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Desensitization of α_{2A}-adrenoceptor signalling by modest levels of adrenaline is facilitated by β_2-adrenoceptor-dependent GRK3 up-regulation

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1 Adrenaline (ADR) and noradrenaline (NA) can simultaneously activate inhibitory α_2- and stimulatory β-adrenoceptors (AR). However, ADR and NA differ significantly in that ADR is a potent β_2-AR agonist while NA is not. Only recently has the interaction resulting from the simultaneous activation of α_2- and β_2-AR been examined at the cellular level to determine the mechanisms of α_2-AR regulation following concomitant activation of both α_2- and β_2-ARs by chronic ADR.

2 This study evaluates β_2-AR regulation of α_{2A}-AR signalling following chronic ADR (300 nM) and NA (1 and 30 μM) treatments of BE(2)-C human neuroblastoma cells that natively express both β_2- and α_{2A}-ARs.

3 Chronic (24 h) treatment with ADR (300 nM) desensitized the response to the α_{2A}-AR agonist, brimonidine, in BE(2)-C cells. Addition of the β-AR antagonist, propranolol, blocked the ADR-induced α_{2A}-AR desensitization. Unlike ADR, chronic NA (1 μM) treatment had no effect on the α_{2A}-AR response. However if NA was increased to 30 μM for 24 h, α_{2A}-AR desensitization was observed; this desensitization was partially reversed by propranolol.

4 Chronic ADR (300 nM) treatment reduced α_{2A}-AR binding levels, contributing to the α_{2A}-AR desensitization. This decrease was prevented by addition of propranolol during ADR treatment. Chronic NA (30 μM), like ADR, treatment lowered specific binding, whereas 1 μM NA treatment was without effect.

5 Chronic ADR treatment produced a significant increase in GRK3 levels and this was blocked by propranolol or GRK2/3 antisense DNA treatment. This antisense DNA, common to both GRK2 and GRK3, also blocked chronic ADR-induced α_{2A}-AR desensitization and down-regulation.

6 Acute (1 h) ADR (300 nM) or NA treatment (1 μM) produced α_{2A}-AR desensitization. The desensitization produced by acute treatment was β-AR independent, as it was not blocked by propranolol.

7 We conclude that chronic treatment with modest levels of ADR produces α_{2A}-AR desensitization by mechanisms that involve up-regulation of GRK3 and down-regulation of α_{2A}-AR levels through interactions with the β_2-AR.
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Abbreviations: AR, adrenoceptor; ARC-239, 2-(2,4-(O-methoxyphenyl)-piperazin-1-yl)-ethyl- 4,4dimethyl-1,3-(2H,4H)-isoquinolindione; brimonidine, 5-Bromo-*N*- (4,5-dihydro-1H-imidazole-2-yl)-6-quinoxalinamine (or UK14,304); G_{α}i, alpha subunit of inhibitory guanine nucleotide binding proteins; G_{α}s, alpha subunit of stimulatory guanine nucleotide binding proteins; $G_{\beta\gamma}$, beta and gamma subunits of guanine nucleotide binding proteins; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRK, G protein coupled receptor kinase; HBSS, Hank's balanced salt solution; IBMX, 3-isobutyl-1- methylxanthine; I-CYP, iodo-cyanopindolol; ODN, oligodeoxynucleotide; PKA, cAMPdependent protein kinase; PKC, phospholipase C-dependent protein kinase; PMSF, phenylmethyl sulfonylflouride; PNS, peripheral nervous system; PVDF, polyvinylidene diflouride; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS/T, tris-buffered saline/tween

Introduction

Adrenaline (ADR) and noradrenaline (NA) are endogenous catecholamines that activate three different families of adrenoceptors (ARs): α_1-ARs (α_{1A}, $_{1B}$, and $_{1D}$) that activate phospholipase C, α_2-ARs (α_{2A}, $_{2B}$, and $_{2C}$) that inhibit adenylate cyclase, and β-ARs (β_1, β_2, and β_3) that activate adenylate cyclase. ADR and NA share similar efficacies and potencies for all these receptors except the β_2-AR, where ADR has a higher affinity than NA.

The interaction between β- and α_2-ARs is especially interesting because these ARs mediate opposing actions on

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adenylate cyclase. The interplay between these two ARs is important for maintaining homeostasis of various physiological mechanisms and, in some cases, causing the manifestation of pathophysiological conditions. In peripheral tissues, α_2 and β-ARs play important roles in modulating uterine smooth muscle tone (Lécrivain *et al*., 1998), insulin release from pancreatic islet cells (John *et al*., 1990), cytokine release (Kalinichenko *et al*., 1999) and vasomotor tone. In addition, neuronal α_2- and β_2-ARs play an important role in the modulation of noradrenergic neurotransmission in the human CNS and PNS. Neuronal α_2-ARs are considered potential therapeutic targets for the treatment of major cardiovascular disorders, such as hypertension and cerebral ischaemia, and CNS disorders, such as pain and depression (Lanier *et al*., 1996). Hence the interaction between α_2- and β-ARs in a single cell after exposure to ADR or NA has potentially great physiological and pathophysiological significance (Meana *et al*., 1992; Shi *et al*., 1996; Kalinichenko *et al*., 1999).

The influence of cross talk between receptors that exert reciprocal effects on a common signal transduction pathway within a single cell has been examined previously. For example, Malbon and co-workers determined that chronic (24 h) activation of adenylyl cyclase by β_2-ARs resulted in a 3 fold increase in the expression of the inhibitory G protein $G_\alpha i2$ in S49 lymphoma cells (Hadcock *et al*., 1990). Moreover, maximal inhibition of forskolin-stimulated adenylyl cyclase activity by somatostatin was increased from 35 to 65% after 24 h isoprenaline (ISO) treatment. In a subsequent report, this same group described cross-talk in the opposite direction (Hadcock *et al*., 1991): chronic stimulation of the inhibitory pathway by somatostatin resulted in an increased expression of the stimulatory G protein $G_\alpha s$ and an increased efficacy of ISO on adenylyl cyclase activity. In summary, this suggests that stimulation of one signalling pathway results in sensitization of the opposing pathway.

The results described above clearly illustrate the potential for cross talk between inhibitory and stimulatory receptors in a single cell. However, there is a paucity of information regarding the consequences of chronic simultaneous activation of both the stimulatory and inhibitory pathways in the same cell. This information is important, since *in vivo* studies suggest that α_2-AR desensitization may occur more readily when the inhibitory α_2-AR and stimulatory β_2-AR are chronically and simultaneously activated by ADR (Schwartz & Eikenburg, 1988; Apparsundaram & Eikenburg, 1996). In contrast, chronic NA treatment that activates only the α_2-AR *in vivo* does not produce α_2-AR desensitization (Eikenburg, 1990). Studies utilizing β_2-AR antagonists during chronic ADR treatment suggest a role for the β_2-AR in ADR-induced α_2-AR desensitization (Apparsundaram & Eikenburg, 1996). These observations are particularly noteworthy when considered with *in vitro* evidence that the α_2-AR is more resistant to desensitization, requiring much higher agonist concentrations for desensitization than other ARs, such as the β_2-AR (Eason & Ligget, 1992; Lanier *et al*., 1996). Therefore, the present study was undertaken to examine the differential regulation of α_2-AR signalling by modest concentrations of ADR (300 nM) and 1 μM NA in a neuronal cell line (BE(2)-C) that endogenously expresses both α_{2A}- and β_2-ARs. The objective was to determine why long-term simultaneous α_2- and β_2-AR activation by ADR (300 nM) produces α_2-AR desensitization, while α_2-AR activation alone by 1 μM NA fails to do so. For the purpose of this study, desensitization is defined as simply a loss of agonist response.

Methods

Materials

The following drugs were purchased or obtained from the indicated sources: (−)adrenaline (ADR), isoprenaline bitartrate (ISO), *p*-aminoclonidine (PAC), guanabenz, oxymetazoline, phenylephrine, yohimbine (RBI, Natick, MA, U.S.A.); ARC-239 (Tocris Cookson, St. Louis, MO, U.S.A.); (±)noradrenaline (NA), agmatine, idazoxan, sodium ascorbate, adrenal cortex acetone powder, IBMX, cAMP, forskolin, hydroxyapatite (Sigma Chemical Co., St. Louis, MO, U.S.A.); oligodeoxynucleotides (ODNs; Sigma-Genosys, Woodlands, TX, U.S.A.); [^{3}H]-rauwolscine (60–80 Ci mmol^{-1}), [^{3}H]-RX821002 (56–60 Ci mmol^{-1}), [^{3}H]-cAMP (35–45 Ci mmol^{-1}) and [^{125}I]-CYP (2000 Ci mmol^{-1}; Amersham Corp., Arlington Heights, IL, U.S.A.); Liquiscint scintillation fluor, (National Diagnostics, Atlanta, GA, U.S.A.); cell culture media (Gibco, Grand Island, NY, U.S.A.); foetal bovine serum (Atlanta Biologicals, Norcross, GA, U.S.A.); antibiotics (Mediatech, Inc., Herndon, VA, U.S.A.); $G_\alpha i1/2$ (AS/7), $G_\alpha i3$ (EC/2, New England Nuclear Corp., Boston MA, U.S.A.), $G_\alpha o$, GRK2 and GRK3 (H-222), GRK2 (C-15) GRK3 (C-14) and GRK5 (C-20) primary antibodies and horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.); and glyceraldehyde-3-phosphate dehydrogenase antibody (GADPH, Research Diagnostics, Inc., Flanders, NJ, U.S.A.).

Brimonidine was provided by RBI as part of the Chemical Synthesis Program of the National Institute of Mental Health, Contract N01MH30003. Rauwolscine was a generous gift from CarlRoth (GmbH, Karlsruhe, Germany).

Cell culture

BE(2)-C (passages 16–58) human neuroblastoma cells (Dr Robert A. Ross, Fordham University, Bronx, NY, U.S.A.) were maintained in a humidified atmosphere (6% CO_2 : 94% air) in a 1 : 1 mixture of Eagle's minimum essential medium with non-essential amino acids and Ham's F-12 containing 10% foetal bovine serum, 100 U ml^{-1} penicillin G and 0.1 mg ml^{-1} streptomycin sulphate. Plates of cells greater than 60% confluence were used throughout the study, and lifted after incubation for 5 min at room temperature with phosphate buffered saline containing 1 mM EGTA. Intact cells were sedimented by centrifugation at $1000 \times g$ for 5 min, and resuspended in the appropriate buffer or reagent, as described below.

Receptor binding

Preparation of cell membranes Cells were homogenized with a polytron (setting 3, 10 s) in 20 volumes of Tris-HCl buffer (50 mM, pH 7.4) containing (mM) NaCl 100, Na_2 EDTA 1 and PMSF 0.1, incubated for 15 min at 25°C, and the membranes sedimented by centrifugation (30 min, $34,000 \times g$) at 4°C.

Pellets were resuspended in 0.32 M sucrose, and aliquots of the membrane fractions were stored frozen (−80°C) until use.

Saturation binding Levels of α_2-ARs in BE(2)-C cell membranes (0.5 mg ml^{-1}) were determined with various concentrations of either [^{3}H]-rauwolscine (0.3–12 nM) or [^{3}H]-RX821002 (0.1–10 nM) in a total volume of 1–2 ml in potassium phosphate buffer (50 mM, pH 7.4) containing $MgSO_4$ (5 mM) at 37°C for 45 min. Thereafter, 2 ml Tris-HCl (5 mM, pH 7.4, 4°C) was added to the mixture to terminate the binding reaction before filtration over no.32 glass fibre filter strips (Schleicher & Schuell, Keene, NH, U.S.A.) using a PHD cell harvester (Cambridge Technology, Cambridge, MA, U.S.A.). The reaction tubes and the filter strips were rinsed twice with a further 2–3 ml of buffer. Levels of radioactivity were determined by scintillation spectroscopy in a Beckman LS6000 liquid scintillation counter. Assays were performed in triplicate, and specific binding was determined by subtracting the binding in the presence of yohimbine or phentolamine (10 μM; non-specific) from the binding in its absence; specific binding comprised 50–75% of total binding. Saturation studies indicated that agonist treatments did not alter the K_d of the radioligand for the α_2-AR, so a single concentration (2 nM) of either [^{3}H]-rauwolscine or [^{3}H]-RX821002 was used to determine levels of binding following catecholamine treatment.

In some studies, plated cells were washed (4 × 5 ml) with serum-free media and treated with an unmodified phosphodiester antisense ODN common to both GRK2 and GRK3: 5′-ACC GCC TCC AGG TCC GCC AT-3′ (20 μM; Shih & Malbon, 1994) for 4 h, before adding sera back for the remainder of the treatment period. Cells treated with ODNs corresponding to the complementary common GRK sense (20 μM) served as controls in those experiments.

β-AR levels were determined as described (Standifer *et al*., 1989) with [^{125}I]-CYP (2-300 pM) and 0.3 mg ml^{-1} of membrane protein at 37°C for 60 min in a total volume of 250 μl. Non-specific binding was determined in the presence of 10 μM alprenolol, while specific binding was determined by subtracting the binding in the presence of alprenolol from the binding in its absence.

Competitive binding Cell membrane fractions were incubated as described above, except that the concentration of [^{3}H]-rauwolscine was fixed (2 nM), and various (5–9) concentrations of unlabelled drugs were included. For β-AR displacement studies, multiple concentrations of competitor were incubated with 100 pM [^{125}I]-CYP.

cAMP accumulation

To determine the effects of α_2-AR agonists on forskolin-induced cAMP accumulation, intact cells were incubated for 5 min at 37°C in HBSS buffer (in mM): NaCl 137, KCl 5, Na_2HPO_4 0.6, KH_2PO_4 0.4, $NaHCO_3$ 4, D-glucose 6, $MgCl_2$ 0.5, $MgSO_4$ 0.4 and $CaCl_2$ 1, containing the phosphodiesterase inhibitor IBMX (0.5 mM). In some experiments, antagonists were also included in this step. To prohibit oxidation, sodium ascorbate (0.11 mM) was included when assaying catecholamines. Upon addition of forskolin (10 μM) and agonist, assay tubes were incubated for an additional 10 min at 37°C. Removing the tubes to a boiling water bath for 5 min terminated the assay. All assays were performed in duplicate in a total volume of 0.5 ml. After boiling, samples were centrifuged for 5 min at 14,000 × g, and cAMP levels from the supernatant fractions were determined in a [^{3}H]-cAMP (0.8 pmol) binding assay as previously described (Standifer *et al*., 1994). β-AR-mediated stimulation of cAMP accumulation was performed in the same manner, except that forskolin was not included in the assay mixture.

Immunoblotting

Membrane proteins (30 μg) were resolved by SDS–PAGE through 10% gels. Proteins were transferred to PVDF membrane, blocked with 5% non-fat dried milk in PBS containing 0.1% Tween (PBST) and incubated overnight at 4°C with dilutions of a rabbit polyclonal antibody directed against $G_\alpha i3$ (1 : 1000), $G_\alpha i1/2$ (1 : 500), $G_\alpha o$ (1 : 500), GRK2 and GRK3 (1 : 1000), GRK2 (1 : 1000), GRK3 (1 : 1000) or GRK5 (1 : 500). Blots were subjected to four washes before incubating for 60 min at room temperature with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1 : 2000) in PBST. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL, U.S.A.). The intensity of each immunoreactive band was determined using a Nucleovision Imaging Workstation (Nucleotech Corp., San Carlos, CA, U.S.A.), and normalized to the GAPDH loading control (1 : 8000).

Protein determination

Bovine serum albumin was used as a standard in the determination of protein levels in intact cells and cell membranes as described (Lowry *et al*., 1951).

Data analysis

K_d, B_{max}, IC_{50} and $LogEC_{50}$ values were determined by nonlinear regression analysis using GraphPad Prism version 3.0 for Windows 95 and 98 (GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com). K_i values were calculated according to the Cheng–Prusoff equation (Cheng & Prusoff, 1973) in which $K_i = (IC_{50}) [(1+S)]^{-1}$, where S = [concentration of radioligand] [K_D of radioligand]$^{-1}$. Comparisons between groups were made by two-way Student's t-tests or ANOVA and Tukey's or Dunnett's *post hoc* test (where appropriate; GraphPad Software), and groups were considered significantly different if $P \leqslant 0.05$.

Results

Chronic ADR induces α_{2A}-AR desensitization and down-regulation

To test the hypothesis that α_2-AR regulation by chronic ADR is β-AR-dependent, α_2-AR function following chronic 300 nM ADR or 1 μM NA pretreatment (16–24 h) was assayed in BE(2)-C cells (passage <50) by measuring the ability of the α_2-AR agonist, brimonidine, to inhibit forskolin (10 μM)-stimulated cAMP accumulation. Chronic 300 nM ADR exposure desensitized the α_2-AR, significantly reducing brimonidine efficacy ($P < 0.0001$) while reducing brimonidine

potency only 2 fold (Figure 1A). The decrease in brimonidine efficacy was prevented when the nonselective β-AR antagonist propranolol (30 nM) was added to the chronic ADR treatment (Figure 1A); 30 nM propranolol alone had no effect. To confirm that 300 nM ADR was sufficient to modulate β-AR activity, the ability of a maximally stimulating concentration of ISO (250 nM) to increase cAMP accumulation after chronic ADR treatment was also determined. Pretreatment with 1 μM ISO desensitized the β-AR response and was included as a positive control. Chronic 300 nM ADR pretreatment desensitized the β-AR response (Figure 2; $P<0.05$), indicating that ADR was activating both α_2- and β-ARs.

Unlike 300 nM ADR, 1 μM NA pretreatment had no effect on brimonidine potency or efficacy (Figure 1B). This concentration of NA also failed to desensitize the β-AR response (Figure 2), suggesting that a concentration of NA producing only activation of the α_2-AR alone was not sufficient to produce α_2-AR desensitization in this cell line. However, while 300 nMM ADR produces a near maximal α_2-AR response (72% inhibition of forskolin-stimulated cAMP accumulation; Figure 1C), 1 μM NA inhibits forskolin-stimulated cAMP accumulation a bit less (53.8%). Also, if the population of β-AR in the cells were primarily of the β_2-AR subtype, NA would be much less effective than ADR at activating those receptors. To achieve equivalent α_2-AR activation as ADR (Figure 1C) and ensure activation of β-ARs, chronic pretreatment was also performed with a higher concentration of NA (30 μM; Figure 1B). This concentration reduced brimonidine efficacy to levels produced by 300 nM ADR treatment, produced a 10 fold loss of brimonidine potency, and desensitized the β-AR response to the same extent as ADR pretreatment (Figure 2). A higher propranolol concentration (1 μM) was only partially effective in preventing the α_2-AR desensitization induced by 30 μM NA pretreatment (Figure 1B), as it reversed the loss of brimonidine potency but not the loss of efficacy. Whether this reflects (1) a significantly higher α_2-AR occupancy than 300 nM ADR, (2) the fact that NA was present in 30 fold excess compared to propranolol, so that complete β-AR blockade was not achieved, or (3) a combination of both is not clear. It is unlikely that differential α_2-AR occupancy played a big role because 300 nM ADR produces an α_2-AR response that is 90% of maximal (Figure 1C, Table 2). It is more likely that there was an insufficient concentration of propranolol to completely antagonize the actions of NA at the β-AR. Unfortunately, higher concentrations of propranolol could not be used without producing nonspecific effects. While both ADR and NA also can activate α_1-ARs, it is unlikely that their ability to desensitize that α_2-AR response in this cell line involves α_1-ARs: the α_1-AR agonist phenylephrine failed to stimulate IP_3 formation in BE(2)-C cells at concentrations up to 100 μM (data not shown) confirming the lack of functional α_1-ARs in this cell line.

To verify the involvement of the β-AR in the process of α_2-AR desensitization by 300 nM chronic ADR, we took advantage of the fact that the β-AR number in our cells significantly declines with increasing passage number while α_2-AR levels and responsiveness are maintained. After passage 50, β-AR responsiveness wanes. By passage 55, both β-AR responsiveness and number are reduced over 80% (Table 1). In BE(2)C cells > passage 50, chronic ADR

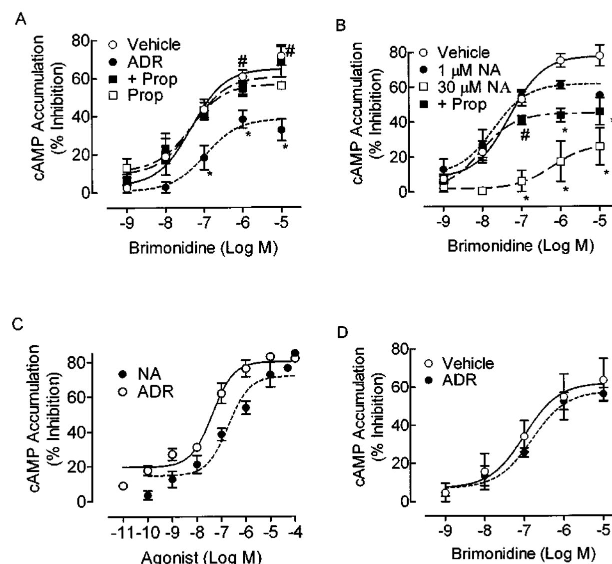

Figure 1 Chronic 300 nM ADR and 30 μM NA desensitizes the α_2-AR response in BE(2)-C cells. The α_2-AR response, as determined by the ability of brimonidine to inhibit forskolin (10 μM)-stimulated cAMP accumulation in intact BE(2)-C human neuroblastoma cells, was measured 16–24 h after pretreatment with any or all of the following: ADR (300 nM), propranolol (30 nM or 1 μM), ADR + 30 nM propranolol, 1 μM NA, 30 μM NA, 30 μM NA + 1 μM propranolol, ISO (1 μM) or vehicle (0.1 mM ascorbate). Forskolin increased cAMP levels over 10 fold (287 ± 109 pmol mg^{-1} protein) compared to the basal state (21.8 ± 3.5 pmol mg $^{-1}$protein), and levels of both were unaltered by any pretreatments. (A) ADR (300 nM) treatment reduced brimonidine efficacy ($n=7$; $*P<0.05$ by ANOVA) compared to vehicle in BE(2)-C cells cultured up to passage 50. Alpha$_2$-AR desensitization was not noted if the β-AR antagonist propranolol (30 nM) was included during chronic ADR exposure ($n=6$; $\#P<0.05$ as compared to ADR by ANOVA). Propranolol (30 nM) alone ($n=5$) had no effect on α_2-AR signalling. (B) Chronic 1 μM NA treatment did not alter the α_2-AR signal as compared to vehicle ($n=6-9$) but 30 μM NA treatment reduced brimonidine potency and efficacy ($n=3$; $*P<0.05$). Inclusion of a higher propranolol concentration (1 μM) blocked 30 μM NA-induced loss of α_2-AR potency, but not efficacy. Neither 1 μM ISO nor 1 μM propranolol alone altered the α_2-AR response (data not shown). (C) To better visualize the ability of different concentrations of ADR and NA to activate α_2-ARs in BE(2)-C cells, the concentration-response curves from which the values in Table 2 were derived for each agonist are shown. Each point represents the mean ± s.e.mean of three (ADR) or five (NA) independent observations, performed in duplicate. (D) Chronic 300 nM ADR treatment does not produce a significant reduction of the brimonidine response as compared to vehicle ($n=3$) in higher passage cells in which β-AR binding and responsiveness has dropped off (Table 1).

treatment does not alter brimonidine efficacy, underscoring the contribution of the β-AR to α_2-AR desensitization by ADR (Figure 1D).

To establish that α_2-AR desensitization was not produced by activation of the β-AR alone, and that it required simultaneous activation of both α_2- and β-ARs, cells were pretreated with the β-AR-specific agonist, ISO. ISO pretreatment (1 μM) had no effect on brimonidine potency (log M EC_{50} vehicle -7.44 ± 0.14 *vs* ISO -7.59 ± 0.23) or efficacy (I_{max} value: ISO 70.2 ± 2.65; vehicle 69.7 ± 7.3; $n=2-6$). None of the pretreatments altered basal or forskolin-stimulated levels of cAMP accumulation compared to vehicle-treated controls. Together, these results confirm previous reports that treatment with very high concentrations

of catecholamines are required to desensitize the α_2-AR response (Eason & Ligget, 1992; Lanier *et al*., 1996) if no β-ARs are present or activated, but that much lower concentrations of catecholamines can produce α_2-AR desensitization if activating both α_2-AR and β-ARs simultaneously.

To confirm the β_2-AR classification, the β-AR response to 100 nM ISO was dose-dependently reduced by the β_2-selective antagonist, ICI 118,551, while the β1-selective antagonist, CGP 20712A was inactive at the highest concentration tested (1 μM; Figure 3). These results indicate that ISO increases cAMP accumulation through β_2-ARs in BE(2)-C cells. In addition, propranolol (30 nM) also blocked β-AR stimulation of cAMP accumulation, supporting its effectiveness at blocking the β-AR in the desensitization studies (Figure 3). Binding studies (Altememi *et al*., 1997; data not shown) further supported the β_2-AR subtype distinction.

To determine whether ADR-induced desensitization of α_2-ARs was a result of receptor down-regulation, changes in levels of specific α_2-AR binding were measured using a single concentration of radioligand. This was sufficient for accurate assessment of changes in receptor number since α_2-AR down-regulation does not change radioligand affinity as observed by our own (K_d values: vehicle 0.95±.03; ADR 0.71±.01 nM) and others' studies (Eason & Liggett, 1992). Alpha$_2$-AR levels were reduced 60% following chronic 300 nM ADR treatment (Figure 4; $P<0.05$), consistent with the functional deficits noted (Figure 1). Furthermore, inclusion of 30 nM propranolol to block activation of β_2-ARs during chronic ADR treatment also prevented the α_2-AR down-regulation; propranolol treatment alone had no effect. Alpha$_2$-AR levels were also reduced following 24 h pretreatment with 30 μM NA (Figure 4; $P<0.05$), but were unaltered by 1 μM NA or ISO treatments that also failed to produce α_2-AR desensitization.

Since down-regulation of the α_{2A}-AR subtype requires pretreatment with higher agonist concentrations than that of α_{2B}- and α_{2C}-ARs (Heck & Bylund, 1997; Deupree *et al*., 2002), various binding and antagonism studies were conducted to determine the α_2-AR subtype expressed in BE(2)-C cells. The nonselective α_2-AR antagonists, rauwolscine and yohimbine, competed for specific [^{3}H]-rauwolscine binding to membranes with high affinity (Table 2; Figure 5A); the two $\alpha_{2B/C}$-selective antagonists, ARC-239 and prazosin, displaced [^{3}H]-rauwolscine from binding sites with 100–400 fold lower affinity than rauwolscine. Hill slope values of antagonist binding did not differ from unity, consistent with binding to a single site (Table 2). The various α-AR agonists showed significant differences in their ability to competitively displace [^{3}H]-rauwolscine from its binding site (Table 2), with the more α_{2A}-AR-preferring agonists (oxymetazoline, brimonidine, guanabenz) and nonselective agonists (ADR, *p*-aminoclonidine) exhibiting higher affinities than the $\alpha_{2B/C}$-AR-preferring agonist, agmatine or the α_1-AR agonist, phenylephrine. Unlike the α_2-AR agonists, phenylephrine

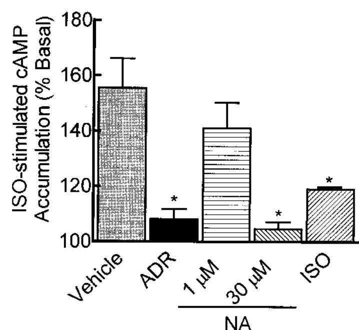

Figure 2 Chronic 300 nM ADR, 30 μM NA, and 1 μM ISO desensitize the β-AR response in BE(2)-C cells. BE(2)-C cells were incubated for 16–24 h with vehicle (0.1 mM ascorbate), 300 nM ADR, 1 μM NA, 30 μM NA or 1 μM ISO. After washing, intact cells were assessed for ISO-stimulated (250 nM) cAMP accumulation. Chronic 300 nM ADR, 30 μM NA, and 1 μM ISO ($n=2-9$; $*P<0.05$), but not 1 μM NA ($n=6$), pretreatment desensitized the β-AR response to ISO compared to the corresponding vehicle-treated control. None of the pretreatments altered basal cAMP levels (27.6±6.1 pmol mg^{-1} protein). Data represent mean±s.e.mean; comparisons were made by ANOVA with Dunnett's *post-hoc* test.

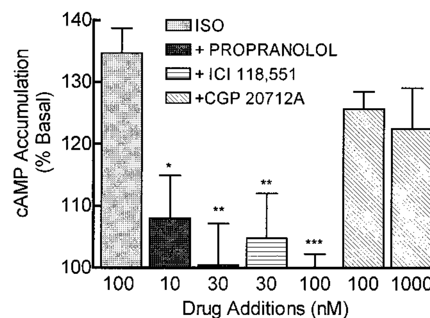

Figure 3 The ISO response in BE(2)-C cells is mediated through β_2-ARs. Cells were assayed for ISO-stimulated accumulation of cAMP in the presence and absence of three different β-AR antagonists. The non-selective antagonist propranolol and the β_2-selective antagonist ICI 118,551 antagonized the stimulatory effect of ISO (100 nM), while the β_1-selective antagonist CGP 20712A was without effect ($*P<0.05$, $**P<0.01$, $***P<0.001$ by ANOVA; $n=3-13$). Antagonists alone had no effect on basal cAMP levels at these concentrations (data not shown).

Table 1 Effect of cell passage number on α_2- and β-ARs in BE(2)-C cells

AR Subtype	*Cell Passage #*	K_d (nM)	B_{max} (fmol/mg)	*Brimonidine* *potency* ($LogEC_{50}$)	*Brimonidine* *Maximal inhibition* (%)	*250 nM ISO* (% Basal)
α_{2A}	16–48	3.2±0.6	40.8±7.0 (6)	−7.33±0.13	70.9±3.9	–
α_{2A}	50–58	1.9±0.2	39.1±9.6 (3)	−6.98±0.53	64.0±11	–
β_2	16–48	0.2±0.1	18.5±6.2 (9)	–	–	170.5±8
β_2	50–58	0.2±0.1	1.3±0.5* (4)	–	–	113.6±14**

Number of independent determinations is given in parentheses following the values (mean±s.e.mean). Values differ significantly compared to the same assay performed in lower passage cells ($*P<0.05$ and $**P<0.005$) by Student's *t*-test.

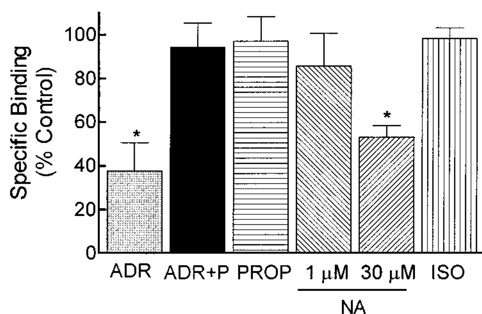

Figure 4 Chronic ADR treatment down-regulates α_2-ARs. BE(2)-C cells were incubated for 16–24 h with vehicle (0.1 mM ascorbate), 300 nM ADR, 30 nM propranolol, ADR + 30 nM propranolol, 1 μM NA, 30 μM NA or 1 μM ISO. Specific binding to cell membrane homogenates was calculated by subtracting the binding of a single concentration of radioligand (2 nM) in the presence of phentolamine (10 μM) from the binding in its absence. ADR profoundly down-regulated α_2-AR levels, but this down-regulation was blocked by the β-AR antagonist, propranolol (*$P<0.05$ by ANOVA). Chronic 30 μM NA treatment also reduced α_2-AR levels while ISO, 1 μM NA or propranolol alone had no effect. Data are presented as mean ± s.e.mean of 3–5 independent experiments; α_2-AR binding in control cells was 1143 ± 283 c.p.m. mg^{-1} protein.

Table 2 Characteristics of α_2-ARs in BE(2)-C cells

Agonist	*log*(K_i)	*log*(EC_{50})	*I.A.*
ADR	−7.74 ± 0.18	−7.37 ± 0.15	1.00
NA	−6.11 ± 0.15	−6.72 ± 0.25	1.00
Brimonidine	−7.77 ± 0.23	−7.22 ± 0.08	0.78
Oxymetazoline	−7.91 ± 0.08	−7.79 ± 0.39	0.55
Guanabenz	−8.15 ± 0.18	−7.73 ± 0.44	0.76
p-aminoclonidine	−7.20 ± 0.18	−6.72 ± 0.11	0.85
Phenylephrine	> −6.0	−5.69 ± 0.40	0.30
Agmatine	> −6.0	N.D.	N.D.
Antagonist	*log*(K_i)	η_H	K_i *Ratios with OXY*
Rauwolscine	−8.38 ± 0.12	1.0 ± 0.4	
Yohimbine	−8.10 ± 0.17	0.9 ± 0.4	1.54
Prazosin	−5.76 ± 0.70	1.1 ± 0.4	141
ARC-239	−6.38 ± 0.09	0.7 ± 0.1	
Idazoxan	−7.06 ± 0.14	2.0 ± 0.5	

Binding displacement and cAMP accumulation studies were performed as described in Methods. The values of the apparent affinity constants Log(K_i) for each competitor were derived from their IC_{50} values ($n=3-9$) using the equation of Cheng & Prusoff (1973). The Log(EC_{50}) values (concentration of the drug that produces 50% of the maximal inhibitory effect of that drug) were calculated by nonlinear regression analysis of the agonist concentration-inhibition curves ($n=3-9$) of each agonist. Affinity ratios for prazosin:OXY and OXY:yohimbine were calculated as a ratio of their respective K_i values. Intrinsic activity (I.A.) was calculated based upon the maximal inhibition of forskolin-stimulated cAMP accumulation (%) by the highest concentration of agonist tested normalized to ADR as a full agonist. Maximal inhibition by ADR was 80 ± 3%. N.D., not determined.

exhibited only weak, partial agonist actions in cyclase assays (Table 2). To further distinguish between the different α_2-AR subtypes that could be expressed in BE(2)-C cells, another process was employed comparing the affinity ratios of prazosin to OXY or OXY to yohimbine (Hieble *et al*., 1997; Table 2). Prazosin : OXY (141) and OXY : yohimbine (1.54) ratios were within the range reported for native and recombinant α_{2A}-ARs, and differ by at least 10 fold from α_{2C}-AR values (from Hieble *et al*., 1997). Apparent K_i values were generated for comparison with previously reported values from cells natively expressing α_{2A}-, α_{2B}, or α_{2C}-ARs (HT29, NG108-15, OK; Gleason & Hieble, 1991; Blaxall *et al*., 1991) or cell lines expressing cloned α_2C10, α_2C2, and α_2C4 (Jansson *et al*., 1994; Jasper *et al*., 1998). Values obtained from our binding studies correlated significantly with those derived from native and cloned α_{2A}-AR-containing cell membranes only (Table 3), with no correlation in cells or membranes containing α_{2B} or α_{2C}-ARs.

The classification of α_2-AR is further borne out by functional antagonism studies. Inhibition of cAMP accumulation by brimonidine (100 nM; Figure 5B) in BE(2)-C cells was reversed by rauwolscine in a concentration-dependent manner, whereas the $\alpha_{2B/C}$-selective antagonist ARC-239 failed to reverse the actions of the agonist. This further supports the classification of the functional α_2-AR subtype in this neuroblastoma cell line as α_{2A}.

Previous studies have shown that chronic activation of the α_2-AR leads to down-regulation of inhibitory G-proteins (G_αi; Jewell-Motz *et al*., 1998; Eason & Liggett, 1992). We observed a small reduction in G_αi3 levels following chronic 300 nM ADR treatment, but this effect never quite achieved significance (Table 4).

Chronic ADR up-regulates GRK3 facilitating α_{2A}-AR desensitization and down-regulation

β-AR-dependent α_2-AR desensitization could occur *via* β_2-AR activation of protein kinase A (PKA; Reutter *et al*., 1998), G-protein-coupled receptor kinase 2 or 3 (GRK2/3; Benovic *et al*., 1987) or PKC (Shih & Malbon, 1994). Cyclic AMP levels in the cell would need to be increased by ADR treatment in order to activate the cAMP-dependent enzyme, PKA. In the absence of forskolin, ADR (300 nM) alone has little effect on cAMP levels (13.8 ± 10.5% inhibition of basal; n = 3), even though β_2-, as well as α_2-ARs, are activated. This suggests that α_2-AR desensitization is not PKA-dependent.

Agonist-occupied α_{2A}-AR is a substrate for GRK2 and GRK3, but not for GRK5 or GRK6 (Jewell-Motz & Liggett, 1996). To examine the role of GRKs in β_2-AR-faciliated α_{2A}-AR desensitization, we utilized antisense DNA that is common to a coding sequence found in both GRK2 and GRK3 (GRK 2/3), but not present in GRK5 or GRK6. Cells were pretreated with either the GRK2/3 antisense or the corresponding sense DNA for 4 h in serum free media (Shih & Malbon, 1994), before adding sera back for the remainder of the treatment period (24 h ± ADR). Under these conditions, 300 nM ADR-induced α_2-AR desensitization was blocked by GRK2/3 antisense, but not sense, treatment (Figure 6A). Antisense and sense treatments alone were without effect on the α_2-AR response. Pretreatment with GRK2/3 antisense also ablated chronic 300 nM ADR-induced α_2-AR down-regulation (Figure 6B), suggesting that GRK regulates both desensitization and downregulation of the α_2-AR following prolonged agonist treatment. This is consistent with the idea that α_2-AR desensitization results from α_2-AR down-regulation. Cell lysates from these experiments were resolved on SDS–PAGE to insure that the GRK 2/3

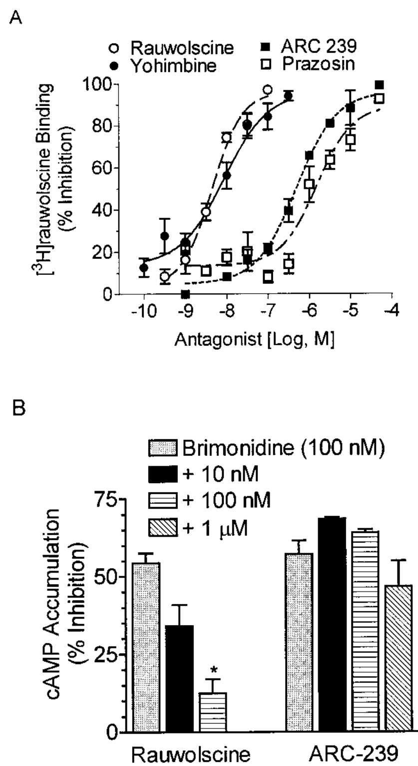

Figure 5 Alpha$_{2A}$-AR antagonists displace [^{3}H]-rauwolscine binding and block brimonidine-mediated inhibition of cAMP accumulation. (A) Binding assays were performed as described in 'Methods' using [^{3}H]-rauwolscine in the presence of 6–8 concentrations of rauwolscine, yohimbine, ARC 239, or prazosin in BE(2)-C cell membrane homogenates. Curves were best fit by nonlinear regression analysis (GraphPad Prism, San Diego, CA, U.S.A.). Each point is the mean±s.e.mean of triplicate determinations from 3–8 experiments. (B) Rauwolscine dose-dependently and significantly antagonized the ability of brimonidine (100 nM) to inhibit cAMP accumulation, while the $\alpha_{2B/C}$-AR-selective antagonist, ARC-239, had no effect. The results represent the mean±s.e. of 3–9 experiments, performed in duplicates.

Table 3 Correlation of human neuroblastoma cell α_2-AR pK_i values with native and cloned α_2-AR subtypes

Comparison	*No. of values compared*	*Correlation coefficient*	*Slope*	P *value*
v. HT29[a,b]	7	0.90*	1.18±0.29	**0.01**
v. NG108-15[a,b]	7	0.31	0.27±0.41	0.55
v. OK[a,b]	7	0.79	0.92±0.44	0.13
v. α_2C10[c]	10	0.95*	0.93±0.14	**0.05**
v. α_2C2[c]	10	0.10	0.08±0.37	0.94
v. α_2C4[c]	10	0.50	0.42±0.33	0.68

Correlation coefficient values (r) were generated by comparing pK_i values from Table 2 with previously published values for one-site models (Gleason & Hieble, 1991[a]; Blaxall *et al.*, 1991[b]; Jansson *et al.*, 1994[c]) using Pearson correlation analysis (GraphPad Prism). The slope of the linear regression line is also included. Correlations were considered significant (*) if $P \leqslant 0.05$.

antisense ODN reduced GRK 2/3 protein levels. Using antibodies recognizing both GRK2 and GRK3, we observed that chronic 300 nM ADR pretreatment alone up-regulated GRK 2/3 (Figure 6A, insert) and GRK 2/3 antisense blocked the up-regulation. Utilizing antibodies specific for GRK2, GRK3, or GRK5 isoforms, we found that chronic ADR preferentially up-regulated GRK3 (Figure 7A); levels of GRK6 in these cells are too low to consistently detect by Western blotting. Inclusion of propranolol (30 nM; Figure 7A) blocked this up-regulation. Neither chronic 1 μM NA nor 250 nM ISO treatment altered GRK3 levels, but after increasing the NA concentration 30 fold (Figure 7B), GRK3 levels were up-regulated as noted with ADR pretreatment. Chronic 30 μM NA-induced GRK3 up-regulation was partially ablated by the addition of 1 μM propranolol, similar to the desensitization results (Figure 1B), though this ablation failed to achieve significance by ANOVA. Levels of GRK5 were neither up-regulated by chronic treatments (Figure 7), nor altered by GRK2/3 antisense treatment (data not shown).

Table 4 G-protein levels following treatment in BE(2)-C cells

Treatment	*G-protein:GADPH Ratio* (% Control) $G_\alpha i3$	$G_\alpha i1/2$	$G_\alpha 0$
300 nM ADR	68.38±4.99 (6) P=0.17	92.08±15.82 (5) P=0.98	105.90±21.79 (6) P=0.61
1μM NA	83.83±3.12 (3) P=0.67	N.D.	N.D.

Number of independent determinations is given in parentheses following the values (mean±s.e.mean). P values were generated by unpaired t-tests comparing the treatment with its respective vehicle. N.D. not determined. Optical density ratios of $G_\alpha i3$, $G_\alpha i1/2$, $G_\alpha 0$ to GADPH in control cells were 2.3±0.6, 0.8±0.2 and 0.6±0.2, respectively.

Acute 300 nM ADR and 1 μM NA induce α_2-AR desensitization

In the present study, chronic co-activation of α_2- and β_2-AR by 300 nM ADR induces α_2-AR desensitization while activation of α_2-AR alone by 1 μM NA has no effect. To determine whether the same mechanism regulates α_2-AR signalling with acute agonist treatment, cells were treated with ADR (300 nM) or NA (1 μM) for only 60 min. Unlike chronic treatments, both acute 300 nM ADR and 1 μM NA pretreatments reduced brimonidine efficacy as compared to vehicle ($P<0.05$; Figure 8) in BE(2)-C cells. As noted with chronic ADR treatment, brimonidine potency was reduced by only 2 fold. Addition of 30 nM propranolol to either acute 300 nM ADR- (Figure 8) or 1 μM NA-treated cells (data not shown) failed to block α_2-AR desensitization. Therefore, α_2-ARs in these cells can desensitize following acute exposure to both 300 nM ADR and 1 μM NA suggesting that co-activation of the β_2-AR is not necessary for acute α_2-AR desensitization by submaximal concentrations of catecholamines. These results also indicate the 1 μM NA is sufficient to produce α_2-AR desensitization, but that mechanisms of α_2-AR desensitization vary with different times of agonist exposure.

Discussion

Cross-regulation of two opposing signalling cascades activated by the same endogenous neurotransmitter is a complex

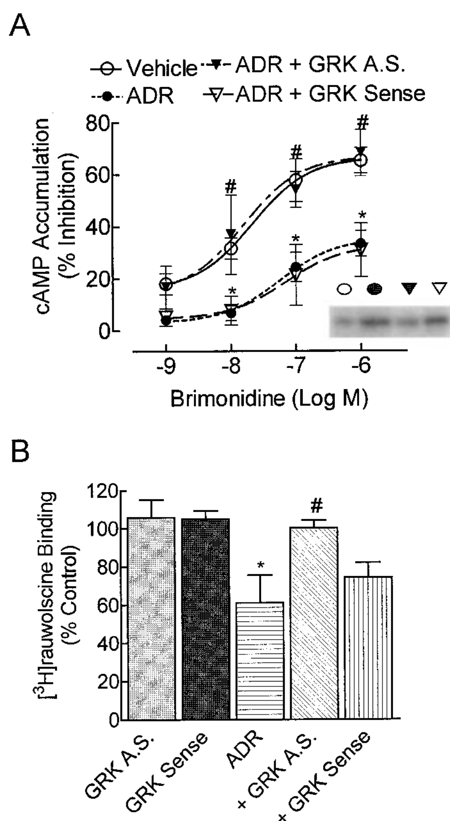

Figure 6 GRK antisense ODN treatment inhibits chronic 300 nM ADR-induced α_2-AR desensitization and down-regulation. BE(2)-C cells were washed with serum-free media and incubated in the presence or absence of an antisense (AS) or sense ODN recognizing sequences common to both GRK2 and 3 (20 μM) for 4 h. Serum was returned to the media for an additional 24 h with ADR (300 nM) or vehicle. (A) Again, 300 nM ADR treatment reduced brimonidine potency and efficacy ($n=8$; $^*P<0.001$ by ANOVA) as compared to vehicle-treated cells. Treatment with GRK antisense ($n=5$), but not GRK sense ($n=3$) ODNs reversed these effects and prevented ADR-induced α_2-AR desensitization ($P<0.05$). Treatment with GRK antisense ($n=3$) or sense ($n=4$) ODNs alone had no effect (data not shown). Insert: 30 μg of whole cell lysate from each treatment group was resolved by SDS–PAGE through a 10% gel as indicated by the representative blot. Chronic 300 nM ADR increased GRK levels using antisera common to GRK2 and GRK3 ($n=4-5$; $^*P\leqslant0.05$) compared to vehicle-treated controls. GRK antisense ODN treatment blocked ADR-induced increases in GRK expression as determined by densitometric analysis of immunoblots normalized to a GAPDH loading control. (B) Specific binding to cell membrane homogenates was calculated by subtracting the binding of [^{3}H]-rauwolscine (2 nM) in the presence of phentolamine (10 μM) from the binding in its absence. GRK antisense ODN ablated chronic ADR-induced α_2-AR down-regulation ($P<0.05$ by ANOVA) while GRK sense ODN had no effect. Neither GRK antisense nor sense ODN alone altered α_2-AR levels. Data are presented as mean$\pm$s.e.mean of four independent experiments; α_2-AR binding in control cells was 2815 ± 525 c.p.m. mg^{-1} protein.

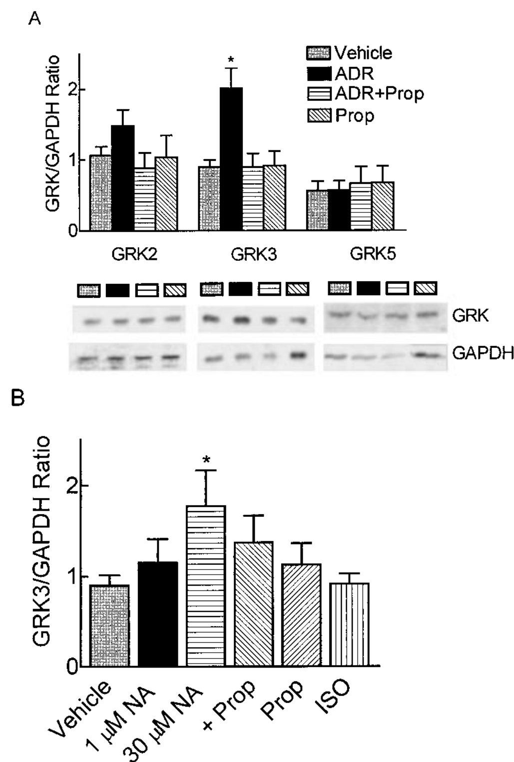

Figure 7 β_2-AR-dependent GRK3 up-regulation. BE(2)-C cells were treated with vehicle (0.1 mM ascorbate), 300 nM ADR, propranolol (30 nM or 1 μM), ADR + 30 nM propranolol, 1 μM NA, 30 μM NA, 30 μM NA + 1 μM propranolol, or 250 nM ISO for 24 h. Thirty μg of whole cell lysate from each treatment group was resolved by SDS–PAGE through a 10% gel. Immunoreactive bands were normalized to the GAPDH loading control. (A) GRK isoform-specific antibodies indicated that the GRK3 isoform, but not GRK2 or GRK5, was significantly up-regulated following chronic 300 nM ADR treatment ($n=4$; $^*P\leqslant0.05$) compared to vehicle-treated controls. Addition of 30 nM propranolol ablated ADR-induced GRK3 up-regulation while 30 nM propranolol alone had no effect. (B) Chronic 30 μM NA treatment up-regulated GRK3 ($^*P\leqslant0.05$) similar to ADR treatment while treatment with 1 μM NA, 250 nM ISO, or 1 μM propranolol alone had no effect on GRK3 levels. Though inclusion of 1 μM propranolol partially prevented 30 μM NA-induced GRK3 up-regulation, it was not significantly different from 30 μM NA by ANOVA with Tukey's *post-hoc* test.

physiological event. Several *in vivo* studies suggested that α_2-AR desensitization following prolonged agonist exposure was regulated by β_2-AR activation (Schwartz & Eikenburg, 1988; Apparsundaram & Eikenburg, 1996), however the mechanism of β_2-AR-faciliated α_2-AR desensitization was not examined. Further elucidation of the cellular mechanisms responsible for this cross-talk required the study of this process in a cell system expressing both β_2- and α_2-ARs, preferably at physiological levels. Therefore, we set out to determine how simultaneous activation of natively expressed β_2- and α_2-ARs by chronic 300 nM ADR in BE(2)-C cells produces α_2-AR desensitization, while activation of the α_2-AR alone by 1 μM NA fails to do so.

β_2-AR involvement in α_2-AR desensitization is clearly implicated by several results in the present study. First, α_2-AR desensitization occurred following chronic 300 nM ADR and 30 μM NA, but not 1 μM NA or ISO treatments. Lands *et al.* (1967) established that ADR has a higher potency at β_2-ARs than NA, while ADR and NA have equal potencies at each α_2-AR subtype (Lanier *et al.*, 1996; Jasper *et al.*, 1998; Deupree *et al.*, 2002). Therefore, chronic 300 nM ADR desensitized the α_2-AR response by activating both α_2- and β_2-ARs simultaneously, while 1 μM NA or ISO activated only α_2- or β_2-ARs, respectively. Only when a higher concentration of NA was used (30 μM), a concentration that activates both α_2- and β-ARs, was α_2-AR desensitization noted.

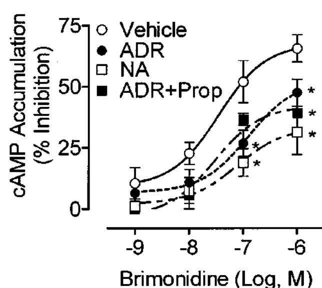

Figure 8 Acute 300 nM ADR and 1 μM NA induce β_2-AR-independent α_2-AR desensitization. The α_2-AR response as determined by the ability of brimonidine to inhibit forskolin (10 μM)-stimulated cAMP accumulation in intact BE(2)-C human neuroblastoma cells was measured 1 h after pretreatment with any or all of the following: vehicle (0.1 mM ascorbate), 300 nM ADR, 30 nM propranolol, ADR + 30 nM propranolol, or 1 μM NA. Both 300 nM ADR and 1 μM NA treatment reduced brimonidine potency and efficacy ($n=5-6$; $*P<0.05$ by ANOVA) compared to vehicle in BE(2)-C cells cultured up to passage 50. Addition of 30 nM propranolol to either ADR or NA (data not shown) treatment failed to block α_2-AR desensitization.

Second, α_2-AR desensitization produced by modest levels of ADR was observed only when the cells expressed both α_2- and β_2-ARs. When BE(2)-C cells are cultured beyond 50 passages, β-AR expression is lost and 300 nM ADR fails to produce α_2-AR desensitization. Finally, the β-AR antagonist, propranolol prevents chronic ADR (300 nM)-promoted α_2-AR desensitization and partially antagonizes α_2-AR desensitization produced by the higher NA concentration (30 μM). Collectively, these observations illustrate that chronic simultaneous activation of α_2- and β_2-ARs enables α_2-AR desensitization at lower concentrations of ADR than NA. Moreover, since the degree of α_2-AR desensitization produced by the higher concentration of NA is partially reduced by β-AR blockade, it suggests that the β-AR may also enhance α_2-AR desensitization at higher concentrations of NA that also activate both α_2- and β-AR.

Acute 300 nM ADR treatment also desensitized the α_2-AR, but unlike chronic treatment, the desensitization was independent of β_2-AR activation as indicated by several results. First, both acute 300 nM ADR (co-activating α_2- and β_2-ARs) and 1 μM NA (activating only α_2-ARs) treatments induced α_2-AR desensitization to a similar extent. Second, inclusion of 30 nM propranolol during 300 nM ADR or 1 μM NA treatments failed to block α_2-AR desensitization. The fact that 1 μM NA desensitizes the α_2-AR after acute, but not chronic, treatment underscores the difference in mechanisms responsible for α_2-AR regulation following short- and long-term agonist exposure.

The present study is the first to provide evidence for the mechanisms that contribute to β_2- AR-dependent α_2-AR desensitization after chronic ADR exposure. When recombinant α_2-ARs were expressed in the absence of β-ARs, long-term (24 h) agonist-induced α_2-AR desensitization was attributed to α_2-AR (Pleus *et al*., 1993; Heck & Bylund, 1997; Jewell-Motz *et al*., 1997) and $G_{\alpha}i/o$ down-regulation (Eason & Liggett, 1992; Liggett *et al*., 1992; Jewell-Motz *et al*., 1998). β_2-AR-dependent α_2-AR desensitization appears to involve α_2-AR down-regulation more than reduction of $G_{\alpha}i/o$. Since the three α_2-AR subtypes have different propensities to down-regulate (Pleus *et al*., 1993), binding and functional studies were utilized to establish the α_2-AR subtype expressed in BE(2)-C cells. Several results support the α_{2A}-AR designation. First, rauwolscine and yohimbine compete for [^{3}H]-rauwolscine binding with much higher affinity than the $\alpha_{2B/C}$-AR selective antagonists ARC-239 and prazosin, that displayed only a weak displacement capability. Second, when apparent p*Ki* values for various α_2-AR agonists and antagonists binding to BE(2)-C membrane homogenates were compared with previously reported values in cell lines expressing a single α_2-AR subtype, a significant correlation was observed only with those cells that expressed α_{2A}-AR. Finally, the inhibitory effect of α_2-AR agonists on cAMP production is readily reversed in a concentration-dependent fashion by the antagonist rauwolscine. The failure of the selective $\alpha_{2B/C}$-AR antagonist ARC-239 to produce any antagonism of brimonidine is consistent with activation of α_{2A}-AR in BE(2)-C cells. Alpha$_{2A}$-ARs require exposure to higher agonist concentration to induce receptor down-regulation than α_{2B} or α_{2C}-ARs (Deupree *et al*., 2002). In the present study, we report down-regulation of the α_{2A}-AR by ADR after exposure to much lower agonist concentrations than previously reported for this subtype (Eason & Liggett, 1992; Pleus *et al*., 1993; Jewell-Motz *et al*., 1997). Whether this is due to more physiological AR levels in our system, a more physiological concentration of ADR (300 nM), a β_2-AR-facilitated effect permitting down-regulation of the α_2-AR at lower ADR concentrations, or a combination of these effects is not clear. Nevertheless, our results suggest the β_2-ARs on BE(2)-C cells regulate the α_2-AR response, in part, by directly modulating the level of α_{2A}-ARs.

The results of the present study differ from previous reports where the effect of β-AR activation on inhibitory receptor number/function has been studied in other cells/tissues. For example, in HT-29 cells, 24 h stimulation of adenylyl cyclase with forskolin or cAMP analogues resulted in an increase in inhibitory α_{2A}-AR expression (Sakaue & Hoffman, 1991). That response was attributed, in part, to increased α_{2A}-AR gene expression by cAMP response elements in the AR gene. A major difference between our study and these previous studies is that we simultaneously activated both the stimulatory (β_2-AR) and inhibitory (α_2-AR) receptors during chronic treatment. This difference is significant for two reasons. First, chronic simultaneous activation of α_2- and β_2-ARs in BE(2)-C cells by ADR resulted in α_{2A}-AR down-regulation. Second ADR treatment does not induce cAMP accumulation even though both α_2- and β_2-AR are simultaneously activated. Therefore it seems unlikely that cAMP or PKA contributed to the response in the present study. The mechanism most likely to contribute to the β_2-AR-dependent α_2-AR desensitization is G protein coupled receptor kinases (GRKs).

Recently several reports have implicated GRKs in regulation of receptor signalling following prolonged agonist treatment (McGraw *et al*., 1998; Thakker & Standifer, 2002). Several lines of evidence support a role for GRKs in the present study. First, chronic ADR treatment of BE(2)-C cells resulted in up-regulation of GRK3 expression. Since the α_{2A}-AR is a substrate for GRK3 when agonist-occupied (Benovic *et al*., 1987; Jewell-Motz & Liggett, 1996), an increase in the expression of GRK3 could render the α_2-AR more readily desensitized upon re-challenge with the α_2-AR agonist (brimonidine). In support of this suggestion, injection of

recombinant GRK3 into isolated chick dorsal root ganglion neurons increased GRK3 levels in those cells and rendered the α_2-AR more susceptible to homologous desensitization (Diverse-Pierluissi *et al.*, 1996). This effect was specific for GRK3 as increasing levels of GRK2 failed to facilitate α_2-AR desensitization. Second, β-AR blockade eliminates α_2-AR desensitization as well as the up-regulation of GRK3 by ADR. Moreover, antisense ODNs, which block GRK3 up-regulation, also prevent ADR-induced α_2-AR desensitization and down-regulation. A recent study by Deupree *et al.* (2002) reports that intact GRK phosphorylation sites are required for the down-regulation of the α_{2C}-AR, suggesting that GRK phosphorylation regulates α_{2C}-AR down-regulation. Similarly in the present study up-regulation of GRK3 is required for the profound α_2-AR down-regulation induced by modest ADR concentrations. In fact, in the present study, 300 nM ADR produced desensitization and down-regulation of the α_{2A}-AR at least as marked as that reported at 30–300 fold higher concentrations of ADR by other investigators (Eason & Liggett, 1992; Jewell-Motz *et al.*, 1997). Therefore, we suggest that chronic simultaneous activation of α_2- and β_2-ARs by ADR renders the α_2-AR more sensitive to homologous desensitization and α_2-AR down-regulation via GRK3 up-regulation.

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