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Ligand-induced α_2-adrenoceptor endocytosis: relationship to G_i protein activation

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Abstract

Most G protein-coupled receptors are desensitized by a uniform two-step mechanism: phosphorylation followed by arrestin binding and internalization. In this study we explored the time-, ligand-, and concentration dependence of α_2-adrenoceptor internalization in human embryonal kidney (HEK-293) cells expressing α_{2A}- and α_{2B}-adrenoceptors. We also explored the relationship between ligand-induced receptor internalization and agonist efficacy, determined with a [^{35}S]GTPγS binding assay. The results showed rapid dose-dependent internalization of both α_{2A}- and α_{2B}-receptors; the extent of internalization was directly proportional to agonist efficacy. The agonist UK 14,304 had a subtype-specific high efficacy at α_{2A}-AR and dexmedetomidine at α_{2B}-AR. Agonist-induced [^{35}S]GTPγS binding was totally blocked by pretreatment with pertussis toxin (PTX) for both receptor subtypes, while only about 50% of the internalization was blocked by PTX. The results indicate that the extent of internalization of α_{2A}-AR and α_{2B}-AR is proportional to agonist efficacy, but only partly dependent on G_i protein coupling.
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Keywords: Internalization; Agonist efficacy; α_2-Adrenoceptors; G protein activation

α_2-Adrenoceptors (α_2-ARs) are potential drug targets in analgesia and anesthesia, cardiovascular disorders, and neuropsychiatric disorders [1]. The three α_2-AR subtypes mediate effects of the endogenous catecholamines norepinephrine and epinephrine and many therapeutic drugs to cellular signalling mechanisms in the central nervous system and in peripheral organs. Inhibition of adenylyl cyclases (ACs) through inhibitory $G_{i/o}$-type G proteins is a main signal transduction mechanism of all α_2-AR subtypes [2,3]. Other $G_{i/o}$ protein dependent signalling pathways activated by α_2-ARs are regulation of ion channel function and release of intracellular Ca^{2+} (reviewed in [4,5]), and modulation of Na^+/H^+ exchange [6]. All three α_2-AR subtypes have also been shown to couple to G_s proteins and to stimulation of ACs in pertussis toxin (PTX)-treated cells, especially in cells with high α_2-AR expression levels [2,3]. Coupling of α_2-ARs to other G protein isoforms [7], as well as activation of extracellularly regulated kinases [8,9], has also been reported.

The relative efficacies of agonists to activate different signalling pathways and their components may vary (e.g., cAMP accumulation vs. GTPγS binding), but even more variable seems to be the ability of agonists to regulate the cell surface receptor number by endocytosis. Agonist-induced μ-opioid receptor (MOR) internalization has been described in vitro and in vivo, and MOR internalization has been reported to be ligand-specific and reversible by antagonists [10–12]. Partial agonists induce less internalization of MOR, β_2-AR, and M3-muscarinic receptors than full agonists, and the

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extent of receptor internalization has been shown to correlate positively with coupling efficiency [13–15]. Some partial MOR agonists and also an antagonist, naloxone, have been shown not only to fail to induce internalization but also to cause increases in cell surface MOR numbers, which was enhanced after PTX-treatment [13].

The efficacies and potencies of various α_2-AR agonists to induce GTPγS binding and inhibition of ACs have been studied previously [3,16,17]. α_2-ARs are similar to many other GPCRs in their ability to adapt to continuous agonist-dependent activation through desensitization, internalization, and down-regulation. Receptor internalization (the loss of receptors from the cell surface) has been implicated in the process of dephosphorylation and resensitization of desensitized receptors (for review, see [18]).

In the field of receptor trafficking it is more likely to be an exception than a rule that the molecular mechanisms first described for one GPCR would equally apply for other members of this broad receptor family. Therefore, we have now studied the time-, ligand-, and concentration dependence of α_2-AR internalization by employing human embryonal kidney (HEK-293) cells expressing α_{2A}- and α_{2B}-ARs, and flow cytometric quantitative analysis. The involvement of G_i proteins in internalization and [^{35}S]GTPγS binding was studied with PTX pretreatment.

Methods

Generation of HEK-293 cell lines expressing human α_{2A}- and α_{2B}-ARs. The cDNAs encoding human α_2-AR subtypes [19,20] were a gift from Dr. R.J. Lefkowitz (Duke University, Durham, NC). The HA-epitope tag was constructed by oligonucleotide-directed PCR in front of the α_2-AR coding sequences, and the cDNAs were subcloned into pMAMneo-based (Clontech Laboratories, Palo Alto, CA) expression vectors for stable expression of human α_{2A}- and α_{2B}-ARs. This HA-epitope has previously been shown not to affect receptor trafficking or ligand binding of other GPCRs and α_2-AR subtypes [21]. Adherent HEK-293 cells (ATCC: CRL 1573) were cultured in monolayers in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in 5% CO_2. HEK-293 cells were transfected with the epitope-tagged constructs with the calcium phosphate precipitation method [22]. Selection of stable HEK-293 cell clones was performed with 500 μg/ml of the neomycin analogue G418 and the resultant clones were screened for α_{2A}- and α_{2B}-AR expression by immunofluorescent staining and radioligand binding. Saturation binding assays with cell homogenates and the α_2-AR antagonist radioligand [^{3}H]RX821002 were performed in K^+-phosphate buffer as described previously [23]. Stable clones expressing receptor densities of 3.9 pmol/mg total cellular protein (α_{2A}-AR) and 5.6 pmol/mg total cellular protein (α_{2B}-AR) were chosen for further studies. Non-transfected HEK-293 cells did not express endogenous α_2-AR as evidenced by immunocytochemistry (investigated with receptor-specific antibodies as previously described [24], and radioligand binding.

Agonist treatment. Subconfluent HEK-293 cell cultures were detached with 0.25% trypsin/0.02% K-EDTA and transferred into 15 ml Falcon tubes. 1×10^6 cells/ml were treated in 2 ml buffer (DMEM + 30 mM Hepes) with or without drugs for 0.5–30 min in a water bath at 37 °C. The agonist treatment was terminated by adding cold buffer into the tubes and placing them on ice.

Immunocytochemical labelling and flow cytometry. Epitope-tagged cell surface α_2-ARs of control and agonist-treated HEK-293 cells were labelled with 10 μg/ml monoclonal anti-HA (12CA5)-antibody in DMEM + 30 mM Hepes + 10% FBS on ice for 1 h. Cells were washed and secondary antibody labelling was performed with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG2b antibody for 30 min in the dark, followed by appropriate washes. Dead cells were labelled with propidium iodide, and 1×10^4 cells/sample, in triplicate, were analyzed on a FACScan flow cytometer with CellQuest 3.0.1 software for data acquisition and analysis (Becton–Dickinson Immunocytometry Systems, Mountain View, CA). Live cells were gated by exclusion of propidium iodide-stained cells. The background autofluorescence of unlabelled cells was subtracted from the mean fluorescence of labelled cells before calculating the change in surface receptor number after agonist treatment (% loss of cell surface receptor number = internalization).

Pertussis toxin treatments. The involvement of G_i proteins in agonist-dependent internalization and [^{35}S]GTPγS binding was studied using PTX pretreatment. HEK-293α_{2A} and -α_{2B} cells were incubated for 18 h in serum-free DMEM containing PTX at 500 ng/ml. For flow cytometric analysis, PTX-treated and non-PTX-treated cells were exposed to agonists as described above at concentrations inducing submaximal levels of internalization ("EC_{90}").

[^{35}S]GTPγS binding. Cells were detached, centrifuged at 520*g* for 5 min at 4 °C, and washed once with phosphate-buffered saline. Cell pellets were suspended in ice-cold homogenization buffer (10 mM Tris–HCl, 0.1 mM EDTA, and 0.32 mM sucrose, pH 7.5) and homogenized with an Ultra-Turrax homogenizer. The homogenates were centrifuged at 380*g* for 15 min at 4 °C and the supernatants were collected. Pellets were re-homogenized and centrifuged as before. The pooled supernatants were centrifuged at 23,300*g* for 30 min at 4 °C. The membrane pellets were washed with sucrose-free Tris–EDTA buffer. The centrifugation procedure was repeated, and the membranes were resuspended in sucrose-free Tris–EDTA buffer. For the [^{35}S]GTPγS binding assay, membrane suspensions were thawed and diluted with binding buffer (50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 10 μM GDP, 1 mM DTT, and 30 μM ascorbic acid, pH 7.4). Incubations were performed on 96-well Millipore MultiScreen MAFB glass fiber filter plates (Millipore, Bedford, MA). The samples containing approximately 10 μg of membrane protein were preincubated with agonists for 30 min at room temperature prior to addition of 0.1 nM [^{35}S]GTPγS. Reactions were terminated after 60 min incubation at room temperature by vacuum filtration using a Millipore MultiScreen Vacuum Manifold. The filter plate was washed three times with ice-cold buffer (20 mM Tris, 1 mM EDTA, and 5 mM $MgCl_2$, pH 7.4), filters were dried, and 50 μl SuperMix scintillation cocktail (Wallac, Perkin–Elmer LifeSciences, Turku, Finland) was added into each well. The incorporated radioactivity was determined using a MicroBeta scintillation counter (Wallac).

Drugs and chemicals. A monoclonal antibody against the viral HA epitope (12CA5) was from Roche Molecular Biochemicals (Basel, Switzerland). Clonidine, GDP, (−)-norepinephrine, (−)-epinephrine, the neomycin analogue G418, and PTX were from Sigma Chemical (St. Louis, MO). UK 14,304 (5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine) was from Research Biochemicals (Natick, MA). The radioligand [^{3}H]RX821002 and [^{35}S]GTPγS were from Amersham (Buckinghamshire, UK). Dexmedetomidine was a gift from Orion Pharma (Turku, Finland). Cell culture reagents were from Gibco (Gaithersburg, MD) unless mentioned otherwise. Other chemicals were of analytical or reagent grade and were purchased from commercial suppliers.

Data analysis. The data were analyzed using GraphPad Prism programs (GraphPad Software, San Diego, CA). The results are mean values ± SEM. Independent samples *t* tests were used to compare treatment effects. A two-sided *p* value of less than 0.05 was considered significant.

Results

Rapid time-course of human α_{2A}- and α_{2B}-AR internalization

The extent of agonist-induced internalization was studied at different time points. The fluorescence remaining on plasma membranes was plotted against time and analyzed with GraphPad Prism programs using single phase exponential decay models. When HEK-293α_{2A} cells were treated with norepinephrine (100 µM), a plateau of the internalization response (about 26%) was seen after 15 min. The time for a half-maximal effect (ET_{50}) for internalization was 1.0 min. When HEK-293α_{2B} cells were treated with norepinephrine (10 µM), a plateau of the internalization response (also about 26%) was seen after 10 min. The time for a half-maximal effect (ET_{50}) of the internalization was 1.6 min. The time-course of internalization was thus rapid and similar for both investigated α_2-AR subtypes (Fig. 1).

Ligand-selective internalization of human α_{2A}- and α_{2B}-ARs

HEK-293α_{2A} and -α_{2B} cells were treated with various α_2-AR ligands for 20 min at +37 °C, chilled on ice, and labelled with an anti-HA-antibody to quantitate the number of cell surface receptors with flow cytometric analysis. Dose-dependence for agonist-induced internalization was clearly evidenced in HEK-293α_{2A} and -α_{2B} cells. Fig. 2 shows the effects of UK 14,304, norepinephrine, epinephrine, clonidine, and dexmedetomidine on internalization of α_{2A}- and α_{2B}-AR. For α_{2A}-AR, epinephrine induced maximal internalization (E_{max} about 28%) similar to that induced by norepinephrine (26%). UK 14,304 induced supramaximal internalization (about 39%, $p<0.01$) compared to norepinephrine, whereas the E_{max} for internalization induced by dexmedetomidine and clonidine was much smaller (7% and 8%, respectively, $p<0.001$ compared to norepinephrine, Table 1). For α_{2B}-AR, epinephrine and UK 14,304 induced similar extents of internalization as norepinephrine (30%, 28%, and 26%, respectively). Dexmedetomidine induced supramaximal internalization of α_{2B}-AR (about 35%, $p<0.01$ compared to norepinephrine), and clonidine had a significantly weaker internalization effect (about 10%, $p<0.001$ compared to norepinephrine) also for this α_2-AR subtype.

UK 14,304 and dexmedetomidine were subtype-preferring agonists regarding their efficacy in inducing internalization. Dexmedetomidine was significantly more efficacious in inducing α_{2B}-AR (35%) than α_{2A}-AR (7%) internalization ($p<0.001$), and UK 14,304 was

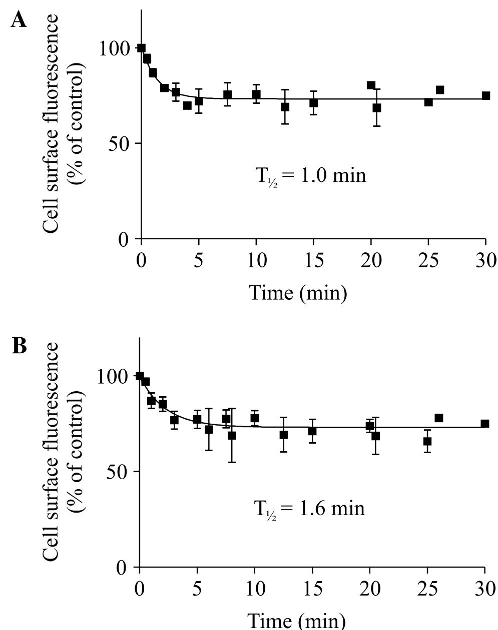

Fig. 1. Time course of norepinephrine-induced internalization of α_{2A}-AR (A) and α_{2B}-AR (B). Cells were treated with 100 µM (HEK-293α_{2A}) or 10 µM (HEK-293α_{2B}) norepinephrine for 0.5–30 min. The fluorescence remaining on plasma membranes was plotted against time and analyzed with single-phase exponential decay models. The time for a half-maximal effect (ET_{50}) for internalization was 1.0 min for α_{2A}-AR and 1.6 min for α_{2B}-AR. Data points are means ± SE of 6–8 independent experiments.

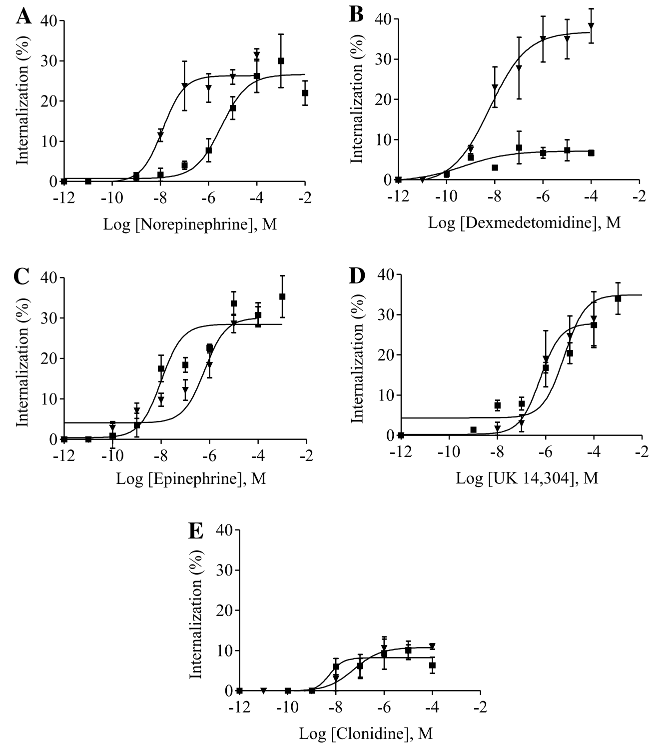

Fig. 2. Dose-dependent effects of different α_2-AR agonists on α_{2A}-AR (squares) and α_{2B}-AR (triangles) internalization. Cells were treated with different concentrations of norepinephrine (A), dexmedetomidine (B), epinephrine (C), UK 14,304 (D), and clonidine (E) for 20 min. Concentration–response curves of agonist-induced loss of cell surface α_2-AR (% internalization) were generated using sigmoidal regression analysis. Results are means ± SE of 8–11 independent experiments.

Table 1
Potencies and efficacies of various α_2-AR agonists for causing internalization of cell surface α_{2A}- and α_{2B}-AR in HEK-293 cells

Compound	HEK-293α_{2A}			HEK-293α_{2B}		
	−log EC_{50}	E_{max}	i.a.	−log EC_{50}	E_{max}	i.a.
Norepinephrine	5.5 ± 0.2[***]	26.2 ± 1.7	100	7.9 ± 0.2	26.3 ± 2.0	100
Epinephrine	7.0 ± 0.2[**,aaa]	27.8 ± 1.7	106	6.2 ± 0.2[aaa]	30.0 ± 1.8	114
UK 14,304	4.8 ± 0.2[a]	39.0 ± 3.3[*,aa]	148	6.2 ± 0.4[aa]	28.0 ± 3.0	106
Dexmedetomidine	9.5 ± 0.6[aaa]	6.7 ± 0.8[***,aaa]	26	8.3 ± 0.2	34.6 ± 2.1[aa]	131
Clonidine	8.3 ± 0.5[aaa]	8.4 ± 1.2[aaa]	32	7.2 ± 0.3	10.6 ± 0.8[aaa]	40

The E_{max}-values for different agonists indicate the maximal percentage reduction of cell surface α_2-AR-specific immunofluorescence compared to non-treated control cells. EC_{50} is the drug concentration (M) causing 50% of the maximal effect. Intrinsic activity (i.a.) is a ratio of E_{max} relative to the endogenous full agonist norepinephrine (NE). The values are means (±SE) of 6–11 independent experiments.

[a] $p < 0.05$ compared to NE.
[aa] $p < 0.01$ compared to NE.
[aaa] $p < 0.001$ compared to NE.
[*] $p < 0.05$ compared to α_{2B}.
[**] $p < 0.01$ compared to α_{2B}.
[***] $p < 0.001$ compared to α_{2B}.

more efficacious in inducing α_{2A}-AR (39%) internalization compared to α_{2B}-AR (28%) ($p < 0.05$).

The rank order of potency of the tested agonists for internalization of α_{2A}-AR was dexmedetomidine = clonidine > epinephrine ≫ norepinephrine > UK 14,304, and that of α_{2B}-AR was dexmedetomidine > clonidine = norepinephrine > epinephrine = UK 14,304. Dexmedetomidine was the most potent drug in inducing internalization of both receptor subtypes. Epinephrine was 10 times less potent at α_{2B}-AR compared to α_{2A}-AR ($p < 0.01$), whereas norepinephrine was 100 times more potent in inducing internalization of α_{2B}-AR than α_{2A}-AR ($p < 0.001$) (Fig. 2 and Table 1).

Potencies and efficacies of α_2-AR agonists for G_i protein activation

Agonist-induced [^{35}S]GTPγS binding reflects the intrinsic efficacy of agonists, and provides a measure of agonist efficacy at the first level of signal transduction:activation of G proteins. The rank order of agonist efficacy (intrinsic activity relative to norepinephrine) for the investigated agonists at the human α_{2A}-AR was epinephrine = norepinephrine > UK 14,304 ≫ dexmedetomidine = clonidine (Table 2 and Fig. 3). The rank order of potency for the same agonists was dexmedetomidine ⩾ UK 14,304 > clonidine ⩾ epinephrine ⩾ norepinephrine.

The rank order of agonist efficacy for the same set of agonists at the human α_{2B}-AR was epinephrine = norepinephrine > dexmedetomidine ≫ UK 14,304 > clonidine (Table 2 and Fig. 3). The rank order of potency for the same agonists at α_{2B}-AR was dexmedetomidine > UK 14,304 ⩾ norepinephrine = epinephrine. The capacity of all studied agonists to induce [^{35}S]GTPγS binding was abolished in PTX-pretreated cell membranes (Fig. 3).

The notable differences between α_{2A}-AR and α_{2B}-AR in agonist-induced [^{35}S]GTPγS binding were the subtype-specific high efficacy of UK 14,304 at α_{2A}-AR and that of dexmedetomidine at α_{2B}-AR. Dexmedetomidine was a partial agonist at α_{2A}-AR and UK 14,304 at α_{2B}-AR, whereas the weak partial agonist clonidine evoked detectable [^{35}S]GTPγS binding only at α_{2A}-AR.

Table 2
Potencies and efficacies of various ligands for inducing [^{35}S]GTPγS binding as a measure of G_i protein activation

Compound	HEK-293α_{2A}			HEK-293α_{2B}		
	−log EC_{50}	E_{max}	i.a.	−log EC_{50}	E_{max}	i.a.
Norepinephrine	6.17 ± 0.1	283 ± 11	100	6.46 ± 0.06	184 ± 4	100
Epinephrine	6.77 ± 0.05[**,aa]	297 ± 5	108 ± 11	6.34 ± 0.07	178 ± 5	97 ± 2
UK 14,304	7.68 ± 0.09[*,aa]	216 ± 5[aa]	82 ± 3[***]	6.67 ± 0.27	46 ± 4[aaa]	25 ± 2
Dexmedetomidine	8.20 ± 0.19[aaa]	54 ± 3[aaa]	19 ± 2[***]	8.22 ± 0.10[aaa]	137 ± 3[aaa]	75 ± 5
Clonidine	6.97 ± 0.25[a]	28 ± 3[aaa]	10 ± 2	n.d.	−4 ± 2[aaa]	0

The values are means (±SE) of three independent experiments. EC_{50} is the drug concentration (M) causing 50% of the maximal effect. Intrinsic activity (i.a.) is a ratio of E_{max} relative to the endogenous full agonist norepinephrine (NE).

[a] $p < 0.05$ compared to NE.
[aa] $p < 0.01$ compared to NE.
[aaa] $p < 0.001$ compared to NE.
[*] $p < 0.05$ compared to α_{2B}.
[**] $p < 0.01$ compared to α_{2B}.
[***] $p < 0.001$ compared to α_{2B}.

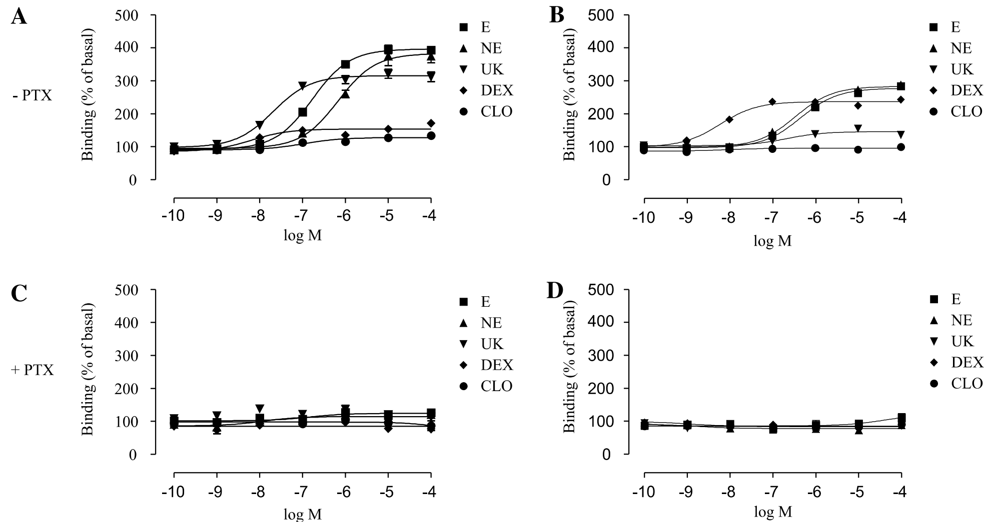

Fig. 3. α_{2A}-AR (A,C) and α_{2B}-AR (B,D) induced stimulation of [^{35}S]GTPγS binding to G_i proteins in HEK-293 cell membranes. Membranes were prepared from non-PTX-pretreated (A,B) and PTX-pretreated (C,D) cells. Membranes were incubated with increasing concentrations of the agonists epinephrine (E), norepinephrine (NE), UK 14,304 (UK), dexmedetomidine (DEX), and clonidine (CLO). Results are means ± SE of three independent experiments.

Effect of PTX on ligand-induced changes in cell surface α_2-AR number

PTX pretreatment (500 ng/ml for 18 h) substantially inhibited internalization induced by partial and full agonists (Fig. 4). The PTX-induced inhibition of internalization was similar for α_{2A}-AR and α_{2B}-AR when epinephrine, norepinephrine, and UK 14,304 were used.

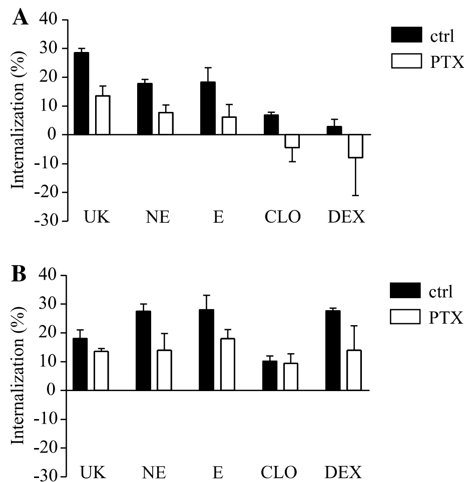

Fig. 4. Effect of PTX treatment on ligand-induced changes in cell surface α_{2A}-AR (A) and α_{2B}-AR (B) number. Cells were pretreated with 500 ng/ml PTX for 18 h. PTX-pretreated and non-PTX-treated (ctrl) HEK-293α_{2A} (A) and HEK-293α_{2B}-cells (B) were treated for 20 min with vehicle or with previously determined concentrations of UK 14,304 (UK), norepinephrine (NE), epinephrine (E), clonidine (CLO), and dexmedetomidine (DEX), inducing submaximal internalization (EC_{90}) of α_{2A}- and α_{2B}-AR. The loss of cell surface α_2-AR labelling, compared to similarly pretreated cells not exposed to agonists (% internalization), is shown as means ± SE of 3–5 independent experiments.

However, when clonidine and dexmedetomidine were used after PTX, an increase in cell surface receptor number was observed in α_{2A}-AR expressing cells (Fig. 4A).

Discussion

Internalization is one of the desensitization mechanisms related to dynamic regulation of GPCRs in response to agonist treatment (for review, see [25]). The molecular processes underlying GPCR desensitization include rapid uncoupling of the receptors from their cognate G proteins by phosphorylation of the receptor, catalyzed by G protein receptor kinases. Phosphorylation is followed by internalization via clathrin-coated pits, a process initiated by binding of β-arrestin and adaptor protein AP2. To regain full responsiveness after agonist exposure, receptor resensitization is required. Although internalization is not a requirement for receptor desensitization, it is thought to be elementary for resensitization of receptors by dephosphorylation induced by protein phosphatases in intracellular vesicles. In addition to clathrin-dependent internalization of α_{2A}- and α_{2B}-ARs, caveolin-rich invaginations of the plasma membrane appear to be involved in α_{2A}-AR internalization [24]. The relationship between G protein activation and internalization is not clear. Different agonists show different efficacies in inducing G protein coupling and internalization, but the relationship of these biological processes is not always predictable [12–14].

We have now investigated the time course and extent of internalization of human α_{2A}- and α_{2B}-ARs expressed in HEK-293 cells. Also the concentration-dependence of internalization, and relationship between agonist-induced

G protein coupling and receptor internalization were analyzed using five different agonist ligands previously known to be full or partial agonists in inducing G protein activation. The results showed rapid dose- and agonist-dependent internalization of both α_{2A}- and α_{2B}-receptors. The relationship between the capacity of the agonists to induce receptor internalization and G protein activation as determined by [^{35}S]GTPγS binding demonstrated subtype-specific proportionality. Pretreatment with PTX, which totally abolished agonist-dependent G_i-activation, did not fully block internalization, which suggests that only part of the internalization response of both studied α_2-AR subtypes is dependent on G_i protein coupling.

Earlier studies on β_2-AR have shown that the time course of internalization after agonist exposure is rapid; a half-maximal internalization response to the full agonist epinephrine was observed in about 3 min and an apparent steady state of internalization was reached by 30 min [14,26]. The internalization of β_2-AR has been shown to be dependent on temperature and cellular ATP but not on de novo synthesis of proteins [26]. Internalization of α_2-ARs also seems to be very rapid with half-maximal effects reached in 1 min for α_{2A}-AR and in 1.6 min for α_{2B}-AR. This suggests that the cellular machinery needed for internalization of these receptors in HEK-293 cells is functional without new protein synthesis.

Subtype-specificity of α_2-AR internalization has earlier been reported: α_{2B}-AR has been shown to internalize similarly to β_2-AR [27], after βARK phosphorylation and β-arrestin binding into endocytic transferrin-positive vesicles [21,28]. Both lack of internalization [21,28] and considerable degrees of internalization of α_{2A}-AR have been reported [29–31]. Using radioligand binding assays, significant agonist-induced α_{2A}-AR internalization was observed in human erythroleukemia cells [29]. In transfected Chinese hamster ovary (CHO) cells, human α_{2A}-AR internalization was 26% and that of human α_{2B}-AR 35% [30], and 20–30% internalization of human α_{2A}- and α_{2B}-ARs was found also previously by us in PC12 and HEK-293 cells [24]. Internalization of mouse α_{2A}-AR in HEK-293 cells, as evidenced by ELISA and induced by 10 or 100 μM norepinephrine, was reported to be only 7% [21]. It is likely that the differences observed for the capacity of α_{2A}-AR to internalize are species-dependent, the human α_{2A}-AR being readily internalized and the mouse α_{2A}-AR being unable to internalize. Since arrestin binding has been shown to be the most important rate-limiting step of α_2-AR sequestration [32], and cell lines are known to differ markedly in their content of arrestins, the extent of internalization may also depend on the employed cell line. HEK-293 cells are known to express large amounts of arrestins and are therefore widely used for receptor regulation studies [33].

Our results indicated concentration-dependence of internalization when both full and partial α_2-AR agonists were used. The extent of the maximal internalization response was identical for α_{2A}- and α_{2B}-ARs when the endogenous full agonists norepinephrine and epinephrine were used. However, the efficacies of UK 14,304 and dexmedetomidine to induce internalization were strongly dependent on the receptor subtype, UK 14,304 being 1.4 times more efficacious at α_{2A}-AR compared to α_{2B}-AR and dexmedetomidine being 5.2 times more efficacious at α_{2B}-AR compared to α_{2A}-AR. In addition, both UK 14,304 and dexmedetomidine proved to be superagonists in inducing internalization of their preferred receptors, with 148% and 131% relative efficacies compared to norepinephrine. These agonist-related internalization responses were in relation to the subtype-specific agonist-related responses measured in the [^{35}S]GTPγS binding assay. In the present study, UK 14,304 was clearly more efficacious than dexmedetomidine at α_{2A}-AR and dexmedetomidine was more efficacious than UK 14,304 at α_{2B}-AR. Superagonistic effects of UK 14,304 and dexmedetomidine were not detected. However, previous studies performed in slightly different assay conditions have shown that both UK 14,304 and dexmedetomidine can have greater efficacy than norepinephrine at their preferred receptors in the [^{35}S]GTPγS binding assay [16,17]. Clonidine was a weak partial agonist in inducing internalization of both subtypes, and it was weak in inducing [^{35}S]GTPγS binding at α_{2A}-AR and evoked no response at α_{2B}-AR in the present assay conditions. These results suggest that strong agonists are capable of inducing or stabilizing conformations that are favorable for both G protein activation and for entry into the endocytic route.

Previously, January et al. [14] have shown proportionality between agonist strength (efficacy in inducing AC activation) and agonist-induced desensitization, internalization, and the rapid, initial phase of phosphorylation of β_2-AR in HEK-293 cells. The proportionality of agonist efficacy in [^{35}S]GTPγS binding assays to internalization of μ- and δ-opioid receptors has also been reported [12]. In the case of MOR, the agonist-induced internalization efficacy was shown to be related to their efficacy to stimulate [^{35}S]GTPγS binding but not to inhibition of cAMP accumulation [13,34], most likely because of the existence of a "receptor reserve" in the latter assay. We found additionally that the potency of norepinephrine in inducing internalization was different between the two α_2-ARs; norepinephrine was 100 times more potent at α_{2B}-AR than at α_{2A}-AR. Similar subtype-specific agonist-related differences have been observed for α_2-AR down-regulation. Down-regulation after 24 h agonist exposure was similar for α_{2B}-AR and α_{2C}-AR, whereas the down-regulation of α_{2A}-AR was more resistant to long-term norepinephrine exposure. The norepinephrine concentration needed to down-regulate α_{2A}-AR by 50% was 100-fold greater than that required for 50% down-regulation of α_{2B}- and α_{2C}-ARs [35–37]. The α_2-AR subtype-dependent differences in the potency of norepinephrine to

induce internalization of α_2-AR in this study, and that of norepinephrine in inducing down-regulation in earlier studies [36,37] may be related to non-G_i protein mediated regulatory effects of the ligand on α_2-ARs, since no differences in the potency of norepinephrine were found between the receptors in the [^{35}S]GTPγS binding assay.

The current study showed that pretreatment with PTX inhibited a significant part of agonist-induced internalization of both α_{2A}- and α_{2B}-ARs. The remaining amount of agonist-induced cell surface receptor loss after PTX-pretreatment was about 50% of the maximal, except for dexmedetomidine and clonidine at α_{2A}-AR. These two weak α_{2A}-AR agonists caused small increments of cell surface receptors after PTX-treatment. Earlier studies with MOR have shown that some ligands induce enhanced cell surface receptor presentation after PTX-treatment [13]. The mechanism may be related to partial agonist effects and enhancement of recycling of the receptors back to the cell surface [13]. The internalization of α_2-AR subtypes may not require activation of G_i-type PTX-sensitive G proteins, but the formation of receptor–G protein complexes may favor the existence of a receptor conformation amenable for internalization. Our results after PTX treatment show that G_i protein coupling is not required for α_2-AR internalization. In line with this, direct interactions of the receptor with other proteins known to be important for internalization, like *N*-ethylmaleimide-sensitive factor (NSF) or the small GTP-binding protein Rab5, have been shown to induce endocytosis of β_2-AR and angiotensin II 1A receptors, respectively (reviewed in [25]).

Taken together, this study shows that the extent of agonist-induced internalization of α_{2A}-AR and α_{2B}-AR is dependent on the duration of exposure, the concentration of agonist, and the pharmacological efficacy of the agonist to induce G_i protein activation. However, a significant part of the α_2-AR internalization response is independent of PTX-sensitive G_i proteins.

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