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Partial Agonist Clonidine Mediates α_2-AR Subtypes Specific Regulation of cAMP Accumulation in Adenylyl Cyclase II Transfected DDT1-MF2 Cells

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ABSTRACT

α2-Adrenergic receptor (α_2-AR) activation in the pregnant rat myometrium at midterm potentiates β_2-AR stimulation of adenylyl cyclase (AC) via G$\beta\gamma$ regulation of the type II isoform of adenylyl cyclase. However, at term, α_2-AR activation inhibits β_2-AR stimulation of AC. This phenomenon is associated with changes in α_2-AR subtype expression (midterm $\alpha_{2A/D}$-AR $\gg$ α_{2B}-AR; term $\alpha_{2B} \geq \alpha_{2A/D}$-AR), without any change in ACII mRNA, suggesting that $\alpha_{2A/D}$- and α_{2B}-AR differentially regulate β_2-cAMP production. To address this issue, we have stably expressed the same density of $\alpha_{2A/D}$- or α_{2B}-AR with AC II in DDT1-MF2 cells. Clonidine (partial agonist) increased β_2-AR-stimulated cAMP production in $\alpha_{2A/D}$-AR-ACII transfectants but inhibited it in α_{2B}-AR-ACII transfectants. In contrast, epinephrine (full agonist) enhanced β_2-stimulated ACII in both α_{2A}- and α_{2B}-ACII clonal cell lines. 4-Azidoanilido-[α-^{32}P]GTP-labeling of activated G proteins indicated that, in α_{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 β_2-AR stimulation of AC by $\alpha_{2A/D}$ and α_{2B}-AR in DDT1-MF2 cells. This may reflect an issue of the amount of G$\beta\gamma$ released upon receptor activation and/or $\beta\gamma$ composition of Gi_3 versus Gi_2.

In pregnant rat myometrium, α_2-adrenoceptor (AR) signaling pathways differentially modulate β_2-AR-mediated regulation of adenylyl cyclase (AC) at midpregnancy and at term (Mhaouty et al., 1995). At midterm, α_2-AR activation potentiates adenylyl cyclase activity stimulated by β_2-AR, thus enhancing uterine relaxation in response to catecholamines. This augmentation of AC activity induced by α_2-AR probably involves the type II family isoform of AC and is caused by the input of G$\beta\gamma$ released from Gi (Gi_2 and/or Gi_3) that synergizes with Gs to further elevate cAMP levels (Mhaouty-Kodja et al., 1997). In contrast, at term, myometrial α_2-AR/Gi signaling pathways reduce the β_2-AR-induced-cAMP generation to allow intracellular Ca^{2+} increase and cell contraction. This switch in the stimulatory versus inhibitory input to β_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 α_2-AR subtypes expression.

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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 time 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 α_2-AR subtypes expressed in rat myometrium, α_{2A}- and α_{2B}-AR, were differentially expressed at midpregnancy and term (midpregnancy $\alpha_{2A/D}$-AR $\gg$ α_{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 α_2-adrenoceptors toward β_2-dependent cAMP production could result from a specific signaling of α_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

ABBREVIATIONS: AR, adrenoceptor; AC, adenylyl cyclase; PAGE, polyacrylamide gel electrophoresis; [α-^{32}P]AA-GTP, 4-azido-anilido-[α-^{32}P]GTP; PTX, pertussis toxin.

deferens smooth muscle cell) cotransfectants stably expressing $\alpha_{2A/D}$-AR (RG20) or α_{2B}-AR (α_2C2) and AC type II isoform and studied the regulation induced by each α_2-AR subtypes on β_2-stimulated AC II activity.

We report, herein, agonist and receptor specific regulation of ACII that involves selective coupling to Gi_2 versus Gi_3. Our results also shed light upon molecular mechanism by which clonidine acts as a partial agonist through α_{2B}-AR.

Materials and Methods

[^{3}H]cAMP (30 Ci/mmol), [^{32}P]ATP (30 Ci/mmol), [^{3}H]rauwolscine (81 Ci/mmol), [α-^{32}P]GTP (3000 Ci/mmol), and [^{125}I]cAMP radioimmunoassay kit were purchased from NEN Life Sciences Products (Les Ulis, France). ARC-239 bichloride (2[2-[4(*O*-methoxy-phen) piperazine-1-y]4,4dimethyl-1,3,2H-4H] isoquinolinedione) was a gift from Karl Thomae (Biberach, Germany). 4-Azidoanilide was obtained from Fluka Biochemicals (Saint Quentin Fallavier, France). 1-Ethyl-3-[3-(dimethylamino)propyl] carbodiimide HCl and Dowex 50W-X4 (100–200 mesh, hydrogen form) were from Bio-Rad (Ivry sur Seine, France). PEI-cellulose plates and aluminum oxide (90 active neutral) were purchased from Merck (Nogent sur Marne, France). Cell culture supplies were obtained from Life Technologies (Cergy-Pontoise, France). Pansorbin cells were supplied from Calbiochem (Meudon, France). GF/C glass-fiber filters were from Millipore (Saint Quentin en Yvelines, France). All remaining drugs were from Sigma-Aldrich (Saint Quentin Fallavier, France).

Cell Culture and Transfection. DDT1-MF2 cells were grown and stably transfected with human α_{2B} (α_2C2) or rat $\alpha_{2A/D}$ (RG20) cDNA as described previously (Duzic and Lanier, 1992). Resistant clones were tested for their α_2-AR binding capacity using the selective tritiated antagonist [^{3}H]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 ^{32}P-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 pepstatin A) and collected by centrifugation (17,000*g* for 15 min at 4°C). Membrane pellet was resuspended in 50 mM HEPES, pH 8.0, for adenylyl 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 Pollack (1973) using bovine serum albumin as standard.

Binding Studies. For saturation experiments, membranes (30–40 μg) were incubated with the required concentrations of [^{3}H]rauwolscine [1–50 nM] for 20 min at 25°C in a final volume of 100 μl. Nonspecific binding was determined in the presence of 10 μM phentolamine. Competition studies were performed in presence of increasing concentrations (10 pM–50 μM) of various competitors and 8 nM [^{3}H]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_{50} were converted to K_i values using the equation of Cheng and Prussof (1973).

Adenylyl Cyclase Assay and Determination of Intracellular cAMP Accumulation. Adenylyl cyclase activity was measured as described previously (E. Duzic and S.M. Lanier, 1992) using 50 μg of crude membrane. For intracellular cAMP accumulation, cells were plated at a concentration of 5×10^5 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-L-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 trichloracetic 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.

Immunoblot and Immunoprecipitation. For immunoblotting, 50 μg of membranes obtained from DDT1-MF2 cells or tissues were resolved on 10% SDS-PAGE and transfer to polyvinylidene difluoride-transfer membrane POLYSCREEN (NEN Research Products, DuPont de Nemours, France). After transfer, the blots were probed with anti-Gα protein subtype specific antibodies as described previously (Cohen-Tannoudji et al., 1995). Briefly, after blocking, the polyvinylidene difluoride blots were incubated with the primary antibody [AS/7 (anti-$Gi\alpha_2$/$Gi\alpha_1$) or EC/2 (anti-$Gi\alpha_3$/Gαo) or GC/2 (anti-Gαo)] for 1 h in a high-detergent 5% nonfat dry milk/Tris-buffered saline at room temperature at a dilution of 1:1000. After four successive washes, the blots were incubated for 1 h with HRP-conjugated secondary antibody in high-detergent 5% nonfat dry milk/Tris-buffered saline. After washing, bound antibodies were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, les Ulis, France). For immunoprecipitation, antisera were raised against the C-terminal decapeptide (amino acids 345–354) of $Gi\alpha_3$ and against the C-terminal decapeptide (amino acids 345–354) that is shared by both $Gi\alpha_1$ and $Gi\alpha_2$ as described previously (Gettys et al., 1994). The antisera were characterized with respect to titer, specificity, and cross-reactivity using lysates from bacteria transformed with cDNA for each of the G proteins. Each antiserum was desalted and purified as described previously (Raymond et al., 1993; Gettys et al., 1994).

Photolabeling of Membrane G Proteins. The 4-azidoanilido-[α-^{32}P]GTP ([α-^{32}P]AA-GTP) was synthesized according to the method described by Offermanns et al., 1990 and 1991), except that [α-^{32}P]AA-GTP was purified using a thin-layer chromatography and finally resuspended in water at a concentration of 4×10^9 cpm/ml. Photoaffinity labeling of G proteins was performed as described by Offermanns et al. (1991). Briefly, cell membranes (50 μg) were preincubated with α_2-AR agonists and/or antagonists for 10 min at 30°C in 50 μl of assay buffer containing 30 mM HEPES, pH 7.5, 100 mM NaCl, 100 mM EDTA, 1 mM benzamidine, 5 mM $MgCl_2$, 50 mM leupeptin, and 3 μM GDP. Then, 10 μl of [α-^{32}P]AA-GTP (0.4×10^9 cpm/ml) diluted in distilled water was added to each sample and the labeling reaction was allowed to proceed for 10 min. The reaction was stopped by the addition of 100 μl of ice-cold assay buffer and immediate 4°C centrifugation at 12,000*g* for 10 min. All subsequent procedures were performed at 4°C. Supernatants containing free [α-^{32}P]AA-GTP were removed. Membrane pellets were rapidly resuspended in 55 μl of assay buffer supplemented with 2 mM dithiothreitol and exposed, on ice, to UV light (254 nm, 15 W) in the dark for 4 min. Before immunoprecipitation, photolabeled membranes (50 μl) were then solubilized by incubation in presence of 0.25% SDS at 60°C for 5 min and addition of 50 μl of an immunoprecipitation buffer (0.5% SDS, 2% Nonidet P40, 1% cholate, 150 mM NaCl). Solubilized samples were first incubated 30 min at 4°C with prewashed Pansorbin cells (25 μl of a 10% solution in 50 mM sodium phosphate, pH

7.4) 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-Giα_2 or -Giα_3 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 700*g*. 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-^{32}P]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-^{32}P]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 β_2 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 (Gi_2 and Gi_3) that exert a similar tonic inhibition of adenylyl cyclase activity in an agonist-independent manner (Tanfin et al., 1991; Cohen-Tannoudji et al., 1995). However, none of α_2-AR subtypes (Philippe et al., 1989; Duzic and Lanier, 1992) nor AC isoforms type II and IV could be detected (Marjamaki et al., 1997). Thus we established, in this cell line, an experimental system expressing $\alpha_{2A/D}$- or α_{2B}-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 α_{2B}-AR or the rat $\alpha_{2A/D}$-AR. After Scatchard analysis of saturation binding studies using [^{3}H]rauwolscine, cell lines expressing ~ 1.5 pmol of receptor/mg of membrane proteins were isolated. No specific binding of [^{3}H]rauwolscine was seen in control DDT1-MF2 cell membranes. As shown Fig. 1A, competition studies revealed that [^{3}H]rauwolscine-specific binding was inhibited by subtype-selective compounds such as oxymetazoline (α_{2A}-specific), chlorpromazine and ARC 239 (α_{2B}- specific) with pK_i values characteristic of human α_{2B}-AR (Bylund et al., 1988), thus indicating that the transfected α_{2B}-AR receptors displayed the expected ligand recognition properties. The α_2-AR selective agonist clonidine inhibited the β_2-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 α_{2B}-AR, because it was prevented by the α_2-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 α_{2B}-AR (data not shown). Maximal reduction of the isoproterenol response was 59% ± 6 (EC_{50} value of ~ 30 nM). These additional data indicated that, besides retaining its binding features, α_{2B}-AR expressed in the plasma membranes of DDT1-MF2 cells was functional and implicated in a negative cross talk with the β_2-AR/Gs cascade through PTX-sensitive G proteins.

DDT1-MF2 cells expressing α_{2B}-AR and $\alpha_{2A/D}$-AR were further stably cotransfected with the cDNA encoding adenylyl cyclase II isoform. RNA screening using AC II cDNA-specific probe indicated that transcripts of expected 4.2-kilobase size were detected in selected hygromycin resistant

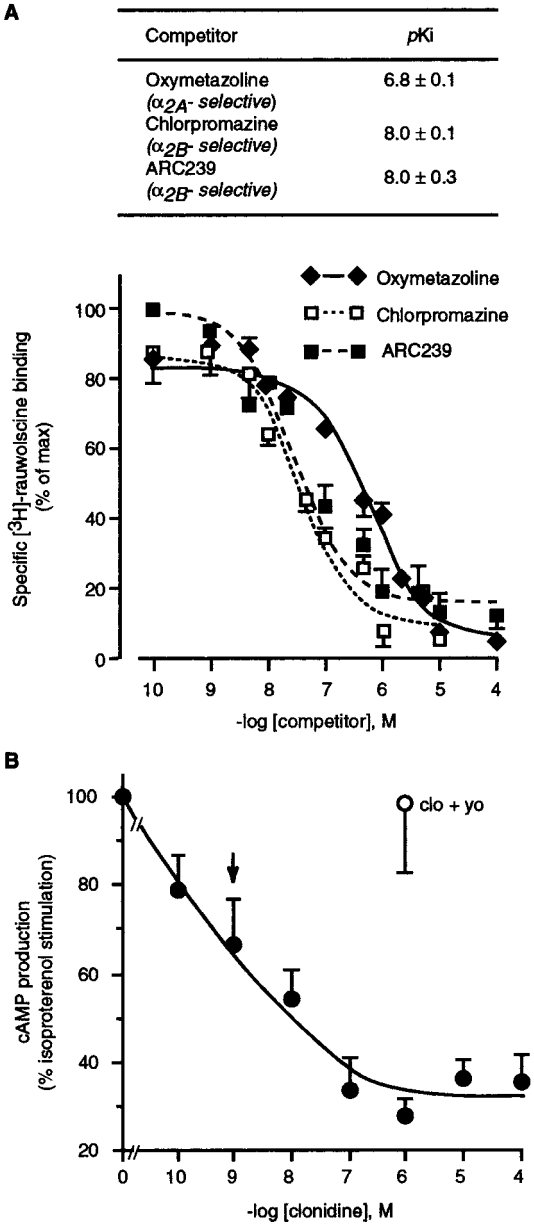

Fig. 1. Analysis of pharmacological properties and functionality of the transfected α_{2B}-AR in DDT1-MF2 cells. A, membranes were prepared from DDT1-MF2 cells stably transfected with the α_{2B}-AR cDNA. Competition studies were performed in the presence of 8 nM [^{3}H]rauwolscine (a concentration near the K_D value) and increasing concentrations (0.1 nM to 1 mM) of various competitors. Values represent the mean ± S.E. of three separate determinations performed in duplicate. The inset indicates pK_i 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 isproterenol control.

clones but not in control cells transfected with vector alone (data not shown). These clonal cell lines coexpressing the α_{2B}-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 α_{2B}-AR only. With regard to DDT1-MF2-α_{2B} and -$\alpha_{2A/D}$ transfectants, stimulation with GTPγS increased adenylyl cyclase activity by ~6 fold in both DDT1-MF2-α_{2B}-ACII and -$\alpha_{2A/D}$-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-α_{2B}-ACII or -$\alpha_{2A/D}$-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 β_2-activated cAMP production in DDT1-MF2-α_{2B}-ACII cotransfectants, clonidine decreased it (Fig. 3A). Indeed, epinephine enhanced β_2-stimulated cAMP production up to 52% ± 2 at 10 μM with an ED_{50} 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 (ED_{50}) occurred at 10 nM clonidine. With higher concentrations of clonidine, negative input persisted, although it was reduced. The inhibitory influence of the clonidine-activated α_{2B}-AR did not seem to be caused by low receptor expression in α_{2B}-ACII cells. Indeed, in these cells, B_{max} was 1.3 ± 1 pmol of receptor/mg of membrane protein, a receptor density equivalent to the one measured in $\alpha_{2A/D}$-ACII cells (1.2 ± 0.045 pmol/mg). Furthermore, we tested six α_{2B}-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-β_2-AR dependent generation. These data demonstrated that in DDT_1-MF_2 cells expressing type II AC isoform, the α_{2B}-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 α_{2B}-AC II cotransfectants, both clonidine and epinephrine increased isoproterenol-stimulated cAMP production in $\alpha_{2A/D}$-AC

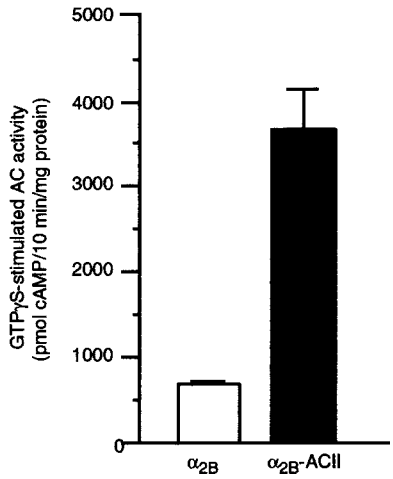

Fig. 2. Effect of GTPγS on adenylyl cyclase activity in DDT1-MF2 cells stably transfected with cDNAs encoding α_{2B}-AR (α_{2B}) alone and α_{2B}-AR and adenylyl cyclase II (α_{2B}-AC II). Adenylyl cyclase activity was measured as described under *Materials and Methods* using 50 μg of membrane protein. Maximally 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-α_{2B}, 73 ± 42; DDT1-MF2-α_{2B}-ACII, 136 ± 7. Values represent the mean ± S.E. of three independent determinations performed in duplicate.

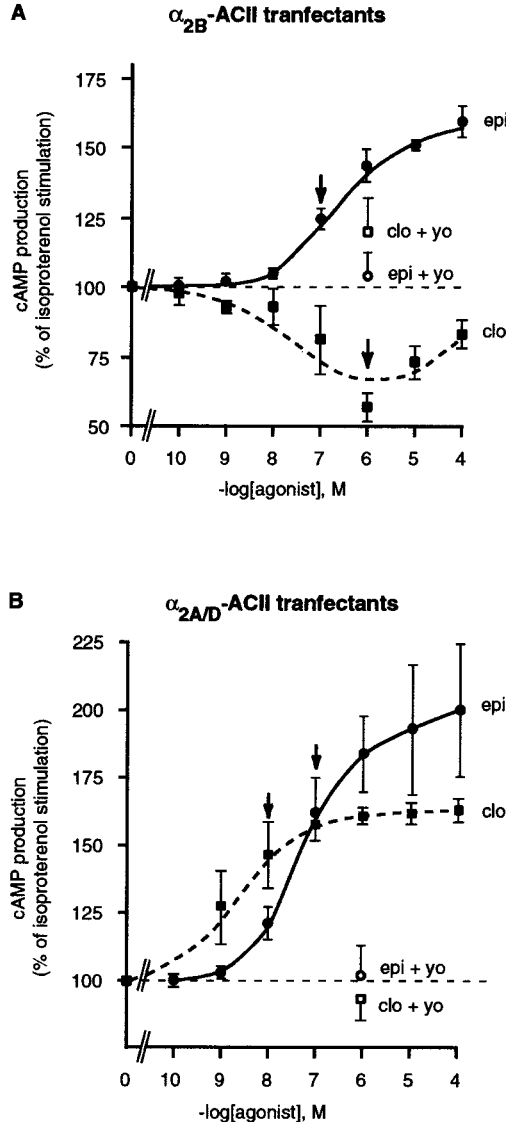

Fig. 3. Effect of clonidine (clo) or epinephrine (epi) on isoproterenol-stimulated cAMP production in DDT1-MF2 -α_{2B}-AC II (A) or $\alpha_{2A/D}$-AC II (B) transfectants. Cells were incubated with isoproterenol (1 μM) and increasing concentrations of clonidine or epinephrine and cAMP concentrations were determined as described under *Materials and Methods*. Data are expressed as the percentage of isoproterenol-stimulated cAMP production (control = 100%). Results are the mean ± S.E. of three independent determinations performed in duplicate. The dose-response significance as well as dose-response curve differences were analyzed by one-way analysis of variance followed by Bonferroni's multiple range test. ● and ■, effects of epinephrine and clonidine, respectively. ○ and □, effects of agonists in presence of yohimbine. Arrows represent the first time point with significance ($p < 0.05$) versus isoproterenol control. Basal cAMP concentrations were 44 ± 5 pmol/mg of protein and 19 ± 3 in α_{2B}-ACII cells and $\alpha_{2A/D}$-ACII cells, respectively. Isoproterenol (1 μM) -stimulated cAMP accumulations were 1959 ± 234 and 1894 ± 187 pmol/mg of protein in α_{2B}-ACII and $\alpha_{2A/D}$-AC II cells, respectively.

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%). ED_{50} values were 9 ± 1.3 and 180 nM ± 48 nM, respectively.

In both cotransfectants (α_{2B}-ACII and $\alpha_{2A/D}$-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 α_2-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 α_{2B}-AR. One possible interpretation of this result is that α_{2B}-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, α_{2B}-AR was able to mediate opposite regulatory effects (positive input versus negative input) on Gs-stimulated cAMP production via PTX-sensitive G proteins. To gain insight on how α_{2B}-AR could switch from a positive to a negative regulation, we compared Gi protein coupling of epinephrine- and clonidine-activated α_2-AR subtypes.

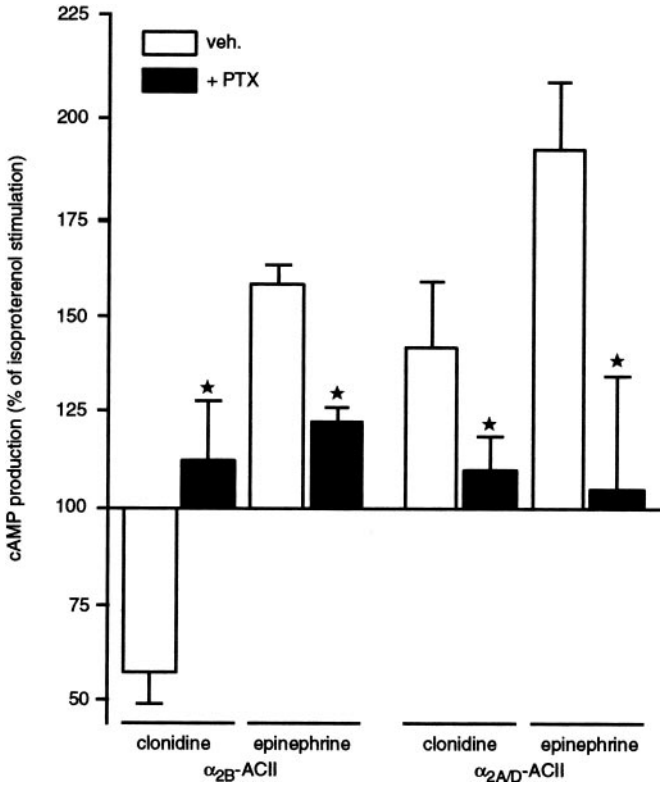

Fig. 4. Effect of clonidine or epinephrine on isoproterenol-stimulated cAMP production in DDT1-MF2-α_{2B}-AC II or $\alpha_{2A/D}$-AC II cotransfectants after cell preincubation in presence or absence of PTX. Confluent plates of cells were pretreated with pertussis toxin (100 ng/ml) or vehicle for 18 h at 37°C in normal culture medium. Cells were further incubated with isoproterenol (1 μM) in the absence and presence of clonidine (1 μM) or epinephrine (1 μM) for 10 min at 37°C. Data are expressed as percentage inhibition or augmentation of isoproterenol-induced elevation of cAMP production and represent the mean ± S.E. of three to six separate experiments performed in duplicate. Values obtained in the presence and absence of pertussis toxin were compared using an unpaired Student's *t* test. *Statistically significant difference ($p < 0.05$).

Selective Recruitment of Gi Proteins by α_{2B}- or $\alpha_{2A/D}$-AR in Response to Clonidine or Epinephrine. Within the family of PTX-sensitive G proteins, DDT_1-MF_2 cells and myometrium express Gi_2 and Gi_3 (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 α_2-AR subtype specific recruitment of Gi_2 and/or Gi_3. So, we questioned whether the differences observed in the receptor coupling to AC for clonidine and epinephrine in the cell models reflected specific coupling to Gi_2 or Gi_3. This issue was addressed by incubation of membranes with the photoreactive GTP analog, 4-azido-anilido-[$\alpha-^{32}$P]GTP ([$\alpha-^{32}$P]AA-GTP) in the presence of ligand, followed by cross-linking, solubilization, and selective immunoprecipitation of Gi_2 or Gi_3.

As shown in Fig. 6A, clonidine induced a dose-dependent labeling of Giα_2 protein exclusively. No significant incorporation of [$\alpha-^{32}$P]AA-GTP was detected in Giα_3 protein. At 1 μM clonidine, maximal labeling of Gαi_2 with [$\alpha-P^{32}$]−AA-GTP (~ 2.5-fold compared with control fraction) was completely inhibited with yohimbine, thus indicating that recruitment of Giα_2 protein was strictly dependent upon α_{2B}-AR activation. It should be noted that 1 μM clonidine elicited maximal inhibition of Gs-stimulated cAMP production (Fig. 3A). In marked contrast, when experiments of similar design were conducted with epinephrine, both Gαi proteins (Giα_2 and Giα_3) were photolabeled (Fig. 6B). Maximal incorporation of [$\alpha-^{32}$P]AA-GTP was obtained at 1 μM epinephrine for each endogenous Gi protein (~2.6-fold compared with control fraction). This epinephrine-dependent [$\alpha-^{32}$P]GTP azidoanilide labeling resulted from α_{2B}-AR activation, because it could be completely blocked by yohimbine. On membranes obtained from DDT1-MF2-$\alpha_{2A/D}$-ACII cotransfectants where AC II potentiation also occurred, clonidine induced activation of both types of Gi proteins [~2.6- and 2-fold, respectively, for Gαi_2 and Gαi_3 proteins compared with unstimulated fraction at 1 μM (Fig. 7)].

Altogether, these data indicated that Gi_3 activation is required for potentiation of β_2-AR stimulation of AC II by $\alpha_{2A/D}$- or α_{2B}-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 α_2-AR subtypes induces multiple cellular effects, including inhibition of adenylyl cyclase or, in some

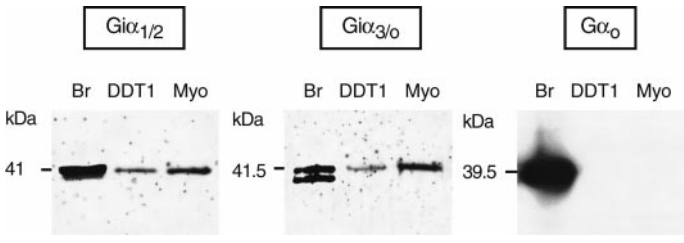

Fig. 5. Identification of Gαi and Goα proteins in DDT1-MF2 cotransfectants cell membranes. Immunoblotting was performed with antibodies directed against Gαi1 and Gαi_2 (AS/7), Gαi_3 and Gαo (EC/2), or Goα (GC/2) as described under *Materials and Methods*. Br is used for rat brain, DDT1 for DDT1-MF2 cells, and Myo for rat pregnant myometrium. Molecular masses (kDa) of protein are given next to molecular size markers.

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 α_2- and β_2-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 $\alpha_{2A/D}$-/α_{2B}-subtypes expression pattern (Bouet-Alard et al., 1997); 2) the drastic changes of Gi_2/Gi_3 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 $\alpha_{2A/D}$- and α_{2B}-AR subtypes could exert different regulatory roles on β_2-AR catalyzed cAMP production. To address this issue, DDT1-MF2 cells provided an interesting context, because they endogenously express some of the molecular entities (β_2-AR, Gi_2, Gi_3, and G_s proteins) involved in myometrium α_2-/β_2-AR cross talk but lack α_2-AR subtypes and AC type II isoform. Thus, we separately expressed α_{2B}- or $\alpha_{2A/D}$-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 α_2-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 α_2-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 β_2-dependent cAMP production in both α_{2A}- and α_{2B}-AR transfectants (at 1 μM clonidine $\alpha_{2A/D}$- and $\alpha_{2B\text{-}AR}$ transfectants: 34 $\pm$ 3% for and

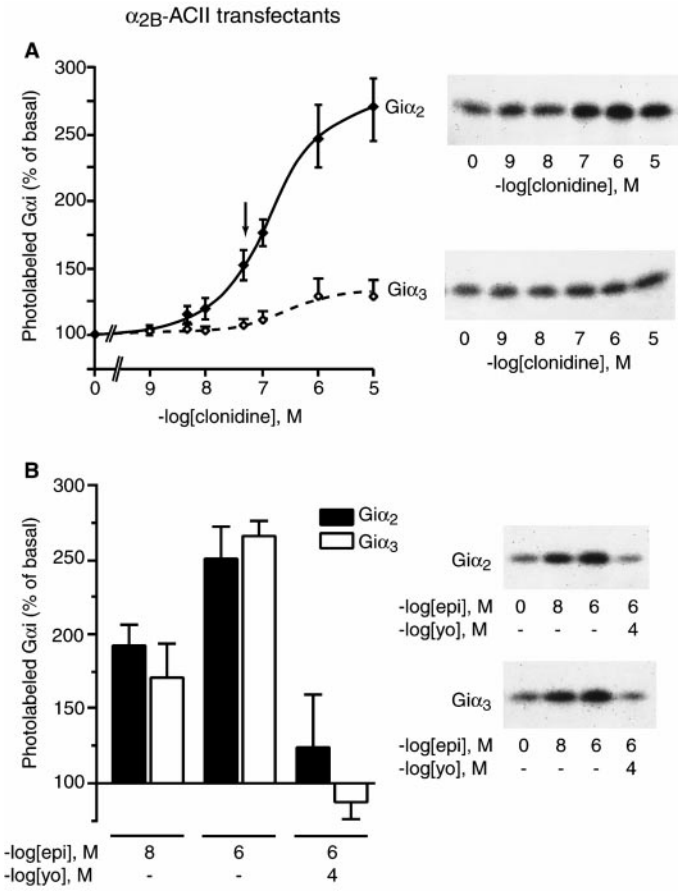

Fig. 6. Effect of clonidine (A) and epinephrine (B) on [α-^{32}P]AA-GTP incorporation into $Gi\alpha_2$ and $Gi\alpha_3$ proteins in membranes obtained from α_{2B}-AC II transfectants. Cell membranes (50 μg) were incubated with [α-^{32}P]AA-GTP and increasing concentrations of clonidine (clo) or epinephrine (epi) as described under *Materials and Methods*. After solubilization, photolabeled aliquots (20 μg) were incubated with anti-$Gi\alpha_{1/2}$ or anti-$Gi\alpha_{3/o}$. 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 Imstar computer-assisted image analyzer. Results are expressed as the percentage of incorporation of [α-^{32}P]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.

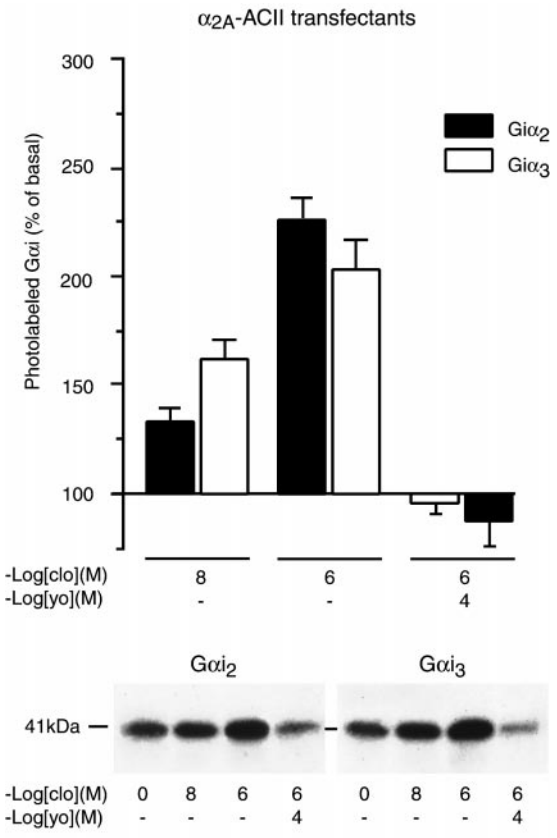

Fig. 7. Effect of clonidine on [α-^{32}P]AA-GTP incorporation into $Gi\alpha_2$ and $Gi\alpha_3$ proteins in membranes obtained from $\alpha_{2A/D}$-AC II transfectants. Cell membranes (50 μg) were incubated with 10 nM or 1 μM clonidine (clo) and [α-^{32}P]AA-GTP as described under *Materials and Methods*. 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-$Gi\alpha_{1/2}$ or anti-$Gi\alpha_{3/o}$. 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 Imstar computer-assisted image analyzer. Results are expressed as percent of incorporation of [α-^{32}P]AA-GTP into immunoprecipitated G proteins α subunits assuming unstimulated controls as 100%. The autoradiograms are representative of seven experiments.

62 ± 2% rspectively). This result is consistent with those reported by Duzic and Lanier (1992) in the same cell line, demonstrating that α_{2B}- and $\alpha_{2A/D}$-AR activation similarly inhibits forskolin-induced increase in intracellular cAMP. When AC II was coexpressed in DDT1-MF2-$\alpha_{2A/D}$ transfectants, epinephrine as well as clonidine were able to switch the inhibitory signal into a stimulatory input through PTX-sensitive G proteins. In DDT1-MF2-α_{2B}-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-^{32}P]AA-GTP followed by selective separation of individual G protein α subunits revealed that clonidine, acting on α_{2B}-AR, mediated an exclusive coupling to Gi_2, whereas the full agonist, epinephrine, led to the recruitment of both PTX-sensitive G proteins, Gi_2 and Gi_3. Thus, using this photoaffinity probe, it seems clear that, in DDT1-MF2 cells overexpressing AC II, the ability of α_{2B}-AR to switch from a negative to a positive input to Gs-stimulated cAMP production greatly depends on the recruitment of Gi_3.

Previous works have established that the potentiation of Gs-stimulated cAMP production is caused by the input of G$\beta\gamma$ 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 Gi$\beta\gamma$ (Tang and Gilman, 1991). Thus, the persistent inhibitory effect observed when Gi_2 is activated alone could be explained by the following hypothesis: the total amount of $Gi_2\beta\gamma$ released upon receptor activation would be insufficient to overcome inhibitory influences exerted by Giα_2 on endogenous AC. On the contrary, when both Gi proteins (Gi_2 and Gi_3) were recruited, the threshold concentration would be reached, thus allowing AC II potentiation. Here, we should note that the possibility of overcoming Gαi_2 inhibition was probably reinforced by the low ability of Giα_3 to induce AC inhibition (Raymond et al., 1993; Gettys et al., 1994). This might also reflect the fact that $Gi_2\beta\gamma$ dimers released upon α_{2B}-AR activation poorly interact or activate AC II compared with Gi_3 $\beta\gamma$. Although there is no direct evidence that $Gi_2\beta\gamma$ and $Gi_3\beta\gamma$ differ in their capacity to potentiate Gs-stimulated AC, such a hypothesis must be taken into account. Indeed, several studies report that the regulation of $\beta\gamma$-sensitive effectors depends on the composition of the G$\beta\gamma$ 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 G$\beta\gamma$ complexes modulate effectors (Ford et al., 1998), differential modifications of $Gi_2\beta$ and/or -γ versus $Gi_3\beta$ 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 Gi_2 to Gi_3 proteins in late pregnant myometrium may play a crucial role for the switch in the stimulatory versus inhibitory input to AC population from the α_2-AR/Gi protein signaling. The down-regulation of Gi_3 protein, together with Gi_2-increased expression (Cohen-Tannoudji et al., 1995), could prevent ACII potentiation, thus allowing the decrease of β_2-AR stimulated cAMP production at term. The inhibition of the synthesis of smooth muscle relaxation factor (cAMP) together with the increase of intracellular Ca^{2+} would promote myometrial contractions at term.

In summary, these data provide the molecular basis of clonidine partial agonist effect when acting through α_{2B}-AR, because they reveal that this compound selectively uncouples the receptor from one of the normally targeted G proteins: Gi_3. 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 Gi_2 in Gi_2/Gi_3 expressing cells. On the other hand, in systems in which clonidine acts as partial agonist or even as an antagonist, Gi_3 would play a determinant or exclusive role. Furthermore, they suggested that Gi_2 and Gi_3 have specific roles in modulating AC II effector through α and/or $\beta\gamma$ subunits. Finally, they shed light upon a possible molecular mechanism that might allow the versatility of signal routed through myometrial α_2-AR during pregnancy involved changes in Gi_2/Gi_3 ratio.

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