ELSEVIER

European Journal of Pharmacology 338 (1997) 293–296

ejp

Short communication

Assessment of α_2-adrenoceptor antagonist potency with GTPase assay

Sami Virolainen, Christian C. Jansson, Mika Scheinin *

Department of Pharmacology and Clinical Pharmacology, University of Turku, Tykistokatu 6A, FIN-20520 Turku, Finland

Received 11 August 1997; revised 17 September 1997; accepted 23 September 1997

Abstract

Membranes from Chinese hamster ovary (CHO) cells expressing high densities of α_{2A}-, α_{2B}- or α_{2C}-adrenoceptor subtypes were used to monitor potencies of α_2-adrenoceptor antagonists with a GTPase assay. Receptor-activated high-affinity GTPase activity was determined by measuring the rate of release of ^{32}P from [γ-^{32}P]GTP. Concentration–response curves to the full agonist (−)-noradrenaline were obtained in the presence of different antagonist concentrations and pA_2 values were calculated by Schild analysis. Three antagonists (rauwolscine, prazosin and chlorpromazine) showed subtype-selectivity, which agrees with earlier radioligand binding results. We suggest that the GTPase assay described here is useful for characterization of the functional potency and subtype-selectivity of α_2-adrenoceptor antagonists. © 1997 Elsevier Science B.V.

Keywords: α_2-Adrenoceptor subtype; Antagonist potency; CHO cell; GTPase

1. Introduction

Pertussis toxin-sensitive G-proteins (G_{i1}, G_{i2}, G_{i3} and G_o) mediate most physiological signalling pathways linked to α_2-adrenoceptors (Limbird, 1988; Kurose et al., 1991; Raymond, 1995), 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). They can therefore regulate several different cellular effectors, including adenylyl cyclases, ion channels (K^+ and Ca^{2+}) and phospholipases (A_2, C and D).

Agonist-activated α_2-adrenoceptors promote the release of GDP from G-proteins and binding and subsequent hydrolysis of GTP. Binding and hydrolysis of GTP can be conveniently detected by measuring receptor-stimulated GTPase activity, monitoring the release of ^{32}P from [γ-^{32}P]GTP. In Chinese hamster ovary (CHO) cells transfected to separately express the α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes, α_2-adrenoceptor-dependent activation of GTPase is mediated solely by pertussis toxin-sensitive G_i-proteins (Jansson et al., data not shown).

In the search for subtype-selective α_2-adrenoreceptor ligands, antagonists have usually been initially evaluated in receptor binding studies. However, receptor binding assays may not give sufficient information. For example, partial agonist and inverse agonist properties can only be detected in functional assays. The aim of this study was to develop a convenient functional in vitro assay to monitor the potency and subtype-selectivity of α_2-adrenoceptor antagonists.

2. Materials and methods

2.1. Materials

[γ-^{32}P]GTP was from Amersham (Amersham, UK). GDPβS [guanosine 5′-*O*-(2-thiodiphosphate)] was from Boehringer-Mannheim (Mannheim, Germany). Rauwolscine was from C. Roth (Karlsruhe, Germany). RX821002 [2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline] was from Research Biochemicals International (Natick, MA, USA). App(NH)p (5′-adenylylimidodiphosphate), ATP, charcoal, chlorpromazine, creatine phosphate, creatine phosphokinase, dithiothreitol, GTP, (−)-noradrenaline, ouabain, phentolamine and prazosin were from Sigma (St. Louis, MO, USA).

* Corresponding author. MediCity Research Laboratory, University of Turku, Tykistökatu 6A, FIN-20520 Turku, Finland. Fax: (358-2) 333-7000; e-mail: mschein@utu.fi

2.2. Methods

2.2.1. Receptor production

The human α_2-adrenoceptor subtypes α_{2A}, α_{2B} and α_{2C} were stably expressed in CHO cells by transfection of the receptor cDNAs with the expression vector pMAMneo (Kobilka et al., 1987; Regan et al., 1988; Lomasney et al., 1991; Pohjanoksa et al., 1997). The expression levels were 1.9 ± 0.4 pmol/mg total cellular protein for α_{2A}, 2.4 ± 0.7 pmol/mg for α_{2B} and 2.0 ± 0.4 pmol/mg for α_{2C}. Cells were harvested into chilled phosphate-buffered saline, centrifuged at $1500\,g$ for 5 min and stored at -20°C. Frozen cells were thawed and suspended in ice-cold 10 mM Tris–HCl and 0.1 mM EDTA (pH 7.5), supplemented with 0.32 mM sucrose. The cells were homogenized on ice with a Potter–Elvehjem homogenizer. The homogenate was centrifuged at $550 \times g$ for 15 min. The supernatant was kept and the pellet was rehomogenized and recentrifuged. The two supernatants were pooled and then 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 the buffer at a concentration of 1–2 mg protein/ml and frozen in aliquots at -70°C. Protein concentrations were determined using the method of Bradford (1976).

2.2.2. GTPase assay

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, 1mM ouabain, 10 mM creatine phosphate, 2.5 units/ml creatine phosphokinase, 0.1–0.2 nM [γ-^{32}P]GTP, 32 μM GDPβS and 4–7 μg membrane protein per tube. Drugs were added in appropriate concentrations into tubes kept on ice before adding the membranes. After 30 min in a 37°C water bath with shaking, the tubes were transferred to ice, and the reaction was terminated by addition of 1 ml of an ice-cold suspension of 5% (w/v) activated charcoal in 20 mM phosphoric acid. After centrifugation (5 min at $11\,000 \times g$) the supernatant was removed, recentrifuged and a 550 μl aliquot was removed for liquid scintillation counting. Low affinity hydrolysis of [γ-^{32}P]GTP was determined in the presence of 100 μM GTP and subtracted to yield the amount of high affinity hydrolysis.

2.2.3. Data analysis

The results were analysed using GraphPAD Prism programs (GraphPAD Software, San Diego, CA, USA). Concentration–response curves to the full agonist (−)-noradrenaline (10 nM–1 mM) were obtained in the presence of three different concentrations of each antagonist. The concentration of (−)-noradrenaline necessary to give a half-maximal response (EC_{50}) in the presence of each concentration of antagonist was divided by the EC_{50} in the absence of antagonist to determine the dose ratio (dr). pA_2 values were calculated by the method of Arunlakshana and Schild (1959) by plotting log(dr − 1) versus log[antagonist]. Statistical analysis was carried out by one-way ANOVA. Results are presented as means ± S.E.M. unless otherwise indicated.

3. Results

All tested antagonists shifted the dose–response curves of (−)-noradrenaline to the right without significant change of slope or maximum effect thus indicating competitive antagonism. pA_2 values given in Table 1 were achieved by Schild analysis. Schild plots are shown in Fig. 1. The basal high-affinity GTPase activities were 1.7 ± 0.7, 1.7 ± 0.8 and 1.6 ± 0.8 fmol/mg per min, the maximal (−)-noradrenaline-stimulated high-affinity activities 8.8 ± 3.3, 7.6 ± 3.5 and 4.0 ± 1.7 fmol/mg per min and the rates of low-affinity GTP hydrolysis (determined in the presence of 100 μM GTP, and not influenced by the drugs) 1.5 ± 0.5, 1.4 ± 0.7 and 1.4 ± 0.4 fmol/mg per min in membranes expressing the α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes, respectively (values are means ± S.D. from at least 15 experiments). The basal high-affinity GTPase activities determined in the absence of (−)-noradrenaline were not changed by the antagonists.

In order to compare potencies of α_2-adrenoceptor antagonists determined by this method to binding affinities determined by radioligand binding experiments, a linear regression analysis was carried out for each receptor subtype using the present results and pK_i values obtained with [^{3}H]rauwolscine as radioligand (Marjamäki et al., 1993). The Pearson correlation coefficients for logarithmic data were 0.97 for α_{2A}, 0.72 for α_{2B} and 0.94 for α_{2C}.

Table 1
pA_2 values derived from Schild analysis are means ± S.E.M. from three independent experiments performed in triplicate

Antagonist	α_{2A}			α_{2B}			α_{2C}		
	pA_2	Slope	pK_i	pA_2	Slope	pK_i	pA_2	Slope	pK_i
Chlorpromazine	6.22 ± 0.13	1.06	5.89	7.40 ± 0.13	0.96	7.85	7.09 ± 0.12	1.02	6.86
Prazosin	6.38 ± 0.23	0.98	5.56	6.62 ± 0.14	1.13	6.97	7.64 ± 0.07	1.24	7.10
Phentolamine	7.52 ± 0.13	1.08	7.75	7.26 ± 0.19	0.91	8.51	6.99 ± 0.24	0.98	7.28
Rauwolscine	8.62 ± 0.07	1.10	8.30	7.83 ± 0.27	1.14	8.30	9.04 ± 0.17	1.15	9.00
RX821002	8.93 ± 0.13	1.14	8.66	8.49 ± 0.10	0.89	8.34	8.71 ± 0.17	0.96	8.60

pK_i values are given for comparison. They were taken from Marjamäki et al. (1993).

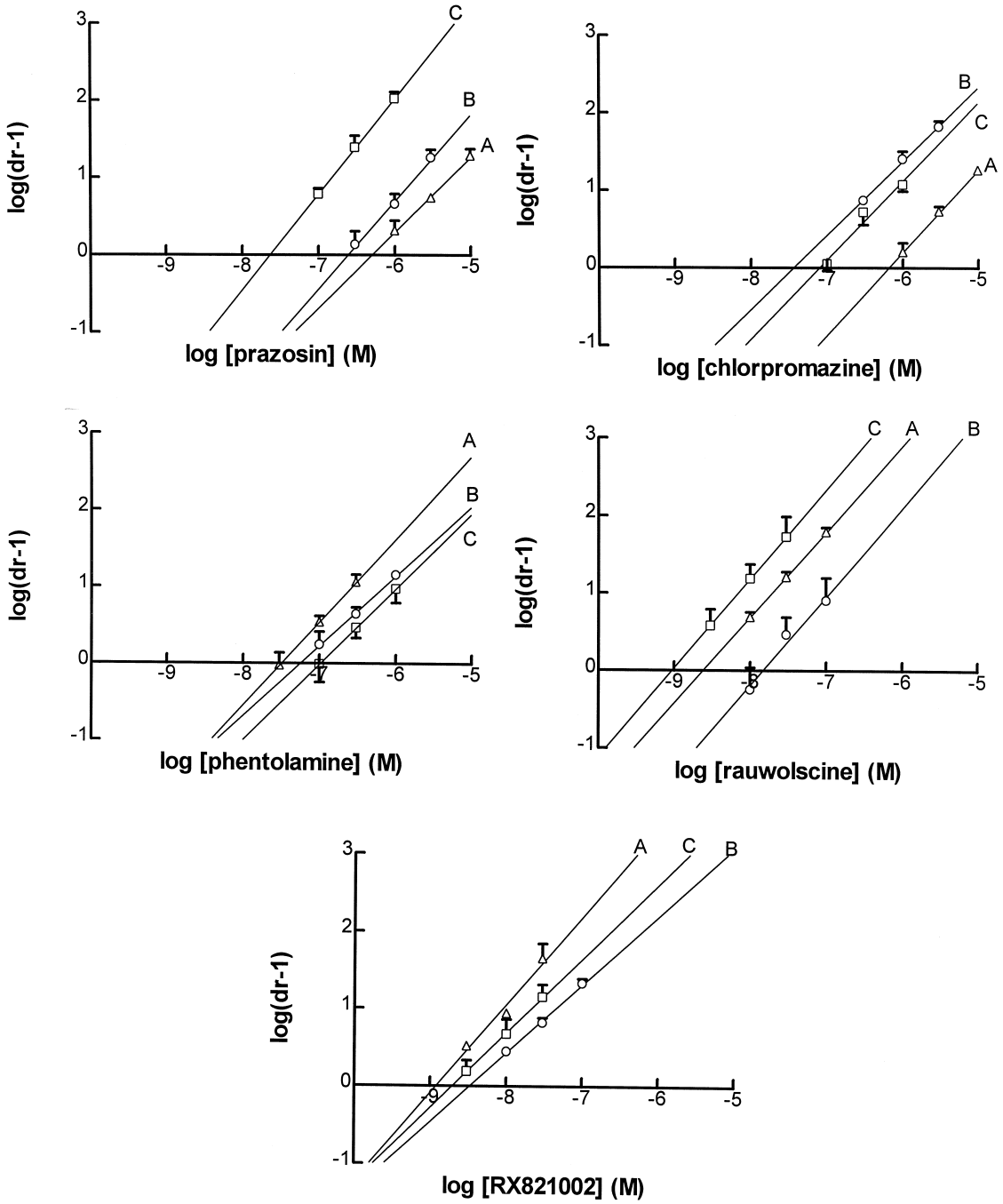

Fig. 1. Schild plots derived from dose–response curves of (−)-noradrenaline in the presence and absence of increasing doses of antagonists. Results are means ± S.E.M. from 3 independent experiments performed in triplicate.

The pK_i values used in this comparison are shown in Table 1.

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 studies on receptor-G-protein coupling. The GTPase assay is technically simple and has been used to study many different G-protein coupled receptors (Aktories and Jakobs, 1981; Koski and Klee, 1981; Brandt and Ross, 1986; Costa and Herz, 1989; Costa et al., 1990, 1992; Schenker et al., 1991; Lazareno et al., 1993; Hasegawa et al., 1996; Seiler et al., 1996). It was recently optimized by Jansson et al. (data not shown) to study α_2-adrenoceptor agonists. In order to increase the relative net signal evoked by (−)-noradrenaline we employed a high concentration (32 μM) of the

nucleotide analog GDPβS (Vachon et al., 1986; Jansson et al., manuscript in preparation), which binds to the GTP-binding site of G-proteins. Under these circumstances we were not able to detect inverse agonist activity in any of the investigated α_2-adrenoceptor antagonists, although it has been detected in some GTPase studies with opioid receptors (Costa and Herz, 1989; Costa et al., 1990, 1992).

The results of this study were in general agreement with results from competitive ligand binding assays (Marjamäki et al., 1993), and high correlations were observed between results generated with the two methods for the α_{2A}- and α_{2C}-adrenoceptor subtypes ($r = 0.97$ and $r = 0.94$). The relatively weak correlation ($r = 0.72$) between GTPase and binding results obtained for the α_{2B}-adrenoceptor subtype was caused by the very limited scatter of the drug potencies at this receptor and by an unexplained difference between the two assays for one test compound, phentolamine (pK_i, 8.51; pA_2, 7.26). Prazosin proved to have highest potency at the α_{2C}-adrenoceptor subtype ($\alpha_{2C} > \alpha_{2B} \approx \alpha_{2A}$), whereas chlorpromazine had almost equal potency at the α_{2B}- and α_{2C}-adrenoceptor subtypes and much lower potency at the α_{2A}-adrenoceptor subtype ($\alpha_{2B} \approx \alpha_{2C} > \alpha_{2A}$). Also rauwolscine was subtype-selective ($\alpha_{2C} > \alpha_{2A} > \alpha_{2B}$). RX821002 and phentolamine showed no significant subtype-selectivity, although both slightly favoured the α_{2A}-adrenoceptor.

In conclusion, we suggest that the GTPase assay described here is useful for characterization of the functional potency and subtype-selectivity of α_2-adrenoceptor antagonists.

Acknowledgements

Anna-Mari Pekuri and Katariina Pohjanoksa are gratefully acknowledged for assistance. The authors are grateful to Dr. R.J. Lefkowitz at the Howard Hughes Medical Institute, Duke University Medical Center (Durham, NC, USA), for permission to use the α_2-adrenoceptor cDNAs.

References

Aktories, K., Jakobs, K.H., 1981. Epinephrine inhibits adenylate cyclase and stimulates a GTPase in human platelet membranes via α-adrenoceptors. FEBS Lett. 130, 235–238.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. 14, 48–58.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brandt, D.R., Ross, E.M., 1986. Catecholamine-stimulated GTPase cycle. J. Biol. Chem. 261, 1656–1664.

Chabre, O., Conklin, B.R., Brandon, S., Bourne, H.R., Limbird, L.E., 1994. Coupling of the α_{2A}-adrenergic receptor to multiple G-proteins. J. Biol. Chem. 269, 5730–5734.

Conklin, B.R., Chabre, O., Wong, Y.H., Federman, A.D., Bourne, H.R., 1992. Recombinant $G_q\alpha$: Mutational activation and coupling to receptors and phospholipase C. J. Biol. Chem. 267, 31–34.

Costa, T., Herz, A., 1989. Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins. Proc. Natl. Acad. Sci. USA 86, 7321–7325.

Costa, T., Lang, J., Gless, C., Herz, A., 1990. Spontaneous association between opioid receptors and GTP-binding regulatory proteins in native membranes: Specific regulation by antagonists and sodium ions. Mol. Pharmacol. 37, 383–394.

Costa, T., Ogino, Y., Munson, P.J., Onaran, H.O., Rodbard, D., 1992. Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: Thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. Mol. Pharmacol. 41, 549–560.

Eason, M.G., Kurose, H., Holt, B.D., Raymond, J.R., Liggett, S.B., 1992. Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects. J. Biol. Chem. 267, 15795–15801.

Hasegawa, H., Negishi, M., Ichikawa, A., 1996. Two isoforms of the prostaglandin E receptor EP3 subtype different in agonist-independent constitutive activity. J. Biol. Chem. 271, 1857–1860.

Kobilka, B.K., Matsui, H., Kobilka, T.S., Yang-Feng, T.L., Francke, U., Caron, M.G., Lefkowitz, R.J., Regan, J.W., 1987. Cloning, sequencing and expression of the gene coding for the human platelet α_2-adrenergic receptor. Science 238, 650–656.

Koski, G., Klee, W.A., 1981. Opiates inhibit adenylate cyclase by stimulating GTP hydrolysis. Proc. Natl. Acad. Sci. USA 78, 4185–4189.

Kurose, H., Regan, J.W., Caron, M.G., Lefkowitz, R.J., 1991. Functional interactions of recombinant α_2 adrenergic receptor subtypes and G proteins in reconstituted phospholipid vesicles. Biochemistry 30, 3335–3341.

Lazareno, S., Farries, T., Birdsall, N.J.M., 1993. Pharmacological characterization of guanine nucleotide exchange reactions in membranes from CHO cells stably transfected with human muscarinic receptors M1–M4. Life Sci. 52, 449–456.

Limbird, L.E., 1988. Receptors linked to inhibition of adenylate cyclase: Additional signaling mechanisms. FASEB J. 2, 2686–2695.

Lomasney, J.W., Cotecchia, S., Lefkowitz, R.J., Caron, M.G., 1991. Molecular biology of α-adrenergic receptors: Implications for receptor classification and for structure–function relationships. Biochim. Biophys. Acta 1095, 127–139.

Marjamäki, A., Luomala, K., Ala-Uotila, S., Scheinin, M., 1993. Use of recombinant human α_2-adrenoceptors to characterize subtype selectivity of antagonist binding. Eur. J. Pharmacol. 246, 219–226.

Pohjanoksa, K., Jansson, C.C., Luomala, K., Marjamäki, A., Savola, J.-M., Scheinin, M., 1997. α_2-Adrenoceptor regulation of adenylyl cyclase in CHO cells: Dependence on receptor density, receptor subtype and present activity of adenylyl cyclase. Eur. J. Pharmacol., in press.

Raymond, J.R., 1995. Multiple mechanisms of receptor-G-protein signaling specificity. Am. Physiol. 269, F141–F158.

Regan, J.W., Kobilka, T.S., Yang-Feng, T.L., Caron, M.G., Lefkowitz, R.J., Kobilka, B.K., 1988. Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype. Proc. Natl. Acad. Sci. USA 85, 6301–6305.

Schenker, A., Goldsmith, P., Unson, C.G., Spiegel, M., 1991. The G protein coupled to the thromboxan A_2 receptor in human platelets is a member of the novel G_q family. J. Biol. Chem. 266, 9309–9313.

Seiler, S.M., Peluso, M., Tuttle, J.G., Pryor, K., Klimas, C., Matsueda, G.R., Bernatowicz, M.S., 1996. Thrombin receptor activation by thrombin and receptor-derived peptides in platelet and CHRF-288 cell membranes: Receptor-stimulated GTPase and evaluation of agonists and partial agonists. Mol. Pharmacol. 49, 190–197.

Vachon, L., Costa, T., Herz, A., 1986. Differential sensitivity of basal and opioid-stimulated low K_m GTPase to guanine nucleotide analogs. J. Neurochem. 47, 1361–1369.

Wong, Y.H., Conklin, B.R., Bourne, H.R., 1992. G_z-mediated hormonal inhibition of cyclic AMP accumulation. Science 255, 339–342.