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FUNCTIONAL α_2-ADRENERGIC RECEPTOR-G_S COUPLING UNDERGOES AGONIST-PROMOTED DESENSITIZATION IN A SUBTYPE-SELECTIVE MANNER

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SUMMARY: Recently it has become clear that α_2-adrenergic receptors (α_2AR) functionally couple to G_S as well as G_i, thus inducing a complex modulation of adenylyl cyclase activity. It is unknown whether α_2AR-G_S coupling undergoes agonist-promoted desensitization. Therefore, in CHO cells expressing the three cloned human α_2AR subtypes (α_2C10, α_2C4, and α_2C2), we assessed the ability of α_2AR-mediated stimulation of adenylyl cyclase activity to undergo short-term agonist-promoted desensitization. To isolate α_2AR-G_S coupling, cells were pretreated with pertussis toxin, which ablates G_i coupling. Following agonist exposure, both α_2C10- and α_2C2-mediated stimulation of adenylyl cyclase activity underwent marked desensitization. In distinct contrast, α_2C4-mediated stimulation of adenylyl cyclase activity underwent no agonist-promoted desensitization. Thus, α_2AR-G_S coupling undergoes agonist-promoted desensitization and does so in a subtype-selective manner.

For many G-protein coupled receptors, exposure to agonist results in desensitization, a state whereby a receptor-mediated response to agonist diminishes regardless of the presence of continuous agonist or subsequent exposure to agonist of the same concentration. α_2AR are traditionally described as being coupled to G_i, eliciting inhibition of adenylyl cyclase activity. Three human α_2AR subtypes have been cloned to date, denoted by their localization to chromosomes 10, 4, and 2 as α_2C10 (1), α_2C4 (2), and α_2C2 (3), respectively. Each of these α_2AR subtypes has been shown to efficiently couple to G_i (5,10). Recent studies from our laboratory have demonstrated that agonist-promoted desensitization of α_2AR-mediated inhibition of adenylyl cyclase activity undergoes substantial desensitization after short-term agonist

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Abbreviations: α_2AR, α_2-adrenergic receptor; CHO, Chinese hamster ovary cells.

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exposure. Furthermore, this desensitization occurs in a subtype-selective manner (4,5).

In addition to the inhibition of cAMP accumulation, several studies have shown the ability of α_2AR to mediated increases in cAMP (6-11). Indeed, our studies (10) and those of Bourne et al. (11) have shown that agonist occupied α_2AR do, in fact, directly couple to G_s, and in certain circumstances mediate stimulation of adenylyl cyclase activity.

As the ability of α_2AR to couple to G_s is a relatively new finding, thus far only regulation of α_2AR-mediated inhibition of adenylyl cyclase activity has been studied. Therefore, in the present study we have utilized clonal cell lines separately expressing the three cloned human α_2AR subtypes and investigated the ability of α_2AR-mediated stimulation of adenylyl cyclase activity to undergo short-term agonist-promoted desensitization.

METHODS

Transfection and cell culture.

CHO cells were grown in monolayers in Ham's F12 media supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 80 µg/ml G418, which provides for selection pressure, at 37°C in a 5% CO_2 atmosphere. CHO cells were co-transfected with 20-40 µg of the cDNA encoding α_2C10, α_2C4, or α_2C2 cloned into the mammalian expression vector pBC12BI and 3.0 µg of psV2-neo (which provides resistance to G-418) as described (4). Selection was performed in 1 mg/ml G418. Clonal cell lines were screened for α_2AR expression using a [^{3}H]yohimbine binding assay.

Pertussis toxin treatment.

In order to ablate α_2AR-G_i coupling, CHO cells at approximately 90% confluency were incubated while in monolayers with 500 ng/ml pertussis toxin in media as described above except without fetal bovine serum for 24 h. This concentration of pertussis toxin has been previously shown to effectively eliminate α_2AR-G_i coupling without detrimental effects to the cells (10).

Agonist-promoted desensitization.

CHO cells expressing the α_2AR subtypes in monolayers were exposed to 100 µM epinephrine in media with 100 µM ascorbic acid for 30 min. Plates were then placed on ice and washed five times with ice-cold phosphate-buffered saline. Membranes were prepared by lysis in ice-cold hypotonic buffer (5 mM Tris, 2 mM EDTA, pH 7.4), pelleted by centrifugation at 42,000 x g, then resuspended in a buffer containing (final concentration in the assay): 25 mM NaCl, 1.6 mM $MgCl_2$, 0.8 mM EDTA, and 40 mM HEPES (pH 7.4). Adenylyl cyclase activities in membranes were determined in the presence of buffer alone, 1.0 µM forskolin, or 1.0 µM forskolin with various concentrations of agonist using the method of Salomon et al. (12) as modified (4).

Radioligand binding assay.

For determination of α_2AR expression levels, membranes were prepared and incubated with 20 nM [^{3}H]yohimbine alone (total binding) or in the presence of 100 µM phentolamine (nonspecific binding) for 30 min at 37°C as described (5). Specific binding was defined as the difference between total and nonspecific binding and was normalized for protein. Protein was measured using the copper bicinchoninic method (13) with bovine serum albumin as the standard. Binding reactions were terminated by

dilution with ice-cold 10 mM Tris buffer followed by rapid fitration over Whatman GF/C glass fiber filters.

RESULTS

Cells expressing the α_2AR subtypes at > 2 pmol/mg were utilized in all studies as we have previously found that functional α_2AR-G_S coupling is more readily observed at these higher expression levels (10). As shown in Figure 1, α_2AR-mediated responses in CHO cells are of a complex, biphasic nature. In membranes from untreated cells, we found that at lower concentrations of agonist in the assay, α_2AR-mediated inhibition of adenylyl cyclase predominated. In contrast, α_2AR-mediated stimulation of adenylyl cyclase activity was noted at higher agonist concentrations. This reflects the ability of α_2AR to couple to both G_i and G_S, respectively (10). Under these circumstances, in membranes from cells preexposed to agonist (100 μM epinephrine), the ability of α_2C10 and α_2C2 to stimulate adenylyl cyclase activity was markedly reduced, whereas that of α_2C4 was not. This suggested that following agonist exposure, α_2AR-G_S coupling becomes desensitized and does so in a subtype-selective manner.

In order to isolate the α_2AR-mediated stimulation of adenylyl cyclase activity, we incubated cells overnight in 500 ng/ml pertussis toxin, which ablates α_2AR-G_i coupling (10). Then, we investigated the ability of α_2AR-G_S coupling to undergo agonist-promoted desensitization. Following pertussis toxin treatment, only monophasic stimulation of adenylyl cyclase was observed (Figure 2). This provided an easily detectable stimulatory component with which to analyze agonist-promoted

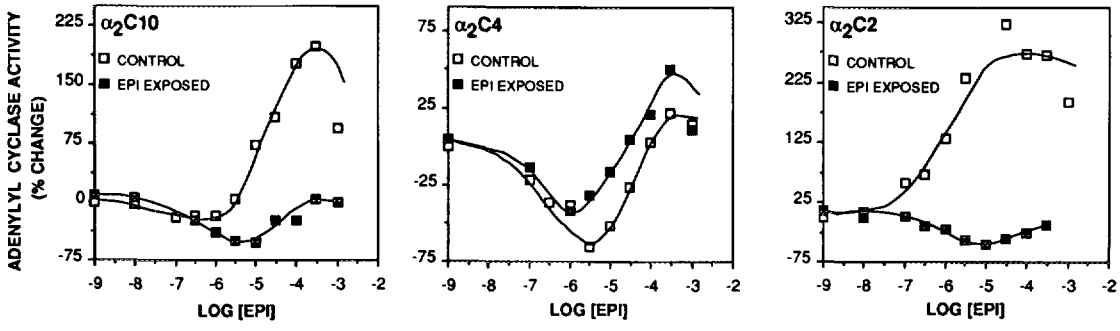

Figure 1. Effects of short-term agonist exposure on α_2AR-mediated inhibition and stimulation of adenylyl cyclase activity. CHO cells permanently expressing α_2C10, α_2C4, and α_2C2 were incubated in media alone (CONTROL), or media with 100 μM epinephrine (EPI EXPOSED) for 30 min., washed extensively, and then membranes were prepared and adenylyl cyclase activities determined as described in Methods. Following short-term agonist exposure, the stimulatory components of α_2C10- and α_2C2-mediated responses were markedly reduced, while the α_2C4-mediated response was unchanged.

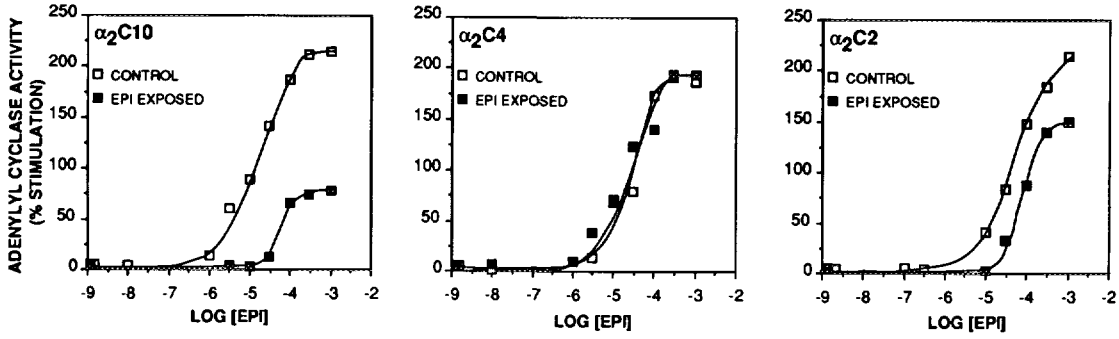

Figure 2. Agonist-promoted desensitization of α_2AR-mediated stimulation of adenylyl cyclase activity. CHO cells permanently expressing α_2C10, α_2C4, and α_2C2 which had been treated with pertussis toxin to ablate G_i coupling, were incubated 30 min. in media alone (CONTROL), or media with 100 μM epinephrine (EPI EXPOSED). Following extensive washing, membranes were prepared and adenylyl cyclase activities determined as described in Methods. Following short-term agonist exposure, α_2C10- and α_2C2-mediated stimulation of adenylyl cyclase activity underwent desensitization, while that of α_2C4 did not. Shown is a single experiment representative of three to four performed.

desensitization of α_2AR-G_S coupling. As shown in Figure 2 and Table 1, α_2C10-mediated stimulation of adenylyl cyclase activity underwent desensitization as manifested by a substantial decrease in the maximal stimulation of adenylyl cyclase activity from ~240% in the control state to ~60% following exposure to agonist. This decrease was accompanied by a rightward shift in the EC_{50} (~3-fold) for epinephrine-induced stimulation of adenylyl cyclase activity. As with α_2C10, α_2C2-mediated stimulation of adenylyl cyclase activity also underwent agonist-promoted desensitization, with a decrease in the maximum stimulation of adenylyl cyclase

Table 1. Subtype-selective agonist-promoted desensitization of α_2AR. Data shown are from experiments described in Figure 2 and are expressed as the mean + SE. Short-term agonist exposure (EPI EXPOSED) promoted significant decreases in the maximal stimulation of adenylyl cyclase by α_2C10 and α_2C2, whereas no change was found in α_2C4-mediated stimulation of adenylyl cyclase activity. For α_2C10, this decrease was accompanied by a rightward shift in the EC50 for epinephrine-mediated stimulation of adenylyl cyclase activity. * = $p<0.02$ as compared to control.

	MAXIMAL ADENYLYL CYCLASE STIMULATION (%)		EC_{50} (μM)	
	CONTROL	EPI EXPOSED	CONTROL	EPI EXPOSED
α_2C10	235.8 ± 21.6	60.3 ± 11.0*	16.0 ± 1.1	46.6 ± 3.8*
α_2C4	200.0 ± 3.9	202.0 ± 3.4	33.7 ± 6.3	32.7 ± 6.4
α_2C2	244.0 ± 20.9	157.0 ± 13.8*	44.3 ± 3.8	61.2 ± 11.0

activity from ~240% in the control state to ~160% following exposure to agonist. The decrease in α_2C2-mediated stimulation of adenylyl cyclase activity was accompanied by only a minimal shift in the EC_{50}. In marked contrast, α_2C4-mediated stimulation of adenylyl cyclase activity did not undergo agonist-promoted desensitization, with no change in the maximal stimulation or in the EC_{50} for epinephrine-induced stimulation of adenylyl cyclase activity.

DISCUSSION

α_2AR-mediated physiological responses have been shown to undergo agonist-promoted desensitization in number of tissues (14), but the mechanisms underlying this process have only recently begun to be understood. Studies from our laboratory have investigated key mechanisms underlying both short- and long-term desensitization (4,5). In studies utilizing α_2C10 permanently expressed in Chinese hamster fibroblasts, both short- and long-term agonist-promoted desensitization of α_2C10-mediated inhibition of adenylyl cyclase activity was shown to occur (4). The key mechanism involved in short-term agonist-promoted desensitization was phosphorylation of serines and threonines in the third intracellular loop region of the receptor, most likely by the β-adrenergic receptor kinase. Additional studies, utilizing CHO cells expressing α_2C10, α_2C4, and α_2C2, demonstrated that α_2AR-G_i coupling undergoes short-term agonist-promoted desensitization in a subtype-selective manner (5). Short-term desensitization was found to occur for both α_2C10- and α_2C2-mediated inhibition of adenylyl cyclase activity, but not for that of α_2C4.

α_2AR-mediated potentiation of cellular cAMP levels has been observed in pancreatic islet cells (8), cortical brain slices (9), and in transfected CHO cells expressing α_2AR (6,7,10). Recently, studies from our laboratory (10) as well as that of Bourne and colleagues (11) have demonstrated that this α_2AR-mediated stimulation of adenylyl cyclase activity is due to direct, agonist dependent coupling of α_2AR to G_s. The stimulation of cAMP by α_2AR-G_s coupling also requires the presence of Type II adenylyl cyclase in the cell (11,15).

While desensitization of α_2AR-G_i coupling has been studied in a large variety of systems, this study is the first to address desensitization of α_2AR-G_s coupling. We found that while both α_2C10- and α_2C2-mediated stimulation of adenylyl cyclase activity underwent substantial agonist-promoted decreases in maximal stimulation after short-term agonist exposure, α_2C4-mediated stimulation of adenylyl cyclase activity did not. Thus, we show that α_2AR-mediated stimulation of adenylyl cyclase activity undergoes short-term agonist-promoted desensitization in a subtype-selective manner.

The subtype-selectivity of short-term desensitization of α_2AR-G_s coupling shows remarkable similarity to that found for agonist-promoted desensitization of α_2AR-G_i coupling. In both studies α_2C10-mediated responses underwent robust desensitization, and α_2C2-mediated responses underwent desenstization to a lesser

degree, whereas α_2C4-mediated responses underwent no desensitization. These parallel patterns of desensitization of α_2AR-G_i and -G_s coupling suggest that agonist-promoted phosphorylation compromises both α_2AR-G_i and -G_s coupling to a similar extent.

Since α_2AR are coupled to both G_i and G_s, under certain circumstances the final cellular response to α_2AR agonists is dependent on both pathways. We have now demonstrated that in addition to α_2AR-G_i coupling (4,5), α_2AR-G_s coupling undergoes agonist-promoted desensitization in a subtype-selective manner. Thus, it appears that differential agonist-promoted regulation of α_2AR subtypes as well as the ability to modulate both stimulation and inhibition of adenylyl cyclase activity may reflect adaptive processes evolved in order to provide selective modulation of intracellular signalling.

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