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α_2-Adrenoceptor agonists stimulate high-affinity GTPase activity in a receptor subtype-selective manner

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Abstract

Transfected Chinese hamster ovary cells expressing human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes were used to monitor α_2-adrenoceptor-stimulated GTP hydrolysis. Incubation with 100 μM (−)-adrenaline resulted in stimulation of pertussis toxin-sensitive GTPase by 380% after activation of the α_{2A}-subtype, by 320% after activation of the α_{2B}-subtype and by 110% after activation of the α_{2C}-subtype. The agonists dexmedetomidine, UK14,304 (5-bromo-6-[2-imidazoline-2-ylamino]quinoxaline) and oxymetazoline showed subtype-dependent efficacy. Dexmedetomidine was a full agonist at the α_{2B}-subtype and a partial agonist at the α_{2A}- and the α_{2C}-subtypes. UK14,304 was a full agonist at the α_{2A}-subtype and a partial agonist at the other two. Oxymetazoline showed strong partial agonism at the α_{2B}-subtype (63% of adrenaline), but did not significantly activate the α_{2A}- and the α_{2C}-subtypes. These results agreed with cAMP accumulation experiments carried out with cell lines endogenously expressing the α_{2A}-subtype (human erythroleukemia, HEL) or the α_{2B}-subtype (neuroblastoma-glioma, NG108-15). The GTPase assay may thus provide a valuable tool for the identification of subtype-selective α_2-adrenoceptor agonists.

Keywords: α_2-Adrenoceptor subtype; GTPase assay; Subtype-selectivity; CHO (Chinese hamster ovary) cell; NG108-15 cell; HEL (human erythroleukemia) cell; cAMP

1. Introduction

Activated G-protein coupled receptors promote the exchange of GTP for GDP in the activated α-subunit of the receptor-associated G-protein. The newly bound GTP is subsequently hydrolysed by the GTPase activity intrinsic to the G-protein α-subunit. Guanine nucleotide exchange and GTP hydrolysis are the first detectable biochemical responses following activation of α_2-adrenoceptors. In principle, receptor-stimulated GTPase activity can be quite conveniently monitored by measuring the release of ^{32}P from [γ-^{32}P]GTP, but the agonist-mediated increase over basal GTP hydrolysis is often quite small compared to downstream responses in the cellular signaling pathways. Enzymatic GTP hydrolysis is not restricted to transmembrane signaling events; there are actually numerous possible sources of basal GTPase activity. However, since a considerable part of the total GTPase activity in cell membranes is eliminated after pretreatment with pertussis toxin (Gierschik et al., 1994), it appears that either receptor activity (ligand-dependent or -independent), or spontaneous receptor-independent $G_{i/o}$-protein activity, or both, actually contribute in a major way.

Physiological signaling pathways linked to the three α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and α_{2C}) are mostly mediated by pertussis toxin sensitive G-proteins (G_{i1}, G_{i2}, G_{i3} and G_o) but α_2-adrenoceptors have also been shown to couple to G_s, G_z, and G_q (Conklin et al., 1992; Eason et al., 1992; Wong et al., 1992; Chabre et al., 1994). Consequently, α_2-adrenoceptors can regulate several different types of effector enzymes, including adenylyl cyclases and phospholipases (A_2, C and D), as well as ion channels (K^+ and Ca^{2+}). The subtype-specific coupling character-

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istics of each α_2-adrenoceptor subtype have not yet been fully established.

Drug discovery projects aimed at the identification of novel receptor ligands usually use competitive radioligand binding assays for the initial characterisation of compounds. Since binding assays do not reliably reveal functional properties, they have to be followed up by functional experiments. Second messenger-based assays are commonly used for this purpose. However, especially when recombinant cell lines expressing high densities of receptors are used, maximal second messenger responses can often be evoked at relatively low degrees of receptor activation, and partial agonists can behave as full agonists (Adham et al., 1993; Pohjanoksa et al., 1997). Direct measurement of receptor-mediated activation of G-proteins would be expected to avoid this problem. The aim of the present study was therefore to develop a convenient assay to monitor α_2-adrenoceptor-stimulated GTPase activity, in order to enable the pharmacological characterisation of α_2-adrenoceptor agonists and their possible subtype-selectivity. For this purpose, we used Chinese hamster ovary (CHO) cells stably transfected to separately express the α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes. The characterisation of the adenylyl cyclase regulation by α_2-adrenoceptor subtypes in transfected CHO cells has already been reported (Pohjanoksa et al., 1997). In order to further validate the GTPase results we now also determined α_2-adrenoceptor-mediated changes in cAMP production in two cell lines endogenously expressing low levels of two α_2-adrenoceptor subtypes: human erytholeukemia (HEL) cells expressing the α_{2A}-subtype and neuroblastoma-glioma (NG108-15) cells expressing the α_{2B}-subtype.

2. Materials and methods

2.1. Materials

[γ-^{32}P]GTP, [^{3}H]adenine and [^{3}H]RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) were from Amersham (Buckinghamshire, UK) and [^{3}H]rauwolscine was from DuPont NEN (Boston, MA). (−)-Adrenaline, App(NH)p (5′-adenylylimidodiphosphate), ATP, charcoal, creatine phosphate, creatine phosphokinase, dithiothreitol, forskolin, IBMX (3-isobuthyl-1-methyl-xanthine), (−)-noradrenaline, oxymetazoline, ouabain, propranolol, quinacrine, pertussis toxin and G418 (Geneticin®) were from Sigma (St. Louis, MO). GDPβS (guanosine 5′-*O*-(2-thiodiphosphate)) and NAD were from Boehringer Mannheim (Mannheim, Germany). Rolipram was a gift from Dr. Wachtel, Schering, Berlin, Germany. UK14,304 (5-bromo-6-[2-imidazoline-2-ylamino]quinoxaline) and RX821002 were from RBI (Natick, MA). Dexmedetomidine and levomedetomidine were gifts from Orion, Orion-Pharma (Turku, Finland). Cell culture reagents were from Gibco (Paisley, UK).

2.2. Methods

2.2.1. Cell culture

The human α_2-adrenoceptor subtypes α_{2A}, α_{2B} and α_{2C} were stably expressed in CHO cells (American Type Culture Collection, Rockville, MD) by transfection of the receptor cDNAs with the expression vector pMAMneo (Clontech, Palo Alto, CA) (Kobilka et al., 1987; Regan et al., 1988; Lomasney et al., 1990; Pohjanoksa et al., 1997). Adherent CHO cells were cultured in α-Modified Eagle Medium supplemented with 2 mM glutamine, 20 mM $NaHCO_3$, 5% heat-inactivated fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) (Nordvacc Media, Stockholm, Sweden). Stable levels of receptor expression were maintained by including the neomycin derivative G418 (150 μg/ml) in the culture medium. For some experiments, cells were grown for 24 h in serum-free medium supplemented with 500 ng/ml pertussis toxin. HEL cells obtained from the American Type Culture Collection were grown in suspension culture in RPMI-1640 medium supplemented with 7.5% heat-inactivated fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml). NG108-15 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium containing 5% fetal calf serum, 100 μM hypoxanthine, 10 μM aminopterin, 17 μM thymidine, penicillin (50 U/ml) and streptomycin (50 μg/ml). All cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.2.2. Radioligand binding experiments

Saturation binding experiments with CHO, HEL and NG108-15 cell homogenates were performed in K^+-phosphate buffer as previously described (Halme et al., 1995). 30–50 μg of protein were incubated with either 0.012–16 nM [^{3}H]rauwolscine (HEL cells) or 0.06–8 nM [^{3}H]RX821002 (CHO and NG108-15 cells). Non-specific binding was determined in the presence of 10 μM (−)-adrenaline in parallel assays.

2.2.3. Preparation of membranes

Frozen cell pellets were thawed and suspended in 20 ml of ice-cold 10 mM Tris–HCl and 0.1 mM EDTA (Tris–EDTA buffer, pH 7.5) supplemented with 0.32 mM sucrose. The cells were homogenised with a Potter–Elvehjem homogeniser (30 strokes at 1000 rpm). The homogenate was centrifuged at $550 \times g$ for 15 min. The supernatant was kept, and the pellet was rehomogenised and recentrifuged. The two supernatants were pooled and centrifuged at $35{,}000 \times g$ for 30 min. The pellet was then suspended in Tris–EDTA buffer and centrifuged at $35{,}000 \times g$ for 30 min. The membranes were finally suspended in Tris–EDTA buffer at a concentration of 1–2 mg protein/ml and frozen in aliquots at −70°C. Separation of plasma membranes and intracellular membranes was performed with sucrose density gradient centrifugation. Cells were lysed at 4°C with 20 mM Tris–HCl and 2 mM EDTA (pH

7.4), homogenised with a Potter–Elvehjem homogeniser, and the lysate (3.5 ml) was layered on top of a sucrose density gradient consisting of 3 ml of 55% (w/v), 3 ml of 32% and 1.5 ml of 5% sucrose, in 12 ml tubes. After centrifugation at 4°C for 60 min at 35,000 × *g*, the membranes at the 5/32% interface were collected as the light vesicle fraction (intracellular membranes) and those at the 32/55% interface were collected as the plasma membrane fraction (Zhu and Toews, 1994). Protein concentrations were determined according to the method of Bradford (1976), using bovine serum albumin as reference.

2.2.4. GTPase assay

The GTPase assay procedure was slightly modified from a previous protocol (McKenzie, 1992). The reaction mixture contained 100 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, 10 mM Tris–HCl, 1 mM App(NH)p, 1 mM ATP, 1 mM ouabain, 10 mM creatine phosphate, 2.5 units/ml creatine phosphokinase, 0.1–0.2 nM [γ-^{32}P]GTP, 30 μM GDPβS (unless indicated otherwise), and 3–5 μg of membrane protein in a final reaction volume of 100 μl. The agonist concentrations ranged from 0.1 nM to 100 μM. After 30 min at 37°C, the tubes were placed on ice, and the reaction was terminated by addition of 1 ml of an ice-cold suspension of 5% activated charcoal in 20 mM phosphoric acid. After centrifugation (5 min at 11,000 × *g*), the supernatant was removed and recentrifuged for 5 min at 11,000 × *g*. 550 μl of the supernatant was mixed with OptiPhase 'HiSafe' III (Wallac Turku, Finland), and its radioactivity was determined by liquid scintillation counting (Wallac 1219 Rackbeta, Wallac). GTPase activity due to low-affinity hydrolysis, determined in the presence of 100 μM GTP, was subtracted to yield the amount of high-affinity hydrolysis.

2.2.5. Pretreatment of membranes with pertussis toxin

Pertussis toxin was preactivated by incubation with 20 mM dithiothreitol for 15 min at 37°C. ADP ribosylation of G-proteins in CHO cell membranes was performed in a reaction mixture (0.3 ml) containing 10 mM NAD, 1 mM ATP, 1 mM GTP, 6 mM dithiothreitol, 100 mM Tris–HCl (pH 8.0) and 18 μg pertussis toxin/mg membrane protein for 60 min at 37°C. The reaction was stopped by addition of 2 ml of ice-cold 10 mM Tris–EDTA buffer (pH 7.5) and centrifugation for 20 min at 40,000 × *g*. Membranes were washed once with 2 ml of 10 mM Tris–EDTA buffer to remove pertussis toxin and nucleotides, and used immediately for the GTPase assay.

2.2.6. Measurement of intracellular cAMP

The growth medium of confluent cultures was replaced with serum-free medium supplemented with 5 μCi/ml [^{3}H]adenine. After incubation for 2 h the cells were collected, pelleted and washed once with NaCl-based medium (137 mM NaCl, 5 mM KCl, 10 mM glucose, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 20 mM TES [2-{3-hydroxyl-1,1-bis(hydroxymethyl)ethyl}ethane sulphonate] and 1 mM $CaCl_2$, adjusted to pH 7.4). Thereafter the cells were resuspended and divided into aliquots of about 1×10^6 (HEL) or 0.1×10^6 (NG108-15) cells in 0.8 ml of the same medium. The cells were preincubated with either 0.5 mM IBMX (HEL) or 60 μM rolipram (NG108-15), 100 μM propranolol and 150 μM quinacrine (a phospholipase A_2 inhibitor) for 10 min at 37°C. Forskolin (5 μM to HEL cells and 0.1 μM to NG108-15 cells) and agonists at different concentrations (1 nM–100 μM) were added. After 10 min the cells were centrifuged for 1 min at 11,000 × g, the medium was immediately removed, and the reaction was terminated by resuspension of the cells in 1 ml of 0.33 M perchloric acid, containing about 1600 dpm [^{14}C]cAMP. The extent of conversion of [^{3}H]ATP to [^{3}H]cAMP was determined by sequential Dowex/alumina ion exchange chromatography to isolate cAMP (Salomon et al., 1974). Conversion to [^{3}H]cAMP was expressed as a percentage of total cell-associated tritium and was normalized to the recovery of [^{14}C]cAMP tracer (generally 70%). Radioactivity was determined by liquid scintillation counting (Wallac 1410) in Optiphase 'HiSafe' III.

2.2.7. Data analysis

The results were analysed using GraphPAD Prism programs (GraphPAD Software, San Diego, CA). pA_2 values were calculated as described by Arunlakshana and Schild (1959). Statistical analysis was carried out with Student's *t*-test with two-tailed probabilities or one-way analysis of variance (ANOVA) followed by Dunnett's test. *P* values smaller than 0.05 were considered to be statistically significant. The results are expressed as mean values ± S.E.

3. Results

3.1. GTPase activity in CHO cell membranes

The basal and (−)-adrenaline stimulated release of ^{32}P from [γ-^{32}P]GTP in membranes from CHO cells transfected to express the three human α_2-adrenoceptor subtypes α_{2A}, α_{2B} and α_{2C} at densities of 1.88 ± 0.40 pmol/mg, 2.40 ± 0.65 pmol/mg and 2.04 ± 0.42 pmol/mg total cellular protein is shown in Fig. 1. All tubes contained 0.1 nM [γ-^{32}P]GTP together with different amounts of unlabelled GTP. Increasing concentrations of unlabelled GTP reduced the hydrolysis of [γ-^{32}P]GTP and abolished the stimulatory effect of (−)-adrenaline, suggesting that (−)-adrenaline-sensitive GTPase activity in this assay derives from high-affinity (low K_m) GTPase. The K_m values for the basal high-affinity GTPase activity were similar in CHO cell membranes containing the three receptor subtypes (α_{2A}, $K_m = 232 \pm 17$ nM; α_{2B}, $K_m = 241 \pm 21$ nM; and α_{2C}, $K_m = 266 \pm 31$ nM), and were slightly increased by 100 μM (−)-adrenaline [α_{2A}, $K_m =$

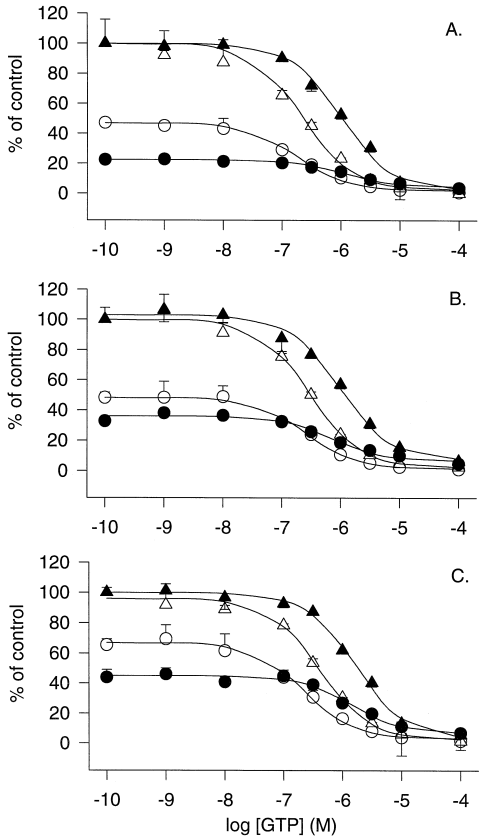

Fig. 1. Hydrolysis of [γ-^{32}P]GTP in membranes prepared from CHO cells transfected to express the α_2-adrenoceptor subtypes α_{2A} (A), α_{2B} (B) and α_{2C} (C), at various concentrations of unlabelled GTP in the absence (○/●) and presence (△/▲) of 100 μM (−)-adrenaline. In some experiments, 30 μM GDPβS was included (●/▲). For calculation of the percent values, the stimulation induced by (−)-adrenaline in the presence of 0.1 nM GTP was taken as 100%; the numerical values are presented in Table 1. The means ± S.E. of 3 separate experiments are presented.

264 ± 22 nM (not statistically significantly different from the K_m for basal activity); α_{2B}, $K_m = 290 \pm 11$ nM ($P < 0.05$); and α_{2C}, $K_m = 405 \pm 27$ nM ($P < 0.01$)]. To calculate the rate of high-affinity hydrolysis (fmol/mg/min), the amount of ^{32}P released in the presence of 100 μM GTP was subtracted from the values obtained from samples containing 0.1 nM–10 μM of GTP. The stimulation of high-affinity hydrolysis induced by 100 μM (−)-adrenaline was not strongly influenced by GTP concentrations of 0.1 μM or less, and was about 90% over the basal activity for the α_{2A}- and α_{2B}-subtypes, and 50% for the α_{2C}-subtype (Fig. 1).

In order to increase the net signal evoked by (−)-adrenaline, we used the non-hydrolysable nucleotide analog GDPβS (Vachon et al., 1986), which binds to the GTP-binding site of G-proteins. The basal and (−)-adrenaline-stimulated rates of high-affinity hydrolysis of GTP were measured by subtracting the activity measured in the presence of 100 μM GTP from the activity measured in the presence of 0.1 nM GTP. This was done in the presence of different concentrations of GDPβS (Table 1). For all three receptor subtypes, both the basal and the (−)-adrenaline (100 μM)-stimulated high-affinity GTP hydrolysis was reduced in a concentration-dependent manner by GDPβS. Since the basal high-affinity activity was reduced more by GDPβS than the receptor-stimulated activity, increases in the percent stimulation by (−)-adrenaline (100 μM) could be seen for all three receptor subtypes. In order to optimise the signal-to-noise ratio of the assay, 30 μM GDPβS was chosen for use in subsequent experiments.

Fig. 1 demonstrates that increasing concentrations of unlabelled GTP decreased both the basal and (−)-adrenaline-stimulated rates of high-affinity GTP hydrolysis in the presence of 30 μM GDPβS in the same manner as in the absence of GDPβS. The addition of 30 μM GDPβS to the assay resulted in a shift of the curves to the right. The K_m values of the basal high-affinity hydrolysis were now 0.92 ± 0.25 μM (α_{2A}), 1.7 ± 0.24 μM (α_{2B}) and 1.1 ± 0.17 μM (α_{2C}), and were not significantly influenced by the addition of 100 μM (−)-adrenaline: 1.1 ± 0.08 μM (α_{2A}), 1.7 ± 0.16 μM (α_{2B}) and 1.0 ± 0.10 μM (α_{2C}).

High-affinity GTP hydrolysis was measured after addition of a saturating concentration (100 μM) of (−)-adrenaline and different concentrations of (−)-noradrenaline, UK14,304, dexmedetomidine, levomedetomidine, clonidine and oxymetazoline to membranes prepared from CHO cells. The basal GTPase activities in CHO cell membranes were 2.2 ± 0.4 fmol/mg/min (non-transfected), 2.2 ± 0.6 fmol/mg/min (α_{2A}-transfected), 2.5 ± 0.6 fmol/mg/min

Table 1
Effects of GDPβS on basal and α_2-adrenoceptor-stimulated high-affinity GTPase activity in membranes prepared from CHO cells transfected to express α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. Results are expressed as fmol/mg protein/min and are means ± S.E. from 3 independent experiments performed in triplicate

[GDPβS] μM	Basal activity	With 100 μM (−)-adrenaline	Percent stimulation by (−)-adrenaline
CHO-α_{2A}			
0	22 ± 0.7	42 ± 0.7	90
3	8.8 ± 0.2	29 ± 1.1	230
10	4.9 ± 0.1	20 ± 0.4	310
30	2.4 ± 0.1	11 ± 0.5	360
100	0.7 ± 0.5	3.9 ± 0.5	460
CHO-α_{2B}			
0	20 ± 0.4	39 ± 0.4	95
3	8.8 ± 0.3	24 ± 0.5	170
10	4.6 ± 0.1	14 ± 0.4	200
30	1.9 ± 0.2	7.4 ± 0.1	290
100	0.6 ± 0.1	3.1 ± 0.1	420
CHO-α_{2C}			
0	25 ± 2.1	38 ± 0.9	50
3	11 ± 0.4	19 ± 2.1	70
10	6.7 ± 0.4	13 ± 0.3	90
30	3.1 ± 0.7	6.2 ± 0.1	100
100	1.1 ± 0.1	2.6 ± 0.1	140

Table 2
Characterization of the high-affinity GTPase activity in membranes from CHO cells transfected to express different α_2-adrenoceptor subtypes. The E_{max} values for the different drugs indicate the maximal percent change from the enzyme activity in the absence of agonists. EC_{50} is the concentration causing 50% of the maximal effect. The values are means ± S.E. from 4–5 experiments performed in triplicate. n.s., not statistically significant; –, not determined

Ligand	E_{max}	Percent of A	EC_{50} (nM)
CHO-α_{2A}			
(−)-Adrenaline (A)	384 ± 65	100	–
(−)-Noradrenaline	322 ± 36	84	3130 ± 802
UK14,304	304 ± 31	79	122 ± 6
Dexmedetomidine	91 ± 14	24	13.3 ± 13.2
Levomedetomidine	−1.5 ± 0.6 (n.s.)	–	–
Clonidine	57 ± 7	15	–[a]
Oxymetazoline	5.4 ± 9.6 (n.s.)	1.4	–
CHO-α_{2B}			
(−)-Adrenaline (A)	324 ± 48	100	–
(−)-Noradrenaline	324 ± 35	100	1700 ± 651
UK14,304	134 ± 24	41	960 ± 80
Dexmedetomidine	268 ± 38	83	7.1 ± 2.7
Levomedetomidine	15 ± 12 (n.s.)	4.6	–
Clonidine	61 ± 17	19	–[a]
Oxymetazoline	205 ± 35	63	649 ± 173
CHO-α_{2C}			
(−)-Adrenaline (A)	108 ± 14	100	–
(−)-Noradrenaline	111 ± 16	103	1340 ± 84
UK14,304	60 ± 9	56	240 ± 69
Dexmedetomidine	58 ± 9	54	41 ± 29
Levomedetomidine	−4.0 ± 10 (n.s.)	–	–
Clonidine	3.0 ± 7.2 (n.s.)	2.8	–
Oxymetazoline	−5.7 ± 11 (n.s.)	–	–

[a]Large scatter due to small effect.

(α_{2B}-transfected) and 2.6 ± 0.8 fmol/mg/min (α_{2C}-transfected). Table 2 presents the relative increases in GTPase activity induced by the different drugs. No statistically significant effects were induced by any of the drugs in membranes from non-transfected CHO cells (data not shown). In membranes from cells expressing the three α_2-adrenoceptor subtypes, the maximal stimulatory effect of (−)-adrenaline was clearly smaller in the α_{2C}-subtype, 108% over basal compared to 384% and 324% in the α_{2A}- and α_{2B}-subtypes.

In order to compare the relative efficacies of different agonists at the receptor subtypes, (−)-adrenaline was used as a reference compound. (−)-Noradrenaline acted as a full agonist at all receptor subtypes, stimulating GTPase activity with the same efficacy as (−)-adrenaline (Table 2). Clonidine was a partial agonist at the α_{2A}- and α_{2B}-subtypes, but appeared inactive at the α_{2C}-subtype. Levomedetomidine was inactive at all subtypes. Clear subtype-selectivity in efficacy was seen for the agonists UK14,304, dexmedetomidine and oxymetazoline. The rank order of efficacy for UK14,304 was α_{2A} (79% of (−)-adrenaline) > α_{2C} (56%) > α_{2B} (41%), and for dexmedetomidine the rank order of efficacy was α_{2B} (83% of (−)-adrenaline) > α_{2C} (54%) > α_{2A} (24%). Oxymetazoline was inactive at the α_{2A}- and α_{2C}-subtypes, but acted as a strong partial agonist at the α_{2B}-subtype (66% of (−)-adrenaline). UK14,304 and dexmedetomidine showed subtype-selectivity also in their potency, UK14,304 by having a lower potency for increasing in the high-affinity hydrolysis at the α_{2B}-subtype (EC_{50} = 960 nM) compared to the α_{2A}- (122 nM) and α_{2C}- (240 nM) subtypes, and dexmedetomidine by having a lower potency at the α_{2C}-subtype (EC_{50} = 41 nM) compared to the α_{2A}- (13 nM) and α_{2B}- (7 nM) subtypes. (−)-Noradrenaline showed nearly equal potency at all three subtypes (EC_{50} = 1300–3100 nM) (Table 2).

The effects of the different agonists on GTPase activity were at all subtypes completely blocked when the α_2-antagonist RX821002 (100 μM) was included in the assays. The antagonist itself had no effect (data not shown). Oxymetazoline shifted the dose–response curve of (−)-noradrenaline to the right for the α_{2A}- and α_{2C}-subtypes without causing a significant change of slope (Fig. 2). The pA_2 values obtained by Schild analysis were 7.99 ± 0.08

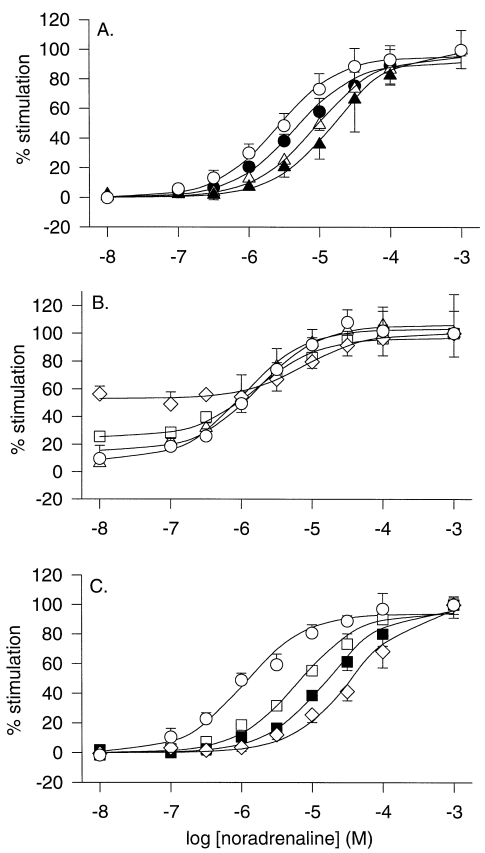

Fig. 2. The antagonistic effect of oxymetazoline (shown as percent of control) on the stimulation of high-affinity GTPase activity by (−)-noradrenaline in three CHO cell lines expressing α_{2A}- (A), α_{2B}- (B) and α_{2C}-adrenoceptors (C). Symbols: (○) control, (●) 10 nM, (△) 30 nM, (▲) 100 nM, (□) 300 nM, (■) 1000 nM and (◇) 3000 nM oxymetazoline. Results are means ± S.E. from 3 separate experiments performed in triplicate.

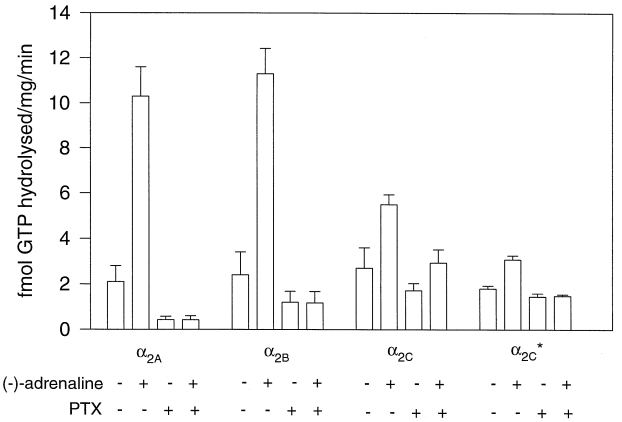

Fig. 3. Effects of pertussis toxin on basal and α_2-adrenoceptor-stimulated (100 μM (−)-adrenaline) high-affinity GTPase activity. Intact cells were pretreated for 24 h with 500 ng/ml pertussis toxin, or membranes were pretreated for 1 h with 0.18 μg/mg pertussis toxin (*). Results are means ± S.E. from 3 experiments performed in triplicate.

for the α_{2A}-subtype and 7.30 ± 0.07 for the α_{2C}-subtype. At the α_{2B}-subtype, oxymetazoline stimulated GTPase activity in the absence of (−)-noradrenaline, and the rightward shift of the dose–response curve of (−)-noradrenaline was small and statistically not significant. No pA_2 value could be calculated.

Pertussis toxin treatment (500 ng/ml in the growth medium for 24 h) of intact cells resulted in a reduction of the basal GTPase activity. Basal activities after such pretreatment were 0.4 ± 0.1 (α_{2A}-transfected), 1.2 ± 0.5 (α_{2B}-transfected) and 1.7 ± 0.3 fmol/mg/min (α_{2C}-transfected). The stimulatory effect of 100 μM (−)-adrenaline was completely blocked by pertussis toxin treatment of cells expressing the α_{2A}- and α_{2B}-receptor subtypes (Fig. 3). In contrast, in membranes with α_{2C}-receptors, 100 μM (−)-adrenaline activated GTPase activity also after pertussis toxin pretreatment of whole cells, albeit to a smaller extent than in membranes from non-pretreated cells (70% stimulation over basal activity ($P < 0.05$) after pertussis toxin versus 110% in non-pretreated cells) (Fig. 3). When pertussis toxin pretreatment (18 μg/mg protein for 60 min at 37°C) was carried out with membranes from CHO cells expressing α_{2C}-adrenoceptors, rather than with whole cells, the stimulatory effect of 100 μM (−)-adrenaline was completely abolished (Fig. 3).

In order to address the question of the subcellular localisation of the α_{2C}-adrenoceptor and its coupling in different environments, an attempt was made to subfractionate the usually used crude membrane preparation with a sucrose gradient into plasma membrane and intracellular membrane fractions. Basal high-affinity GTPase activity was similar in both membrane fractions and the addition of 100 μM (−)-adrenaline resulted in 80–90% increases over basal activity in both fractions (data not shown).

3.2. cAMP production in HEL and NG108-15 cells

The density of endogenous α_{2A}-receptors in HEL cells was 54 ± 11 fmol/mg total cellular protein. In NG108-15 cells, the density of α_{2B}-receptors was 100 ± 14 fmol/mg total cellular protein. The basal conversion of [^{3}H]ATP to [^{3}H]cAMP was in HEL cells 0.10 ± 0.02% in 10 min and in NG108-15 cells 0.9 ± 0.2% in 10 min. Addition of 5 μM forskolin to HEL cells resulted in an 8-fold increase in the rate of cAMP production and addition of 0.1 μM forskolin to NG108-15 cells resulted in a 4-fold increase. The inhibition of forskolin-stimulated cAMP production by (−)-adrenaline, UK14,304, dexmedetomidine and oxymetazoline in the two cell lines is presented in Table 3. The maximal inhibitory effect of (−)-adrenaline was 67% in HEL cells and 54% in NG108-15 cells. The inhibitory effects of the other ligands were compared to that of (−)-adrenaline, and their rank order of efficacy was in HEL cells UK14,034 (84% of (−)-adrenaline) > dexmedetomidine (65%) > oxymetazoline (13%). In NG108-15 cells the rank order of efficacy was oxymetazoline (91% of (−)-adrenaline) = dexmedetomidine (90%) > UK14,304 (50%).

4. Discussion

A receptor-mediated increase in high-affinity GTPase activity is among the first detectable biochemical events that follow activation of G-protein coupled receptors and is in this respect a potentially useful indicator for the pharmacological characterisation of agonists. Kim et al. (1994) have shown that the effector enzyme, adenylyl cyclase, is quantitatively the limiting factor in the coupling of receptors via G_s-proteins to activation of adenylyl cyclase. This

Table 3
Inhibition of cAMP production in intact HEL and NG108-15 cells. The percent conversion of [^{3}H]ATP to [^{3}H]cAMP was measured in the absence and presence of forskolin (5 μM in HEL cells and 0.1 μM in NG108-15 cells). The effects of different agonists were tested, and the maximal responses (E_{max}) are expressed as percent inhibition of forskolin-stimulated cAMP production. The values are means ± S.E. from 3–6 experiments performed in triplicate

Ligand	HEL (α_{2A})			NG108-15 (α_{2B})		
	E_{max} (%)	Percent of A	EC_{50} (nM)	E_{max} (%)	Percent of A	EC_{50} (nM)
(−)-Adrenaline (A)	66.8 ± 4.7	100	1630 ± 1440	54.5 ± 1.2	100	660 ± 60
UK14,304	56.3 ± 3.6	84	357 ± 1.52	27.3 ± 2.6	50	420 ± 175
Dexmedetomidine	43.4 ± 7.1	65	22.6 ± 12.3	48.5 ± 4.5	90	6.0 ± 2.0
Oxymetazoline	8.7 ± 0.7	13	–	49.7 ± 2.0	91	440 ± 75

limiting step is avoided by using the GTPase assay, which is technically simple to perform and has been used for investigation of several different receptors (Aktories and Jakobs, 1981; Koski and Klee, 1981; Brandt and Ross, 1986; Higashijima et al., 1987; Costa and Herz, 1989; Costa et al., 1990, 1992; Schenker et al., 1991; McKenzie, 1992; Lazareno et al., 1993; Odagaki and Fuxe, 1995; Hasegawa et al., 1996; Opperman et al., 1996; Seiler et al., 1996). The GTPase assay has also been used in some studies on α_2-adrenoceptors. In cell lines endogenously expressing α_2-adrenoceptors, the stimulation of GTPase activity by a supramaximal concentration of adrenaline amounts to about 40% (Odagaki et al., 1993; Musgrave and Seifert, 1995), while 150% increases have been reported in transfected cells (McClue and Milligan, 1991; MacNulty et al., 1992; Carr et al., 1994), probably as a consequence of the higher levels of receptor expression. In the present study performed with CHO cells transfected to express α_{2A}-, α_{2B}- and α_{2C}-receptor subtypes at about 2 pmol receptor/mg total cellular protein, the high-affinity GTPase activity was increased by 50–90% by a supramaximal concentration of (−)-adrenaline, but the agonist signal could be readily increased 2- to 4-fold by addition of the non-hydrolyzable guanine nucleotide analog GDPβS (Vachon et al., 1986) to the assay.

These results indicate that a smaller number of G-proteins are activated in CHO cell membranes containing the α_{2C}-subtype than in membranes containing one of the other two subtypes. At the second messenger level, no indication for such a difference has been seen (Jansson et al., 1994; Pohjanoksa et al., 1997), but, observed against a common host cell background, it probably has its origin in differences between the α_2-receptor subtypes. Transfected α_{2A}- and α_{2B}-receptors mainly reside in the plasma membrane, while most α_{2C}-receptors are seen in intracellular vesicles (Von Zastrow et al., 1993). The weaker stimulation of GTPase activity by the α_{2C}-subtype compared to the other two subtypes could therefore depend on the intracellular α_{2C}-receptors having restricted access to G-proteins. This hypothesis was not substantiated by experiments performed on fractionated plasma membranes and intracellular membranes, since (−)-adrenaline stimulated GTPase activity equally in both fractions.

The stimulatory effect of 100 μM (−)-adrenaline was completely blocked by pertussis toxin treatment of cells expressing the α_{2A}- and α_{2B}-receptor subtypes. But in membranes with α_{2C}-receptors, 100 μM (−)-adrenaline still activated GTPase after pertussis toxin pretreatment of whole cells, albeit to a smaller extent than in membranes from non-pretreated cells (Fig. 3). This residual GTPase activation was completely abolished when membranes from CHO cells expressing α_{2C}-adrenoceptors, instead of whole cells, were incubated with pertussis toxin. This suggests that pertussis toxin treatment of whole cells did not inactivate intracellular G-proteins associated with intracellular α_{2C}-adrenoceptors. However, as control membranes treated similarly but without pertussis toxin also showed reduced GTPase responses (Fig. 3), this conclusion remains tentative and may depend on the insufficient sensitivity of the assay.

We have earlier demonstrated that α_{2B}-receptors can stimulate cAMP production in CHO cells through a pertussis toxin-insensitive mechanism, probably via G_s-proteins (Pohjanoksa et al., 1997). In the present study, the increase in GTPase activity mediated by the α_{2B}-subtype was completely blocked by pertussis toxin pretreatment, indicating that the GTPase assay selectively monitors coupling of the α_{2B}-adrenoceptor to pertussis toxin-sensitive G-proteins.

Some α_2-adrenoceptor ligands show significant subtype-selectivity in binding assays, e.g., prazosin ($\alpha_{2B} = \alpha_{2C} > \alpha_{2A}$), chlorpromazine ($\alpha_{2B} > \alpha_{2C} > \alpha_{2A}$) and oxymetazoline ($\alpha_{2A} > \alpha_{2C} > \alpha_{2B}$) (Marjamäki et al., 1993). These three ligands are commonly used for the pharmacological characterisation of α_2-adrenoceptor subtypes in competition binding assays. Radioligand binding assays are clearly insufficient for agonist characterisation, and functional assays are needed. We have now optimised the high-affinity GTPase assay for this purpose. Our results argue that some agonists possess marked subtype-selectivity in their efficacy. This phenomenon was particularly evident for dexmedetomidine, UK14,304 and oxymetazoline. Dexmedetomidine was a full agonist at the α_{2B}-subtype, but was a partial agonist at the α_{2A}- and the α_{2C}-subtypes. UK14,304 was a full agonist at the α_{2A}-subtype, but was a partial agonist at the other two. Oxymetazoline was a rather strong partial agonist at the α_{2B}-subtype, but was inactive at the others. Thus, the intrinsic efficacy patterns emerging from the GTPase assay were clearly different from those we had previously obtained in cAMP experiments with transfected S115 (mouse mammary tumour) cells (Jansson et al., 1994) and CHO cells (Pohjanoksa et al., 1997), where no subtype-dependence was seen in agonist efficacy. Eason et al. (1994) have reported that UK14,304 inhibits adenylyl cyclase in transfected CHO cell membranes with a clearly lower potency through α_{2B}-adrenoceptors than through α_{2A}- or α_{2C}-adrenoceptors. A similar rank order was also seen for oxymetazoline, but with smaller differences between the subtypes (Eason et al., 1994). To further validate our results obtained with the GTPase assay and relatively high levels of expression of recombinant receptors, we used HEL and NG108-15 cells, which endogenously express low densities of α_{2A}-adrenoceptors (HEL) or α_{2B}-adrenoceptors (NG108-15).

The results from the HEL and NG108-15 cAMP assays were in good agreement with the GTPase data, and qualitatively confirmed the α_{2A}-/α_{2B}-subtype-dependent intrinsic efficacies of dexmedetomidine, UK14,304 and oxymetazoline. However, closer inspection of the α_{2A}-adrenoceptor GTPase results (CHO) and the cAMP results (HEL) shows that the two data sets are not a perfect match.

While oxymetazoline was inactive in the α_{2A}-GTPase assay, it had weak but significant inhibitory activity in the HEL cell cAMP assay. This difference may appear puzzling at first sight, since current thinking in receptor-mediated signal transduction considers G-protein activation as a mandatory requirement for the subsequent activation (or here, inhibition) of the effector enzyme. However, at least three possible explanations can be offered for the apparent lack of activity at the G-protein level despite some agonistic effects in a second messenger-based assay.

Firstly, it is possible that a weak agonist may cause some G-protein activation, too little to be picked up as a G-protein signal but detectable at the second messenger level due to additional signal amplification at the G-protein/effector interface. This hypothesis would also explain the almost 3-fold higher apparent efficacy of dexmedetomidine in the HEL cell cAMP assay (65% efficacy relative to (−)-adrenaline) than in the α_{2A}-GTPase assay in CHO cells (24%).

A second possible explanation for the apparent inactivity of oxymetazoline in the α_{2A}-GTPase assay may be that the GTPase assay does not allow the detection of the complete spectrum of activated G proteins. However, no evidence exists for the inhibition of adenylyl cyclase through mechanisms other than pertussis toxin-sensitive G-proteins. HEL cells, like CHO cells, express $G_{i\alpha 2}$ and $G_{i\alpha 3}$, but HEL cells also express a low level of $G_{i\alpha 1}$ (Williams et al., 1990; Gerhardt and Neubig, 1991). Neither cell line is immunoreactive for $G_{o\alpha}$ (Michel et al., 1989; A. Marjamäki, unpublished).

Third, we have obtained new results in a closely related G-protein-based assay (^{35}S-GTPγS binding) that strongly argue that the absence of agonist activity for oxymetazoline in the α_{2A}-GTPase assay may be due to experimental conditions. Na^+ ions and GDP can reduce the potency of agonists in GTPase assays, while Mg^{2+} ions can increase it (Gierschik et al., 1994). Experiments in our laboratory indicate that Na^+ and guanine nucleotides in addition can depress the intrinsic activity of partial agonists to such an extent that these compounds may appear inactive. A detailed report of this phenomenon is in preparation and will be presented elsewhere (S. Wurster et al.).

The functional subtype-selectivity of oxymetazoline for the α_2-adrenoceptor subtypes identified in the current investigation also offers an explanation for the discrepancies in the binding selectivity of oxymetazoline observed by Gleason and Hieble (1991) and MacKinnon et al. (1993). These authors reported that oxymetazoline displayed clear subtype-selectivity for the α_{2A}- over the α_{2B}-subtype when ^{3}H-antagonists ([^{3}H]rauwolscine or [^{3}H]RS-15385-197) were used to label the two receptor subtypes. When ^{3}H-agonists ([^{3}H]UK14304 or [^{3}H]adrenaline) were used for this purpose, the affinity of oxymetazoline for the α_{2A}-subtype remained unchanged, whereas that for the α_{2B}-subtype increased, becoming almost equal to that for the α_{2A}-subtype. Thus, oxymetazoline was subtype-selective when the receptors were labelled with ^{3}H-antagonists but not when they were labelled with ^{3}H-agonists. Within experimentally feasible concentrations, ^{3}H-agonists preferentially or even exclusively label only agonist high-affinity receptor forms. These receptor forms are considered to consist of activated receptor species and in most cases represent only a subset of the total receptor population. Radiolabelled antagonists label the entire receptor population (Gleason and Hieble, 1991). The minor affinity difference for oxymetazoline at the α_{2A}-subtype when ^{3}H-agonists or ^{3}H-antagonists were used as radioligands shows that oxymetazoline has only a relatively small preference for activated over resting α_{2A}-receptors. In contrast, the large affinity difference at the α_{2B}-adrenoceptor argues that, for this receptor subtype, oxymetazoline possesses a much larger preference for activated over resting receptors.

Within the framework of 'two-state' G-protein-coupled receptor models, the preference of an agonist for activated over resting receptor forms is the equivalent of 'intrinsic activity' in classical pharmacology (Leff, 1995). Consequently, the affinity results for oxymetazoline reported by Gleason and Hieble (1991) and MacKinnon et al. (1993) can be interpreted as indicating that oxymetazoline has greater intrinsic efficacy at the α_{2B}-adrenoceptor than at the α_{2A}-subtype. This is an interpretation completely in line with the results of the current investigation.

The GTPase assay was optimised and used for the pharmacological characterisation of some selected α_2-adrenoceptor agonists. Unlike the situation with classical second messenger assays (Pohjanoksa et al., 1997), the high receptor expression levels in transfected CHO cells did not mask partial agonist characteristics in the GTPase assay. The agonists dexmedetomidine, UK14,304 and oxymetazoline stimulated GTPase activity in a receptor subtype-selective manner. This subtype-selectivity was supported by the results from cAMP accumulation assays performed with intact HEL and NG108-15 cells endogenously expressing low densities of α_2-adrenoceptors. While this manuscript was in preparation, similar α_2-adrenoceptor subtype-dependent efficacy profiles were reported for these agonists in a study which used another method to detect G-protein activation, i.e., agonist-stimulated [^{35}S]GTPγS binding (Jasper et al., 1998; Peltonen et al., 1998), which further confirms the validity of the current results. In terms of efficacy, dexmedetomidine and oxymetazoline clearly preferred the α_{2B}-subtype in both assays, and UK14,304 preferred the α_{2A}-subtype. Some quantitative differences in partial agonist activity were, however, noted between the present study and that of Jasper et al. (1998). This is presumably due to differences in the host cell lines (HEK-293 vs. CHO), and/or different assay conditions. In conclusion, we would like to suggest that GTPase assays represent a useful method for the investigation of $G_{i/o}$-protein coupled receptors, particularly in the quest for subtype-selective agonists.

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