Reciprocal regulation of β_2-adrenoceptor-activated cAMP response-element binding protein signalling by arrestin2 and arrestin3

Alexander Pearce, Lucy Sanders, Paul J. Brighton, Shashi Rana, Justin C. Konje and Jonathon M. Willets*

Department of Molecular Cell Biology, University of Leicester, Henry Wellcome Building, Lancaster Road Leicester, LE1 9HN, United Kingdom

*Corresponding author: Jonathon M. Willets, Department of Molecular Cell Biology, University of Leicester, Henry Wellcome Building, Lancaster Road, Leicester, LE1 9HN, United Kingdom

Tel: +44 116-229 7148; Fax: +44 116-252-; E-mail: jmw23@le.ac.uk

Word count 6654

Figures: 7

Abstract

Activation of Gs coupled receptors (e.g. β_2-adrenoreceptor (β_2AR)) expressed within the uterine muscle layer (myometrium), promotes intracellular cAMP generation, inducing muscle relaxation through short-term inhibition of contractile proteins, and longer-term modulation of cellular phenotype to promote quiescence. In the myometrium cAMP-driven modulation of cell phenotype is facilitated by CREB activity, however despite the importance of CREB signalling in the promotion of myometrial quiescence during pregnancy, little is currently known regarding the molecular mechanisms involved. Thus, we have characterised β-adrenoceptor-stimulated CREB signalling in the immortalised ULTR human myometrial cell line.

The non-selective β-adrenoceptor agonist isoprenaline induced time- and concentration-dependent CREB phosphorylation, which was abolished by the β_2AR selective antagonist ICI118,551. β_2AR-stimulated CREB phosphorylation was mediated through a short-term PKA-dependent phase, and longer-term Src/p38 MAPK-dependent/PKA-independent phase. Since in model cells, arrestin2 can facilitate β_2AR-mediated Src/p38 recruitment, we examined whether CREB signalling was activated through a similar process in myometrial cells. Depletion of arrestin2 attenuated p38 phosphorylation, whilst arrestin3 depletion enhanced and prolonged isoprenaline-stimulated p38 signals, which was reversed following inhibition of Src. Knockdown of arrestin2 led to enhanced short-term (up to 10 min), and attenuated longer-term (>10 min) isoprenaline-stimulated CREB phosphorylation. Contrastingly, removal of arrestin3 enhanced and prolonged isoprenaline-stimulated CREB phosphorylation, whilst depletion of both arrestins abolished CREB signals at time points greater than 5 min.

In summary, we have delineated the molecular mechanisms coupling β_2AR activity to CREB signalling in ULTR myometrial cells, revealing a biphasic activation process encompassing

short-term PKA-dependent, and prolonged Src/arrestin2/p38-dependent components. Indeed, our data highlight a novel arrestin-mediated modulation of CREB signalling, suggesting a reciprocal relationship between arrestin2 and arrestin3, wherein recruitment of arrestin3 restricts the ability of β_2AR to activate prolonged CREB phosphorylation by precluding recruitment of an arrestin2/Src/p38 complex.

Key words: Myometrium, Arrestin, CREB, Myometrial quiescence, β_2-adrenoceptor, MAPK

Abbreviations: ATF-1, activation of transcription factor-1; β_1AR, β_1 adrenoceptor β_2AR, β_2 adrenoceptor; CGP-20712A, 1-[2-((3-Carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride; CREB, cAMP response element binding protein; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; PKAI, protein kinase A inhibitor, myristolated PKI 14-22 amide; PP1, pyrazolo pyrimidine type 1; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA.

1 Introduction

Throughout pregnancy the uterus undergoes dramatic remodelling in order to accommodate the growing foetus. During this time it is vital that the uterine muscle (myometrium) remains in a quiescent state to prevent unwanted or premature contractions that may lead to preterm delivery [1]. Myometrial quiescence is induced and maintained through many divergent changes in cellular physiology, many of which are mediated by the actions of sex hormones. Moreover, previous evidence highlights a specific sex hormone-induced down-regulation of contractile associated proteins such as $G\alpha_q$-coupled receptors like the oxytocin receptor, connexin 43 and cyclooxygenase 2 [2]. Furthermore, sex hormones promote the increased expression of proteins whose actions mediate myometrial relaxation. Indeed, previous studies show that $G\alpha_S$ expression [3-5] is elevated in the pregnant myometrium and that progesterone prevents cAMP breakdown by inhibiting phosphodiesterase activity [6]. The net result is to increase the cellular concentrations of cAMP which activates a diverse array of signalling pathways within cells, and in the myometrium is known to mediate long and short term effects upon muscle contractile tone.

In the short term cAMP is known to drive PKA-mediated inactivation of myosin light-chain kinase [2, 7], inhibition of phospholipase C activity and inhibition of calcium channels, which prevents muscle contraction [2]. However, cAMP is known to mediate longer term changes in smooth muscle physiology induced through regulation of gene expression. This process has been linked to activation of cAMP response-element binding protein (CREB) family in the human myometrium [8, 9]. CREB is a member of the basic region leucine-zipper (bZIP) family of transcription factors which include cAMP-response element modulator (CREM) and the activation of transcription factor-1 (ATF-1), all of which bind to DNA at cAMP response element motifs upstream of relevant promoter regions [10]. Like CREM and ATF-1, CREB

requires phosphorylation usually mediated by PKA, to promote its translocation to the nucleus and consequently enable DNA binding and transcriptional activity [10]. Although PKA activity may be central to CREB activation, it should be noted that several other kinases are known to phosphorylate CREB enabling a diversity of initial signalling pathways to mediate CREB transcriptional regulation [10-12]. Indeed, CREB has been shown to be phosphorylated following activation of many different G protein-coupled receptors, whether this be as a direct result of $G\alpha_s$-coupling or via the increased activity of other kinases [10-13].

The combination of long and short term alterations of myometrial physiology, via CREB and PKA provide a potential mechanism by which cAMP mediates its quiescent effects upon the myometrium during pregnancy. Indeed, CREB and ATF-1 expression is known to be suppressed dramatically in the myometrium shortly before and during labour [8], an effect that could conceivably signal the change from quiescent to contractile phenotype. However, despite the potential importance of CREB signalling to myometrial physiology, the molecular mechanisms which control and mediate agonist-driven CREB signalling have not been fully determined in myometrial cells.

Since previous studies have highlighted the importance of β-adrenoceptor signalling in cAMP-mediated myometrial relaxation [14], we chose to examine the effects of β-adrenoceptor (Gs-coupled) stimulation on CREB signalling in the immortalised human ULTR myometrial cell line. Established through retroviral immortalisation, ULTR cells [15] express the smooth muscle markers α-actin and calponin [16], but to a lesser extent that primary myometrial cells. Both primary myometrial and ULTR cell types have similar signalling profiles for the following GPCRs, histamine H_1 [17], oxytocin [16], angiotensin type 1, cannabinoid receptors 1 [18, 19], although the expression of oxytocin receptors in ULTR cells is more stable than

primary cells [16]. Our unpublished data show that both ULTR and primary myometrial cells express equivalent levels of progesterone A and B receptors, oestrogen α and β receptors, connexin 43 and relaxin LGR7/8 and β_2-adrenergic receptors. Indeed, our work and that of others [20-23] suggest that the ULTR cell line is a useful and valid model for non-pregnant human myometrial. Furthermore, β-adrenoceptor agonists have previously been utilised to inhibit preterm uterine contractions, although it should be noted that their success is somewhat limited due to associated receptor desensitization [24]. Since arrestin proteins are integrally linked with β-adrenoceptor desensitization [25], and also act as agonist-regulated adaptor scaffolds for GPCR-activated signalling pathways including mitogen-activated protein kinases (MAPK) [26], we investigated whether they played a role in the regulation of β-adrenoceptor-mediated CREB signalling.

2 Materials and methods

2.1 Cell culture

The immortalised human ULTR myometrial cell line [15], extensively characterised by us [16-19, 27] and others [20, 28], which shows a high degree of similarity to primary myometrial cells, was cultured in Dulbecco's minimal essential medium, supplemented with 10% foetal calf serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml) and Glutamax-1. Cells were maintained under humidified conditions at 37°C, in air/5% CO_2.

2.2 Tissue collection

All protocols for human tissue collection and experimental use were approved by the University Hospitals of Leicester R&D, and the Leicestershire, Northamptonshire, and Rutland Research Ethics Committees (Reference Number 06/Q2501/48). All tissue donors gave signed informed consent and were between 30 and 48 years (mean 39 ± SD 5.8) of age. Uterine samples were obtained from non-pregnant, pre-menopausal women at hysterectomy from non-pregnant pre-menopausal women undergoing surgery for non-neoplastic indications, for example, dysfunctional uterine bleeding, or from pregnant term non-labouring women undergoing elective caesarean section.

2.3 Detection of CREB phosphorylation

Agonist-driven CREB phosphorylation was detected using standard western blotting techniques and a specific anti-phospho-(p)CREB antibody. Briefly, ULTR cells were seeded into 6-well plates and grown to confluency. Cells were then serum-starved for 24 h prior to agonist addition. Signalling was terminated with the addition of 1x SDS lysis buffer (125 mM Tris-HCl, pH 6.8, 0.01% (w:v) bromophenol blue, 1% (w:v) sodium dodecyl sulphate, 20% (v:v) glycerol and 25 mM ditiothreitol, 500 µM phenylmethanesulfonylfluoride, 0.1 mg/ml leupeptin, 0.2 mg/ml benzamidine, and 0.1 mg/ml pepstatin). Samples were heated (5 min, 100°C) before gel loading, separated by SDS-PAGE and transferred to nitrocellulose using Western blotting techniques. Phospho-CREB was detected using a specific pCREB antibody (Cell Signaling, Madison, WI, USA), which detects the phosphorylation of S133, followed by enhanced chemiluminescence detection as per manufacturers' instructions, and exposure to autoradiography film. Densitometric analysis of the resultant autoradiographs was undertaken using the GeneGnome image analysis system and software (Syngene, Cambridge, UK).

To ensure that all samples contained the same levels of protein, total CREB levels were determined by running additional gels in parallel with the detection of pCREB. For pCREB

samples, uniform protein loading was confirmed by detection of total CREB protein using an anti-total CREB antibody (Cell Signaling, Madison, WI, USA). Immunoreactive bands were visualized and quantified as described above. pCREB absorbance levels for each treatment were corrected for differences in total CREB immunoreactivity.

2.4 Detection of p38 phosphorylation

ULTR cells were seeded into 6-well plates and grown to confluency and then serum-starved for 24 h prior to agonist addition. Signalling was terminated with the addition of lysis buffer (composition; 20 mM Tris-HCl (pH 7.4), 1% (v:v) Triton X-100, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanidate, 500 µM phenylmethanesulfonylfluoride, 0.1 mg/ml leupeptin, 0.2 mg/ml benzamidine, and 0.1 mg/ml pepstatin). Samples were then centrifuged to clear insoluble material and an equal volume of 2x sample buffer (composition; 250 mM Tris-HCl, pH 6.8, 0.01% (w:v) bromophenol blue, 2% (w:v) sodium dodecyl sulphate, 40% (v:v) glycerol and 50 mM ditiothreitol) and added before heating (5 min, 100^{o}C) and gel loading. Next, samples were separated by SDS-PAGE and transferred to nitrocellulose using Western blotting techniques. Phospho-p38 MAPK (pTGpY) was detected using a specific pp38 antibody (Cell Signaling, Madison, WI, USA) followed by enhanced chemiluminescence detection as per manufacturers' instructions, and exposure to autoradiography film. Densitometric analysis of the resultant autoradiographs was undertaken using the GeneGnome image analysis system and software (Syngene, Cambridge, UK). To ensure that all samples contained the same levels of protein, total p38 levels were determined by running additional gels in parallel with the detection of phospho-p38. Uniform protein loading was confirmed by detection of p38 using an anti-p38 antibody (Cell Signaling, Madison, WI, USA). Immunoreactive bands were visualized and quantified as described above. Phospho-p38

MAPK absorbance levels for each treatment were corrected for differences in total p38 immunoreactivity.

2.5 siRNA targeted arrestin depletion

Endogenous arrestin2 or arrestin3 (otherwise known as β-arrestin1 and β-arrestin2) and were targeted for depletion using the following previously characterised [19, 29] siRNA constructs designed to target either human arrestin2 (ARRB1) (5'-GGAGAUCUAUUACCAUGGtt-3') or arrestin3 (ARRB2) siRNA (5'-CGAACAAGAUGACCAGGUAtt-3') (Ambion, Applied Biosystems, Warrington, UK). For MAPK experiments ULTR cells (150,000 per well) were transfected with either negative control (non-targeting 100 nM), anti-arrestin2 (100 nM), or anti-arrestin3 (10 nM) siRNA constructs using the Interferin™ transfection reagent (Polyplus, NY, USA), as per manufacturers' instructions. Depletion of siRNA targeted arrestin expression was confirmed by immunoblotting using either the A1CT (which preferentially detects arrestin2 [19, 30] or A2CT (which preferentially detects arrestin3) antibodies as described previously [30].

2.6. Data analysis

All concentration-response curves were generated and EC_{50} values determined using non-linear regression analysis software Prism, version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Data were analysed using One-way or Two-way ANOVA, followed by appropriate *post-hoc* testing (GraphPad Software Inc., San Diego, CA, USA). Significance was accepted when $p<0.05$.

3 Results

3.1 Temporal and concentration-dependency of βAR-stimulated CREB phosphorylation

Addition of 1 μM of the non-selective βAR agonist isoprenaline (a concentration that caused maximal production of cAMP, data not shown), produced a time dependent increase in CREB phosphorylation that peaked after 20-30 min and returned towards basal after 60 min (Fig 1A, C). Pre-incubation (20 min) with the β_2AR specific antagonist ICI 118,551 (100 nM) blocked isoprenaline-stimulated CREB phosphorylation (Fig 1B, C), whilst inclusion of the β_1AR selective antagonist CGP-20712A (300 nM) had no effect (data not shown). Isoprenaline-induced CREB phosphorylation was concentration-dependent (curves generated at the peak of pCREB, 20 min), with maximal CREB phosphorylation at 1 μM and EC_{50} of 19 ± 2.7 nM (mean ± S.E.M., n=4) (Fig 1D, E).

3.2 Delineating the signalling pathway linking βAR-stimulation to CREB phosphorylation

An array of kinases are known to phosphorylate CREB in other cell lines [11-13], and as this mechanism has not been identified in myometrial cells, a series of specific inhibitors, for proteins and enzymes known to be involved in GPCR-mediated CREB-activation were investigated. Initial studies carried out at the peak time of isoprenaline-stimulated CREB phosphorylation (20 min) showed that inclusion of the PKA inhibitor KT5720 (5 μM) had no effect (data not shown). In contrast, pre-incubation (30 min) with the PKA inhibitor peptide (5 μM, myristolated PKI 14-22 amide, Tocris, Bristol, UK) prevented isoprenaline-stimulated CREB phosphorylation at 5 min, but not at 20 min (Fig 2A, B). Pre-treatment with Src kinase inhibitor pyrazolo pyrimidine type 1 (PP1) (5 μM, 30 min), attenuated isoprenaline-induced CREB phosphorylation at 20 min (Fig 2C, D). Since at the 5 min time point isoprenaline-induced CREB phosphorylation was PKA-dependent, we constructed concentration-response curves to assess the isoprenaline dose-dependency of the PKA activation of CREB. As with

the concentration-curves generated at 20 min, maximal responses were observed with 1 μM isoprenaline, and an EC_{50} of 22 ± 5.4 nM (Fig 2E, F). In an attempt to bias CREB signalling towards the PKA pathway, we next examined the temporal-dependency of isoprenaline-stimulated CREB signals to a low concentration (10 nM). This low dose of isoprenaline induced a transient phosphorylation of CREB peaking at 5 min, which rapidly decayed to basal levels within 10 min, and was blocked by the PKA inhibitor (PKAI) peptide (myristolated PKI 14-22 amide, 5 μM, 30 min pre-treatment) (Fig 2G, H).

As both ERK1/2 and p38 MAPK have been reported to induce CREB phosphorylation [11, 12] we assessed whether they played a similar role in ULTR cells. Here, isoprenaline (1 μM) induced a time-dependent inhibition of ERK phosphorylation (data not shown) and the inclusion of the MEK inhibitor PD98059 (Sigma, UK) had no effect upon isoprenaline stimulated CREB phosphorylation (data not shown). Conversely, isoprenaline stimulated p38 phosphorylation, peaking at 5 min and declining to basal by 60 min (Fig 3A, B). Inclusion of the p38 inhibitor SB203580 (20 μM, 30 min pre-incubation) not only ablated isoprenaline-induced p38 phosphorylation (Fig 3A, B), but significantly attenuated the time-dependent isoprenaline-induced CREB phosphorylation at all time points ≥10 min (Fig 3C, D). In addition, inclusion of both SB203580 and PKAI completely inhibited isoprenaline-stimulated CREB phosphorylation (Fig 3C, D). To further characterise βAR-stimulated p38 signalling we constructed concentration-response curves to isoprenaline at 5 min, the peak of p38 phosphorylation. Isoprenaline induced maximal p38 phosphorylation at concentrations >100 nM and EC_{50} of 10 ± 4.9 nM (Fig 4A, B). As PKA mediated isoprenaline-stimulated CREB phosphorylated at 5 min we next examined whether PKA was able to phosphorylate p38 over the same time period. However, inclusion of PKAI (5 μM, 30 min pre-treatment) failed to inhibit isoprenaline-stimulated p38 phosphorylation over 5 min (Fig 4C, D). Since inhibition

of Src kinase attenuated βAR-stimulated CREB phosphorylation (Fig 2C, D), and as Src has been previously reported to phosphorylate p38 [31], we next examined whether inhibition of Src had any effect upon p38 activity. Indeed as hypothesised, pre-treatment with the Src kinase inhibitor PP1, prevented isoprenaline-stimulated p38 phosphorylation (Fig 4E, F).

3.3 Arrestin dependency of βAR-stimulated p38 MAPK signalling

Previous studies [19, 27] have shown that arrestin proteins can play a part in regulating GPCR-driven MAPK signalling in ULTR cells. Therefore, we utilised our previously characterised siRNA depletion techniques [19, 27] to determine whether arrestins 2 or 3 could regulate βAR-stimulated p38 signalling. In comparison with our previous findings transfection with anti-arrestin2 (100 nM) siRNA depleted 72 ± 2.6 % (mean ± S.E.M., n=5) of the targeted protein, whilst anti-arrestin3 siRNA depleted 84 ± 5.2 % (mean ± S.E.M., n=5) of arrestin3 (see Fig 5A, B), when compared to negative-control transfected cells [19, 27]. Transfection of ULTR cells with negative-control siRNA had no effect on basal p38 phosphorylation levels (basal means ± S.E.M. for negative-control 6762 ± 1253, and non-transfected cells 4991 ± 1417), or the magnitude or time-course of isoprenaline-stimulated p38 MAPK responses compared to non-transfected cells (Figs 1, 5). In contrast, depletion of arrestin2 markedly attenuated isoprenaline-stimulated p38 phosphorylation, whilst, knockdown of arrestin3 greatly enhanced βAR-stimulated p38 phosphorylation (Fig 5C, D).

Previous evidence suggests that arrestins can act as agonist-adapter scaffolds recruiting Src kinase to GPCR complexes to activate p38 signalling [32, 33]. Therefore, to investigate whether this process underlies the observed increase in p38 signalling in the absence of arrestin3, isoprenaline-stimulated p38 phosphorylation was examined in arrestin3 depleted cells in the presence and absence of the Src kinase inhibitor PP1. Interestingly, in arrestin3

siRNA treated cells inclusion of PP1 reversed the effects of arrestin3 depletion on p38 phosphorylation (Fig 5C).

3.4 Arrestin-dependency of βAR-stimulated CREB signalling

As our data show that p38 plays a central role in βAR-stimulated phosphorylation of CREB, and this process is mediated by arrestin proteins, it is conceivable that arrestins should regulate CREB signalling in a similar manner. Therefore, we examined the effects of arrestin depletion on the ability of isoprenaline to activate CREB signalling in ULTR cells. Reassuringly, in negative-control siRNA treated cells the time-course of isoprenaline-induced CREB phosphorylation was similar to that in non-transfected cells (Figs 1, 6). In contrast, depletion of arrestin2 increased the initial phase (5-10 min) of CREB phosphorylation, however the sustained phase of isoprenaline-stimulated CREB phosphorylation was dramatically attenuated (Fig 6). Furthermore, depletion of arrestin3 markedly enhanced and prolonged isoprenaline-stimulated CREB phosphorylation over the experimental time-course (Fig 6). Interestingly, following knockdown of both arrestins isoprenaline-stimulated CREB phosphorylation peaked at 5 min producing similar levels of phosphorylation as in negative-control siRNA treated cells. However more strikingly, depletion of both arrestins virtually ablated β_2AR-driven CREB signalling at time points >5 min (Fig 6). Moreover, combined arrestin2/3 depletion and PKA inhibition blocked the residual isoprenaline-stimulated CREB signals observed at 5 min (Fig 6), further suggesting short term CREB signalling is mediated by PKA, and longer-term CREB signals via arrestin-dependent mechanisms.

3.5 Comparison of arrestin expression in non-pregnant and pregnant myometrium

Since no previous studies have examine how pregnancy affects myometrial arrestin expression, and as our data indicate that arrestin proteins can regulate β_2AR-stimulated CREB signalling,

a process important in maintaining uterine quiescence during gestation [9], we examined the expression of arrestin proteins in the myometrium of non-pregnant and pregnant term non-labouring women. Immunoblotting revealed that in the myometrium of pregnant term non-labouring women arrestin2 expression was approximately twice that of non-pregnant myometrium (Fig 7). Arrestin3 expression in pregnant term non-labouring women was approximately double that found in non-pregnant women (Fig 7).

4 Discussion

Elevated cAMP levels within myometrial cells are thought to play a vital role in the induction and maintenance of quiescence during pregnancy, inducing short term and longer term effects upon the muscle phenotype [8, 9]. The primary signalling pathways linking cAMP to suppression of myometrial contraction are those mediated by PKA, including direct activation of myosin light chain phosphatase, inhibition of Ca^{2+}-channels and inhibition of agonist-stimulated phospholipase C activity [34, 35]. However, it is clear that cAMP activates many other signalling pathways within cells [36], including the CREB family of transcription factors [9]. CREB-family signalling has been reported to alter the expression of a plethora of different proteins in myometrial cells, many of which are implicated in promoting myometrial relaxatory phenotype [1, 9], a process that may underlie some of the long term changes in myometrial physiology seen during pregnancy. Despite the potential importance of cAMP/CREB signalling in this context, relatively little was known regarding the regulation of agonist-driven CREB signalling in myometrial cells. To address this we examined the effects of the non-selective βAR agonist isoprenaline to stimulate CREB signals in the human ULTR myometrial cell line.

Initial experiments suggested that isoprenaline caused a concentration-dependent increase in both CREB and ATF-1 phosphorylation, which followed similar temporal profiles. Although myometrial cells are reported to express a mixed population of βARs, and the profile of their expression appears to alter depending upon the gestational age [37, 38], our data show that isoprenaline-stimulated CREB/ATF-1 signals are completely attenuated upon inclusion of the β_2AR antagonist ICI 118,551, suggesting that at least in ULTR cells isoprenaline signals are mediated by β_2AR. It is noteworthy here to state that activation of CREB-family signalling in myometrial cells can promote β_2AR transcription [9], which may highlight a positive feed-forward mechanism between cAMP production and myometrial relaxatory phenotype. Previous studies have identified cAMP-stimulated PKA activity as the central mediator of CREB activation [10], and in agreement with these findings we show that PKA plays a role in agonist-stimulated phosphorylation of CREB in ULTR myometrial cells. However, surprisingly PKA involvement is restricted to relatively short time periods (5-10 min), playing no part in the prolonged phase of isoprenaline mediated CREB phosphorylation, which suggests that other kinases must be responsible for this period of CREB signalling. Indeed, as existing studies suggest that several other kinases are capable of phosphorylating CREB, including the stress activated pathways such as ERK1/2 and p38 MAPKs [11, 12], we examined their potential involvement in β_2AR-stimulated CREB signalling. Our data indicate that β_2AR signalling stimulates p38 phosphorylation, but inhibits ERK1/2 phosphorylation (data not shown), suggesting a key role for p38 but not ERK in the regulation of isoprenaline-stimulated CREB signalling. Moreover, analysis of the temporal profile of CREB phosphorylation revealed that inhibition of p38 activity only suppressed CREB phosphorylation at time periods >5 min. Taken together our data reveal that β_2AR-stimulated CREB phosphorylation is mediated through a short-term PKA-dependent phase, and longer-term p38 MAPK-dependent, PKA-independent phase.

A number of different signalling pathways have been reported to link β_2AR activity and p38 phosphorylation, ranging from classical PKA-mediated p38 phosphorylation [13, 39], to implication of cAMP as a direct activator of p38 phosphorylation in a PKA- and EPAC-independent manner [40]. Alternatively, Src-family kinases have also been reported to mediate 17β oestradiol-induced p38 phosphorylation in skeletal muscle [41], and prostaglandin E_2 receptor mediated p38 activation in HEK293 cells [31]. Therefore, as the previously identified signalling pathways linking Gs/GPCR to p38 phosphorylation appear to be diverse and cell specific, we sought to characterise this pathway in ULTR cells. In our hands inhibition of PKA activity had no effect upon p38 phosphorylation (data not shown), whilst in agreement with Chun & Shim, [31] inhibition of Src completely attenuated isoprenaline-induced p38 phosphorylation. Furthermore, inhibition of Src attenuated CREB phosphorylation at time periods >5 min, confirming Src as the key kinase linking β_2AR activity to the sustained phase of p38 and CREB phosphorylation. Thus, here we report that β_2AR-induced CREB signalling is mediated through both short term PKA-dependent, and longer term Src-p38 dependent/PKA-independent phosphorylation. Interestingly, these observations are reminiscent of the effects that PKA and arrestins play in the regulation of β_2AR-driven ERK signalling [30], and further highlight the importance of this dual regulation process in MAPK signalling.

Aside from their classical roles in the desensitization and internalisation of GPCRs such as the β_2AR, arrestins can modulate the activity a wide array of intracellular signalling pathways [26, 42]. Central to this process is the ability of arrestins to act as agonist-adaptor scaffolds, recruiting and supporting such diverse signalling pathways as ERK, p38, and JNK [26, 42]. Notably in model cells systems, arrestin2 can act in this capacity to facilitate the recruitment of Src to β_2AR [31, 43], underlying its ability to recruit and promote a sustained phase of

cytosolic ERK signalling [43]. In addition, enhanced expression of arrestin3 has been reported to mediate an increased level of CREB phosphorylation in a mouse model of cystic fibrosis [44]. Therefore, as both arrestin proteins are known to interact with β_2AR, and either can regulate Src function [43, 44], we examined their potential involvement in Src-mediated p38 and CREB signalling. RNAi-depletion of individual arrestins revealed that arrestin2 knockdown resulted in attenuation of β_2AR-stimulated p38 phosphorylation. Conversely, suppression of arrestin3 expression led to marked increase in the isoprenaline-induced p38 phosphorylation signal. Collectively, these data suggest a reciprocal relationship exists whereby arrestin3 expression possibly limits the recruitment of arrestin2 to agonist-occupied β_2AR, to regulate the ability of Src to activate p38 signalling. Interestingly, our findings are reminiscent of the roles that arrestins play in mediating angiotensin II type 1 ERK signalling [45]. However, it should be noted that in our study arrestin2 and arrestin3 play opposite roles to those reported by Ahn et al., [45]. As p38 activity is essential for the PKA-independent, prolonged phase of CREB phosphorylation mediated by β_2AR activation in ULTR cells, alteration of p38 activity via manipulation of arrestin expression should produce similar alterations in CREB signals. Indeed, as hypothesised depletion of arrestin3 led to a marked increase in isoprenaline-stimulated CREB phosphorylation throughout the whole experimental time course, further suggesting that removal of arrestin3 enhances arrestin2 recruitment to agonist-occupied β_2AR, thus facilitating Src recruitment and p38 phosphorylation. However, depletion of arrestin2 enhanced agonist-driven CREB phosphorylation at early time points (i.e. up to 10 min), yet in the absence of arrestin2 the prolonged phase of CREB signalling was attenuated. Since the early time period of β_2AR-stimulated CREB signalling is PKA-dependent, it is likely that the enhanced CREB signalling observed during this period is reflective of an inability to desensitise β_2AR/PKA signalling following arrestin2 knockdown.

Moreover, as p38 phosphorylation is largely absent following arrestin2 knockdown, p38 is unlikely to contribute significantly to CREB phosphorylation under these conditions.

In addition to the essential role that arrestins play in the regulation of GPCR signalling, increasing evidence suggests that they also play a role in regulating gene transcription. Indeed, the distribution of arrestin2 and arrestin3 are different within cells. Arrestin2 is equally distributed between cytoplasm and nucleus, whilst in contrast due to its nuclear export sequence arrestin3 is almost exclusively found in the cytoplasm [46]. Interestingly, arrestin2 is known to accumulate within the nucleus following GPCR stimulation [47, 48], where it can interact with CREB to regulate c-fos and p27 promoted gene expression [47]. Furthermore, arrestin proteins are known to dimerise [49, 50], which raises the potential possibility that through hetero-oligomerization, arrestin3 has the ability to sequester and displace arrestin2 from the nucleus via its nuclear export sequence [46, 51]. The potential for arrestin3 to pump arrestin2 out of the nucleus thus preventing arrestin2/CREB interactions, offers an alternative mechanism by which arrestin3 may negatively regulate β_2AR/arrestin2 mediated CREB signalling.

5 Conclusions

In summary, we have characterised the molecular mechanisms linking β_2AR stimulation to CREB signalling in ULTR myometrial cells, uncovering a biphasic activation process comprising of short-term PKA-dependent, and longer-term Src/arrestin2/p38-dependent components. Moreover, our data show a novel arrestin-mediated regulation of CREB signalling, highlighting a reciprocal relationship between arrestin2 and arrestin3, wherein recruitment of arrestin3 limits the ability of β_2AR to activate prolonged CREB phosphorylation by preventing recruitment of an arrestin2/Src/p38 complex. Finally, we show that when

compared to non-pregnant tissues, the expression of both arrestin2/3 proteins is enhanced in term non-labouring pregnant myometrium. As the level of arrestin2/3 expression is roughly equivalent (i.e. double), any effects of increased arrestin2 expression on β_2AR-stimulated CREB signalling is likely to be negated by a proportional increase of arrestin3 expression. However, it is worth noting that arrestin proteins play vital roles in the acute desensitization of several contractile GPCRs within the myometrium, including histamine H_1, oxytocin [19] and bradykinin B_2 [27]. Although modest, the change in myometrial arrestin expression during pregnancy is similar to that found in arterial smooth muscle during the development of hypertension [52], and in arteries this conferred a two-fold increase in GPCR desensitization. Thus, equivalent changes in myometrial arrestin expression could reflect a similarly enhanced ability to attenuate GPCR signalling. Therefore increasing myometrial arrestin expression during pregnancy may perhaps provide an extra layer of control to prevent unwanted preterm contractions induced by infections [53, 54] (via H_1 and B_2 receptors), and/or provide increased provision of proteins that mediate the desensitization and re-sensitization of oxytocin receptor mediated contractions during labour.

Acknowledgements

We thank Robert Lefkowitz (Duke University, USA) for kindly providing the A1CT and A2CT antibodies.

References

[1] Price SA, Bernal AL, Expt. Physiol. 2001;86:265-272.

[2] Challis JR, Lye SJ, Gibb W, Whittle W, Patel F, Alfaidy N, Ann. New York Acad. Sci. 2001;943:225-234.

[3] Europe-Finner GN, Phaneuf S, Tolkovsky AM, Watson SP, Lopez Bernal A, J. Clin. Endocrinol. & Metabol. 1994;79:1835-1839.

[4] Europe-Finner GN, Phaneuf S, Watson SP, Lopez Bernal A, Endocrinol. 1993;132:2484-2490.

[5] Lopez Bernal A, Europe-Finner GN, Phaneuf S, Watson SP, Trends Pharmacol. Sci. 1995;16:129-133.

[6] Kofinas AD, Rose JC, Koritnik DR, Meis PJ, J. Reprod. Med. 1990;35:1045-1050.

[7] Yu X, Li F, Klussmann E, Stallone JN, Han G, Am. J. Physiol. Endocrinol. & Metabol. 2014;307:E398-407.

[8] Bailey J, Sparey C, Phillips RJ, Gilmore K, Robson SC, Dunlop W, Europe-Finner GN, Mol. Human Reprod. 2000;6:648-660.

[9] Bailey J, Tyson-Capper AJ, Gilmore K, Robson SC, Europe-Finner GN, J. Mol. Endocrinol. 2005;34:1-17.

[10] Sands WA, Palmer TM, Cellular Signalling. 2008;20:460-466.

[11] Deak M, Clifton AD, Lucocq LM, Alessi DR, EMBO J. 1998;17:4426-4441.

[12] Vermeulen L, Vanden Berghe W, Beck IM, De Bosscher K, Haegeman G, Trends Biochem. Sci. 2009;34:311-318.

[13] Delghandi MP, Johannessen M, Moens U, Cellular signalling. 2005;17:1343-1351.

[14] Liu YL, Nwosu UC, Rice PJ, Am. J. Obstet. & Gynecol. 1998;179:895-898.

[15] Perez-Reyes N, Halbert CL, Smith PP, Benditt EP, McDougall JK, Proc Natl Acad Sci U S A. 1992;89:1224-1228.

[16] Willets JM, Brighton PJ, Mistry R, Morris GE, Konje JC, Challiss RA, Mol. Endocrinol. 2009;23:1272-1280.

[17] Willets JM, Taylor AH, Shaw H, Konje JC, Challiss RA, Mol Endocrinol. 2008;22:1893-1907.

[18] Brighton PJ, McDonald J, Taylor AH, Challiss RA, Lambert DG, Konje JC, Willets JM, Mol. Endocrinol. 2009;23:1415-1427.

[19] Brighton PJ, Rana S, Challiss RJ, Konje JC, Willets JM, Br. J. Pharmacol. 2011;162:1603-1617.

[20] Ball A, Wang JW, Wong S, Zielnik B, Mitchell J, Wang N, Stemerman MB, Mitchell BF, Am. J. Physiol. Endocrinol. & Metabol. 2006;291:E922-928.

[21] Miggin SM, Kinsella BT, Biochim Biophys Acta. 2001;1539:147-162.

[22] Zaragoza DB, Wilson RR, Mitchell BF, Olson DM, Biol Reprod. 2006;75:697-704.

[23] Hertelendy F, Molnar M, Romero R, J. Soc. Gynecol. Invest. 2002;9:215-219.

[24] Engelhardt S, Zieger W, Kassubek J, Michel MC, Lohse MJ, Brodde OE, J. Clin. Endocrinol. & Metabol. 1997;82:1235-1242.

[25] Drake MT, Violin JD, Whalen EJ, Wisler JW, Shenoy SK, Lefkowitz RJ, J. Biol. Chem. 2008;283:5669-5676.

[26] DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK, Ann. Rev. Physiol. 2007;69:483-510.

[27] Willets JM, Brighton PJ, Windell LN, Rana S, Nash CA, Konje JC, Mol. & Cell. Endocrinol. 2015;407:57-66.

[28] Olson DM, Zaragoza DB, Shallow MC, Cook JL, Mitchell BF, Grigsby P, Hirst J, Placenta. 2003;24 Suppl A:S47-54.

[29] Brighton PJ, Marczylo TH, Rana S, Konje JC, Willets JM, Br. J. Pharmacol. 2011;164:1479-1494.

[30] Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ, J. Biol. Chem. 2006;281:1261-1273.

[31] Chun KS, Shim M, Biomol. & Therapeut. 2015;23:539-548.

[32] Sun H, Xu B, Inoue H, Chen QM, Cellular Signalling. 2008;20:1952-1959.

[33] Bruchas MR, Macey TA, Lowe JD, Chavkin C, J. Biol. Chem. 2006;281:18081-18089.

[34] Lopez Bernal A, Watson SP, Phaneuf S, Europe-Finner GN, Bailliere's Clin. Obstet. & Gynaecol. 1993;7:523-552.

[35] Sanborn BM, Ku CY, Shlykov S, Babich L, J. Soc. Gynecol. Invest. 2005;12:479-487.

[36] Yuan W, Lopez Bernal A, BMC Preg. & Childbirth. 2007;7 Suppl 1:S10.

[37] Bardou M, Rouget C, Breuiller-Fouche M, Loustalot C, Naline E, Sagot P, Frydman R, Morcillo EJ, Advenier C, Leroy MJ, Morrison JJ, BMC Preg. & Childbirth. 2007;7 Suppl 1:S14.

[38] Breuiller M, Rouot B, Leroy MJ, Blot P, Kaplan L, Ferre F, Gynecol. & Obstet. Invest. 1987;24:28-37.

[39] Gong K, Li Z, Xu M, Du J, Lv Z, Zhang Y, J. Biol. Chem. 2008;283:29028-29036.

[40] Yin F, Wang YY, Du JH, Li C, Lu ZZ, Han C, Zhang YY, J. Mol. & Cell. Cardiol. 2006;40:384-393.

[41] Ronda AC, Buitrago C, Boland R, J. Steroid Bioch. & Mol. Biol. 2010;122:287-294.

[42] Kovacs JJ, Hara MR, Davenport CL, Kim J, Lefkowitz RJ, Dev. Cell. 2009;17:443-458.

[43] Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ, Science. 1999;283:655-661.

[44] Manson ME, Corey DA, Rymut SM, Kelley TJ, Biochemistry. 2011;50:6022-6029.

[45] Ahn S, Shenoy SK, Wei H, Lefkowitz RJ, J. Biol. Chem. 2004;279:35518-35525.

[46] Scott MG, Benmerah A, Muntaner O, Marullo S, J. Biol. Chem. 2002;277:3552-3559.

[47] Kang J, Shi Y, Xiang B, Qu B, Su W, Zhu M, Zhang M, Bao G, Wang F, Zhang X, Yang R, Fan F, Chen X, Pei G, Ma L, Cell. 2005;123:833-847.

[48] Rosano L, Cianfrocca R, Tocci P, Spinella F, Di Castro V, Spadaro F, Salvati E, Biroccio AM, Natali PG, Bagnato A, Oncogene. 2013;32:5066-5077.

[49] Milano SK, Kim YM, Stefano FP, Benovic JL, Brenner C, J. Biol. Chem. 2006;281:9812-9823.

[50] Storez H, Scott MG, Issafras H, Burtey A, Benmerah A, Muntaner O, Piolot T, Tramier M, Coppey-Moisan M, Bouvier M, Labbe-Jullie C, Marullo S, J. Biol. Chem. 2005;280:40210-40215.

[51] Wang P, Wu Y, Ge X, Ma L, Pei G, J. Biol. Chem. 2003;278:11648-11653.

[52] Willets JM, Nash CA, Rainbow RD, Nelson CP, Challiss RA, Am. J. Physiol. Cell. Physiol. 2015;309:C179-189.

[53] Wassdal I, Nicolaysen G, Iversen JG, Acta Physiol. Scand. 1998;164:47-52.

[54] Bytautiene E, Romero R, Vedernikov YP, El-Zeky F, Saade GR, Garfield RE, Am. J Obstet. & Gynecol. 2004;191:1356-1361.

Figure legends

Figure 1. Temporal-profile and concentration-dependency of isoprenaline-stimulated CREB phosphorylation in ULTR cells. Confluent ULTR cells were serum-starved for 24 h prior to agonist challenge. Representative immunoblots depicting the temporal prolife of CREB phosphorylation after addition of a maximal concentration of isoprenaline (1 μM) in the absence (A) or presence (B) of the β_2AR-selective antagonist ICI 118,551 (100 nM) are shown (upper blots). To verify equal gel loading all blots were stripped and reprobed with anti-CREB antibody (lower panels). C) Cumulative data (means ± S.E.M., for n=5 separate experiments) showing the temporal-profile of isoprenaline-stimulated CREB phosphorylation. Inclusion of ICI 118,551 significantly (*p<0.05; **p<0.01, by two-way ANOVA and Tukey's *post-hoc* test) attenuated isoprenaline-stimulated CREB phosphorylation. D) Representative immunoblots showing concentration-dependent CREB-phosphorylation in confluent serum-starved ULTR cells undertaken at the peak of isoprenaline-stimulated CREB phosphorylation (20 min). D) Cumulative concentration-response data are shown (data are means ± S.E.M., for n=4 separate experiments).

Figure 2. Isoprenaline-stimulated CREB signalling is PKA and Src-dependent. Confluent ULTR cells were serum-starved for 24 h prior to agonist challenge. Representative immunoblots depicting isoprenaline-stimulated (1 μM) CREB phosphorylation in the presence or absence of or presence of (A) the PKA inhibitor, PKI 14-22 amide (5 μM), or (C) Src-family kinase inhibitor PP1 (5 μM). Cumulative densitometric analysis data (means ± S.E.M., for n=5 separate experiments) show that PKA inhibition blocks CREB phosphorylation at 5 min (B) and Src-inhibition prevented CREB phosphorylation at 20 min (D). *p<0.05, ***p<0.001, one-way ANOVA, Dunnett's *post-hoc* test). Representative immunoblots (E) and cumulative data

(F) show isoprenaline-stimulated concentration-response curves (means ± S.E.M., for n=3 separate experiments) generated at the peak of PKA activity (5 min). Representative immunoblots (G) and cumulative data (H) (means ± S.E.M., for n=3 separate experiments) show the time-course of CREB phosphorylation induced by 10 nM isoprenaline, in the presence or absence PKAI (5 μM). **p<0.01 (one-way ANOVA, Dunnett's *post-hoc* test) indicates a significant increase in CREB phosphorylation vs PKAI treated cells.

Figure 3. p38 MAPK and PKA-Dependency of isoprenaline-stimulated CREB signalling. Serum-starved cells were stimulated with isoprenaline (1 μM) for up to 60 min, following a 30 min pre-incubation with the p38 inhibitor SB203580 (20 μM), or a combination of SB203580 and the PKA inhibitor, PKI 14-22 amide (5 μM; C, D). Following isoprenaline treatment cells were lysed and immunoblotted for either (A) phospho-p38 or phospho-CREB (C) upper panels). To verify equal gel loading all blots were stripped and reprobed with anti-p38 (A) or anti-CREB (C) antibodies (lower panels). Cumulative densitometric analysis data are shown for the time-course of isoprenaline-stimulated p38 phosphorylation (B) and CREB phosphorylation (D). Data are expressed as means ± S.E.M., for n=4-5 separate experiments. *p<0.05; **p<0.01, when compared to non-inhibitor treated responses (two-way ANOVA and Sidak's *post-hoc* test).

Figure 4. Isoprenaline-stimulated p38 phosphorylation is Src-, but not PKA-dependent. Representative immunoblots (A) and cumulative data (B) showing isoprenaline concentration-dependent p38 phosphorylation in serum-starved ULTR cells (data are means ± S.E.M., for n=4 separate experiments). Representative immunoblots (C) and cumulative data (means ± S.E.M., for n=3 separate experiments) (D) show that inclusion of the PKA inhibitor (PKAI) PKA inhibitor, PKI 14-22 amide (5 μM) did not prevent isoprenaline-stimulated (1

μM, 5 min) p38 phosphorylation. Serum-starved cells were challenged with isoprenaline (1 μM) for up to 60 min, either in the absence (E), or presence of (F) a 30 min pre-incubation with the Src-kinase inhibitor PP1 (5 μM). Representative immunoblots indicating the temporal-profile of isoprenaline-induced p38 phosphorylation (upper panels). To verify equal gel loading all blots were stripped and reprobed an anti-p38 antibody (lower panels). Cumulative densitometric analysis of p38 phosphorylation signals are shown (F) indicating that isoprenaline-stimulated p38 phosphorylation is mediated by Src kinase. Data are expressed as means ± S.E.M., for n=3 separate experiments. *$p<0.05$; **$p<0.01$, when compared to non-inhibitor treated responses (two-way ANOVA and Sidak's *post-hoc* test).

Figure 5. Arrestin-dependency of isoprenaline-induced p38 signalling. ULTR cells were transfected with either negative control (NC), anti-arrestin2 (AR2) or anti-arrestin3 (AR3) siRNAs as described in the Method Section. After 24 h cells were serum-starved for a further 24 h prior to isoprenaline (1 μM) challenge. A) Representative immunoblots show the extent of arrestin2 or arrestin3 knockdown 48 h after transfection using either the A1CT (upper panel) or A2CT (lower panel) antibodies, which preferentially recognise arrestin2 or arrestin3, respectively. B) Cumulative data show the percentage of either arrestin2 or arrestin3 depletion following siRNA treatment. Data are expressed as means± S.E.M., for n=6 separate experiments. **$p<0.01$ Student's unpaired *t*-test, when compared negative control treated cells. C) Representative immunoblots show the temporal-profiles of isoprenaline-stimulated p38 phosphorylation in ULTR cells transfected with NC, anti-AR2, anti-AR3 siRNAs or anti-AR3+PP1 (Src inhibitor, 5 μM) treated cells (upper panels). To verify equal gel loading all blots were stripped and reprobed an anti-p38 antibody (lower panels). Cumulative densitometric analysis of p38 phosphorylation signals are shown (D) indicating that isoprenaline-stimulated p38 phosphorylation is attenuated when arrestin2 is

knockdown and enhanced when arrestin3 is knockdown. Inclusion of the Src inhibitor PP1 reversed the effects of AR3 knockdown. Data are expressed as means ± S.E.M., for n=4-6 separate experiments. *p<0.05; **p<0.01, when compared to negative-control transfected cells (two-way ANOVA and Sidak's *post-hoc* test).

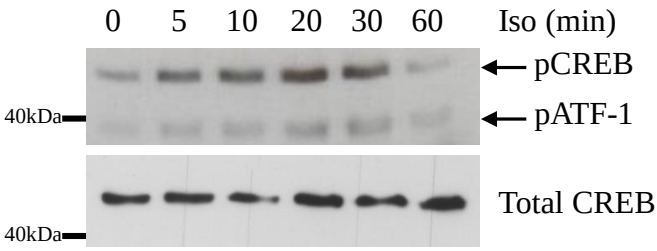
Figure 6. Arrestin-dependency of isoprenaline-stimulated CREB signalling. ULTR cells were transfected with either negative-control, anti-arrestin2, anti-arrestin3 or both anti-arrestin2/3 siRNAs as described in the Method Section. The PKA inhibitor, PKI 14-22 amide (5 μM; 30 min pre-treatment) was also included in some experiments where cells were treated with anti-arrestin2 and anti-arrestin3 siRNAs. After 24 h cells were serum-starved for a further 24 h prior to isoprenaline (1 μM) challenge for up to 1 h. Representative immunoblots (A) show the temporal-profile of agonist-induced CREB phosphorylation following depletion of individual or both arrestin proteins (upper panels). To verify equal gel loading all blots were stripped and reprobed with anti-CREB (lower panels). Cumulative densitometric analysis of CREB phosphorylation signals are shown (B) indicating that depletion of arrestin2 enhanced the initial phase and attenuated the later phase of CREB phosphorylation, whilst isoprenaline-stimulation CREB signals were enhanced and prolonged following depletion of arrestin3. In contrast, knockdown of both arrestins suppressed isoprenaline-stimulated CREB phosphorylation in ULTR cells at time points >5 min. Data are expressed as means ± S.E.M., for n=4 separate experiments. *p<0.05; **p<0.01, when compared to negative-control transfected cells (two-way ANOVA and Sidak's *post-hoc* test).

Figure 7. Arrestin expression is increased in the pregnant myometrium. Myometrial tissue samples were homogenised and prepared as described in the Methods section and 40 μg of protein loaded per lane. A representative immunoblot is shown indicating arrestin2 and

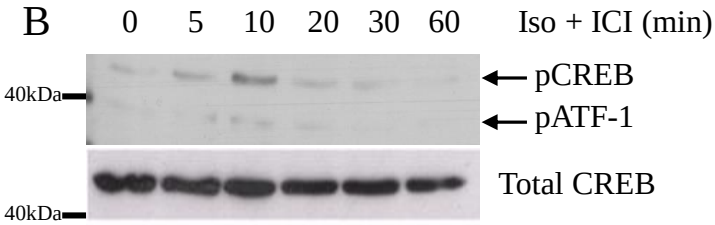
arrestin3 expression in non-pregnant (NP) and pregnant (PM) myometrium (A). Cumulative data (B) (means ± S.E.M. from n=6 separate patient donors) shows that arrestin expression is increased in pregnant term non-labouring women when compared to non-pregnant controls (*$p<0.05$; **$p<0.01$ by Student's *t-test*).

Figure 1

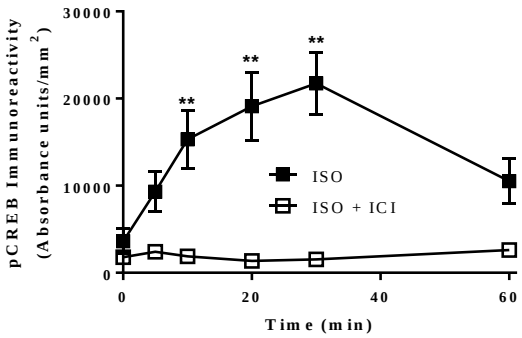
A



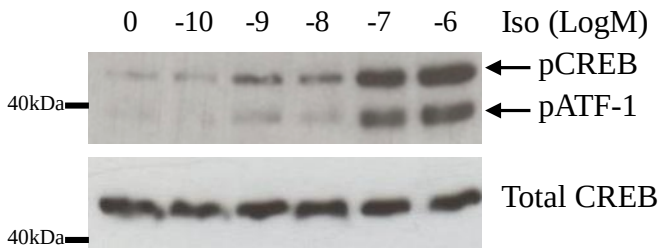
B



C



D



E

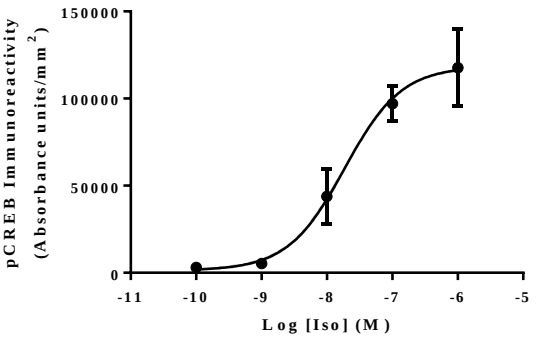


Figure 2

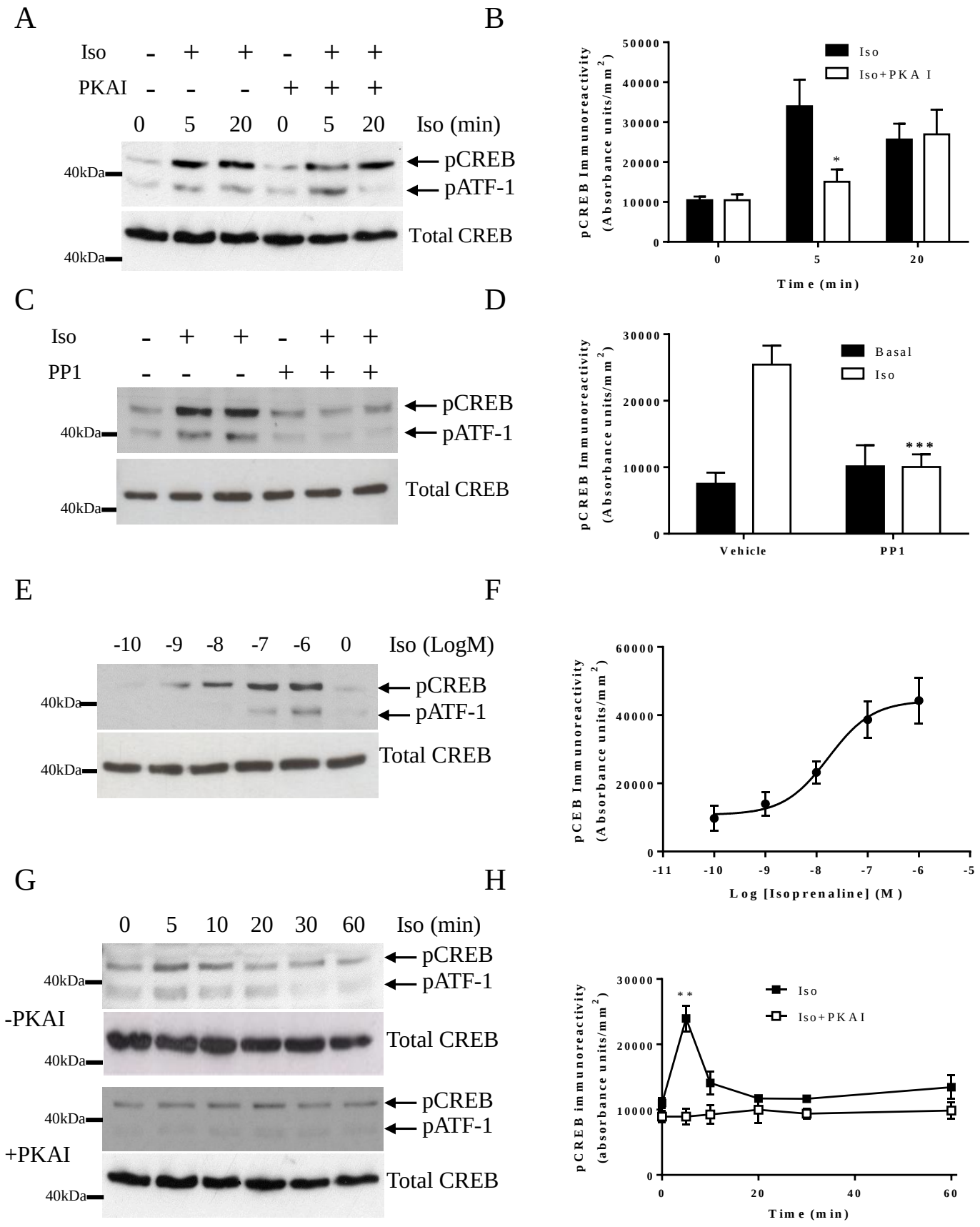

A
Iso - + + - + +
PKAI - - - + + +
0 5 20 0 5 20 Iso (min)
40kDa
pCREB
pATF-1
Total CREB
40kDa
B
pCREB Immunoreactivity (Absorbance units/mm2)
Iso
Iso+PKA I
Time (min)
C
Iso - + + - + +
PP1 - - - + + +
pCREB
pATF-1
Total CREB
D
pCREB Immunoreactivity (Absorbance units/mm2)
Basal
Iso
Vehicle
PP1
E
-10 -9 -8 -7 -6 0 Iso (LogM)
pCREB
pATF-1
Total CREB
F
pCEB Immunoreactivity (Absorbance units/mm2)
Log [Isoprenaline] (M)
G
0 5 10 20 30 60 Iso (min)
-PKAI
+PKAI
pCREB
pATF-1
Total CREB
H
pCREB immunoreactivity (absorbance units/mm2)
Iso
Iso+PKA I
Time (min)

Figure 3

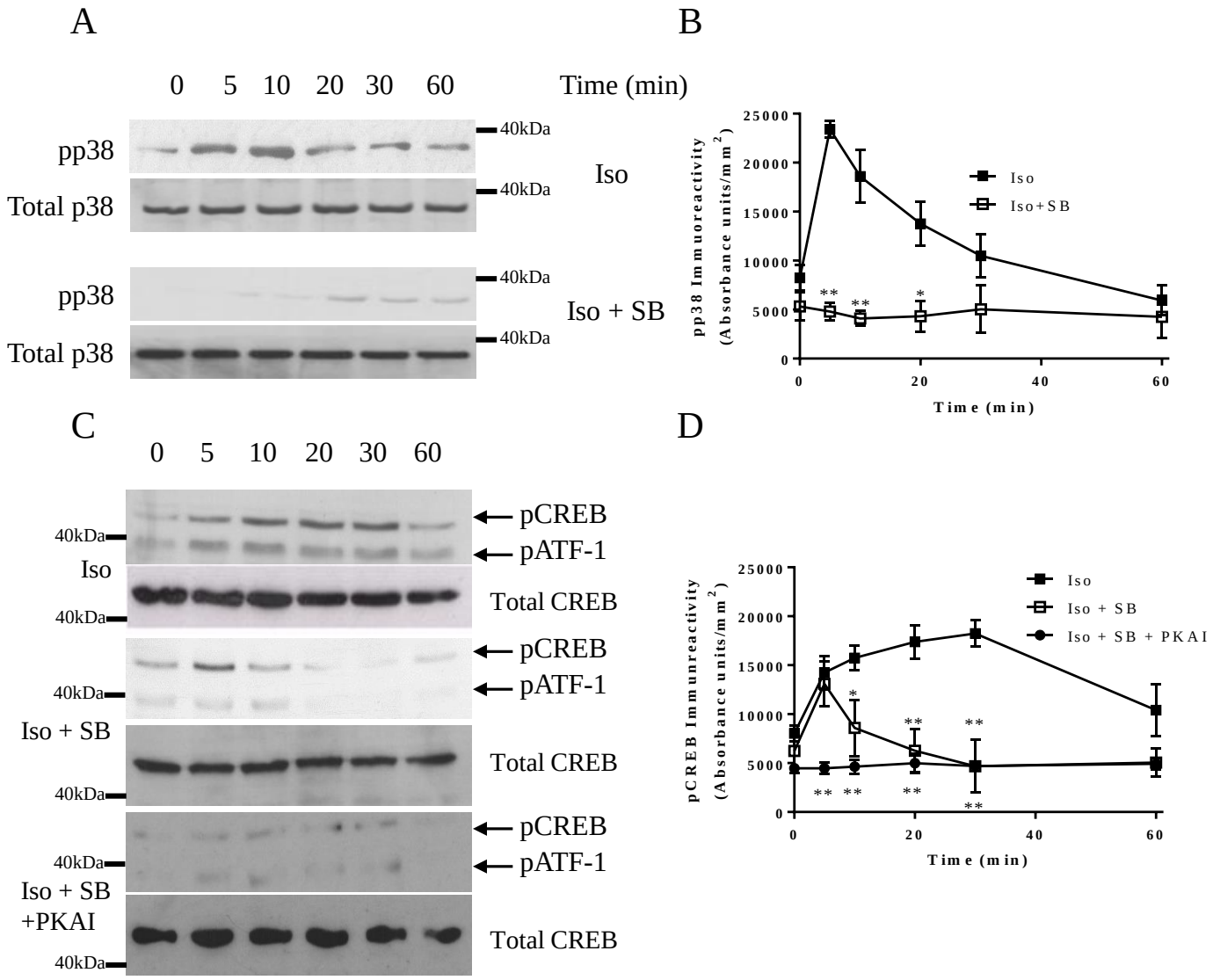

A
0 5 10 20 30 60
Time (min)
pp38
Total p38
40kDa
Iso
Iso + SB
B
pp38 Immuoreactivity (Absorbance units/mm2)
Iso
Iso+SB
Time (min)
C
pCREB
pATF-1
Total CREB
Iso
Iso + SB
Iso + SB +PKAI
D
pCREB Immunreactivity (Absorbance units/mm2)
Iso
Iso + SB
Iso + SB + PKAI
Time (min)

Figure 4

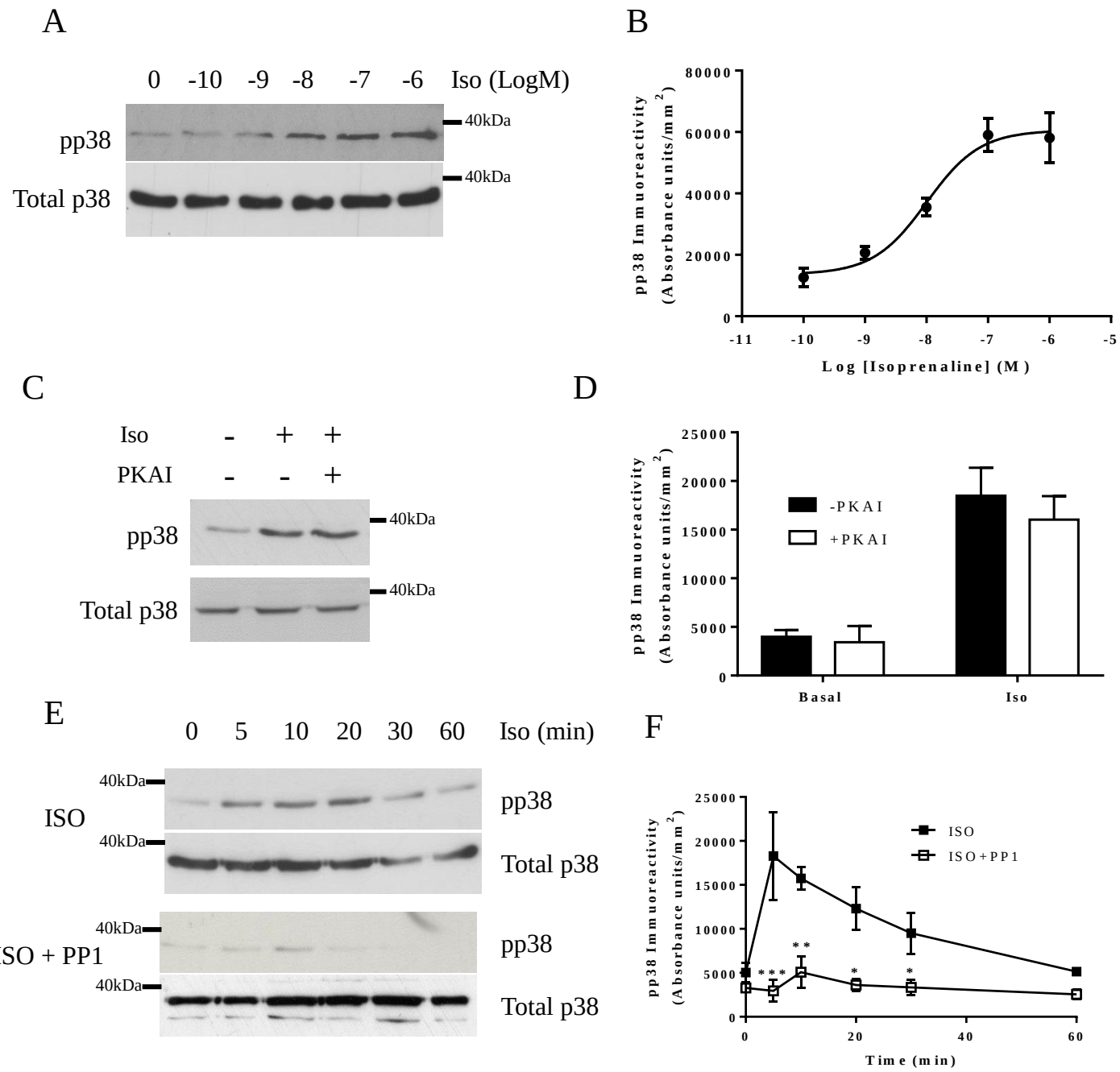

A
0 -10 -9 -8 -7 -6 Iso (LogM)
pp38
Total p38
40kDa
40kDa
B
pp38 Immuoreactivity (Absorbance units/mm2)
80000
60000
40000
20000
0
-11 -10 -9 -8 -7 -6 -5
Log [Isoprenaline] (M)
C
Iso - + +
PKAI - - +
pp38
Total p38
40kDa
40kDa
D
pp38 Immuoreactivity (Absorbance units/mm2)
25000
20000
15000
10000
5000
0
-PKAI
+PKAI
Basal
Iso
E
0 5 10 20 30 60 Iso (min)
ISO
ISO + PP1
40kDa
pp38
Total p38
F
pp38 Immuoreactivity (Absorbance units/mm2)
25000
20000
15000
10000
5000
0
ISO
ISO+PP1
0 20 40 60
Time (min)

Figure 5

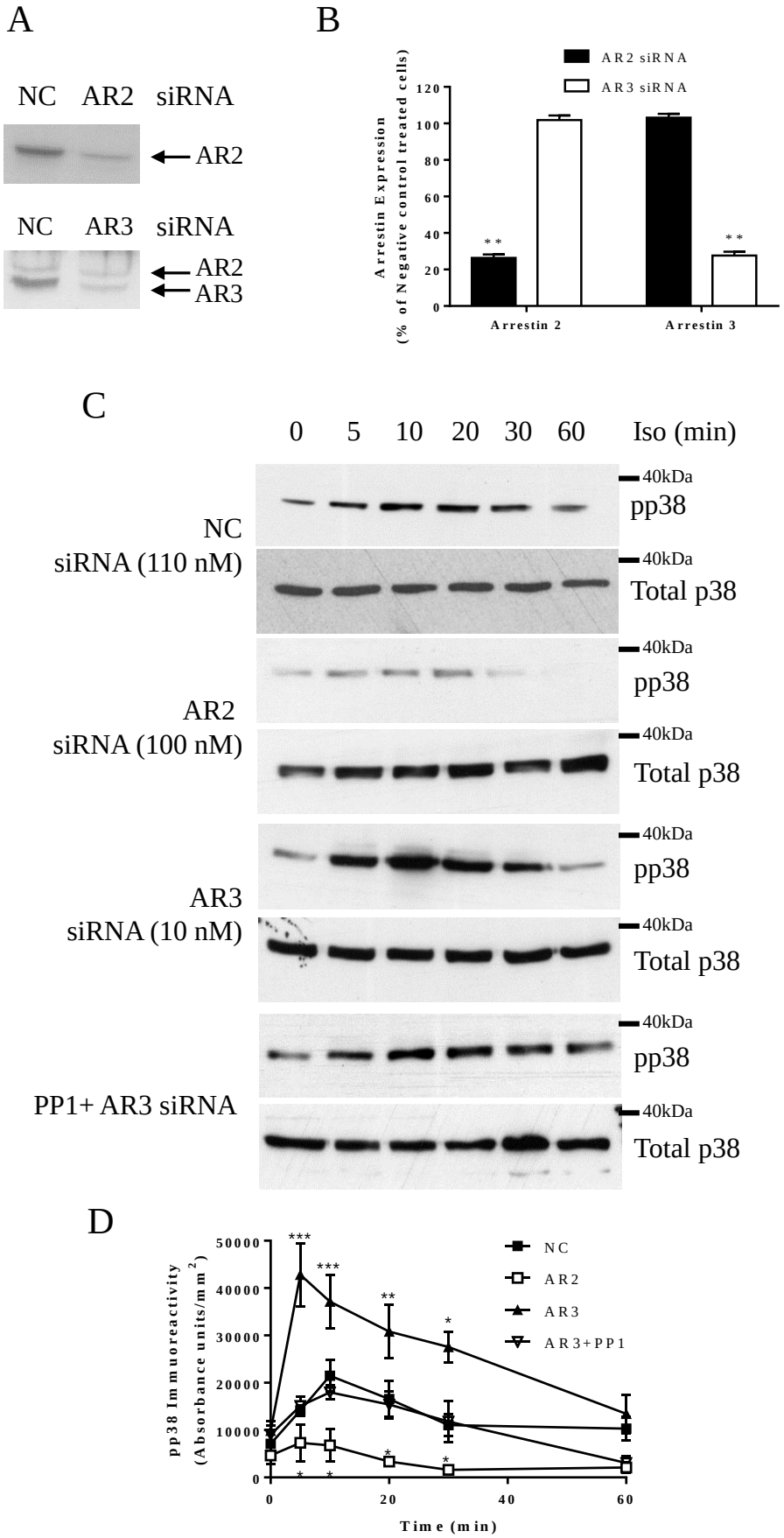
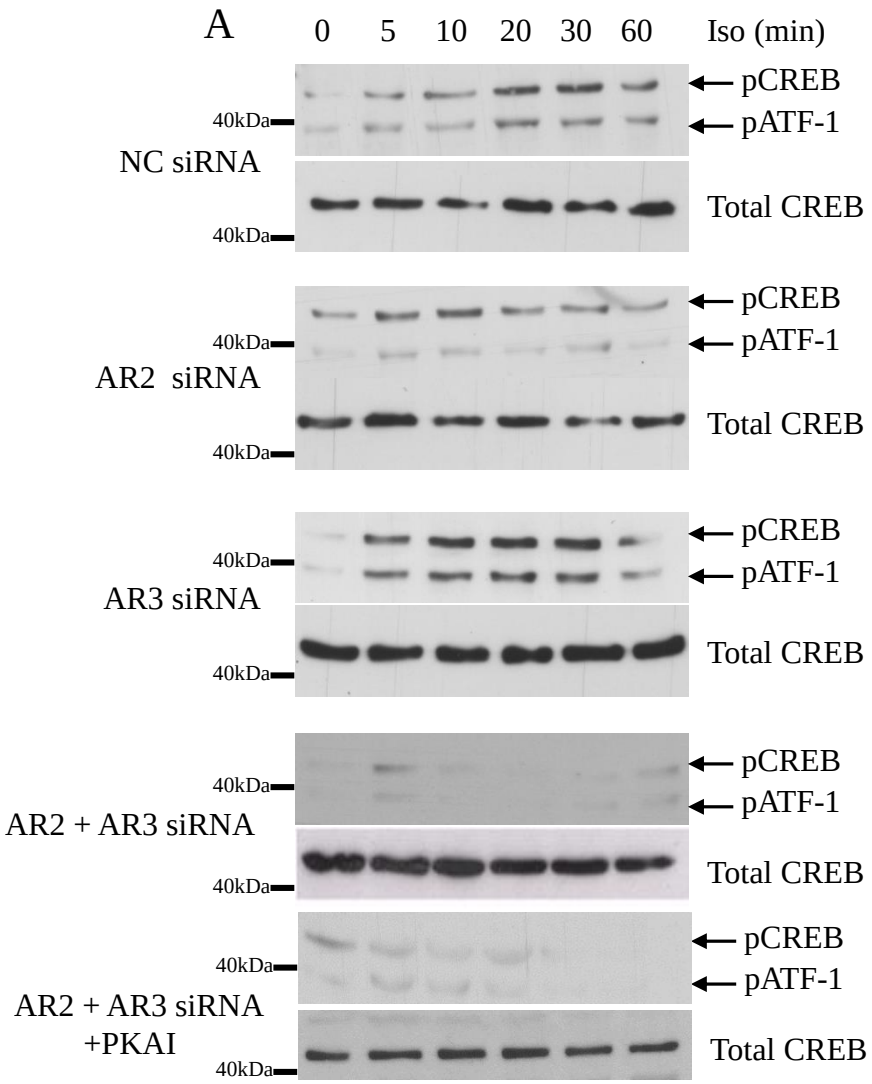

A
NC AR2 siRNA
AR2
NC AR3 siRNA
AR2
AR3
B
AR2 siRNA
AR3 siRNA
Arrestin Expression (% of Negative control treated cells)
120
100
80
60
40
20
0
**
**
Arrestin 2
Arrestin 3
C
0 5 10 20 30 60 Iso (min)
NC siRNA (110 nM)
AR2 siRNA (100 nM)
AR3 siRNA (10 nM)
PP1+ AR3 siRNA
40kDa
pp38
Total p38
D
pp38 Immuoreactivity (Absorbance units/mm2)
50000
40000
30000
20000
10000
0
***
***
**
*
*
*
*
NC
AR2
AR3
AR3+PP1
0
20
40
60
Time (min)

Figure 6


A
0
5
10
20
30
60
Iso (min)
40kDa
NC siRNA
pCREB
pATF-1
Total CREB
AR2 siRNA
AR3 siRNA
AR2 + AR3 siRNA
AR2 + AR3 siRNA
+PKAI

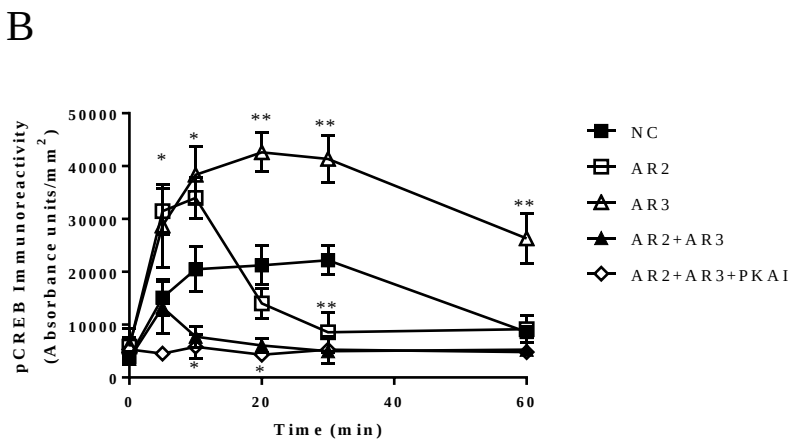
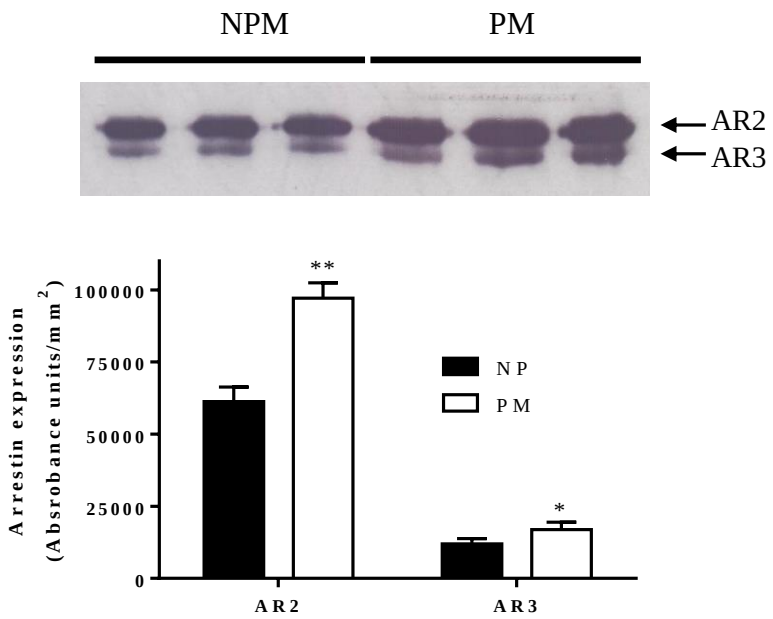
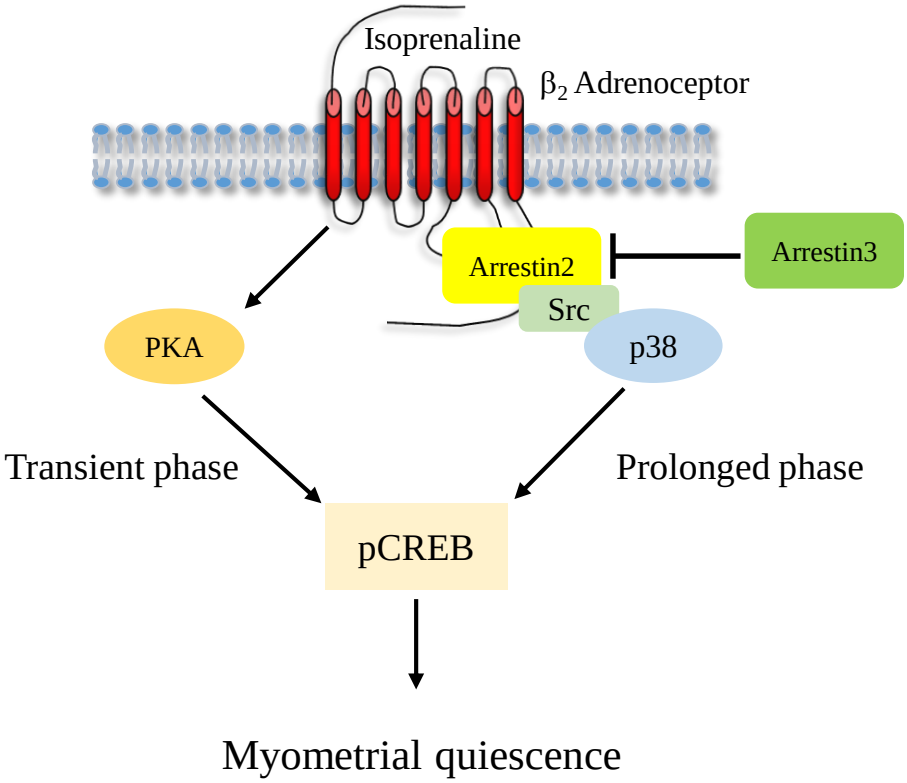

B
pCREB Immunoreactivity
(Absorbance units/mm²)
50000
40000
30000
20000
10000
0
0
20
40
60
Time (min)
NC
AR2
AR3
AR2+AR3
AR2+AR3+PKAI

Figure 7


NPM
PM
AR2
AR3
Arrestin expression (Absrobance units/mm2)
100000
75000
50000
25000
0
**
*
NP
PM
AR2
AR3


Isoprenaline
β2 Adrenoceptor
Arrestin2
Arrestin3
Src
PKA
p38
Transient phase
Prolonged phase
pCREB
Myometrial quiescence