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N-terminal truncation of human α_{1D}-adrenoceptors increases expression of binding sites but not protein

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Abstract

The role of the N-terminus of human α_{1D}-adrenoceptors was examined by deleting the first 79 amino acids (Δ^{1-79}) and epitope-tagging to facilitate immunoprecipitation and detection. Following transfection into HEK293 cells, 6- to 13-fold increases in the density of specific [^{125}I]BE 2254 binding sites were observed for both tagged and untagged $\Delta^{1-79}\alpha_{1D}$- compared to full-length α_{1D}-adrenoceptors, while agonist and antagonist affinities remained unchanged. In contrast, immunoprecipitation of tagged receptors showed that full-length α_{1D}-adrenoceptor protein was at least twice as abundant as $\Delta^{1-79}\alpha_{1D}$-adrenoceptor protein. Photoaffinity labelling with [^{125}I]arylazidoprazosin showed much more intense labelling of tagged $\Delta^{1-79}\alpha_{1D}$- than of full-length α_{1D}-adrenoceptors. Substantial N-linked glycosylation of tagged $\Delta^{1-79}\alpha_{1D}$-adrenoceptors was observed, although full-length α_{1D}-adrenoceptors contain two consensus glycosylation sites but are not glycosylated. These results suggest that N-terminal truncation of α_{1D}-adrenoceptors enhances processing of a binding competent form in HEK293 cells; and show a clear dissociation between abundance of receptor protein and density of receptor binding sites.

Keywords: Noradrenaline; Adrenoceptor; N-terminal; Radioligand binding; Protein expression

1. Introduction

Noradrenaline and adrenaline act through three distinct α_1-adrenoceptor subtypes (α_{1A}, α_{1B}, and α_{1D}) to alter cell growth, differentiation, proliferation, excitability, secretion, and vascular and nonvascular smooth muscle contraction (Zhong and Minneman, 1999a). The α_1-adrenoceptors couple to multiple signaling mechanisms in different cells, including activation of phospholipases C (Minneman, 1988), A_2 (Kanterman et al., 1990; Nishio et al., 1996; Perez et al., 1993; Xing and Insel, 1996) and D (Llahi and Fain, 1992; Balboa and Insel, 1998; Ruan et al., 1998), transglutaminase (G_h) (Nakaoka et al., 1994), mitogen-activated protein kinases (Zhong and Minneman, 1999b), and cation channels (Inoue et al., 2001).

Recently, intense effort has focused on identifying α_1-adrenoceptor subtypes involved in responses to catecholamines in animals and isolated tissues. However, this task has been complicated by the small number of subtype-selective ligands available and the absence of subtype-specific antibodies. Alternatively, heterologous expression of recombinant α_1-adrenoceptors in cell lines has been used to study properties of individual subtypes. Although this approach has been useful in studying human α_{1A}- and α_{1B}-adrenoceptors, study of α_{1D}-adrenoceptors has been more difficult. Human α_{1D}-adrenoceptors are expressed at low densities and show poor coupling efficiency following heterologous expression (Zhong and Minneman, 1999b; Esbenshade et al., 1995; Theroux et al., 1996; Taguchi et al., 1998). The molecular determinants for these low expression levels and poor coupling are unknown.

α_{1D}-Adrenoceptors have an unusually long extracellular N-terminus (95 amino acids) compared to α_{1A}- and α_{1B}-adrenoceptors (25 and 45 amino acids) or to other α_2- and β-adrenoceptors. Although some information is available concerning the roles of intracellular loops and the cytoplasmic C-tail in receptor function, much less is known about the role of the extracellular N-terminus. To study this, we generated an N-truncated human α_{1D}-adrenoceptor, in which the first 79 amino acids were deleted ($\Delta^{1-79}\alpha_{1D}$) and the remaining N-terminus tagged with different epitopes

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to facilitate receptor immunoprecipitation and detection. We show that deletion of the N-terminal 79 amino acids of the α_{1D}-adrenoceptor causes a dramatic increase in receptor binding site density (detected by radioligand binding) without increasing receptor protein expression (detected by Western blot). The implications of these findings in understanding the properties of α_{1D}-adrenoceptors are discussed.

2. Materials and methods

2.1. Constructs

The cDNA for the human α_{1D}-adrenoceptor was cloned in our lab (Esbenshade et al., 1995). $\Delta^{1-79}\alpha_{1D}$ mutants were generated by polymerase chain reaction using specific primers (forward ACATCTAGAGTGAATGGCACGGCGGGTC and reverse CATGATGGCTGGGTACTTGAGTGA), and full-length and N-truncated coding sequences subcloned into the mammalian expression plasmid pDoubleTrouble (pDT), containing N-terminal sequential hexahistidine and FLAG epitopes (Vicentic et al., 2002), or pCytomegalovirus (pCMV) containing an in-frame N-terminal haemagglutinin epitope. Alternatively, full-length and N-truncated coding sequences were subcloned into the expression plasmid RousSarcomaVirus (pRSV) with no epitope tag.

2.2. Cell culture and transfections

Human embryonic kidney 293 (HEK293) cells were propagated in Dulbecco's modified Eagle's Medium with sodium pyruvate supplemented with 10% heat inactivated fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere with 5% CO_2. Confluent plates were subcultured at a ratio of 1:3. Cells were transfected with 50 μg cDNA encoding tagged or non-tagged full-length or truncated α_{1D}-adrenoceptors by calcium phosphate precipitation, and stably transfected cells selected with geneticin (400 μg/ml).

2.3. Radioligand binding and intracellular Ca^{2+} measurements

For radioligand binding, confluent 150 mm plates were washed with phosphate buffered saline (PBS; 20 mM $NaPO_4$, 154 mM NaCl, pH 7.6) and harvested by scraping. Cells were collected by centrifugation, homogenized with a Polytron, centrifuged at 30,000 × *g* for 20 min, and resuspended in 1 × buffer A (25 mM HEPES, 150 mM NaCl, pH 7.4) supplemented with protease inhibitors (1 mM benzamidine, 3 μM pepstatin, 3 μM phenylmethylsulfonylfluoride, 3 μM aprotinin, 3 μM leupeptin, and 5 mM ethylenediamine tetraacetic acid). Radioligand binding sites were measured by saturation analysis of specific binding of the α_1-adrenoceptor antagonist radioligand [^{125}I]BE 2254 (20–800 pM). Nonspecific binding was defined as binding in the presence of 10 μM phentolamine. The pharmacological specificity of radioligand binding sites was determined by displacement of [^{125}I]BE 2254 (50–70 pM) by noradrenaline, prazosin and 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY 7378), and data was analyzed by nonlinear regression (Theroux et al., 1996). Intracellular Ca^{2+} mobilization was measured after preloading with fura-2 acetoxymethylester as described previously (Theroux et al., 1996).

2.4. Immunoprecipitation and Western blots

HEK293 cells expressing Flag- or haemagglutinin-tagged full-length or $\Delta^{1-79}\alpha_{1D}$-adrenoceptors were harvested by scraping and washed by repeated centrifugation and homogenization. Cell lysates containing 2 mg protein were solubilized with 2% *n*-Dodecyl-β-D-maltoside (DβM) for 90 min at 4 °C in 1 × buffer A with protease inhibitors as described above. Samples were centrifuged, the supernatant diluted 10 × with buffer A containing protease inhibitors, and incubated with 100–200 μl of anti-FLAG M2 affinity resin or anti-haemagglutinin affinity resin for 90 min at 4 °C with gentle rotation. Immunoprecipitated material was recovered by centrifugation and washed at least four times with buffer A containing protease inhibitors. After washing, samples were eluted with 100 to 200 μl buffer A containing 400 μg/ml Flag peptide or loading buffer. For deglycosylation, immunoprecipitated samples were treated with peptide *N*-glycosidase F (2.5 U/μl) for 2 h at room temperature. Thirty microliters of each sample was separated by 4–20% SDS polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane and blotted with anti-FLAG M2 antibodies or anti-haemagglutinin antibodies conjugated to horseradish peroxidase (1:600). Proteins were visualized by enhanced chemiluminescence. For comparison purposes, intensities of immunoreactive bands were estimated by densitometry.

2.5. Photoaffinity labelling

Photoaffinity labelling was performed on membranes from HEK293 cells expressing Flag- or haemagglutinin-tagged full-length or N-truncated α_{1D}-adrenoceptors. Membranes were prepared as described above for radioligand binding (1 μg protein/μl), and treated in the dark for 1 h at room temperature with 6 nM [^{125}I]arylazidoprazosin. Nonspecific labelling was determined in the presence of 1 μM unlabelled prazosin. While still in the dark, open tubes were exposed to 6000 μJ/cm^2 ultraviolet light for 3 min using a Stratalinker. Membranes were then washed once with 1 ml of 1 × buffer A with protease inhibitors, centrifuged at 16,000 × *g* for 5 min and the pellet homogenized in 0.5 ml of 2 × buffer A (50 mM HEPES, 300 mM NaCl, pH 7.4) containing protease inhibitors. Membranes were solubilized and immunoprecipitated as described above, and immuno-

precipitates run in parallel on SDS-PAGE. One gel was transferred to nitrocellulose for Western blotting and the parallel gel analyzed for radioactivity in a phosphorimager.

2.6. Materials

Materials were obtained from the following sources: HEK293 cells, ATCC (Manassas, VA); fura-2/acetoxymethyl ester, Calbiochem (La Jolla, CA); (−)-arterenol bitartrate (noradrenaline), Dulbecco's modified Eagle's medium, penicillin, streptomycin, FLAG peptide, anti-FLAG M2 affinity resin, and horseradish peroxidase-conjugated anti-FLAG M2 antibody, Sigma (St. Louis, MO); prazosin, Pfizer (Groton, CT); BMY 7378, RBI (Natick, MA); carrier-free $Na^{125}I$ and enhanced chemiluminescence reagent, Amersham (Chicago, IL); anti-haemagglutinin affinity resin, Roche (Indianapolis, IN) and anti-haemagglutinin polyclonal antibody, Clontech (Palo Alto, CA). Pre-cast Tris–Glycine gels were obtained from Novex (Carlsbad, CA). Radio-iodinated arylazidoprazosin was obtained from New England Nuclear (Boston, MA).

3. Results

3.1. Pharmacological properties of $\Delta^{1-79}\alpha_{1D}$ expressed in HEK293 cells

HEK293 cells were transfected with cDNAs encoding a truncated mutant in which the N-terminal 79 amino acids of human α_{1D}-adrenoceptors were deleted ($\Delta^{1-79}\alpha_{1D}$, Fig. 1A) and selected with geneticin. In membranes from stably transfected cells, the α_1-adrenoceptor antagonist radioligand [^{125}I]BE 2254 labelled a homogeneous population of binding sites with K_d values similar to those observed with full-length α_{1D}-adrenoceptors (Table 1), suggesting that the N-terminus does not control affinity for [^{125}I]BE 2254. However, the density of binding sites (B_{max}) in membranes from $\Delta^{1-79}\alpha_{1D}$ expressing cells was about 7-fold higher than in membranes from cells stably expressing full-length α_{1D}-adrenoceptors (Fig. 1B; Table 1).

The binding properties of $\Delta^{1-79}\alpha_{1D}$ were studied by analysis of displacement of specific [^{125}I]BE 2254 binding by the agonist (−)-noradrenaline, the non-subtype selective antagonist prazosin, and the α_{1D}-selective antagonist BMY 7378. The apparent affinities (pK_i) of these ligands for $\Delta^{1-79}\alpha_{1D}$ were not different from their affinities for full-length α_{1D}-adrenoceptors (Table 1), supporting the conclusion that the N-terminus plays little role in the pharmacological specificity of α_{1D}-adrenoceptors.

3.2. Pharmacological properties of epitope tagged α_{1D}-adrenoceptor constructs

These results suggest that deletion of the N-terminal 79 amino acids from human α_{1D}-adrenoceptors increases

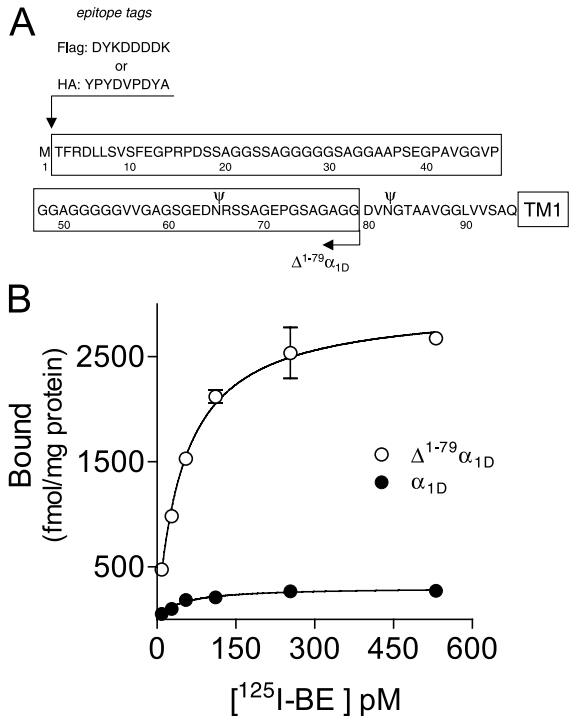

Fig. 1. (A) Amino acid sequence of the extracellular N-terminus of the human α_{1D}-adrenoceptor showing the position at which the epitope tags were introduced (arrow), the residues deleted to generate the $\Delta^{1-79}\alpha_{1D}$ (inside box) and the predicted N-linked glycosylation sites (ψ). (B) Typical experiment representing the saturation of [^{125}I]BE 2254 specific binding to membranes from HEK293 cells stably transfected with full length (●) and $\Delta^{1-79}\alpha_{1D}$-adrenoceptors (○).

either receptor expression or stability. To examine this, we generated constructs in which Flag or haemagglutinin-epitopes were introduced into the N-terminus (Fig. 1A) to facilitate identification of receptor protein by immunoprecipitation and Western blot. Table 1 shows that introduction of these tags did not affect the pharmacological properties of either full-length or $\Delta^{1-79}\alpha_{1D}$-adrenoceptors expressed in HEK293 cells. Neither the K_d of the radioligand [^{125}I]BE 2254, nor the pK_i for noradrenaline, prazosin or BMY 7378 were affected by the presence of epitope tags. Again, binding site densities (B_{max}) were 6- to 13-fold higher with N-truncated constructs than with full-length α_{1D}-adrenoceptors (Table 1).

3.3. Immunoprecipitation and detection of Flag-tagged α_{1D}- and $\Delta^{1-79}\alpha_{1D}$-adrenoceptor protein

Membranes from cells expressing Flag-tagged full-length or $\Delta^{1-79}\alpha_{1D}$-adrenoceptors were solubilized and immunoprecipitated with anti-Flag affinity resin as previously described (Vicentic et al., 2002). Western blots (Fig. 2) showed that full-length Flag-α_{1D}-adrenoceptors migrated as ≅80 kDa proteins consistent with the expected molecular weight of this 572 amino acid protein. However, immunoreactive bands migrating at ≅160 and

Table 1
Binding of the α_1-adrenoceptor antagonist [^{125}I]BE 2254 to membrane preparations from HEK293 cells stably transfected with different constructs

	Saturation of [^{125}I]BE 2254 specific binding		Fold increase in B_{max}	Inhibition of [^{125}I]BE 2254 specific binding (pK_i)		
	K_d (pM)	B_{max} (fmol/mg protein)		Prazosin	BMY 7378	Noradrenaline
α_{1D}	76 ± 32	450 ± 37		9.69 ± 0.09	8.62 ± 0.16	6.69 ± 0.14
$\Delta^{1-79}\alpha_{1D}$	97 ± 21	3170 ± 538[a]	7	9.65 ± 0.03	8.61 ± 0.04	6.77 ± 0.05
Flag-α_{1D}	90 ± 19	266 ± 27		9.70 ± 0.12	8.57 ± 0.10	6.86 ± 0.14
Flag-$\Delta^{1-79}\alpha_{1D}$	79 ± 13	1714 ± 113[a]	6	9.87 ± 0.04	8.85 ± 0.12	6.48 ± 0.10
HA-α_{1D}	102 ± 38	265 ± 35		9.57 ± 0.08	8.48 ± 0.10	6.64 ± 0.09
HA-$\Delta^{1-79}\alpha_{1D}$	115 ± 15	3420 ± 643[a]	13	9.53 ± 0.20	8.68 ± 0.05	6.71 ± 0.10

Saturation analysis of specific binding was used to determine B_{max} and K_d values for [^{125}I]BE 2254. pK_i values for prazosin, BMY 7378 and noradrenaline were determined by competition for specific [^{125}I]BE 2254 binding as described in Materials and methods. Each value represents mean ± S.E.M. of at least three experiments performed in duplicate.

[a] $P < 0.05$ when compared to the respective untagged or tagged α_{1D}-adrenoceptor.

≅240 kDa were also detected, corresponding to dimeric and trimeric forms of the receptor, as previously described (Vicentic et al., 2002). Flag-$\Delta^{1-79}\alpha_{1D}$-adrenoceptors also migrated as multiple bands comprised of monomers at ≅65 kDa, dimers at ≅130 kDa, and trimers at ≅200 kDa. Surprisingly, immunoprecipitation of full-length Flag-α_{1D}-adrenoceptors resulted in stronger specific bands on Western blots than were obtained with Flag-$\Delta^{1-79}\alpha_{1D}$ (Fig. 2A), despite the 6-fold higher binding site density observed with $\Delta^{1-79}\alpha_{1D}$ (Table 1). Quantitation of band intensities revealed that protein expression of N-truncated receptors was about half that of full-length receptors (Fig. 2B). In both cases, about 25% appeared as monomers, while the remaining 75% was composed equally of dimers and trimers (Fig. 2C). Similar results were observed with haemagglutinin-tagged constructs (not shown).

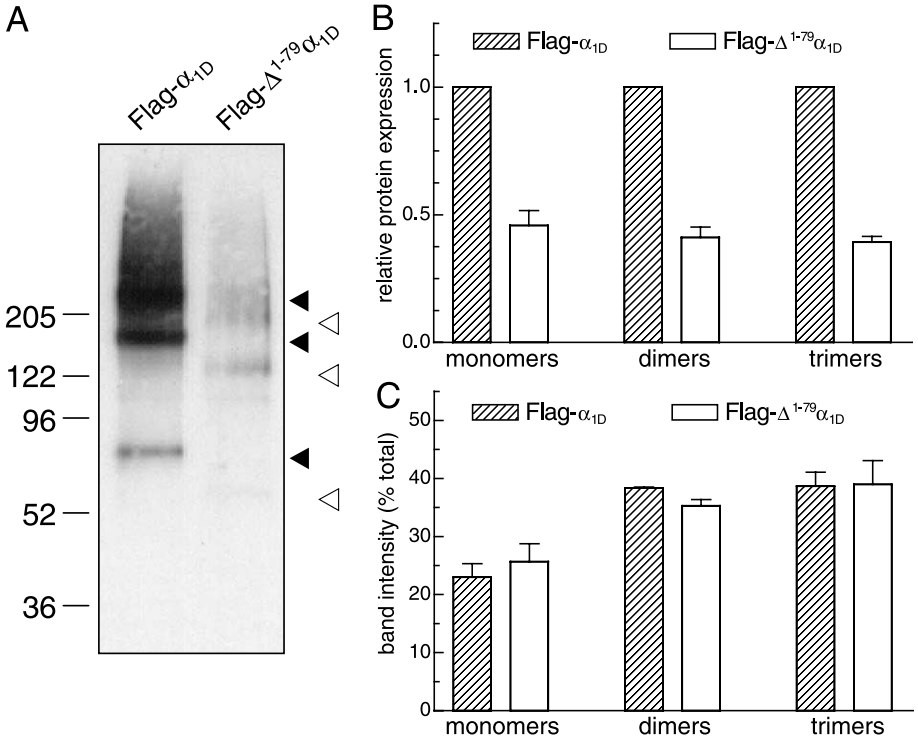

Fig. 2. Representative Western blot of immunoprecipitated Flag-tagged full length and $\Delta^{1-79}\alpha_{1D}$-adrenoceptors. (A) HEK293 cells expressing Flag-α_{1D}- or Flag-$\Delta^{1-79}\alpha_{1D}$-adrenoceptors (2 mg protein each) were solubilized in 2% DβM and immunoprecipitated with anti-Flag affinity resin. Immunoprecipitated material was treated with *N*-glycosidase F, run on SDS-PAGE, transferred, and Western blotted with anti-Flag M2 antibody conjugated to horseradish peroxidase. Arrowheads at the right side of the Western blot indicate monomeric, dimeric and trimeric forms of full length Flag-tagged α_{1D}- (◀) and Flag-$\Delta^{1-79}\alpha_{1D}$-adrenoceptors (◁). (B) Relative expression of Flag-$\Delta^{1-79}\alpha_{1D}$ species. For quantitation, density of each band corresponding to full-length constructs was taken as 1.0, and that of Flag-$\Delta^{1-79}\alpha_{1D}$ as a fraction thereof. (C) Percent distribution of monomers, dimers and trimers, taking as 100% the sum of the densities of all three species. Data in B and C represent mean ± S.E.M. of three independent experiments.

3.4. Photoaffinity labelling of epitope-tagged full-length α_{1D}- and $\Delta^{1-79}\alpha_{1D}$-adrenoceptors with [^{125}I]arylazidoprazosin

To further examine the properties of immunoreactive bands, receptors were photoaffinity-labelled with the α_1-adrenoceptor radioligand [^{125}I]arylazidoprazosin prior to solubilization and immunoprecipitation. Equal amounts of membranes from HEK293 cells stably expressing Flag- or haemagglutinin-tagged full-length or $\Delta^{1-79}\alpha_{1D}$-adrenoceptors were treated in the dark with [^{125}I]arylazidoprazosin in the absence or presence of 1 μM unlabelled prazosin, transilluminated, washed, solubilized and immunoprecipitated (Fig. 3). The immunoprecipitated material was resolved in parallel gels for Western blotting with anti-haemagglutinin or anti-FLAG M2 antibody and radioactivity detected by phosphorimager. An intense radioactive band was easily detected in immunoprecipitates from HEK293 cells expressing Flag- or haemagglutinin-tagged $\Delta^{1-79}\alpha_{1D}$ while very little photoaffinity labelling of Flag- or haemagglutinin-tagged full-length α_{1D}-adrenoceptors was detected (Fig. 3). Essentially all photoaffinity labellings were blocked by the presence of 1 μM unlabelled prazosin. Previous experiments have shown that detection of photoaffinity-labelled full-length Flag-α_{1D}-adrenoceptors requires long exposure times and large amounts of protein (Vicentic et al., 2002), consistent with the low expression of receptor binding sites. Although bands corresponding to monomers, dimers and trimers of the Flag- or haemagglutinin-tagged $\Delta^{1-79}\alpha_{1D}$ were present in comparable intensities in Western blots (see Fig. 2; data not shown), only a single major band corresponding to receptor monomers and an extremely weak band corresponding to dimers were detected in the phosphorimager. A slightly larger species than the monomer was observed with both Flag- and haemagglutinin-tagged $\Delta^{1-79}\alpha_{1D}$, which may represent partially deglycosylated $\Delta^{1-79}\alpha_{1D}$ receptors (see below). This suggests that the primary species binding [^{125}I]arylazidoprazosin is the monomeric $\Delta^{1-79}\alpha_{1D}$, similar to previous observations with all three full-length α_1-adrenoceptor subtypes (Vicentic et al., 2002).

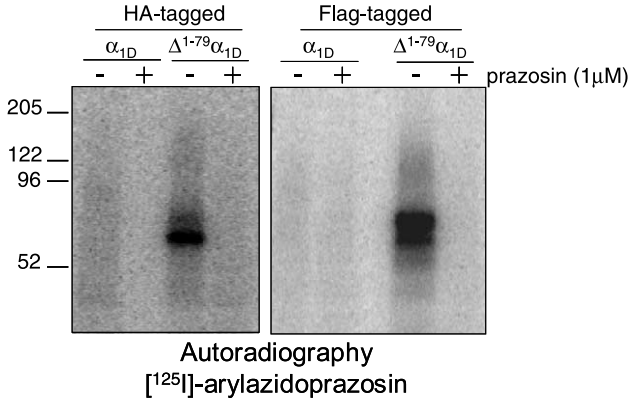

Fig. 3. Representative autoradiography of immunoprecipitated Flag- and haemagglutinin (HA)-tagged full-length α_{1D}- and $\Delta^{1-79}\alpha_{1D}$-adrenoceptors photoaffinity-labelled with [^{125}I]arylazidoprazosin. HEK293 cells expressing each construct (2 mg protein) were photoaffinity labelled in the presence (+) or absence (−) of 1 μM-unlabelled prazosin, washed, solubilized in 2% DβM, and immunoprecipitated with anti-HA (left) or anti-Flag (right) affinity resin. Immunoprecipitated material was treated with *N*-glycosidase F, resolved by SDS-PAGE, and radioactivity detected in a phosphorimager.

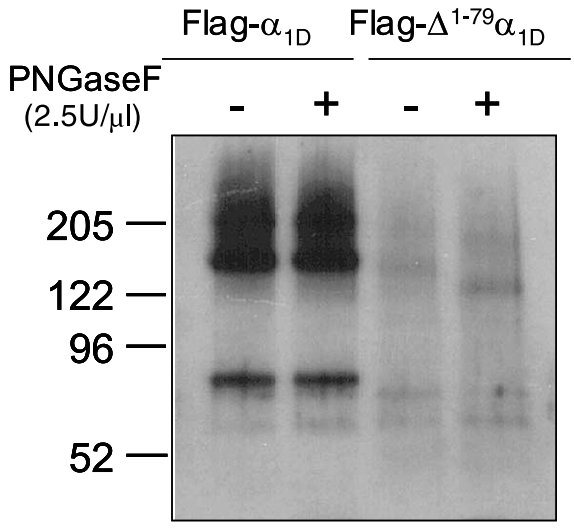

Fig. 4. Western Blot of immunoprecipitated Flag-tagged full length and $\Delta^{1-79}\alpha_{1D}$-adrenoceptors before and after treatment with *N*-glyosidase F. Membranes from HEK293 cells expressing Flag-α_{1D}- or Flag-$\Delta^{1-79}\alpha_{1D}$ (2 mg protein) were solubilized in 2% DβM and immunoprecipitated with anti-Flag affinity resin. Immunoprecipitated material was divided into two aliquots and treated with or without *N*-glyosidase F for 2 h at room temperature and Western blotted with anti-Flag horseradish peroxidase conjugated antibodies. The experiment shown is representative of two other experiments with similar results.

3.5. Glycosylation state of Flag-tagged full-length and $\Delta^{1-79}\alpha_{1D}$-adrenoceptors

The residues Asn^{65} and Asn^{82} in the N-terminus of α_{1D}-adrenoceptors are consensus glycosylation sites (Asn, any amino acid, Ser/Thr, Fig. 1A). However, previous work suggests that full-length Flag-α_{1D}-adrenoceptors are not significantly glycosylated in HEK293 cells, while Flag-α_{1A}- and Flag-α_{1B}-adrenoceptors show substantial N-linked glycosylation (Vicentic et al., 2002). Fig. 4 also shows that treatment of immunoprecipitated full-length Flag-α_{1D}-adrenoceptors with *N*-glycosidase F did not change the migration of this protein in SDS-PAGE. On the other hand, the migration of immunoprecipitated Flag-$\Delta^{1-79}\alpha_{1D}$ was significantly affected by treatment with *N*-glycosidase F (Fig. 4), showing that this protein is subject to N-linked glycosylation in HEK293 cells. Note that the Western blot in Fig. 4 shows this effect most strongly for dimers and trimers of Flag-$\Delta^{1-79}\alpha_{1D}$ because a short exposure time was chosen for clarity. However, clear shifts in migration of the bands corresponding to monomers of Flag-$\Delta^{1-79}\alpha_{1D}$ after treatment with *N*-glycosidase F are visible in films exposed for longer periods (not shown).

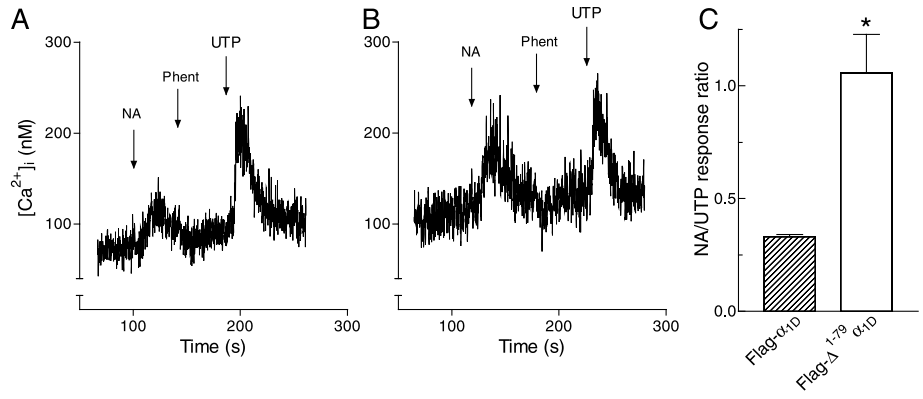

Fig. 5. Effects of noradrenaline (NA, 100 μM) phentolamine (Phent, 10 μM) and UTP (1 mM) on intracellular Ca^{2+} concentration in HEK293 cells transfected with cDNAs encoding Flag-tagged full-length α_{1D}- (A) or $\Delta^{1-79}\alpha_{1D}$-adrenoceptors (B). Stably transfected cells were loaded with the calcium fluorescent indicator fura-2-acetoxymethylester. For comparison purposes, the graph in C shows the ratio between responses to NA and UTP shown in A and B (mean ± S.E.M. of three experiments, *different from the ratio found in cells transfected with cDNAs encoding Flag-tagged α_{1D}-adrenoceptors, $P < 0.05$). Similar results were obtained with untagged or haemagglutanin-tagged receptors (not shown).

3.6. Increases in intracellular [Ca^{2+}] induced by noradrenaline in HEK293 cells expressing Flag-tagged full-length or $\Delta^{1-79}\alpha_{1D}$-adrenoceptors

To determine whether truncation of the N-terminus also resulted in increased functional responses, we examined the effect of noradrenaline on mobilization of intracellular Ca^{2+} in HEK293 cells expressing Flag-tagged full-length or $\Delta^{1-79}\alpha_{1D}$-adrenoceptors. The effects of noradrenaline were normalized against those of UTP, which acts through endogenous P2Y purinoceptors to also increase Ca^{2+}. Consistent with the higher receptor density, the effect of noradrenaline on intracellular Ca^{2+} in cells expressing Flag-$\Delta^{1-79}\alpha_{1D}$ was not different from that obtained with UTP, while in cells expressing full-length Flag-α_{1D}-adrenoceptors the response to noradrenaline corresponded to only 35% of the response to UTP (Fig. 5). Similar differences were observed between the effects of noradrenaline in cells transfected with untagged or haemagglutinin-tagged constructs (data not shown), suggesting that these differences are not due to the N-terminal tag.

4. Discussion

This study examined the role of the extracellular N-terminus in expression, function and ligand binding properties of human α_{1D}-adrenoceptors. Because the 95 amino acid N-terminus is substantially larger than that of other adrenoceptor subtypes, the effect of N-terminal truncation was examined. Epitope tags were used to facilitate immunoprecipitation and detection of receptor protein, and the biochemical, pharmacological and functional properties of tagged and untagged constructs were compared following expression in HEK293 cells.

Expression of recombinant α_{1D}-adrenoceptors results in a much lower receptor density than does expression of α_{1A}- or α_{1B}-adrenoceptors under similar conditions (Esbenshade et al., 1995; Theroux et al., 1996; Taguchi et al., 1998; Vicentic et al., 2002). We found that the B_{max} for the radioligand [^{125}I]BE 2254 in HEK293 cells expressing full-length human α_{1D}-adrenoceptors was relatively low (≅450 fmol/mg protein), consistent with previous results. Remarkably, truncation of the N-terminal 79 amino acids caused a 6- to 13-fold increase in receptor binding sites. This was caused by an increase in B_{max} with no effect on K_d for radioligand, demonstrating that it is due to increased expression of receptor binding sites and not changes in affinity. Also, the affinities of noradrenaline, prazosin, and BMY 7378 in competing for specific [^{125}I]BE 2254 binding were the same for full-length and $\Delta^{1-79}\alpha_{1D}$-adrenoceptors, suggesting that the additional binding sites resulting from N-terminal truncation show the same pharmacological specificity.

Subtype-specific antibodies for α_1-adrenoceptor subtypes are not yet available, so studies of receptor proteins must rely on epitope tags. As reported previously (Hirasawa et al., 1997), we found that Flag or haemagglutinin epitopes fused to the N-terminus of full-length α_{1D}- or $\Delta^{1-79}\alpha_{1D}$-adrenoceptors had no discernible effect on phenotype. Tagged and untagged receptors showed similar expression, pharmacological specificity, and mobilization of intracellular Ca^{2+}, allowing us to use these N-terminal tags to study the molecular properties of the recombinant proteins.

The higher B_{max} observed with $\Delta^{1-79}\alpha_{1D}$ suggested that N-terminal truncation either increases receptor expression or reduces its degradation. However, Western blots of immunoprecipitated receptors suggest that these are not satisfactory explanations. In fact, full-length α_{1D}-adrenoceptor protein expression was actually higher than that observed with the $\Delta^{1-79}\alpha_{1D}$ construct, despite the 6- to 12-fold lower density of radioligand binding sites. This suggests that only part of the protein detected in Western blots of full-length α_{1D}-adrenoceptors may reflect *binding competent* receptors, while a much larger fraction of the truncated receptor can bind radioligand. This does not appear to be due to

incomplete synthesis or partial degradation of full-length receptors, since the protein appears primarily as discrete, specific bands of expected sizes.

Radioligand binding sites are often assumed to be equivalent to receptor protein, and the fact that different conclusions about expression levels of α_{1D}-adrenoceptors are obtained from radioligand binding and Western blots is surprising. The discrepancy is not due to technical artefacts related to protein measurement, since full-length and N-truncated receptors had identical tags, were immunoprecipitated and detected with the same antibodies, and similar results were obtained using two different epitopes. Although expression of untagged receptor protein could not be measured, N-terminal truncation caused similar large increases in binding site density with untagged α_{1D}-adrenoceptors. These results suggest that $\Delta^{1-79}\alpha_{1D}$ may be more effectively "processed" than full-length α_{1D}-adrenoceptors in HEK293 cells, although neither protein appears to be incompletely synthesized or partially degraded. Thus, additional factors may be required for formation of receptor binding pockets other than synthesis of receptor protein. It is unlikely that the 95 amino acid N-terminus influences formation of receptor binding sites by a bulk effect, since G protein coupled receptors such as metabotropic glutamate (Ciruela et al., 2001), calcium sensing (Chang et al., 2001) and $GABA_B$ (Ng et al., 1999) receptors with very long (>400 amino acids) N-termini are well expressed in HEK293 cells.

Several recent reports have suggested that α_{1D}-adrenoceptors may be primarily intracellular. Recent studies on localization of α_1-adrenoceptor subtypes conjugated with green fluorescent protein in HEK293 cells suggest that α_{1D}-adrenoceptors are mainly cytosolic and poorly coupled to mitogen activated protein kinase activation (Chalothorn et al., 2002). This raises the possibility that this receptor subtype cannot be properly folded and translocated to the cell membrane. It will be interesting to determine the cellular localization of the N-truncated α_{1D}-adrenoceptor using similar fusion proteins.

Attempts to photoaffinity label α_{1D}-adrenoceptors with [^{125}I]arylazidoprazosin have yielded conflicting results. When the rat α_{1D}-adrenoceptor was first cloned, it was found to be resistant to photoaffinity labelling by this radioligand (Perez et al., 1991). More recent studies however, have reported successful labelling of α_{1D}-adrenoceptors with [^{125}I]arylazidoprazosin (Garcia-Sainz et al., 2001; Vicentic et al., 2002). Our results also suggest that α_{1D}-adrenoceptors are poorly labelled by [^{125}I]arylazidoprazosin, but that this may be due to a relative inability of the full-length protein to form binding sites. Labelling of equal amounts of membrane protein resulted in very strong signals for $\Delta^{1-79}\alpha_{1D}$ but little or no detectable signal for full-length α_{1D}-adrenoceptors, further supporting dissociation between expression of receptor protein and binding site density. It should be noted that use of larger amounts of material results in photoaffinity labelling of full-length α_{1D}-adrenoceptors (Vicentic et al., 2002).

Full-length α_{1D}-adrenoceptors exist as both monomers and SDS-resistant dimers and trimers, as reported previously for all three α_1-adrenoceptor subtypes (Vicentic et al., 2002). In fact, dimers and trimers of α_{1D}-adrenoceptors are more abundant than monomers, comprising more than 70% of total immunoreactivity, and a similar pattern was observed with $\Delta^{1-79}\alpha_{1D}$. However, only monomeric bands were photoaffinity-labelled by [^{125}I]arylazidoprazosin, raising the possibility that monomers are the only pharmacologically active species. Although the large increase in binding sites caused by N-terminal truncation greatly increased photoaffinity labelling of monomeric $\Delta^{1-79}\alpha_{1D}$, it did not change receptor oligomerization or result in photoaffinity labelling of higher molecular weight species. This suggests the N-terminus is not involved in oligomerization, but strongly influences the ability of the monomeric α_{1D}-adrenoceptor proteins to bind radioligand.

We found that the full-length α_{1D}-adrenoceptor does not undergo significant N-linked glycosylation in HEK293 cells, as reported previously (Vicentic et al., 2002), despite the presence of two consensus sites in its N-terminus. α_{1D}-Adrenoceptors are also not glycosylated when expressed in PC12 cells (unpublished data), suggesting this may be a common property of these receptors. Glycosylation has been used as an index of translocation of the N-terminus of the endothelin B receptor across the endoplasmic reticulum membrane during posttranslational modification (Kochl et al., 2002), and the lack of glycosylation supports the idea that α_{1D}-adrenoceptors are not properly processed in HEK293 cells. Surprisingly, we found that $\Delta^{1-79}\alpha_{1D}$ showed substantial glycosylation in HEK293 cells, despite elimination of one of two consensus glycosylation sites. This supports the idea that $\Delta^{1-79}\alpha_{1D}$ and full-length α_{1D}-adrenoceptors are differentially processed in these cells. Although the coupling efficiency of the N-truncated α_{1D} to release of intracellular $[Ca]_i$ is increased, it is still lower than $[Ca]_i$ responses observed in cells with similar expression of α_{1A} or α_{1B} adrenoceptors (not shown). In fact, the relationship between glycosylation and formation of α_{1D}-adrenoceptor binding sites is unclear, since glycosylation is not critical for expression and processing of most other G protein coupled receptors. It has been shown that glycosylation-deficient mutants of hamster α_{1B}-adrenoceptors are expressed at binding densities similar to those of the wild type, and are photoaffinity-labelled by [^{125}I]arylazidoprazosin (Bjorklof et al., 2002).

These studies show that deletion of the N-terminal 79 amino acids from human α_{1D}-adrenoceptors greatly increases expression of receptor binding sites but not receptor protein. Both epitope-tagged wild type and N-terminally truncated receptors appear to be synthesized to their full length, neither appears to be proteolytically degraded, and both show similar proportions of monomers and SDS-resistant dimers and trimers. In both cases, however, only monomers are photoaffinity-labelled by [^{125}I]arylazidoprazosin, suggesting that this may be the only

pharmacologically active species. Finally, N-terminal truncation resulted in a surprising *increase* in N-linked glycosylation, despite the loss of one consensus glycosylation site, raising the possibility of more efficient processing of the N-truncated construct. The mechanisms by which the N-terminus influences formation of α_{1D}-adrenoceptor binding sites are currently being examined.

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