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Addition of a signal peptide sequence to the α_{1D}-adrenoceptor gene increases the density of receptors, as determined by [^{3}H]-prazosin binding in the membranes

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1 Both in mammalian tissues and in transfected cells, only low levels of α_{1D}-adrenoceptors are detected in radioligand binding studies. It has been implicated that the comparatively long N-terminal tail of the α_{1D}-adrenoceptor is responsible for the inefficient surface expression of the receptor.

2 In the present study, we created gene constructs for six N-terminally truncated variants of the human α_{1D}-adrenoceptor. These constructs were used to transfect Neuro2A cells. We show that the density of α_{1D}-adrenoceptors, observed by [^{3}H]-prazosin binding, gradually increased with longer truncations of the N-terminus. This seems to indicate that the long N-terminal tail nonspecifically interferes with receptor translocation to the plasma membrane.

3 The addition of a 16 amino acids long signal peptide to the N-terminus of the wild-type α_{1D}-adrenoceptor increased the density of receptor binding sites 10-fold in Neuro2A and COS-7 cells. This indicates that, after the addition of a signal peptide, the long N-terminal tail of the α_{1D}-adrenoceptor does not interfere with proper translocation of the receptor to the plasma membrane. This, in turn, indicates that the N-terminal tail of the wild-type α_{1D}-adrenoceptor, merely by its long length, hinders the first transmembrane helix of the receptor from being a signal anchor.

4 Neither the wild-type α_{1D}-adrenoceptor (for which the expression level of [^{3}H]-prazosin binding sites is low) nor the truncated α_{1D}-adrenoceptor variant (for which the expression level of [^{3}H]-prazosin binding sites is high) showed any constitutive activity in stimulating inositol phosphate accumulation. This indicates that the low expression level of [^{3}H]-prazosin binding sites, after transfection with the wild-type α_{1D}-adrenoceptor, is not caused by constitutive activity of the receptor and subsequent receptor downregulation.
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Abbreviations: α_{1D}-AR, α_{1D}-adrenoceptor; DMEM, Dulbecco's modified Eagle's medium; GPCR, G-protein-coupled receptor; IPs, inositol phosphates; PE, phenylephrine

Introduction

The α_1-adrenoceptors are G-protein-coupled receptors (GPCRs) in the body responsive to the hormone adrenaline and the transmittor substance noradrenaline. There exist three subtypes of α_1-adrenoceptors, denoted α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR (Zhong & Minneman, 1999; Hague *et al*., 2003). α_1-Adrenoceptors are abundantly present in the brain, with as yet unclear functions, and they play an important role in the control of blood pressure, by influencing contraction and growth of smooth and cardiac muscle (Zhong & Minneman, 1999). Regarding the α_{1D}-subtype, it has been shown that α_{1D}-adrenoceptors are present in the aorta (Gisbert *et al*., 2003), and that they regulates arterial blood pressure *via* vasoconstriction (Tanoue *et al*., 2002). However, the functional role of the α_{1D}-adrenoceptor is obscured by the low level of α_{1D}-adrenoceptor binding sites in mammalian tissues (Yang *et al*., 1997; see Garcia-Sainz & Villalobos-Molina, 2004). Even after transfection of cultured mammalian cells with the gene for α_{1D}-AR, only few receptors are detected by radioligand binding in membranes (Pupo *et al*., 2003). Instead, it seems like the α_{1D}-adrenoceptor protein is localized mainly intracellularly (McCune *et al*., 2000; Chalothorn *et al*., 2002), in a form that apparently does not bind radioligand (Pupo *et al*., 2003). Recently, it has been proposed that the α_{1D}-adrenoceptor is poorly expressed in membranes because it is not properly translocated to the plasma membrane after its synthesis (Hague *et al*., 2004a). The initial step in the translocation of a membrane protein to the plasma membrane is the insertion of the maturing protein into the membrane of the endoplasmatic reticulum. To accomplish this, the majority of GPCRs have short N-tails (about 40 amino acids) and use the first transmembrane domain as signal anchor, while GPCRs with longer N-tails often contain an additional cleavable signal peptide sequence (Wallin & von Heijne, 1995). Therefore, poor translocation efficieny of α_{1D}-AR might be caused by its fairly long extracellular N-terminal tail (95 amino acids), combined

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with its lack of a signal peptide sequence. Indeed, truncation of the N-terminal tail of the α_{1D}-AR increased the density of specific α_{1D}-AR binding sites in the membranes quite dramatically compared to the wild-type receptor (Pupo *et al*., 2003). Studies with other GPCRs clearly show that the N-terminus of the receptor is important for their expression pattern. Thus, it has been shown that removal of the inherent signal peptide from the endothelin B receptor decreased surface expression, while further truncation of the residual tail rescued it (Köchl *et al*., 2002). Also, truncation of the long N-terminal tail of the cannabinoid receptor 1, or supplementing it with an N-terminal signal peptide sequence, increased expression of receptors in the plasma membrane (Andersson *et al*., 2003).

In the present study, we made gene constructs for six N-terminally truncated variants of the human α_{1D}-adrenoceptor. We also added a 16 amino acids long signal peptide to the N-terminus of the wild-type α_{1D}-adrenoceptor. These constructs were used to investigate the B_{max} levels of the receptors obtained after transient transfections of Neuro2A cells.

Earlier, it has been hypothesized that the α_{1D}-AR is constitutively active and therefore continuously becomes downregulated (McCune *et al*., 2000). In order to study if constitutive activity is responsible for the observed low density of α_{1D}-AR binding sites in the plasma mambrane, we investigated basal, agonist-stimulated, and antagonist-modified levels of inositol phosphates (IPs) accumulation. This was carried out in Neuro2A cells stably expressing wild-type α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors, and two truncated α_{1D}-adrenoceptors.

Methods

Materials

[7-methoxy-^{3}H]-prazosin (84 Ci mmol^{-1}) and myo-[2-^{3}H]-inositol (15 Ci mmol^{-1}) were from Amersham Biosciences, Uppsala, Sweden. Cirazoline was a gift from Synthelabo, Paris, France. Metitepine, phenylephrine, and prazosin were from Sigma-Aldrich, Stockholm, Sweden. Primers were purchased from TAG Copenhagen A/S, Denmark, or Thermo Hybaid GmbH, Ulm, Germany. COS-7 (African green monkey kidney fibroblast) and Neuro2A (mouse neuroblastoma) cells were obtained from ATCC (Rockville, U.S.A.). All other chemicals and molecular biology reagents were purchased from the appropriate commercial sources.

Gene constructs

Truncated variants of the human α_{1D}-adrenoceptor were obtained by PCR, using Platinum Pfx DNA polymerase, on the α_{1D}-adrenoceptor gene inserted in pCI-neo with *Eco*RI and *Xba*I.

The forward primers used were as follows: wt α_{1D}-AR: 5′-ga gaa ttc atg act ttc cgc gat ctc ct; Δ1–5α_{1D}-AR: 5′-ga gaa ttc atg ctc ctg agc gtc agt ttc gag-3′; Δ1–17α_{1D}-AR: 5′-ga gaa ttc atg agc agc gca ggg ggc tcc ag-3′; Δ1–30α_{1D}-AR: 5′-ga gaa ttc atg agc gcg ggc ggc gcg gcc cc-3′; Δ1–58α_{1D}-AR: 5′-ga gaa ttc atg gca ggc agc ggc gag gac aac-3′; and Δ1–89α_{1D}-AR: 5′-ga gaa ttc atg ctg gtg gtg agc gcg cag gg-3′. The reverse primer was 5′-agg ttc acg atg aaa tag ttg gt-3′.

This reverse primer is located with its 5′-end at the position of the base 413 in the α_{1D}-gene. These primer pairs amplified between 157 to 421 bp bands, which could be cut with *Eco*RI (site present in the forward primers) and *Bst*EII (site at position 338 in the α_{1D}-gene). The cut bands were purified by agarose gel electrophoresis, and then ligated into the *Eco*RI- and *Bst*EII-cut wild-type α_{1D} construct.

The N-terminal 95 amino acids of the human α_{1D}-adrenoceptor can be represented as shown below. The wild-type α_{1D}-adrenoceptor starts at the first Met, the first truncated variant (Δ1–5α_{1D}-AR) had the first five amino acids exchanged for a Met at the first * (below), (Δ1–17α_{1D}-AR) had 17 amino acids exchanged for a Met at the second *, (Δ1–30α_{1D}-AR) had 30 amino acids exchanged for a Met at the third *, accidentally the Δ1–30α_{1D}-AR primer gave rise to an extra truncated clone (Δ1S–49α_{1D}-AR), which had 49 amino acids exchanged for Met-Ser at the **, (Δ1–58α_{1D}-AR) had 58 amino acids exchanged for a Met at the fourth *, and (Δ1–89α_{1D}-AR) had 89 amino acids exchanged for a Met at the fifth *.

mtfrd * llsvsfegprpd * ssaggssaggggg * saggaapsegpavggvpgg*
*agggggvvg * agsgednrssagepgsagaggdvngtaavgg * lvvsaq

An α_{1D}-adrenoceptor supplemented with an N-terminal cleavable signal peptide (α_{1D}-AR-SP) was constructed by inserting a modified Influenza hemaglutinin signal sequence (Jou *et al*., 1980) before the start codon of the α_{1D}-adrenoceptor gene. The same sequence has been used previously for adding signal peptide to the hamster β_2-adrenoceptor (Guan *et al*., 1992). The signal peptide-inducing primer was tctt (*Nhe*I>) gct agc (signal peptide>) atg aag acg atc atc gcc ctg agc tac atc ttc tgc ctg gta ttc gcc (α_{1D}>) atg act ttc cgc gat ctc ctga. A wild-type α_{1D}-AR construct, having the same kozak sequence (Kozak, 1991) as the α_{1D}-AR-SP construct, was made using the corresponding forward primer, but devoid of the 48 bases encoding-the signal peptide sequence. The same reverse primer as was described previously (three sections above) (with its 5′-end at position 413 bp in the α_{1D}-gene) was used. Inserts obtained from the PCR bands were cloned with *Nhe*I and *Bst*EII, yielding the new wild-type α_{1D}-AR construct, and the full-length α_{1D}-AR-signal peptide (α_{1D}-AR-SP) construct. The procedure described here added the peptide sequence MKTIIALSYI FCLVFA N-terminally to the wild-type α_{1D}-adrenoceptor.

Cell culture

The COS-7 as well as Neuro2A cells were grown at 37°C, 95% air, 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) (Sigma D5796), supplemented with 10% fetal calf serum and 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin, in standard tissue culture plastic material. Cells were subcultured every 3 to 4 days, after detachment with 0.5 mg ml^{-1} trypsin and 0.2 mg ml^{-1} EDTA (Sigma T3924).

Transient expression

The genes encoding the α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors, as well as the truncated α_{1D} variants, were cloned into the expression vector pCI-neo. For transfection, 15 μg of construct DNA was mixed with 200 μl of lipofectin and 600 μl of OptiMEM, and after 20 min the mixture was added to an

about 70% confluent layer of COS-7 or Neuro2A cells in a 13.5 cm Petri dish, immediately followed by the addition of 20 ml of OptiMEM (in some experiments,10 μg of construct DNA was used to transfect 9 cm plates). Next day, the medium was exchanged to DMEM, with 10% fetal calf serum and 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin, and the cells were further cultured for 48 h. The transfections, membrane preparations, and determinations of B_{max} values by radioligand binding were matched, so that one plate of cells was transfected with 15 μg of the corresponding wild-type α_{1D} construct, and simultaneously other plates were transfected with the truncated α_{1D}-AR variants or with the α_{1D}-AR-SP construct. In matched transfections with the two wild-type α_{1D} constructs, having Kozak sequences gaattcatga and gctagcatga, respectively, the membranes showed similar levels of [^{3}H]-prazosin binding sites, indicating that this difference in Kozak sequence was not of critical importance for the expression levels of α_{1D}-AR (S Uhlén, personal communication).

Establishment of stable cell lines

Clonal cell lines, expressing the truncated α_{1D} variant Δ1-17α_{1D}-AR, the truncated α_{1D} variant Δ1-58α_{1D}-AR, as well as the wild-type α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors, were established by transfection of Neuro2A cells with the gene constructs, as described in the section above. At 12 h after transfection, OptiMEM was exchanged for DMEM and the cells were cultured for 24 h. Then, the cells were trypsinized, diluted into medium including 800 μg ml^{-1} of geneticin, and seeded into a 48-well microtiter plate (about 10 cells well^{-1}). The medium was exchanged every day or every other day, and wells containing a single attached cell were identified. After about 10 days, clonal islands had developed, and the cultures were trypsinized, transferred, and expanded in six-well plates. After 4–5 days, full monolayers had developed. These cells were then cultured in 10 ml flasks and the concentration of geneticin was sequentially decreased (first 500 μg ml^{-1}, then 300 μg ml^{-1}, and all subsequent 200 μg ml^{-1}). Cell lines obtained were screened for α_1-adrenoceptors with [^{3}H]-prazosin. For each receptor variant, several clones were obtained, among themselves expressing similar levels of binding sites, which were high for the α_{1D} variant Δ1–58α_{1D}-AR, as well as for the α_{1B}-adrenoceptor, but fairly low for the α_{1A}- and α_{1D}-adrenoceptors and for the α_{1D} variant Δ1–17α_{1D}-AR.

Measurement of inositol phosphate accumulation

Accumulation of [^{3}H]-IPs was determined by a modification of the protocol described by Berridge *et al.* (1983). Cells stably expressing the different receptors were loaded with myo-[^{3}H]-inositol for 18 h. Thereafter, cells were detached with Versene (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.54 mM EDTA, pH 7.4) at 37°C, spun at 2500 × *g* for 2 min, and resuspended in Na-Elliot buffer (137 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.4), including 10 mM lithium. Lithium inhibits the breakdown of inositol phosphates, which are second messenger molecules for the α_1-AR. The cell suspension was incubated for 10 min at 37°C, and then 100 μl of cells were added to a 96-well plate, containing 50 μl of varying concentrations of the agonists cirazoline (Horie *et al.*, 1995) or phenylephrine (Obika *et al.*, 1995), or the antagonist prazosin. Aliquots (100 μl) of the cell preparations were also added to scintillation vials in order to measure the total amount of [^{3}H]-inositol taken up by the cells. The incubation in the 96-well plate then proceeded for 50 min. Cells were spun down at 2500 × *g* for 90 s, the supernatant discarded, the cells lyzed with 100 μl of 0.4 M perchloric acid ($HClO_4$), and immediately frozen at −80°C. After 1 h, the suspensions were thawed, immediatelly neutralized with 50 μl of cold 0.36 M KOH + 0.3 M $KHCO_3$, and cell debris was spun down at 2000 × *g* for 10 min. Based on the method used by Berridge *et al.* (1983), the total amount of [^{3}H]-IPs from each well was isolated on a Dowex column (BioRad AG1-X8 anion exchange column, formate form, about 1.2 ml packed suspension), equilibrated with 4 ml 0.1 M formic acid + 1 M NH_4-formate, followed by 4 ml ^dH_2O. Each sample was added to a column, and then the column was washed two times with 4 ml ^dH_2O and two times with 4 ml 5 mM Na_2tetraborate + 60 mM NH_4-formate, and then the [^{3}H]-IPs were eluted with 4 ml 0.1 M formic acid + 1 M NH_4-formate. A measure of 10 ml of scintillation cocktail (OptiPhase HiSafe 3) was added, and radioactivity was measured in the β-scintillation counter. The EC_{50} values for cirazoline and phenylephrine were calculated by the four-parameter logistic function, using the BindAid radioligand binding analysis package (Wan System, Umeå, Sweden).

Membrane preparations

COS-7 and Neuro2A cells transiently or stably expressing the different receptors were detached from 13.5 cm plastic culture dishes in 50 mM Tris, 5 mM EDTA. The cells were homogenized by ultraturraxing (IKA T25, 8 mm probe) for 15 s at 15,000 r.p.m. The homogenate was centrifuged at 400 × *g*, and the supernatant was then recentrifuged at 38,000 × *g* for 10 min. The final pellets were resuspended in 2.8 ml of 50 mM Tris and 1.5 mM EDTA, and was used directly in binding experiments. The protein concentrations of the membrane preparations were 0.2–1 mg ml^{-1}, as determined by the method of Lowry *et al.* (1951) with the inclusion of SDS (Markwell *et al.*, 1978).

Radioligand binding

In order to determine the B_{max} values of α_1-adrenoceptors in the membranes, saturation experiments with [^{3}H]-prazosin were performed. Each saturation experiment involved distributing membranes, obtained from one 13.5 cm diameter culture dish, into 24 wells of a 96-well microtiter plate at about 10–100 μg of protein well^{-1}, in 150 μl of 33 mM Tris-HCl, 1 mM EDTA, at pH 7.5 (a four-fold dilution was made of the membranes obtained from plates transfected with constructs yielding high levels of binding sites, that is, the Δ1–58α_{1D}-AR, α_{1D}-AR-SP, and α_{1B}-AR). Also present in the assays were different concentrations of [^{3}H]-prazosin, ±2 μM metitepine (to define nonspecific binding). The incubations lasted for 1 h at room temperature, and then the suspensions were filtered and washed on Whatman GF/C filters, using a Brandel cell harvester. Radioactivity retained on the filters were counted in a Beckman β-scintillation counter. All assays were performed in duplicate. The nonspecific binding of [^{3}H]-prazosin was lower in Neuro2A membranes as compared to COS-7

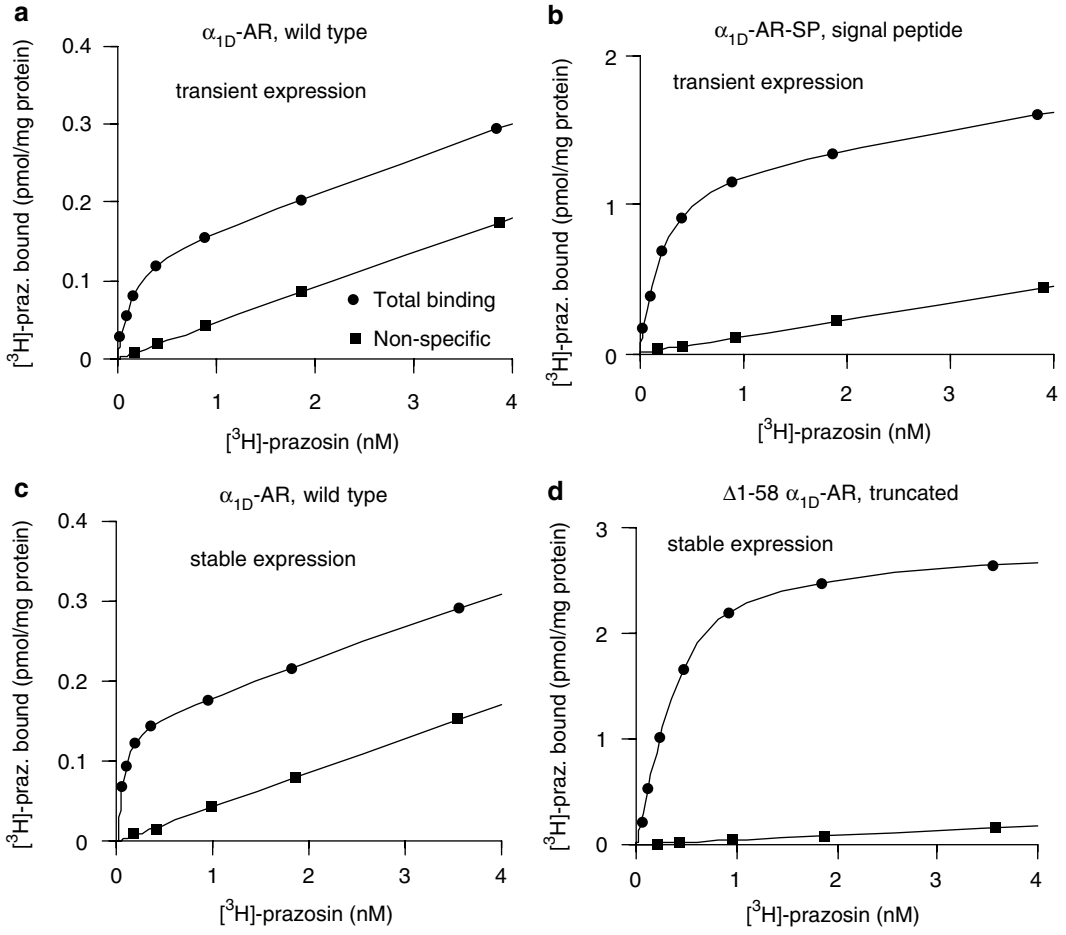

Figure 1 Saturation curves for [^{3}H]-prazosin on membranes after transient or stable transfections of Neuro2A cells with different α_{1D}-AR constructs. Nonspecific binding was defined by 2 μM of metitepine.

membranes. Curves were drawn using the BindAid radioligand binding analysis package (Wan System, Umeå, Sweden). Figures 1 and 2 were constructed using DeltaGraph® Pro 3.5. Figure 3 was constructed using Power Point.

Results

Transient expression of the truncated α_{1D}-AR variants

After transient expression of the α_{1D}-AR variants in Neuro2A cells, the wild-type α_{1D}-AR and the minutely truncated α_{1D} variants (Δ1–5α_{1D}-AR and Δ1–17α_{1D}-AR) showed low levels of [^{3}H]-prazosin binding sites. When the inherently 95 amino acids long N-terminal tail of the α_{1D}-adrenoceptor was further shortened, there was a graded increase in the density of [^{3}H]-prazosin binding sites (Table 1). Thus, Δ1–30α_{1D}-AR, Δ1S–49α_{1D}-AR, and Δ1–58α_{1D}-AR showed increasingly higher B_{max} values. However, the α_{1D} variant with a very short N-terminal, Δ1–89α_{1D}-AR showed a lower level than Δ1S–49α_{1D}-AR and Δ1–58α_{1D}-AR. The α_{1D} variant with the highest density of [^{3}H]-prazosin binding sites (Δ1–58α_{1D}-AR) showed a 6.4-fold higher level compared to the wild-type α_{1D}-adrenoceptor (Table 1). Thus, the levels of detectable α_{1D}-adrenoceptors gradually increased as the N-terminal became shorter, at least in the span between truncation by 17, 30, 49, and 58 amino acids.

Transient expression of the α_{1D}-AR supplemented with a signal peptide

In a separate set of experiments, the wild-type α_{1D}-AR and the α_{1D}-AR supplemented with a 16 amino acids long signal sequence (α_{1D}-AR-SP) were transiently expressed in COS-7 and Neuro2A cells. The densities of [^{3}H]-prazosin binding sites were about 70 and 100 fmol mg^{-1} membrane protein for the wild-type α_{1D}-AR in COS-7 and Neuro2A cells, respectively (Table 2). In comparison, the α_{1D}-AR-SP receptor showed binding densities of about 800 and 1100 fmol mg^{-1} membrane protein in the COS-7 and Neuro2A cells. In matched experiments, this represented a 12- to 13-fold increase in the expression level of [^{3}H]-prazosin binding sites for the α_{1D} variant supplemented with signal peptide (α_{1D}-AR-SP) as compared to the wild-type α_{1D}-AR (Table 2; Figure 1). This shows that adding a signal peptide to the N-terminus of the wild-type α_{1D}-adrenoceptor dramatically increased the density of detectable receptors in the membranes.

Basal level of IPs accumulation in transfected Neuro2A cells

After stabilizing the expression of the α_{1A}-, α_{1B}-, and α_{1D}-AR, as well as the two truncated α_{1D}-AR variants Δ1–17α_{1D}-AR and Δ1–18α_{1D}-AR with gentamicin treatment, we measured

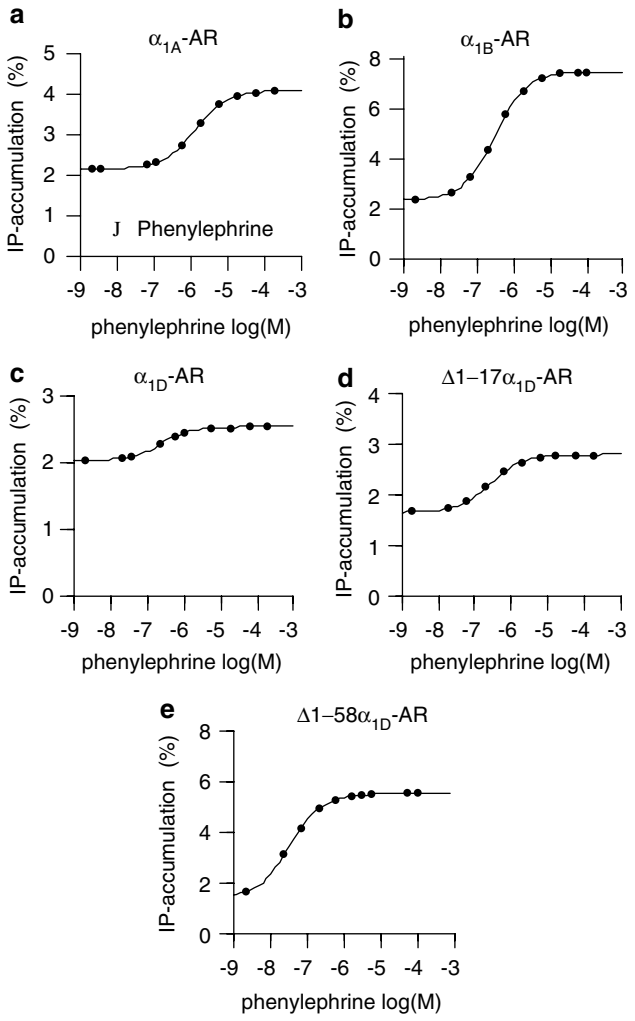

Figure 2 Dose–response curves for the α_1-agonist phenylephrine on [^{3}H]-inositol phosphate accumulation in Neuro2A cells stably transfected with the wt α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors, or with the truncated α_{1D}-AR variants Δ1–17α_{1D} and Δ1–58α_{1D}.

receptor-activated stimulation of second messenger production, that is, IPs accumulation. The percent conversion of [^{3}H]-inositol to inositol phosphates (IPs accumulation) was determined by measuring how much of the [^{3}H]-inositol, taken up by the cells, was transformed to [^{3}H]-inositol phosphates during 1 h of incubation with lithium (which inhibits the breakdown of IPs). The basal level of IPs accumulation in one set of experiments was about 4% for untransfected Neuro2A-cells, as well as for cells transfected with the wild-type or truncated α_{1D}-AR or with α_{1A}-AR (Table 3A). In another set of experiments, the basal level of IPs accumulation was about 2% for the α_{1A}-, α_{1B}-, and α_{1D}-AR, as well as for the two truncated α_{1D}-AR variants (Table 3B). The equal basal levels of IPs accumulation, within the experimental sets, even within untransfected cells, show that none of the receptors was constitutively active in the Neuro2A cells.

Agonist induced stimulation of IPs accumulation

Dose–response curves for cirazoline or phenylephrine were obtained on plain Neuro2A cells, and on cells stably expressing the wild-type α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors, and the truncated Δ1–17α_{1D}-AR and Δ1–58α_{1D}-AR variants. The stimulated levels of IPs accumulation and the EC_{50} values for cirazoline and phenylephrine, at the different receptors, are reported in Table 3A and B. The α_1-agonist/partial agonist cirazoline stimulated IPs accumulation in cells expressing the α_{1A}- and α_{1D}-AR, as well as the truncated α_{1D} variants, but not in control Neuro2A cells. Also, the full agonist phenylephrine stimulated IPs accumulation in cells expressing the α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors, as well as the truncated α_{1D} variants (Figure 3). Altogether, these results show that all the tested receptors mediate agonist-dependent stimulation of IPs production in the cells. The pEC_{50} values (potencies) for both cirazoline and phenylephrine were higher at Δ1–58α_{1D}-AR compared to, for example, the wild-type α_{1D}-AR (Table 3A and B). This might be explained by the presence of a receptor reserve for the highly expressed Δ1–58α_{1D}-AR, leading to the fact that only a fraction of the receptors needs to be stimulated by the agonist for inducing maximal effect.

Blockade of potential constitutively active receptors with the inverse agonist prazosin

Dose–response curves for prazosin were obtained on cells stably expressing the wild-type α_{1A}- and α_{1D}-adrenoceptors, and the Δ1–17α_{1D}-AR and Δ1–58α_{1D}-AR α_{1D} variants. The results showed that the α_1-antagonist prazosin did not influence the IPs accumulation in the transfected cells; the percent conversion was about 4% both in the presence and in the absence of prazosin ($n=3$). This shows that neither the wild-type α_{1D}-AR nor the truncated variants of the α_{1D}-AR showed any constitutive activity.

Discussion

Most GPCRs have a short N-terminal (at an average about 40 amino acids), which enables the first TM domain to function as a reverse signal anchor targeting sequence (see Andersson *et al*., 2003). However, the α_{1D}-adrenoceptor has a relatively long extracellular N-terminus (95 amino acids), and lacks signal peptide sequence. While the α_{1A}- and α_{1B}-subtypes, which have shorter N-terminal tails, are well expressed in the plasma membrane, the α_{1D}-adrenoceptor protein is poorly expressed in the plasma membrane, and instead seems to be localized mainly intracellularly (McCune *et al*., 2000; Chalothorn *et al*., 2002). Using radioligand ligand binding, it was shown that truncation of the N-terminal tail of α_{1D}-AR increased the density of detectable α_{1D}-adrenoceptors in the membranes, indicating that the N-terminal tail is responsible for the low expression level of binding competent receptors (Pupo *et al*., 2003). The same research group proposed that the α_{1D}-adrenoceptor is not properly translocated to the plasma membrane after its synthesis (Hague *et al*., 2004a).

In the present study, we have investigated by what means the N-terminal tail of the α_{1D}-adrenoceptor is responsible for the low receptor numbers in the plasma membrane. As working tools, we constructed 6 N-terminally truncated variants of the α_{1D}-adrenoceptor, as well as the α_{1D}-adrenoceptor supplemented with a signal peptide sequence. The truncated variants were made in order to investigate if there was a specific function residing in the N-terminal (mediating a decrease in the density of α_{1D}-adrenoceptors detected by radioligand binding), which

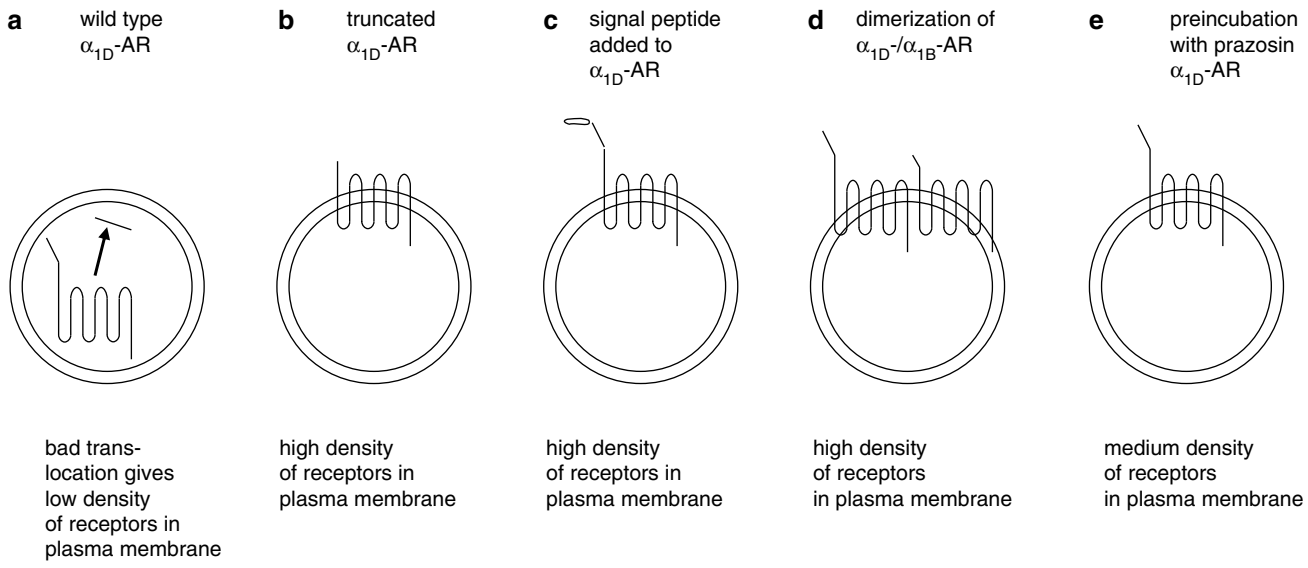

Figure 3 Schematic illustration of α_{1D}-adrenoceptor surface expression in transfected cells. The wt α_{1D}-AR is poorly translocated to the plasma membrane (a). Truncated α_{1D}-AR variant (b) and the α_{1D}-AR supplemented with a cleavable signal peptide (c) are well expressed in the membranes. Coexpression of α_{1D}-AR with the α_{1B}-subtype increases surface expression of α_{1D}-adrenoceptors (d). The well-expressed receptors (b–d) show a six to 10-fold increase in the density of binding sites in the plasma membrane compared to the wt α_{1D}-AR. Incubation of α_{1D}-AR-expressing cells with the α_1-antagonist prazosin induces an increased density of receptors in the plasma membrane (e). The figure is based on results reported in the present study (c), and in Pupo *et al*. (2003) (a and b), Hague *et al*. (2004b) (d), and McCune *et al*. (2000) (e).

Table 1 Density of receptors for the wild-type α_{1D}-adrenoceptor and six gradually shorter trunctated α_{1D}-adrenoceptor variants

Transient expression in Neuro2A cells of wt and truncated α_{1D}-adrenoceptors	A. B_{max} *receptors* (fmol mg^{-1} *protein)*	B. B_{max} *receptors (% compared to wt)*	C. pK_d *[^{3}H]-prazosin*	n
α_{1D}-AR (wt)	18.9±6.6	100	10.14±0.10	3
Δ1–5α_{1D}-AR	10.3±3.3	56±12	10.12±0.13	3
Δ1–17α_{1D}-AR	11.8±6.0	56±10	10.26±0.05	3
Δ1–30α_{1D}-AR	34.7±18.3	156±39	10.40±0.00	3
Δ1S–49α_{1D}-AR	106±75	426±198	10.32±0.06	3
Δ1–58α_{1D}-AR	151±94	642±224	10.32±0.08	3
Δ1–89α_{1D}-AR	52.6±27.2	238±57	10.32±0.02	3

The B_{max} values were determined in Neuro2A cell membranes by [^{3}H]-prazosin binding, following transient transfection of cells with 10 μg of construct DNA per 9 cm Petri dish. Column A shows the amounts of receptors (B_{max}) in membrane preparations. Column B shows the B_{max} in percent of the wt B_{max} values, from experimentally paired plates. Column C shows the pK_d values of [^{3}H]-prazosin for the receptors.

Table 2 Column A shows the amounts of receptors (B_{max}) in membrane preparations from COS-7 and Neuro2A cells after transient transfection with the wt α_{1D}-adrenoceptor (wt), and with the signal peptide-supplemented α_{1D}-adrenoceptor (SP)

Transient expression in COS-7 or Neuro2A cells of wt and signal peptide-supplemented α_{1D}-AR	A. B_{max} *receptors* (fmol mg^{-1} *protein)*	B. B_{max} *receptors (% compared to wt)*	C. pK_d *[^{3}H]-prazosin*	n
α_{1D}-AR (wt) COS-7	69.3±6.4	100	9.93±0.10	4
α_{1D}-AR (SP) COS-7	838±200	1190±220	9.83±0.07	4
α_{1D}-AR (wt) Neuro2A	103±31	100	10.13±0.07	4
α_{1D}-AR (SP) Neuro2A	1120±80	1300±240	9.90±0.09	4

Column B shows B_{max} of the signal peptide-supplemented α_{1D}-AR (SP) in percent of that of the wild type α_{1D}-AR (wt), from experimentally paired plates. Column C shows the pK_d values of [^{3}H]-prazosin for the receptors.

would be cut off at a certain point in the amino-acid sequence. After transient transfection of Neuro2A cells with the α_{1D}-AR constructs, the B_{max} and pK_d values in the membranes were determined with the radioligand [^{3}H]-prazosin. The results showed that the pK_d values of [^{3}H]-prazosin were almost identical for all the α_{1D}-adrenoceptor variants (Table 1), indicating that the binding pocket of the labelled receptors were conformationally intact. However, the B_{max} levels of the α_{1D}-adrenoceptor variants gradually increased as the N-terminal was made shorter, at least in the span between truncation by 17, 30, 49, and 58 amino acids (Table 1). In a previous study, it has also been reported that a 79 amino acids truncated α_{1D}-AR induced a high level of receptor binding sites (Pupo *et al*., 2003). However, our 89 amino acids truncated variant (Δ1–89α_{1D}-AR) showed an intermediate B_{max} level, probably reflecting a somewhat too short N-terminus (seven amino acids) to be well translocated to the plasma membrane. The graded response, that is, the increases in B_{max} values as a result of the serial truncations, seems to exclude that certain amino acids in the N-terminus specifically induce a translocation-interfering activity. Rather, it seems as though the long N-terminal tail nonspecifically interferes with the translocation of

Table 3 IPs accumulation in Neuro2A cells, and in Neuro2A cells stably expressing wt α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors, and two truncated α_{1D}-adrenoceptor variants

(A) Cirazoline-stimulated IPs accumulation

Receptor	*IPs/total I (basal level) (% conversion)*	*IPs/total I (+cirazoline) (% conversion)*	*Stimulation (individual exp.) (fold)*	*pEC_{50} (cirazoline)*	n
α_{1D}-AR (wt)	3.90±0.35	7.11±1.30	1.81±0.23	7.16±0.12	5
Δ1–17α_{1D}-AR	4.22±0.68	9.05±1.16	2.21±0.22	7.18±0.20	5
Δ1–58α_{1D}-AR	4.82±1.67	19.74±3.29	5.22±1.05	7.58±0.09	6
α_{1A}-AR	3.33±0.39	13.68±3.09	4.45±1.09	7.43±0.16	6
Neuro2A cells	4.67±1.10	no effect		N.C.	3

(B) Phenylephrine (PE)-stimulated IPs accumulation

Receptor	*IPs/total I (basal level) (% conversion)*	*IPs/total I (+ PE) (% conversion)*	*Stimulation (individual exp.) (fold)*	*pEC_{50} (PE)*	n
α_{1D}-AR (wt)	2.02±0.19	2.53±0.24	1.25±0.02	6.63±0.12	6
Δ1–17α_{1D}-AR	1.65±0.27	2.82±0.68	1.65±0.16	6.72±0.10	4
Δ1–58α_{1D}-AR	1.43±0.10	5.51±0.72	3.80±0.34	7.43±0.05	6
α_{1A}-AR	2.17±0.30	4.12±0.44	1.94±0.07	5.80±0.08	6
α_{1B}-AR	2.32±0.22	7.38±0.76	3.27±0.36	6.48±0.04	6

The basal-, cirazoline-, and phenylephrine-stimulated percent conversion of [^{3}H]-inositol to [^{3}H]-inositol phosphates, and the fold increase in IPs accumulation induced by the agonists are shown. Also shown are the pEC_{50} values of cirazoline and phenylephrine. In (A) the results with cirazoline, and in (B) the results with phenylephrine as shown. The incubation time with drugs was 50 min.

the receptor to the plasma membrane. The graded response also indicates that the N-terminal tail does not specifically bind to the receptor itself to induce constitutive activity. Such a concept has been described for the thrombin receptor, where an N-terminal stretch of six amino acids in the cleaved N-terminal of the thrombin receptors acts as activating ligand of the receptor (MacFarlane *et al*., 2001). Thus, if the α_{1D}-AR tail bound to the receptor and activated it, we believe that there would be a crucial short amino-acid stretch in the tail, important for binding, activation, and downregulation of the receptor. The model implies that such downregulation would disappear abruptly for those receptor variants for which that stretch had been truncated. However, our results seem to indicate that such a crucial stretch, that is, receptor-activating moiety, does not exist in the α_{1D}-AR N-terminal tail.

As mentioned above, we also constructed an α_{1D}-adrenoceptor with a signal peptide added to its N-terminal, that is, the α_{1D}-AR-SP construct. The idea was to prolong the 95 amino acids long N-terminal with 16 extra amino acids of signal peptide character. In both COS-7 and Neuro2A cells, the addition of the signal peptide increased the levels of detectable α_{1D}-adrenoceptors more than 10-fold (Table 2). This indicates that, after addition of a signal peptide, the long N-terminal tail of the α_{1D}-adrenoceptor does not interfere with proper translocation of the receptor to the plasma membrane. It can be noted that there is a public database available, where one can estimate the probability of a protein being translocated to the plasma membrane (http://www.cbs.dtu.dk/services/SignalP-2.0/). In our case, this gave a hint that the N-terminal of the wild-type α_{1D}-adrenoceptor, including the first TM domain, was not an appropriate signal anchor, while the truncated Δ1–58α_{1D}-AR was predicted to be an excellent signal anchor. Our results seem to indicate that the long N-terminus of the α_{1D}-adrenoceptor interferes with the translocation of the receptor to the plasma membrane, by hindering the first transmembrane helix of the receptor from being an appropriate signal anchor (see Andersson *et al*., 2003; Hague *et al*., 2004a).

It has been suggested that α_{1D}-AR is constitutively active. This was first shown in rat-1 fibroblast cells stably expressing α_{1D}-AR, where the inverse agonist BMY7378 decreased basal $[Ca^{2+}]_i$ (Garcia-Sainz & Torres-Padilla, 1999). It was also suggested that constitutive activity of the α_{1D}-AR continuously downregulates the receptor, since incubation of α_{1D}-expressing cells with the inverse agonist prazosin induced a slight decrease in basal IPs accumulation, as well as a redistribution of immunocytochemically detected α_{1D}-adrenoceptors from intracellular sites to the plasma membrane (McCune *et al*., 2000). However, in HEK 293 and SK-N-MC cells, no constitutive activity of α_{1D}-AR was detected (Theroux *et al*., 1996). In the present study, we established stable expression in Neuro2A cells of the wild-type α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors, and the truncated α_{1D}-adrenoceptors Δ1–17α_{1D}-AR and Δ1–58α_{1D}-AR. These cell lines were then used to investigate putative constitutive activity on IPs production. We measured both the basal level of IPs accumulation, the cirazoline- and phenylephrine-stimulated IPs accumulation, and the effect of prazosin (which potentially might decrease basal IPs accumulation, by blocking constitutive receptor activity). Our working hypothesis was that the wild-type α_{1D}-AR (which shows a low density of [^{3}H]-prazosin binding in the membranes, that is, which seems to be downregulated) would induce a high basal level of IPs accumulation. On the other hand, the truncated variant of the α_{1D}-AR (which shows a high density of [^{3}H]-prazosin binding in the membranes, that is, which does not seem to be downregulated) would induce a low basal level of IPs accumulation. However, we show that both wild-type and N-terminally truncated variants of the α_{1D}-adrenoceptor stimulate IPs accumulation, but *nota bene,* only in the presence of agonist (Table 3A and B). We also show that α_{1D}-AR is not constitutively active, since the inverse agonist prazosin did not decrease IPs accumulation. This seems to indicate that neither

the wild-type α_{1D}-adrenoceptor (which is present at low density in the membranes) nor the truncated α_{1D} variant (which is present at high density in the membranes) is constitutively active in the Neuro2A cells. However, maybe the low density of the wild-type α_{1D}-AR in the membranes made it difficult to observe constitutive activity in the Neuro2A cells.

After transient transfection of Neuro2A cells, the α_{1D}-AR supplemented with signal peptide (α_{1D}-AR-SP) showed a 13-fold increase in [^{3}H]-prazosin binding as compared to the wild-type α_{1D}-AR (Table 2). It seems unlikely that this increased level of detectable binding sites for α_{1D}-AR-SP as compared to the wild-type α_{1D}-AR is a reflection of constitutive activity and downregulation only of the wild-type α_{1D}-AR. Note that α_{1D}-AR-SP, after the presumed cleavage of its signal peptide and once present in the membranes, is supposed to be identical to the wild-type α_{1D}-AR. In future studies, α_{1D}-AR-SP will be an excellent tool for investigating if the α_{1D}-AR is constitutively active, owing to its high expression level in the membranes. At present, we favor the hypothesis that the main reason the wild-type α_{1D}-AR shows low expression of binding sites is that the translocation of the receptor protein to the plasma membrane does not work efficiently (Pupo *et al*., 2003; Hague *et al*., 2004a). Then, the best explanation for the much higher density of receptors observed after transfection with Δ1–58α_{1D}-AR and α_{1D}-AR-SP, compared to wild-type α_{1D}-AR, is that these former receptors are well translocated to the plasma membrane. Whether α_{1D}-AR is constitutively active is still an open question.

It has been shown previously that coexpression of the α_{1D}-adrenoceptor with the α_{1B}-adrenoceptor increased the number of functional α_{1D}-adrenoceptors in the cells (Uberti *et al*., 2003; Hague *et al*., 2004b). This indicates that heterologuos receptor dimerization can mediate an increased translocation of the α_{1D}-adrenoceptor to the plasma membrane. There thus seems to be at least four ways to increase the density of α_{1D}-adrenoceptors in the plasma membrane: truncation of the N-terminal (Pupo *et al*., 2003), addition of a signal peptide (present study), coexpression with α_{1B}-adrenoceptors (Hague *et al*., 2004b), and preincubation with antagonists (McCune *et al*., 2000). In Figure 3, we display a schematic presentation of these mechanisms. Whether the same mechanism of action underlies all of these four increases in the α_{1D}-AR populations is uncertain. A new concept is that GPCRs may have inherent instability and therefore become downregulated, and that antagonists may stabilize receptor conformations that are less prone to downregulation (Prinster *et al*., 2003; Zeng *et al*., 2003). Thus, not only blocking of constitutively active receptors but also stabilization of unstable receptors, could explain the previously observed antagonist-mediated increment in membrane-localized α_{1D}-adrenoceptors (McCune *et al*., 2000; see Garcia-Sainz & Villalobos-Molina, 2004).

It seems like the long N-terminal tail of the α_{1D}-AR is responsible for less efficient translocation of the receptor to the plasma membrane, while this can be overcome by truncation of the tail or by the addition of a signal peptide sequence to the α_{1D}-AR gene. A fundamental question is why nature has provided signal peptides to some GPCRs with long N-terminal tails (i.e. the endothelin B receptor), but not to others (i.e. the α_{1D}-adrenoceptor and the cannabinoid receptor 1). Further studies may show whether the inefficient translocation of α_{1D}-adrenoceptors to the plasma membrane has functional importance, for example, if the α_{1D}-adrenoceptors under physiological circumstances are activated by interactions with some regulatory proteins (see Pupo & Minneman, 2003; Hague *et al*., 2004b). The present study highlights the importance of the N-terminus for the proper expression of binding competent receptors in the plasma membrane.

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