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Mutations of Tyr^{326} in the β_2-adrenoceptor disrupt multiple receptor functions

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Abstract

A tyrosine residue at the cytoplasmic end of the seventh transmembrane helix is conserved in many G-protein-coupled receptors. In the human β_2-adrenoceptor, this tyrosine (Tyr^{326}) has been proposed to be a specific determinant for agonist-induced receptor sequestration. In order to probe its contribution to the sequestration process we have replaced this tyrosine by alanine (Y326A) or phenylalanine (Y326F). Wild-type and mutant receptors were stably expressed in Chinese hamster ovary cells. Agonist-induced sequestration was essentially abolished in Y326A receptors and only slightly reduced in Y326F receptors. However, cells expressing Y326A receptors displayed a high percentage of internal receptors under basal conditions while cells expressing wild-type receptors did not. In addition, high-affinity agonist binding and the ability to activate adenylyl cyclase were markedly reduced in Y326A receptors and slightly reduced in Y326F receptors. We conclude that Tyr^{326} is required for the functional integrity of the β_2-adrenoceptor and that it may be involved in multiple agonist-induced effects.

Keywords: β_2-Adrenoceptor; Adenylyl cyclase; Receptor sequestration; CHO (Chinese hamster ovary) cell

1. Introduction

Stimulation of β-adrenoceptors results not only in the generation of the second messenger, cAMP, but also in the initiation of a series of adaptive processes which alter the coupling, the expression and the subcellular distribution of these receptors (Levitzki, 1988; Hausdorff et al., 1990; Lohse, 1993). Specific structural domains have been assigned to several of these properties of receptors. These include the identification of the ligand binding domain, which is formed by the transmembrane helices, and of the G-protein-coupling domain located in membrane-associated segments of the second and third intracellular loops and the C-terminus (Dohlman et al., 1991). Among the diverse agonist-induced alterations, the sequestration of receptors to an as yet ill-defined intracellular compartment is one of the most intriguing processes. No structural and functional determinants required for this process have yet been identified.

Sequestered receptors are translocated to an intracellular compartment based on several lines of evidence: (1) they become inaccessible to hydrophilic ligands but remain accessible for hydrophobic ligands (Staehelin and Simons, 1982); (2) upon cell fractionation and sucrose density centrifugation they are recovered in a fraction lighter than the plasma membrane fraction (Harden et al., 1980); and (3) in some (but not all) immunofluorescence studies agonist treatment results in the appearance of the receptors in intracellular aggregates which might represent endosomes (Raposo et al., 1989; Wang et al., 1989; Von Zastrow and Kobilka, 1992), and upon agonist removal the receptors reappear at the cell surface. Receptor sequestration has been discussed in the context of at least three possible functions: sequestration might contribute to receptor *de*sensitization (Cheung et al., 1989; Hertel et al., 1990), it might on the contrary be involved in receptor *re*sensitization (Yu et al., 1993; Pippig et al., 1995), and it might be a first step for receptor degradation in lysosomes (Von Zastrow and Kobilka, 1992).

The receptor domains which might be involved in receptor sequestration are still unknown. Strader et al. (1987) and Bouvier et al. (1988) showed that the C-terminus of the β_2-adrenoceptor, which is the target for phosphorylation by β-adrenergic receptor kinase and subsequent de-

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sensitization, is not required for receptor sequestration. These data were confirmed by studies showing that receptor phosphorylation and sequestration were independent events (Hausdorff et al., 1989; Lohse et al., 1990). In contrast to these findings, Tsuga et al. (1994) proposed that for the muscarinic m2 receptor receptor phosphorylation might be required for or at least facilitate the sequestration process. However, very recently Pals-Rylaarsdam et al. (1995) showed for the same muscarinic m2 receptor that sequestration could occur without receptor phosphorylation. Cheung et al. (1989) proposed that the regions involved in β_2-adrenoceptor sequestration might be the same ones which are required for G-protein coupling. This was challenged in a later report by Cheung et al. (1990) and also by Campbell et al. (1991) who generated a series of β_2-adrenoceptor mutants with impaired coupling but normal sequestration properties.

More recently, Barak et al. (1994) proposed that a highly conserved tyrosine residue thought to be localised at the cytoplasmic end of the seventh transmembrane α-helix is required for agonist-mediated sequestration of β_2-adrenoceptor. They reported that mutation of this Tyr^{326} to alanine resulted in a receptor with normal coupling properties but completely abolished sequestration. Interestingly, overexpression of β-adrenergic receptor kinase appeared to 'rescue' this reduced sequestration behaviour, suggesting, as discussed above, a possible link between receptor phosphorylation and sequestration (Ferguson et al., 1995).

This tyrosine is contained in a conserved motif NPxxY (**NPLIY** in the case of the β_2-adrenoceptor) which is reminiscent of the internalization motif NPxY contained in the low-density lipoprotein receptor (Chen et al., 1990). In the latter case, tyrosine can be substituted by phenylalanine without loss of sequestration, whereas other mutations cause impaired sequestration, suggesting that the aromatic side-chain rather than tyrosine phosphorylation is important. The present study was undertaken in order to investigate the possibility for similar structural requirements in the case of Tyr^{326} in the β_2-adrenoceptor and in order to assess the contribution of this amino acid to agonist-induced receptor sequestration.

2. Materials and methods

2.1. Generation and expression of wild-type and mutant β_2-adrenoceptors

Oligonucleotide-directed mutagenesis as described by Kunkel (1985) was used to introduce nucleotide substitutions into the cDNA of the human β_2-adrenoceptor (Kobilka et al., 1987) in order to change the Tyr^{326} to Ala (Y326A mutant) or to Phe (Y326F mutant). The identities of the mutations were confirmed by automated sequencing on an ABI sequencer.

Wild-type and mutant receptor coding sequences were cloned into the pBC-CMV-SK expression plasmid (Lohse, 1992). The plasmids were transfected into Chinese hamster ovary (CHO) cells together with the selection plasmid pSV2-neo by coprecipitation with calcium phosphate. Transfected clones were selected in the presence of 1 mg/ml geneticin (G418, Boehringer Mannheim). Colonies originating from single cells were subcloned and evaluated for receptor expression using ^{125}I-cyanopindolol (Amersham) binding. Permanent lines of stably transfected cells were mantained in monolayer culture following clonal selection in Dulbecco's modified Eagle's medium (DMEM F-12, GIBCO) supplemented with 10% fetal calf serum (Pan Systems), 2 mM glutamine (GIBCO), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO) and 0.5 mg/ml of G418 in a 7.5% CO_2 incubator at 37°C. The cells were maintained in serum-free DMEM overnight prior to all the experiments. Several clones expressing the wild-type and mutant receptors were isolated and characterised.

2.2. Preparation of crude cell membranes

Cells were washed three times with ice-cold phosphate-buffered saline, scraped in 24 ml of ice-cold lysis buffer (5 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.4), homogenised with an UltraTurrax for 30 s at full speed and centrifuged at $1000 \times g$ for 10 min (4°C). The supernatants were then centrifuged at $50000 \times g$ for 15 min (4°C), and the resulting pellets were washed once with incubation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EGTA). The final pellets were resuspended in an appropriate volume of incubation buffer to give a final protein concentration of 60 μg/ml. Protein content was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

2.3. Radioligand binding assays

For saturation assays, 0.2 ml of fresh membranes were incubated in duplicate with eight different concentrations of ^{125}I-cyanopindolol (5–400 pM) for 2 h at 37°C. Non-specific binding was determined in the presence of 10 μM (−)-propranolol (Sigma). For inhibition assays, the same volume of membranes was incubated with 80 pM ^{125}I-cyanopindolol and various concentrations of (−)-isoprenaline (Sigma) (10^{-12}–10^{-3} M; 19 concentrations) in the absence or presence of 100 μM Gpp(NH)p (Sigma). The incubations were terminated by dilution of the samples with ice-cold 50 mM Tris-HCl, pH 7.4, filtration through Whatman GF/C filters and washing with the same buffer.

2.4. Receptor sequestration assays

CHO cells were incubated in serum-free DMEM medium with or without 10 μM (−)-isoprenaline for 20

min at 37°C. After the incubation, the cells were placed on ice, washed three times with ice-cold DMEM and resuspended in an appropriate volume of ice-cold DMEM for the binding assay. The percentage of sequestered receptors was determined as previously described (Lohse et al., 1990). In brief, the receptor concentration was measured in 0.2 ml aliquots of whole cells by binding of 35 pM ^{125}I-cyanopindolol at 16°C during 4 h. Unspecific binding was determined by adding 10 μM (−)-propranolol. Internalised receptors were quantified by competition with 0.3 μM CGP 12177 (Ciba Geigy).

2.5. Adenylyl cyclase assays

For determination of adenylyl cyclase activity, crude membranes were prepared, and the activity was measured as described earlier (Pippig et al., 1993). 50 μg of protein were incubated for 30 min at 37°C in a final volume of 100 μl containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 μM cAMP, 50 μM GTP, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase, 1 mg/ml bovine serum albumin, 100 μM [α-^{32}P]ATP (0.2 μCi/tube, NEN/DuPont) and various concentrations of the agonist (−)-isoprenaline (10^{-9}–10^{-4} M, 8 concentrations) or of 10 mM NaF. The resulting [^{32}P]cAMP was purified by precipitation of ATP and chromatography on alumina.

2.6. Data analysis

Determination of ligand binding parameters (K_D, dissociation constant and B_{max}, maximum density of binding sites) and inhibition parameters (K_{iH} and K_{iL}, dissociation constants of the high- (H) and low-affinity (L) binding sites for the agonist isoprenaline, and R_H and R_L, the percentages of each affinity state of the receptors) as well as the fitting of data to the appropriate binding model were performed by computer-assisted non-linear regression using the LIGAND programs (Munson and Rodbard, 1980; McPherson, 1985).

Adenylyl cyclase activity was normalised to the protein content and concentration-response curves were analysed as described earlier (Lohse et al., 1986) by curve-fitting to the equation:

$$E = E_o + E_{max} \cdot A^n/(EC_{50}^n + A^n) \quad (1)$$

with E denoting the effect, E_o the basal activity, E_{max} the maximum effect, A the agonist concentration, EC_{50} the concentration of agonist that produces 50% of the E_{max}, and n a slope factor which was not significantly different from 1 in any of the experiments presented here.

Simultaneous fitting of the concentration-response curves for wild-type and mutant receptors was used to determine the coupling efficiency. This coupling efficiency was calculated as the transducer ratio τ (Black et al., 1985) using the following algorithm:

$$E = E_o + E_m \cdot (\tau * A)/((K_A + A) + \tau * A) \quad (2)$$

with E denoting the effect, E_o the basal effect, E_m the maximum possible effect (which was shared between all curves), A the agonist concentration, K_A the dissociation constant of the agonist-receptor complex (determined in binding experiments). τ is a parameter describing the signal transduction efficacy of the system, and is estimated individually for each curve. The details of this quantitation procedure have been described earlier (Lohse, 1990).

2.7. Sequence and structure analysis

The UWGCG sequence analysis package was used to search the NRL3D database (release 20.00) for structures containing NPxxY sequence motifs. Secondary structure of these motifs was determined with DSSP (Kabsch and Sander, 1983) from the coordinates deposited in the Brookhaven Protein Database.

3. Results

3.1. Receptor expression and binding properties

Human wild-type β_2-adrenoceptor and the mutants produced by replacement of Tyr^{326} in the seventh transmembrane sequence NPLIY by Ala (Y326A) or Phe (Y326F) were transfected and stably expressed in CHO cells. Screening of the expression level in the different resulting clones was performed by binding experiments with saturating concentrations of ^{125}I-cyanopindolol (400 pM), and three clones with similar expression levels were selected. Further complete saturation experiments in membranes from these cells with ^{125}I-cyanopindolol showed that the density of β_2-adrenoceptors was comparable in all three cell lines (Table 1). The affinities of both mutant receptors for the antagonist radioligand ^{125}I-cyanopindolol was not different from the corresponding value of the wild-type receptors (Table 1).

3.2. Receptor sequestration

Incubation of cells expressing the wild-type β_2-adrenoceptor with 10 μM (−)-isoprenaline for 20 min promoted the sequestration of 10.8% of cell surface receptors

Table 1
^{125}I-Cyanopindolol binding to membranes from CHO cells expressing wild-type or mutant human β_2-adrenoceptors

Receptor	K_D (pM)	B_{max} (fmol/mg protein)
Wild type	40.9 ± 1.8	293 ± 24
Y326A	40.1 ± 4.2	202 ± 17
Y326F	45.5 ± 6.6	268 ± 27

Binding parameters were determined from complete saturation experiments (5–400 pM) using the non-linear regression program LIGAND. Each value represents the mean ± S.E.M. of 6 experiments.

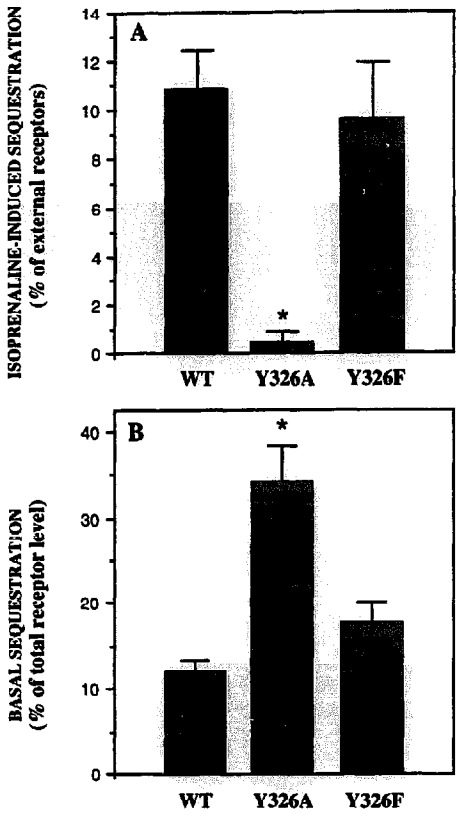

Fig. 1. Sequestration of wild-type and mutant β_2-adrenoceptor clones. A: Stably transfected CHO cells were incubated with 10 μM (−)-isoprenaline for 20 min at 37°C. Agonist-stimulated receptor sequestration was calculated as the percentage of cell surface receptors that were internalised after incubation with (−)-isoprenaline. B: The basal level of sequestered receptors represents the percentage of internal receptors detected without exposure to (−)-isoprenaline (fraction of specific ^{125}I-cyanopindolol binding displaced by (−)-propranolol but not by CGP 12177). The bars represent mean values ± S.E.M. derived from 13–15 independent experiments. * $P < 0.001$ when compared with cells expressing wild-type β_2-adrenoceptor (ANOVA followed by Scheffé's test).

(Fig. 1A). The same experiments performed with Y326A mutant cells showed the complete absence of sequestration in receptors containing this point mutation. In contrast, in the more conservative Y326F mutant the ability to sequester was almost completely maintained (Fig. 1A). The absolute extent of receptor sequestration was lower than in earlier studies by ourselves (Lohse et al., 1990) and others (Barak et al., 1994). This is due to the relatively higher concentration of the competitor CGP 12177 (0.3 μM) and lower concentration of ^{125}I-cyanopindolol (35 pM) which we used in the present study in order to minimise binding to cell surface receptors when determining sequestered receptors.

In non-stimulated cells, the basal level of internal receptors (12 ± 1.3% for wild-type receptors) showed a significant 2.8 ± 0.6-fold increase in the case of the Y326A mutant. In Y326F mutant cells, the basal level of internal receptors was slightly higher than for the wild-type receptors (1.5 ± 0.2-fold vs. wild-type receptors). These data suggest that not only sequestration but possibly also incorporation into the plasma membrane might be affected by mutations of Tyr^{326} in the β_2-adrenoceptor.

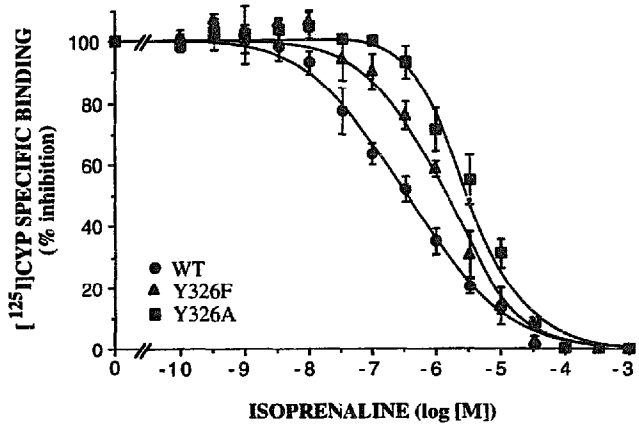

Fig. 2. Inhibition of ^{125}I-cyanopindolol binding by (−)-isoprenaline in membranes from CHO cells expressing wild-type or mutant β_2-adrenoceptor. Analysis of the experiments by non-linear curve-fitting gave the binding parameters shown in Table 2. Note that a two-site model gave significantly better results than a one-site model for wild-type receptors ($P < 10^{-6}$ by F-test) and for Y326F mutant receptors ($P < 0.02$), but not for Y326A mutant receptors ($P = 0.78$). Each data set represents mean values ± S.E.M. derived from 3 independent experiments with duplicate samples. The solid lines represent the computer-assisted curve-fitting of experimental data to the appropiate binding model.

3.3. Agonist binding

In order to test whether the decreased ability of the mutant receptors to undergo agonist-induced sequestration corresponds to a decreased ability to bind agonist ligands and/or to interact with G-proteins, competition binding experiments with ^{125}I-cyanopindolol were performed using (−)-isoprenaline as agonist competitor.

The competition experiments showed biphasic curves for wild-type and Y326F mutant receptors (Fig. 2). Analysis by non-linear curve-fitting assuming two types of binding sites showed a reduction of high-affinity sites in the

Table 2
Inhibition of ^{125}I-cyanopindolol binding by (−)-isoprenaline in membranes from CHO cells expressing wild-type or mutant human β_2-adrenoceptors

Receptor	K_{iH} (nM)	R_H (%)	K_{iL} (nM)	R_L (%)
Wild type	17 ± 7	51 ± 7	681 ± 210	49 ± 8
Y326A	–	–	1101 ± 178	100
Y326F	44 ± 15	25 ± 13	815 ± 256	75 ± 16

Binding parameters were obtained by non-linear curve-fitting of the data shown in Fig. 2. The parameters (K_{iH}, K_{iL} and the percentages of high- and low-affinity binding sites for (−)-isoprenaline) were determined using the non-linear regression program LIGAND. For each clone, all competition data were simultaneously analysed assuming initially a one-site model of binding and then assuming a two-site model. The selection between models was made statistically using the extra sum of squares principle (F-test). Each value represents the mean ± S.E.M. of 3 experiments.

Y326F mutant (Table 2). These biphasic curves could be shifted to low-affinity monophasic curves by the presence of the guanine nucleotide Gpp(NH)p (not shown), which indicates coupling between the receptors and their G-proteins.

In contrast, the competition curve obtained with Y326A receptors was clearly monophasic even in the absence of guanine nucleotides (Fig. 2), and only the low-affinity binding state of the receptor was identified by curve-fitting (Table 2).

These results are consistent with an inability of the Y326A mutant to couple to G-proteins while the Y326F mutant is capable of G-protein coupling. Nevertheless, both the affinity and the proportion of high-affinity binding sites for Y326F mutant receptors were lower than those of the wild-type receptors, suggesting that the G-protein coupling of this mutant receptor is also impaired.

3.4. Adenylyl cyclase activity

To further analyse the functionality of these mutant β_2-adrenoceptors, their ability to stimulate adenylyl cyclase activity in response to the agonist (−)-isoprenaline was tested. Concentration-response curves were performed in membranes prepared from cells expressing the wild-type and the mutant receptors (Fig. 3, Table 3).

The basal levels of cAMP production were not significantly different between the membranes containing wild-type and mutant receptors. In membranes containing wild-type receptors (−)-isoprenaline was able to promote a ~6-fold stimulation of adenylyl cyclase activity, which represented 48.6% of the activity caused by direct stimulation of G-proteins with 10 mM NaF.

The Y326A mutant receptors revealed almost no ability to stimulate adenylyl cyclase activity in response to high concentrations of (−)-isoprenaline. The maximum response was less than a fifth of the response of wild-type receptors and amounted to only 8.9% of the NaF stimulation in these membranes. Furthermore, the EC_{50} value was 2.5-fold higher than in wild-type receptors. Both the rightward shift and the decrease of the maximum of the concentration-response curve are indicative of impaired receptor coupling. Analysis of the coupling efficiency by estimation

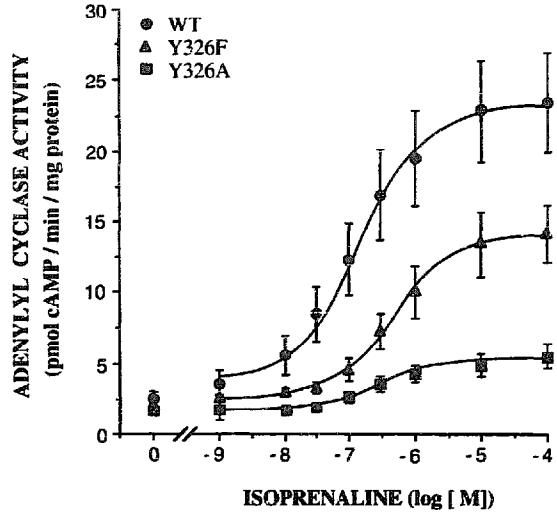

Fig. 3. Adenylyl cyclase activity in membranes from CHO cells expressing the wild-type or mutant β_2-adrenoceptor. The activity was determined under basal conditions and in the presence of the indicated concentrations of (−)-isoprenaline. Each data set represents the mean values ± S.E.M. of 5 separate experiments. The solid lines represent the computer-assisted curve-fitting of experimental data to Equation 1 described under Materials and methods. Parameters obtained from this analysis are given in Table 3.

of the transducer ratio τ (see Materials and methods) indicated a coupling efficiency in the Y326A mutant of 4 ± 2% of the wild-type receptors. This large decrease in the functionality of the mutant receptors is in agreement with the lack of coupling to G-proteins detected in competition binding assays.

The Y326F mutant receptors showed an intermediate ability to stimulate adenylyl cyclase activity in response to (−)-isoprenaline, with their maximum effect being about half that of the wild-type receptors, corresponding to 24.4% of the NaF-promoted activity. Once more, the EC_{50} value was higher (4.2-fold shift) than for the wild-type receptors. Calculation of the transducer ratio τ as above indicated that the Y326F mutant had a coupling efficiency of 20 ± 7% of the wild-type receptors. This decrease in the capacity to stimulate adenylyl cyclase activity may correspond to the decreased affinity (2.6-fold shift) and proportion of high-affinity binding sites (25 vs. 51%) detected in competition binding assays.

Table 3
Adenylyl cyclase stimulation by (−)-isoprenaline in membranes from CHO cells expressing wild-type and mutant human β_2-adrenoceptors

Receptor	E_o (pmol cAMP/min/mg membrane protein)	E_{max}	EC_{50} (μM)	τ
Wild type	3.4 ± 1.5	19.3 ± 2.0	0.12 ± 0.05	4.6 ± 1.9
Y326A	1.6 ± 0.3	3.6 ± 0.4 [b,c]	0.30 ± 0.16	0.19 ± 0.09 [b,c]
Y326F	2.6 ± 0.6	11.5 ± 1.1 [a]	0.50 ± 0.20	0.93 ± 0.30 [a]

Adenylyl cyclase activity parameters were calculated from data shown in Fig. 3 and are expressed as the best fit values (mean ± S.E.M.) calculated according to Equation 1 as described under Materials and methods. Statistical comparisons were made by comparing the goodness of fit of simultaneous analysis with and without a set of constraints by means of an F-test. Coupling efficiency was calculated as the transducer ratio τ according to Equation 2 described under Materials and methods.

[a] $P < 0.005$; [b] $P < 10^{-6}$ vs. wild type; [c] $P < 10^{-6}$ vs. Y326F.

4. Discussion

A tyrosine in the seventh transmembrane helix is conserved almost throughout the whole family of G-protein-coupled receptors (Probst et al., 1992). Oliveira et al. (1994) have hypothesised that this residue might be involved in agonist-induced conformational changes of such receptors. In contrast, Barak et al. (1994) have provided data which argue for a specific involvement in agonist-induced receptor sequestration. In an attempt to delineate its function, we have mutated the corresponding residue in the β_2-adrenoceptor, Tyr^{326}, to either alanine or phenylalanine, and expressed the mutant receptors in stably transfected CHO cells. We found that, in addition to its loss of agonist-induced sequestration, the alanine mutant was defective in G-protein coupling and exhibited a high percentage of internal receptors even in the absence of agonists. The G-protein interaction of the phenylalanine mutant was much less impaired and its sequestration behavior was almost normal. Antagonist binding was unperturbed in both mutations.

Barak et al. (1994) reported only minor differences between the Y326A mutant and the wild-type β_2-adrenoceptor in terms of high-affinity binding (from 37% in wild type to 15% in the mutant). In adenylyl cyclase activation the Y326A receptor had the same E_{max} as the wild-type receptor, but the EC_{50} was an order of magnitude higher. In our experiments, the Y326A mutant receptors did not show any detectable high-affinity binding and the E_{max} for (−)-isoprenaline was dramatically reduced (81% less than the wild type) with a smaller reduction in the EC_{50} (2.5 times). These differences can be largely attributed to different levels of receptor expression. While we have used clones which expressed about 200–300 fmol β_2-adrenoceptor/mg membrane protein, their clones show an expression in the range of 1 pmol/mg. At higher receptor densities, impaired receptor coupling causes a rightward shift of the concentration-response curve, while only at lower receptor levels a drop in the E_{max} becomes apparent. In fact, the 10-fold shift in the EC_{50} observed by Barak et al. (1994) for the Y326A mutant would indicate a coupling efficiency of less than 10%, which is not dramatically different from the 4% coupling efficiency which we observed.

Another abnormality that we found for the Y326A mutant β_2-adrenoceptor was an increase in the proportion of internal receptors in non-stimulated cells. In cells with Y326F mutant receptors this increase in basal levels of internal receptors was less evident. These results could mean either the existence of constitutive sequestration for the Y326A receptors or may be due to a reduced ability of newly synthesised receptors to reach the cell surface. The inability of the Y326A mutant receptors to undergo agonist-induced sequestration makes the second hypothesis more plausible. A related observation has been made by Keefer et al. (1994) who introduced the homologous mutation in the α_{2A}-adrenoceptor and found that traffic to the basolateral membrane was severely impaired in Madin-Darby canine kidney cells. These data suggest that this highly conserved tyrosine residue may be important for the proper folding of these receptors and their incorporation into the cell membrane.

Our initial assumption was that, as has been reported for the NPxY internalization motif in the low-density lipoprotein receptor (Chen et al., 1990), replacement of the Tyr^{326} by alanine would result in disruption of receptor sequestration whereas replacement by phenylalanine would not. Indeed, the impairment in sequestration was much less pronounced for the more conservative Y326F mutant than for the Y326A mutant. The same was true for all other changes in receptor properties. Rather than interpreting this as a specific function of the tyrosine residue we believe that the lack of the bulky side-chain is the reason for an alteration of the overall structure. However, even the relatively small change from tyrosine to phenylalanine resulted in an impairment of G-protein coupling (even though sequestration was essentially unaffected). This supports the hypothesis of Oliveira et al. (1994) that this conserved tyrosine is somehow involved in agonist-induced conformational changes within the receptor and indicates that the tyrosine-OH group may form a hydrogen bond with an as yet unidentified partner residue.

The possibility that the conserved NPxxY motif surrounding Tyr^{326} might represent an internalization signal has recently also been addressed for two other receptors. Mutations of the corresponding tyrosine have been reported for the receptors for the gastrin-releasing peptide (where it is actually NPxxxY, Slice et al., 1994) and angiotensin II (Hunyady et al., 1995; LaPorte et al., 1996). While the mutated gastrin-releasing peptide receptor was indistinguishable from its wild-type counterpart, the mutated AT_1 receptor was reported to be defective in high-affinity agonist binding and agonist-stimulated second messenger production, but sequestration was only slightly affected. These data are more compatible with a role of this tyrosine in receptor activation than with a specific involvement in sequestration.

This contention is supported by structural considerations. The NPxY internalization motif of the low-density lipoprotein receptor probably folds into a tight turn. This conformation is required for its function (Vaux, 1992). Baldwin (1993) has demonstrated by comparative sequence analysis of G-protein-coupled receptors that the NPxxY motif is very likely located in the seventh transmembrane helix with the tyrosine at the cytoplasmic end facing the ligand binding pocket. A search of the Brookhaven protein database showed that of fourteen unrelated NPxxY motifs, only one was located in a turn but seven in a helix. This suggests that the NPxxY sequence will not fold into a turn, thereby indicating that it will probably not function as an internalization signal related to the NPxY motif.

While this paper was in preparation, Barak et al. (1995) reported additional experiments with the Y326A mutant. In this paper they also find defects in the signalling of the Y326A mutant receptor in intact cells, which they interpret in the context of its reduced sequestration and, hence, resensitization. They also conclude that Tyr^{326} has a general role in the β_2-adrenoceptor. It will be interesting to further delineate its functional importance in agonist-dependent conformational change and to identify its potential hydrogen-bonding partner in the receptor.

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References

Baldwin, J.M., 1993, The probable arrangement of the helices in G protein-coupled receptors, EMBO J. 12, 1693.

Barak, L.S., L. Ménard, S.S.G. Ferguson, A.-M. Colapietro and M.G. Caron, 1995, The conserved seven-transmembrane sequence $NP(X)_{2,3}Y$ of the G-protein-coupled receptor superfamily regulates multiple properties of the β_2-adrenergic receptor, Biochemistry 34, 15407.

Barak, L.S., M. Tiberi, N.J. Freedman, M.M. Kwatra, R.J. Lefkowitz and M.G. Caron, 1994, A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated β_2-adrenergic receptor sequestration, J. Biol. Chem. 269, 2790.

Black, J.W., P. Leff and N.P. Shankley, 1985, An operational model of pharmacological agonism: the effect of E/[A] curves shape on agonist dissociation constants, Br. J. Pharmacol. 84, 561.

Bouvier, M., W.P. Hausdorff, A. DeBlasi, B.F. O'Dowd, B.K. Kobilka, M.G. Caron and R.J. Lefkowitz, 1988, Removal of phosphorylation sites in the β_2-adrenergic receptor delays onset of agonist-promoted desensitization, Nature (London) 333, 370.

Bradford, M.M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248.

Campbell, P.T., M. Hnatowich, B.F. O'Dowd, M.G. Caron, R.J. Lefkowitz and W.P. Hausdorff, 1991, Mutations of the human β_2-adrenergic receptor that impair coupling to G_s interfere with receptor down-regulation but not sequestration, Mol. Pharmacol. 39, 192.

Chen, W.-J., J.L. Goldstein and M.S. Brown, 1990, NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor, J. Biol. Chem. 265, 3116.

Cheung, A.H., R.A.F. Dixon, W.S. Hill, I.S. Sigal and C.D. Strader, 1990, Separation of the structural requirements for agonist-promoted activation and sequestration of the β-adrenergic receptor, Mol. Pharmacol. 37, 775.

Cheung, A.H., I.S. Sigal, R.A.F. Dixon and C.D. Strader, 1989, Agonist-promoted sequestration of the β_2-adrenergic receptors requires regions involved in functional coupling with G_s, Mol. Pharmacol. 34, 132.

Dohlman, H.G., J. Thorner, M.G. Caron and R.J. Lefkowitz, 1991, Model systems for the study of seven-transmembrane-segment receptors, Annu. Rev. Biochem. 60, 653.

Ferguson, S.S.G., L. Ménard, L.S. Barak, W.J. Koch, A.-M. Colapietro and M.G. Caron, 1995, Role of phosphorylation in agonist-promoted β_2-adrenergic receptor sequestration. Rescue of a sequestration-defective mutant receptor by βARK1, J. Biol. Chem. 270, 24782.

Harden, T.K., C.V. Cotton, G.L. Waldo, J.K. Lutton and J.P. Perkins, 1980, Catecholamine-induced alteration in sedimentation behavior of membrane bound β-adrenergic receptors, Science 210, 441.

Hausdorff, W.P., M. Bouvier, B.F. O'Dowd, G.P. Irons, M.G. Caron and R.J. Lefkowitz, 1989, Phosphorylation sites on two domains of the β_2-adrenergic receptor are involved in distinct pathways of receptor desensitization, J. Biol. Chem. 264, 12657.

Hausdorff, W.P., M.G. Caron and R.J. Lefkowitz, 1990, Turning off the signal: desensitization of β-adrenergic receptor function, FASEB J. 4, 2881.

Hertel, C., M. Nunnally, S.K.-F. Wong, E.A. Murphy, E.M. Ross and J.P. Perkins, 1990, A truncation mutation in the avian β-adrenergic receptor causes agonist-induced internalization and GTP-sensitive agonist binding characteristic of mammalian receptors, J. Biol. Chem. 265, 17988.

Hunyady, L., M. Bor, A.J. Baukal, T. Balla and K.J. Catt, 1995, A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for the type I angiotensin II receptor, J. Biol. Chem. 270, 16602.

Kabsch, W. and C. Sander, 1983, Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features, Biopolymers 22, 2577.

Keefer, J.R., M.E. Kennedy and L.E. Limbird, 1994, Unique structural features important for stabilization versus polarization of the α_{2A}-adrenergic receptor on the basolateral membrane of Madin-Darby canine kidney cells, J. Biol. Chem. 269, 16425.

Kobilka, B.K., R.A.F. Dixon, T. Frielle, H.G. Dohlman, M.A. Bolanowski, I.S. Sigal, T.L. Yang-Feng, U. Francke, M.G. Caron and R.J. Lefkowitz, 1987, cDNA for the human β_2-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor, Proc. Natl. Acad. Sci. USA 84, 46.

Kunkel, T.A., 1985, Rapid and efficient site-specific mutagenesis without phenotypic selection, Proc. Natl. Acad. Sci. USA 82, 488.

LaPorte, S.A., G. Servant, D.E. Richard, E. Escher, G. Guillemette and R. Leduc, 1996, The tyrosine within the NPX_nY motif of the human angiotensin II type 1 receptor is involved in mediating signal transduction but is not essential for internalization, Mol. Pharmacol. 49, 89.

Levitzki, A., 1988, From epinephrine to cyclic AMP, Science 241, 800.

Lohse, M.J., 1990, Quantitation of receptor desensitization by an operational model of agonism, J. Biol. Chem. 265, 3210.

Lohse, M.J., 1992, Stable overexpression of β_2-adrenergic receptors in mammalian cells, Naunyn-Schmiedeberg's Arch. Pharmacol. 345, 444.

Lohse, M.J., 1993, Molecular mechanisms of membrane receptor desensitization, Biochim. Biophys. Acta 1179, 171.

Lohse, M.J., J.L. Benovic, M.G. Caron and R.J. Lefkowitz, 1990, Multiple pathways of rapid β_2-adrenergic receptor desensitization: delineation with specific inhibitors, J. Biol. Chem. 265, 3202.

Lohse, M.J., K.-N. Klotz and U. Schwabe, 1986, Agonist photoaffinity labeling of A_1 adenosine receptors: persistent activation reveals spare receptors, Mol. Pharmacol. 30, 403.

McPherson, G.A., 1985, Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC, J. Pharmacol. Methods 14, 213.

Munson, P.J. and D. Rodbard, 1980, LIGAND: a versatile computerized approach for characterization of ligand binding systems, Anal. Biochem. 107, 220.

Oliveira, L., A.C.M. Paiva, C. Sander and G. Vriend, 1994, A common step for signal transduction in G protein-coupled receptors, Trends Pharmacol. Sci. 15, 1355.

Pals-Rylaarsdam, R., Y. Xu, P. Witt-Enderby, J.L. Benovic and M.M.

Hosey, 1995, Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms, J. Biol. Chem. 270, 29004.

Pippig, S., S. Andexinger, K. Daniel, M. Puzicha, M.G. Caron, R.J. Lefkowitz and M.J. Lohse, 1993, Overexpression of β-adrenergic receptor kinase and β-arrestin augment homologous desensitization of β_2-adrenergic receptors, J. Biol. Chem. 268, 3201.

Pippig, S., S. Andexinger and M.J. Lohse, 1995, Sequestration and recycling of β_2-adrenergic receptors permit receptor resensitization, Mol. Pharmacol. 47, 666.

Probst, W.C., L.A. Snyder, D.I. Schuster, J. Brosius and S.C. Sealfon, 1992, Sequence alignment of the G-protein coupled receptor superfamily, DNA Cell Biol. 11, 1.

Raposo, G, I. Dunia, C. Delavier-Klutchko, S. Kaveri, A.D. Strosberg and E.L. Benedetti, 1989, Internalization of β-adrenergic receptor in A431 cells involves non-coated vesicles, Eur. J. Cell. Biol. 50, 340.

Slice, L.W., H.C. Wong, C. Sternini, E.F. Grady, N.W. Bunnett and J.H. Walsh, 1994, The conserved NPX_nY motif present in the gastrin-releasing peptide receptor is not a general sequestration sequence, J. Biol. Chem. 269, 21755.

Staehelin, M and P. Simons, 1982, Rapid and reversible disppearance of β-adrenergic cell surface receptors, EMBO J. 1, 187.

Strader, C.D., I.S. Sigal, A.D. Blake, A.H. Cheung, R.B. Register, E. Rands, B.A. Zemcik, M.R. Candelore and R.A.F. Dixon, 1987, The carboxyl terminus of the hamster β-adrenergic receptor expressed in mouse L-cells is not required for receptor sequestration, Cell 49, 855.

Tsuga, H., K. Kameyama, T. Haga, H. Kurose and T. Nagao, 1994, Sequestration of muscarinic acetylcholine receptor m2 subtypes. Facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2, J. Biol. Chem. 269, 32522.

Vaux, D., 1992, The structure of an endocytosis signal, Trends Cell Biol. 2, 189.

Von Zastrow, M. and B.K. Kobilka, 1992, Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors, J. Biol. Chem. 267, 3530.

Wang, H.. M. Berrios and C.C. Malbon, 1989, Localization of β-adrenergic receptors in A 431 cells in situ, Biochem. J. 263, 553.

Yu, S.S., R.J. Lefkowitz and W.P. Hausdorff, 1993, β-Adrenergic receptor sequestration. A potential mechanism of receptor resensitization, J. Biol. Chem. 268, 337.