Superfusion Experiments

In view of the above problems of desensitisation, the technique was changed to that of superfusion, using the method of Boura and Olley (1981).

(i) Effect of Enkephalin, 5HT and Acetylcholine on Superfused Colon

The sensitivity of the colon was first checked by the addition of acetylcholine (10^{-8} to 10^{-4} $mol.^{-1}$, EC50 = 5 x 10^{-7} $mol.l^{-1}$, Figure 49). The responses to this agonist were competitively antagonised by atropine (pA_2 = 8.1), Figure 50, but not by 10^{-5} $mol.l^{-1}$ naloxone.

Leucine enkephalin and methionine enkephalin (10^{-9} -

10^{-5} mol.l^{-1}) produced contractions (EC50 = 3 x 10^{-8} mol.l^{-1} and 6 x 10^{-8} mol.l^{-1}, respectively). The maximum attained by either peptide was only 30 - 40% of the maximum response attained by acetylcholine (Figure 49). Naloxone (10^{-9} - 10^{-4} mol.l^{-1}) antagonised the responses to both leucine and methionine enkephalin in a competitive manner (pA_2 = 7.1 and 7.0 respectively, Figure 51). The responses to the opiates were unaffected by 10^{-6} mol.l^{-1} atropine, whereas 10^{-5} mol.l^{-1} methysergide did antagonise the opiate responses (Figure 52).

The addition of 5-hydroxytryptamine (10^{-8} to 10^{-4} mol.l^{-1}) produced contractions (EC50 = 6.1). These responses were antagonised by the addition of 10^{-5} mol.l^{-1} methysergide, but not by the addition of 10^{-5} mol.l^{-1} naloxone or 10^{-6} mol.l^{-1} atropine (Figure 52).

(ii) Effect of External Calcium Concentration on the Colon Responses

Reducing the concentration of calcium in the superfusing fluid from 2.5 mmol.l^{-1} to 0.25 mmol.l^{-1} was without effect on the responses to acetylcholine, but abolished the responses to leucine enkephalin (Figure 49).

(iii) Effect of 'Calcium Antagonists' on the Responses of the Colon

The responses to acetylcholine (10^{-8} to 10^{-4} mol.l^{-1}) were unaffected by the addition of 10^{-7} mol.l^{-1} nifedipine or 10^{-6} mol.l^{-1} PY 108 068 (Figure 53), whereas the responses to leucine enkephalin were antagonised in a non-competitive manner by both nifedipine (Figure 54) and PY 108 068 (Figure

55), 10^{-9} to 10^{-7} mol.l^{-1}). The responses to 5-hydroxytryptamine were abolished by nifedipine (10^{-5} mol.l^{-1}); however, a lower dose of nifedipine (10^{-6} mol.l^{-1}), whilst completely abolishing the opiates responses, only partially antagonised the responses to 5-hydroxytryptamine (Figure 56).

(iv) Effects of Calcitonin on the Colon

The addition of calcitonin (10^{-12} to 10^{-4} mol.l^{-1}) was without effect on the colon when administered alone or in conjunction with acetylcholine or the opiates, leucine or methionine enkephalin.

Discussion

The colon appears to be unique in its response to the opiates, in that a contractile response is obtained. The problem of rapid desensitisation to the opiates (and also to 5-hydroxytryptamine) has been reported by other workers using the rat colon (Gillan and Pollock, 1980), although not by the work of Nijkamp and van Ree (1980) or by Huidobro-Toro and Way (1981).

The action of opiates has been reported to be mediated by activation of serotonergic neurones (Huidobro-Toro and Way, 1981). The results obtained here also implicate 5-hydroxytryptamine, in that methysergide, a specific 5-hydroxytryptamine antagonist, antagonises the action of the opiates. The relationship between the opiates and 5-hydroxytryptamine is further indicated in that both are antagonised by the calcium antagonists, nifedipine and PY 108 068, (although the opiates are more sensitive to these

substances). In addition, the maximum attained by the opiates and 5-hydroxytryptamine is only 30 - 40% that of the maximum attained by acetylcholine.

Huidobro-Toro and Way (1981) and Nijkamp and van Ree (1980) have proposed that enkephalin is released from neurones to act on serotonergic neurones. This neuronal location of enkephalin is inferred from experiments using tetrodotoxin, a substance which by blocking sodium channels inhibits neuronal depolarisation; this agent antagonises the action of enkephalin. It was also demonstrated that the opiates are sensitive to a reduction in the external calcium concentration, a fact which has also been shown here. The authors, Huidobro-Toro and Way (1981), concluded that this was consistent with the idea that enkephalin was released from neurones. The action of the calcium antagonists in antagonising enkephalin but not acetylcholine, may also indicate that enkephalin is released from neurones.

Finally, results, not presented here, have shown that acetylcholine, but not enkephalin, is capable of inducing a contractile response on a tissue already depolarised by potassium, which again may indicate that the opiates act on the neurones, while acetylcholine acts directly on the muscle.

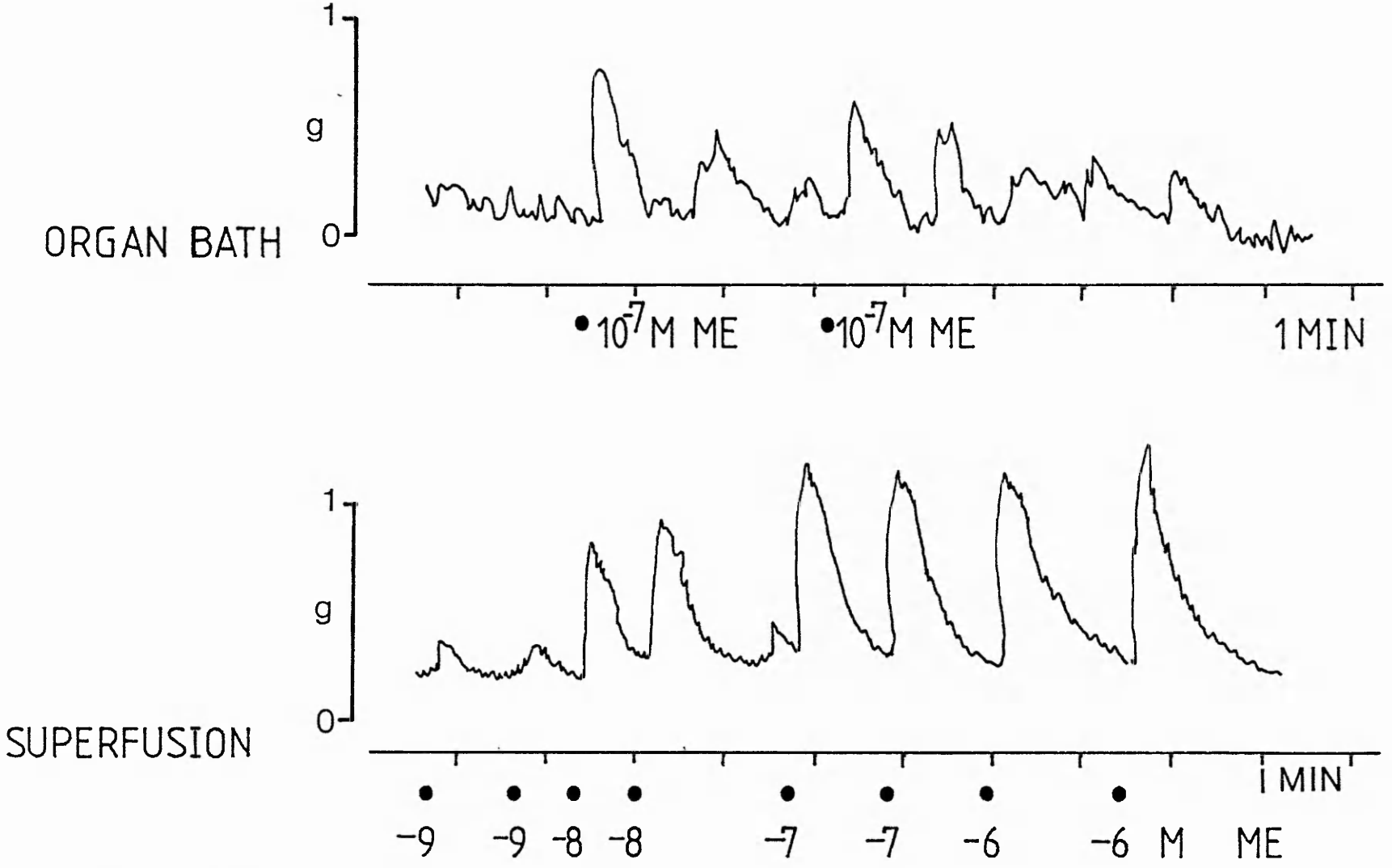
The absence of any effect of calcitonin in this preparation is in agreement with the work of Braga et al (1978) who showed that the hormone is inactive in opiate bioassays. The guinea-pig ileum contains opiate receptors mainly of the mu tupe (Kosterlitz and Paterson, 1980),

whilst the colon possesses opiate receptors primarily of the delta type (Boura and Olley, 1981). One can, therefore, conclude that calcitonin does not interact directly with opiate receptors of the mu type to induce an antinociceptive action, since the action of the opiates in the abdominal constriction test is mediated by the mu receptor type (Kosterlitz and Paterson, 1980).

Calcitonin does not possess antagonist properties of the mu and delta receptors, since it did not affect the activity of the opiates in the colon preparation. This finding is also in agreement with the work of Braga et al (1978) who found that calcitonin did not affect the binding of enkephalin to the guinea-pig brain homogenates. The concept that calcitonin may act as a functional (but not pharmacological) opiate antagonist has been implied by Levine and Morley (1982), in their model of the regulation of feedng by the hypothalamus.

Finally, Dreyfus, Gershon, Hamovitz and Nunez (1976) propose that calcitonin exerts antimuscarinic actions on the intestinal tissue of the guinea-pig and rabbit. Their work has not been duplicated by this author (unpublished observations) using the same experimental protocol. The lack of effect of calcitonin on the acetylcholine responses on the colon also refutes the work of Dreyfus et al (1976).

Figure 48 Responses to methionine enkephalin by rat colon using both organ bath and superfusion techniques.



ME = methionine enkephalin.

Figure 48

Figure 49 Responses of superfused rat colon to acetylcholine (Ach) and leucine enkephalin (LE) in krebs solution containing either 2.5 $mmol.l^{-1}$ or 0.25 $mmol.l^{-1}$ $CaCl_2$. Values are mean ± s.e.m.; n = 4.

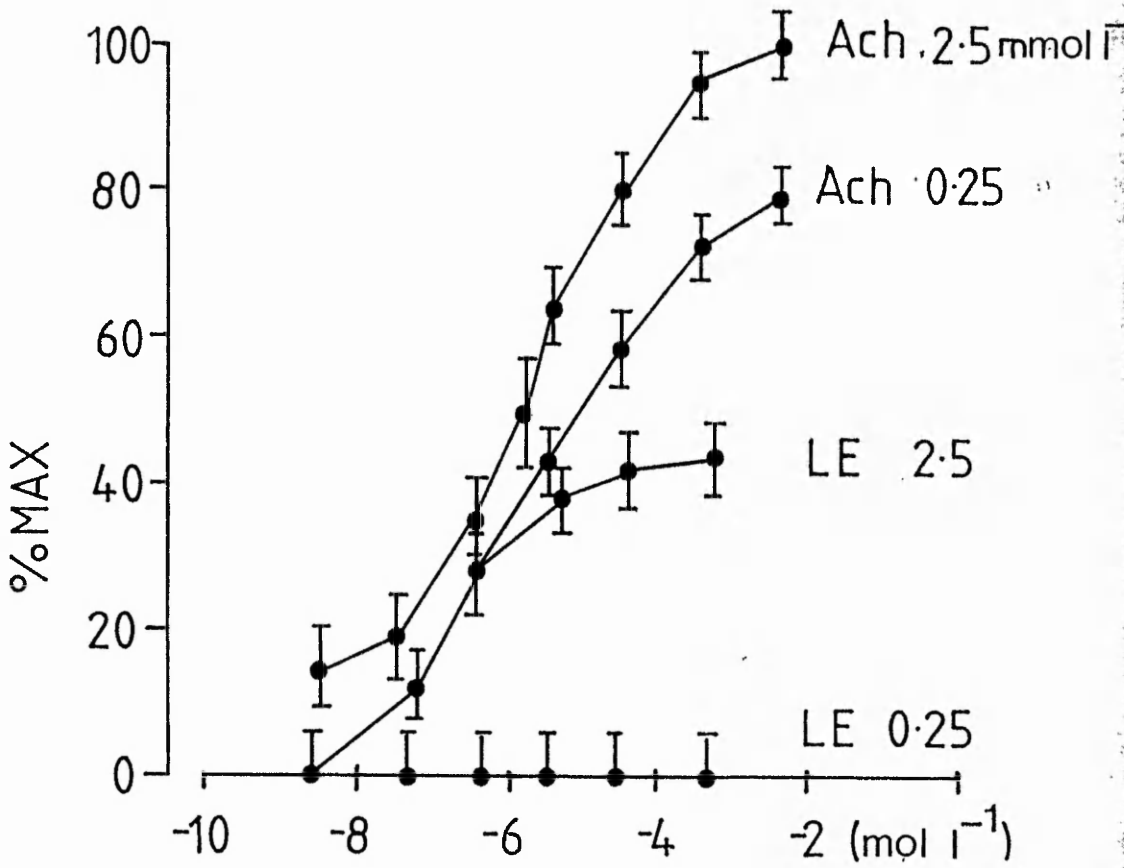

100
80
60
40
20
0
%MAX
-10
-8
-6
-4
-2 (mol l^{-1})
Ach 2·5 mmol l^{-1}
Ach 0·25
LE 2·5
LE 0·25

Figure 49

Figure 50 Schild plot of antagonism of responses to acetylcholine by atropine in superfused rat colon. pA_2 = 7.1.

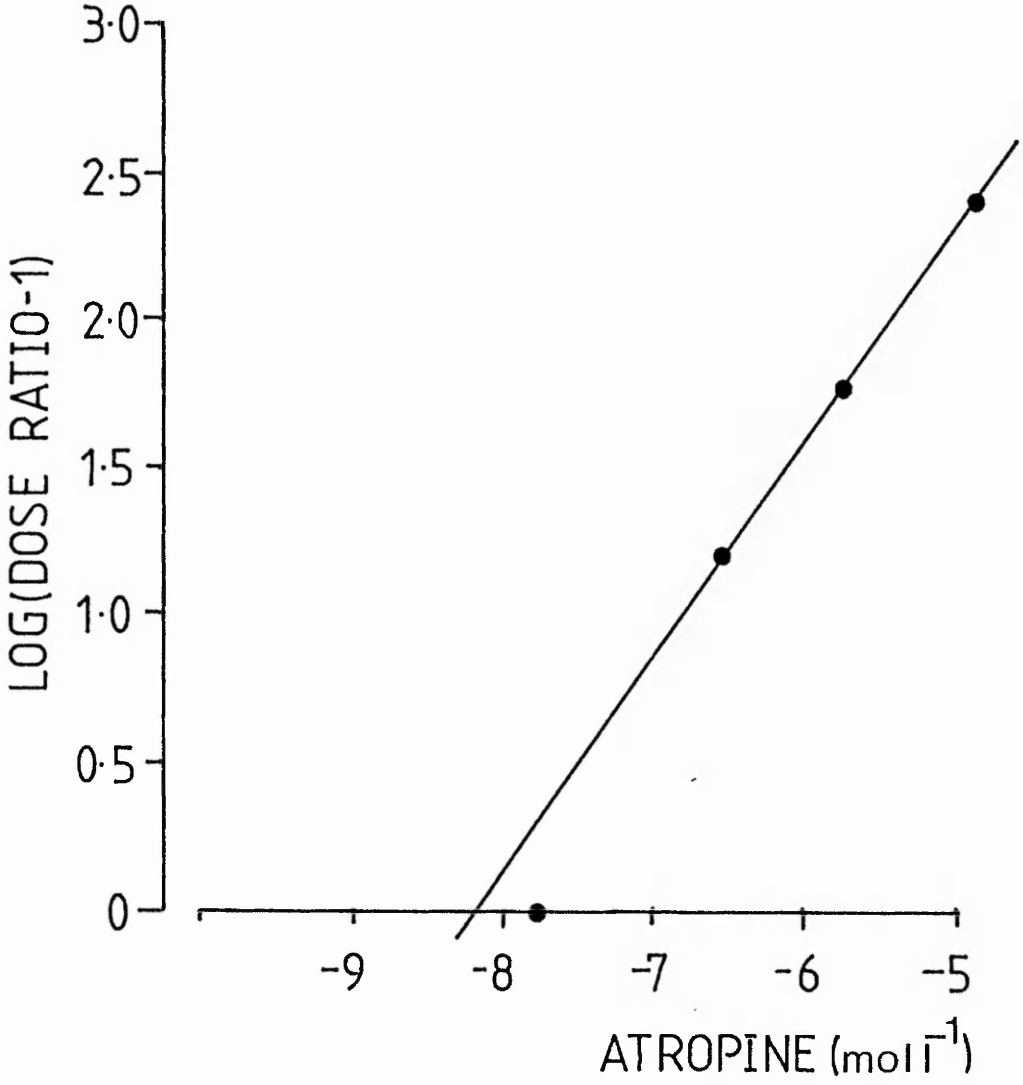


Figure 50

Figure 51 Schild plot of antagonism of responses to leucine or methionine enkephalin by naloxone in superfused rat colon. pA_2 = 7.1 (leucine enkephalin), 7.0 methionine enkephalin).

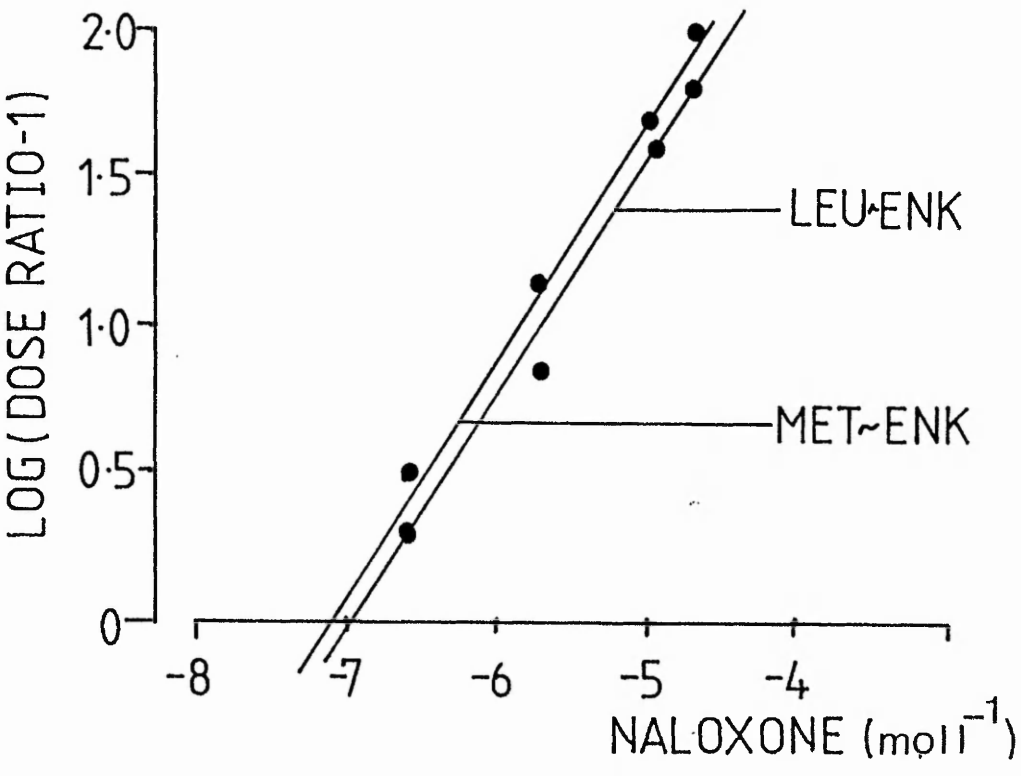

2·0
1·5
1·0
0·5
0
LOG (DOSE RATIO-1)
LEU-ENK
MET-ENK
-8
-7
-6
-5
-4
NALOXONE (mol l^{-1})

Figure 51

Figure 52 Responses, by superfused rat colon, to leucine enkephalin (LE) and 5-hydroxytryptamine (5HT), and antagonism of both these agents by methy sergide (10^{-5} mol. l^{-1}). Values are mean ± s.e.m.; n = 4.

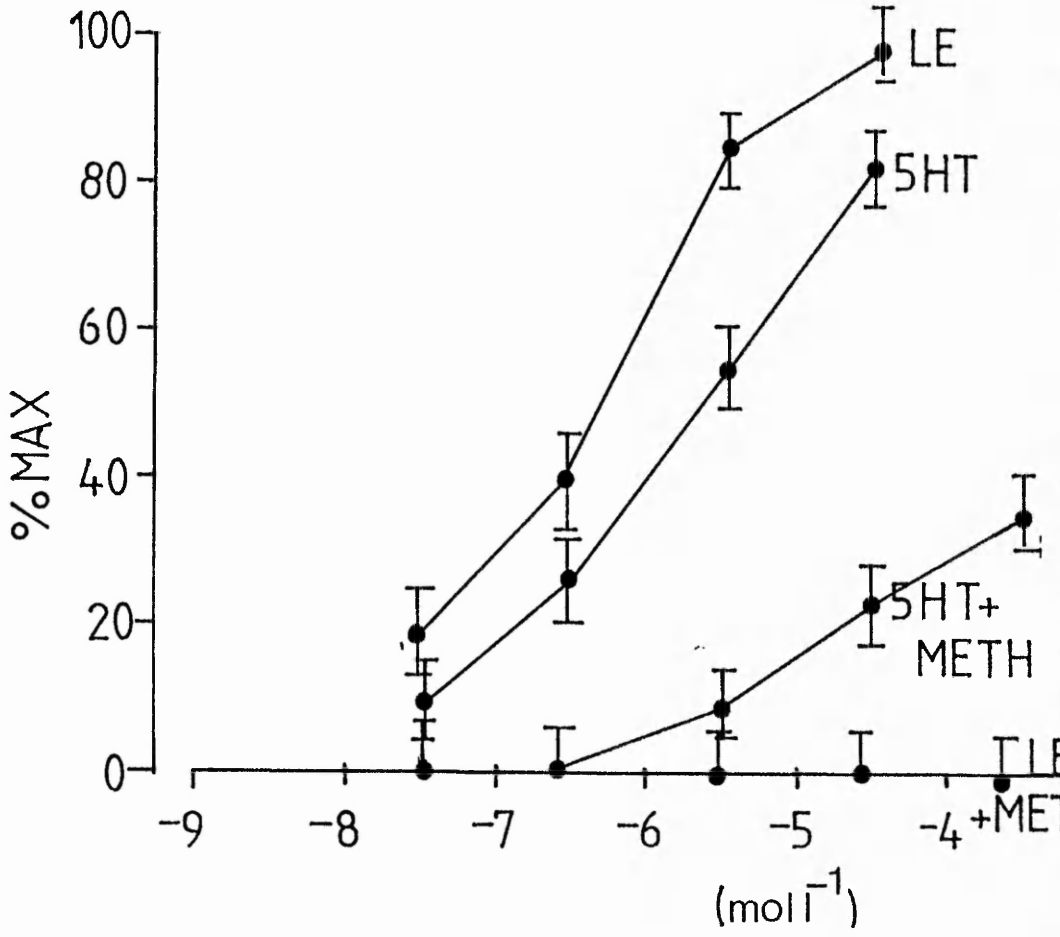


Figure 52

Figure 53 Effect of nifedipine (10^{-7} $mol.l^{-1}$) on responses to acetylcholine in superfused rat colon. Values are mean ± s.e.m.; n = 4.

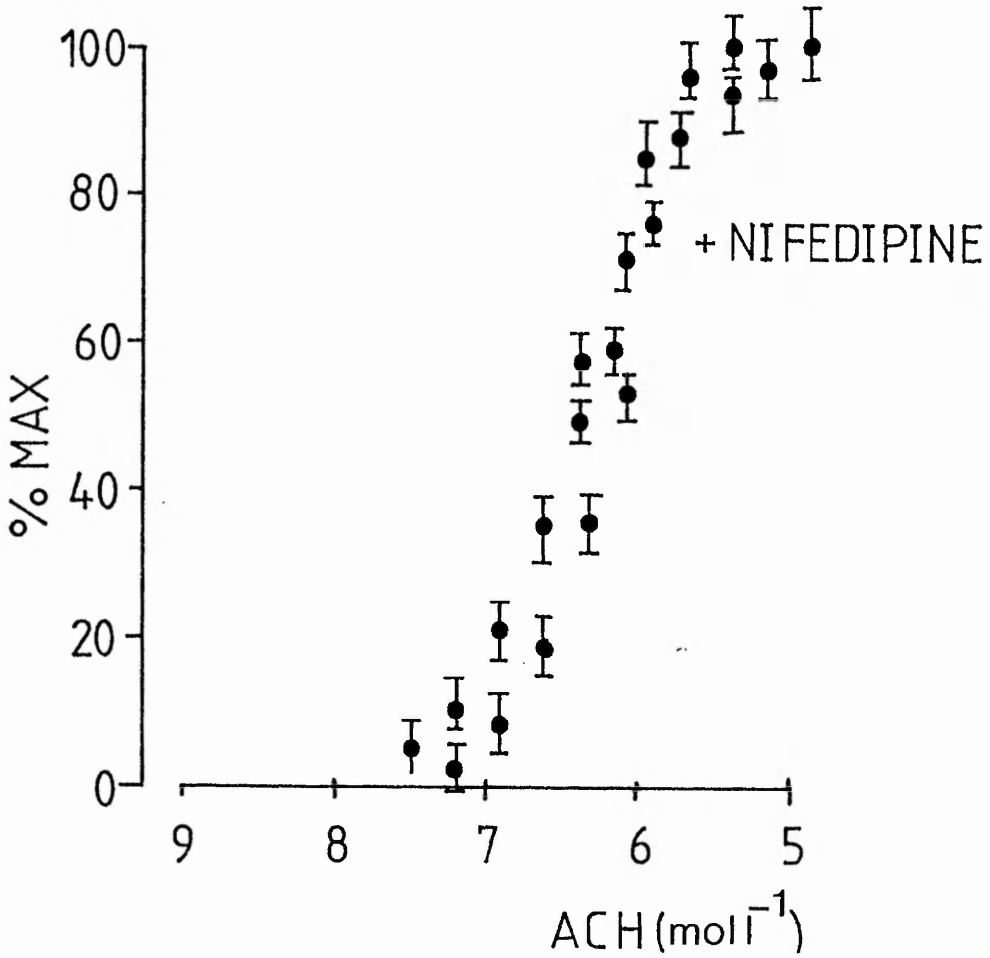


Figure 53

Figure 54 Effect of nifedipine on responses to leucine enkephalin in superfused rat colon. Values are mean ± s.e.m.; n = 4.

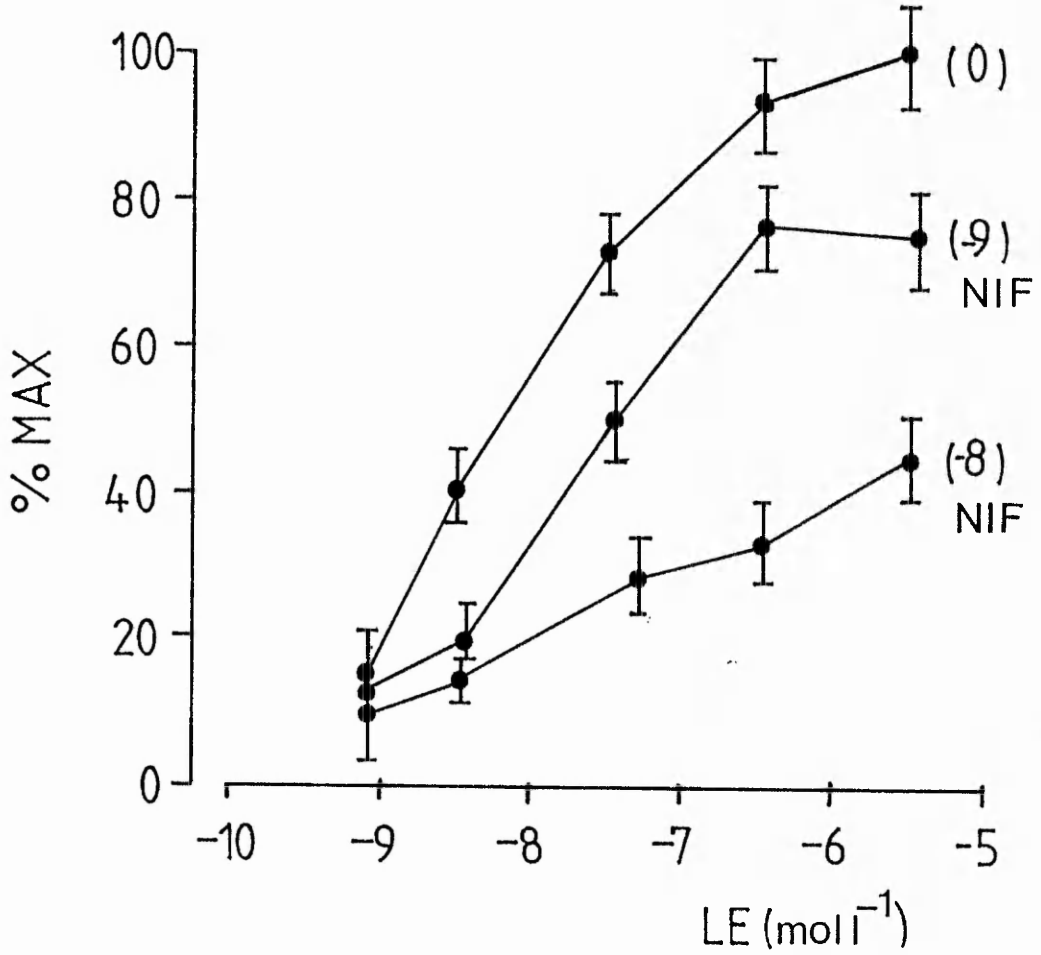


Figure 54

Figure 55 Effect of PY 108 068 on responses to leucine enkephalin in superfused rat colon. Values are mean ± s.e.m.; n = 4.

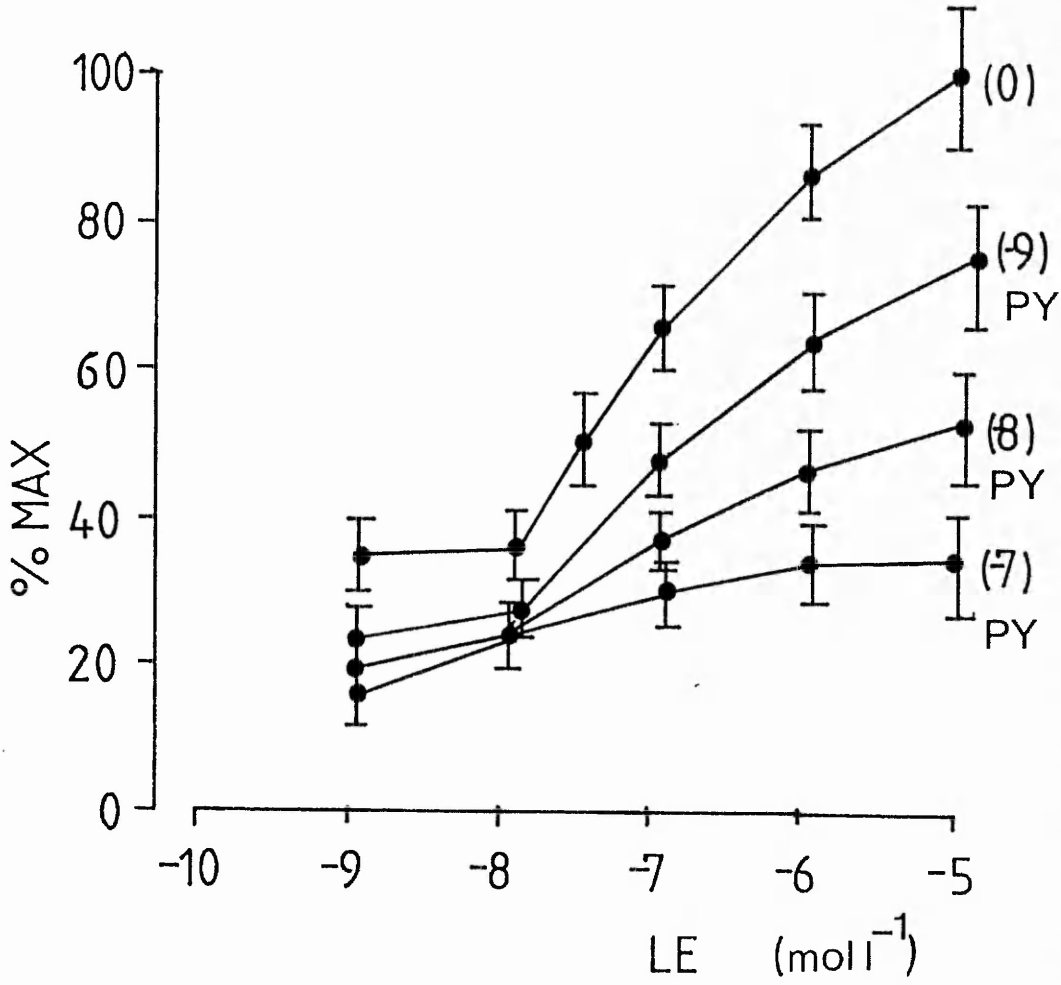


Figure 55

Figure 56 Effect of nifedipine (10^{-7} $mol.l^{-1}$) on responses to 5-hydroxytryptamine in superfused rat colon. Values are mean ± s.e.m.; n = 4.

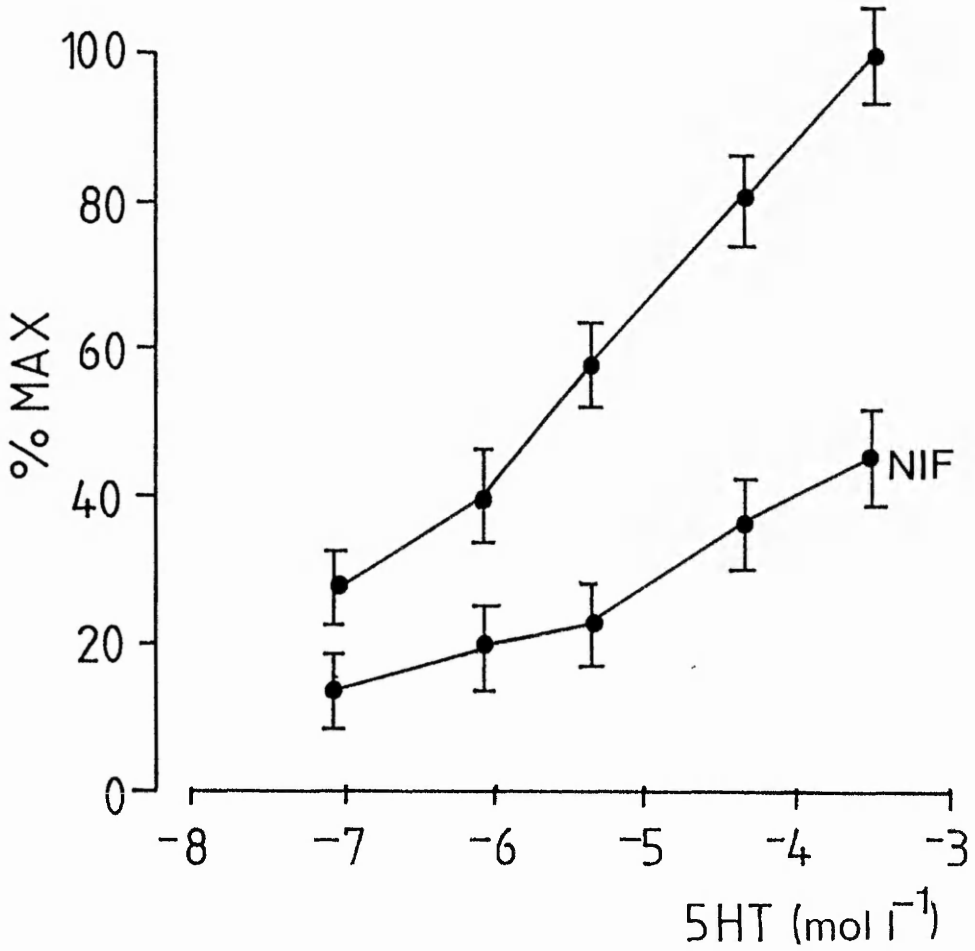


Figure 56

3.12 HAEMODYNAMIC PROPERTIES OF CALCITONIN

This thesis, so far, has concentrated almost exclusively on the antinociceptive actions of calcitonin. Similarities have been shown between the action of the opiates and calcitonin to induce antinociception with regard to the calcium ion. However, the hormone is unlikely to act via activation of the opiate receptor for reasons already outlined in the Introduction. However, to date, the hormone has not been tested in many non-analgesic opiate systems, athough it has been applied in two opiate bioassays, i.e. the transmurally stimulated guinea-pig ileum (Braga et al, 1978) and the superfused rat colon (see previous section). This section is devoted to a comparison between the effects of calcitonin and the opiates in firstly, the regulation of blood pressure in the normotensive animal and secondly, in animals subjected to haemorrhagic shock.

Opiates and opioid peptides elicit profound changes in the cardiovascular system following either central or peripheral administration (Laubie, Schmitt, Vincent and Remond, 1977; Bellet, Elghozi, Meyer, Pernollet and Schmitt, 1980; Moore III and Dowling, 1981). It has been proposed that forebrain delta opiate receptors mediate cardio-excitatory effects (such as tachycardia and hypertension), whilst brain stem mu receptors mediate cardiodepressor responses (Hassen, Feuerstein, Pfeiffer and Faden, 1982). The peripheral injection of opiates generally results in hypotension, athough it is uncertain whether this is an effect mediated by central mechanisms or by peripheral

sensory receptors (Moore III and Dowling, 1981). Opiates have been shown to influence the baroreceptor reflex, either acting in the nucleus of the tractus solitarius, or in the cardiac vagal nuclei (Schaz, Stock, Simon, Schlor, Unger, Rockhold and Ganten, 1980).

In animals rendered hypotensive by either haemorrhage (Faden and Holaday, 1979) or by spinalisation (Holaday and Faden, 1980) the opiate antagonist, naloxone, improves recovery, whilst the hypotension is exacerbated by morphine. It is thought that during shock there is a large release of opioids which contribute to the hypotension. The beneficial effect of naloxone in shock, therefore, is achieved by antagonising the actions of these endogenous opioids (Faden and Holaday, 1979).

Calcitonin has been studied little with regard to its action on the cardiovascular system (see Introduction). It has been show to cause vasoconstriction in isolated blood vessels from bone tissue (Driessens and Vanhoutte, 1981). Calcitonin has also been shown to antagonise vasodilator responses in the hepatic and renal circulation in the dog (Charbon and Piepper, 1972). However, there are no reports concerning any pressor or depressor effects of the hormone in the normal animal.

Results

Normotensive Animals

Leucine enkephalin (2×10^{-9} mol.kg^{-1} - 2×10^{-7} mol.kg^{-1}), by i.v. injection produces a transient, dose dependent reduction in both diastolic and systolic pressure. The

action of this agent was completely antagonised by pretreating the animals wth 10 mg.kg^{-1} naloxone (Figure 57). Calcitonin (0.01 to 100 i.u. kg^{-1}) by i.v. injection, was without effect in this preparation.

Haemorrhaged Animals

In a series of experiments designed to test the response to naloxone or calcitonin, rats were prepared as the above animals and blood was withdrawn until the mean arterial pressure was reduced by 20%. The mean arterial pressure (MAP) twenty minutes post haemorrhage was adopted as the control pressure for that animal. Each animal was given an injection of either vehicle, calcitonin or naloxone at 20.5 minutes post haemorrhage. The change in the MAP was recorded continuously for sixty minutes.

Animals injected with vehicle did not recover from post haemorrhage values for at least sixty minutes. Animals injected with naloxone (1 mg.kg^{-1}), Figure 58, showed good recovery with the MAP at fifteen minutes being significantly increased. This was sustained for a further forty-five minutes. Calcitonin (0.1 to 10 i.u. kg^{-1}) produced a dose dependent increase in MAP, compared to the post haemorrhage value at fifteen minutes and which was sustained for a further forty-five minutes. At fifteen minutes through to sixty minutes (post haemorrhage) both naloxone and all concentrations of calcitonin were significantly different from vehicle injected animals (Figure 59).

Discussion

These results in normotensive animals, using leucine

enkephalin and antagonism of the hypotensive effects by naloxone, are in agreement with the work of Moore III and Dowling (1981). In rats rendered hypotensive by haemorrhage, naloxone produced a significant pressor response, in agreement with the work of Faden and Holaday (1979). Although calcitonin was without effect in normotensive animals, it did produce a pressor effect in haemorrhaged animals. It is unlikely that the effects of calcitonin are due to the use of anaesthetised animals, since the responses to naloxone and to enkephalin are in agreement with work undertaken using conscious animals (e.g. Moore III and Dowling, 1981). However, it should be borne in mind that anaesthetics can modulate cardiovascular reponses to drugs in general and opiates in particular.

Calcitonin then resembles narcotics such as meptazinol in that it produces antinociception via central mechanisms but in haemorrhagic shock it induces pressor responses similar to naloxone. The results provide further evidence that the action of calcitonin is independent of the opiate system. The physiological significance of this action of calcitonin is unclear and further experiments are required to elucidate the site of action of the hormone.

Figure 57 Effect of leucine enkephalin (i.v.) on blood pressure in normotensive rats. Values are mean ± s.e.m.; n = 6 *$p < 0.05$.

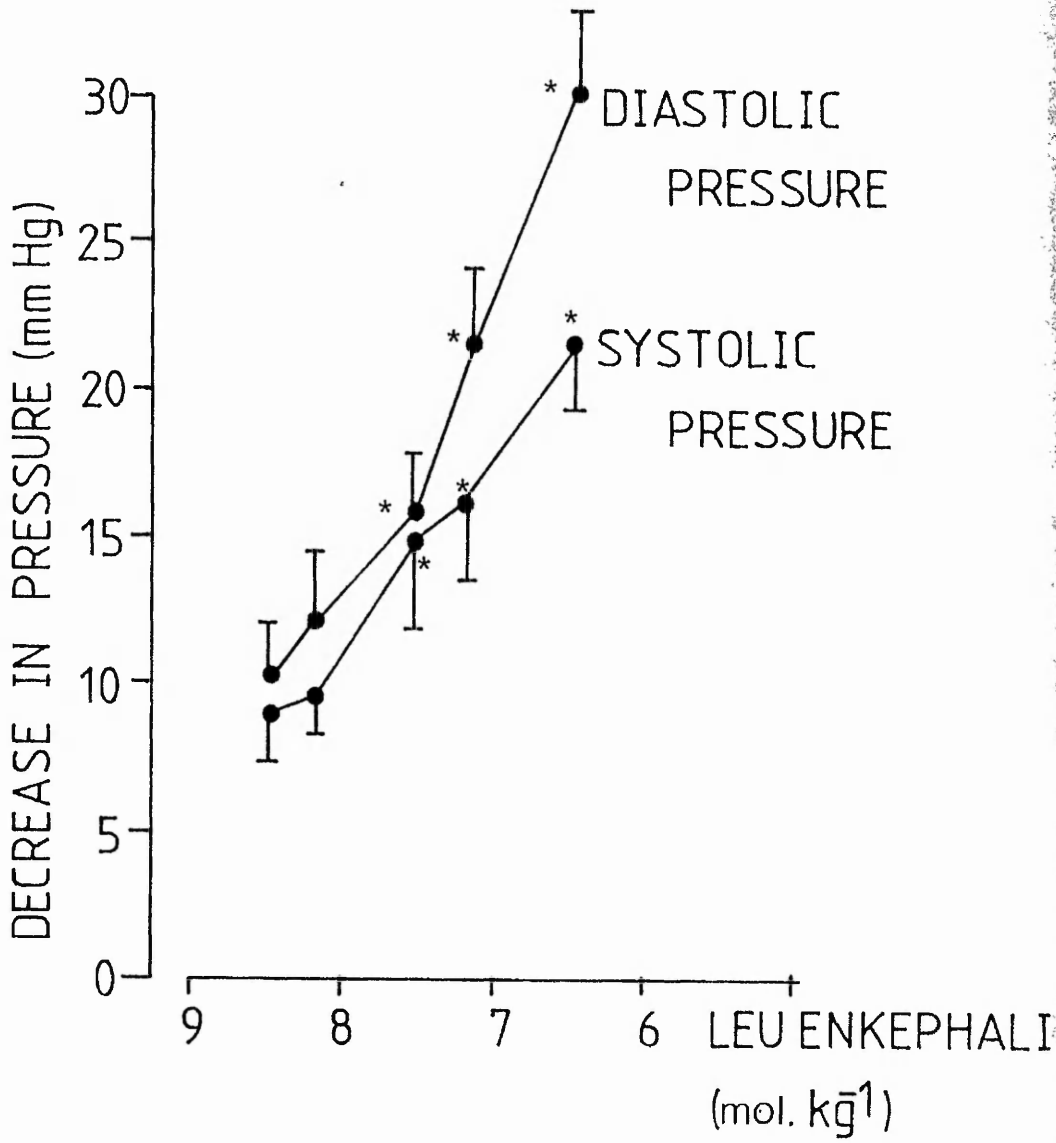

DIASTOLIC
PRESSURE
SYSTOLIC
PRESSURE
DECREASE IN PRESSURE (mm Hg)
30
25
20
15
10
5
0
9
8
7
6
LEU ENKEPHALI
(mol. kg⁻¹)

Figure 57

Figure 58 Effect of naloxone (1 $mg.kg^{-1}$), or vehicle, on mean arterial pressure in rats subjected to haemorrhagic shock. Values are mean ± s.e.m.; n = 4 *$p < 0.05$.

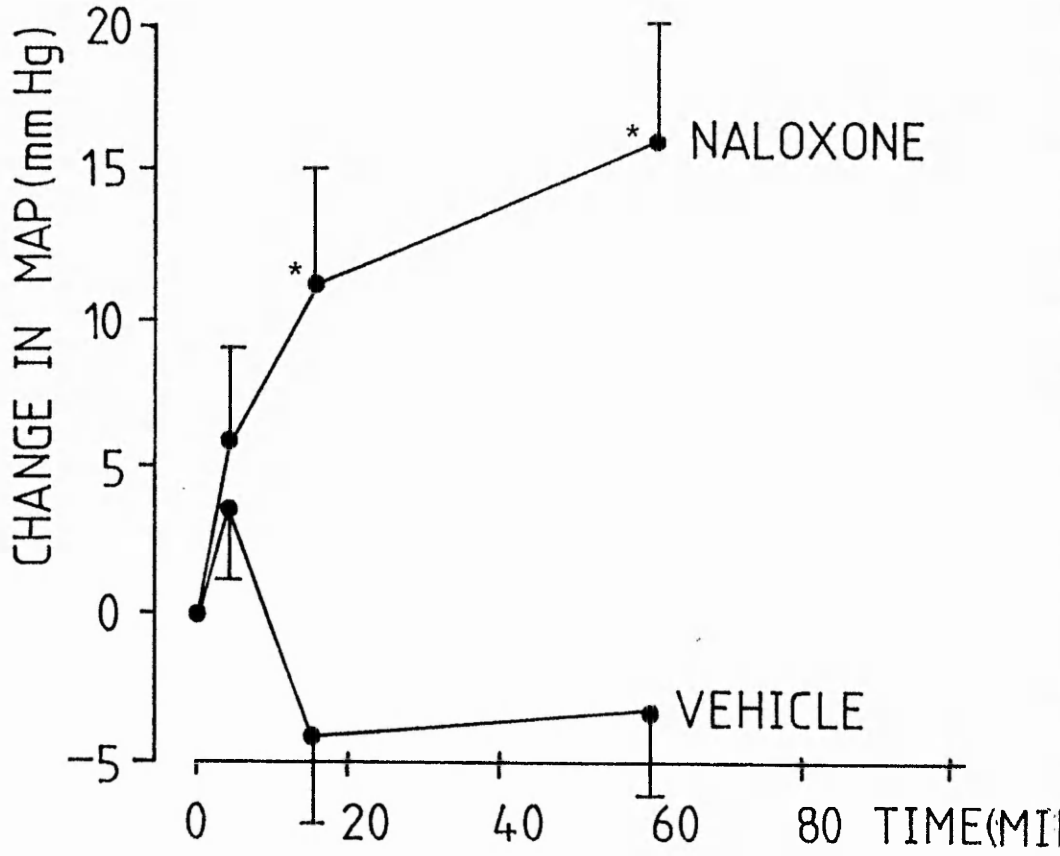

CHANGE IN MAP (mm Hg)
20
15
10
5
0
−5
NALOXONE
VEHICLE
*
*
0
20
40
60
80
TIME(MI

Figure 58

Figure 59 Effect of salmon calcitonin, or vehicle, on mean arterial pressure in rats subjected to haemorrhagic shock. Values are mean ± s.e.m.; n = 4 *$p < 0.05$.

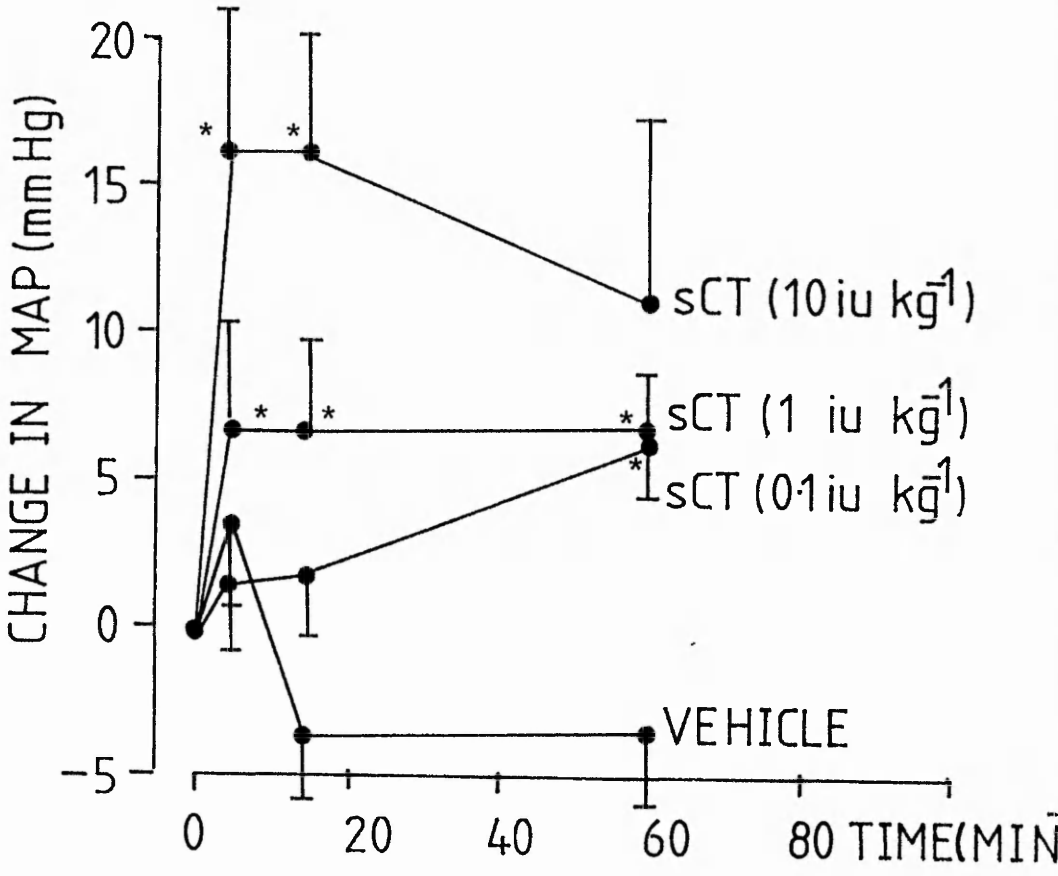

20
15
10
5
0
−5
CHANGE IN MAP (mm Hg)
sCT (10 iu kg⁻¹)
sCT (1 iu kg⁻¹)
sCT (0·1 iu kg⁻¹)
VEHICLE
0
20
40
60
80 TIME(MIN

Figure 59

3.13 EFFECT OF CALCITONIN ON ACETYLCHOLINESTERASE ACTIVITY OF THE BRAIN

The action of calcitonin on cerebral acetylcholinesterase (Achase) has been studied by the group of Nakhla and co-workers and reported in a series of papers (Majumdar and Nakhla, 1977; Nakhla and Majumdar, 1978; Nakhla, 1979; Nakhla, 1980). These workers have purported to show that calcitonin increases the uptake of plasma tryptophan from the plasma to the brain, which is then converted into 5-hydroxytryptamine. This elevation in brain 5-hydroxy-tryptamine then stimulates the synthesis of Achase. Evidence in support of this includes the finding that administration of tryptophan (p.o.) results in an elevation in the activity of Achase and conversely that administration of parachlorophenylalanine (pCPA), an agent which inhibits the synthesis of 5-hydroxytryptamine, also reduces the activity of Achase.

This section describes the effects of calcitonin on central Achase activity after administration of the hormone by both peripheral or central routes. This is of interest since opiates can modulate the activity of Achase (Dewey, Harris, Howes, Kennedy, Nuite and Hayhurst, 1969). The authors found that opiates were mixed inhibitors of pseudo-cholinesterase activity in vivo. They concluded that there was no correlation between these results and their anti-nociceptive potency. Eserine has been used as a positive control in order to calibrate the technique.

Results (Table 8)

The s.c. injection of calcitonin (20 i.u. kg^{-1}) resulted in no change in the Achase activity in any of the regions studied, i.e. cortex, cerebellum and the remainder. The exception was that the s.c. injection 50 $i.u.kg^{-1}$ produced a significant ($p < 0.05$) reduction in the activity of the area remaining after the cortex and cerebellum were removed.

The i.c.v. injection of calcitonin (10 - 50 $i.u.kg^{-1}$) again produced no change in Achase activity of any of the regions studied, with the exception of the administration of 50 i.u. kg^{-1}, which produced a significant ($p < 0.05$) increase in the Achase activity in the cerebellum.

In an attempt to duplicate the work of Majumdar and Nakhla exactly, the effect of calcitonin on Achase activity in the cortex and cerebellum was assessed using Wistar rats, and the enzyme activity was measured at pH 8.0 and at 37^{o}C. There was no change in the enzyme activity in any of the regions studied.

Eserine (2.13 $mg.kg^{-1}$), which was used as a positive control, produced a significant reduction in enzyme activity in the cortex and remaining areas. There was no change in the activity in the cerebellum.

To assess the accuracy of the assay, the regional distribution of the Achase activity was measured in normal untreated mice. The highest activity was seen in the midbrain area, followed by the cortex, brain stem, hypothalamus and the cerebellum (Table 9). This order is similar to that reported in the literature.

Discussion

The work undertaken in this section was designed specifically to duplicate and extend the work undertaken by Nakhla and co-workers. The results indicate that, in contrast to the work of Nakhla, calcitonin does not stimulate the activity of Achase after any route of administration. The two positive effects observed with calcitonin - a reduction in the activity in the midbrain after an s.c. injection of 50 i.u. kg^{-1} and an increase in the cerebellum after a i.c.v. injection of 50 i.u. kg^{-1} - are considered to be spurious, since at the 5% probability limit there are likely to be one or two positives, by chance. Further evidence that calcitonin does not influence brain Achase activity is shown in the experiment where the experimental conditions of Nakhla's work were duplicated exactly, and again no effect was seen.

Baumann, Perey, Martin-du-Pau and Rousille (1980) can find no depletion in the concentrations of plasma tryptophan after calcitonin injection (s.c. in man), in contrast to the work of Majumdar and Nakhla (1977).

In conclusion, it is unlikely that the antinociceptive actions of calcitonin are elicited by an increase in Achase activity.

Table 8 Effect of Calcitonin and Eserine on Acetylcholinesterase Activity in Mouse and Rat Brain

Treatment	Achase Activity ($\mu mol.mg\ protein^{-1}.hr^{-1}$) Cerebellum	Cortex	Remainder
Calcitonin (Mice)			
(20 i.u. kg^{-1} s.c.)	1.02 ± 0.12	4.15 ± 0.21	3.90 ± 0.05
Vehicle	1.05 ± 0.08	4.37 ± 0.27	3.91 ± 0.18
Calcitonin (Mice)			
50 i.u. kg^{-1} s.c.)	1.50 ± 0.16	5.08 ± 0.23	3.88 ± 0.20*
Vehicle	1.57 ± 0.13	5.22 ± 0.12	4.79 ± 0.23
Calcitonin (Mice)			
(10 i.u. kg^{-1} i.c.v.)	2.01 ± 0.21	7.75 ± 0.33	5.03 ± 0.22
Vehicle	3.25 ± 0.25	7.94 ± 0.26	5.15 ± 0.18
Calcitonin (Mice)			
(50 i.u. kg^{-1} i.c.v.)	1.45 ± 0.14*	6.44 ± 0.30	5.48 ± 0.18
Vehicle	1.76 ± 0.19	6.45 ± 0.32	5.48 ± 0.20
Calcitonin (Rats)			
(20 i.u. kg^{-1} s.c.)	4.41 ± 0.21	7.53 ± 0.34	7.07 ± 0.32
Vehicle	4.33 ± 0.27	7.18 ± 0.37	7.23 ± 0.43
Eserine (Mice)			
(2 $mg.kg^{-1}$ s.c.)	1.77 ± 0.20	3.30 ± 0.25*	3.16 ± 0.24*
Vehicle	2.06 ± 0.24	5.65 ± 0.25	4.48 ± 0.41

Table 8 Effect of calcitonin and eserine on mouse brain acetylcholinesterase activity. Values are mean ± s.e.m.; n = 7-10 *$p < 0.05$.

Table 9 Regional Activity of Acetylcholinesterase in Mouse Brain

Region	Activity (μmol.mg protein^{-1} hour^{-1})
Cerebellum	1.27 ± 0.12
Brain Stem	4.22 ± 0.19
Cortex	5.42 ± 0.55
Mid Brain	6.18 ± 0.44
Hypothalamus	3.30 ± 0.32

Table 9 Regional activity of acetylcholinesterase activity in mouse brain. Values are mean ± s.e.m.; n = 5.

DISCUSSION

This thesis is concerned with the antinociceptive actions of calcitonin. The aims of the project were defined as follows:

(i) to characterise the antinociceptive action of calcitonin wth regard to its site of action, duration, potency and relationship to changes in the plasma calcium concentration;

(ii) to investigate any interaction between calcitonin and the opiate system to produce the antinociceptive response;

(iii) to study any potential relationship between calcitonin-induced antinociception and the calcium flux within the CNS.

The following discussion is firstly concerned with a comparison of calcitonin with other neuropeptides, which are capable of inducing antinociception. Secondly, the antinociceptive actions of calcitonin wll be compared and contrasted with the two main categories of analgesic agents - the narcotic and antipyretic classes. These sections will try to set in context calcitonin as an antinociceptive agent.

The other sections in the Discussion are concerned with the relationship between calcitonin and the calcium concentration of the various compartments in the CNS. In order to discuss this aspect fully, the mechanisms by which the calcium concentration of the CNS is regulated are described. Therefore, the way in which the calcium concentration in the CSF interstitial fluid, neurones and glial cells is controlled will be described in some detail.

The postulated action of calcitonin on the CNS calcium concentration will be compared to that of the two other agents involved in calcium regulation, PTH and vitamin D.

Finally, the relationship between the opiates and the calcium ion is not identical to the relationship between calcitonin and the calcium ion. The similarities and differences will be discussed in some detail.

4.1 COMPARISON OF CALCITONIN WITH OTHER ANTINOCICEPTIVE NEUROPEPTIDES

Calcitonin is not unique among peptides associated with the CNS in producing antinociception. A list of these substances is shown below:

PEPTIDE	EC50 ($nmol.kg^{-1}$)	NALOXONE ANTAGONIST CONCENTRATION ($mg.kg^{-1}$)
Caerulin (s.c.)	83	0.05
CCK-8 (s.c.)	790	0.05 - 1
Substance P (i.c.v.)	25	7 - 40
Leucine enkephalin (i.c.v.)	5000	10
Neurotensin (i.c.v.)	1000	No effect
Morphine (i.c.v.)	1	0.01
Calcitonin (i.c.v.)	0.06	0.1

Calcitonin, when administered i.c.v. and compared to the effects of morphine, is a highly potent agent. This conclusion must be qualified by considering that the stability of these peptides differ (which contributes to the surprisingly low potency of enkephalin), and also that the antinociceptive test used differs (CCK-8 is much less potent in the tail - flick test compared to the hot plate test, Zettler, 1980). Also the route of administration may affect the potency of the peptide.

The use of naloxone to antagonise antinociception is

frequently considered to indicate that the peptide in question may have some affinity for the opiate receptor. This may be erroneous since substances such as CCK-8 possess receptors different from the opiate system, yet naloxone is relatively potent in antagonising the antinociceptive effects of this peptide (Zettler, 1980). Calcitonin appears to be in the middle range, with regard to the ability of naloxone to antagonise its antinociceptive properties. Naloxone at concentrations greater than 1 $mg.kg^{-1}$, has been shown to be hyperalgesic when administered alone, which makes the interpretation of such data difficult.

Calcitonin, when viewed in the context of such peptides as listed in the above table, appears to be exceptional only with regard to its potency. However, the effects of the hormone on the CNS are exerted at concentrations similar to those found in the brain. This fact makes it more likely that the effects observed in this project may be a reflection of a physiological role, and not due to the use of high concentrations (relative to the actual in vivo concentrations) of other peptides listed in the table.

The duration of the action of calcitonin (about 60 minutes) is similar to that observed for other peptides, such as substance P (90 - 120 minutes, Zettler, 1980). It would be instructive to compare the effect of calcium in the antinociception induced by these agents.

The concept that central antinociception is the result of a complex interaction of a range of neuropeptides is not new and at least two theories have been proposed:

Frederickson et al (1980) suggest that peptides such as substance P or somatostatin facilitate the release of endogenous opioid peptides. Alternatively it is possible that the brain possesses a number of independent systems to induce analgesia. Some evidence for this concerns the work reported using neurotensin (Osbahr, Nemeroff, Luttinger, Mason and Prange, 1981). These authors found that antinociception induced by neurotensin was not antagonised by naloxone. The explanation put forward was that since stimulation of the periaquaductal grey matter (an area thought to be responsible for analgesia) produces antinociception, only part of which is reversed by naloxone, then other non-opiate peptides may be involved in antinociception. These authors ascribed this role to neurotensin. It is tempting to speculate that this antinociception not antagonised by naloxone may equally be due to calcitonin. However, a brief report (no experimental data or results were presented) by Yamamoto et al (1979) in the discussion states that microinjection of calcitonin into the periaquaductal grey matter does not produce antinociception.

4.2 COMPARISON OF CALCITONIN WITH 'CLASSICAL' ANALGESICS

Agents which reduce sensitivity to pain are known as analgesics and have been classified into three main types (Bowman and Rand, 1980): narcotic, e.g. morphine; anti-inflammatory, e.g. aspirin and finally local anaesthetics. The clinical use of calcitonin, as an analgesic agent, has provoked speculation as to which class the hormone belongs. As will be seen, calcitonin cannot be classified into such a simplistic system, since it possesses properties of all types.

(i) Anti-inflammatory Analgesics

Agents of this class are thought to act primarily at the periphery, and they induce analgesia by an inhibition of prostaglandin synthesis (Guzman, Braun, Lim, Potter and Rodgers, 1964; Ferreira, 1972). Prostaglandins act primarily by sensitising the nociceptors to noxious stimuli (Ferreira, Moncada and Vane, 1973). In some cases prostaglandins are hyperalgesic when injected alone (Karim, 1971) and this effect may be related to an effect on the intracellular calcium concentration (Ferreira and Nakamura, 1979). In addition to their analgesic action, many of the effects of the anti-inflammatory analgesics may be ascribed to their action on prostaglandin synthesis, e.g. antipyrexia (a process which may be related to an increase in CNS prostaglandin concentration), gastrointestinal irritation (prostaglandins play an important role in protecting the gastric mucosa from erosion) and inhibition of platelet aggregation (prostaglandins G_2 and H_2 induce platelet

aggregation; Lewis, 1976).

Calcitonin has been shown to possess few of the properties outlined above, although it does exert a potent anti-inflammatory action (Strettle, Bates and Buckley, 1980). However, the hormone is an effective antiulcer agent, the opposite action of the antipyrexic analgesic.

The relationship between calcitonin and the prostaglandin system is uncertain. It has been shown that the hormone may inhibit prostaglandin synthesis (Ceserani, Colombo, Olgiati and Pecile, 1979), by influencing cyclo-oxygenase activity. However, Clopath and Sinzinger (1980) have shown that it increases vascular prostacyclin (PGI_2) formation. In addition, it is unlikely that the anti-inflammatory action of calcitonin is due to a lowering of the plasma calcium concentration (Strettle, 1980).

The action of calcitonin on Paget's disease may also be due, in part, to its anti-inflammatory properties (Ceserani et al, 1979). The potent effect of the hormone in alleviating the pain associated with this condition may be due to a long term anti-inflammatory action, since alleviation does not occur until after 2 - 3 weeks of treatment (MacIntyre, 1981). The reduction in skin temperature over the affected joint may also be due to an anti-inflammatory action of the hormone.

Calcitonin, then, only in part resembles this class of analgesic agents. The next section considers how far calcitonin resembles the narcotics.

(ii) Narcotic Analgesics

The characteristics of this class of analgesic agents are that they are highly potent, and are effective against severe forms of pain. They are thought to act primarily on the CNS and, in addition, to analgesia they induce respiratory depression, reduction in motor activity and a decrease in general anxiety (Goodman and Gillman, 1981). These agents are also highly addictive and tolerance to repeated administration occurs.

Calcitonin resembles this class of substances in that the site of action is mainly the CNS. In addition, the hormone is effective against animal models of severe pain (Pecile et al, 1978; Braga et al, 1979). As has been extensively discussed, there are a number of similarities between calcitonin and the opiates with regard to the calcium flux in the CNS. Finally, withdrawal from chronic calcitonin treatment results in the development of hyperalgesia, which is also seen after chronic opiate treatment.

However, the similarities are limited, and there are major differences both on a gross behavioural level and in the mechanism of antinociception. Calcitonin, in contrast to the narcotic analgesics, only exerts a few specific effects on the CNS. There is no evidence of respiratory depression, reduction of locomotor activity, etc. In fact, some actions of the hormone resemble the action of opiate antagonists (e.g. the anorexic effect (Levine and Morley, 1980) and the pressor action of the hormone in haemorrhage).

Finally, there is convincing evidence (i.e. that calcitonin does not affect the binding of opiates to brain homogenates, and vice versa; calcitonin is inactive in opiate bioassays in vitro; calcitonin has opposite effects to the opiates in haemorrhagic shock; calcitonin-induced antinociception is relatively insensitive to naloxone; the attenuation by calcitonin of evoked cortical potentials to painful stimuli is not antagnised by naloxone) which further points to the fact that the action of calcitonin is not totally dependent on the opiate system.

4.3 REGULATION OF CNS CALCIUM CONCENTRATION

The action of calcitonin on the CNS to produce anti-nociception may involve changes in the calcium fluxes within the brain. In order to understand how the hormone may do this, it is important to discuss how the calcium concentration within the brain is controlled. It will be seen that whilst the plasma calcium concentration is controlled within precise limits, primarily by endocrine control, the brain calcium content is controlled, as precisely by transport processes, both active and passive in nature. Figure 60 shows the fluid compartments of the CNS.

Figure 60 Diagram showing the relationship between neurones, glia interstitial fluid (ISF), cerebrospinal fluid (CSF) and blood. Thick arrows represent direction of fluid flow. Double ended arrows indicate sites where exchange of water and solutes may occur.

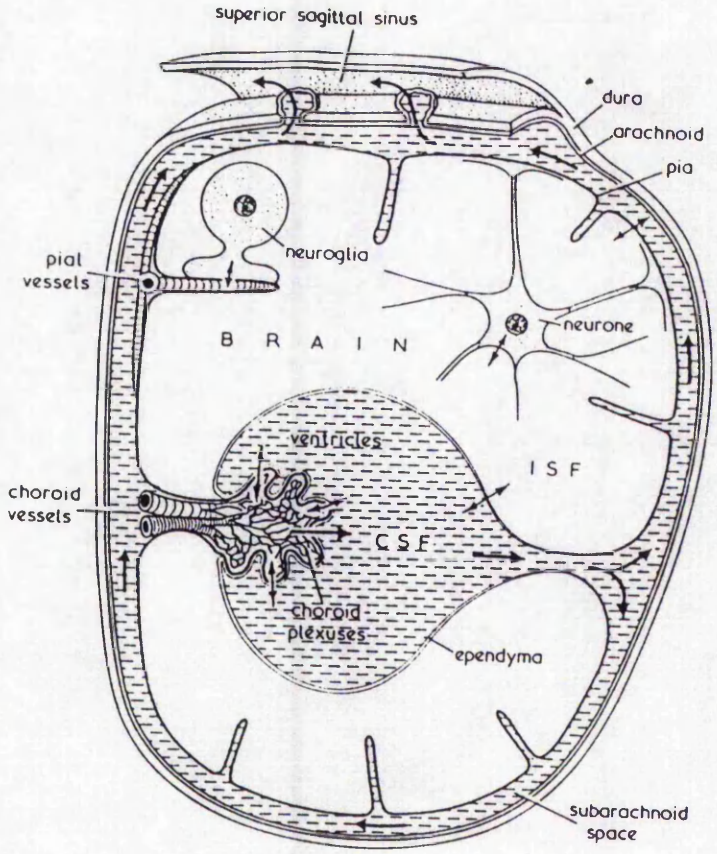

superior sagittal sinus
dura
arachnoid
pia
neuroglia
pial vessels
neurone
B R A I N
ventricles
I S F
choroid vessels
C S F
choroid plexuses
ependyma
subarachnoid space

Figure 60

4.3.1 CSF Calcium Regulation

The normal calcium concentration in the CSF may vary between 1 and 1.5 $mmol.l^{-1}$ whereas the serum calcium concentration may vary between 2 and 2.8 $mmol.l^{-1}$. Calcium ions are transported from the choroid plexus and the concentration may be altered by diffusional exchange with the surrounding tissues in the passage from the ventricle to the lumbar sac.

About one third to one half of plasma calcium is protein bound, and it was suggested that the CSF calcium concentration represents the unbound portion of plasma calcium (Cameron and Moorehouse, 1925). However, the actual unbound portion of calcium in the plasma is still greater than that in the CSF. It has also been shown that the plasma can exhibit large changes in calcium concentration, which are reflected in the CSF. These facts indicate that the CSF calcium concentration is determined by active secretory processes, and does not represent the dialyzable, diffusible calcium content of the plasma.

The CSF calcium concentration is regulated in a precise homeostatic fashion, although minor changes may be seen in animals undergoing parathyroidectomy (Merrit and Bauer, 1931). In such animals, the calcium concentration in the CSF was significantly reduced 5 weeks after the operation. The infusion of $CaCl_2$ into these animals, to raise the plasma calcium concentration from 3 $mmol.l^{-1}$ to 9 $mmol.l^{-1}$ induced a rise of only 0.5 $mmol.l^{-1}$ in the CSF. The homeostatic mechanism which maintains the CSF calcium

concentration is, therefore, good, but not perfect. It has since been shown that calcium exchange between the blood and the CSF is primarily a carrier-mediated process, but that there is also a small diffusional component.

The mechanism by which calcium is transferred from the blood to the CSF has not been extensively studied. However, it has been shown that when the plasma calcium concentration is reduced, the transport of ^{45}calcium is increased, and vice versa.

The rate of efflux of calcium from the CSF is directly related to the CSF calcium concentration. This is, however, unlikely to be due to passive diffusion, since the plasma calcium concentration is greater than that of the CSF. The mechanism is thought to be an active transport mechanism not functioning near its maximum.

4.3.2 INTERSTITIAL FLUID CALCIUM REGULATION

The CNS differs from most other organs in that the cellular elements are tightly packed together. The volume of the extracellular space may be as little as 5 - 15% of the total volume, compared to the 20 - 40% found in most other tissues (Iversen and Iversen, 1975). The interstitial fluid of the CNS may be envisaged as being composed of narrow clefts between the neurones and glial cells. Such clefts possess high sodium and low potassium and calcium concentrations (Orkland, 1982). In this respect, it resembles the composition of the CSF, with which it is often assumed, the brain interstitial fluid readily equilibrates. However, the exchange of calcium between the CSF and the brain may be less than that of other potassium ions.

The action of glial cells in acting as calcium buffers to maintain the ionic environment of the neurone wll be discussed more fully later.

4.3.3 NEURONAL CALCIUM REGULATION

The large resting membrane potential difference of -70 mV means that a low intracellular calcium concentration (10^{-7} $mol.l^{-1}$) compared to the outside (10^{-5} $mol.l^{-1}$) is actively maintained. This concentration difference is maintained by processes includng mitochondrial buffering, and binding by protein. A rise in intracellular calcium stimulates active efflux, via a sodium-calcium exchange mechanism.

Elevations in the intracellular calcium ion concentration occurs when the neurone is depolarised and this results from two phases of calcium entry. A rapid initial phase, where the calcium enters the cell by voltage-sensitive sodium channels and a slower phase of entry, where the ion enters by voltage-insensitive calcium channels. The inactivation of calcium entry occurs as a result of a critical value in the intracellular concentration being reached (10^{-7} - 10^{-6} $mol.l^{-1}$), which causes the channels to close.

4.3.4 GLIAL CELL CALCIUM REGULATION

Until recently, the role of glial cells in determining the extracellular fluid composition of the brain had not been extensively studied. However, it has been shown (Orkland, 1982) that the relationship between neurone and glial cell is extremely close and the two are thought to be functionally linked. Neurones and glia are anatomically distinct but gap junctions permit the rapid exchange of ions. Over most adjacent surfaces the glial cells and neurones are separated by a narrow cleft. It has been proposed (Orkland, 1982) that these cells communicate by altering the ionic composition of this cleft. Nerve impulses are thought to produce a small influx in sodium ions and calcium ions into the cell and a small efflux of potassium into the cleft (10^{-12} mol.cm^{-2}) of sodium ions and the same amount of potassium ions (Katz, 1966). Because of the small volume of the synaptic cleft, these ion fluxes significantly affect the ionic concentration of the interstitial fluid. With intense neuronal activity the concentration of potassium in the interstitial fluid increased by fivefold and the calcium concentration decreased by a third. This change in the external potassium concentration effects neuronal excitability, induces the glial cell to depolarise and influences a variety of metabolic processes in the glial cell (the fact that glial cells depolarise is still, however, unresolved (Orkland, 1982).

The elevation in the external potassium concentration is also thought to signal the glial cells to restore the

ionic composition of the cleft. The external potassium concentration is restored by an active transport mechanism. Glial cells are also thought to restore the calcium concentration of the cleft (Sellstrom, 1981) since the efflux of calcium from glial cels is dependent upon the external potassium concentration.

4.4 ACTION OF CALCITONIN ON THE CNS

The above discussions show that calcium metabolism in the CNS is a complex interaction between the blood, CSF and the various tissue compartments. Each compartment possesses homeostatic mechanisms to a greater or lesser extent. It is possible, therefore, that agents that modify neuronal activity, by reducing the available calcium for entry into the neurone, may act on a number of mechanisms.

General Considerations

The ability of substances to affect the CNS is dependent on their ability to enter the CNS. The entry of substances into the CNS is strictly governed. The structure of the capilliaries in the brain is such that the walls are devoid of any fenestrations, so that substances may only leave the blood vessels by traversng the wall. In addition, 80% of the external capilliary is covered by a glial cell sheath. Consequently, only small or highly lipophilic molecules are thought to enter the CNS in this fashion. The penetration of substances into the CSF occurs either by passage into the interstitial fluid of the brain from the cerebral capilliaries or by passage into the blood vessels of the choroid plexus, the structure responsible for secreting the CSF. However, the distribution of this 'blood-brain barrier' is not homogenous, in that some areas of the brain possess capilliaries which share permeability properties of capilliaries in general. These areas include the floor of the median eminence, the preoptic recess of the hypothalamus and the area postrema at the caudal extreme

of the 4th ventricle. In these areas, agents which achieve low concentrations in the other areas of the CNS, achieve relatively high concentrations in the interstitial fluid in these regions (Oldendorf, 1978).

The next section describes the effects of the other agents involved in the regulation of the plasma calcium concentration, parathyroid hormone and vitamin D on the calcium metabolism of the brain.

4.5 CALCITONIN AND CNS CALCIUM REGULATION

Calcitonin, or a molecule very similar to the peptide in structure, is phylogenetically, a primitive neurohormone. Its sustained presence in neural tissue throughout evolution implies that calcitonin must exert a physiological influence over neural function. Calcitonin, in the periphery, is an important regulator of the intracellular calcium concentration of a number of tissues. In view of the pivotal role of calcium in the CNS function, it is reasonable to suggest that the hormone may influence the concentration of calcium in the CNS. The previous section describes how the calcium concentration in several compartments in the CNS is controlled. To influence neural function calcitonin may act at one or more of these compartments, to alter the calcium concentration.

Calcitonin, after peripheral injection, has been shown to alter the concentration of CSF calcium (Stekol'nikov and Abdukarimov, 1969; Carmen and Wyatt, 1979). It could do this either by altering the calcium transport mechanisms to or from the CSF to the blood/interstitial fluid or vice versa. There is no evidence to suggest how this may occur. In the periphery, calcitonin inhibits the efflux of calcium from various tissues. In the CNS calcium may inhibit the efflux of calcium from the CSF, to the blood, to the interstitial fluid, or both. At present, the only studies concerning the action of calcitonin on the CSF calcium concentration have reported diammetrically opposing results. Stekol'nikov and Abdukarimov (1969) have reported that a

rise in the CSF calcium concentration occurs, whilst the converse is reported by Carmen and Wyatt (1979). No studies have been published concerning the effect of centrally administered calcitonin on the calcium concentration of the CSF.

The results presented in this project imply that antinociception induced by calcitonin, resembles that of the opiates, in that the main event is reduction in the calcium concentration at the nerve terminal. It is unlikely, however, that calcitonin inhibits the uptake of calcium by nerve terminal (in a similar fashion to the opiates), since this is the opposite of its action in the periphery. Calcitonin may, however, do this indirectly by reducing the concentration of available calcium which may enter the nerve. The role of glial cells, in extruding calcium, to buffer the reduction in calcium concentration which occurs after neuronal depolarisation, has been described. Calcitonin may inhibit the efflux of calcium by glial cells, and thus reduce their buffering capacity. The calcium available in the narrow cleft between the glial cell and neurone is therefore reduced. There is no evidence for such speculation, althought Loffler et al (1982) have shown that calcitonin can influence glial cell function, by binding to specific receptor sites on these cells. It is obvious that further experiments would need to be undertaken to study this proposal.

4.6 EFFECT OF PTH AND VITAMIN D ON CNS CALCIUM METABOLISM

Parathyroid hormone, when administered into hypocalcaemic subjects, has been reported (Graziani, Escrive and Katzman, 1965), to reduce the influx of ^{45}calcium from the blood to the CSF. (These animals were rendered hypocalcaemic by removal of the parathyroid and thyroid glands.) When these animals were given calcium gluconate, by infusion, and the flux plotted as a function of the plasma calcium concentration, the active component was significantly smaller than in animals made hypocalcaemic by the injection of EDTA. In hypoparathyroid animals, the passive component of calcium flux into the CSF was unchanged, so that calcium gluconate increased the flux from the blood to the CSF towards the normal value. The authors also showed that in hypoparathyroid animals, not perfused, the calcium concentration in the CSF was reduced 5 weeks after gland removal, whereas from a period greater than 5 weeks, the CSF calcium concentration gradually increased towards control values. It was concluded that the PTH is not essential for the maintenance of normal CSF calcium concentration when the serum calcium concentration is normal, but does play a role at very low plasma calcium concentrations.

The s.c. injection of either PTH or parathyroid gland extract (Arieff and Massry, 1974) produced an increase in the calcium content of brain tissue, unrelated to a rise in the calcium concentration of either the CSF or the plasma. These experiments were undertaken in uraemic dogs, and the authors concluded that the changes were unrelated to the

uraemia per se, but to the presence of an excess of PTH. (This hormone is elevated in uraemia.)

Harris, Carnes and Forte (1981) have reported that parathyroidectomy or infusion of PTH did not affect the total calcium of the brain. However, animals fed on a calcium deficient diet or vitamin D (which caused a sevenfold elevation in plasma PTH concentration) showed a decrease in the brain calcium concentration (24% in the brain stem and 10% in the striatum). It was concluded that long-term hypocalcaemia produces changes in brain calcium concentration, which is not seen under short-term hypocalcaemia. However, the authors did not state that PTH exerts a modulatory role on brain calcium function. Infusion of calcium elevated brain calcium concentrations without reducing plasma calcium concentrations. After reduction of plasma PTH by thyroid/parathyroidectomy, the infusion of calcium was ineffective in changing brain calcium concentration. Since the infusion of calcium caused only a slight change in plasma calcium concentration, but restored brain calcium concentrations to control values, it was concluded that PTH directly stimulates the uptake of calcium into the brain.

Recently, the action of vitamin D on CNS function has become more complicated, because of the isolation of a specific vitamin D dependent binding protein for calcium. This substance has been found in tissues associated with calcium transport, such as the kidney (Taylor and Wasserman, 1972), bone (Christakos and Norman, 1978) and shell gland

(Corradino, Wasserman, Pubols and Chang, 1968). The function of this substance is uncertain, although it may act as an intracellular buffering agent (Spencer, Charman, Wilson and Lawson, 1976) or it may be an essential requirement for calcium transport to occur (Wasserman and Taylor, 1973). This protein, in the chick CNS, was found to be present in highest concentrations in the cerebellum (11 times more than in the cortex). The substance was thought to be present on neurones, since the use of kainic acid (a substance which destroys neurones) produced a reduction in the activity of the protein (Baimbridge and Owen Parkes, 1981). These authors, thus, consider that the protein is not present on the glia.

Calcitonin is similar to these agents in being able to influence CNS function. It is possible that as well as maintaining the peripheral calcium concentration relatively consistent, these agents may act, coordinately, to control the calcium content of the CNS.

4.7 POSSIBLE SIGNIFICANCE OF THE CHRONIC ACTION OF CALCITONIN

This project has shown that chronic administration of calcitonin results in the development of hyperalgesia, after withdrawal of the calcitonin. This may be a central effect and may involve changes in the cellular calcium concentration. In view of the previous discussions, calcitonin, a polar polypeptide hormone, is unlikely to cross the blood brain barrier in appreciable concentrations after s.c. injection. The hyperalgesia may, therefore, be due to mobilisation of an intermediary, which is able to cross the blood brain barrier and can influence the CNS. As discussed previously, PTH can do this by increasing the uptake of calcium into the brain when the plasma calcium concentration is reduced. In the experiments described in this project it was shown that the plasma calcium concentration is lowered after the s.c. injection of calcitonin. This reduction would increase the secretion of PTH, which may in turn increase the calcium content of the brain tissue. However, this indirect effect of calcitonin would be only short lived, snce 4 or more injections of calcium are required to induce hyperalgesia. This could be because repeated injections of calcitonin are needed to lower plasma calcium, and thus to increase PTH secretion, to eventually alter brain calcium concentrations sufficiently to induce hyperalgesia. An elevation of brain calcium is a distinct possibility since the antinociceptive effects of EGTA are attenuated in calcitonin pretreated animals.

The development of hyperalgesia after chronic calcitonin treatment by central injection has not been reported. It is possible that repeated central injection does not lower the plasma calcium concentration sufficiently to mobilise an intermediary which then alters brain calcium concentration. However, it is fair to state that Braga et al did not administer the hormone as long as the chronic peripheral administration undertaken in this project and that these authors did not look for the development of hyperalgesia during the withdrawal period.

4.8 RELATIVE ROLES OF CALCIUM IN OPIATE AND CALCITONIN ANTINOCICEPTION

(i) Calcium and Opiate Action

The previous discussions have set in context the regulation of calcium within the CNS. Changes in neuronal calcium ion concentration induced by the opiates are presumably localised to the areas associated with the opiate receptor. The changes induced are presumed to be at the neurones only, although no evidence has been reported as to the effects of the opiates on the permeability of the blood brain barrier, cerebral capilliary permeability, etc. The action of the opiates on the CNS is thought to be related to an effect on the calcium concentration in the brain. This has been discussed at length previously and will be briefly reviewed here.

Opiates are thought to induce a reduction in the neuronal calcium concentration, which reduces the release of neurotransmitter and analgesia eventually results. A homeostatic mechanism then occurs which reverses the reduction in neuronal calcium and produces a compensatory increase. Repeated addition of the opiates produces a similar effect and the calcium concentration increases as a result. Therefore, greater concentrations of opiate are required to elicit the same effect. The sudden withdrawal of opiate reveals greatly elevated calcium concentrations and neurotransmitter release is greatly facilitated (Chapman and Way, 1980).

This model, though consistent with many known actions

of opiates, implies that neuronal calcium concentration is adjusted to new levels after every administration of the opiates. However, there is no evidence to suggest that the machinery involved in regulating neuronal calcium concentration is so adjusted during chronic opiate administration. The large reduction in the calcium content of the brain which occurs (about 30% in all regions) should presumably be reflected in the calcium concentration of the CSF. This also implies that the calcium transport mechanism in the CSF, interstitial fluid and cellular compartments are affected. However, no reports have been published on this aspect. Furthermore, the amount of morphine which enters the brain after peripheral administration is very small (about 2% of the administered dose, Oldendorf, 1978) and such a massive reduction in the calcium concentration is hard to reconcile with the highly localised action of the opiates.

The current theory (Chapman and Way, 1980) also suggests that the opiates inhibit calcium uptake by inhibiting the binding of calcium to the neuronal membrane. Calcium also inhibits opiate binding (Pasternak, Snowman and Snyder, 1975), although not as effectively as manganese. It has, therefore, been suggested that the opiate receptor and the calcium channel are functionally linked. This may not necessarily be the case since calcium alters the fluorescence of morphine which may be due to complex formation (Lin, Sutherland and Way, 1975) and could also account for the ability of calcium ions to antagonise opiate

actions.

(ii) Calcium and Calcitonin Action

The relationship between calcium and calcitonin is similar to that of the opiates, yet there are some important differences. Calcitonin-induced antinociception is not antagonised by magnesium in contrast to the opiates. EGTA potentiates both opiate and calcitonin antinociception. Similarly calcium ionophores will antagonise both morphine and calcitonin antinociception. Calcium antagonists potentiate the action of both morphine and calcitonin in producing antinociception. The withdrawal of either opiates or calcitonin produces hyperalgesia - it would be instructive to see whether there is any degree of cross tolerance between opiate addicted animals and calcitonin. In animals withdrawn from chronic calcitonin treatment, EGTA is less effective in producing antinociception, which is similar to that observed in opiate addicted animals. However, in animals withdrawn from chronic calcitonin treatment, calcium or magnesium ions are ineffective in enhancing the state of hyperalgesia observed. This is in contrast to the actions of these ions in animals withdrawn from chronic opiate treatment. Finally, acute administration of calcitonin does not affect the calcium content of the brain, whereas large reductions are seen with opiate administration. Similarities may exist between calcitonin and morphine with regard to their effect on brain slice calcium uptake.

The above similarities and differences between opiate

and calcitonin antinociception allow one to conclude that the mechanism of action of calcitonin antinociception may be different from that of morphine. Similar conclusions have been made by other authors (e.g. Braga et al, 1978) to the effect that calcitonin and opiate systems are essentially independent but may share overlapping pools of neurones. Alternatively, it may be suggested that opiates and calcitonin act on different cell types within the brain.

The absence of any side effects of calcitonin suggests that calcitonin exerts a specific localised action. This may be reflected in the lack of effect of calcitonin on the total brain calcium concentration. The basis for such a localised specific action of the hormone presumably resides in a discrete distribution of receptor sites. In the Introduction there was a discussion of the possible local regulatory role of calcitonin (Deftos, 1981).
Calcitonin may be secreted from the periventricular areas into the CSF, possibly in response to changes in the calcium concentration. Once in the CSF calcitonin may affect CNS function in a number of ways by either altering the calcium transport mechanisms in the CNS or by affecting cellular function directly.

REFERENCES

S.E. Abdullahi, E. Arrigoni-Martelli, E. Bramm, L. Franco and G.P. Velo (1977) Effect of Calcitonin on Different Inflammatory Models. Agents and Actios 7 533-538.

S.G. Amara, V. Jonas, M.G. Rosenfeld, E.D. Ong and R.M. Evans (1982) Alternative RNA Processing in Calcitonin Gene Expression Generates mRNA's Encoding Different Polypetide Products. Nature 298 240-244.

A. Ames and F.E. Nesbett (1958) A Method for Multiple Electrolyte Analyses on Small Samples of Nervous Tissue. J. Neurochem 3 116-121.

R. Ardaillo, J.P. Fillastre, G. Milhaud, F. Rousselet, F. Delaunay and G. Richet (1969) Renal Excretion of Phosphate Calcium and Sodium During and After Prolonged Thyrocalcitonin Infusion in Man. Proc. Soc. Exp. Biol. Med. 131 56-60.

A.I. Arieff and S.G. Massry (1974) Calcium Metabolism of Brain in Acute Renal Failure - Effects of Uraemia, Haemodialysis and Parathyroid Hormone. J. Clin. Invest. 53 387- 392.

L.A. Austin (1981) Calcitonin Physiology and Pathophysiology. New Eng. J. Med. 304 269-278.

R.F.L. Bates and J.P. Barlet (1974) The Preventative Effect of Porcine Calcitonin Given by Mouth on Restraint Induced Gastric Ulcers in Rats. Horm. Metab. Res. 6 332-333.

K.L. Becker, S. Snider and C.F. Moore (1979) Calcitonin in Extrathyroidal Tissues of Man. Acta. Endocrinol. 92 746-751.

K.G. Baimbridge and C. Owen Parkes (1981) Vitamin D Dependent Calcium Binding Protein in the Chick Brain. Cell Calcium 2 65-76.

H.A. Bern (1980) Primitive Control of Endocrine Systems. In: Hormones Adaptation and Evolution. Ed. S. Ishit. Springer-Verlag, Berlin, 25-33.

V.V. Barabanova and Y.Y. Yegorov (1980) Effect of Calcitonin on Electrical Activity of the Sinoauricular Node and Contractile Myocardium in Warm Blooded Animals. Fiziol ZH SSSR IM. Sechenova 66 1222-1223.

F. Blanquet, M. Bouvier and J. Gonella (1982) Effects of Enkephalin and Morphine on Spontaneous Electrical Activity and on Junction Potentials Elicited by Parasympathetic Nerve Stimulation in Cat and Rabbit Colon. Br. J. Pharmacol. 77 419-429.

G.R. Brobalik, R.R. Kleszynski, J.P. Aldred and J.W. Bastian (1974) Differential Effects of Salmon Porcine and Human Calcitonin on Gastric Acid Secretion and Gastric Emptying in Rats. Proc. Exp. Biol. Med. 147 284-288.

A.B. Borle (1975a) Regulation of Cellular Calcium Metabolism and Calcium Transport by Calcitonin. J. Membr. Biol. 21 125-146.

A.B. Borle (1975b) Regulation of Mitochondrial Control of Cellular Calcium Homeostasis and Calcium Transport by Phosphate Parathyroid Hormone Calcitonin Vitamine D and Cyclic AMP. In: 5th Parathyroid Conference. Excerpta Medica, Amsterdam 217-228.

A.B. Borle (1976) Cyclic AMP Stimulation of Calcium Efflux from Islated Mitochondria: A Negative Report. J. Membr. Biol. 29 209-210.

A.L. Boura and J.E. Olley (1981) Characterisation of the Excitatory Opiate Receptors in the Rat Large Intestine. Br. J. Pharmacol. 74 246P.

W.C. Bowman and M.J. Rand (1980) Textbook of Pharmacology Blackwell, U.K.

P. Braga, S. Ferri, A. Santagostino, V.R. Olgiati and A. Pecile (1978) Lack of Opiate Receptor Involvement in Centrally Induced Analgesia. Life. Sci. 22 971-978.

P. Baumann, M. Perey, R. Martin Du Pau and I. Rouselle (1980) Effect of Calcitonin on Free and Total Plasma Tryptophan in Humans. Acta Vitamin. Enzymol. 2 6-8.

R.C. Campbell (1967) Statistics for Biologists. Cambridge University Press, Cambridge.

D.B. Chapman and E.L. Way (1980) Metal Ion Interaction With Opiates. Ann. Rev. Pharmacol. Toxicol. 20 553-579.

D.B. Chapman, J. Hu and E.L. Way (1980) Methionine Enkephalin Antagonism and Endorphin Potentiation of Narcotic-Induced Analgesia. Eur. J. Pharmacol. 65 369-377.

A.T. Cameron and M. Moorehouse (1925) The Tetany of Parathyroid Deficiency and the Calcium of the Blood and Cerebrospinal Fluid. J. Biol. Chem. 63 687-720.

H.L. Cardenas and D.H. Ross (1975) Morphine Induced Depletion in Discrete Regions of the Rat Brain. J. Neurochem. 24 487-493.

H.L. Cardenas and D.H. Ross (1976) Calcium Depletion of Synaptosomes After Morphine Treatment. Br. J. Pharmacol. 57 521-526.

J. Carmen and T. Wyatt (1979) Calcium: Bivalent Cation in the Bivalent Psychoses. Biol. Psych. 14 295-326.

C.H. Cashin and C.E. Heading (1968) The Assay of Anti-Pyrexic Drugs in Mice Using Intracerebral Injection of Pyretogenins. Br. J. Pharmacol. 34 148-150.

R.A. Carradino and R.H. Wasserman (1968) Vitamin D_3 Induction of a Calcium Protein in the Uterus of the Laying Hen. Arch. Biochem. Biophys. 125 378-380.

R. Cesarini, M. Colombo, V.R. Olgiati and A. Pecile (1979) Calcitonin and Prostaglandin Synthesis. Life Sci. 25 1851-1856.

G.A. Charbon and E.E.M. Piepper (1972) Effect of Calcitonin on Parathyroid Hormone Induced Vasodilatation. Endocrinol. 91 828-831.

A. Chausmer, P. Weiss and S. Wallach (1965) Effect of Thyrocalcitonin on Calcium Exchange in Rat Tissues. Endocrinol. 77 1151-1154.

R.E. Chipkin, D.H. Morris, M.G. English, J.D. Rosamund and J.M. Stewart (1981) Potent Tetrapeptide Enkephalins. Life Sci. 28 1517-1522.

S. Christakos and A.W. Norman (1978) Vitamin D_3 induced Calcium Binding Protein in Bone Tissue. Science 202 70-71.

P. Clopath and H. Sinzinger (1980) Calcitonin Increases Porcine Vascular Prostacyclin Formation. Prostaglandins 19 1.

H.O.J. Collier (1964) Analgesics In: Pharmacometrics of Drugs. Laurence and Bacharach, Springer-Verlag.

H.O.J. Collier, A.R. Hammond, S. Horwood-Barrett and C. Schneider (1964b) Rapid Induction by Acetylcholine Bradykinin and Potassium of a Nociceptive Response in Mice and Its Selective Antagonism by Aspirin. Nature 204 1316-1318.

H.O.J. Collier, L.C. Dineen, C.A. Johnson and C. Schneider (1968) The Abdominal Constriction Response and Its Suppression by Analgesic Drugs in the Mouse. Br. J. Pharmacol. 32 295-310.

W.J. Cooke and J.D. Robinson (1971) Factors Influencing Calcium Movements in Rat Brain Slices. Amer. J. Physiol. 221 218-225.

D.H. Copp, E.C. Cameron, B.A. Cheney, A.G.H. Davidson and K.G. Henze (1962) Evidence for Calcitonin - a New Hormone from the Parathyroid that Lowers Plasma Calcium. Endocrinol.70 638-649.

C.W. Cooper and L.J. Deftos (1980) Presence by Radioimmunoassay of a Calcitonin-Like Substance in the Porcine Pituitary Glands. Endocrinol. 106 1886-1891.

C.W. Cooper, T.C. Peng, J.F. Obie and S.C. Garner (1980) Calcitonin Like in Rat and Human Pituitary Glands: Histochemical in vitro and in vivo Studies. Endocrinol. 107 98-107.

C.W. Cooper, J.F. Obie, D.L. Margules, J.J. Flynn and J. Walker (1980) Differences in the Ability of Calcitonin to Inhibit Food Consumption in the Rat. Calcif. Tissue Int. 31 58-62.

B.D. Catherwood and L.J. Deftos (1980) Presence by Radioimmunoassay of a Calcitonin-Like Substance in Porcine Pituitary Glands. Endocrinol. 106 1886-1891.

L.J. Deftos (1981) Calcitonin as a Drug. Ann. Intern. Med. 95 192-197.

L.J. Deftos (1981) Effects of Age and Sex on Calcitonin Secretion In: Calcitonin 1980 Elsevier Press, Amsterdam.

L.J. Deftos, D. Burton, B.D. Catherwood, H.G. Bone, J.G. Parthmore, R. Guillemin, W.B. Watkins and R.Y. Moore (1978) Demonstration by Immunoperoxidase Histochemistry of Calcitonin in the Anterior Lobe of the Rat Pituitary. J. Clin. Endocrinol. Metab. 47 457-460.

L.J. Deftos, D.W. Burton, W.B. Watkins and B.D. Catherwood (1980) Immunohistological Studies of Artiodactyl and Teleost Pituitaries with Antisera to Calcitonin.

W.C. Dermody, M.A. Rosen, R. Anathaswamy, A.G. Levy, C.V. Hickson, P.H. Aldenderfer, W.A. Nelson Rees, P.J. Marangos and E.C. Swanson (1981) Characterisation of Calcitonin and Adrenocorticotropin Producing Human Cloned Cell Lines. J. Endocrinol. Metab. 53 970-977.

W.L. Dewey, L.S. Harris, J.F. Howes, J.S. Kennedy, J.A. Nuite and V. Hayhurst (1969) The Effects of Narcotic Antagonist Analgesics on Rat Brain Cholinesterases. Arch. Int. Pharmacodyn. 182 471-479.

M. De Luise, T.J. Martin and R.A. Melick (1972) Inactivation and Degradation of Porcine Calcitonin by Rat Liver and Relative Stability of Salmon Calcitonin. J. Endocrinol. 48 181-188.

M. Driessens and P. Vanhoutte (1981) Effect of Calcitonin, Hydrocortisone and Parathyroid Hormone on Canine Bone Blood Vessels. Am. J. Physiol. 241 H91-H94.

W.E. Doepfner and E.E. Ohnhaus (1972) Gastrointestinal Effects of Synthetic Salmon Calcitonin in the Rat. Biol. Gastroenterol. (Paris) 5 456c.

C.F. Dreyfuss, M.D. Gerschon, A. Haymovitz and E. Nunez (1976) Calcitonin: Antagonism at Intestinal Muscarinic Receptors. Br. J. Pharmacol. 57 155-157.

N.W. Dunham and T.S. Miya (1957) A Note on a Simple Apparatus for Detecting Neurological Deficit in Mice and Rats. J. Am. Pharm. Assoc. 46 208-209.

G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone (1961) A New and Rapid Colourmetric Determination of Acetylcholinesterase Activity. Biochem. Pharmacol. 7 88-95.

A. Fabbri, C. Santoro, C. Moretti, M. Capa, F. Fraioli, G.P. DiJulio, T. Galluzzi and V. LaManna (1981) The Analgesic Effects of Calcitonin in Humans: Studies on the Role of Opioid Peptides. Int. J. Clin. Pharmacol. Ther. Toxicol. 19 509-511.

W. Feldberg and S.L. Sherwood (1953) A Permanent Cannula for Intracerebroventricular Injection in Cats. J. Physiol. 120 3P.

A.I. Faden and J.W. Holaday (1979) Opiate Antagonists: A Role in the Treatment of Hypovolemic Shock. Science 205 317-318.

S.H. Ferreira and M. Nakamura (1979) Prostaglandin Hyperalgesia-A cAMP/Ca^{2+} Dependent Process. Prostaglandins 18 179-190.

J.J. Flynn, D.L. Margules and C.W. Cooper (1981) Presence of Immunoreactive Calcitonin in the Hypothalamus and Pituitary Lobes of Rats. Brain. Res. Bull. 6 547-549.

K. Forslund, P. Slanina, M. Stridsberg and L.-E. Appelgren (1980) Whole Body Autoradiography of ^{3}H and ^{125}I Labelled Calcitonin in Young Rats. Acta. Pharmacol. Toxicol. 46 398-401.

J.A.Fischer, S.M. Sagar and J.B. Martin (1981) Characterization and Regional Distribution of Calcitonin Binding Sites in the Rat Brain. Life Sci. 29 663-671.

R.C.A. Frederickson and P.D. Gesellchen (1980) Analgesic and Other Activities of Substance P and Fragments. In: Neuropeptides and Neural Transmission Ed: C.Ajmone Marsan and W.Z. Traczyk, Raven Press, N.Y.

R.C.A. Frederickson, V. Burgis and J.D. Burgis (1977) Hyperalgesia induced by Naloxone Follows a Diurnal Rhythm in Responsivity to Painful Stimuli. Science 198 756-760.

W.J.Freed, M.J. Perlow and R.D. Wyatt (1979) Calcitonin: Inhibitory Effect on Eating in Rats. Science 206 850-852.

H.A.Fritsch, S. Van Noorden and A.G.E. Pearse (1980) Calcitonin Like Immunochemical Staining in the Alimentary Tract of Ciona intestinalis L. Cell Tissue Res. 205 439-444.

F.Fraioli, A.Fabbri, L. Gnessi, C. Moretti, C. Santoro and M. Felci (1982) Subarachnoid Injection of Salmon Calcitonin Induces Analgesia in Man. Eur. J. Pharmacol. 78 381-382.

Ibid (1982) Subarachnoid Calcitonin for Intolerable Pain. Lancet 2 (8302) 831.

S.E.File and J. Wardill (1975) Validity of Head Dipping as a Measure of Exploration in a Modified Hole Board. Psychopharm. (Berlin) 44 53-56.

H.A.R. Fritsch, S. Van Noorden and A.G.E. Pearse (1979) Localisation of Somatostatin Substance P and Calcitonin Like Immunoreactivity in the Neural Ganglion of Ciona intestinalis L (Ascidaceae). Cell Tissue Res. 202 263-274.

S.I. Girgis, F. Galan Galan, T.R. Arnett, R.M. Rogers, Q. Bone, M. Ravazzola and I. MacIntyre (1980) Immunoreactive Calcitonin Like Molecule in the Nervous System of Protochordates and a Cyclostome, Myxine. J. Endocrinol. 87 375-382.

F. Galan Galan, R.M. Rogers, S.I. Girgis, T.R. Arnett, M. Ravazzola, L. Orci and I. MacIntyre (1981) Immunochemical Characterisation and Distribution of Calcitonin in the Lizard. Acta Endocrinol. 97 427-432.

F. Galan Galan, R.M. Rogers, S.I. Girgis and I. MacIntyre (1981) Immunoreactive Calcitonin in the Central Nervous System of the Pigeon. Brain Res. 212 59-66.

R.F. Gagal, E.F. Voelkel, H.J. Wolfe, R.A. DeLellis and D.S. O'Brian (1979) Pituitary Calcitonin Like Material: Radioimmunoassay and immunohistochemical studies. Proc. 61 rst. Meeting of Endocrine Soc. 87.

D. Goltzman (1980) Examination of Interspecies Differences in Renal and Skeletal Receptor Binding and Adenylate Cyclase Stimulation with Human Calcitonin. Endocrinol. 106 510-516.

L.S. Goodman and A. Gilman (1975) The Pharmacological Basis of Therapeutics. 5th Edition, MacMillan, N.Y.

J. Glowinski and L. Iversen (1966) Regional Studies of Catecholamines in the Rat Brain. I. J. Neurochem. 13 665-672.

M.G.C. Gillan and D. Pollock (1980) Acute Effects of Morphine and Opioid Peptides on the Motility and Responses of Rat Colon to Electrical Stimulation. Br. J. Pharmacol. 68 381-392.

R.J. Griebel, E.C. Knoblock and T.R. Koch (1979) Fluorometric Titration: Interference from Haemolysis Estimated. Cli. Chem. 25 1983-1984.

F. Guzman, C. Braun, R.K.S. Lim, G.D. Potter and D.W. Rogers (1964) Narcotic and Non Narcotic Analgesics which block Visceral Pain Evoked by Intra-arterial Injections of Bradykinin and Other Analgesic Agents. Arch. Int. Pharmac. Ther. 149 571-588.

L. Graziani, A. Escriva and R.A. Katzman (1965) Exchange of Fluid between Blood, Brain and Cerebrospinal Fluid. Amer. J. Physiol. 208 1058-1064.

F. Guerro-Munoz, K.V. Cerrata, M.L. Guerro and E.L. Way (1979) Effect of Morphine on Synaptosomal Calcium Uptake. J. Pharmacol. Exp. Ther. 209 132-136.

R.A. Harris, E.T. Iwamoto, H.H. Loh and E.L. Way (1975) Analgetic Effects of Lanthanum: Cross Tolerance with Morphine. Brain Res. 100 221-225.

R.A. Harris, H.H. Loh and E.L. Way (1976) Antinociceptive Effects of Lanthanum and Cerium in Non-Tolerant and Morphine Tolerant Animals. J. Pharmacol. Exp. Ther. 196 288-297.

R.A. Harris, H.H. Loh and E.L. Way (1975) Effects of Divalent Cations, Cation Chelators and an Ionophore on Morphine Analgesia and Tolerance. J. Pharmacol. Exp. Ther. 195 488-498.

R.A. Harris, H. Yamoto, H.H. Loh and E.L. Way (1977) Discrete changes in Brain Calcium with Morphine Analgesia, Tolerance Development and Abstinence. Life Sci. 20 501-506.

J.F. Habemer, F.R. Singer, R.M. Neer, L.J. Deftos and J.T. Potts (1972) Metabolism of Salmon Calcitonin and Porcine Calcitonin: An Explanation for the Increased Potency of Salmon Calcitonin. In: Talmage and Munson (eds) Calcium Parathyroid Hormone and the Calcitonins, 152-156. Excerpta Medica, Amsterdam.

A.H. Hassen, G. Feuerstein, A. Pfeiffer and A.I. Faden (1982) Delta Versus Mu Receptors: Cardiovascular and Respiratory Effects of Opiate Agonists Microinjected into Nucleus Tractus Solitarus of Cats. Regulatory Peptides 4 299-309.

T.J. Haley and C. McCormick (1957) Pharmacological Effects Produced by Intracerebral Injection of Drugs in the Conscious Mouse. Br. J. Pharmacol. 12 12-15.

P.F. Hirsch, E.F. Voelkel and P.L. Munson (1964) Thyrocalcitonin: Hypocalcaemic Hypophosphataemic Principle of the Thyroid Gland. Science 146 412-413.

T. Hokfelt, O. Johansson, A. Ljungdahl, J.M. Lundberg and M. Schultzberg (1980) Peptidergic Neurones. Nature 284 515-521.

J.W. Holaday (1981) Endorphins in Shock and Trauma. Abstracts, World Pharmacology Conference, Tokyo.

J. Hotz, H. Minne, H. Goebell and R. Zeigler (1974) Persistent Inhibition of Gastric Secretion by an Intravenous 12 Hour Infusion of Calcitonin in Normal. Peptic Ulcer and High Risk Patients. Digestion 11 311-314.

J.P. Huidoboro-Toro and E.L. Way (1981) Contractile Effect of Morphine, B-Endorphin and Methionine Enkephalin on the Islated Colon from Long Evans Rats. Br. J. Pharmacol. 74 681-694.

S.D. Iversen and L.L. Iversen (1977) Behavioural Pharmacology, Oxford University Press, Oxford.

E.T. Iwamoto, R.A. Harris, H.H. Loh and E.L. Way (1976) Antinociceptive Effects after Microinjection of Lanthanum in Discrete Rat Brain Sites. J. Pharmacol. Exp. Ther. 206 46-55.

Y. Iwasaki, K. Chiara, J. Iwasaki, H. Abe and T. Fujita (1979) Effect of Calcitonin on Prolactin Release in Rats. Life Sci. 25 1243-1248.

J.J. Jacob, J.T. Potts, N.H. Bell and J.F. Habener (1981) Calcitonin Precursors. In: Calcitonin 1980. Ed. A. Pecile, Excerpta Medica.

J.J. Jacob, E.C. Tremblay and M. Colombel (1974) Facilitation de Reactions Nociceptives par le Naloxone chez la Souris et chez le Rat. Psychopharmacology 37 217-223.

S.M.M. Karim (1971) Action of Prostaglandin in the Pregnant Woman. Ann. N.Y. Acad. Sci. 180 483-498.

T. Kakunga, H. Kaneto and K. Hano (1966) Pharmacologic Studies on Analgesics VII. Significance of the Calcium Ion

in Morphine Analgesia. J. Pharmacol. Exp. Ther. 153 134-141.

B. Katz (1966) Nerve Muscle and Synapse. McGraw-Hill, N.Y.

A.D. Kenny and C.A. Heiskell (1965) Effect of Crude Thyrocalcitonin on Calcium and Phosphorous Metabolism in Rats. Proc. Soc. Exp. Biol. Med. 120 269-271.

H. Kosterlitz and S.J. Paterson (1980) Characterisation of Opioid Receptors in Nervous Tissue. Proc. R. Soc. Lond. B 210 113-122.

M. Koida, H. Nakamuta, S. Furukawa and R.C. Orlowski (1980) Abundance and Location of 125-1-Salmon Calcitonin Binding Sites in Rat Brain. Jpn. J. Pharmacol. 30 575-577.

M. Laubie, H. Schmitt, M. Vincet and G. Remond (1977) Central Cardiovascular Effects of Morphinomimetic Peptides in Dogs. Eur. J. Pharmacol. 46 67-71.

H. Laurence Shaw (1982) Subarachnoid Administration of Calcitonin: A Warning. Lancet 2 (8294) 390.

A.S. Levine and J.E. Morley (1981) Reduction of Feeding in Rats by Calcitoniin. Brain Res. 222 187-191.

A.S. Levine and J.E. Morley (1981) Peptidergic Control of Insulin Induced Feeding. Peptides 2 261-264.

G.P. Lewis and P.J. Piper (1975) Inhibition of Release of Prostaglandins as an Explanatiion of Some of the Actions of Anti Inflammatory Corticosteroids. Nature 254 308-311.

R.K.S. Lim, F. Guzman, D.W. Rogers, K. Goto, C. Braun G.D. Dickerson and R.J. Engle (1964) Site of Narcotic and Non Narcotic Action Determined by Blocking Bradykinin Evoked Visceral Pain. Arch. Int. Pharmac. Dynam. 153 25-58.

F. Loffler, D. van Caulker and B. Hamprecht (1982) Parathyrin and Calcitonin Stimulate cAMP Accummulation in Cultured Murine Brain Cells. E.M.B.O. Journal 1 2997-303.

R.N. Lolley (1963) The Calcium Content of Isolated Cerebral Tissues and their Study State Exchange of Calcium. J. Neurochem. 10 665-676.

S.W.J. Lambert, W.H.L. Hackeng amd T.J. Visser (1980) Is Calcitonin Part of the 31K Common Precursor Molecule? Horm. Res. 13 325-326.

O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall (1951) Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193 265-275.

A.P.N. Majumdar and A.M. Nakhla (1977) Influence of Tryptophan on the Activity of Acetylcholinesterase in the Brain of Well Fed and Adrenalectimised Rats. Biochem. Biophysss. Res. Comm. 76 71-77.

I. MacIntyre (1978) The Action and Control of the Calcium Regulating Hormones. J. Endocrinol. Invest. 1 277-284.

I. MacIntyre, C.L. Hillyard, P.K. Murphy, J.J. Reynolds, R.E. Gaines and R.K. Craig (1982) A Second Plasma Lowering Peptide from the Human Calcitonin Presursor. Nature 300 460-462.

T.J. Martin and N.J.Y. Woodhouse (1976) Calcitonin in the Treatment of Paget's Disease. In: Bone Disease and Calcitonin. Armour Pharmaceutical Corp., Eastbourne, 11-18.

J.M. McDonald, D.E. Burns, L. Jarrett and J.E. Davis (1977) A Rapid Microtechnique for the Preparation of Biological Material for the Simultaneous Analysiiis of Calcium, Magnesium and Protein. Ann. Biochem. 82 485-492.

G. Mendelssohn, R. D'Agostino, J.C. Eggleston and S.B. Baylin (1979) Distribution of B Endorphin Immunoreactivity in Normal Human Pituitary. J. Clin. Invest. 63 1297-1301.

H.H. Merritt and W. Bauer (1931) The Equilibrium Between Cerebrospinal Fluid and Plasma IV The Calcium Content of Serum, Cerebrospinal Fluid and Aqueous Humour at Different Levels of Parathyroid Activity. J. Biol. Chem. 90 233-246.

C. Minkin, J. Newbrey and M. Walling (1977) Mononucleur Phagocytes and Bone Resorption: Parathyroid Hormone and Calcitonin Stimulation of Adenyl Cyclase Activityy in Murine Peritoneal Mononuclear Phagocytes. J. Dent. Res. 56 All.

S. Miyahara and Y. Oomura (1981) Inhibitory Effect of Calcitonin on Neurones in the Lateral Hypothalamus of the Rat. Abstracts, World Pharmacology Conference, Tokyo.

R.H. Moore III and D.A. Dowling (1980) Effects of Intravenously Administered Leu- or Met-Enkephalin on Arterial Blood Pressure. Regul. Pept. 1 77-87.

J.E. Morley and A.S. Levine (1981) Intraventricular Calcitonin Inhibits Gastric Acid Secretion. Science 214 671-673.

J.E. Morley, A.S. Levine and J. Kneip (1981) Muscimol Induced Feeding: A Model to Study the Hypothalamic Regulation of Appetite. Life Sci. 29 1213-1218.

J.E. Morley, A.S. Levine, S.S. Murray and J. Kneip (1982) Peptidergic Regulation of Norepinephrine Induced Feeding. Pharmacol. Biochem. and Behav. 16 225-228.

F.Murad, H.B. Brewer and M. Vaughan (1970) Effect of Thyrocalcitonin on Adenosiiine 3' 5' cyclic phosphate.

Formation by Rat Kidney and Bone. Proc. Natl. Acad. Sci. 65 446-453.

H. Nakamuta, S. Furukawa, M. Koida, H. Yajuma, R.C. Orlowski R. Sehleuter and I. Nagasaki. (1980) Jpn. J. Pharmacol. 31 253-260.

S. Nakanishi, I. Inove, T. Kita, M. Nakamura, A.C.Y. Chang, S.N. Cohen and S. Numa (1979) Nucleotide Sequence of Cloned cDNA for Bovine Corticotripin-B-Lipotropin Precursor. Nature 278 423-427.

A.M. Nakhla and A.P.N. Majumdar (1978) Calcitonin Mediated Changes in Plasma Tryptophan and Brain 5-hydroxytryptamine and Acetylcholinesterase Activity. Biochem. J. 170 445-448.

A.M. Nakhla (1980) Stimulation of Cerebral Acetylcholinesterase Activity by Calcitonin: A Possible Mediation by 5-hydroxytryptamine. Horm. Res. 12 161-164.

A.M. Nakhla (1979) Calcitonin: Effect on Protein Synthesis in Different Rat Tissues. Experientia 35 1525.

F.P. Nijkamp and J.M. van Ree (1980) Effects of Endorphiins on Different Parts of the Gastrointestinal Tract of Rat and Guinea-Piig in vitro. Br. J. Pharmacol. 68 599-606.

W.G. Nayler and P. Poole-Wilson (1981) Calcium Antagonists: Definition and Mode of Action. Basic Res. Cardiol. 76 1-15.

W.H. Oldendorf (1978) Factors Affecting Passage of Opiates through the Blood-Brain Barrier. In: Factors Affecting the Action of Narcotics. Ed. M.L. Alder, Raven Press, N.Y.

V.R. Olgiati, C. Netti, F. Guidobono and A. Pecile (1981) Calcitonin Inhibition of Physiological and Stimulated Prolactin Secretion in Rats. Life Sci. 29 585-588.

F.A. Opmeer and J.M. van Ree (1979) Competitive Antagonism of Morphine Action in vitro by Calcium. Eur. J. Pharmacol. 53 395-397.

D.K. Orkland (1982) In: Neuronal-Glial Cell Interrelationships. Ed. T.A. Sears, Springer-Verlag, N.Y. 147-158.

A.J. Osbahr, C.S. Nemeroff, D. Luttiinger, G.A. Mason and A.J. Prange (1981) Neurotensin-Induced Antinociception in Mice: Antagonism by Thyrotropin-Releasing Hormone. J. Pharmacol. Exp. Ther. 217 645-651.

A. Pecile, S. Ferri, P.C. Braga and V.R. Olgiati (1978) Effects of Intracerebroventricular Calcitonin in the Conscious Rabbit. Experientia 31 332-333.

A. Pecile, V.R. Olgiati, G. Luisetto, F. Guidobone, C. Netti and D. Ziliotto (1981) Calcitonin and the Control of Prolactin Secretion. In: Calcitonin 1980 Ed. A. Pecile, Excerpta Medica.

C.B. Pert and S.H. Snyder (1974) Opiate Receptor Binding of Agonists and Antagonists Affected Differentially by Sodium. Mol. Pharmacol. 10 868-879.

R. Perez-Cano, S.I. Girgis, F. Galan Galan and I. MacIntyre (1982) Identification of Both Human and Salmon Calcitonin-Like Molecules in Birds Suggesting the Existence of Two Calcitonin Genes. J. Endocrinol. 92 351-355.

P.W. Reed and H.A. Lardy (1972)) Antibiotic A23187 as a Probe for the Study of Calcium and Magnesium Function in Biological Systems. In: The Role of Membranes in Metabolic Regulation. Ed. M.A. Mehlman and R.W. Hanson. Academic Press, N.Y. 111-132.

A.J. Rizzo and D. Goltzman (1981) Calcitonin Receptors in the Central Nervous System of the Rat. Endocrinol. 108 1672-1677.

D.H. Ross (1978) Effects of Opiate Drugs on the Metabolism of Calcium in Synaptic Tissus. In: Calcium and Drug Action. Ed. G.B. Weiss Plenium Press, N.Y. 241-259.

D.H. Ross, M.A. Medina and H.L. Cardenas (1974) Morphine and Ethanol: Selective Depletion of Regional Brain Calcium. Science 186 63-65.

D.H. Ross (1977) Calcium Content and Binding in Synaptosomal Subfractions During Chronic Morphine Treatments. Neurochem. Ress. 2 581-593.

J. Roth (1982)) New Theory of Hormones Described. Science 215 1383-1384.

N.A. Samaan and M.E. Leavens (1981) Calcitonin-Like Immunoreactivity in the Pituitary Vascular Bed of Man. J. Clin. Endocrinol. Metab. 53 985-989.

G. Sander, T. Giles, A. Kasten, A, Kaneish and D. Cosy (1982) Leucine-enkephalin: Reversal of Intrinsic Cardiovascular Stimulation by Pentobarbital. Eur. J. Pharmacol. 78 467-470.

M. Satoh, H. Amano, T. Nakazawa and H. Takagi (1979) Inhibition by Calcium of Analgesia Induced by Intracisternal Injection of Porcine Calcitonin in Mice. Res. Comm. Chem. Pathol. Pharmacol. 26 213-216.

K. Schaz, G. Stock, H. Strumpf, W. Simon, K-H. Schlor Unger, R. Rockold and D. Ganten (1980) Enkephalin Effects on Blood Pressure, Heart Rate and Baroreceptor Reflex. Hypertension 2 395-407.

T. Shikimi, H. Kaneto and K. Hano (1967) Effect of Morphine on the Liberation of Acetylcholine from the Mouse Cerebral Cortical Slices in Relation to the Calcium Concentration in the Medium. Jpn. J. Pharmacol. 17 136-137.

L.I. Stekol'nikov and A. Abdukarimov (1969) Physicochemical Investigation of the Cerebrospinal Fluid on Treatment with Thyrocalcitonin. Biofizika 14 921-925.

R.J. Strettle (1980) Ph.D. Thesis Pharmacology of Calcitonin. Trent Polytechnic, Nottingham.

A.Sellstrom (1981) On the Calcium Permeability of Neurones ad Glia. Act. Physiol. Scand. 113 253-258.

R. Spencer, M. Charman and D.E. Lawson. (1976) Production and Properties of Vitamin D Induced in RNA for Chick Calcium Binding Protein. Eur. J. Biochem. 71 (2) 399-409.

A.N. Taylor and R.H. Wasserman (1972) Vitamin D Induced Calcium Binding Protein: Comparative Aspects in Kidney and Intestine. Amer. J. Physiol. 223 110-114.

M. Van Houten, D. Goltzman and B.I. Posner (1980) Calcitonin Receptors in Brain: Localization in Normals and Deficiency in Hereditary Diabetes Insipidus. Clin. Res. 28 A676.

M. Van Houten, A.J. Rizzo, D. Goltzman and B.I. Posner (1981) Brain Receptors for Blood-Borne Calcitonin in Rats: Localisation in Normals and Deficiency in Hereditary Diabetes Insipidus. Clin. Res. 29 A546.

F.J. Vocci, S.P. Welch and W.L. Dewey (1980) Differential Effects of Cations, Cation Chelators and an Ionophore (A23187)) on Morphine and Dibutryl Guanosine 3':5' Cyclic

Monophosphate Antinociception. J. Pharm. Exp. Ther. 214 463-466.

E.T.Wallick, J.S. Allen and A. Schwartz (1973) Differential Effects of Metal Chelators on Na^+, K^+-ATPase Activity, Arch. Biochem. Biophys. 158 149-153.

W. Watkins, R.Y. Moore, D. Burton, H.G. Bone, B.D. Catherwood and L.J. Deftos (1980) Distribution of Immunoreactive Calcitonin in the Rat Pituitary Gland. Endocrinol. 106 1966-1970.

A.W. Wase, A. Peterson, E. Rickes and J. Solewski (1966) Some Effects of Thyrocalcitonin on the Calcium Metabolism of the Rat. Endocrinol. 79 687-691.

R.W. Wasserman, C.S. Fuller and A.N. Taylor (1978) The Vitamin D Dependent Calcium Binding Proteins. In: Vitamin D. Ed. D.E.M. Lawson, Academic Press, London 133-166.

E. Weber, K.H. Boight, R.E. Mains and B.A. Eipper (1979) Calcitonin is not Contained within the Common Precursor to Corticotrophin and Endorphin in the Rat. Biochem. Biophys. Res. Comm. 89 360-365.

M. Widman, D. Rosin and D.L. Dewey (1978) Effects of Divalent Cations Lanthanum Cation Chelators and an Ionophore on Acetylcholine Antinociception. J. Pharmacol. Exp. Ther. 205 311-318.

H.J. Woolfe, R.A. DeLellis, D.S. O'Brian and A.H. Tashjian (1979) Immunocytochemical Localization of Calcitonin-Like Material in the Pituitary Gland. Lab. Invest. 40 292-295.

M.Yamaguichi, Y. Takei and T. Yamamoto (1975) Effect of Thyrocalcitonin on Calcium Concentration in Liver and Intact

and Thyroparathyroidectomised Rats. Endocrinol. 96 1004-1008.

M. Yamamoto, F. Kumagi, S. Tachikawa and S. Murayama (1978) Effects of Porcine Calcitonin on Various Analgesic Responses in Mice and Rabbits. Chiba. Med. J. 54 27.

M. Yamamoto, F. Kumagi, S. Tachikawa and H. Maeno (1978) Lack of Effect of Levallorphan on Analgesia Induced by Intraventricular Application of Porcine Calcitonin in Mice. Eur. J. Pharmacol. 55 211-213.

M. Yamamoto, S. Tachikawa and H. Maeno (1981) Evoked Potential Studies of Porcine Calcitonin in Rabbits. Neuropharmacol. 20 83-86.

R. Yui, Y. Yamada, R. Kayamori and T. Fujita (1981) Calcitonin Immunoreactive Neurons in the Brain of the Bullfrog Rana Catesbeiana with Special Reference to their Liquor-Contacting and Neurosecretory Nature. In: Immunochemical and Immunohistochemical Study. Biomed. Res. 2 208-216.

G. Zetler (1980) Analgesia and Ptosis Caused by Caeruliein and Cholecystokinin Octapeptide (CCK-8). Neuropharmacol. 19 415-422.

D. Ziliotto, G. Luisetto, M. Cecchini, M. Gastaldo, G. Francia, L. Padovan and G. Heynen (1979) Calcitonin Serum Levels in Different Pituitary Diseases. A Preliminary Report. J. Endocrinol. Invest. 3 71-72.

APPENDICES

APPENDIX I

STATISTICS

(i) The data was assessed for normal distribution using Filliben's test for normality. This test (Filliben, 1975) is amenable to computer programming in BASIC and was used to test the data prior to undertaking the tests of significance listed below.

(ii) The data from the antinociceptive testing was assessed for significance using the Mann - Whitney U test (Cambell, 1967).

(iii) The remaining data was assessed for signiticance using the paired or non-paired t test where appropriate.

In both tests the data was considered to be significant at the p 0.05 level.

APPENDIX II

HISTOLOGY

Mouse brains were removed and washed as described in the Methods. They were then placed in a formol - saline solution for at least 48 hours. The samples were then embedded in wax after dehydration in alcohol and xylene using a Histokinette machine. Ribbons of the wax section were then cut using a sledge microtome. The thickness of the sections were 5 µm.

The tissues were stained using haemotoxylin and eosin stain according to standard procedures. Briefly, the sections were first hydrated by taking them through graded alcohols (100, 90, 70, 50, water). They were then placed in haemotoxylin for 5 - 10 minutes and differentiated by placing them in acid alcohol for 5 seconds. Counterstaining was done by staining with eosin for one minute. The sections were washed of excess stain and dehydrated by passing the sections through the graded alcohols (water, 50, 70, 90, 100%) and were then cleared in xylene. Coverslips were applied and mounted in DPX mounting medium.

APPENDIX III

Br. J. Pharmac. (1981) 72, 575P

COMPARISON OF THE ANALGESIC EFFECTS OF SUBCUTANEOUS AND INTRACEREBROVENTRICULAR CONSTRICTIONS IN THE MOUSE.

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Calcitonin possesses analgesic activity after administered by injection into the lateral ventricles of the rabbit (Pecile, Ferri, Braga and Olgiati, 1975) by an effect independent of the opiate receptor (Braga, Ferri, Santagostino, Olgiati and Pecile, 1978; Yamamoto, Kumagai, Tachikawa and Maeno, 1979). However, calcitonin also possesses peripheral anti-inflammatory activity (Reisterer & Jacques, 1969; Abdullahi, Bastiani, Nogarin and Velo, 1975; Strettle, Bates and Buckley, 1980) and inhibits the synthesis of prostaglandins and thromboxane (Cesarini, Colombo, Olgiati and Pecile, 1979).

We have investigated the possibility that the analgesic effect of centrally administered calcitonin may be due to a peripheral action.

Acetic acid in 0.154 $mol.dm^{-3}$ NaCl was injected into the peritoneal cavity of CPLA mice (male and female, 25-45 gm BW) (Collier, Dineen, Johnson and Schneider, 1968) and the number of abdominal constrictions was counted between the 10th-14th minute inclusive, after the injection of acid.

Salmon calcitonin was administered by subcutaneous or by intracerebroventricular (ICV) injection. Calcitonin, dissolved in a solution containing 0.154 $mol.dm^{-3}$ NaCl and 1 $gm.dm^{-3}$ bovine serum albumin (BSA), was given by subcutaneous injection either 10 or 20 minutes prior to the injection of acetic acid. Calcitonin, dissolved in 10 µl of a solution containing 0.100 $mol.dm^{-3}$ Tris buffer pH 7.35, was injected into the ventricle by the method of Haley & McCormick (1957) 10 minutes prior to the injection of acetic acid. Aspirin suspended in 0.154 $mol.dm^{-3}$ NaCl plus 100 $gm.dm^{-3}$ gum acacia was injected S.C. 30 minutes prior to acetic acid. Control animals received the appropriate vehicle.

Abdominal constrictions began 5-8 min after the injection of acetic acid and the frequency of constrictions was proportional to the concentration of acid (0.25-2.4% w/v) between the 10th - 14th minutes after the injection. 1 - 1.2% acetic acid was selected for further work. Aspirin (0.125 - 1.0 $mol.kg^{-1}$) caused a dose related inhibition of

the abdominal constrictions with an ED_{50} of 0.44 $mmol.kg^{-1}$.

Salmon calcitonin (0.01 - 1000 MRC U kg^{-1}, 20 minutes prior to acid or 10 - 50 MRC U kg^{-1} 10 minutes prior to acid) by subcutaneous injection did not affect the response to acetic acid. Subcutaneous injection of 10 MRC U kg^{-1} SCT caused a significant fall of 0.46 $mmol.dm^{-3}$ ($p < 0.005$) in the plasma calcium concentration. Salmon calcitonin at doses of 0.040, 0.20, 2.0, 10 and 50 MRC U kg^{-1} by ICV injection caused 14 ± 6.4, 37 ± 9.3 , 38.1 ± 5.7 , 50 ± 8.0 , and 31 ± 8.2 (mean ± SE) % inhibition in the frequency of abdominal constrictions (* = $p < 0.005$; control group n = 26, test groups n = 10). SCT 10 MRC U kg^{-1} by ICV injection caused a fall of 0.18 $mmol.dm^{-3}$ in the plasma calcium concentration ($p < 0.05$).

In conclusion, SCT by subcutaneous injection does not produce analgesia. SCT by ICV injection produced a potent analgesic effect by a central action upon cells accessible to diffusion from the ventricular system. Salmon calctonin, after subcutaneous injection, does not readily cross the blood brain barrier.

REFERENCES

Abdullahi, S.E. et al. (1975) Effect of calcitonin on carageenan foot oedema. Agents and Actions 5, 371-373.

Braga, P. et al. (1978) Lack of opiate receptor involvement in centrally induced calcitonin analgesia. Life Sci. 22, 971-978.

Cesarini, R. et al. (1979) Calcitonin and prostaglandin system. Life Sci. 25, 1851-1856.

Collier, H.O.J. et al. (1968) The abdominal cnstriction response and its suppression by analgesic drugs in the mouse. Br. J. Pharmac. 32, 295-310.

Haley, T.J. and McCormick, W.G. (1957) Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. Br. J. Pharmac. 12, 12-15.

Pecile, A. et al. (1975) Effects of intracerebroventricular calcitonin in the conscious rabbit. Experentia 31, 332-333.

Reisterer, L. and Jacques, R. (1969) Reduction of increased vascular permeability by calcitonin. Pharmacol. 3, 53.

Strettle, R.J. et al. (1980) Evidence for a direct anti-inflammatory action of cacitonin; inhibition of histamine-induced mouse pinnal oedema by porcine calcitonin. J. Pharm. Pharmacol. 32, 192-195.

Yamamoto, M. et al. (1979) Lack of effect of levallorphan on analgesia induced by intraventricular application of porcine calcitonin in the mouse. Europ. J. Pharmacol. 55, 211-213.

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INTERACTION OF CALCIUM IONS AND SALMON CALCITONIN IN THE PRODUCTION OF ANALGESIA IN THE MOUSE.

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Calcitonin produces analgesia when injected centrally (Pecile, Ferri, Braga and Olgiati, 1975) by mechanisms independent of the opiate receptor (Braga, Ferri, Santagostino, Olgiati and Pecile, 1978). The action of opiate drugs are strongly influenced by changes in neuronal calcium ion concentration (Chapman and Way, 1980). We have investigated the effect of changes in brain calcium ion concentration upon calcitonin-induced analgesia.

Abdominal constrictions were induced by i.p. injection of acetic acid (1% in 0.154 mol.dm^{-3} NaCl) in male and female CFLP mice (30g) after the method of Collier, Dineen, Johnson and Schneider (1968). Intracerebroventricular (ICV) injections of 10 µl/mouse were given by the method of Haley and McCormick (1957). Salmon calctonin (SCT), ethylene glycol-bis-(β-amino ethyl) N,N' tetra acetic acid (EGTA) and $CaCl_2$ were dissolved in tris-saline pH 7.35 (Bates, Buckley, Eglen and Strettle, 1981). Control animals received tris-saline alone.

Intracerebroventricular injections of $CaCl_2$ (0.1 µmol/mouse) caused an increase in the frequency of abdominal constrictions. Intracerebroventricular injections of EGTA (0.05 µmol/mouse) decreased the frequency of abdominal constrictions. These findings are similar to those of Schmidt and Way (1980) using the tail-flick test. The ICV injection of an equimolar concentration of $CaCl_2$ together with the EGTA restored the frequency of abdominal constrictions to control values (3.63 ± 0.39 abdominal constrictions min^{-1}; mean ± standard error). Intracerebroventricular injection of 10 MRC U kg^{-1} SCT decreased the frequency of abdominal constrictions from 3.24 ± 0.2 to 2.36 ± 0.21 constrictions min^{-1} (mean ± standard error). When SCT (10 MRC U kg^{-1}) was injected simultaneously with EGTA (0.05 µmol/mouse) no additional decrease in the frequency of abdominal constrictions was seen.

The effect of ICV SCT (2 MRC U kg^{-1}) upon the frequency of abdominal constrictions was antagonised by the simultaneous ICV injection of $CaCl_2$ (0.001 - 0.05 µmol/mouse).

In summary, central administration of calcium ions induces hyperalgesia and antagonizes the analgesic effects of EGTA and SCT. Similar findings have been reported (Chapman and Way, 1980) for the interaction of calcium ions

and opiate induced analgesia.

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REFERENCES

Bates, R.F.L. et al. Comparison of the analgesic effects of subcutaneous and intracerebroventricular injection of calcitonin on acetic acid induced abdominal constrictions in the mouse. Br. J. Pharmac., 72, 575P (1981).

Braga, P.et al. Lack of opiate receptor involvement in centrally induced calcitonin analgesia. Life Sciences, 22, 971-978 (1978).

Chapman, D.B. and Way, L.E. Metal ion interactions with opiates. Ann. Rev. Pharmacol. Toxicol., 20, 553-579 (1980).

Collier, H.O.J. et al. The abdominal constriction response and its suppression by analgesic drugs in the mouse. Br. J. Pharmac., 32, 295-310 (1968).

Haley, T.J. and McCormick, W.G. Pharmacological effects produced by intracerebral injection of calcitonin in the conscious mouse. Br. J. Pharmac., 12, 12-15 (1957).

Pecile, A. et al. Effects of intracerebroventricular calcitonin in the conscious rabbit. Experimentia, 31, 332-333 (1975).

Schmidt, W.K. and Way, E.L. Hyperalgesic effects of divalent cations and antinociceptive effects of a calcium chelator in aive and morphine dependent mice. J. Pharmacol. Exp. Ther., 212 (1), 22 (1980).

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THE INTERACTION OF NALOXONE AND CALCITONIN IN THE PRODUCTION OF ANALGESIA IN THE MOUSE

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Calcitonin produces analgesia when injected centrally (Pecile, Ferri, Braga and Olgiati, 1975). Similarities have been observed between opiate and calcitonin-induced analgesia, with regard to their interactions with calcium ions (Bates, Buckley, Eglen and Strettle, 1981 a). However, two differences have been reported. Repeated central administration of calcitonin does not induce tolerance (Braga, Ferri, Santagostino, Olgiati and Pecile, 1978) and Braga et al (1978) have shown that calcitonin is not antagonised by the opiate antagonist, naloxone. This has also been shown using levallorphan (Yamamoto, Kumagai, Tachikawa and Maeno, 1979). We have reinvestigated the interactions between naloxone and calcitonin-induced analgesia using several doses of each agent.

Abdominal constrictions were induced by i.p. injection of acetic acid (1% solution in 0.154 $mol.dm^{-3}$ NaCl) in CFLP mice (male and female, 30g) after the method of Collier, Dineen, Johnson and Schneider (1968) as modified by Bates et al (1981 b). The number of abdominal constrictions was counted between the 10th and 14th minute inclusive after the injection of acetic acid. Ten µl of salmon calcitonin (SCT) was administered by intracerebroventricular (ICV) injection (Haley and McCormick, 1957) 10 min prior to the injection of acetic acid. SCT was dissolved in tris-saline, pH 7.35. Control animals received tris-saine alone. Naloxone hydrochloride (0.10-10 $mg.kg^{-1}$) was injected sub-cutaneously in 0.154 $mol.dm^{-3}$ NaCl both alone or simultaneously with ICV SCT. Twenty-four animals were used in the control group and ten used in each test group. Statistical significance was assessed using the Mann-Whitney U test.

The frequency of abdominal constrictions induced by acetic acid in the control animals was 3.36 ± 0.11 min^{-1} (mean ± s.e.). SCT (0.04 - 10 MRC U kg^{-1}) produced a dose related inhibition of abdominal constrictions (Bates et al, 1980 a).

The lower naloxone doses of 0.01 and 0.1 $mg.kg^{-1}$ had no significant effect upon the frequency of abdominal constrictions, but higher doses of naloxone (1 and 10 mg. kg^{-1}) induced a significant ($p < 0.001$) increase in the frequency of abdominal constrictions (5.3 ± 0.1 and 5.2 ± 0.11 min^{-1} respectively, mean ± s.e.).

The lowest dose of naloxone (0.01 $mg.kg^{-1}$) did not

affect the analgesia induced by SCT. However, doses of 0.1 and 1.0 mg.kg^{-1}, naloxone totaly reversed the analgesic effect of doses of SCT up to and including 2 MRC U kg^{-1}. The dose of 10 mg.kg^{-1} naloxone was required to reverse the analgesic effects of 10 MRC U kg^{-1} SCT.

In summary, the effects of naloxone upon SCT - induced analgesia, in this preparation, are more complex than has been previously reported (Braga et al, 1978). The analgesic effects of SCT can be reversed by naloxone but only using doses 10-1000 fold greater than that required to reverse the effects of morphine in the mouse (AMD 50 = 0.01 mg.kg^{-1}; Collier and Schneider, 1972). The reversal of SCT induced analgesia is not entirely due to the hyperalgesic effect of naloxone, since doses which are not hyperalgesic (0.1 mg. kg^{-1}) antagonise the analgesic effect of SCT.

The salmon calcitonin was generously donated by Drs. J.W. Bastian and J.P. Aldred, Armour Pharmaceutical Corp. Kankakee III, USA.

Naloxone hydrochloride was generously donated by Endo Laboratories Inc., New York, USA.

REFERENCES

Bates, R.F.L. et al. (1981 a) Comparison of the analgesic effects of subcutaneous and intracerebrovenrtricular injection of calcitonin on acetic acid induced abdominal constrictions in the mouse. Br. J. Pharmac. 72 575P.

Bates, R.F.L. et al. (1981 b) Interaction of calcium ions and salmon calcitonin in the production of analgesia in the mouse. Br. J. Pharmac. 73, 302-303P.

Braga, P. et al. (1978) Lack of opiate receptor involvement in centrally induced calcitonin analgesia. Life Sciences 22, 971-978.

Collier, H.O.J. et al. (1968) The abdominal constriction response and its suppression by analgesic drugs in mouse. Br. J. Pharmac. 32, 295-310.

Collier, H.O.J. and Schneider, C. (1972) Some agonist and antagonist properties of morphine antagonists in rodents. In: Agonist and antagonist actions of narcotic drugs. Ed. Kosterlitz, H.W., Collier, H.O.J. and Villareal, J.E. MacMillan, London.

Haley, T.J. and McCormick, W.G. (1957) Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. Br. J. Pharmac. 12, 12-15.

Pecile, A. et al. (1975) Effects of intracerebroventricular calcitonin in the conscious rabbit. Experienta 31, 332-333.

Yamamoto, M. et al. (1979) Lack of effect of levellorphan on analgesia induced by intraventriclar application of porcine calcitonin in mice. Europ. J. Pharmacol. 55, 211-213.

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HYPERALGESIA INDUCED BY CHRONIC SUBCUTANEOUS INJECTION OF CALCITONIN.

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Single injections of calcitonin into the cerebral ventricles induce a transient analgesic response (Pecile, Ferri, Braga and Olgiati, 1975; Yamamoto, Kumagai and Tachikawa, 1979). Daily intracerebroventriclar injection of calcitonin does not induce tolerance to the analgesic properties of calcitonin (Braga, Ferri, Santagostino, Olgiati and Pecile, 1978). Single subcutaneous injections of calcitonin does not induce analgesia (Bates, Buckley, Eglen and Strettle, 1981). In the present work, we have studied the effect of repeated subcutaneous injections of calcitonin.

Groups of 8 - 10 CFLP mice (male and female, 30g) were given subcutaneous injections of salmon calcitonin (SCT, 10 MRC U kg^{-1} in 0.154 $mol.dm^{-3}$ NaCl plus 1g dm^{-3} BSA) on alternate days for periods up to 14 days. Control animals received vehicle alone. The animals were allowed access to food and water ad libitum and the amount of food and water consumed was recorded daily. There was a statistically significant decrease in food intake during the 24 hours after each SCT injection ($p < 0.02$). Water intake and weight gain were not different from the controls.

The sensitivity to peritoneal irritation by acetic acid was assessed at varying times following the final injection of SCT or vehicle using the method of Collier, Dineen, Johnson and Schneider (1968) as modified by Bates et al (1980). The statistical significances of the results were assessed using the Mann-Whitney U test.

Control animals, pretreated with vehicle, gave 3.30 - 3.78 abdominal constrictions min^{-1} between the 10th - 14th minute inclusive after the injection of the acetic acid. Mice receiving either 1, 2 or 3 injections of SCT and tested with acetic acid 48 hours after the final injection showed no change in the frequency of abdominal constrictions. In contrast, animals receiving 4 injections of SCT exhibited a gradual increase in the sensitivity to acetic acid. The frequency of abdominal constriction was not significantly different from the controls at 6 hours, but at 12, 24 and 48 hours after the final SCT injection the frequency of abdominal constrictions had increased by 27% ($p < 0.001$), 45% ($p < 0.001$) and 46% ($p < 0.001$) respectively.

After 8 injections of SCT the frequency of abdominal constrictions was again not significantly altered at 10

minutes after the final injection but it had increased by 37% ($p < 0.001$) 48 hours after the final injection.

Animals pretreated with SCT showed a decrease of 0.28 $mmol.dm^{-3}$ in the plasma calcium concentration 1 hour after the fourth injection but the plasma calcium concentration was restored to control values after 12 hours.

It is concluded that withdrawal of SCT after 4 or more subcutaneous injections at 48 hours intervals produces a long-lasting hyperalgesia in mice which is not coincident with the changes in plasma calcium concentration.

The salmon calcitonin was generously donated by Drs. W.J. Bastian and J.P. Aldred, Armour Pharmaceutical Corp., Kankakee III, USA.

REFERENCES

Bates, R.F.L. et al. (1981) Comparison of the analgesic effects of subcutaneous and intracerebroventricular injection of calcitonin on acetic acid induced abdominal constrictions in the mouse. Br. J. Pharmac. 72 575P.

Braga, P.C. et al. (1978) Lack of opiate receptor involvement in centrally induced calcitonin analgesia. Life Sciences 22, 971-978.

Collier, H.O.J. et al. (1968) The abdominal constriction response and its suppression by analgesic drugs in the mouse. Br. J. Pharmac. 32, 295-310.

Pecile, A. et al. (1975) Effects of intracerebroventricular calcitonin in the conscious rabbit. Experienta 31, 332-333.

Yamamoto, M. et al. (1979) Lack of effect of Levellorphan on analgesia induced by intraventriclar application of porcine calcitonin mice. Europ. J. Pharmacol. 55, 211-213.

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ANTAGONISM OF CALCITONIN INDUCED ANALGESIA BY IONOPHORE A23187

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Calcitonin (CT), by intracerebroventricular (i.c.v.) injection, induced analgesia in several species (Pecile, Ferri, Braga and Olgiati, 1975; Bates, Buckley, Eglen and Strettle, 1981a). The analgesic action of CT can be antagnised in mice by the s.c. injection of naloxone (Bates et al, 1981b). The ionophore, A23187, has been shown to faclitate the movement of calcium and magnesium ions across membranes (Reed and Lardy, 1972). We have investigated the effects of A23187, calcium and magnesium on the analgesia induced by i.c.v. injection of CT.

Groups of 6-10 mice (male, female, CFLP, 30-40g) were given unilateral i.c.v. injections (volume = 10µl) of calcium ($CaCl_2$), magnesium ($MgCl_2$), ionophore A23187 or salmon calcitonin (SCT) 2 $IU.kg^{-1}$ = 1.18 x 10^{-10} $moles.kg^{-1}$). These agents were dissolved in a solution of 50% dimethyl sulphoxide in tris-saline pH 7.4 (Bates et al, 1981a, DMSO vehicle). Control animals received vehicle alone. Analgesia was assessed 10 minutes later by the acetic acid - abdominal constriction test (Bates et al, 1981a). Statistical analyses were performed with the Mann-Whitney U test.

The sensitivity of the control animals receiving DMSO vehicle to peritoneal irritation by acetic acid (3.05 ± 0.11 constrictions min^{-1}, $\bar{x}$ ± s.e.), was not significantly different from that of animals receiving the tris-saline vehicle (Bates et al, 1981a).

SCT (2 $IU.kg^{-1}$) produced a significant ($p < 0.001$) decrease of 30% in the frequency of abdominal constriction. However, 3.3 $\mu moles.kg^{-1}$ calcium ions significantly ($p < 0.001$) increased the frequency of abdominal constriction by 36%. Magnesium ions (3.3 $\mu moles.kg^{-1}$) had no effect on the frequency of abdominal constricton. A23187 (117 $nmoles.kg^{-1}$) caused a significant ($p < 0.05$) increase of 26% in the frequency of constrictions. Doses of 11.7 $nmoles.kg^{-1}$ and 1.17 $nmoles.kg^{-1}$ had no effect. The hyperalgesic effects of A23187 (117 $nmoles.kg^{-1}$) and calcium ions (3.3 $\mu moles.kg^{-1}$) were not additive. Administration of magnesium ions (3.3 $\mu moles.kg^{-1}$) did not affect the response to either ionophore or SCT. In contrast, magnesium ions have been shown to be equipotent with calcium ions in antagonising morphine-induced analgesia (Harris, Loh and Way, 1975).

SCT analgesia was antagonised by A23187 (117 nmoles.kg^{-1} and 11.7 nmoles.kg^{-1}). The analgesic potency of SCT was reduced approximately 100 fold by the simultaneous injection of 11.7 nmoles.kg^{-1} A23187. This dose of A23187 alone was not hyperalgesic and did not affect neuromuscular co-ordination or exploratory behaviour as assessed by standard rotating drum and head-dipping tests respectively.

We conclude that the analgesic effect of SCT can be antagonised by A23187. This antagonism is possibly produced by alteration of the fluxes of calcium ion, rather than magnesium ion, within the brain.

The salmon calcitonin was generously donated by Drs. J.W. Bastian and J.P. Aldred, Armour Pharmaceutical Corp., Kankakee III, USA.

REFERENCES

Bates, R.F.L. et al. (1981) Interaction of calcium ions and salmon calcitonin in the production of analgesia in the mouse. Br. J. Pharmac. 73 302-303P.

Bates, R.F.L. et al. (1981a) Comparison of the analgesic effects of subcutaneous and intracerebroventricular injection of calcitonin on acetic acid-induced abdominal constrictions in the mouse. Br. J. Pharmac. 72 (3), 575P.

Bates, R.F.L. et al. (1981b) The interaction of naloxone and calcitonin in the production of analgesia in the mouse. British Pharmacology Society Meeting, Bradford, April 1981.

Harris, R.A. et al. (1975) Effects of divalent cations, cation chelators and an ionophore in morphine analgesia and tolerance. J. Pharmacol. Exp. Ther. 195, 488-498.

Pecile, A. et al. (1975) Effects of intracerebroventricular calcitonin in the conscious rabbit. Experienta 31, 332-333.

Reed, P.W. and Lardy, H.A. (1972) Antibiotic A23187 as a probe for the study of calcium and magnesium function in biological system. In: The role of membranes in metabolic regulation. Ed: Mehlman, M.A. and Hanson, R.W. pp 111-132. London and New York: Academic Press.

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DESENSITISATION TO CALCITONIN INDUCED ANALGESIA BY WITHDRAWAL OF CHRONIC CALCITONIN TREATMENT.

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Calcitonin, by intracerebroventricular (i.c.v.) injection, possesses analgesic properties, in several species, which are not observed when the hormone is given by subcutaneous (s.c.) injection (Pecile et al, 1975; Bates et al, 1981a). Repeated daly i.c.v. injection of calcitonin for 5 days does not induce tolerance (Braga et al, 1978). In mice withdrawal of chronic treatment with salmon calcitonin (SCT) by s.c. injection induces a prolonged hyperalgesic response (Bates et al, 1981b).

We have investigated the effect of chronic pretreatment with SCT by s.c. injection upon the analgesic action of either SCT, given by i.c.v. injection, or aspirin, given by s.c. injection.

Groups of 10 CFLP mice (male, female, 30g) were pretreated with s.c. injections of SCT (10 i.u. kg^{-1}) or vehicle (0.9% NaCl + 1 mg ml^{-1} BSA) given on alternate days for a period of 8 days. The sensitivity to peritoneal irritation by acetic acid was assessed 48 hours after the last s.c. injection of SCT. (Each animal was given 0.3 mls 1% acetic acid in 0.8% NaCl by i.p. injection and the number of abdominal constrictions were counted between the 10th and 14th minute inclusive.) This treatment induces a rate of 3 to 4 constrictions per minute in the control animals and is taken as the baseline response. Substances to be tested for analgesia were administered 10 min prior to acetic acid. SCT (0.04-10 i.u. kg^{-1}) was given by i.c.v. injection as described previously (Bates et al, 1981a). Aspirin (0.125-0.5 mmol.kg^{-1}) was given by s.c. injection. Control animals received injections of the appropriate vehicle. In animals, not pretreated with SCT, SCT by i.c.v. injection produced a dose dependent inhibition of abdominal constrictions, with a threshold of 0.04 i.u. kg^{-1} and a maximum response at a dose of 10 i.u. kg^{-1}. The ED_{50} was .36 i.u. kg^{-1}. Aspirin (0.125 mmol.kg^{-1} by s.c. injection also produced a dose dependent inhibition of abdominal constrictions with an ED_{50} of 0.44 mmol.kg^{-1}.

In animals, pretreated with SCT, the frequency of abdominal constrictions was increased by 32-38% ($p < 0.005$) 48 hours after the last injection of SCT. In these pretreated animals, SCT by i.c.v. injection (0.04-10 i.u. kg^{-1}) was approxmately 100 fold less effective in decreasing the frequency of abdominal constrictions (ED_{50} = 66 i.u. kg^{-1}). In contrast, the sensitivity of the pretreated animals to

aspirin associated with withdrawal of chronic treatment with SCT may result from a central effect of the hormone.

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

REFERENCES

Bates, R.F.L. et al (1981a) Br.J. Pharmac. 72 (3), 559P.
Bates, R.F.L. et al (1981b) Br.J. Pharmac. 74, 279P.
Braga, P. et al (1978) Life Sciences 22, 971-978.
Pecile, A. et al (1975) Experientia 31, 332-333.

EIGHTH INTERNATIONAL SOCIETY FOR NEUROCHEMISTRY, NOTTINGHAM 1981

ANALGESIA INDUCED BY CENTRAL INJECTION OF CALCITONIN INTO MICE

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The central analgesic action of salmon calcitonin (SCT) was assessed in mice by the inhibition of abdominal constrictions induced by acetic acid. The acetic acid (0.3 ml, 1% w/v in 0.9% NaCl) was injected i.p. and the abdominal constrictions were counted between the 10th - 14th min. inclusive. SCT was given either by intracerebroventricular (i.c.v.) injection (10µl in Tris-saline buffer pH 7.35 + 1mg ml^{-1} BSA) or by s.c. injection 0.1ml 0.9% NaCl + 1mg ml^{-1} BSA) 10 min. prior to the acetic acid. Control animals received the appropriate injection of vehicle.
SCT given by i.c.v. injection (0.04 - 10 IU kg^{-1}) but not by s.c. injection (10 - 1000 IU kg^{-1}) produced a dose related inhibition of abdominal constrictions. These doses did not affect neuromuscular co-ordination as indicated by the ability of the mice to maintain position on a rotating sandpaper drum. EGTA (0.05µM/mouse in 10µl, i.c.v.) produced analgesia equivalent to 10 IU SCT (i.c.v.). These maximal effects of SCT and EGTA were not additive. The analgesic action of EGTA was reversed by the simulataneous injection of equimolar doses of calcium ion. The analgesic action of SCT 2 IU kg^{-1} was also reversed in a dose related manner by the simultaneous injection of calcium ion (0.01 - 0.05 [moles/mouse i.c.v.) but not by magnesium ion (0.1 moles/mouse i.c.v.)
Thus SCT produces analgesia by a central action which can be mimicked by EGTA and antagonised by small doses of calcium ion.

EIGHTH INTERNATIONAL SOCIETY FOR NEUROCHEMISTRY, NOTTINGHAM 1981.

WITHDRAWAL HYPERALGESIA FOLLOWING CHRONIC SUBCUTANEOUS INJECTION OF CALCITONIN

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Salmon calcitonin (SCT) by intracerebroventricular (i.c.v.) injection, but not by single subcutaneous (s.c.) injection, to mice induces a dose dependent analgesia which can be reversed by calcium ion (Eglen et al, this symposium). We have now investigated the effects of chronic s.c. treatment with SCT. Groups of 10 mice (CFLP, 30 g) were given SCT (10 $IU.kg^{-1}$ by s.c. injection on alternate days for up to 14 days. Control animals were injected with vehicle (0.3ml 0.9% NaCl + 1 mg ml^{-1} BSA). The sensitivity to peritoneal irritation by acetic acid (Eglen et al, this symposium) was assessed at 10 min, 6, 12, 24, 48 or 96 hr after the last injection of SCT. Mice treated with 1, 2 or 3 s.c. injections of SCT showed no significant change in he frequency of abdominal constrictions. In contrast, animals receiving 4 or more s.c. injections exhibited a gradual increase in the sensitivity to acetic acid. Thus, up to 6 hours there was no significant change, but at 12, 24, 48 and 96 hrs after the 4th s.c. injection of SCT the frequency of constrictions was increased by 27%, 45%, 46% and 17% ($P = <0.001$) respectively. The hyperalgesic response was not co-incident with the transient hypocalcaemia induced by SCT. The sensitivity of the mice to analgesic effects of SCT by i.c.v. injection was reduced by 100 fold 48 hr after the 4th subcutaneous injection of SCT.

We conclude that during the period following the withdrawal of chronic treatment with SCT by s.c. injection there is a persistent hyperalgesia which counteracts the central analgesic effect of SCT.

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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 antagonsim 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 (male, female, 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 4 ml 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 calctonin ($2.5 \times 10^{-12} - 2.5 \times 10^{-4}$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 δ or μ 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 wth opiate receptors of the μ type.

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

REFERENCES

Bates, R.F.L. et al (1981a) Br. J. Pharmac. 72, 575P.

Bates, R.F.L. et al (1981b) Br. J. Pharmac. 74, 279P.

Boura, A.L. and Olley, J.E. (1981) Br.J. Pharmac. 74, 246P.

Braga, P. et al (1978) Life Sciences 22, 971-978.

Gillan, M.G.C. and Pollock, D. (1980) Br. J. Pharmac. 68, 381-392.

Huidoboro-Toro, J.P. and Way, E.L. (1981) Br. J. Pharmac. 74, 681-694.

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

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The analgesia produced 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 (male, female, 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} ($\bar{x}$ ± s.e.). 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% (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 antagnist. 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 injecton 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. injecton of magnesium ion.

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

REFERENCES

Bates, R.F.L. (1981) Br. J. Pharmac. 72, 575P.

Chapman, D.B. and Way, E.L. (1980) Ann. Rev. Pharmacol. Toxicol. 20, 553-579.

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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 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 (male and female, 30g) or Wistar rats (male and female (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 accordng to the method of Lowry et al (1951) and AchE activity was determined accordng to Ellman et al (1961) at pH 7.4 ^{o}C and 21 ^{o}C for mouse or pH 8 and 37 ^{o}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 $\mu.mol.hr^{-1}$ (mg protein)$^{-1}$, $\bar{x} \pm$ 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.

REFERENCES

Bates, R.F.L. et al (1981) Br. J. Pharmac. 72, 559P.

Ellman, G.L. et al (1961) Biochem. Pharmacol. 7, 88-95.

Lowry, O.H. et al (1951) J. Biol. Chem. 193, 265-275.

Nakhla, A.M. and Majumdar, A.P.N. (1978) Biochem. J. 170, 445-448.

Pecile, A. et al (1975) Experientia 31, (3) 332-337.

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CALCIUM ANTAGONISTS - DIFFERENTIAL EFFECTS OF 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 slution 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.

Cumulative 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 antagonised non-competitively by all four agents. The responses of both tissues were completely abolished by 10^{-7}M of the organic antagonists and 2 $mmol.l^{-1}$ 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 tissues 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 were generously donated by the following: verapamil (Abbott Laboratories Ltd.,), nifedipine (Bayer UK Ltd.,) and Py 108-068 (Sandoz Ltd.).

REFERENCES

Nayler, W.G. and Poole-Wilson, P. (1981) Basic Res. Cardiol. 76, 1-15.

Ticku, M.K. and Triggle, D.J. (1976) Gen. Pharmacol. 7, 133-140.

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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 and 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 response 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-Haemorrhage 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.

REFERENCES

Bates, R.F.L. et al (1981) Br. J. Pharmac. 72 (3), 575P.

Chance, E. et al (1981) Br. J. Pharmac. 74 (4), 930-931P.

Faden, A.I. and Holaday, J.W. (1979) Science 205, 317-318.