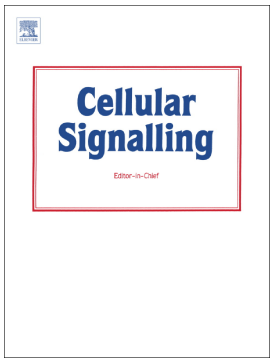

Differential regulation of β2-adrenoceptor and adenosine A2B receptor signalling by GRK and arrestin proteins in arterial smooth muscle

Craig A. Nash, Carl P. Nelson, Rajendra Mistry, Christian Moeller-Olsen, Elena Christofidou, R.A. John Challiss, Jonathon M. Willets

PII: S0898-6568(18)30170-0
DOI: doi:10.1016/j.cellsig.2018.07.013
Reference: CLS 9150

To appear in: *Cellular Signalling*

Received date: 19 April 2018
Revised date: 13 July 2018
Accepted date: 29 July 2018

Please cite this article as: Craig A. Nash, Carl P. Nelson, Rajendra Mistry, Christian Moeller-Olsen, Elena Christofidou, R.A. John Challiss, Jonathon M. Willets , Differential regulation of β2-adrenoceptor and adenosine A2B receptor signalling by GRK and arrestin proteins in arterial smooth muscle. Cls (2018), doi:10.1016/j.cellsig.2018.07.013

Differential regulation of β_2-adrenoceptor and adenosine A_{2B} receptor signalling by GRK and arrestin proteins in arterial smooth muscle

Craig A. Nash[a,b], Carl P. Nelson[a,c], Rajendra Mistry[a], Christian Moeller-Olsen[a], Elena Christofidou[a], R. A. John Challiss[a], and Jonathon M. Willets[a,*]

[a] *Department of Molecular and Cell Biology, University of Leicester, Henry Wellcome Building, Lancaster Road, Leicester, LE1 7RH, United Kingdom*

[b] *Present address: Department of Pharmacology, University of Michigan Medical School, 1150 West Medical Center Drive, Ann Arbor, MI 48109-5632, USA*

[c] *Present address: School of Science & Technology, Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, United Kingdom*

[*] Correspondence to: Jonathon M. Willets, Department of Molecular and Cell Biology, University of Leicester, Henry Wellcome Building, Lancaster Road, Leicester, LE1 7RH, U.K., E-mail: jmw23@leicester.ac.uk

Word count 7499

Figures: 9

ABSTRACT

Generation of cAMP through G_s-coupled G protein-coupled receptor (GPCR) [e.g. β_2-adrenoceptor (β_2AR), adenosine A_{2B} receptor (A_{2B}R)] activation, induces arterial smooth muscle relaxation, counteracting the actions of vasoconstrictors. G_s-coupled GPCR signalling is regulated by G protein-coupled receptor kinases (GRK) and arrestin proteins, and dysregulation of Gs/GPCR signalling is thought play a role in the development of hypertension, which may be a consequence of enhanced GRK2 and/or arrestin expression. However, despite numerous studies indicating that β_2AR and A_{2B}R can be substrates for GRK/arrestin proteins, currently little is known regarding GRK/arrestin regulation of these endogenous receptors in arterial smooth muscle. Here, endogenous GRK isoenzymes and arrestin proteins were selectively depleted using RNA-interference in rat arterial smooth muscle cells (RASM) and the consequences of this for β_2AR- and A_{2B}R-mediated adenylyl cyclase (AC) signalling were determined by assessing cAMP accumulation. GRK2 or GRK5 depletion enhanced and prolonged β_2AR/AC signalling, while combined deletion of GRK2/5 has an additive effect. Conversely, activation of AC by A_{2B}R was regulated by GRK5, but not GRK2. β_2AR desensitization was attenuated following combined GRK2/GRK5 knockdown, but not by depletion of individual GRKs, arrestins, or by inhibiting PKA. Arrestin3 (but not arrestin2) depletion enhanced A_{2B}R-AC signalling and attenuated A_{2B}R desensitization, while β_2AR-AC signalling was regulated by both arrestin isoforms. This study provides a first demonstration of how different complements of GRK and arrestin proteins contribute to the regulation of signalling and desensitization of these important receptors mediating vasodilator responses in arterial smooth muscle.

Key words: Vascular smooth muscle, GRK, arrestin, adenosine A_{2B} receptor, β_2-adrenoceptor

Abbreviations: AC, adenylyl cyclase; A_{2B}R, adenosine A_{2B} receptor; βAR, β-adrenoceptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; RASM, rat aortic smooth muscle cells; siRNA, small interfering RNA; VSMC, vascular smooth muscle cells

1. Introduction

Maintaining a balance between the actions of vasoconstrictor and vasodilator inputs is vital for the control of vascular tone and blood vessel diameter [1]. Disturbances in this balance can lead to hypertension, which is an important risk factor in the development of major cerebral, cardiovascular and renal diseases [2]. Vascular tone is tightly regulated by an array of opposing vasoconstrictor and vasodilator molecules, many of which mediate their effects via specific G protein-coupled receptors (GPCRs) [2]. In general, vasoconstrictors (e.g. endothelin, angiotensin II, noradrenaline) bind to, and activate $G\alpha_{q/11}$/phospholipase C-coupled GPCRs to increase intracellular Ca^{2+} concentrations and bring about their vaso-regulatory actions. Conversely, vasodilators (e.g. noradrenaline, adenosine) interact with $G\alpha_s$-coupled GPCRs, to activate adenylyl cyclase, producing cyclic-3',5'-adenosine monophosphate (cAMP), which mediates smooth muscle relaxation through plasma membrane hyperpolarization and reduced myosin light chain phosphorylation [3, 4].

GPCR desensitization plays a critical role in limiting the duration and magnitude of GPCR activation, thus preventing prolonged or potentially damaging signalling. Desensitization is primarily mediated through the actions of a family of serine/threonine protein kinases, the G protein-coupled receptor kinases (GRKs). GPCR phosphorylation by GRKs enhances receptor affinity for arrestins 2 and/or 3, which can sterically suppress further interactions between receptor and G proteins [5, 6]. While vascular smooth muscle has been shown to express at least four of the seven members of the GRK family, accumulating evidence suggests that GRK2 may play a dominant role in regulating a number of vasoconstrictor GPCRs, including the α_{1D}-adrenoceptor [7], angiotensin II type 1 [8], ET_A endothelin [9], and $P2Y_2$ [10] receptors. Moreover, we have found that in arterial smooth muscle, despite both being substrates for GRK2, $P2Y_2$ and ET_A receptor phosphorylation leads to preferential recruitment of arrestin2 or arrestin3, respectively [9-11].

Previous studies, conducted in a number of cell backgrounds, have highlighted GRK2 as a key regulator of β-adrenoceptor (βAR) and A_2 adenosine receptor (A_2R) function [12-16]. Limited evidence is presently available supporting a role for GRK2 as a key regulator of βAR or A_2R activity in vascular smooth muscle cells (VSMC). For example, βAR-stimulated cAMP generation in mouse aortic smooth muscle cells has been shown to be suppressed when GRK2 is over-expressed [17], and increased when GRK2 is depleted using a shRNAi

approach [18]. At present, a potential role for arrestin proteins in the regulation of βAR or adenosine A_2R signalling in arterial smooth muscle has not been investigated. Furthermore, the molecular mechanisms by which endogenous GRK and/or arrestin proteins regulate signalling by the endogenous βAR or A_2R populations have not been delineated in the vasculature. Considering that in rodent models of hypertension, the disease phenotype is accompanied by an arterial smooth muscle-specific increase in GRK2 [19, 20] and arrestin2/3 protein expression [20], it is important to determine whether these proteins regulate βAR- and/or A_2R-mediated vasodilator signalling in arterial smooth muscle. Therefore, we have utilized RNAi approaches specifically to deplete GRK and/or arrestin isoforms to identify their respective roles in the regulation of βAR and A_2R signalling in arterial smooth muscle.

2. Methods

2.1 Isolation of aortic smooth muscle cells from Wistar rats

Adult Wistar rats (>300 g) were culled by stunning and cervical dislocation. The care and Schedule 1 killing of animals conformed to the requirements of the United Kingdom Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039). Smooth muscle cells were isolated from the aorta of animals by enzymatic dissociation as previously described [21]. Cells were separated by gentle trituration in Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX supplemented with 10% fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotercin B. Cells were maintained in 5% CO_2 in humidified air at 37°C in flasks and used for experiments between passages 3-6.

2.2 Measurement of cAMP generation

Rat aortic smooth muscle (RASM) cells were plated in 24-well plates and grown until near confluency. Cells were washed twice in Krebs-Henseleit buffer (KHB) (mM: HEPES, 10; $NaHCO_3$, 1.3; D-glucose, 11.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; KCl, 4.7; NaCl, 118; $CaCl_2$, 1.3; pH 7.4) and left in KHB to recover for 10 min. Cells were treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 300 µM) during this period. Cells were then stimulated with agonist as indicated for each experiment before the reaction was terminated by the addition of trichloroacetic acid (TCA; 0.5 M). Samples were then processed and cAMP concentrations determined as described previously [22]. Since IBMX has been reported to bind to and inhibit adenosine receptors [23], rolipram (10 µM) was used instead in experiments assessing NECA-stimulated cAMP signalling.

2.3 Assessing receptor desensitization using cAMP-based assays

RASM were plated as described in the previous sub-section. To assess βAR desensitization cells were washed with KHB without IBMX, before stimulation for 15 min with isoprenaline (1 µM) as a desensitizing stimulus, or vehicle. Next cells were washed 3 times with KHB containing IBMX (300 µM) and re-stimulated with a range of concentrations of isoprenaline in the presence of IBMX to define a concentration-response relationship, before the reaction was terminated with TCA (0.5 M). A similar protocol was applied to assess A_{2B}R desensitization, however cells were pre-treated with the non-selective adenosine agonist NECA (10 µM) as a desensitizing stimulus, or vehicle for 30 min. Cells were then washed 3 times with KHB containing rolipram (10 µM) and re-stimulated with NECA. Samples were then processed and cAMP concentrations determined as described previously

[22]. Receptor desensitization was determined as a percentage for the responses in isoprenaline pre-treated cells compared to vehicle-treated cells.

2.4 Knockdown of endogenous GRK2, and arrestin proteins in RASM cells

RASM (2×10^6) cells were transfected with 10 nM negative-control (non-targeting sequence) or GRK2 (5′-GCAGGUACCUCCAGAUCUCtt-3′), GRK5 (5'-CAGUGGAAAUGGUUAGAAAtt-3'), GRK6 (5'-GACCAAGACUUCUACCAGAtt-3'); arrestin2 (5′-GCCACUGACUCGGCUACAAtt-3′) or arrestin3 (5′-GCCUUCUGUGCCAAAUCUAtt-3′) small interfering (si)RNAs (Applied Biosystems, UK), as previously validated by our group [10, 11]. siRNAs were introduced using the Lonza nucleofection system (Lonza, Cologne, Germany) according to the manufacturer's instructions. Transfected cells were grown for 48 h to allow for efficient knockdown of the target protein expression before experiments were performed. Specific protein depletion was quantified using standard western blotting techniques, exactly as described previously [9, 10].

2.5 Data and statistical analysis

Data presented are from a minimum of 3 different cell preparations each obtained from a different rat, and are expressed as means ± SEM. Parametric data were analysed using one-way or two-way ANOVA as indicated, with appropriate *post-hoc* testing, outlined in the corresponding figure legends (GraphPad Prism v7.2, San Diego, CA). Where data were normalized, non-parametric ANOVA analysis using Kruskal-Wallis was applied. In all cases *post-hoc* tests were only applied when initial ANOVA testing revealed a significant ($p<0.05$) result.

3. Results

3.1 Characterization of βAR and adenosine receptor signalling in RASM cells

The non-selective β-adrenoceptor agonist, isoprenaline caused a concentration-dependent increase in cAMP accumulation in isolated primary RASM cells, with a maximal effect observed at ≥300 nM and an EC_{50} value of 79 nM (Fig. 1A). Since previous reports suggested that vascular smooth muscle cells express a mixed population of β-adrenoceptor subtypes [24] and our preliminary studies indicated the presence of β_1AR and β_2AR mRNA, but the absence of β_3AR transcripts (data not shown), we assessed the contributions made by β_1AR and β_2AR to isoprenaline-induced cAMP accumulation. Pre-incubation with a range of concentrations of the β_2AR-selective antagonist ICI118,551 resulted in a biphasic inhibition of isoprenaline-induced cAMP accumulation, with two-site curve-fitting revealing pK_B values of 9.23 ± 0.17 and 6.61 ± 0.14 (Fig. 1B), corresponding closely to published β_2AR (pK_B = 9.26) and β_1AR (pK_B = 6.52) affinity estimates, respectively [25]. However, inclusion of the β_1AR-selective antagonist CGP20712A (300 nM, 30 min pre-treatment), had no significant effect on isoprenaline-stimulated cAMP accumulation (Fig. 1C). Together, these data indicate that isoprenaline-stimulated cAMP accumulation in RASM is primarily mediated by β_2AR (>80%), with a minor β_1AR contribution.

Adenosine mediates vaso-relaxatory responses in a variety of vascular beds [26], and RASM cells are reported to express a number of adenosine receptor subtypes [27]. Therefore, we first characterized which receptor subtype(s) could generate cAMP. The non-selective adenosine receptor agonist 5'-*N*-ethylcarboxamidoadenosine (NECA) induced concentration-dependent cAMP accumulation with EC_{50} value of 3 μM (Fig. 1D), which is in agreement with previously published values for A_{2B} adenosine receptor (A_{2B}R) activation [28]. NECA-stimulated cAMP accumulation was significantly attenuated by pre-incubation of RASM cells with the A_{2B}R-selective antagonist, MRS1754 (10 μM), while addition of the A_{2A}R-selective agonist CGS21680 did not stimulate cAMP accumulation (Fig. 1D). NECA-induced cAMP accumulation was unaffected by pre-incubation of RASM with PTx (100 ng/mL; 18 h), suggesting that $G_{i/o}$-coupled adenosine A_1 or A_3 receptors are not involved (Fig. 1E). Collectively, these data suggest that cAMP generation in RASM is primarily mediated by the A_{2B}R subtype.

3.2 *GRKs selectively regulate β_2AR and A_{2B}R coupling to adenylyl cyclase*

Previous studies have indicated involvements of GRK2, GRK5 and/or GRK6 in the regulation of β_2AR signalling [19, 29-31] and GRK2 in the regulation of A_{2B}R signalling [16]. We have examined the potential of these GPCR regulatory proteins to regulate native β_2AR signalling in the RASM cell background. Exposure of RASM to individual anti-GRK2, anti-GRK5 or anti-GRK6 siRNAs (each at 10 nM) resulted in isoenzyme-selective ≥75% reductions in GRK2, GRK5 or GRK6, compared to cells transfected with negative-control siRNA (see Fig. 2A, B). Depletion of GRK5 (but not GRK2 or GRK6) significantly, though transiently enhanced NECA-stimulated cAMP accumulation, with the most marked effect being observed at 5 min (Fig. 2C). Similar experiments performed with isoprenaline showed significantly enhanced β_2AR-stimulated cAMP accumulations following knockdown of GRK2 or GRK5 (but not GRK6) (Fig. 2D). Moreover, depletion of both GRK2 and GRK5 increased and prolonged the enhancement in isoprenaline-stimulated cAMP accumulation in RASM cells (Fig. 2D).

To determine further the specificity of GRK interactions with β_2AR/A_{2B}R-mediated cAMP signalling we examined the effects of GRK inhibition on β_2AR and A_{2B}R-activated cAMP generation. NECA (10 μM) produced a time-dependent increase in cAMP accumulation that was not significantly altered by pre-incubation with the GRK2/3 inhibitor, compound 101 (30 μM, for 20 min; Fig. 2E); this was in contrast to the response stimulated by isoprenaline, where compound 101 caused a significant enhancement of cAMP accumulation throughout the time-course (Fig. 2E). In addition, depletion of GRKs 2, 5 or 6 had no effect upon forskolin-stimulated cAMP production (Fig. 2F), suggesting that the enhanced agonist-mediated cAMP accumulation following GRK2 or GRK5 depletion was stimulus-specific. These data indicate a degree of GRK/receptor-specific selectivity, with GRK2/GRK5 required for modulation of β_2AR/AC signalling, while A_{2B}R/AC activity is only regulated by GRK5.

3.3 *Arrestin dependency of A_{2B}R- and β_2AR-stimulated cAMP accumulation in RASM*

As arrestins are reported to regulate signalling downstream of both A_{2B}R and β_2AR [30, 32], we therefore used isoform-specific anti-arrestin siRNAs to deplete arrestin expression, either alone or in combination, to investigate their roles in A_{2B}R- and β_2AR-

stimulated cAMP accumulation in RASM cells. In agreement with our previous studies [11, 20], application of anti-arrestin2/3 siRNAs induced isoform-selective ≥75% reductions of each arrestin isoform (Fig. 3A, B). In the presence of PDE inhibition, depletion of either arrestin2 or arrestin3 resulted in a marked increase in isoprenaline- and NECA-stimulated cAMP accumulation, respectively (Fig. 3C, E). In the absence of pharmacological PDE inhibition, enhancement of the isoprenaline-stimulated cAMP accumulation was only observed in RASM cells where arrestin2 (and not arrestin3) had been depleted (Fig. 3D). In contrast, enhanced NECA-stimulated cAMP generation was only observed after arrestin3, but not arrestin2 knockdown (Fig. 3E). Moreover, although PDE inhibition enhanced and prolonged the temporal profile of NECA-stimulated cAMP accumulation (Fig. 3E), the selectivity of A_{2B}R/arrestin3 interaction was maintained in the presence or absence of PDE inhibition (Fig. 3E, F).

3.4 Differential effects of PDE isoenzyme-selective inhibitors on isoprenaline-stimulated adenylyl cyclase activity in RASM cells

Arrestins can act as scaffold proteins, promoting the recruitment of specific phosphodiesterase (PDE) isoenzymes to active βARs, a process that contributes to the regulation and compartmentalization of cAMP [33]. Therefore, the possibility that arrestins enhance β_2AR-stimulated cAMP signalling by mediating the recruitment of specific PDE isoenzymes was investigated. Isoprenaline-stimulated cAMP accumulation was determined in the absence or presence of isoenzyme-selective PDE inhibitors, in RASM cells after arrestin2 or arrestin3 knockdown. In agreement with data already reported (Fig. 3D), in the absence of PDE inhibition an enhanced isoprenaline-stimulated cAMP production was only observed in arrestin2-depleted cells (Fig. 4). However, in the presence of a broad spectrum PDE inhibitor (IBMX), cAMP accumulations were greater in both arrestin2- and arrestin3-depleted cells (Fig. 4). Inclusion of the PDE3-selective inhibitor siguazodan had little effect on the observed cAMP responses, however, inclusion of the PDE4-selective inhibitor rolipram caused cAMP accumulations similar to those observed in the presence of IBMX (Fig. 4).

3.5 β_2AR desensitization is regulated by a combination of GRK2 and GRK5

β_2AR desensitization was initially investigated in primary RASM cells utilizing a protocol involving pre-treatment of cells with a maximal concentration (1 μM, 15 min) of the

non-selective βAR agonist isoprenaline; cells were then washed and re-challenged with a range of concentrations of isoprenaline. This pre-treatment protocol led to a 47 ± 5% (mean ± SEM; for n=5) attenuation of the cAMP response (to 1 μM isoprenaline re-challenge), with no apparent change in agonist potency (Fig. 5A). To assess whether the observed receptor desensitization was attributable to β_1AR and/or β_2AR subtypes, RASM cells were pre-treated with the β_1AR-selective antagonist CGP20712A (300 nM, 30 min), prior to application of the desensitization protocol. Inclusion of CGP20712A had no significant effect on isoprenaline-stimulated cAMP accumulation in agonist-naïve cells (Fig. 5B), or on the extent of receptor desensitization (Fig. 5C). These data strongly suggest that this protocol allows us to assess predominantly β_2AR desensitization in a native arterial cell background. Comparison of the concentration-response curves indicated that agonist-pre-treatment only attenuated isoprenaline-stimulated cAMP accumulation at concentrations above 100 nM, and maximal cAMP accumulation was achieved with addition of 1 μM (Fig. 5A). Thus for all future desensitization experiments we simplified our protocol, applying an isoprenaline (1 μM; 15 min) pre-treatment, followed by washing and a further application of a single concentration of isoprenaline (1 μM) for 10 min in the presence of IBMX.

As previous reports implicate GRK2, GRK5 and/or GRK6 in the regulation of β_2AR signalling [19, 29-31], our initial studies examined the effects of depleting endogenous GRKs on the desensitization of β_2AR in RASM. Isoprenaline-stimulation of cells transfected with negative-control siRNA yielded a similar profile of cAMP accumulation to that observed in untransfected RASM cells (*cf.* Fig. 5A, D). Equivalent degrees of β_2AR desensitization were also observed in negative-control siRNA-transfected and untransfected RASM cells (*cf.* Fig. 5A, D). In agonist-naïve cells, isoprenaline-stimulated cAMP accumulation was enhanced after GRK2 or GRK5 depletion, whereas depletion of GRK6 was without effect (Fig. 5E). Following agonist pre-treatment, the level of cAMP was significantly greater in RASM with depleted GRK5, compared to NC, anti-GRK2 and anti-GRK6 siRNA-treated cells (Fig. 5E). However, despite these changes the extent of receptor desensitization following pre-treatment with isoprenaline (1 μM, 15 min) was similar in all siRNA-treated cells, suggesting that depletion of individual GRKs (2, 5 or 6) failed to prevent β_2AR desensitization in RASM cells (Fig. 5F). In contrast, depletion of both GRK2 and GRK5 resulted in a significant reduction in β_2AR desensitization in RASM cells (Fig. 5F).

Since PKA is also reported to play a role in β_2AR desensitization [29], we pre-incubated RASM cells with the cell-permeant PKA inhibitor, myristoylated 14-22 amide (PKAI, 5 μM), before applying the standard desensitization protocol. Inclusion of the PKA inhibitor had no effect on extent of receptor desensitization following isoprenaline pre-treatment (1 μM, 15 min; Fig. 5D, E). Furthermore, addition of the cell-permeant PKAI did not alter the β_2AR desensitization observed in GRK2/GRK5-depleted RASM cells (Fig. 5E, F). These data indicate that combined GRK2/GRK5 depletion is required to observe a significant attenuation in isoprenaline-induced β_2AR desensitization (from a 45 ± 2% to 29 ± 2% decline in maximal responsiveness; Fig. 5F), and that pharmacological inhibition of PKA has no detectable effect *per se*, or in combination with GRK2/GRK5 depletion.

To complement our siRNA GRK knockdown approach, we also studied β_2AR desensitization in the absence and presence of the GRK2/3 inhibitor, compound 101 [34, 35]. Addition of compound 101 (30 μM) produced similar results to those obtained using the anti-GRK2 siRNA approach, enhancing isoprenaline-mediated cAMP accumulation in both agonist-naïve and isoprenaline-pre-treated RASM cells (Fig. 5G). However, compound 101 (30 μM) had no effect on the extent of isoprenaline-induced β_2AR desensitization (1 μM, 15 min; Fig. 5H).

3.6 A_{2B}R desensitization is regulated by GRK5

As no previous studies have examined A_{2B}R desensitization in RASM, or indeed the roles that GRKs play in this process, we investigated A_{2B}R desensitization in control or GRK-depleted cells. Initially, we utilized a pre-treatment protocol where cells were pre-incubated with vehicle-control or a high concentration of NECA (10 μM), for various time-periods. Next cells were extensively washed and re-challenged with NECA (10 μM), for 10 min in the presence of rolipram, before cAMP concentrations were measured and compared to those obtained in agonist-naïve cells. These data indicated a time-dependent desensitization of A_{2B}R-stimulated AC activity, with maximal effects after 30 min pre-incubation with NECA (Fig. 6A). Thus, in all subsequent experiments A_{2B}R desensitization was induced by a 30 min NECA (10 μM) pre-treatment. When NC siRNA transfected RASM were subjected to the desensitization protocol, the degree of A_{2B}R desensitization was similar to that obtained in non-transfected cells (44 ± 4% non-transfected *vs* 52 ± 2% NC siRNA-transfected cells). In agreement with our previous data, NECA-induced cAMP accumulation was enhanced in

agonist-naïve cells following knockdown of GRK5, but not after GRK2 or GRK6 depletion (Fig. 6B). Furthermore, depletion of GRK5 attenuated NECA-induced $A_{2B}R$ desensitization with respect to the AC readout (Fig. 6C), whereas knockdown of GRK2 or GRK6 had no effect.

3.7 Comparison of the roles played by arrestin proteins in β_2AR versus $A_{2B}R$ desensitization

Utilizing the simplified protocol (see above) we examined the effects of isoprenaline or NECA pre-treatment on β_2AR and $A_{2B}R$ desensitization of AC activity in the presence of PDE inhibition. As expected, knockdown of arrestin2 and/or arrestin3, alone or a combination, enhanced the ability of isoprenaline to stimulate cAMP accumulation in agonist-naïve cells (Fig. 7A). Although the degree of β_2AR desensitization following knockdown of arrestins was unaffected (Fig. 7B), the level of cAMP produced after agonist pre-treatment was significantly greater in RASM with depleted arrestin expression, than that observed in negative-control transfected RASM (Fig. 7A).

In comparison, in agonist-naïve cells NECA-driven cAMP accumulation was enhanced after knockdown of arrestin3 when compared to NC siRNA-treated RASM (Fig. 7C). Furthermore, combined suppression of arrestin2 and arrestin3 expression produced a similar effect to arrestin3 knockdown alone, and arrestin2 knockdown had no effect on on NECA-stimulated cAMP accumulation (Fig. 7C). Unlike for the β_2AR, where suppression of arrestin2 and/or 3 expression failed to reverse desensitization of β_2AR/AC coupling, knockdown of arrestin3 did produce a significant reversal of $A_{2B}R$/AC desensitization (Fig. 7D).

3.8 Dynamin inhibition attenuates $A_{2B}R$, but not β_2AR desensitization

To examine whether receptor endocytosis plays a role in the desensitization of β_2AR or $A_{2B}R$, we pre-incubated RASM with the dynamin inhibitor Dyngo 4A (30 µM, 15 min) [37], before applying the standard desensitization protocols to examine each receptor subtype. Inclusion of Dyngo 4A alone caused a 50% reduction in the accumulation of cAMP produced by isoprenaline (1 µM) or NECA (10 µM) challenge in agonist-naïve cells over a 10 min period (Fig. 8A, B). Interestingly, following agonist pre-treatments, the presence of Dyngo 4A significantly reduced the attenuation in cAMP response seen for NECA-challenged cells

(p<0.01; Fig 8B, C). In contrast, the desensitization observed as the cAMP readout following isoprenaline pre-challenge was not altered by the presence of the dynamin inhibitor (Fig. 8B, C).

3.9 Temporal profile of β_2AR and A_{2B}R re-sensitization

To examine time-courses of re-sensitization of β_2AR- and A_{2B}R-stimulated cAMP responses, RASM cells were subject to standard desensitization protocols (i.e. β_2AR, isoprenaline (1 µM) for 15 min; A_{2B}R, NECA (10 µM) for 30 min) before extensive buffer washing over a 3 min period beforer re-challenge. Cells were also allowed to re-sensitize for up to an additional 60 min before agonist re-challenge. PDE inhibitors were included 5 min prior to the second agonist stimulation. Our data show that β_2AR re-sensitization after pre-treatment with isoprenaline is rapid with near full recovery within 30 min (Fig 9). In contrast, A_{2B}R re-sensitization after pre-treatment with NECA is considerably slower (Fig. 9).

4. Discussion

GPCR-mediated cAMP production plays a vital role in the regulation of arterial tone, inducing vessel relaxation [38], counteracting the effects of vasoconstrictors and preventing the switch to a contractile hypertensive phenotype. Indeed, dysregulation of G_s-coupled receptors such as the βARs and A_2Rs has long been implicated in the development of hypertension [39-41]. The majority of GPCRs are negatively regulated by GRKs and arrestin proteins, and previous studies both in model cells and animal models have implicated various GRKs, arrestins and PKA in the regulation of βAR and A_2R desensitization and downstream signalling pathways [16, 18, 29, 30, 42, 43]. Although three GRK isoforms [9], two arrestins [11] and PKA [21] expressed in RASM have the potential to regulate βAR/A_2R signalling [16, 29, 30, 42, 44], a thorough understanding of which kinases and/or arrestins are required to mediate receptor signalling and/or desensitization in this tissue has yet to be fully determined. Therefore, we utilized previously characterized RNAi approaches and pharmacological inhibitors to address this unanswered question.

VSMC are reported to express β_1AR, β_2AR and β_3AR receptors [24], therefore we determined the relative contributions of each receptor to the production of cAMP in response to the non-selective βAR agonist isoprenaline. In agreement with previous findings [45], we observed that a mixed β_1AR/β_2AR population (with ≥4 times more β_2AR than β_1AR) contributed to the total isoprenaline-stimulated cAMP response. Furthermore, the β_1AR-selective antagonist CGP20712A did not alter the extent of isoprenaline-stimulated cAMP accumulation. Collectively, these data suggest that the β_1AR population plays at most a minor role in isoprenaline-stimulated cAMP production, with the majority role played by the β_2AR, implying that we examined β_2AR (rather than β_1AR) activity in this study. VSMC are also reported to express various adenosine receptors [27], therefore we characterised their contributions to cAMP accumulation in response to the non-selective adenosine agonist NECA. Our data show that NECA-induced cAMP production was unaffected by PTx pre-treatment and the A_{2A}R-selective agonist CGS21680 produced a weak response only at concentrations reported to interact with A_{2B}R [16]. These findings, combined with the ability of the A_{2B}R-selective antagonist MRS1754 to inhibit NECA-driven cAMP production, strongly suggests that NECA-mediated cAMP generation in RASM is mediated by A_{2B}R activity.

Using agonist pre-treatment protocols [29, 42, 46], we report robust desensitization of β_2AR- and A_{2B}R-stimulated AC activity. Concurring with previous reports where removal of PKA phosphorylation sites within the β_2AR failed to prevent receptor desensitization in HEK293 cells [29, 46], inhibition of PKA activity failed to affect acute cAMP production or desensitization of β_2AR/AC activity. Contrastingly, GRK2 knockdown dramatically enhanced β_2AR-induced AC activity, findings that are qualitatively similar to observations in murine VSMC [18] and human airway smooth muscle following GRK2 depletion [47]. In the heart GRK2-mediated β_2AR phosphorylation is known to promote G_i coupling [48, 49], which may explain why cAMP levels are enhanced in RASM following GRK2 depletion. Nevertheless, the requirement for GRK2-mediated phosphorylation (presumably of the β_2AR) appears central to mediating β_2AR/AC desensitization, since the GRK2 inhibitor, compound 101, mimicked the effects of GRK2 knockdown. Conversely, despite previous work highlighting a role for GRK2 in the regulation of A_{2B}R/AC activity in neuroblastoma cells [16], in RASM, NECA-driven cAMP accumulation was enhanced after GRK5 knockdown, yet unaffected by GRK2 inhibition or depletion. Collectively, these data demonstrate an exclusive and novel role for GRK5 in the regulation of A_{2B}R/AC coupling and hint at cell background-dependent differences in selective GRK-GPCR interactions [50].

Interestingly, GRK5 depletion also enhanced acute β_2AR-stimulated cAMP accumulation, suggesting that both kinases are able to phosphorylate and desensitize β_2AR/G_s/AC coupling. Moreover, depletion of both GRK2 and GRK5 was necessary to reduce desensitization, suggesting that either kinase can substitute for each other to induce β_2AR desensitization. We were unable to completely reverse the robust desensitization of β_2AR/AC coupling, even after depletion of both GRK2 and GRK5. However, our ~50% reversal of receptor desensitization is similar to previous observations with the β_2AR [29, 42] A_{2B}R [16] and other G_s-coupled receptors [50-52] and could simply reflect incomplete (typically 75-80%) depletion or inhibition of GRK isoforms. Although alternative kinases may contribute to β_2AR or A_{2B}R desensitization, we found no evidence to support a role for either GRK6 or PKA in β_2AR desensitization, or GRK2 or GRK6 in A_{2B}R desensitization. Furthermore, previous reports question the presence of GRK3 in arterial smooth muscle [7, 9]. Moreover, since compound 101 inhibits both GRK2 and 3 [34] and no change in β_2AR or

$A_{2B}R$ desensitization was observed in the presence of this compound, it seems that combined inhibition of GRK2/3 cannot prevent β_2AR/AC or $A_{2B}R$/AC uncoupling.

Analysis of GRK/β_2AR interactions in model cell systems may provide possible explanations for our findings. Despite having multiple potential GRK phosphorylation sites, the relative importance of each site to subsequent β_2AR desensitization and/or recruitment of different signalling pathways is a subject of debate. While previous studies have identified S355/S356 residues as vital for GRK-mediated desensitization of human β_2AR/AC activity [29], mass spectrometry analysis of the potential GRK phosphorylation sites suggested that GRK2 phosphorylates T360, S364, S396, S401, S407 and S411, whereas GRK6 phosphorylates S355/S356 [42]. Although equivalent data are not available for the rat β_2AR, with the exception of S407, human and rat β_2ARs share the same putative phosphorylation sites, suggesting that the rat β_2AR may show a similar GRK-mediated phosphorylation pattern to the human receptor.

In HEK293 cells, both GRK2 and GRK6 were able to desensitize human β_2AR/AC coupling, with some degree of cooperativity observed [42]. In contrast, we have shown that although individual knockdown of GRK2 or GRK5 enhanced acute cAMP production, β_2AR desensitization was only attenuated following depletion of both kinases. These data imply that although there could be some overlap between GRK2 and GRK5 phosphorylation sites in the rat β_2AR, unique sites may also be present. As evidence indicates that differential GRK phosphorylation patterns promote differential recruitment of arrestins, and in turn dictate the differential activation of downstream signalling pathways [42, 53], our findings highlight the possibility that GRK2 and GRK5 may differentially regulate β_2AR signalling pathways in RASM. The rat $A_{2B}R$ contains 12 potential GRK phosphorylation sites (8 serine, 4 threonine), however, no studies have identified which are required for GRK-mediated desensitization. Nonetheless, deletion of the last 4 amino acids (^{329}SLSL^{332}) of the C-terminal tail, prevented desensitization of $A_{2B}R$/AC signalling, implying that this region might be a key GRK-phosphorylation site [32].

Previous studies have identified arrestins as regulators of β_2AR and $A_{2B}R$ signalling pathways in a variety of cellular backgrounds [30, 32, 33, 43, 54], but this has not been studied in RASM. Initial experiments suggested that both arrestin2 and arrestin3 are equally adept at regulating β_2AR/AC signalling, since knockdown of either protein enhanced and

prolonged agonist-driven cAMP accumulation. Surprisingly, our desensitization studies indicate that arrestins do not play a role in the desensitization of β_2AR/AC signalling, as even depletion of both arrestin isoforms failed to reverse receptor desensitization. It should be noted that although the percentage desensitization in all treatments was similar, the relative amount of cAMP produced was significantly greater in arrestin-depleted cells, even after desensitization. Therefore, it is conceivable that the effects of arrestins on β_2AR/AC desensitization are masked due their ability to inhibit acute β_2AR/AC activity. In this regard both arrestins can act as agonist-adaptor scaffolds, to facilitate PDE4 recruitment to the β_2AR [44, 55]. However, in contrast to previous studies [56] it would appear that in RASM it is arrestin2, rather than arrestin3, that performs this function. In RASM cells, in the absence of PDE inhibition, elevated cAMP production was only observed following arrestin2 knockdown. Moreover, the isoprenaline-stimulated cAMP response was enhanced in the presence of PDE4, but not PDE3 inhibitors, suggesting a selective recruitment of PDE4. Interestingly, cAMP production was further enhanced following combined arrestin2 depletion and pharmacological inhibition of PDE4, suggesting that perhaps arrestin2 facilitates the targeting of PDE4 to the agonist-bound receptor. The interaction between arrestin2 and β_2AR appears to be receptor-specific as arrestin2 plays no role in the regulation of A_{2B}R/AC activity, which appears to be solely mediated by arrestin3. This raises the possibility that arrestin3 may play an equivalent role in recruiting PDE isoenzymes to the A_{2B}R. However, unlike the β_2AR a significant reversal of A_{2B}R desensitization was observed in arrestin3 depleted cells, which suggests that arrestin3 may also play a more traditional role in attenuating A_{2B}R/G_s/AC coupling.

It is noteworthy that receptor desensitization can differ depending upon whether the desensitization stimulus is chronic or acute, and that G_s-coupled receptors may be able to continue to signal at the cell-surface [36], and/or following internalization [37]. In the case of the β_1AR, which shows continued signalling at the cell-surface, receptor activity is masked by the recruitment of PDE enzymes to the receptor microdomain. These findings chime with our data, where acute (over a 10 min time-course) signalling can be substantially enhanced following GRK or arrestin knockdown, possibly as a result of decreased receptor phosphorylation and arrestin/PDE recruitment. Interestingly, inhibition of dynamin-induced receptor internalization reduced the ability of either β_2AR or A_{2B}R to stimulate cAMP accumulation in agonist-naïve cells. At present it is unclear whether this is due to a non-specific action, or alternatively, in agreement with previous data [37], both β_2AR and A_{2B}R

may continue to signal upon internalization. In addition, inhibition of dynamin reduced $A_{2B}R$, but not β_2AR desensitization in agonist pre-treated cells, indicating that $A_{2B}R$ desensitization is dependent on receptor internalization. β_2AR [37] and $A_{2B}R$ [57] receptor internalization has been reported previously to be arrestin- and dynamin-dependent. Therefore, it is possible that endogenous RASM β_2ARs are differently regulated, internalizing via a dynamin-independent mechanism, or perhaps more radically that endogenous RASM β_2ARs are processed at the plasma membrane – a finding that might also account for the rapid β_2AR re-sensitization that we have been observed.

The development of hypertension results, at least in part, through an imbalance in the relative signalling inputs generated by contractile and relaxatory agents in VSMC, which are mediated through activation of their cognate GPCRs. Here, we confirm that GRK2, GRK5 and arrestin isoforms are negative regulators of β_2AR/AC signalling, and highlight a similar novel role for GRK5 and arrestin3 in the regulation of $A_{2B}R$ signalling. Since the expression of GRK2 and arrestin proteins is known to increase in the early stages of hypertension [20], our findings suggest that, as has previously been postulated [18, 19], β_2AR/AC activity would likely be suppressed during hypertension, leading to a reduced capacity to relax arteries. Logically, therapeutic strategies targeting GRK2 activity would seem beneficial and with recent developments in small molecule GRK inhibitors may eventually be possible. However, since GRK2 also negatively regulates the majority of contractile GPCRs [7, 9, 10, 18] it is debatable whether such therapies would be beneficial. In contrast, our findings suggest that targeting GRK5 to prolong $A_{2B}R$ mediated vessel relaxation might provide a more selective treatment for hypertension.

5. Conclusion

In summary GRK2, GRK5 and arrestin2 negatively regulate β_2AR-AC signalling in RASM, while GRK5/arrestin3 play similar roles in the regulation of A_{2B}R signalling, implying that these mechanisms play a key role in arterial relaxation. Furthermore, arterial GRK2 and arrestin2/3 expression is enhanced in hypertension, which likely contributes to reduced arterial relaxation observed in this disorder. However, since GRK2 also negatively regulates a number of contractile GPCRs, our findings point to GRK5 providing a more promising target for the development of novel anti-hypertensives.

Funding

This work was supported by the British Heart Foundation of the United Kingdom by grants to J.M.W. and R.A.J.C. [RG06/008/22062, PG/11/60/29007, PG/13/95/30603].

References

[1] Hill-Eubanks DC, Werner ME, Heppner TJ, Nelson MT, Calcium signaling in smooth muscle. Cold Spring Harb Perspect Biol. 2011;3:a004549.

[2] Brinks HL, Eckhart AD, Regulation of GPCR signaling in hypertension. Biochim Biophys Acta. 2010;1802:1268-1275.

[3] Nelson MT, Quayle JM, Principal role of adenylyl cyclase 6 in K^+ channel regulation and vasodilator signalling in vascular smooth muscle cells. Am J Physiol. 1995;268:C799-822.

[4] Quayle JM, Standen NB, K_{ATP} channels in vascular smooth muscle. Cardiovasc Res. 1994;28:797-804.

[5] Willets JM, Challiss RA, Nahorski SR, Non-visual GRKs: are we seeing the whole picture? Trends Pharmacol Sci. 2003;24:626-633.

[6] DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK, β-arrestins and cell signaling. Annu Rev Physiol. 2007;69:483-510.

[7] Cohn HI, Harris DM, Pesant S, Pfeiffer M, Zhou RH, Koch WJ, Dorn GW, 2nd, Eckhart AD, Inhibition of vascular smooth muscle G protein-coupled receptor kinase 2 enhances α_{1D}-adrenergic receptor constriction. Am J Physiol. Heart Circ Physiol. 2008;295:H1695-1704.

[8] Kim J, Ahn S, Rajagopal K, Lefkowitz RJ, J Biol Chem. Independent β-arrestin2 and Gq/protein kinase Cξ pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. 2009;284:11953-11962.

[9] Morris GE, Nelson CP, Standen NB, Challiss RA, Willets JM, Endothelin signalling in arterial smooth muscle is tightly regulated by G protein-coupled receptor kinase 2. Cardiovasc Res. 2010:424-433.

[10] Morris GE, Nelson CP, Everitt D, Brighton PJ, Standen NB, Challiss RA, Willets JM, G protein-coupled receptor kinase 2 and arrestin2 regulate arterial smooth muscle P2Y-purinoceptor signalling. Cardiovasc Res. 2011;89:193-203.

[11] Morris GE, Nelson CP, Brighton PJ, Standen NB, Challiss RA, Willets JM, Arrestins 2 and 3 differentially regulate ET_A and $P2Y_2$ receptor-mediated cell signaling and migration in arterial smooth muscle. Am J Physiol Cell Physiol. 2012;302:C723-734.

[12] Hata JA, Koch WJ, Phosphorylation of G protein-coupled receptors: GPCR kinases in heart disease. Mol Interv. 2003;3:264-272.

[13] Penela P, Murga C, Ribas C, Tutor AS, Peregrin S, Mayor F, Jr., Mechanisms of regulation of G protein-coupled receptor kinases (GRKs) and cardiovascular disease. Cardiovasc Res. 2006;69:46-56.

[14] Black JB, Premont RT, Daaka Y, Feedback regulation of G protein-coupled receptor signaling by GRKs and arrestins. Semin Cell Dev Biol. 2016;50:95-104.

[15] Dorn GW, 2nd, J Mol Med (Berl). GRK mythology: G-protein receptor kinases in cardiovascular disease. 2009;87:455-463.

[16] Mundell SJ, Kelly E, Evidence for co-expression and desensitization of A_{2a} and A_{2b} adenosine receptors in NG108-15 cells. Biochem Pharmacol. 1998;55:595-603.

[17] Eckhart AD, Ozaki T, Tevaearai H, Rockman HA, Koch WJ, Vascular-targeted overexpression of G protein-coupled receptor kinase-2 in transgenic mice attenuates β-adrenergic receptor signaling and increases resting blood pressure. Mol Pharmacol. 2002;61:749-758.

[18] Tutunea-Fatan E, Caetano FA, Gros R, Ferguson SS, GRK2 targeted knock-down results in spontaneous hypertension, and altered vascular GPCR signaling. J Biol Chem. 2015;290:5141-5155.

[19] Gros R, Chorazyczewski J, Meek MD, Benovic JL, Ferguson SS, Feldman RD, G-Protein-coupled receptor kinase activity in hypertension: increased vascular and

lymphocyte G-protein receptor kinase-2 protein expression. Hypertension. 2000;35:38-42.

[20] Willets JM, Nash CA, Rainbow RD, Nelson CP, Challiss RA, Defining the roles of arrestin2 and arrestin3 in vasoconstrictor receptor desensitization in hypertension. Am J Physiol Cell Physiol. 2015;309:C179-189.

[21] Hayabuchi Y, Dart C, Standen NB, Evidence for involvement of A-kinase anchoring protein in activation of rat arterial K_{ATP} channels by protein kinase A. J Physiol. 2001;536:421-427.

[22] Brown BL, Albano JD, Ekins RP, Sgherzi AM, A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. Biochem J. 1971;121:561-562.

[23] Fredholm BB, Persson CG, Xanthine derivatives as adenosine receptor antagonists. Eur J Pharmacol. 1982;81:673-676.

[24] Daly CJ, McGrath JC, Previously unsuspected widespread cellular and tissue distribution of β-adrenoceptors and its relevance to drug action. Trends Pharmacol Sci. 2011;32:219-226.

[25] Baker JG, The selectivity of β-adrenoceptor antagonists at the human β_1, β_2 and β_3 adrenoceptors. Br J Pharmacol. 2005;144:317-322.

[26] Olsson RA, Pearson JD, Cardiovascular purinoceptors. Physiol Rev. 1990;70:761-845.

[27] Sousa JB, Diniz C, The Adenosinergic System as a Therapeutic Target in the Vasculature: New Ligands and Challenges. Molecules. 2017;22.

[28] Alexander SP, Losinski A, Kendall DA, Hill SJ, A comparison of A_2 adenosine receptor-induced cyclic AMP generation in cerebral cortex and relaxation of pre-contracted aorta. Br J Pharmacol. 1994;111:185-190.

[29] Seibold A, January BG, Friedman J, Hipkin RW, Clark RB, Desensitization of β_2-adrenergic receptors with mutations of the proposed G protein-coupled receptor kinase phosphorylation sites. J Biol Chem. 1998;273:7637-7642.

[30] Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ, beta-arrestin-dependent, G protein-independent ERK1/2 activation by the β_2 adrenergic receptor. J Biol Chem. 2006;281:1261-1273.

[31] Simon V, Robin MT, Legrand C, Cohen-Tannoudji J, Endogenous G protein-coupled receptor kinase 6 triggers homologous β-adrenergic receptor desensitization in primary uterine smooth muscle cells. Endocrinology. 2003;144:3058-3066.

[32] Matharu AL, Mundell SJ, Benovic JL, Kelly E, Rapid agonist-induced desensitization and internalization of the A_{2B} adenosine receptor is mediated by a serine residue close to the COOH terminus. J Biol Chem. 2001;276:30199-30207.

[33] Houslay MD, Baillie GS, Maurice DH, cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. Circ Res. 2007;100:950-966.

[34] Thal DM, Yeow RY, Schoenau C, Huber J, Tesmer JJ, Molecular mechanism of selectivity among G protein-coupled receptor kinase 2 inhibitors. Mol Pharmacol. 2011;80:294-303.

[35] Lowe JD, Sanderson HS, Cooke AE, Ostovar M, Tsisanova E, Withey SL, Chavkin C, Husbands SM, Kelly E, Henderson G, Bailey CP, Role of G Protein-Coupled Receptor Kinases 2 and 3 in μ-Opioid Receptor Desensitization and Internalization. Mol Pharmacol. 2015;88:347-356.

[36] Fu Q, Kim S, Soto D, De Arcangelis V, DiPilato L, Liu S, Xu B, Shi Q, Zhang J, Xiang YK, A long lasting β_1 adrenergic receptor stimulation of cAMP/protein kinase A (PKA) signal in cardiac myocytes. J Biol Chem. 2014;289:14771-14781.

[37] Irannejad R, Tomshine JC, Tomshine JR, Chevalier M, Mahoney JP, Steyaert J, Rasmussen SG, Sunahara RK, El-Samad H, Huang B, von Zastrow M, Conformational biosensors reveal GPCR signalling from endosomes. Nature. 2013;495:534-538.

[38] Morgado M, Cairrao E, Santos-Silva AJ, Verde I, Cyclic nucleotide-dependent relaxation pathways in vascular smooth muscle. Cell Mol Life Sci. 2012;69:247-266.

[39] Asano M, Masuzawa K, Matsuda T, Asano T, Reduced function of the stimulatory GTP-binding protein in beta adrenoceptor-adenylate cyclase system of femoral arteries isolated from spontaneously hypertensive rats. J Pharmacol Exp Ther. 1988;246:709-718.

[40] Gros R, Ding Q, Chorazyczewski J, Andrews J, Pickering JG, Hegele RA, Feldman RD, The impact of blunted β-adrenergic responsiveness on growth regulatory pathways in hypertension. Mol Pharmacol. 2006;69:317-327.

[41] Headrick JP, Ashton KJ, Rose'meyer RB, Peart JN, Cardiovascular adenosine receptors: expression, actions and interactions. Pharmacol Ther. 2013;140:92-111.

[42] Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang TY, Bressler EA, Hara MR, Shenoy SK, Gygi SP, Lefkowitz RJ, Distinct phosphorylation sites on the β_2-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. Sci Signal. 2011;4:ra51.

[43] Lymperopoulos A, Negussie S, βArrestins in cardiac G protein-coupled receptor signaling and function: partners in crime or "good cop, bad cop"? Int J Mol Sci. 2013;14:24726-24741.

[44] Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, Miller WE, McLean AJ, Conti M, Houslay MD, Lefkowitz RJ, Targeting of cyclic AMP degradation to β_2-adrenergic receptors by β-arrestins. Science. 2002;298:834-836.

[45] Nelson CP, Rainbow RD, Brignell JL, Perry MD, Willets JM, Davies NW, Standen NB, Challiss RA, Principal role of adenylyl cyclase 6 in K^+ channel regulation and vasodilator signalling in vascular smooth muscle cells. Cardiovasc Res. 2011;91:694-702.

[46] Seibold A, Williams B, Huang ZF, Friedman J, Moore RH, Knoll BJ, Clark RB, Localization of the sites mediating desensitization of the β_2-adrenergic receptor by the GRK pathway. Mol Pharmacol. 2000;58:1162-1173.

[47] Kong KC, Gandhi U, Martin TJ, Anz CB, Yan H, Misior AM, Pascual RM, Deshpande DA, Penn RB, Endogenous Gs-coupled receptors in smooth muscle exhibit differential susceptibility to GRK2/3-mediated desensitization. Biochemistry. 2008;47:9279-9288.

[48] Zhu W, Petrashevskaya N, Ren S, Zhao A, Chakir K, Gao E, Chuprun JK, Wang Y, Talan M, Dorn GW, 2nd, Lakatta EG, Koch WJ, Feldman AM, Xiao RP, G_i-biased β_2AR signaling links GRK2 upregulation to heart failure. Circ Res. 2012;110:265-274.

[49] Xiao RP, Avdonin P, Zhou YY, Cheng H, Akhter SA, Eschenhagen T, Lefkowitz RJ, Koch WJ, Lakatta EG, Coupling of β_2-adrenoceptor to G_i proteins and its physiological relevance in murine cardiac myocytes. Circ Res. 1999;84:43-52.

[50] Mundell SJ, Benovic JL, Kelly E, A dominant negative mutant of the G protein-coupled receptor kinase 2 selectively attenuates adenosine A2 receptor desensitization. Mol Pharmacol. 1997;51:991-998.

[51] Willets JM, Parent JL, Benovic JL, Kelly E, Selective reduction in A2 adenosine receptor desensitization following antisense-induced suppression of G protein-coupled receptor kinase 2 expression. J Neurochem. 1999;73:1781-1789.

[52] Nagayama Y, Tanaka K, Hara T, Namba H, Yamashita S, Taniyama K, Niwa M, Involvement of G protein-coupled receptor kinase 5 in homologous desensitization of the thyrotropin receptor. J Biol Chem. 1996;271:10143-10148.

[53] Liggett SB, Phosphorylation barcoding as a mechanism of directing GPCR signaling. Sci Signal. 2011;4:pe36.

[54] Mundell SJ, Loudon RP, Benovic JL, Characterization of G protein-coupled receptor regulation in antisense mRNA-expressing cells with reduced arrestin levels. Biochemistry. 1999;38:8723-8732.

[55] Baillie GS, Sood A, McPhee I, Gall I, Perry SJ, Lefkowitz RJ, Houslay MD, β-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates β-adrenoceptor switching from G_s to G_i. Proc Natl Acad Sci U S A. 2003;100:940-945.

[56] Berthouze-Duquesnes M, Lucas A, Sauliere A, Sin YY, Laurent AC, Gales C, Baillie G, Lezoualc'h F, Specific interactions between Epac1, -arrestin2 and PDE4D5 regulate β-adrenergic receptor subtype differential effects on cardiac hypertrophic signaling. Cell Signal. 2013;25:970-980.

[57] Claing A, Perry SJ, Achiriloaie M, Walker JK, Albanesi JP, Lefkowitz RJ, Premont RT, Multiple endocytic pathways of G protein-coupled receptors delineated by GIT1 sensitivity. Proc Natl Acad Sci U S A. 2000;97:1119-1124.

Figure Legends

Fig. 1. Characterization of isoprenaline- or adenosine-induced cAMP generation in RASM. (A) RASM cells were stimulated with isoprenaline or forskolin at the indicated concentrations in the presence of IBMX (300 µM) for 10 min. (B) To determine the relative contributions that β_1AR and β_2AR play in determining total cAMP accumulation, RASM cells were pre-incubated with increasing concentrations of the β_2AR-selective antagonist ICI118,551 (for 30 min) prior to the addition of isoprenaline (1 µM, 10 min), in the presence of IBMX (300 µM). (C) Isoprenaline concentration-response curves were generated in the presence or absence of the β_1AR-selective antagonist CGP20712A (300 nM, 30 min pre-treatment). (D) RASM cells were pre-treated with either vehicle-control or the A_{2B} receptor-selective antagonist MRS1754 (10 µM) for 30 min prior to the addition of increasing concentrations of the non-selective adenosine receptor agonist NECA, for a further 10 min, in the presence of the PDE4 inhibitor rolipram. Concentration-response curves were generated following addition of the selective A_{2A} receptor agonist CGS21680. MRS1754 significantly inhibited NECA-stimulated cAMP accumulation $*p<0.05$, $**p<0.01$ (two-way ANOVA; Tukey's *post hoc* test; when compared to vehicle control treated cells). E) RASM were pre-treated with PTx (100 ng/mL; 18 h) prior to stimulation with NECA (10 µM), in the presence of rolipram. In all cases cAMP accumulation was determined as described previously (see *Methods*). Data are shown as means ± SEM for at least duplicate values each obtained in 6 (panels A, B) or 4 (panels C-E) RASM preparations from separate donor animals.

Fig. 2. Suppression of GRK expression enhances β_2AR- and A_{2B}-stimulated adenylyl cyclase activity. Representative immunoblots (A) and cumulative data (B) show the extent of siRNA-mediated GRK2, GRK5 or GRK6 depletion following transfection of RASM with specific anti-GRK siRNAs (10 nM, 48 h; data are means ± SEM for at least duplicate values from 4 separate animal preparations). $*p<0.01$ (Kruskal-Wallis, Dunn's *post hoc* test) indicates a significant reduction of targeted GRK expression vs control siRNA-treated cells. The effects of simultaneous transfection with GRK2 and GRK5 siRNAs (10 nM, 48 h; data are means ± SEM for at least duplicate values from 4 separate animal preparations). $*p<0.01$ (Kruskal-Wallis, Dunn's *post hoc* test) indicates a significant reduction of targeted GRK expression vs control siRNA-treated cells are shown (C, D). The temporal-profiles of NECA (E) and isoprenaline (F) -stimulated cAMP accumulation in the absence of PDE inhibition are shown,

following siRNA-mediated depletion of GRK isoenzymes (see *Methods*). Data are means ± SEM for at least duplicate values from 6-8 separate animal preparations. GRK5 depletion enhanced NECA-stimulated adenylyl cyclase activity (E) *p<0.05; **p<0.01; vs control cells (two-way ANOVA; Tukey's *post hoc* test). Knockdown of GRK2 or GRK5 enhanced isoprenaline-stimulated adenylyl cyclase activity ($^{\#}p$<0.05 vs control cells; two-way ANOVA; Tukey's *post hoc* test), while combined knockdown of GRK2 and GRK5 further increased isoprenaline-stimulated cAMP accumulation (F) (*p<0.05; **p<0.01; vs other treatments, two-way ANOVA; Tukey's *post hoc* test). Inclusion of the GRK2/3-selective inhibitor compound 101 (30 μM, 20 min pre-treatment) enhanced and prolonged the temporal-profile of isoprenaline (1 μM), but not NECA (10 μM) stimulated cAMP accumulation (G) in the presence of PDE inhibitor (means ± SEM for at least duplicate values from 6 separate animal preparations; **p<0.01; vs control cells, two-way ANOVA; Tukey's *post hoc* test). H) Depletion of individual GRK expression has no effect on forskolin-stimulated AC activity. RASM cells were transfected with negative-control, anti-GRK2, anti-GRK5 or anti-GRK6 siRNAs (10 nM) for 48 h prior to addition of the AC activator forskolin (10 μM) for 10 min, in the presence of IBMX (300 μM). Data are means ± SEM, n=6, from 6 separate animal preparations.

Fig. 3. Time-course profiles of isoprenaline- or NECA-stimulated adenylyl cyclase activity in RASM following knockdown of arrestin2 and/or arrestin3. RASM were transfected with 10 nM of negative-control (NC), anti-arrestin2 (AR2), anti-arrestin3 (AR3) or AR2/AR3 siRNAs, for 48 h prior to the addition of agonists. (A) Representative immunoblots show arrestin2 (upper panel) or arrestin3 (lower panel) knockdown in RASM cells 48 h after transfection with AR2 or AR3 siRNAs. Cumulative data (separate experiments from 4 animal preparations) are shown in (B) (**p<0.01 vs NC siRNA-treated cells, Kruskal-Wallis, Dunn's *post hoc* test). The prolife of isoprenaline-stimulated cAMP accumulation was determined in the presence (C) or absence (D) of the PDE inhibitor IBMX (300 μM); and NECA-stimulated cAMP generation in the presence (E) or absence (F) of the PDE inhibitor rolipram (10 μM). Data are expressed as means ± SEM for at least duplicate values from 4 separate animals, *p<0.05; **p<0.01 two-way ANOVA, Sidak's *post hoc* test compared to NC-treated RASM.

Fig. 4. Differential effects of PDE isoenzyme-selective inhibitors on isoprenaline-stimulated adenylyl cyclase activity in RASM. The effect of PDE3-selective inhibitor siguazodan (1 μM) or PDE4-selective inhibitor rolipram (10 μM) on isoprenaline-stimulated cAMP accumulation was determined in AR2- or AR3-depleted cells. PDE inhibitors were added 15 min prior to isoprenaline challenge (1 μM, 10 min). cAMP accumulation was determined as described previously (see *Methods*). Data are means ± SEM for at least duplicate values from 5 separate animal preparations; $^{**}p<0.01$, $^{***}p<0.001$ two-way ANOVA; Tukey's *post hoc* test compared to negative-control siRNA-treated cells.

Fig. 5. Desensitization of β_2AR-induced adenylyl cyclase activity in RASM is mediated by GRK2 and GRK5. A) β_2AR-stimulated cAMP generation in non-transfected RASM, is shown in the presence or absence of isoprenaline pre-treatment (1 μM, 15 min, see *Methods*). Data are means ± SEM for n=5 separate preparations from 5 animals; $p<0.01$ (two-way, ANOVA, Tukey's *post hoc* test), indicates a significant reduction in isoprenaline-driven cAMP generation following agonist pre-treatment. B) To delineate the relative contributions played by β_1AR and β_2AR receptors in the overall isoprenaline induced desensitization of adenylyl cyclase activity, RASM were pre-incubated with the β_1AR selective antagonist CGP20712 (300 nM, 30 min) prior to application of our standard receptor desensitization protocol. Data are means ± SEM for n=4 separate preparations from 4 animals, and show a significant reduction ($p<0.01$ two-way ANOVA, Tukey's *post hoc* test) in isoprenaline-driven cAMP production when compared to their relative non-pre-treated controls (** +CGP samples / $^{\#}$-CGP samples). (C) Cumulative data showing that the degree of receptor desensitization was similar in the presence and absence of CGP20712 (data are means ± SEM for n=5 separate preparations from 5 animals). The effects of GRK2 depletion or PKA inhibition on acute isoprenaline-stimulated cAMP production is shown in (D). Data are expressed as means ± SEM for n=6-10 from up to 10 separate animal preparations ($^{**}p<0.01$, two-way ANOVA; Sidak's *post hoc* test, when compared to negative-control (NC) siRNA- or PKAI-treated cells). The extent of isoprenaline (1 μM, 15 min)-induced β_2AR (E, F) of cAMP generation are shown, following the depletion of GRK2, 5 or 6 (data are means ± SEM for n=6-8, from 6-8 separate animal preparations). Depletion of GRK2 or GRK5 enhanced cAMP generation in non-pre-treated RASM ($^{**}p<0.01$ vs NC; two-way ANOVA; Tukey's *post hoc* test), while GRK5 and combined GRK2/5 knockdown increased isoprenaline-stimulated cAMP accumulation in agonist pre-treated RASM ($^{\#}p<0.01$ vs NC; two-way ANOVA; Tukey's *post*

hoc test). The extent of β_2AR desensitization of isoprenaline-stimulated cAMP generation is shown (G) when RASM cells were pre-incubated with vehicle-control or the GRK2/3-selective inhibitor compound 101 (30 μM) for 20 min prior to application of the standard desensitization protocol. Data are means ± SEM for n=4, from 4 separate animal preparations. Inclusion of compound 101 enhanced (**p<0.01; $^{\#}p$<0.05 vs control cells, two-way ANOVA; Tukey's *post hoc* test) isoprenaline-stimulated cAMP accumulation in both naïve (**) and isoprenaline pre-treated ($^{\#}$) cells. Cumulative data (H) show the degree of β_2AR desensitization, measured as the percent change in cAMP generated by 1 μM isoprenaline in agonist-pre-treated (R2) compared to non-pre-treated (R1) RASM.

Fig. 6. Desensitization of A_{2B}R-stimulated adenylyl cyclase activity in RASM is mediated by GRK5. A) RASM were stimulated with NECA (10 μM) for varying time periods prior to washing and reapplication of NECA (10 μM) for a further 10 min in the presence of rolipram (10 μM). Data (means ± SEM n=6, from 6 separate animal preparations) show the time-dependent desensitization (**p*<0.01* vs vehicle pre-treated cells; one-way ANOVA; Tukey's *post hoc* test) of A_{2B}R-stimulated AC activity. The extent of NECA (10 μM, 30 min)-induced A_{2B}R desensitization (B) of cAMP generation are shown, following the depletion of GRK2, 5 or 6 (data are means ± SEM for n=6-8, from 6-8 separate animal preparations). Depletion of GRK5 enhanced NECA-stimulated cAMP generation in non-pre-treated RASM (**p<0.01 vs NC; two-way ANOVA; Tukey's *post hoc* test). Cumulative data show the degree of A_{2B}R (C) desensitization, measured as the percent change in cAMP generated by 10 μM NECA, in agonist-pre-treated (R2) compared to non-pre-treated (R1) RASM cells (means ± SEM, n=6-8 from 6-8 separate animal preparations). GRK5 knockdown attenuated A_{2B}R desensitization (C) (*p<0.05 vs NC-treated cells; Kruskal-Wallis, Dunn's *post hoc* test).

Fig. 7. Differential effects of arrestin depletion on the desensitization of β_2AR- and A_{2B}R-stimulated adenylyl cyclase signalling. To determine whether arrestins regulate isoprenaline-induced β_2AR (A, B) or NECA-induced A_{2A}R (C, D) desensitization, RASM were transfected with 10 nM of negative-control (NC), anti-arrestin2 (AR2), anti-arrestin3 (AR3) or anti-arrestin2 and anti-arrestin3 siRNAs, for 48 h before application of the standard desensitization assays (see *Methods*). Cumulative data show the degree of β_2AR (B) or A_{2B}R (D)

desensitization. Data are expressed as means ± SEM (n=7-10 separate experiments from 7-10 separate animals preparations) for the percent change in cAMP generated by 1 μM isoprenaline for of β_2AR and 10 μM NECA for A_{2B}R in agonist-pre-treated (R2) compared to naïve (R1) RASM cells.

Fig 8. The dynamin inhibitor Dyngo 4A attenuates A_{2B}R but not β_2AR desensitization. RASM cells were pre-treated with vehicle control or Dingo 4A (30 μM) for 15 min prior to being subjected to the standard desensitization protocols (A) isoprenaline 1 μM, 15 min or (B) NECA 10 μM, 30 min pre-treatments. Cells were then washed and stimulated for 10 min with either (A) isoprenaline (1 μM) or (B) NECA (10 μM) in the presence of IMBX (300 μM) or rolipram (10 μM), respectively. The data (means ± SEM; n=3 separate experiments from 3 separate animals preparations) show that agonist pre-treatment caused significant decreases in cAMP accumulation (**p<0.01 vs non-pre-treated cells; two-way ANOVA; Tukey's *post hoc* test). Dyngo 4A treatment inhibited acute isoprenaline and NECA-stimulated cAMP accumulation ($^{\#}p$<0.01 vs vehicle control; two-way ANOVA; Tukey's *post hoc* test). Cumulative data (C) show the degree of β_2AR and A_{2B}R desensitization, measured as the percent change in cAMP generated by 1 μM isoprenaline or 10 μM NECA in agonist-pre-treated (R2) compared to non-pre-treated (R1) RASM. Data are expressed as means ± SEM (n=3 separate experiments from 3 separate animals preparations). Dyngo 4A also attenuated A_{2B}R but not β_2AR desensitization (**p<0.01 vs vehicle control; two-way ANOVA; Tukey's *post hoc* test).

Fig 9. Time-course of β_2AR and A_{2B}R re-sensitization. RASM cells were subjected to the standard desensitization protocols (β_2AR- 15 min isoprenaline 1 μM; A_{2B}R- 30 min NECA 10 μM), before washing 3 times with KREB buffer. Cells were then allowed to recover for up to 45 min before a second agonist challenge (1 μM isoprenaline, or 10 μM NECA) for a further 10 min in the presence of phosphodiesterase inhibitors. Cumulative data show the degree of β_2AR or A_{2B}R desensitization. Data are expressed as means ± SEM (n=3 separate experiments from 3 separate animals preparations) for the percent change in cAMP generated by 1 μM isoprenaline for of β_2AR and 10 μM NECA for A_{2B}R in agonist-pre-treated (R2) compared to

naïve (R1) RASM cells (*p<0.05, **p<0.01 vs agonist naïve cells; Kruskal-Wallis, Dunn's *post hoc* test).

Highlights

- Depletion of GRK2 or GRK5 enhanced and prolonged β_2AR adenylyl cyclase activity
- Combined GRK2/GRK5 knockdown further enhanced β_2AR adenylyl cyclase activity
- Combined knockdown of GRK2 and GRK5 attenuated β_2AR receptor desensitization
- Depletion of GRK5 enhanced acute adenosine A_{2B}-stimulated adenylyl cyclase activity
- Adenosine A_{2B} receptor desensitization was GRK5 and arrestin3 dependent

Formatted: Font: Times New Roman, 12 pt

Formatted: List Paragraph, Bulleted + Level: 1 + Aligned at: 0.25" + Indentat: 0.5"

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt

Formatted: Font: Times New Roman, 12 pt

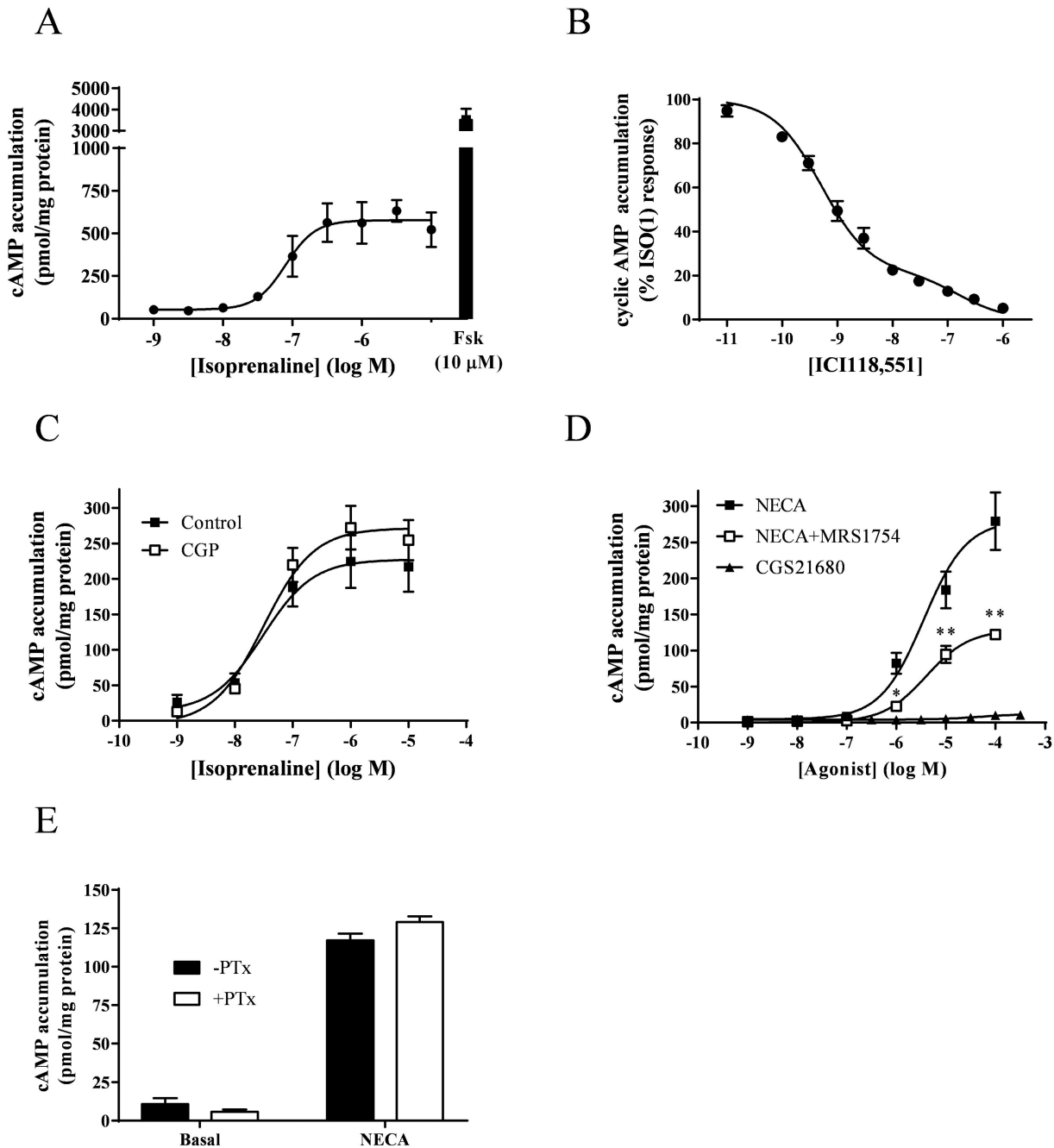
A
cAMP accumulation (pmol/mg protein)
5000
4000
3000
1000
750
500
250
0
-9
-8
-7
-6
Fsk (10 μM)
[Isoprenaline] (log M)
B
cyclic AMP accumulation (% ISO(1) response)
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
[ICI118,551]
C
cAMP accumulation (pmol/mg protein)
Control
CGP
300
250
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
[Isoprenaline] (log M)
D
cAMP accumulation (pmol/mg protein)
NECA
NECA+MRS1754
CGS21680
*
**
**
**
-10
-9
-8
-7
-6
-5
-4
-3
[Agonist] (log M)
E
cAMP accumulation (pmol/mg protein)
150
125
100
75
50
25
0
-PTx
+PTx
Basal
NECA

Figure 1

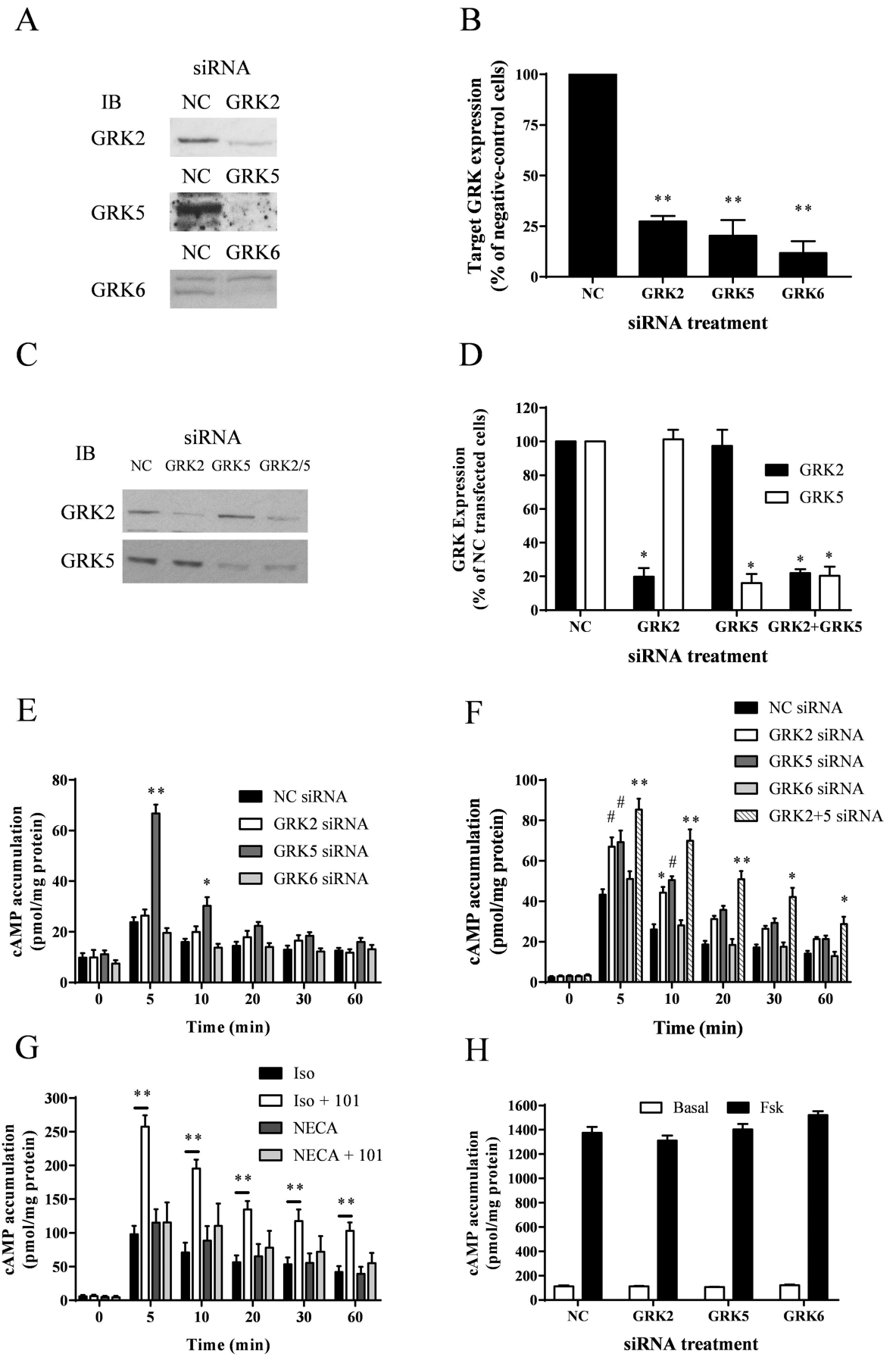
A
siRNA
IB
NC GRK2
GRK2
NC GRK5
GRK5
NC GRK6
GRK6
B
Target GRK expression (% of negative-control cells)
100
75
50
25
0
**
**
**
NC
GRK2
GRK5
GRK6
siRNA treatment
C
siRNA
IB
NC GRK2 GRK5 GRK2/5
GRK2
GRK5
D
GRK Expression (% of NC transfected cells)
120
100
80
60
40
20
0
*
*
*
*
GRK2
GRK5
NC
GRK2
GRK5
GRK2+GRK5
siRNA treatment
E
cAMP accumulation (pmol/mg protein)
80
60
40
20
0
**
*
NC siRNA
GRK2 siRNA
GRK5 siRNA
GRK6 siRNA
0
5
10
20
30
60
Time (min)
F
NC siRNA
GRK2 siRNA
GRK5 siRNA
GRK6 siRNA
GRK2+5 siRNA
cAMP accumulation (pmol/mg protein)
100
80
60
40
20
0
#
#
**
*
#
**
**
**
*
*
0
5
10
20
30
60
Time (min)
G
cAMP accumulation (pmol/mg protein)
300
250
200
150
100
50
0
**
**
**
**
**
Iso
Iso + 101
NECA
NECA + 101
0
5
10
20
30
60
Time (min)
H
cAMP accumulation (pmol/mg protein)
1600
1400
1200
1000
800
600
400
200
0
Basal
Fsk
NC
GRK2
GRK5
GRK6
siRNA treatment

Figure 2

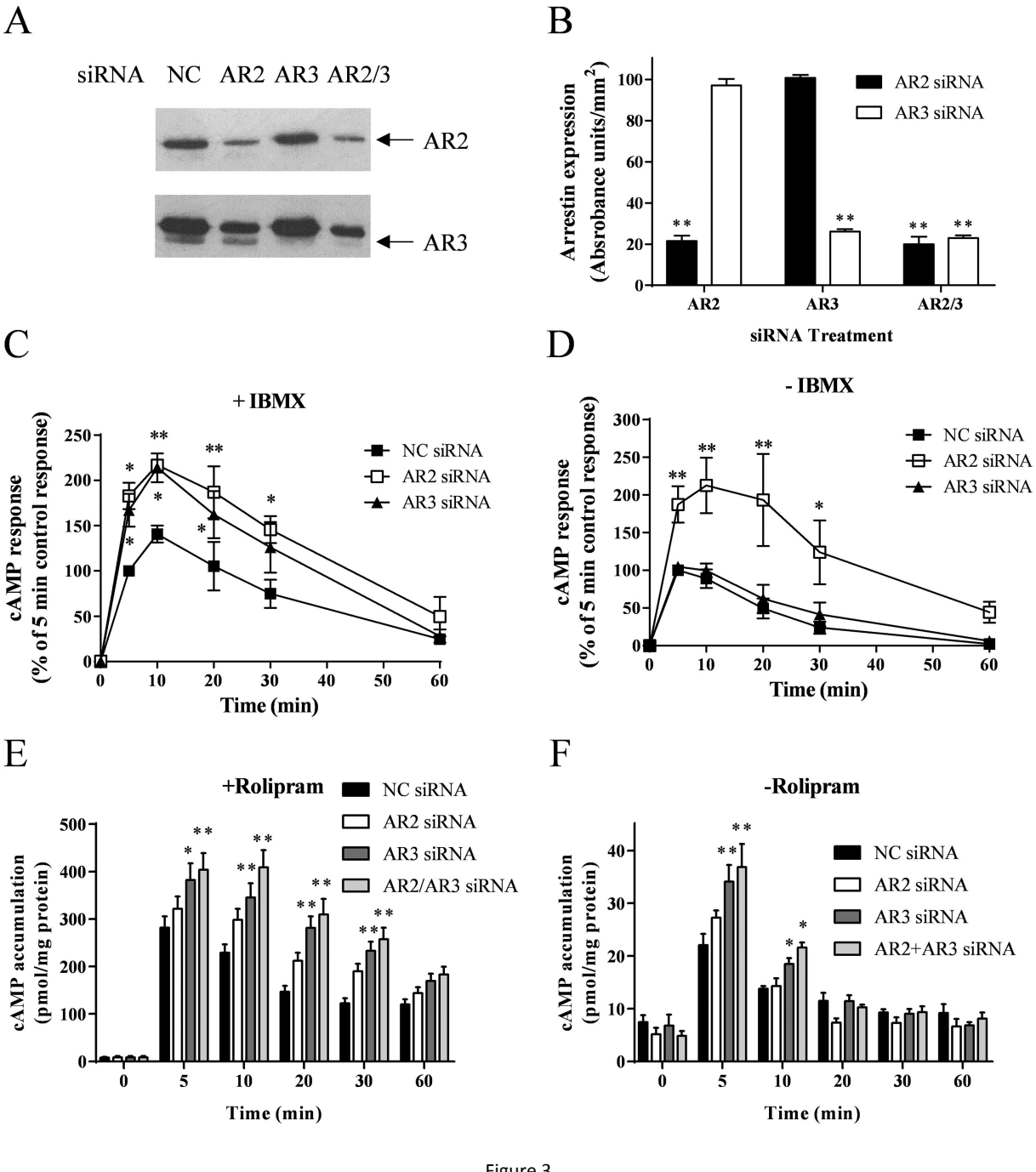
A
siRNA NC AR2 AR3 AR2/3
AR2
AR3
B
Arrestin expression (Absrobance units/mm2)
AR2 siRNA
AR3 siRNA
AR2
AR3
AR2/3
siRNA Treatment
C
+ IBMX
cAMP response (% of 5 min control response)
NC siRNA
AR2 siRNA
AR3 siRNA
Time (min)
D
- IBMX
cAMP response (% of 5 min control response)
NC siRNA
AR2 siRNA
AR3 siRNA
Time (min)
E
+Rolipram
cAMP accumulation (pmol/mg protein)
NC siRNA
AR2 siRNA
AR3 siRNA
AR2/AR3 siRNA
Time (min)
F
-Rolipram
cAMP accumulation (pmol/mg protein)
NC siRNA
AR2 siRNA
AR3 siRNA
AR2+AR3 siRNA
Time (min)

Figure 3

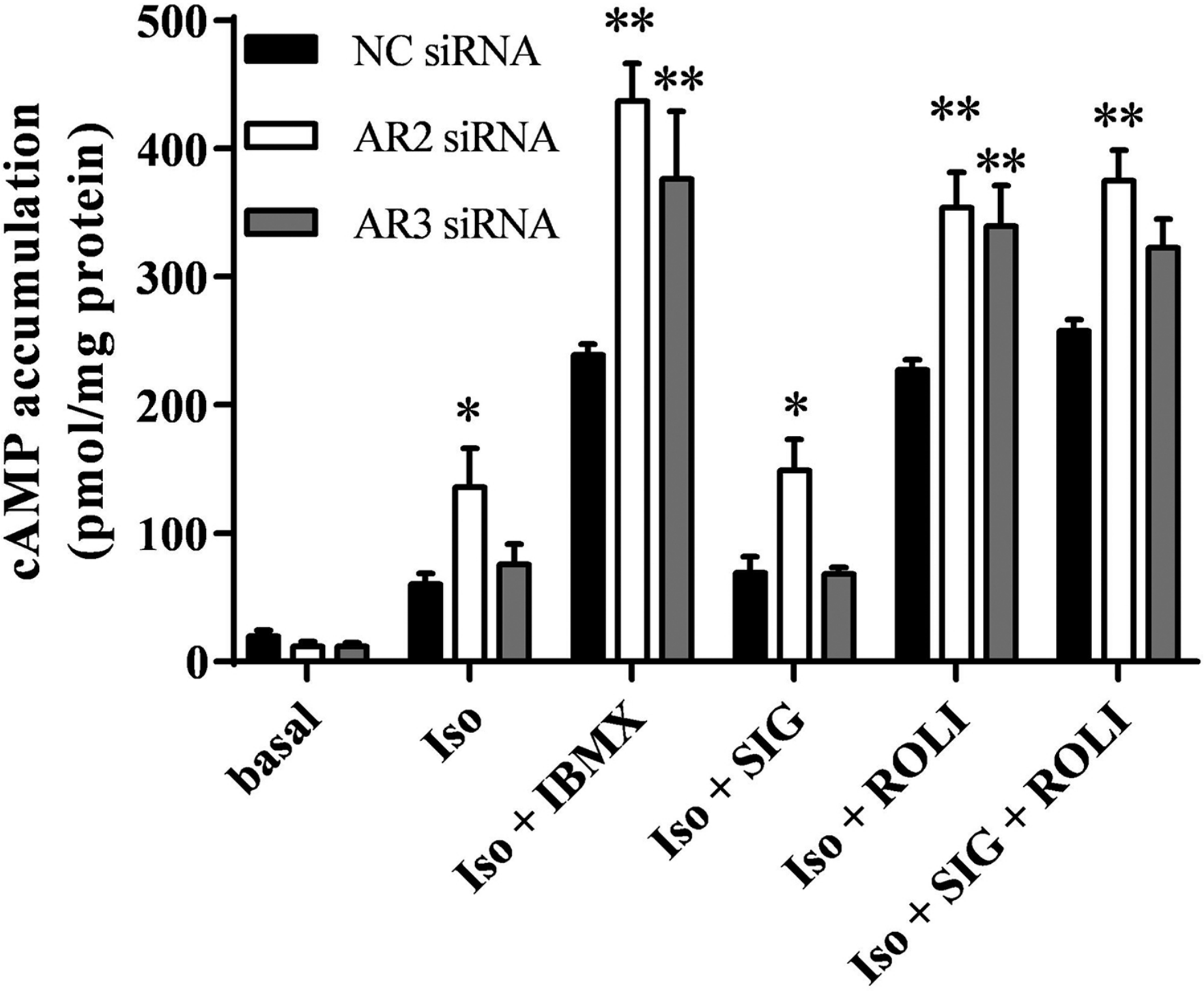
cAMP accumulation
(pmol/mg protein)
500
400
300
200
100
0
NC siRNA
AR2 siRNA
AR3 siRNA
*
**
**
*
**
**
**
basal
Iso
Iso + IBMX
Iso + SIG
Iso + ROLI
Iso + SIG + ROLI

Figure 4

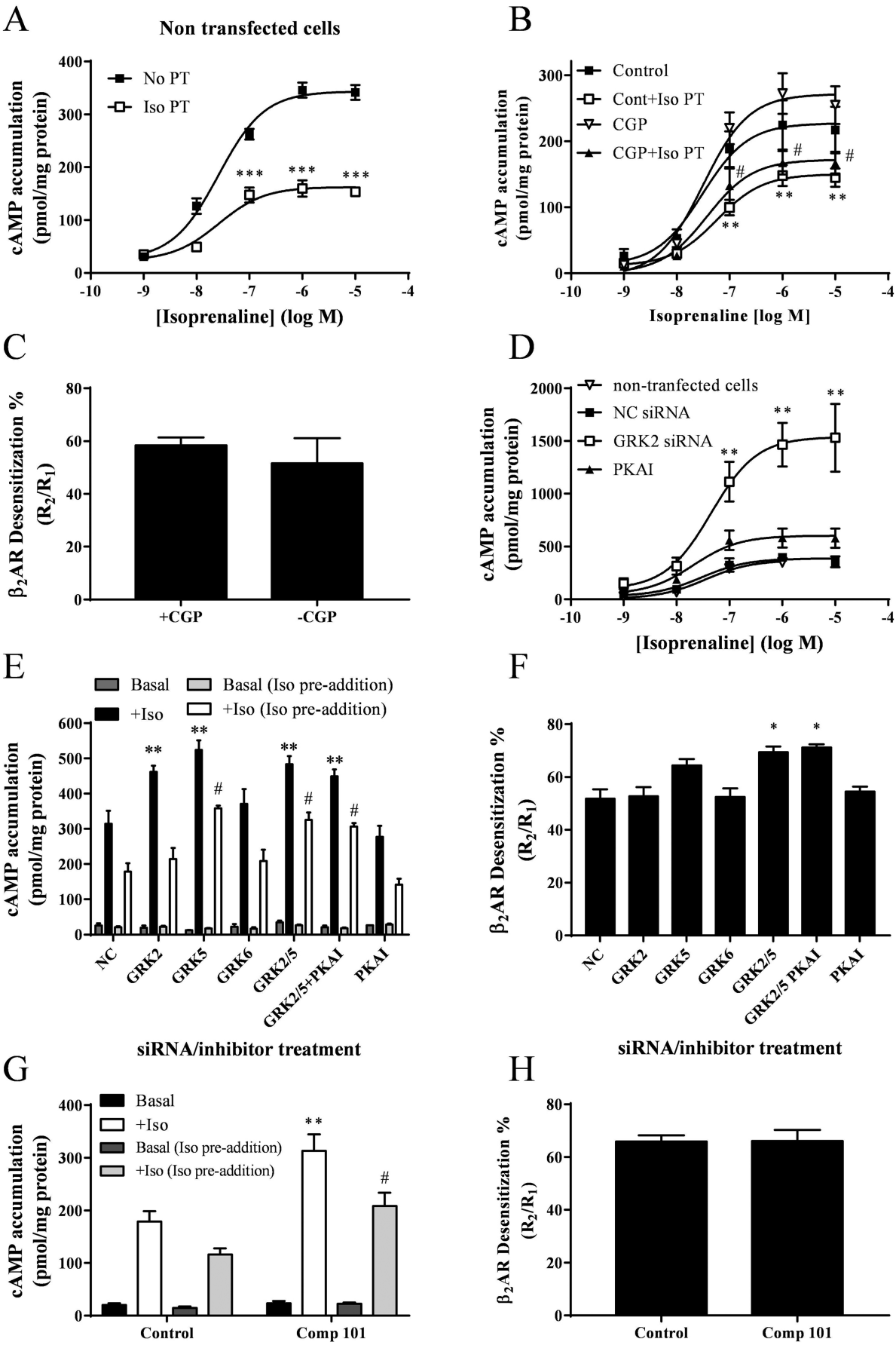
A
Non transfected cells
No PT
Iso PT
cAMP accumulation (pmol/mg protein)
[Isoprenaline] (log M)
B
Control
Cont+Iso PT
CGP
CGP+Iso PT
cAMP accumulation (pmol/mg protein)
Isoprenaline [log M]
C
β2AR Desensitization % (R2/R1)
+CGP
-CGP
D
non-tranfected cells
NC siRNA
GRK2 siRNA
PKAI
cAMP accumulation (pmol/mg protein)
[Isoprenaline] (log M)
E
Basal
+Iso
Basal (Iso pre-addition)
+Iso (Iso pre-addition)
cAMP accumulation (pmol/mg protein)
NC
GRK2
GRK5
GRK6
GRK2/5
GRK2/5+PKAI
PKAI
F
β2AR Desensitization % (R2/R1)
NC
GRK2
GRK5
GRK6
GRK2/5
GRK2/5 PKAI
PKAI
siRNA/inhibitor treatment
G
Basal
+Iso
Basal (Iso pre-addition)
+Iso (Iso pre-addition)
cAMP accumulation (pmol/mg protein)
Control
Comp 101
siRNA/inhibitor treatment
H
β2AR Desensitization % (R2/R1)
Control
Comp 101

Figure 5

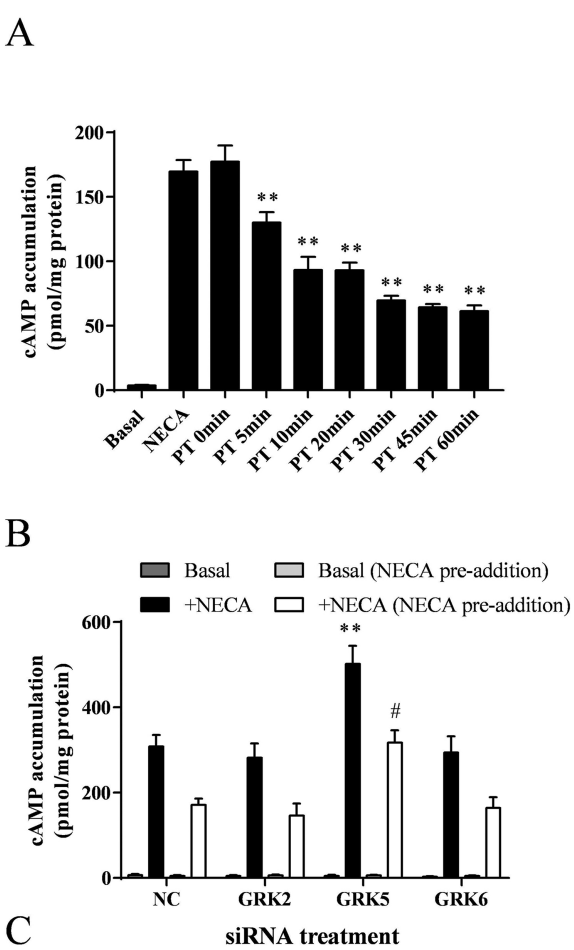
A
cAMP accumulation (pmol/mg protein)
200
150
100
50
0
**
**
**
**
**
**
Basal
NECA
PT 0min
PT 5min
PT 10min
PT 20min
PT 30min
PT 45min
PT 60min
B
Basal
Basal (NECA pre-addition)
+NECA
+NECA (NECA pre-addition)
cAMP accumulation (pmol/mg protein)
600
400
200
0
**
#
NC
GRK2
GRK5
GRK6
siRNA treatment
C
A2BR Desensitization % (R2/R1)
80
60
40
20
0
*
NC
GRK2
GRK5
GRK6
siRNA treatment

Figure 6

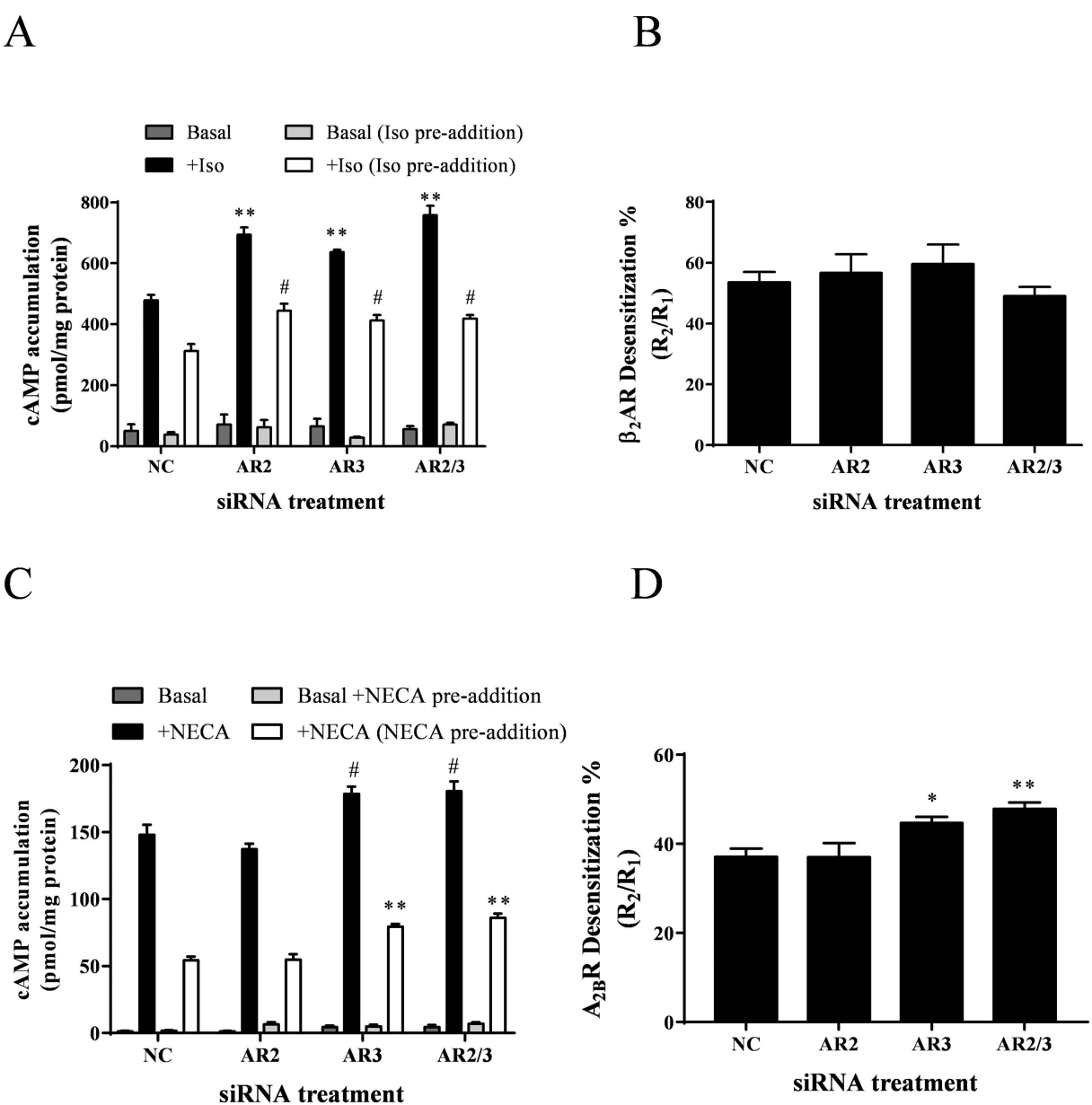
A
Basal
Basal (Iso pre-addition)
+Iso
+Iso (Iso pre-addition)
cAMP accumulation (pmol/mg protein)
800
600
400
200
0
**
**
**
#
#
#
NC
AR2
AR3
AR2/3
siRNA treatment
B
β2AR Desensitization % (R2/R1)
80
60
40
20
0
NC
AR2
AR3
AR2/3
siRNA treatment
C
Basal
Basal +NECA pre-addition
+NECA
+NECA (NECA pre-addition)
cAMP accumulation (pmol/mg protein)
200
150
100
50
0
#
#
**
**
NC
AR2
AR3
AR2/3
siRNA treatment
D
A2BR Desensitization % (R2/R1)
60
40
20
0
*
**
NC
AR2
AR3
AR2/3
siRNA treatment

Figure 7

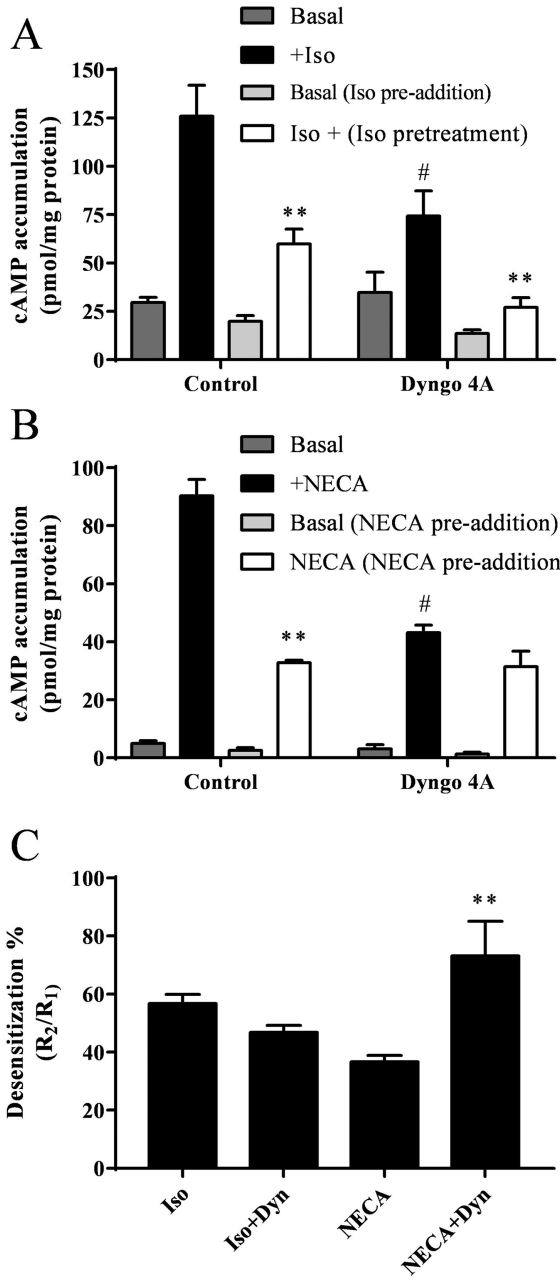
A
Basal
+Iso
Basal (Iso pre-addition)
Iso + (Iso pretreatment)
cAMP accumulation (pmol/mg protein)
150
125
100
75
50
25
0
**
#
**
Control
Dyngo 4A
B
Basal
+NECA
Basal (NECA pre-addition)
NECA (NECA pre-addition)
cAMP accumulation (pmol/mg protein)
100
80
60
40
20
0
**
#
Control
Dyngo 4A
C
Desensitization % (R2/R1)
100
80
60
40
20
0
**
Iso
Iso+Dyn
NECA
NECA+Dyn

Figure 8

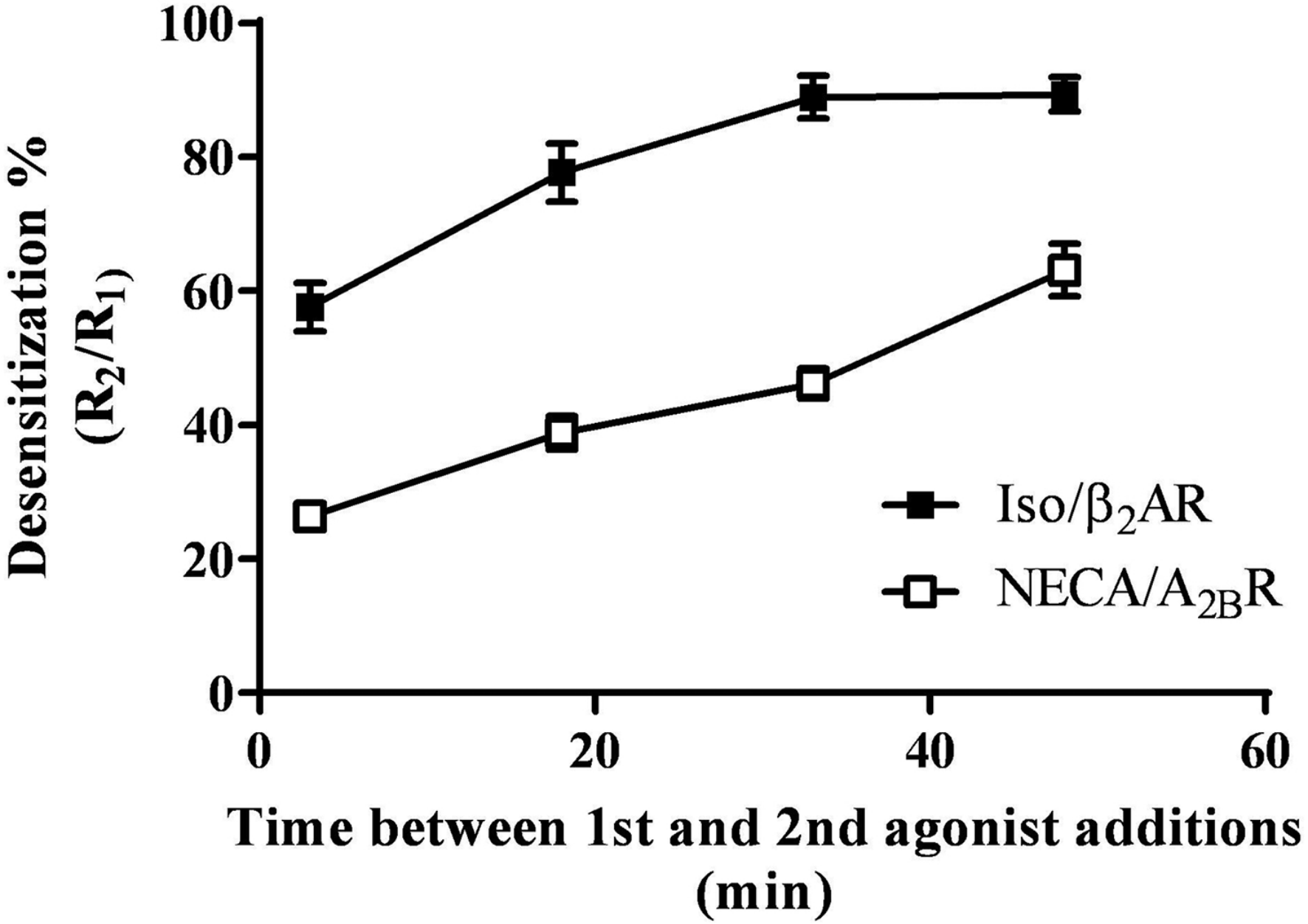
Desensitization %
(R2/R1)
100
80
60
40
20
0
0
20
40
60
Time between 1st and 2nd agonist additions
(min)
Iso/β2AR
NECA/A2BR

Figure 9