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Functional effects of long-term activation on human β_2- and β_3-adrenoceptor signalling

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1 The functional effects of long-term activation of β-adrenoceptors were investigated by measuring adenylyl cyclase activity, cyclic AMP accumulation and cyclic AMP-dependent protein kinase activity in CHW and L cells expressing either human β_2- or β_3-adrenoceptors.

2 Pre-incubation of CHW and L cells expressing β_2-adrenoceptors with 10 μM isoprenaline for 24 h produced a marked reduction in the total receptor number and dramatically reduced the capacity of the receptor to stimulate adenylyl cyclase maximally.

3 In contrast, the ability of β_3-adrenoceptors to stimulate adenylyl cyclase maximally was not affected by pre-incubation with the agonist in either cell type. However, a significant reduction of isoprenaline potency and a sustained down-regulation of β_3-adrenoceptor number was observed in L but not in CHW cells.

4 Maximal levels of intracellular cyclic AMP concentrations were reached during the first hour of receptor activation with isoprenaline in all four cell lines. In the absence of phosphodiesterase inhibitors, cyclic AMP decreased to basal levels within 24 h of continuous stimulation. This phenomenon occurred more rapidly in cells expressing the β_2- than the β_3-adrenoceptors.

5 These results confirm that, at the level of adenylyl cyclase stimulation and cyclic AMP accumulation, the β_3-adrenoceptor is more resistant than the β_2-adrenoceptor to long-term desensitization. However, when cyclic AMP-dependent protein kinase activity was considered, a 24 h stimulation of β_2- and β_3-adrenoceptor expressing cells led to the desensitization of the kinase in L but not in CHW cells.

6 In conclusion, long-term desensitization may have distinct functional effects on cell signalling depending on the receptor subtype and the cell type considered. These findings might have practical implications for future strategies involving long-term therapies with receptor agonists.

Keywords: Atypical β-adrenoceptors; β_3-adrenoceptors; desensitization; down-regulation; adenylyl cyclase; cyclic AMP

Introduction

Based on the clinical observation that bronchodilator β_2-sympathomimetic drugs, prescribed to asthmatic patients, may induce down-regulation of leukocyte β-adrenoceptors (Galant *et al.*, 1978), and that contractility improvement produced by β-adrenoceptor agonists in patients with cardiomyopathy wanes with time (Packer, 1990), it is generally admitted that the loss of pharmacological effect is a potential shortcoming for long-term treatments with receptor agonists (Hausdorff *et al.*, 1990). Indeed, signal transduction of most G protein-coupled receptors is tightly controlled by regulatory processes which, on the one hand, prevent the hormonal overload (desensitization) and which, on the other hand, reset the signalling pathways for further hormonal stimuli (resensitization). However, clinically important tachyphylaxis has not always been observed in asthmatic patients upon sustained treatment with β-adrenoceptor agonists (Tattersfield, 1985). More recently, sustained treatment with β-agonists has been shown to promote tolerance to their non-bronchodilator actions but not to their direct bronchorelaxant effects (O'Connor *et al.*, 1993). This raises the intriguing possibility that the clinical manifestation of agonist-promoted desensitization may be tissue-specific.

Desensitization is a multifactorial process which limits the effects of receptor activation by impairing the signal-transmission pathway at receptor and/or post-receptor levels. The β_2-adrenoceptor (β_2AR) is one of the most thoroughly investigated models of receptor desensitization (Lefkowitz, 1993). After a few minutes of incubation with an agonist, the β_2ARs is phosphorylated by the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase A (PKA) and by the β-adrenoceptor kinase (βARK), causing the functional uncoupling of the receptor from the signalling pathway (Benovic *et al.*, 1988; Hausdorff *et al.*, 1989). Phosphorylation of the β_2AR by PKA, activated by the increase of intracellular cyclic AMP levels, constitutes a heterogeneous negative feedback loop affecting all β_2AR present while βARK phosphorylates only those receptors occupied by the agonist (Benovic *et al.*, 1986). Resensitization of the β_2AR-mediated response occurs following the sequestration of the phosphorylated β_2AR and its subsequent dephosphorylation and recycling to the cell surface (Yu *et al.*, 1993). When receptor activation is sustained for longer periods of time (h), receptor down-regulation contributes to the reinforcement of desensitization through the degradation of pre-existing receptors (Doss *et al.*, 1981; Homburger *et al.*, 1984) and the destabilization of receptor mRNA (Hadcock *et al.*, 1988; Bouvier *et al.*, 1989). Under these conditions, *de novo* synthesis of receptor proteins is necessary for cellular responsiveness to be fully recovered (Doss *et al.*, 1981).

In rodents, β_3-adrenoceptors (β_3AR) play a major role in the control of white adipose tissue lipolysis and brown adipose tissue thermogenesis (Arch & Kaumann, 1993). In man, it has been shown that β_3AR distribution is mostly restricted to adipose tissues and gallbladder (Krief *et al.*, 1993) and that β_3-selective agonists may promote lipolysis in

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samples of human adipose tissues (Lönnqvist *et al.*, 1993). The β_3AR thus represents a target for anti-obesity therapy using adrenoceptor agonists (Connacher *et al.*, 1988; MacLahan *et al.*, 1991; Howe, 1993). However, desensitization of the cellular response might hamper the therapeutic effect of these compounds.

In contrast to the well documented rapid desensitization of the β_2AR, recent studies have shown the β_3AR to be almost completely resistant to short-term desensitization (Granneman, 1992; Liggett *et al.*, 1993; Nantel *et al.*, 1993), presumably because this receptor does not contain PKA or βARK phosphorylation sites (Liggett *et al.*, 1993; Nantel *et al.*, 1993). Several observations also suggest that the β_3AR could be less prone that the β_2AR to desensitization which occurs following longer activation with agonists. Indeed, little or no agonist-induced down-regulation of the β_3AR was found in CHW cells (Liggett *et al.*, 1993; Nantel *et al.*, 1994), in 3T3-F442A adipocytes (Thomas *et al.*, 1992) and in human SK-N-MC cells (F.N. and S.M., unpublished observation). Similarly, β_3AR-mediated adenylyl cyclase activation in hamster adipocytes was unaffected by *in vivo* infusion of adrenaline for up to six days (Carpéné *et al.*, 1993).

While long-term desensitization has been extensively investigated at the level of the receptor, little attention has been given to the functional consequences of this regulation on distal signalling events such as cyclic AMP accumulation and PKA activation. Therefore, in the present study, we have compared the adenylyl cyclase activation, the intracellular cyclic AMP levels and the PKA activity, upon sustained agonist activation, in cells expressing βAR subtypes with different desensitization profiles.

Methods

Cell culture

The coding regions of human β_2AR cDNA and human β_3AR genomic DNA were cloned into the pBC12BI plasmid and transfected into either chinese hamster fibroblasts (CHW-β_2AR and CHW-β_3AR) or murine L cells (L-β_2AR and L-β_3AR) as previously described (Nantel *et al.*, 1993). Cells were grown in 75 cm^2 Corning flasks at 37°C in an atmosphere of 95% air/5% CO_2. The cell culture medium consisted of Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) FBS, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 0.25 μg ml^{-1} fungizone and 1 mM glutamine. Geneticin, 150 or 450 μg ml^{-1}, was added to the medium for CHW or L cell clones, respectively.

Membrane preparation

Nearly confluent cells, grown in 75 cm^2 flasks, were washed twice with ice-cold PBS, mechanically detached and resuspended in 10 ml of 5 mM Tris, 2 mM EDTA, pH 7.4 supplemented with protease inhibitors (5 μg ml^{-1} soybean trypsin inhibitor, 5 μg ml^{-1} leupeptine, 10 μg ml^{-1} benzamidine). Cells suspensions were homogenized with a polytron homogenizer (Janke & Undel Ultra-Turrax T25) for 5 s at maximal setting. The lysate was centrifuged at 500 *g* for 5 min at 4°C (to eliminate the nucleus and unbroken cells). The supernatant was centrifuged at 43,000 *g* for 20 min at 4°C, and the pellet was resuspended in 10 ml of 5 mM Tris, 2 mM EDTA, pH 7.4. After an additional centrifugation at 43,000 *g* for 20 min at 4°C the pelleted membranes were resuspended in 75 mM Tris (pH 7.4), 5 mM $MgCl_2$, 2 mM EDTA supplemented with protease inhibitors (as above).

Radioligand binding

To determine total β_2AR number, 150 ml of membrane preparation (10 μg protein) was incubated in the presence of 250 pM [^{125}I]-cyanopindolol ([^{125}I]-CYP) in the absence or presence of 10 μM (−)-alprenolol (to define non-specific binding). In β_3AR-expressing cells, 50 μg protein were used with 1 nM [^{125}I]-CYP as previously described (Nantel *et al.*, 1993). The binding assays were conducted for 90 min at 25°C in a final volume of 500 ml of 75 mM Tris (pH 7.4), 5 mM $MgCl_2$, 2 mM EDTA supplemented with protease inhibitors (as above). The reaction was terminated by rapid filtration through Whatman GF/C glass fibre filters previously soaked for 30 min in 25 mM Tris (pH 7.4), 0.3% polyethyleneimine (to reduce non-specific binding). Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad protein assay system. Bovine serum albumin was used as standard.

Adenylyl cyclase assays

Adenylyl cyclase activity was measured by the method of Salomon *et al.* (1974). Briefly, 0.02 ml membrane preparation (as above, 2–6 μg protein), 45 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1.2 mM EDTA, 0.12 mM ATP, 0.053 mM GTP, 0.1 μM cyclic AMP, 0.1 mM isobutylmethylxanthine, 1 μCi [α-^{32}P]-ATP, 2.8 mM phosphoenolpyruvate, 0.2 u pyruvate kinase and 1 u myokinase were mixed in a final volume of 50 μl. Enzymatic activity was determined in the presence of 0–100 μM isoprenaline for 15 min at 37°C. The reactions were terminated by the addition of 1 ml ice-cold stop solution containing 0.4 mM ATP, 0.3 mM cyclic AMP and 25,000 c.p.m. [^{3}H]-cyclic AMP. Cyclic AMP was then isolated by sequential chromatography on a Dowex cation exchange resin and aluminium oxide.

Determination of intracellular cyclic AMP levels

Cells grown in 75 cm^2 flasks were exposed to 10 μM isoprenaline for 1–24 h at 37°C. The cells were washed twice with 5 ml of ice-cold PBS and resuspended in 6 (CHW) or 1.5 (L cells) ml of ice-cold 50 mM Tris (pH 7.4), 5 mM EDTA. A 1 ml aliquot was boiled for 3 min and centrifuged in a microfuge at maximum speed for 5 min. The supernatant was used for cyclic AMP determination using an [^{3}H]-cyclic AMP radio-immunoassay system (Amersham).

Protein kinase A assay

PKA activity was measured by using a variant of the protocol described by Corbin & Reimann (1974) as modified by Giembycz & Diamond (1990). Briefly, cells grown to near confluency were exposed, or not, to 10 μM isoprenaline for 10 min or 24 h. The cells were washed thrice with 5 ml ice-cold PBS and homogenized in 5 ml of ice-cold buffer A (5 mM KH_2PO_4, 5 mM K_2PO_4 pH 6.8, 10 mM EDTA, 10 mM dithiothreitol, 0.5 mM isobutylmethylxanthine (IBMX), 500 mM NaCl) for 5 s with a polytron homogenizer. The homogenate was centrifuged at 50,000 *g* for 30 min at 4°C and the supernatant was used for the determination of soluble protein kinase A activity. PKA activity was measured in duplicate using kemptide as substrate for the enzyme. The enzymatic reaction was conducted using 3–5 μg of soluble protein in a final volume of 100 μl containing 10 mM KH_2PO_4, 10 mM K_2HPO_4, 10 mM $Mg(CH_3COO)_2$, 0.5 mM IBMX, 500 mg ml^{-1} BSA, 100 μM ATP, 71 μM kemptide and 100 c.p.m. per pmol [α-^{32}P]-ATP, in the presence or absence of 10 μM cyclic AMP. The reaction was continued for 20 min at 30°C and terminated by cooling the samples on ice. Aliquots of 70 μl were spotted onto 2.5 × 2.5 cm phosphocellulose papers (Whatman, P81) which were subsequently washed four times in 75 mM phosphoric acid (4 × 5 min), once in ethanol and once in ether. The paper squares were allowed to dry and the incorporation of ^{32}P were determined in a scintillation counter. The ratio of ^{32}P incorporation in the absence over that observed in the presence of excess cyclic AMP was used as an index of PKA activity.

Data analysis

The adenylyl cyclase activity data were fitted by non-linear least squares regression analysis with the computer programme ALLFIT (De Léan *et al.*, 1978). Differences between data were evaluated by the Bonferroni *t* test with the computer programme PRIMER. Differences were considered statistically significant with $P<0.05$.

Materials

[α-^{32}P]-ATP and [^{3}H]-cyclic AMP were from DuPont-New England Nuclear. [^{125}I]-CYP was either from DuPont-New England Nuclear or Amersham. Isoprenaline, (−)-alprenolol, ATP, GTP, cyclic AMP, BSA, kemptide, and phosphoenolpyruvate were from Sigma. Pyruvate kinase was from Calbiochem. DMEM, PBS, trypsin-EDTA, geneticin (G418), penicillin, streptomycin and fungizone were from GIBCO/BRL. The FBS was purchased either from GIBCO/BRL or from Immunocorps.

Results

CHW and L cells, which do not express endogenous βARs and are unresponsive to catecholamines, were transfected with either the human β_2 AR cDNA or the human β_3 AR gene as previously described (Nantel *et al.*, 1993). Cell lines expressing an equivalent number of receptors (200–350 fmol mg^{-1} membrane protein) were used throughout this study.

Effect of sustained receptor activation on adenylyl cyclase activity and receptor number

In membrane preparations from CHW-β_2AR and L-β_2AR cells, the binding of the agonist isoprenaline to the β_2AR induced the activation of adenylyl cyclase. Pre-incubation of both CHW and L cells with 10 μM isoprenaline induced a time-dependent decrease in the ability of the β_2AR to stimulate the enzyme maximally (Figure 1 and Table 1). This desensitization was rapid as most of the decrease in adenylyl cyclase stimulation over the 24 h period occurred within the first 3 h of isoprenaline treatment. Following 24 h of continuous incubation with the agonist, the ability of the β_2AR to stimulate the adenylyl cyclase activity was reduced by ≈85% in both cell lines. The agonist pretreatment also induced a time-dependent down-regulation in the total number of [^{125}I]-CYP binding sites: after 24 h of continuous stimulation, β_2AR levels decreased by 68% and 83% in CHW-β_2AR and L-β_2AR cells respectively (Table 1). Post-receptor desensitization was evident in CHW-β_2AR cells as a significant reduction in the capacity of NaF and of forskolin to activate the adenylyl cyclase (Table 1) was observed following sustained incubation with the agonist. In contrast, no

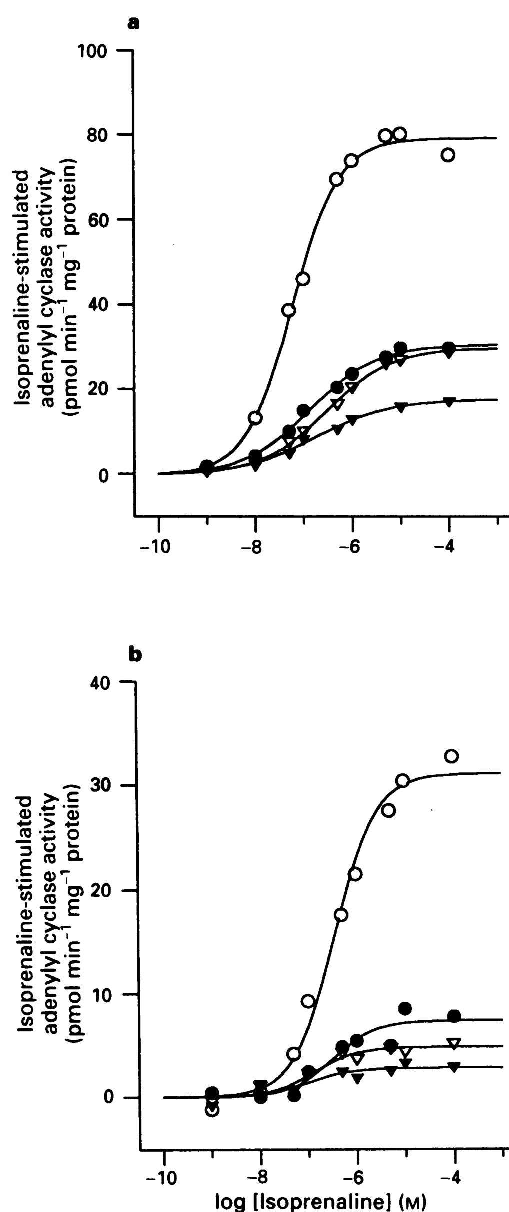

Figure 1 Isoprenaline-induced stimulation of adenylyl cyclase activity in cells expressing β_2-adrenoceptors (β_2AR). Membranes were prepared from CHW-β_2AR (a) or L-β_2AR (b) previously incubated in the presence of 10 μM isoprenaline for 0 (○), 3 (●), 6 (▽) or 24 h (▼). The isoprenaline-stimulated adenylyl cyclase activity is expressed as pmol of cyclic AMP produced min^{-1} mg^{-1} membrane protein. Data represent the mean of 3–4 experiments done in duplicate.

Table 1 β_2-Adrenoceptor (β_2AR) number and stimulation of adenylyl cyclase

Cell line	*Time* (h)	*β_2AR number* (fmol mg^{-1} prot.)	*AC basal activity* (pmol min^{-1} mg^{-1} prot.)	*Iso-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)	*Iso EC_{50}* (nM)	*NaF-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)	*FK-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)
CHW-β_2AR	0	354 ± 7	20.2 ± 10	120.9 ± 18.2	58 ± 22	102 ± 13	241 ± 52
	3	264 ± 36	5.4 ± 1.3	36.1 ± 14.9*	110 ± 50	50 ± 11*	154 ± 42*
	6	186 ± 28*	3.9 ± 2.7	29.8 ± 2.4*	310 ± 20	57 ± 10*	152 ± 39*
	24	113 ± 6*	3.2 ± 1.0	17.7 ± 2.9*	300 ± 100	56 ± 11*	142 ± 34*
L-β_2AR	0	202 ± 6	6.8 ± 1.9	38.3 ± 1.8	340 ± 90	47 ± 6	63 ± 8
	3	52 ± 2*	3.9 ± 1.5	11.7 ± 2.9*	950 ± 880	52 ± 5	68 ± 5
	6	48 ± 5*	1.5 ± 0.7	6.8 ± 1.2*	250 ± 110	44 ± 8	55 ± 7
	24	26 ± 0*	1.5 ± 0.8	5.8 ± 0.6*	983 ± 790	45 ± 9	53 ± 11

Cells were incubated with 10 μM isoprenaline for 0–24 h and membranes were prepared as described under the Methods section. Data represent the mean ± s.e.mean of 3–4 experiments done in duplicate. *$P<0.05$.

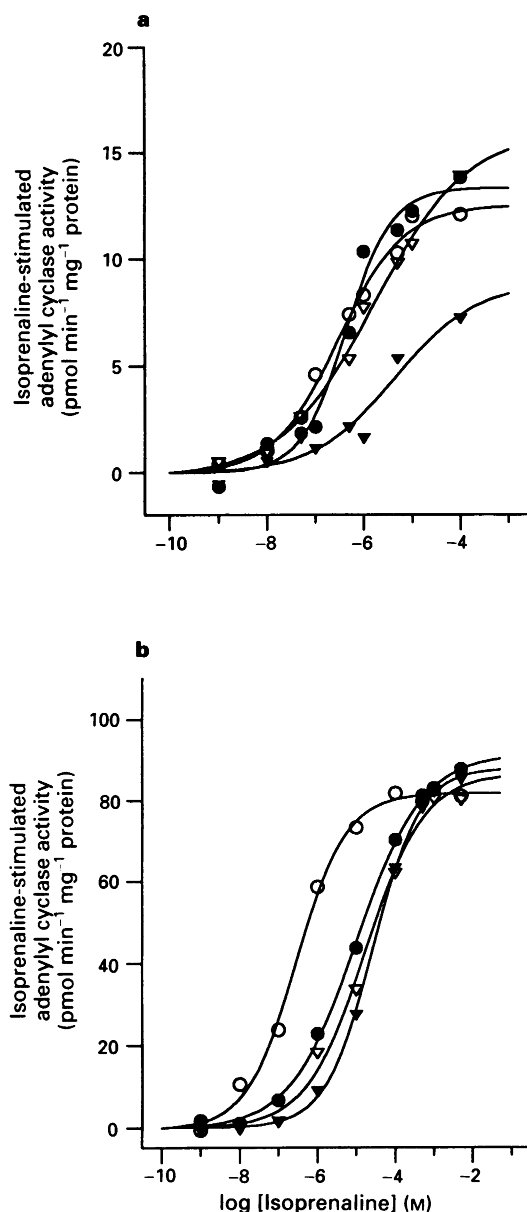

Figure 2 Isoprenaline-induced stimulation of adenylyl cyclase activity in cells expressing β_3-adrenoceptors (β_3AR). Membranes were prepared from CHW-β_3AR (a) or L-β_3AR (b) previously incubated in the presence of 10 μM isoprenaline for 0 (○), 3 (●), 6 (▽) or 24 h (▼). The isoprenaline-stimulated adenylyl cyclase activity is expressed as pmol of cyclic AMP produced min^{-1} mg^{-1} membrane protein. Data represent the mean of 3–4 experiments done in duplicate.

such reduction was observed in L-β_2AR cells, suggesting that the agonist-promoted desensitization occurred mainly at the level of the receptor in these cells.

Contrary to what was observed in the β_2AR-expressing cells, incubation with isoprenaline for 3, 6 and 24 h did not significantly affect the maximal β_3AR-mediated adenylyl cyclase stimulation in either CHW-β_3AR or L-β_3AR cells (Figure 2 and Table 2). It should be pointed out, however, that although it did not reach statistical significance, the 24 h pretreatment caused an apparent modest reduction of the β_3AR-mediated adenylyl cyclase stimulation in CHW cells. This was accompanied, in that cell type, by a significant decrease in NaF- and forskolin-stimulated adenylyl cyclase activity (Table 2) suggesting that the blunted responsiveness might reflect changes occurring down-stream of the receptor. A significant reduction in the potency of isoprenaline in stimulating adenylyl cyclase was found in L-β_3AR cells at the three time points studied. This agonist-promoted reduction in isoprenaline potency may result, in part, from the time-dependent down-regulation of the β_3AR number observed. No such down-regulation was observed in CHW-β_3AR cells.

Effect of sustained receptor activation on intracellular cyclic AMP concentrations

Intracellular concentrations of cyclic AMP were measured following 1, 3, 6 and 24 h of β-adrenoceptor stimulation (Table 3). In the four cell lines studied, a significant elevation in cyclic AMP levels was found following a 1 h isoprenaline treatment. Subsequently, cyclic AMP levels decreased progressively toward basal concentrations despite the continuous presence of the agonist. This decrease can be considered as a cellular manifestation of desensitization since a sustained stimulation fails to maintain a constant level of second messenger. Thus, this decrease was expressed as a percentage of desensitization considering that reaching pre-stimulation levels would represent 100% desensitization. As illustrated in Figure 3, the desensitization tended to be faster and more pronounced in the β_2AR than in the β_3AR expressing cells for the two cell types studied.

Effects of sustained receptor activation on PKA activity

Phosphorylation of target substrates by PKA is, ultimately, one of the major intracellular events leading to the proper cell response following β-adrenoceptor activation. To evaluate the consequences of β-adrenoceptor/adenylyl cyclase pathway desensitization on the subsequent step of signal transduction, PKA activity was assessed *in vitro* following short-term or sustained cell stimulation with isoprenaline (Figure 4). In the four cell lines, a 10 min stimulation led to a 2–3 fold increase in the activity of PKA. In CHW cells, irrespective of the receptor subtype being expressed, this level of activity was maintained for at least 24 h in the presence of

Table 2 β_3-Adrenoceptor (β_3AR) number and stimulation of adenylyl cyclase

Cell line	*Time* (h)	*β_2AR number* (fmol mg^{-1} prot.)	*AC basal activity* (pmol min^{-1} mg^{-1} prot.)	*Iso-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)	*Iso EC_{50}* (nM)	*NaF-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)	*FK-stimulated AC activity* (pmol min^{-1} mg^{-1} prot.)
CHW-β_3AR	0	264 ± 63	27.9 ± 3.5	39.8 ± 5.6	440 ± 160	104 ± 16	247 ± 52
	3	232 ± 26	19.6 ± 2.9	34.1 ± 8.1	452 ± 110	95 ± 16	231 ± 42
	6	188 ± 34	19.4 ± 3.5	33.0 ± 7.2	618 ± 20	92 ± 23	224 ± 42
	24	217 ± 19	18.5 ± 5.6	27.4 ± 8.3	2900 ± 200*	73 ± 16*	176 ± 37*
L-β_3AR	0	192 ± 32	9.0 ± 2.7	82.2 ± 2.9	297 ± 57	93 ± 13	81 ± 25
	3	102 ± 6*	7.8 ± 2.0	94.8 ± 3.8	10400 ± 2380*	101 ± 10	67 ± 8
	6	83 ± 1*	7.7 ± 1.7	86.9 ± 3.6	17600 ± 3890*	97 ± 15	55 ± 5
	24	47 ± 5*	7.7 ± 1.4	88.1 ± 3.0	27500 ± 4770*	110 ± 18	54 ± 3

Cells were incubated with 10 μM isoprenaline for 0–24 h and membranes were prepared as described under the Methods section. Data represent the mean ± s.e.mean of 3–4 experiments done in duplicate. *$P < 0.05$.

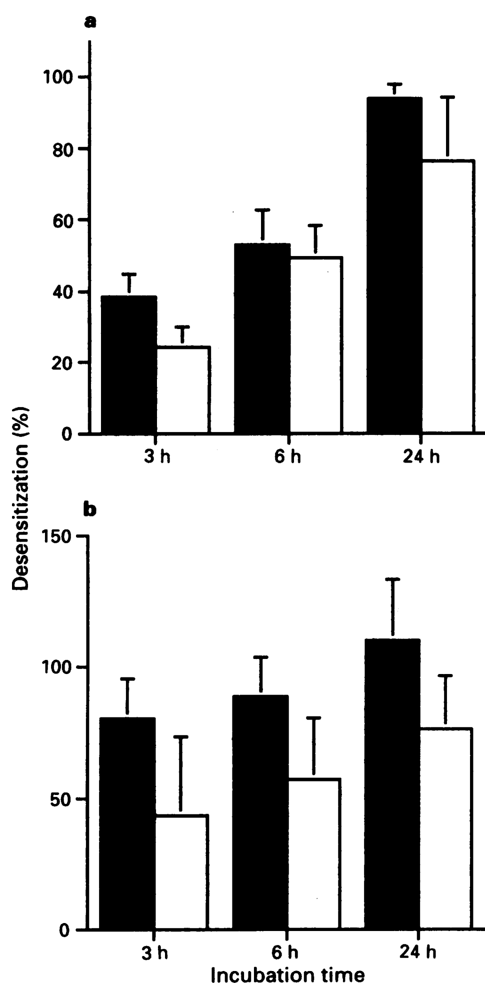

Figure 3 Reduction of intracellular cyclic AMP concentrations in CHW (a) and L cells (b) expressing β_2-adrenoceptors (β_2AR) (solid columns) or the β_3AR (open columns). The reduction in cyclic AMP levels, observed between 3–24 h of continuous stimulation with 10 μM isoprenaline, is expressed as a percentage of desensitization of the maximal cyclic AMP levels observed after 1 h of stimulation (see Table 3) and considering that reaching pre-stimulation levels would represent 100% desensitization. Data represent the mean ± s.e.mean of 3–4 experiments done in duplicate.

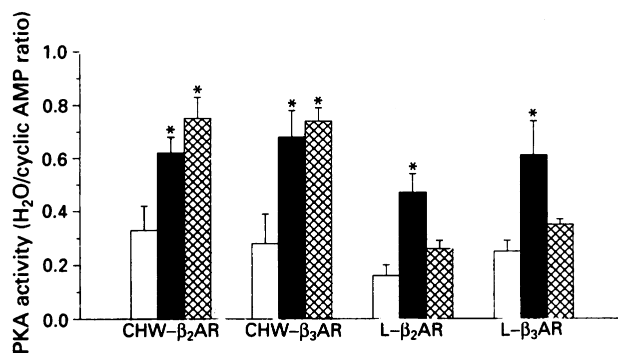

Figure 4 Protein kinase A (PKA) activity in cells incubated with 10 μM isoprenaline for 0 (□), 10 min (■) or 24 h (▩). PKA activity is expressed as the ratio of ^{32}P incorporation in the absence over that observed in the presence of excess cyclic AMP. Data represent the mean ± s.e.mean of 3–5 experiments done in duplicate.

Table 3 Intracellular cyclic AMP concentrations

	Cell type			
Time	*CHW-β_2AR*	*CHW-β_3AR*	*L-β_2AR*	*L-β_3AR*
Control	23.2 ± 8.5	26.5 ± 5.5	7.6 ± 1.7	7.9 ± 0.9
Iso 1 h	82.6 ± 7.4*	60.0 ± 5.0*	18.3 ± 5.1*	20.3 ± 6.8*
Iso 3 h	59.8 ± 3.8*	51.9 ± 1.9*	9.7 ± 1.6	14.9 ± 3.7*
Iso 6 h	51.2 ± 5.8*	43.5 ± 3.0*	8.8 ± 1.6	13.2 ± 2.9*
Iso 24 h	26.8 ± 2.5	34.4 ± 6.0	6.5 ± 2.5	10.8 ± 2.5

Cells were incubated with 10 μM isoprenaline (Iso) for 0–24 h and intracellular cyclic AMP concentrations were determined as described under Methods. Data are expressed in pmol mg^{-1} protein and represent the mean ± s.e.mean of 6–7 experiments done in duplicate.

a saturating concentration of agonist, suggesting that no desensitization could be detected at this level. This contrasted with the kinase activity profile observed in L cells. Indeed, the PKA activity returned to near basal values in both β_2AR and β_3AR expressing cells following 24 h of sustained stimulation. This indicates that, in this cell type, desensitization was also reflected at the PKA level.

Discussion

Previous studies have shown that desensitization processes which occur at the level of the receptor affect more readily the β_2AR than the β_3AR (Granneman, 1992; Liggett *et al.*, 1993; Nantel *et al.*, 1993; 1994). The results presented here indicate that, following long-term treatment with agonists, β-adrenoceptor-mediated stimulation of adenylyl cyclase reflects primarily the changes in βAR number and activity and, thus, that β_3AR-mediated adenylyl cyclase stimulation undergoes only very modest desensitization. Despite this lack of desensitization at the level of the signal transducing apparatus, the level of second messenger did not increase steadily during long-term stimulation of the β_3AR. Instead, the intracellular cyclic AMP levels reached a peak and then gradually declined, suggesting that processes acting downstream of the second messenger production are also involved in blunting the cellular response. When a more distal event in the signalling cascade, the PKA activity, was monitored, desensitization was evident for the two receptor subtypes but only in one of the two cell types being studied. This suggests that distal manifestation of the various desensitization processes are highly dependent on the cell type being considered.

The modest desensitization of isoprenaline-stimulated adenylyl cyclase activity observed in the β_3AR expressing cells, following 24 h of stimulation, contrasted with the 85% decrease in maximal β_2AR-dependent adenylyl cyclase activation seen under the same conditions. The relative resistance of the β_3AR-stimulated adenylyl cyclase to long-term desensitization probably reflects two intrinsic properties of the β_3AR: unlike β_2AR, (1) sustained stimulation of the β_3AR does not promote its functional uncoupling resulting from receptor phosphorylation (Granneman, 1992; Liggett *et al.*, 1993; Nantel *et al.*, 1993), (2) long-term stimulation of this receptor subtype leads to slower and more modest reduction in receptor number. The agonist-induced down-regulation of the β_3AR has recently been shown to result exclusively from a cyclic AMP-mediated reduction of receptor mRNA content (Nantel *et al.*, 1994). The absence of desensitization of the maximal isoprenaline-stimulated adenylyl cyclase activity suggests that the number of receptors remaining following down-regulation still exceeds that required to stimulate the enzyme maximally. In CHW cells, no statistically significant down-regulation of the β_3AR was observed and, thus, no change in either the efficacy or the potency of isoprenaline was observed following stimulation for 3 or 6 h. This observation is consistent with the recent finding that no sustained reduction in β_3AR mRNA content is observed upon long-

term stimulation in that cell line (Nantel *et al.*, 1994). The modest desensitization, observed after 24 h of continued stimulation in CHW-β_3AR cells, probably reflects post-receptor regulation (Unelius *et al.*, 1993) since both NaF- and forskolin-stimulated activity were significantly reduced at that time point.

The difference in response between the two receptor subtypes to agonist-promoted desensitization was also evident, although to a lesser extent, at the level of cyclic AMP accumulation. Indeed, cyclic AMP levels returned toward control values more rapidly in cells expressing the β_2AR than in cells expressing the β_3 subtype. However, in contrast to what was observed for the β_3AR-stimulated adenylyl cyclase activity measured in membrane preparations, the β_3AR-promoted cyclic AMP accumulation clearly became desensitized. This suggests that, even in the absence of desensitization at the level of the receptor itself, other cellular processes may contribute to the reduction of cyclic AMP content during prolonged stimulation. Activation of an inducible cyclic AMP phosphodiesterase (Barber *et al.*, 1992a; Houslay *et al.*, 1992) could contribute to the rapid decrease in cyclic AMP concentrations, despite the continued stimulation of the adenylyl cyclase. The presence of a phosphodiesterase with a strong positive cooperativity toward cyclic AMP in L cells (Barber *et al.*, 1992b) is consistent with such an hypothesis.

The functional consequences of long-term stimulation observed at the level of PKA activity were unexpected. After 24 h of continuous stimulation with a saturating concentration of isoprenaline, although intracellular cyclic AMP fell to near basal levels in all cell-lines, PKA was still fully activated in both CHW-β_2AR and CHW-β_3AR cells. These results indicate that, in these cells, a modest elevation of cyclic AMP concentrations above basal values is sufficient to maintain the dissociation and the activation of the catalytic subunit of the PKA (Cadd *et al.*, 1990). Maximal PKA activation has been shown to occur at relatively low levels of cyclic AMP in some cellular systems (Feldman, 1989; Lohse *et al.*, 1990). The existence of multiple catalytic and regulatory PKA subunits, which may combine to form different isoforms of the holoenzyme (Tasken *et al.*, 1993), could provide variable levels of sensitivity to the system. The functional consequences of this diversity are not completely understood. However, judging from the PKA activity patterns observed in the present study, it is clear that regulation of the kinase activity varies among cell lines and may lead to distinct patterns of cellular responsiveness regulation. Indeed, under identical conditions, the 24 h stimulation which led to no desensitization of the isoprenaline-stimulated PKA activity in CHW cells almost completely desensitized this activity in L-β_2AR and L-β_3AR cells.

This nearly complete desensitization of the agonist-stimulated PKA activity, in L-β_3AR cells, occurred despite no detectable desensitization of the receptor-stimulated adenylyl cyclase activity in membrane preparations. This suggests that, in these cells, the other processes contributing to the reduction in cyclic AMP levels are sufficient to promote desensitization. Therefore, the final phenotype of desensitization appears to be highly dependent on the cell type being considered. The precise nature of the mechanisms leading to such desensitization remain largely unknown. Whether it reflects a heterologous desensitization of all the PKA stimulating pathways or is restricted to the β-adrenoceptor-mediated activation of this kinase remains to be determined.

In the present study, the βAR coding regions were under the control of a viral promoter. For native receptors, modulation of gene transcription might increase further the diversity of βAR regulation: in DTT-MF$_2$ cells, positive PKA-mediated regulation of βAR genes drives a transient (1–2 h) increase of β_2AR mRNA (Collins *et al.*, 1989) while, in 3T3-F442A cells, elevated levels of β_3AR are still measured following 30 h of exposure to the agonist.

Our results provide biochemical support for previous observations of clinical pharmacology suggesting that receptor desensitization does not occur in all tissues. For example, β_2-adrenoceptor agonists are effective in the treatment of asthma by causing bronchodilatation and by protecting against the release of bronchoconstrictor stimuli. The former effect is due to the direct relaxation of airways smooth muscle, while the latter results from the stabilization of mast cells. Long-term exposure to β-agonists does not seem to induce a loss of their airway-specific effects, although tolerance to their protective effects against bronchoconstriction may blunt their beneficial effects in asthma, thus suggesting that βAR desensitization may occur in mast cells (O'Connor *et al.*, 1993) and in polymorphonuclear leukocytes (Galant *et al.*, 1978) but not in the airway smooth muscle cells. Lack of receptor desensitization in various tissues is also illustrated by the observation that in several diseases, normal G protein-coupled receptors are chronically activated by abnormal hormone (or hormone-like) overload, with deleterious consequences. Chronic autoimmune thyrotropin receptor activation in Graves-Basedow disease (McGregor, 1990) and sustained adrenoceptor activation by catecholamines in phaeochromocytoma (Bravo & Gifford, 1984) are well known examples. These observations indicate that some receptor-mediated pathways of signal transmission may escape long-term desensitization in man.

In conclusion, the desensitization of βAR following long-term activation may have distinct functional effects on cell signalling depending on both the receptor subtype and on the cell type considered. The physiological relevance, in man, of variable signalling regulation requires further studies, since these findings might have practical implications for future strategies involving long-term therapies with receptor agonists.

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References

ARCH, J.R.S. & KAUMANN, A.J. (1993). β_3 and atypical β-adrenoceptors. *Med. Res. Rev.*, **13**, 663–729.

BARBER, R., GOKA, T.J. & BUTCHER, R.W. (1992a). Cyclic AMP turnover in intact tissue: role of cyclic nucleotide phosphodiesterases. *Adv. Cyc. Nucl. Protein Phosphorylation*, **25**, 1–11.

BARBER, R., GOKA, T.J. & BUTCHER, R.W. (1992b). Postively cooperative cAMP phosphodiesterase attenuates cellular cAMP responses. *Second Messengers Phosphoproteins*, **14**, 77–97.

BENOVIC, J.L., BOUVIER, M., CARON, M. & LEFKOWITZ, R.J. (1988). Regulation of adenylyl cyclase-coupled β-adrenergic receptors. *Annu. Rev. Cell. Biol.*, **4**, 405–427.

BENOVIC, J.L., STRASSER, R.H., CARON, M.G. & LEFKOWITZ, R.J. (1986). β-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 2797–2801.

BOUVIER, M., COLLINS, S., O'DOWD, B.F., CAMPBELL, P.T. DE BIASI, A., KOBILKA, B.K., MACGREGOR, C., IRONS, G.P., CARON, M.G. & LEFKOWITZ, R.J. (1989). Two distinct pathways for cAMP-mediated down-regulation of the β_2-adrenergic receptor. Phosphorylation of the receptor and regulation of its mRNA level. *J. Biol. Chem.*, **264**, 16786–16792.

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,** 248–254.

BRAVO, E.L. & GIFFORD, R.W.J. (1984). Pheochromocytoma: diagnosis, localization and management. *New Engl. J. Med.*, **311,** 1298–1303.

CADD, G.C., UHLER, M.D. & MCKNIGHT, G.S. (1990). Holoenzymes of cAMP-dependent protein kinase containing the neural form of type I regulatory subunit have an increased sensitivity to cyclic nucleotide. *J. Biol. Chem.*, **265,** 19502–19506.

CARPÉNÉ, C., GALITZKY, J., COLLON, P., ESCLAPEZ, F., DAUZATS, M. & LAFONTAN, M. (1993). Desensitization of β_1- and β_2- but not β_3-adrenoceptor-mediated lipolytic responses of adipocytes after long-term norepinephrine infusion. *J. Pharmacol. Exp. Ther.*, **265,** 237–247.

COLLINS, S., BOUVIER, M., BOLANOWSKI, M., CARON, M. & LEFKOWITZ, R. (1989). cAMP stimulates transcription of the β_2-adrenergic receptor gene in response to short-term agonist exposure. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 4853–4857.

CONNACHER, A.A., JUNG, R.T. & MITCHELL, P.E.G. (1988). Weight loss in obese subjects on a restricted diet given BRL 26830A, a new atypical β adrenoceptor agonist. *Br. Med. J.*, **296,** 1217–1220.

CORBIN, J.D. & REIMANN, E.M. (1974). Assay of cyclic AMP-dependent protein kinases. *Methods Enzymol.*, **38,** 271–283.

DE LÉAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.*, **235,** E97–E102.

DOSS, R.C., PERKINS, J.P. & HARDEN, T.K. (1981). Recovery of β-adrenergic receptors following long term exposure of astrocytoma cells to catecholamine: role of protein synthesis. *J. Biol. Chem.*, **256,** 12281–12286.

FELDMAN, R.D. (1989). Beta-adrenergic desensitization reduces the sensitivity of adenylate cyclase for magnesium in permeabilized lymphocytes. *Mol. Pharmacol.*, **35,** 304–310.

GALANT, S.P., DURISETI, L., UNDERWOOD, S. & INSEL, P.A. (1978). Decreased β-adrenergic receptors on polymorphonuclear leukocytes after adrenergic therapy. *New Engl. J. Med.*, **299,** 933–936.

GIEMBYCZ, M.A. & DIAMOND, J. (1990). Evaluation of Kemptide, a synthetic serine-containing heptapeptide, as a phosphate acceptor for the estimation of cyclic AMP-dependent protein kinase activity in respiratory tissues. *Biochem. Pharmacol.*, **39,** 271–283.

GRANNEMAN, J.G. (1992). Effects of agonist exposure on the coupling of β_1 and β_3 adrenergic receptors to adenylyl cyclase in isolated adipocytes. *J. Pharmacol. Exp. Ther.*, **261,** 638–642.

HADCOCK, J.R., WANG, H. & MALBON, C. (1988). Agonist-induced destabilization of β-adrenergic receptor mRNA: attenuation of glucocorticoid-induced up-regulation of β-adrenergic receptors. *J. Biol. Chem.*, **264,** 19928–19933.

HAUSDORFF, W.P., BOUVIER, M., O'DOWD, B.F., IRONS, G.P., CARON, M.G. & LEFKOWITZ, R.J. (1989). Phosphorylation sites on two domains of the β_2-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J. Biol. Chem.*, **264,** 12657–12665.

HAUSDORFF, W.P., CARON, M.G. & LEFKOWITZ, R.J. (1990). Turning off the signal – desensitization of beta-adrenergic receptor function. *Fed. Am. Soc. Exp. Biol.*, **4,** 2881–2889.

HOMBURGER, V., PANTALONI, C., LUCAS, M., GOZLAN, H. & BOCKAERT, J. (1984). β-adrenergic receptor repopulation of C_6 glioma cells after irreversible blockade and down regulation. *J. Cell. Physiol.*, **121,** 589–597.

HOUSLAY, M.D., GRIFFITHS, S.L., HORTON, Y.M., LIVINGSTONE, C., LOBBAN, M., MACDONALD, F., MORRIS, N., PRYDE, J., SCOTLAND, G., SHAKUR, Y., SWEENEY, G. & TANG, E.K.Y. (1992). Regulation of intracellular cyclic AMP concentrations in hepatocytes involves the integrated activation and desensitization of adenylyl cyclase coupled with the action and activation of specific isoforms of cyclic AMP phosphodiesterase. *Biochem. Soc. Trans.*, **20,** 140–146.

HOWE, R. (1993). β_3-adrenergic agonists. *Drugs of the Future*, **18,** 529–549.

KRIEF, S., LÖNQVIST, F., RAIMBAULT, S., BAUDE, B., VAN SPRONSEN, A., ARNER, P., STROSBERG, A.D., RICQUIER, D. & EMORINE, L.J. (1993). Tissue distribution of β_3-adrenergic receptors mRNA in man. *J. Clin. Invest.*, **91,** 344–349.

LEFKOWITZ, R.J. (1993). G-protein-coupled receptor kinases. *Cell*, **74,** 409–412.

LIGGETT, S.B., FREEDMAN, N.J., SCHWINN, D.A. & LEFKOWITZ, R.J. (1993). Structural basis for receptor subtype-specific regulation revealed by a chimeric β_3/β_2-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **90,** 3665–3669.

LOHSE, M.J., BENOVIC, J.L., CARON, M.G. & LEFKOWITZ, R.J. (1990). Multiple pathways of rapid β_2-adrenergic receptor desensitization. *J. Biol. Chem.*, **265,** 3202–3209.

LÖNNQVIST, F., KRIEF, S., STROSBERG, A.D., NYBERG, B., EMORINE, L.J. & ARNER, P. (1993). Evidence for functional β_3-adrenoceptor in man. *Br. J. Pharmacol.*, **110,** 929–936.

MACLAHAN, M., CONNACHER, A.A. & JUNG, R.T. (1991). Psychological aspects of dietary weight loss and medication with the atypical β-agonist BRL 26830A in obese subjects. *Int. J. Obesity*, **15,** 27–35.

MCGREGOR, A.M. (1990). Autoantibodies to the TSH receptor in patients with autoimmune thyroid disease. *Clin. Endocrinol.*, **33,** 683–685.

NANTEL, F., BONIN, H., EMORINE, L.J., ZILBERFARB, V., STROSBERG, A.D., BOUVIER, M. & MARULLO, S. (1993). The human β_3-adrenergic receptor is resistant to short-term agonist-promoted desensitization. *Mol. Pharmacol.*, **43,** 548–555.

NANTEL, F., MARULLO, S., KRIEF, S., STROSBERG, A.D. & BOUVIER, M. (1994). Cell-specific down-regulation of the β_3-adrenergic receptor. *J. Biol. Chem.*, **269,** 13148–13155.

O'CONNOR, B.J., AIKMAN, S.L. & BARNES, P.J. (1993). Tolerance to the nonbronchodilator effects of inhaled β_2-agonist in asthma. *New Engl. J. Med.*, **327,** 1204–1208.

PACKER, M. (1990). Pathophysiological mechanisms underlying the effects of beta-adrenergic agonists and antagonists on functional capacity and survival in chronic heart failure. *Circulation*, **82,** 77–88.

SALOMON, Y., LONDOS, C. & RODBELL, M. (1974). A highly sensitive adenylate cyclase assay. *Analyt. Biochem.*, **58,** 541–548.

TASKEN, K., SKALHEGG, B.J., SOLBERG, R., ANDERSSON, K.B., TAYLOR, S.S., LEA, T., BLOMHOFF, H.K., JAHNSEN, T. & HANSSON, V. (1993). cAMP-dependent protein kinase exist in human cells due to formation of RIa-RI' heterotrimeric complexes. *J. Biol. Chem.*, **268,** 21276–21283.

TATTERSFIELD, A.E. (1985). Tolerance to β-agonists. *Bull. Eur. Physiolpathol. Respir.*, **21,** 1S–5S.

THOMAS, R.F., HOLT, B.D., SCHWINN, D.A. & LIGGETT, S.B. (1992). Long-term agonist exposure induces upregulation of β_3-adrenergic receptor expression via multiple cAMP response elements. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 4490–4494.

UNELIUS, L., BRONNIKOV, G., MOHELL, N. & NEDERGAARD, J. (1993). Physiological desensitization of beta(3)-adrenergic response in brown fat cells – involvement of a postreceptor process. *Am. J. Physiol.*, **265.**

YU, S.S., LEFKOWITZ, R.J. & HAUSDORFF, W.P. (1993). β-adrenergic receptor sequestration. A potential mechanism of receptor resensitization. *J. Biol. Chem.*, **268,** 337–341.

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