Partial Agonist Clonidine Mediates \(\alpha_2\)-AR Subtypes Specific Regulation of cAMP Accumulation in Adenyl Cyclase II Transfected DDT1-MF2 Cells

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Received July 26, 2000; accepted October 4, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

\(\alpha_2\)-Adrenergic receptor (\(\alpha_2\)-AR) activation in the pregnant rat myometrium at midterm potentiates \(\beta_2\)-AR stimulation of adenylyl cyclase (AC) via \(\beta\gamma\) regulation of the type II isoform of adenylyl cyclase. However, at term, \(\alpha_2\)-AR activation inhibits \(\beta_2\)-AR stimulation of AC. This phenomenon is associated with changes in \(\alpha_2\)-AR subtype expression (midterm \(\alpha_{2A/D}\)-AR \(\gg\) \(\alpha_{2B}\)-AR; term \(\alpha_{2B} \geq \alpha_{2A/D}\)-AR), without any change in ACII mRNA, suggesting that \(\alpha_{2A/D}\)- and \(\alpha_{2B}\)-AR differentially regulate \(\beta_2\)-cAMP production. To address this issue, we have stably expressed the same density of \(\alpha_{2A/D}\)- or \(\alpha_{2B}\)-AR with AC II in DDT1-MF2 cells. Clonidine (partial agonist) increased \(\beta_2\)-AR-stimulated cAMP production in \(\alpha_{2A/D}\)-AR-ACII transfectants but inhibited it in \(\alpha_{2B}\)-AR-ACII transfectants. In contrast, epinephrine (full agonist) enhanced \(\beta_2\)-stimulated ACII in both \(\alpha_{2A}\) and \(\alpha_{2B}\)-ACII clonal cell lines. 4-Azidoanilido-[\(\alpha^{32}\text{P}\)]GTP-labeling of activated G proteins indicated that, in \(\alpha_{2B}\)-AR transfectants, clonidine activated only Gi\(_2\), whereas epinephrine, the full agonist, effectively coupled to Gi\(_2\) and Gi\(_3\). Thus, partial and full agonists selectively activate G proteins that lead to drug specific effects on effectors. Moreover, these data indicate that Gi\(_3\) activation is required for potentiation of \(\beta_2\)-AR stimulation of AC by \(\alpha_{2A/D}\) and \(\alpha_{2B}\)-AR in DDT1-MF2 cells. This may reflect an issue of the amount of \(\beta\gamma\) released upon receptor activation and/or \(\beta\gamma\) composition of Gi\(_3\) versus Gi\(_2\).

In pregnant rat myometrium, \(\alpha_2\)-adrenoceptor (AR) signaling pathways differentially modulate \(\beta_2\)-AR-mediated regulation of adenylyl cyclase (AC) at midpregnancy and at term (Mhaouty et al., 1995). At midterm, \(\alpha_2\)-AR activation potentiates adenylyl cyclase activity stimulated by \(\beta_2\)-AR, thus enhancing uterine relaxation in response to catecholamines. This augmentation of AC activity induced by \(\alpha_2\)-AR probably involves the type II family isoform of AC and is caused by the input of \(\beta\gamma\) released from Gi (Gi\(_2\) and/or Gi\(_3\)) that synergizes with Gs to further elevate cAMP levels (Mhaouty-Kodja et al., 1995). In contrast, at term, myometrial \(\alpha_2\)-AR/Gi signaling pathways reduce the \(\beta_2\)-AR-induced-cAMP generation to allow intracellular \(Ca^{2+}\) increase and cell contraction. This switch in the stimulatory versus inhibitory input to \(\beta_2\)-AR-dependent cAMP generation that occurs between mid- and late pregnancy may be influenced by changes in the expression of AC isoforms, G proteins, and/or \(\alpha_2\)-AR subtypes expression.

When comparing mid- and late pregnancy, no substantial modification in the amounts of specific types of AC transcripts and no alteration in the basal activity of the AC system (Mhaouty-Kodja et al., 1997) could be found. In particular, the expression of transcripts encoding for members of AC type II family, which are involved in this potentiation process, persisted throughout the course of pregnancy up to parturition (Mhaouty-Kodja et al., 1997). Conversely, as shown by pharmacological data (Bouet-Alard et al., 1997) and Northern blot analysis (Mhaouty et al., 1995), the two \(\alpha_2\)-AR subtypes expressed in rat myometrium, \(\alpha_{2A}\) and \(\alpha_{2B}\)-AR, were differentially expressed at midpregnancy and term (midpregnancy \(\alpha_{2A/D}\)-AR \(\gg\) \(\alpha_{2B}\)-AR; term \(\alpha_{2B} \geq \alpha_{2A/D}\)-AR). Also, significant changes in the Gi\(_2\)/Gi\(_3\) ratio could be detected by immunoblot analysis (Tanfin et al., 1991; Cohen-Tannoudji et al., 1995). Altogether, these data suggested that the switch of regulation mediated by \(\alpha_2\)-adrenoceptors toward \(\beta_2\)-dependent cAMP production could result from a specific signaling of \(\alpha_2\)-AR subtypes toward AC II activity and/or alteration in receptor coupling to G proteins.

As an initial approach, we used DDT1-MF2 (hamster vas...
deferens smooth muscle cell) cotransfectants stably expressing \( \alpha_{2D,TR}-2 \)-AR (RG20) or \( \alpha_{2B}-2 \)-AR (\( \alpha_{2B}C2 \)) and AC type II isof orm and studied the regulation induced by each \( \alpha_{2}-2 \)-AR subtypes on \( \beta_{3} \)-stimulated AC II activity.

We report, herein, agonist and receptor specific regulation of ACII that involves selective coupling to Gi2 versus Gi3. Our results also shed light upon molecular mechanism by which clonidine acts as a partial agonist through \( \alpha_{2B}-2 \)-AR.

Materials and Methods

\([\text{3H}]\text{cAMP (30 Ci/mmol), [32P]}\text{ATP (30 Ci/mmol), [3H]}\text{rauwolscine (81 Ci/mmol), [3-32P]}\text{GTP (3000 Ci/mmol), and [3H]}\text{cAMP radioimmunoassay kit were purchased from NEN Life Sciences Products (Les Ulis, France). ARC-239 bichloride (2-[2-[4-(O-Clonidine acts as a partial agonist through results also shed light upon molecular mechanism by which clonidine acts as a partial agonist through \( \alpha_{2B}-2 \)-AR.}

Cell Culture and Transfection. DDT1-MF2 cells were grown and stably transfected with human \( \alpha_{2B} \) (\( \alpha_{2B}C2 \)) or rat \( \alpha_{2B,2D}-2 \) (RG20) cDNA as described previously (Duzic and Lanier, 1992). Resistant clones were tested for their \( \alpha_{2B}-2 \)-AR binding capacity using the selective tritiated antagonist [3H]rauwolscine as described under binding studies. Clones expressing a receptor density between 1 and 1.5 pmol/mg of membrane proteins, were further cotransfected with the ACII cDNA expression vector and the drug resistance cassette pHYG according to the transfection strategy described by Gorman (1986). Transfected cells were selected by their resistance to hygromycin B (750 mg/ml). Each clone was then analyzed for AC II expression by Northern blot using a 32P-labeled cDNA AC II probe (rat full-length (4 kilobases)] as described previously (Marjamaki et al., 1997) and tested for enzyme activity.

Partially Purified Membrane Preparation. Membranes were prepared by hypotonic lysis in ice-cold lysis buffer (5 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml peptides) and collected by centrifugation (17,000 g for 15 min at 4°C). Membrane pel let was resuspended in 50 mM HEPES, pH 8.0, for adenyl cyclase assay or 50 mM Tris, pH 7.5, 5 mM MgCl\(_2\), 0.6 mM EDTA for binding studies. Protein concentration was determined according to the method of Schacterle and Polack (1973) using bovine serum albumin as standard.

Binding Studies. For saturation experiments, membranes (30–40 mg) were incubated with the required concentrations of [3H]rauwolscine [1–50 nM] for 20 min at 25°C in a final volume of 100 \( \mu \)l. Non-specific binding was determined in the presence of 10 \( \mu \)M phentolamine. Competition studies were performed in presence of increasing concentrations (10 pM–50 \( \mu \)M) of various competitors and 8 nM [3H]rauwolscine (a concentration near the \( K_{d} \) value). Bound radioligand was separated from the free by vacuum filtration over GF/C glass-fiber filters as described previously (Bouet-Alard et al., 1997). Radioactivity was counted by liquid scintillation in a 1214 Rack-β spectrometer (LKB, Rockville, MD) with a counting efficiency of approximately 30%.

Data for saturation and competition studies were analyzed by a nonlinear least-squares, curve-fitting GraphPad program (Graph Pad Software, San Diego, CA). Iterative curve fitting to experimental data from one site model provided IC\(_{50}\). IC\(_{25}\) were converted to \( K_{d} \) values using the equation of Cheng and Prusoff (1973).

Adenylyl Cyclase Assay and Determination of Intracellular cAMP Accumulation. Adenylyl cyclase activity was measured as described previously (G. Duzic and S.M. Lanier, 1992) using 50 \( \mu \)g of crude membrane. For intracellular cAMP accumulation, cells were plated at a concentration of 5 × 10\(^4\) cells/well in six-well plates and incubated at 37°C for 24 h. One hour before starting the experiment, the medium was removed and replaced with 4 ml of serum-free DMEM containing 20 mM HEPES, pH 7.5, and 250 mM isobutyl-1-methylxanthine. Then, cells were incubated with drugs to be tested for 10 min at 37°C. The reaction was stopped by aspiration of the medium and cells were disrupted by the addition of 1 ml of 10% ice-cold trichloroacetic acid per well. After recovering the cellular lysate by scrapping the wells, samples were centrifuged (10,000 g, 15 min at 4°C). The supernatants were then extracted 3 times with diethyl ether (1v/4v) and cAMP contents were determined by a cAMP radioimmunoassay system obtained from NEN Life Sciences Products.
to further minimize the nonspecific antibody binding. After removing Pansorbin cells by centrifugation (700 g), each sample was divided in two aliquots and incubated in presence of anti-Gia or -Gib IgG (1:50) over night at 4°C under constant rotation. The immunocomplexes were collected by the addition of 25 µl of Pansorbin cell suspension and centrifugation at 700g. Then, pellets were washed two times in PBS and resuspended in 30 µl of 1.5% SDS and 30 µl of Laemmli buffer (Laemmli, 1970). Samples were boiled for 5 min before 10% SDS-PAGE analysis. After drying the gel, photolabeled proteins were visualized by autoradiography on Kodak X-Omat AR-5 films (Sigma-Aldrich). Incorporation of [α-32P]AA-GTP into immunoprecipitated G proteins α subunits was quantified by densitometric analysis of autoradiograms with an Imstar computer-assisted image analyzer. Results are expressed as fold incorporation of [α-32P]AA-GTP into immunoprecipitated G protein α subunits compared with unstimulated control subunits.

Results

Establishment of the Experimental System. DDT1-MF2 cells express useful common and distinct signaling entities in comparison with pregnant myometrium. Indeed, they display a similar density of β2 adrenoceptors and Gs proteins (Hadcock et al., 1991; Vivat et al., 1992). They also express the same isoforms of pertussis toxin (PTX)-sensitive G proteins (Gia and Gib) that exert a similar tonic inhibition of adenyl cyclase activity in an agonist-independent manner (Tanfin et al., 1991; Cohen-Tannoudji et al., 1995). However, none of α2-AR subtypes (Philippe et al., 1989; Duzic and Lanier, 1992) nor AC isofoms type II and IV could be detected (Marjamaki et al., 1997). Thus we established, in this cell line, an experimental system expressing α2A/AR or α2B-AR subtype in presence of AC II using stable gene transfection method to further assess their functional characterization.

DDT1-MF2 cells were transfected with the cDNA encoding the human α2B-AR or the rat α2A/D-AR. After Scatchard analysis of saturation binding studies using [3H]rauwolscine, cell lines expressing ~ 1.5 pmol of receptor/mg of membrane proteins were isolated. No specific binding of [3H]rauwolscine was seen in control DDT1-MF2 cell membranes. As shown Fig. 1A, competition studies revealed that [3H]rauwolscine-specific binding was inhibited by subtype-selective compounds such as oxymetazoline (α2A-specific), chlorpromazine and ARC 239 (α2B-specific) with pKi values characteristic of human α2B-AR (Bylund et al., 1988), thus indicating that the transfected α2B-AR receptors displayed the expected ligand recognition properties. The α2A-selective agonist clonidine inhibited the β2-AR-stimulated-cAMP production in a dose-dependent manner (significant at concentrations as low as 1 nM, p < 0.05) (Fig. 1B). This inhibitory effect was mediated through α2B-AR, because it was prevented by the α2A-AR antagonist yohimbine and was not observed in cells transfected with vector alone. Incubation of the cells with PTX completely abolished the inhibition of stimulated cAMP accumulation elicited by the activation of the expressed α2B-AR (data not shown). Maximal reduction of the isoproterenol response was 59% ± 6 (EC50 value of ~ 30 nM). These additional data indicated that, retaining its binding features, α2B-AR expressed in the plasma membranes of DDT1-MF2 cells was functional and implicated in a negative cross talk with the β2-AR/Gs cascade through PTX-sensitive G proteins.

DDT1-MF2 cells expressing α2B-AR and α2A/D-AR were further stably cotransfected with the cDNA encoding adenyl cyclase II isofom. RNA screening using AC II cDNA-specific probe indicated that transcripts of expected 4.2-kilobase size were detected in selected hygromycin resistant cell lines expressing AC II.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>pKi</th>
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<tr>
<td>Oxymetazoline</td>
<td>6.8 ± 0.1</td>
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<tr>
<td>Chlorpromazine</td>
<td>8.0 ± 0.1</td>
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<tr>
<td>ARC239</td>
<td>8.0 ± 0.3</td>
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Fig. 1. Analysis of pharmacological properties and functionality of the transfected α2B-AR in DDT1-MF2 cells. A, membranes were prepared from DDT1-MF2 cells stably transfected with the α2B-AR cDNA. Competition studies were performed in the presence of 9 nM [3H]rauwolscine (a concentration near the Ki value) and increasing concentrations (0.1 mM to 1 mM) of various competitors. Values represent the mean ± S.E. of three separate determinations performed in duplicate. The inset indicates pKi values. B, cells were incubated with 1 µM isoproterenol and increasing concentrations of clonidine (0.1 nM to 1 mM). cAMP accumulation was determined as described under Materials and Methods. The specificity of clonidine (1 µM) was evaluated in the presence of yohimbine (0.1 mM). Basal cAMP and isoproterenol (1 µM) stimulated-cAMP accumulation in presence of GTP (pmol/mg of protein) were, respectively: 55.6 ± 12.2 and 2499 ± 493. Data are expressed as the percentage of isoproterenol-stimulated cAMP production (control = 100%) and represent the mean ± S.E. of three independent experiments performed in duplicate. ■ and □, clonidine effect alone and clonidine effect in presence of yohimbine, respectively. Arrow represents the first time point with significance (p < 0.05) versus isoproterenol control.
clones but not in control cells transfected with vector alone (data not shown). These clonal cell lines coexpressing the α2B-AR and adenylyl cyclase II were also assessed for functional evaluation of enzyme activity in response to a saturating dose (10 μM) of GTPγS in comparison with DDT1-MF2 cells expressing α2B-AR only. With regard to DDT1-MF2-α2B and -α2A/D transfectants, stimulation with GTPγS increased adenylyl cyclase activity by 6-fold in both DDT1-MF2-α2B-ACII and -α2A/D-ACII cotransfectants [from 650 ± 27 to 3650 ± 460 and 570 ± 70 to 4100 ± 330 pmol cAMP/10 min/mg of protein, respectively (Fig. 2)]. Similar observations have been made in previous experiments in DDT1-MF2 cells stably transfected with adenylyl cyclase II cDNA alone (Marjamaki et al., 1997). These results clearly indicated that the adenylyl cyclase II transcript expressed in DDT1-MF2 cells did not seem to be caused by low receptor expression in α2B-ACII or -α2A/D-ACII cotransfectants encoded for an enzyme exhibiting the expected functional properties.

**Effect of α2-AR Activation on Cellular cAMP in DDT1-MF2 Cotransfectants.** Whereas epinephrine stimulation potentiated the β2-activated cAMP production in DDT1-MF2-α2B-ACII cotransfectants, clonidine decreased it (Fig. 3A). Indeed, epinephrine enhanced β2-stimulated cAMP production up to 52% ± 2 at 10 μM with an ED50 value of 114 ± 33 nM. Conversely, clonidine produced a dose-dependent attenuation of cAMP accumulation over a concentration range of 1 nM to 0.1 mM. Maximal inhibition (~43%) was obtained at 1 μM (p < 0.05). Half-maximal inhibition (ED50) occurred at 10 nM clonidine. With higher concentrations of clonidine, negative input persisted, although it was reduced. The inhibitory influence of the clonidine-activated α2B-AR did not seem to be caused by low receptor expression in α2B-ACII cells. Indeed, in these cells, Rmax was 1.3 ± 1 pmol of receptor/mg of membrane protein, a receptor density equivalent to the one measured in α2A/D-ACII cells (1.2 ± 0.045 pmol/mg). Furthermore, we tested six α2B-ACII clones ranging in receptor density from 1 to 3 pmol/mg of protein; none produced significant potentiation of isoproterenol-stimulated cAMP accumulation using clonidine (data not shown).

Conversely, they all reduced cAMP-β2-AR dependent generation. These data demonstrated that in DDT1-MF2 cells expressing type II AC isoform, the α2B-AR could translate into opposite response depending on the type of agonist used (epinephrine or clonidine).

In contrast to the divergent agonist effects observed in α2B-AC II cotransfectants, both clonidine and epinephrine increased isoproterenol-stimulated cAMP production in α2A/D-ACII cotransfectants (Fig. 3B). Indeed, isoproterenol enhanced cAMP accumulation using clonidine (data not shown). These data clearly demonstrated that in DDT1-MF2 cells expressing type II AC isoform, the α2B-AR could translate into opposite response depending on the type of agonist used (epinephrine or clonidine).

![Fig. 2. Effect of GTPγS on adenylyl cyclase activity in DDT1-MF2 cells stably transfected with cDNAs encoding α2B-AR (α2B) and adenylyl cyclase II (α2A/D-ACII). Adenylyl cyclase activity was measured as described under Materials and Methods using 50 μg of membrane protein. Maximal stimulated enzyme activity was determined in the presence of 10 μM GTPγS. Basal AC activity (pmol of cAMP/10 min/mg of protein): DDT1-MF2-α2B, 73 ± 42; DDT1-MF2-α2A/D-ACII, 136 ± 7. Values represent the mean ± S.E. of three independent determinations performed in duplicate.](image-url)
II cotransfectants (Fig. 3B). The maximal stimulation of cAMP accumulation was produced at 0.1 μM clonidine (60 ± 1%) and 1 μM epinephrine (115 ± 41%). ED₅₀ values were 9 ± 1.3 and 180 nM ± 48 nM, respectively.

In both cotransfectants (α₂B-ACII and α₂A/D-ACII), clonidine as well as epinephrine effects were blocked by yohimbine (Fig. 3, A and B) and prior treatment of cells with PTX (Fig. 4). These data were consistent with the fact that these two agonists potentiated or inhibited cAMP production acting on α₂-ARs through Gi/o family members endogenously expressed in DDT1-MF2. However, epinephrine also produced a small PTX-insensitive potentiation of cAMP levels when acting through α₂B-AR. One possible interpretation of this result is that α₂B-AR, when present in high density in the membrane, may also cross-react, to a low extent, with endogenous Gs proteins, as reported previously in other cell lines (Eason et al., 1992, 1994; Pepperl and Regan, 1993).

Altogether, these results indicated that, in the presence of transfected AC II, α₂B-AR was able to mediate opposite regulatory effects (positive input versus negative input) on Ga-stimulated cAMP production via PTX-sensitive G proteins. To gain insight on how α₂B-AR could switch from a positive to a negative regulation, we compared Gi protein coupling of epinephrine- and clonidine-activated α₂-AR subtypes.

Selective Recruitment of Gi Proteins by α₂B or α₂A/D-AR in Response to Clonidine or Epinephrine. Within the family of PTX-sensitive G proteins, DDT1-MF2 cells and myometrium express Gi₂ and Gi₃ (Fig. 5) (Hadcock et al., 1991; Tanfin et al., 1991, Cohen-Tannoudji et al., 1995). Thus, agonist-specific adenylyl cyclase II response could be the consequence of a differential α₂-AR subtype specific recruitment of Gi₂ and/or Gi₃. So, we questioned whether the differences observed in the receptor coupling to AC for clonidine and epinephrine in the cell models represented specific coupling to Gi₂ or Gi₃. This issue was addressed by incubation of membranes with the photoactive GTP analog, 4-azido-anilido-[α-³²P]GTP ([α-³²P]AA-GTP) in the presence of ligand, followed by cross-linking, solubilization, and selective immunoprecipitation of Gi₂ or Gi₃.

As shown in Fig. 6A, clonidine induced a dose-dependent labeling of Gi₂ protein exclusively. No significant incorporation of [α-³²P]AA-GTP was detected in Gi₃ protein. At 1 μM clonidine, maximal labeling of Gi₂ with [α-³²P]AA-GTP (~2.5-fold compared with control fraction) was completely inhibited with yohimbine, thus indicating that recruitment of Gi₂ protein was strictly dependent upon α₂B-AR activation. It should be noted that 1 μM clonidine elicited maximal inhibition of Ga-stimulated cAMP production (Fig. 3A). In marked contrast, when experiments of similar design were conducted with epinephrine, both Gαi proteins (Gαi₂ and Gαi₃) were photolabeled (Fig. 6B). Maximal incorporation of [α-³²P]AA-GTP was obtained at 1 μM epinephrine for each endogenous Gi protein (~2.6-fold compared with control fraction). This epinephrine-dependent [α-³²P]AA-GTP azidoanilide labeling resulted from α₂AR activation, because it could be completely blocked by yohimbine.

On membranes obtained from DDT1-MF2-α₂A/D-ACII cotransfectants where AC II potentiation also occurred, clonidine induced activation of both types of Gi proteins (~2.6- and 2-fold, respectively, for Gαi₂ and Gαi₃ proteins compared with unstimulated fraction at 1 μM (Fig. 7A)).

Altogether, these data indicated that Gi₂ activation is required for potentiation of β-AR stimulation of AC II by α₂A/D- or α₂B-AR in DDT1-MF2 cells. Furthermore, they suggested that heterotrimeric Gi2 and Gi3 proteins may have specific roles in modulating stimulated AC II activity in a given cell type.

Discussion

Activation of α₂-AR subtypes induces multiple cellular effects, including inhibition of adenylyl cyclase or, in some
physiological models or cell systems, an increase of cAMP levels. The mechanisms responsible for inhibitory or stimulatory input on adenylyl cyclase activity/cAMP production greatly depend on their interaction with PTX-sensitive G inhibitory proteins and, additionally, also reflect the type of adenylyl cyclases expressed in various cells.

The present work was motivated by the observation that, in the midpregnancy and term myometrium, the cross talk between activated α2- and β2-ARs differently affect the degree of intracellular cAMP generation (Mhaouty et al., 1995) and, consequently, the relaxed or contractile state of the uterus (Do Khac et al., 1986). Molecular events that underlie these subtle changes in sensitivity of the smooth muscle to catecholamines may result from 1) the alteration of the α2A/β2-ARs expression pattern (Bouet-Alard et al., 1997); 2) a drastic changes of Gi2/Gi3 protein ratio (Tanfin et al., 1991; Cohen-Tannoudji et al., 1995); and/or 3) the functional properties of the pregnant myometrium adenylyl cyclase population (Mhaouty-Kodja et al., 1997; Suzuki et al., 1997). As an initial approach, we investigated whether α2A- and α2B-AR subtypes could exert different regulatory roles on β2-AR catalyzed cAMP production. To address this issue, DDT1-MF2 cells provided an interesting context, because they endogenously express some of the molecular entities (β2-AR, Gi2, Gi3, and Gs proteins) involved in myometrium α2-/β2-AR cross talk but lack α2-AR subtypes and AC type II isoform. Thus, we separately expressed α2A- and α2B-AR subtype in this cell line and, further on, cotransfected each clone with the adenylyl cyclase II isoform that potentiated cAMP production in response to α2-AR agonists in pregnant myometrium. Selected clonal cell lines with comparable functional pools of ARs and adenylyl cyclase have provided useful test models for a careful examination on how α2-AR differ in their ability to modulate adenylyl cyclase and to couple to endogenous Gi protein.

In this context, we found that, without any ACII expression, clonidine induced an inhibition of β2-dependent cAMP production in both α2A- and α2B-AR transfectants (at 1 μM clonidine α2A/ and α2B-AR transfectants: 34 ± 3% for and

![Fig. 6. Effect of clonidine (A) and epinephrine (B) on [α-32P]AA-GTP incorporation into Gi2 and Gi3 proteins in membranes obtained from α2B-AC II transfectants. Cell membranes (50 μg) were incubated with [α-32P]AA-GTP and increasing concentrations of clonidine (clo) or epinephrine (epi) as described under Materials and Methods. After solubilization, photolabeled aliquots (20 μg) were incubated with anti-Gi2α or anti-Gi3α. Immunocomplexes were precipitated and analyzed on SDS-PAGE as described under Materials and Methods. Gels were submitted to autoradiography with intensifying screens for 5 to 7 days. The specificity of epinephrine (1 μM) was determined in the presence of 100 μM yohimbine (Yo). The autoradiograms were scanned with Instar computer-assisted image analyzer. Results are expressed as the percentage of incorporation of [α-32P]AA-GTP into immunoprecipitated G protein α subunits assuming unstimulated controls as 100%. The curves were fit by least-squares and the autoradiograms are representative of four to seven experiments.

![Fig. 7. Effect of clonidine on [α-32P]AA-GTP incorporation into Gi2 and Gi3 proteins in membranes obtained from α2A/AC II transfectants. Cell membranes (50 μg) were incubated with 10 nM or 1 μM clonidine (clo) and [α-32P]AA-GTP as described under Materials and Methods. The specificity of clonidine (1 μM) was determined in presence of 100 μM yohimbine (yo). After solubilization, photolabeled aliquots (20 μg) were incubated with anti-Gi2α or anti-Gi3α. Immunocomplexes were precipitated and analyzed on SDS-PAGE as described under Materials and Methods. Gels were submitted to autoradiography with intensifying screens for 5 to 7 days. The autoradiograms were scanned with Instar computer-assisted image analyzer. Results are expressed as percent of incorporation of [α-32P]AA-GTP into immunoprecipitated G proteins α subunits assuming unstimulated controls as 100%. The autoradiograms are representative of seven experiments.
62 ± 2% respectively). This result is consistent with those reported by Duzic and Lanier (1992) in the same cell line, demonstrating that α2B-AR and α2A/Δ-AR activation similarly inhibits forskolin-induced increase in intracellular cAMP. When AC II was coexpressed in DDT1-MP2-α2AR transfectants, epinephrine as well as clonidine were able to switch the inhibitory signal into a stimulatory input through PTX-sensitive G-proteins. In DDT1-MP2-α2B-AC II cotransfectants, despite a similar functional pool of AC II and an equivalent density of receptor, clonidine was unable to trigger such a switch, in contrast with epinephrine. Direct measurement of G protein activation by photoaffinity labeling with [α-32P]AA-GTP followed by selective separation of individual G protein α subunits revealed that clonidine, acting on α2B-AR, mediated an exclusive coupling to Gi2, whereas the full agonist, epinephrine, led to the recruitment of both PTX-sensitive G proteins, Gi2 and Gi3. Thus, using this photoaffinity probe, it seems clear that, in DDT1-MP2 cells overexpressing AC II, the ability of α2AR to switch from a negative to a positive input to Ga-stimulated cAMP production greatly depends on the recruitment of Gi3.

Previous works have established that the potentiation of Ga-stimulated cAMP production is caused by the input of Gβγ released from Gi to AC II that synergizes with Gs to further elevate cAMP levels (Tang and Gilman, 1991; Federman et al., 1992). Nevertheless, this phenomenon can only occur from a threshold concentration of released Gβγ (Tang and Gilman, 1991). Thus, the persistent inhibitory effect observed when Gi3 is activated alone could be explained by the following hypothesis: the total amount of Gβγ released upon receptor activation would be insufficient to overcome inhibitory influences exerted by Gi2 on endogenous AC. On the contrary, when both Gi proteins (Gi2 and Gi3) were recruited, the threshold concentration would be reached, thus allowing AC II potentiation. Here, we should note that the possibility of overcoming Gi2 inhibition was probably reinforced by the low ability of Gi3 to induce AC inhibition (Raymond et al., 1993; Gettys et al., 1994). This might also reflect the fact that Gi2Gαβγ dimers released upon α2B-AR activation poorly interact or activate AC II compared with Gi3. Although there is no direct evidence that Gi2Gβγ and Gi3Gβγ differ in their capacity to potentiate Ga-stimulated AC, such a hypothesis must be taken into account. Indeed, several studies report that the regulation of βγ-sensitive effectors depends on the composition of the Gβγ dimers with which they are interacting (Müller et al., 1997; Bayewitch et al., 1998; Maier et al., 2000). Finally, because post-translational modifications are considered important criteria in determining the potency by which Gβγ complexes modulate effectors (Ford et al., 1998), differential modifications of Gi2Gβ and/or -γ versus Gi3Gβ and/or -γ might also contribute to the clonidine-specific effect.

The present work also brings some evidence as to whether changes in the ratio of Gi2 to Gi3 proteins in late pregnant myometrium may play a crucial role for the switch in the stimulatory versus inhibitory input to AC population from the α2-AR/Gi protein signaling. The down-regulation of Gi3 protein, together with Gi3-increased expression (Cohen-Tannoudji et al., 1995), could prevent ACII potentiation, thus allowing the decrease of βγ-AR stimulated cAMP production at term. The inhibition of the synthesis of smooth muscle relaxation factor (cAMP) together with the increase of intracellular Ca2+ would promote myometrial contractions at term.

In summary, these data provide the molecular basis of clonidine partial agonist effect when acting through α2B-AR, because they reveal that this compound selectively uncouples the receptor from one of the normally targeted G proteins: Gi3. From this finding, it can be predicted that, in the situation where clonidine behaves as the full agonist, the second messenger pathway would be exclusively regulated by Gi2 in Gi2/Gi3 expressing cells. On the other hand, in systems in which clonidine acts as partial agonist or even as an antagonist, Gi3 would play a determinant or exclusive role. Furthermore, they suggested that Gi2 and Gi3 have specific roles in modulating AC II effector through a and/or β subunits.

References


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