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Short communication

Subtype-specific stimulation of [^{35}S]GTPγS binding by recombinant α_2-adrenoceptors

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Abstract

We measured agonist-stimulated binding of the stable GTP-analog guanosine-5′-*O*-(3-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) as a functional assay to monitor G-protein activation by recombinant human α_2-adrenoceptor subtypes α_{2A}, α_{2B} and α_{2C}. (−)-Noradrenaline was a full agonist in all receptors. Dexmedetomidine, UK14,304, clonidine and oxymetazoline showed subtype-selectivity in efficacy. Dexmedetomidine was a full agonist at α_{2B} and a partial agonist at α_{2A}; UK14,304 was a full agonist at α_{2A} and a partial agonist at α_{2B}. Clonidine and oxymetazoline were weak partial agonists at the α_{2B}-subtype, but appeared inactive at α_{2A} and α_{2C}. The [^{35}S]GTPγS binding assay provides functional information on pertussis toxin-sensitive G-protein activation, complementing radioligand binding assays and conventional second messenger assays.

Keywords: α_2-Adrenoceptor subtype; Guanosine-5-*O*-thiotriphosphate; Efficacy; Potency; G-protein

1. Introduction

Three distinct human α_2-adrenoceptor subtypes have been cloned, designated α_{2A}, α_{2B} and α_{2C}. The agonist-induced coupling of α_2-adrenoceptors to heterotrimeric GTP-binding proteins (G-proteins) promotes the release of GDP from the α-subunit of G-proteins and binding and subsequent hydrolysis of GTP. Poorly hydrolyzable GTP analogs have been shown to be useful in monitoring the agonist-dependent activation of G-proteins. One of these analogs is guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS). Binding assays using radiolabeled [^{35}S]GTPγS have been used to measure the basal and agonist-induced GTP-affinity of G-proteins (Hilf and Jakobs, 1992; Lazareno et al., 1993; Tian et al., 1994; Stanton and Beer, 1997).

The aim of this study was to evaluate the [^{35}S]GTPγS binding assay as a method to characterize α_2-adrenoceptor agonists and their functional subtype-selectivity. Due to signal amplification, agonists which only partially activate receptors and G-proteins can act as full agonists later in the signal transduction pathway, especially in the presence of a significant receptor reserve. Therefore functional assays directly monitoring receptor-mediated G-protein activation are considered important and provide valuable information to complement results from radioligand binding assays and conventional second messenger assays. We employed Chinese hamster ovary (CHO) cells transfected to separately express relatively high densities of the human α_{2A}, α_{2B} and α_{2C}-adrenoceptor subtype: such clonal cell lines may provide useful test models for drug discovery and development.

2. Materials and methods

2.1. Materials

[^{35}S]GTPγS was from DuPont NEN (Wilmington, DE, USA). [^{3}H]RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) was from Amersham (Amersham, UK). Dexmedetomidine, levomedetomidine and atipamezole were gifts from Orion-Farmos (Turku, Finland). Rauwolscine was from Carl Roth (Karlsruhe, Germany). RX821002 and UK14,304 (5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine) were from Research Biochemicals (Natick, MA, USA). (−)-Noradrenaline, clonidine, oxymetazoline, chlorpromazine, pertussis toxin,

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GDP and GTPγS were from Sigma (St. Louis, MO, USA). Cell culture reagents were supplied by Gibco (Gaithersburg, MD, USA).

2.2. Cell culture and transfection

CHO cell lines stably expressing cDNAs encoding human α_2-adrenoceptor subtypes α_{2A}, α_{2B} and α_{2C} were generated as described previously (Marjamäki et al., 1992; Pohjanoksa et al., 1997). The receptor densities (B_{max}) of the cell clones used in this study and the corresponding radioligand K_d values were determined using [^{3}H]RX821002 as radioligand (Halme et al., 1995). They were: B_{max} 1.3 ± 0.2 pmol/mg, K_d 0.89 ± 0.27 nM for α_{2A}, B_{max} 2.6 ± 0.5 pmol/mg, K_d 4.37 ± 0.55 nM for α_{2B} and B_{max} 3.0 ± 0.4 pmol/mg, K_d 1.28 ± 0.12 nM for α_{2C}. For some experiments, cells were grown for 18 h in serum-free medium supplemented with 200 ng/ml pertussis toxin.

2.3. [^{35}S]GTPγS binding assay

Cell membranes were prepared by centrifugation (Pohjanoksa et al., 1997). Agonist-induced stimulation of [^{35}S]GTPγS binding was measured essentially as described previously (McKenzie, 1992; Tian et al., 1994). Membrane pellets were thawed and resuspended in hypotonic lysis buffer (5 mM Tris–HCl, 5 mM EDTA, pH 7.4). The reaction was started by adding an aliquot of membrane suspension (15 μg of membrane protein per tube) to reaction buffer (final concentrations 25 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 1 μM GDP, and 2 nM [^{35}S]GTPγS) with or without agonist in a total volume of 100 μl. The tubes were incubated for 10 min at 25°C. The incubation was terminated by rapid filtration through Whatman GF/B filters, which were then placed in scintillation vials for counting in a liquid scintillation counter (Wallac 1410, Turku, Finland). Non-specific binding was measured in the presence of 10 μM GTPγS and subtracted from total bound radioactivity.

2.4. Data analysis

The results were analyzed using GraphPad Prism programs (GraphPad Software, San Diego, CA, USA). The results are expressed as mean values ± SE.

3. Results

High-affinity [^{35}S]GTPγS binding to CHO cell membranes was measured after addition of increasing concentrations of (−)-noradrenaline, dexmedetomidine, clonidine and UK14,304 (Fig. 1). The maximal stimulatory effect of (−)-noradrenaline was clearly smaller in the α_{2C}-subtype,

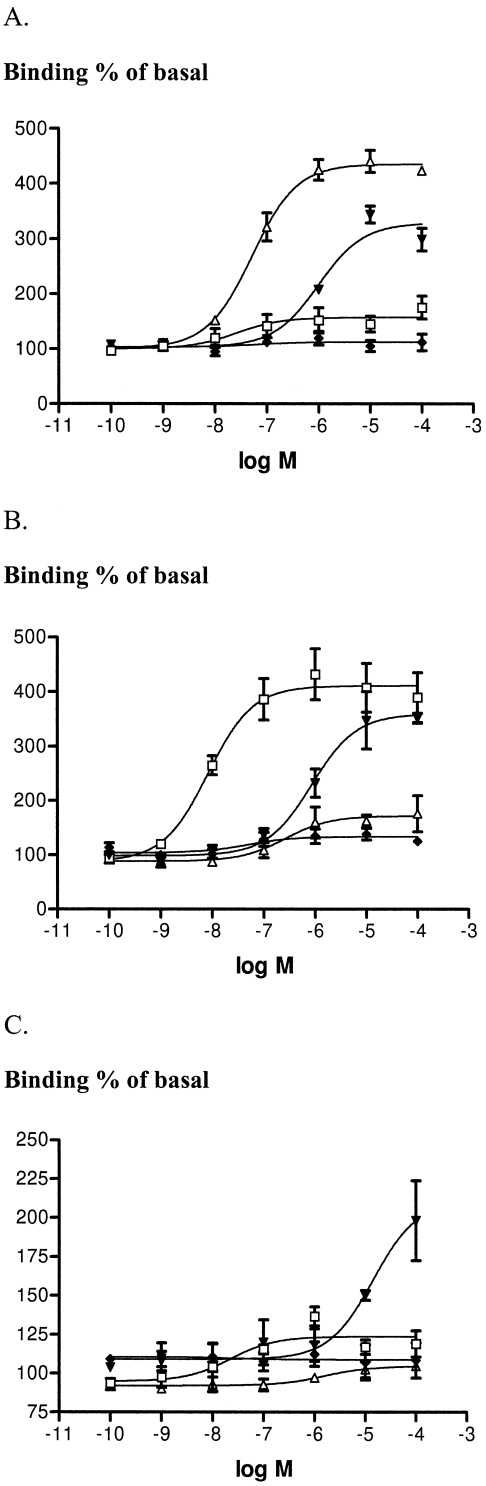

Fig. 1. Characterization of the agonist-stimulated [^{35}S]GTPγS binding to membranes from CHO cells transfected to express the α_2-adrenoceptor subtypes α_{2A} (A), α_{2B} (B) and α_{2C} (C). Dose–response curves of (−)-noradrenaline (▼), dexmedetomidine (□), UK14,304 (△) and clonidine (◆). Results are presented as means ± SE of three independent experiments performed in duplicate.

110%, compared to 229% and 260% in the α_{2A}- and α_{2B}-subtypes, respectively. In order to compare the relative efficacies of different agonists at the receptor sub-

Table 1
Characterization of [^{35}S]GTPγS binding to membranes from CHO cells transfected to express different α_2-adrenoceptor subtypes

Compound	CHO-α_{2A}			CHO-α_{2B}			CHO-α_{2C}		
	E_{max} % change	% of NA	$-\log EC_{50}$	E_{max} % change	% of NA	$-\log EC_{50}$	E_{max} % change	% of NA	$-\log EC_{50}$
(−)-Noradrenaline	229 ± 12	100	6.00 ± 0.13	260 ± 18	100	6.06 ± 0.17	110 ± 20	100	4.85 ± 0.31
Dexmedetomidine	57.3 ± 9.2	25	7.58 ± 0.60	311 ± 17	120	8.08 ± 0.20	23.6 ± 3.5	21	7.64 ± 0.45
UK14,304	336 ± 8	147	7.28 ± 0.07	71.8 ± 11.0	28	6.58 ± 0.39	4.5 ± 2.9	4 (n.s.)	n.d.
Clonidine	12.5 ± 5.8	5.5 (n.s.)	n.d.	33.6 ± 5.6	13	7.31 ± 0.65	8.8 ± 4.3	8 (n.s.)	n.d.

n.s., not statistically significant; n.d., not determined. The E_{max} values for the different agonists indicate the maximal % change in the binding over the basal level in the absence of agonists. EC_{50} is the drug concentration (M) causing 50% of the maximal effect. The values are means ± SE of three independent experiments performed in duplicate. Statistical significance was assessed with ANOVA followed by Dunnett's test.

types, (−)-noradrenaline was used as reference. Clear subtype-selectivity could be seen for dexmedetomidine, oxymetazoline, clonidine and UK14,304. Dexmedetomidine acted as a full agonist at the α_{2B}-subtype (efficacy 120% of (−)-noradrenaline) and as a partial agonist at α_{2A} (25%) and α_{2C} (21%). UK14,304 acted as full agonist at the α_{2A}-subtype (147% of (−)-noradrenaline), as a partial agonist at α_{2B} (28%), and had no statistically significant effect at the α_{2C}-subtype. Clonidine acted as a weak partial agonist at the α_{2B}-subtype (13% of (−)-noradrenaline), but had no significant effect at the α_{2A}- and α_{2C}-subtypes. Oxymetazoline stimulated [^{35}S]GTPγS binding by 79 ± 14% (30% of NA) over basal at the α_{2B}-subtype; it had no significant effects at the α_{2A}- and α_{2C}-subtypes (Table 1).

There were also subtype-dependent differences in agonist potency. (−)-Noradrenaline had significantly lower potency for the stimulation of [^{35}S]GTPγS binding in the α_{2C}-subtype (EC_{50} = 14120 nM) compared to α_{2A} (99 nM) and α_{2B} (860 nM). Dexmedetomidine was most potent at the α_{2B}-subtype (EC_{50} = 8 nM), and equally potent at α_{2A} (26 nM) and α_{2C} (23 nM). UK14,304 was most potent at the α_{2A}-subtype (EC_{50} = 52 nM), compared to α_{2B} (265 nM) and α_{2C} (inactive).

In membranes from non-transfected CHO cells, no stimulation of [^{35}S]GTPγS binding was detected in the presence of 100 μM of any of the drugs. The stimulatory effects of different agonists on [^{35}S]GTPγS binding were also completely blocked by addition of the α_2-adrenoceptor antagonist RX821002 (10 μM) to the assays (data not shown). No stimulation of [^{35}S]GTPγS binding was seen in any receptor subtype in the presence of 100 μM rauwolscine, RX821002, atipamezole, levomedetomidine or chlorpromazine.

Several previous reports have demonstrated the coupling of α_2-adrenoceptors also to pertussis toxin-insensitive G-proteins (Wong et al., 1992; Eason et al., 1992; Chabre et al., 1994). To assess the role of pertussis toxin-sensitive G_i-type G-proteins in the agonist-stimulated [^{35}S]GTPγS binding, intact cells were pretreated with pertussis toxin (200 ng/ml in the growth medium for 18 h). This blocked completely the stimulatory effect of 100 μM (−)-noradrenaline, dexmedetomidine, clonidine and UK14,304 in all α_2-adrenoceptor subtypes, thus indicating that the observed stimulation of [^{35}S]GTPγS binding was entirely due to the coupling of α_2-adrenoceptors to pertussis toxin-sensitive G-proteins (G_i/G_o).

4. Discussion

Activation of G-proteins is the first biochemically detectable step in the cellular signal transduction pathways of G-protein coupled receptors. [^{35}S]GTPγS binding assays have been widely used to study the functional activity of several G-protein coupled receptors. In contrast to assays measuring changes in second messenger concentrations, this method gives direct information on receptor-mediated G-protein activation. Thus, the results are not affected by signal amplification resulting from subsequent steps in the intracellular signal transduction pathways, and the influence of receptor reserve is smaller than in second messenger assays. In adenylyl cyclase assays high receptor densities often mask partial agonist activity (Adham et al., 1993; Pohjanoksa et al., 1997), but the [^{35}S]GTPγS binding assay can discriminate between partial and full agonists even at high receptor densities (Stanton and Beer, 1997).

In the current study, we used stably transfected CHO cells, which expressed separately the three α_2-adrenoceptor subtypes α_{2A}, α_{2B} and α_{2C}. Interestingly, we found that the [^{35}S]GTPγS binding assay was able to demonstrate clear functional subtype-selectivity for the agonists dexmedetomidine, UK14,304, clonidine and oxymetazoline. The assay was also able to discriminate between full and partial agonists. (−)-Noradrenaline acted as a full agonist at all three receptor subtypes and was used as a reference substance. Dexmedetomidine acted as a full agonist at the α_{2B}-subtype and as a partial agonist at the α_{2A}- and α_{2C}-subtypes. UK14,304 acted as a full agonist at the α_{2A}-subtype, even exceeding the efficacy of (−)-noradrenaline. It was a partial agonist at the α_{2B}-subtype and appeared inactive at the α_{2C}-subtype. Clonidine and oxymetazoline were weak partial agonists at the α_{2B}-subtype, but appeared inactive at α_{2A} and α_{2C}. The responses elicited by these partial agonists have been clearly larger in

adenylyl cyclase assays and in various in vitro and in vivo preparations compared to the current [^{35}S]GTPγS binding assay (Marjamäki et al., 1992; MacMillan et al., 1996; Pohjanoksa et al., 1997). This is probably the result of several amplification steps in the intracellular signaling cascade following G-protein activation. In addition, partial agonist efficacy may have been underestimated in the current study due to the experimental conditions (presence of 1 μM GDP and 150 mM Na^+ in the incubation medium). Lower GDP concentrations have been reported to result in higher relative partial agonist efficacies in [^{35}S]GTPγS binding assays (Selley et al., 1997).

Very similar efficacy profiles were recently reported for these agonists in another study, which employed membranes from transfected HEK-293 cells to study α_2-adrenoceptor-dependent [^{35}S]GTPγS binding (Jasper et al., 1998). Dexmedetomidine and oxymetazoline preferentially activated the α_{2B}-subtype and UK14,304 had highest efficacy at the α_{2A}-subtype. The partial agonists oxymetazoline and clonidine were, however, clearly discernible from inactive compounds in this extensive investigation. This may have been due to differences in the two cell lines (HEK-293 vs. CHO), or, more likely, somewhat different assay conditions. Most importantly, the incubation time was 60 min (vs. 10 min in this study), which favor the detection of GDP-releasing activity of partial agonists (Lorenzen et al., 1993).

Treatment of cells with pertussis toxin completely blocked the agonist-induced stimulation of [^{35}S]GTPγS binding, indicating the coupling of all α_2-adrenoceptor subtypes to pertussis toxin sensitive G_i-type G-proteins. In another study performed on CHO cells, the α_{2B}-subtype was demonstrated to stimulate cAMP production through a pertussis toxin-insensitive mechanism, probably through activation of G_s-proteins (Pohjanoksa et al., 1997). It has previously been reported that all three subtypes have the potential to couple physically and functionally to G_s-proteins (Eason et al., 1992). In the current study, binding of [^{35}S]GTPγS to pertussis toxin-insensitive G-proteins could not be shown for any receptor subtype. In all subtypes the pertussis toxin pretreatment of intact cells completely abolished the signal evoked by the addition of agonists. It has been reported that the GTPase assay is unable to demonstrate receptor stimulation of G_S-proteins, probably because of the lower rate of GTP binding and GTPase activity of G_s compared to G_i (McKenzie, 1992). This may be the reason why we were not able to detect any activation of pertussis toxin-insensitive G-proteins by α_2-adrenoceptors.

In conclusion, the [^{35}S]GTPγS binding assay was demonstrated to be a convenient functional test to screen potential subtype-selective agonists and to discriminate between full and partial agonists. Functional assays are essential for the characterization of agonists. Conventional second messenger assays may be influenced by signal amplification, especially in the presence of a significant receptor reserve. Therefore functional assays directly measuring receptor-mediated G-protein activation are considered important and provide valuable information to complement results from radioligand binding assays and conventional second messenger assays.

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