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Influence of receptor density on the patterns of β_2-adrenoceptor desensitization

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Abstract

Sustained stimulation of the β_2-adrenoceptor leads to a desensitization of the receptor-mediated adenylyl cyclase stimulation. While desensitization promoted by nanomolar concentrations of isoproterenol involves the phosphorylation of the β_2-adrenoceptor by protein kinase A alone, both protein kinase A- and β-adrenoceptor kinase-mediated phosphorylation leading to the binding of β-arrestin contribute to the desensitization evoked by micromolar concentrations of agonist. In the present study, we assessed the influence of receptor density on the patterns of desensitization induced by these two different levels of stimulation. Murine L cells were transfected with a cDNA encoding the human β_2-adrenoceptor and clonal cell lines expressing various levels of β_2-adrenoceptor were used for the study. In cell lines expressing the highest number of receptor, approx. 150 000 sites/cell (approx. 3000 fmol/mg of membrane proteins), pretreatment with micromolar concentrations of isoproterenol causes a desensitization pattern characterized by a reduction in both the potency and the efficacy of isoproterenol to further stimulate the adenylyl cyclase activity. In contrast, desensitization induced by 10 nM isoproterenol resulted only in a decrease in the potency of isoproterenol. This distinct pattern of desensitization is not seen in cells expressing 12 000 receptors/cell (approx. 200 fmol/mg of membrane proteins) and, in that case, pretreatment with 10 nM isoproterenol leads to a reduction in both the sensitivity and the maximal response. Similar effects on the β-adrenoceptor-stimulated adenylyl cyclase were observed in these cells following treatment with dibutyryl cAMP. Receptor density therefore dramatically influences the pattern of desensitization evoked by low level of stimulation. The results also demonstrate that although different molecular events are involved in the desensitization evoked by different levels of stimulation, its phenotypic expression can be qualitatively identical in cells expressing a relatively small number of receptors. Hence, protein kinase A-mediated desensitization cannot be qualitatively distinguished from the β-adrenoceptor kinase-mediated process in these cells.

Keywords: Desensitization; β-Adrenoceptor; Adenylyl cyclase; Protein kinase, cAMP dependent; Receptor density

1. Introduction

Agonist-mediated stimulation of the β_2-adrenoceptor activates the adenylyl cyclase via a stimulatory GTP-binding protein (G_s). This enzymatic response rapidly wanes upon sustained stimulation. The molecular processes involved in such desensitization have been the object of a number of studies (for reviews, see Benovic et al., 1988; Dohlman et al., 1991; Kobilka, 1992). The fastest event to occur is a functional uncoupling of the receptor from G_s, which is believed to result from the phosphorylation of the β_2-adrenoceptor by two protein kinases (Sibley et al., 1984; Strasser et al., 1986; Bouvier et al., 1988a; Hausdorff et al., 1989; Lohse et al., 1990). Stimulation of cells with low concentrations of agonist (10 nM isoproterenol), while occupying less then 10% of the receptor sites, nearly maximally activates the cAMP-dependent protein kinase. Desensitization induced by this stimulation intensity has been shown to result in part from the phosphorylation of the β_2-adrenoceptor by protein kinase A (Hausdorff et al., 1989; Clark et al., 1987). At higher level of stimulation (1 μM isoproterenol), most receptors are ligand-bound and thus become substrate for the β-adrenoceptor kinase. Therefore, phosphorylation by both protein kinase A and β-adrenoceptor kinase is believed to contribute to the

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desensitization elicited by micromolar concentrations of isoproterenol (Bouvier et al., 1988a; Hausdorff et al., 1989; Lohse et al., 1990). β-Adrenoceptor kinase-mediated phosphorylation of the β_2-adrenoceptor has been suggested to promote the association of the protein β-arrestin with the receptor and thereby to prevent the proper coupling of the receptor to G_s (Lohse et al., 1990; Pitcher et al., 1992). In contrast, phosphorylation of the β_2-adrenoceptor by protein kinase A does not increase the affinity of the receptor for β-arrestin (Pitcher et al., 1992) and the exact mechanism by which protein kinase A phosphorylation leads to desensitization remains elusive. One possible hypothesis is that the change in the charge distribution imposed by the addition of negatively charged phosphates could be sufficient to reduce the coupling efficacy between the receptor and G_s. This idea is supported by the fact that the protein kinase A phosphorylation site implicated in the desensitization evoked by low level of stimulation has been mapped to the N-terminal portion of the third cytoplasmic loop of the receptor, a domain involved in receptor-G protein coupling. A site-directed mutagenesis study by Yuan et al. (1994), however, cast some doubts on that simple charge distribution hypothesis. Indeed, substitution of a positively charged aspartate for the putatively protein kinase A phosphorylated serine was without effect on receptor coupling.

Independently of the precise mechanism involved in the protein kinase A-mediated desensitization of the β_2-adrenoceptor, several studies have reported that desensitization evoked by protein kinase A alone and that involving β-adrenoceptor kinase have distinct characteristics (Hausdorff et al., 1989; Lohse et al., 1990; Clark et al., 1989). It has been proposed that phosphorylation of the receptor by protein kinase A leads strictly to a reduction in the potency of agonists with no change in their efficacy to maximally stimulate the adenylyl cyclase activity. In contrast, desensitization involving phosphorylation by β-adrenoceptor kinase is characterized by a reduction of both the efficacy and the potency of agonists to activate adenylyl cyclase. These distinct desensitization phenotypes have been taken as an illustration that the molecular processes involved in protein kinase A- and β-adrenoceptor kinase-mediated desensitization, affected receptor coupling in different manners. An alternative explanation is the presence of a receptor reserve and the additive desensitizing effects of protein kinase A and β-adrenoceptor kinase. Indeed, in their formalisation of the influence of receptor density on agonist potency and efficacy, Whaley et al. (1994), predicted that a decrease in coupling efficiency that occurs through receptor phosphorylation would manifest itself as an increase in the EC_{50} with very little change in V_{max} in cells with high receptor levels but that at lower receptor density it would be seen as a reduction in V_{max} and an elevation in EC_{50}. The same group then presented data suggesting that this prediction may be true but concluded that the extent of protein kinase A-mediated desensitization is not dramatically affected by receptor density (Yuan et al., 1994). However, the effect of different receptor density on desensitization patterns was never systematically assessed in cells having the same genetic background.

In the present study we assess the influence of receptor expression level on the desensitization phenotype evoked by stimuli leading to the activation of protein kinase A alone or to the activation of protein kinase A and β-adrenoceptor kinase. For this purpose, clonal lines of murine L cells expressing the human β_2-adrenoceptor at levels varying between 10 000 and 200 000 sites per cell were used.

2. Materials and methods

2.1. Materials

[^{125}I]Cyanopindolol, [α-^{32}P]ATP and [^{3}H]cAMP were obtained from New England Nuclear. Isoproterenol, ATP, GTP, cAMP, dibutyryl-cAMP, butyric acid, phospho-*enol*pyruvate, myokinase, and dexamethasone were purchased from Sigma. Pyruvate kinase and isobutylmethylxanthine were from Calbiochem. Geneticin (G418), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, streptomycin, amphotericin B and trypsin were purchased from Gibco. Alprenolol was a generous gift of Hassle Pharmaceutical (Sweden). ICI-118551 and betaxolol were gifts from Imperial Chemical Industries and Synthelabo, respectively.

2.2. Plasmid construction and cell culture

The human β_2-adrenoceptor clone pTF (Kobilka et al., 1987) was subcloned into the eucaryotic vector pMAM*neo* that contains a dexamethasone-inducible MMTV-LTR promoter or pBC12BI (Cullen, 1987) that contains a constitutive SV40 promoter. The two constructs were transfected in mouse fibroblast L cells by calcium phosphate precipitation procedures (Mellon et al., 1981). Positive clones were selected for their resistance to geneticin (G418; 450 μg/ml) (Southern and Berg, 1982) and screened for β_2-adrenoceptor expression in a radioligand binding assay, using 400 pM [^{125}I]cyanopindolol as the radioligand and 10 μM alprenolol to define non-specific binding. The transfected L cells were grown as monolayer in 75 cm^2 flasks containing DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), and glutamine (1 mM) in an atmosphere of 95% air and 5% CO_2 at 37°C.

2.3. Cell desensitization

Nearly confluent cells were incubated with fresh media supplemented as above and containing or not the desensitizing agent (isoproterenol or dibutyryl-cAMP) at the spec-

ified concentration and for the indicated period of time. When cells transfected with the pMAM*neo* β_2-adrenoceptor vector were used, a preincubation of 20 h with 2 μM dexamethasone was carried out in all cases to induce the expression of the β_2-adrenoceptor.

2.4. Membranes preparation

Cells were washed three times with 10 ml of phosphate-buffered saline at room temperature and mechanically detached in 10 ml of ice-cold buffer containing 5 mM Tris-HCl (pH 7.4), 2 mM EDTA, leupeptin (5 μg/ml), soybean trypsin inhibitor (5 μg/ml), and benzamidine (10 μg/ml). Cells were then lysed with a polytron homogenizer (1 burst of 5 s at maximum speed) and the lysates were centrifuged at $45\,000 \times g$ for 20 min at 4°C. The pelleted membranes were washed twice in the same buffer, resuspended in 0.5 ml of a buffer containing 75 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM EDTA, leupeptin (5 μg/ml), soybean trypsin inhibitor (5 μg/ml), and benzamidine (10 μg/ml), and used immediately for adenylyl cyclase activity determination and radioligand binding assays as described below. Protein content was determined according to the method of Bradford (1976) using a Bio-Rad kit.

2.5. Adenylyl cyclase assay

Adenylyl cyclase activity was measured according to the method of Salomon et al. (1974). Briefly, approx. 3 μg of membrane proteins were used in a total volume of 50 μl. The incubation mixture included 120 μM ATP, 1 μCi [α-^{32}P]ATP, 100 μM cAMP, 53 μM GTP, 2.8 mM phospho*enol*pyruvate, 0.2 U of pyruvate kinase, 1 U of myokinase, 30 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 0.8 mM EDTA, and 0.1 mM isobutylmethylxanthine. The final free Mg^{2+} concentration in the assay was 1.1 mM as calculated by the method of Iyengar and Birnbaumer (1982). Enzyme activity was determined in duplicate in the absence (basal activity) or in the presence of activators (isoproterenol 1 nM–100 μM or forskolin 100 μM).

2.6. Sequestration assay

Agonist-promoted sequestration was assessed by whole-cell binding assay as previously published (Suzuki et al., 1992). Briefly, nearly confluent cells were detached with trypsin (0.25%), washed three times with supplemented DMEM and incubated at 37°C for 15 min in 5 ml of the same media in the presence or absence of 10 nM or 1 μM isoproterenol. After incubation, 10 ml of ice-cold PBS was added to the cells which were then centrifuged at $500 \times g$ for 5 min and washed twice with ice-cold PBS. The cells were then resuspended in PBS and 0.05 ml of suspension was used with 225 pM [^{125}I]cyanopindolol for radioligand binding assays. Binding reactions were performed in supplemented DMEM at 13°C for 3.5 h in a final volume of 0.5 ml, and were terminated by rapid filtration over GF/C glass fiber filters (Whatman). Total cellular β-adrenoceptor was defined as the amount of radioligand binding inhibited by 0.3 μM (−)-propranolol, whereas cell surface receptor number was defined as the amount of [^{125}I]cyanopindolol binding displaced by 0.1 μM CGP-12177 at 13°C. The number of sequestered receptor was defined as the difference between total cellular and cell surface receptor and expressed as % of the total receptor number.

2.7. Radioligand binding assay

Radioligand binding assays were conducted essentially as described (Bouvier et al., 1988b) using approx. 8 μg of membrane proteins in a total volume of 0.5 ml. For saturation experiments, triplicate assay tubes contained 2–400 pM [^{125}I]cyanopindolol in the presence or absence of 10 μM alprenolol to define non-specific binding. For competition experiments, duplicate assay tubes contained approx. 50 pM [^{125}I]cyanopindolol and 0–100 μM of displacing agent (isoproterenol, epinephrine, norepinephrine, alprenolol, ICI 118551, or betaxolol). The binding reactions were incubated at room temperature 90 min and terminated by rapid filtration with ice-cold 25 mM Tris-HCl (pH 7.4) over Whatman GF/C glass fiber filters preincubated for at least 30 min in a buffer containing 25 mM Tris-HCl (pH 7.4), 0.1% bovine serum albumin, and 0.3% polyethylenimine. Data from competition and saturation experiments were analyzed by non-linear least-squares regression using the computer program SCATFIT (DeLean et al., 1982). For the competition studies on LD1 cells (Table 3), the data from 11 experiments were fitted individually according to a two-site model, the values of the fitted asymptotes (i.e., $f(x)$ as $x \rightarrow 0$ and $f(x)$ as $x \rightarrow$) were averaged, and each set of data was normalized to the mean asymptotes. The normalized data from the control experiments then were analyzed simultaneously to yield estimates of K_H, K_L, and the total capacity associated with each affinity (R_H and R_L, respectively); the latter values were used to calculate the proportion of receptors associated with each affinity. For data acquired after treatment of cells with either 1 μM isoproterenol or 1 mM dibutyryl-cAMP, the normalized data were fitted simultaneously assuming the affinities obtained from the control experiments; in each case, fixing K_H and K_L to the control values did not increase the sum of squares relative to that observed if the parameters were allowed to float ($P > 0.25$). The values of R_H and R_L obtained were used to calculate the proportion of receptors associated with each affinity. If both the affinities and the binding capacities were fixed to the values obtained from control experiments, a significant increase in the sum of squares was observed ($P < 0.01$).

Table 1
Pharmacological characterization of the human β_2-adrenoceptor expressed in cells

Antagonists	K_i (nM)	Agonists	K_i (nM)
Alprenolol	0.3	Isoproterenol	19.9
ICI-118551	1.4	Epinephrine	83.3
Betaxolol	6.1	Norepinephrine	543

Membranes derived from LD1 cells were prepared and assayed for [^{125}I]cyanopindolol binding. A K_d value of 22 ± 7 pM for [^{125}I]cyanopindolol binding was obtained from saturation experiments. The competition binding assays were carried out in the presence of ~ 50 pM of [^{125}I]cyanopindolol and increasing concentrations (1×10^{-10} to 1×10^{-4} M) of antagonists (alprenolol, ICI-118551 and betaxolol) and agonists ((−)-isoproterenol, (−)-epinephrine, (−)-norepinephrine). Similar data were obtained with membranes derived from LD10 cells.

2.8. Statistical analysis

All data are reported as group means ± S.E.M. Statistical significance of the differences between treated and control groups were assessed by paired *t*-test.

3. Results

3.1. Expression of the human β_2-adrenoceptor in L cells

The human β_2-adrenoceptor coding sequence was cloned into a dexamethasone-inducible vector (pMAM*neo* β_2-adrenoceptor) and transfected in mouse fibroblast L cells which lack β-adrenergic-stimulated adenylyl cyclase activity (data not shown). Cellular clones were selected for their G418 resistance. The β_2-adrenoceptor expression level was then assessed in the resistant clones by [^{125}I]cyanopindolol binding following a 20 h induction with 2 μM dexamethasone. Cell lines expressing either a low or a high level of receptor (LD1: 0.22 ± 0.01 pmol/mg of protein; LD10: 2.7 ± 0.5 pmol/mg of protein) were se-

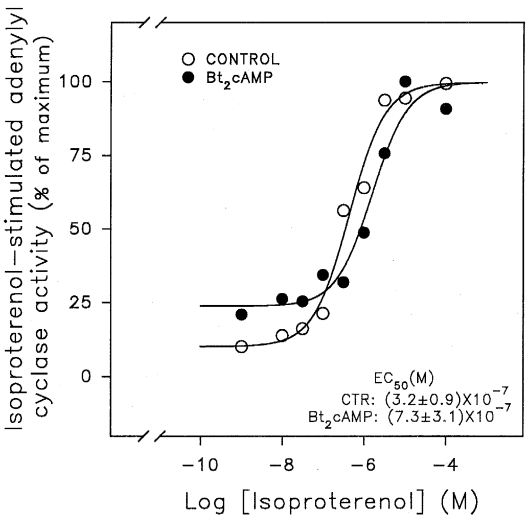

Fig. 2. Bt_2cAMP-induced desensitization of the β_2-adrenoceptor in L cells expressing a high number of receptor (LD10). LD10 cells were incubated in the absence (○) or the presence of 1 mM (●) Bt_2cAMP for 10 min at 37°C. Adenylyl cyclase activity was then measured in membrane preparations derived from these cells. The isoproterenol dose-response curve shown is representative of three independent experiments. The data are expressed as a percentage of the response observed in control membranes.

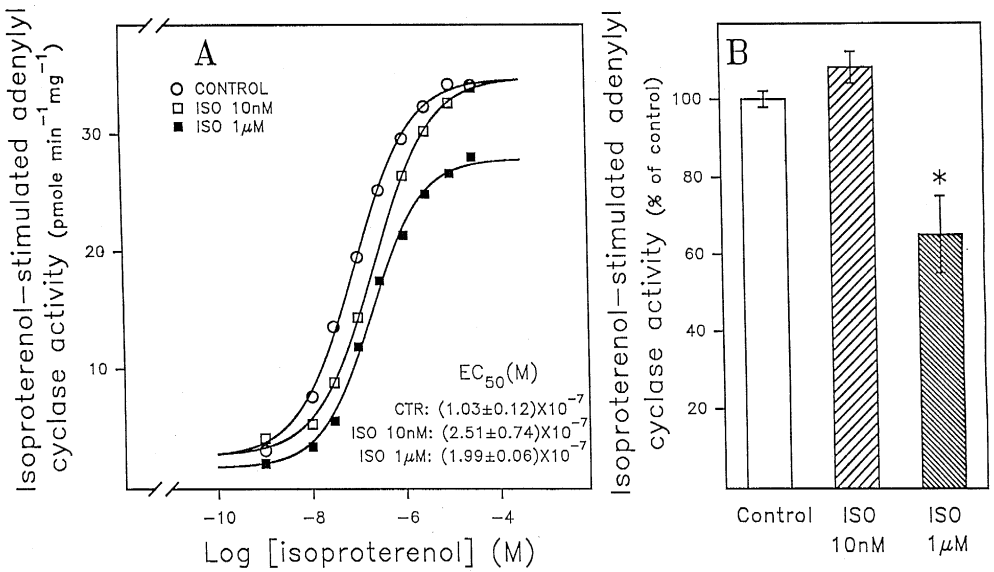

Fig. 1. Agonist-induced desensitization of the β_2-adrenoceptor in L cells expressing a high number of receptor (LD10). LD10 cells were incubated in the absence (○) or the presence of 10 nM (□) or 1 μM (■) isoproterenol for 10 min at 37°C. Adenylyl cyclase activity was then measured in membrane preparations derived from these cells. (A) Dose-response curve of the isoproterenol-stimulated adenylyl cyclase activity expressed in pmol of cAMP produced per min per mg of membrane protein. This figure is representative of three independent experiments. The EC_{50} indicated represent the mean ± S.E.M. of these experiments. (B) Maximal isoproterenol-stimulated activity expressed as a percentage of the response observed in control membranes. The data shown represent the mean ± S.E.M. of three distinct experiments. [a] $P < 0.05$.

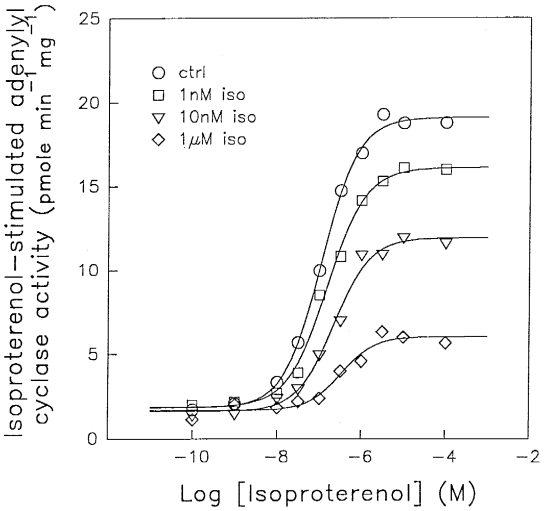

Fig. 3. Desensitization induced by increasing concentrations of isoproterenol on L cells expressing low number of receptor (LD1). LD1 cells were pretreated or not (○) with increasing concentrations of isoproterenol 1 nM (□), 10 nM (▽) and 1 μM (◇) for 10 min at 37°C. Adenylyl cyclase activities were then measured in membrane preparation derived from these cells. The data shown are expressed in pmol of cAMP produced per minute per mg of membrane protein and are representative of two independent experiments.

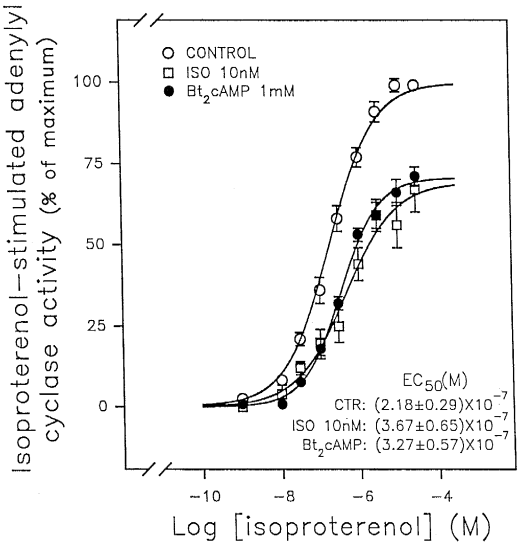

Fig. 5. cAMP-induced desensitization. LD1 cells were incubated in the absence (○) or presence of 10 nM isoproterenol (□) or 1 mM Bt_2cAMP (●) for 10 and 15 min respectively. The isoproterenol-stimulated adenylyl cyclase activity was measured in membrane preparations and expressed as the percentage of maximal stimulation observed in membranes derived from control cells. Data represent the mean ± S.E.M. of seven independent experiments.

lected for thorough characterization of their desensitization patterns. These correspond to cell lines expressing approx. 12 000 and 150 000 receptors/cell respectively. The pharmacological properties of the human β_2-adrenoceptor expressed in L cells were identical to those observed in other transfected cell lines (Fraser et al., 1987; Tate et al., 1991; Suzuki et al., 1992). A K_d value of 22 ± 7 pM was found for the radioligand [^{125}I]cyanopindolol in saturation isotherm experiments. In competition binding experiments, the order of potency of agonists to inhibit [^{125}I]cyanopindolol binding was: (−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine whereas for antagonists the order was: alprenolol > ICI-118551 > betaxolol (Table 1). No specific [^{125}I]cyanopindolol binding was detected in L cells transfected with the pMAM*neo* vector alone.

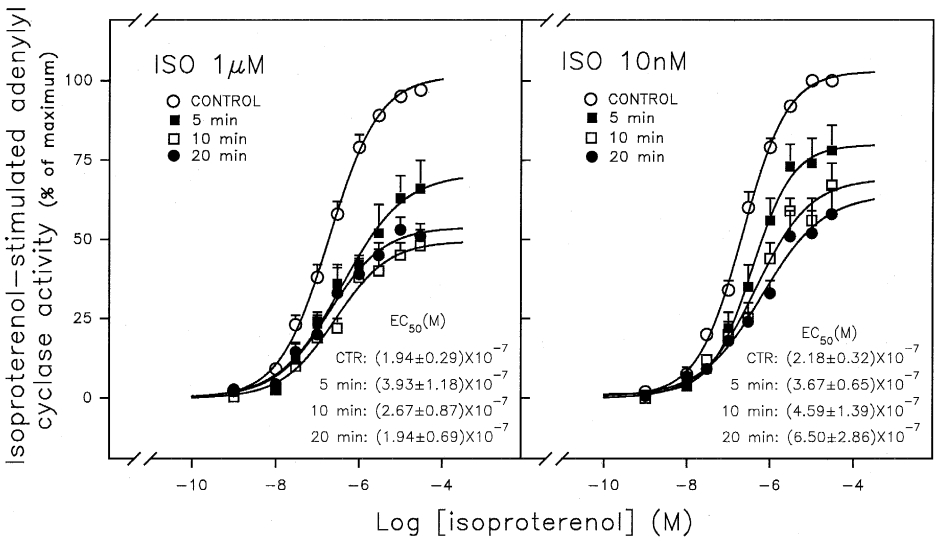

Fig. 4. Time-course of agonist-induced desensitization in L cells expressing a low number of receptors (LD1). LD1 cells were pretreated with 1 μM (left panel) or 10 nM (right panel) isoproterenol for the indicated period of time at 37°C. Adenylyl cyclase activities were measured in membrane preparations derived from these cells. For each experiment, the maximal isoproterenol response minus the basal response of the control cells was set to 100%, and all other cyclase activities were normalized to that. Adenylyl cyclase activities are presented as the means ± S.E.M. of four independent experiments.

3.2. Agonist- and dibutyryl-cAMP-induced desensitization of the β_2-adrenoceptor-stimulated adenylyl cyclase activity

First, the desensitization patterns induced by nanomolar and micromolar concentrations of isoproterenol were characterized in the highly β_2-adrenoceptor expressing LD10 cells. Cells were incubated with 10 nM or 1 μM isoproterenol for 10 min at 37°C, washed extensively at 4°C and membranes prepared and assayed for agonist-stimulated adenylyl cyclase activity as described in Section 2. As shown in Fig. 1, pretreatment of the cells with 10 nM isoproterenol led to a twofold decrease in the potency of isoproterenol to stimulate adenylyl cyclase activity but did not alter the efficacy of isoproterenol to maximally stimulate the enzyme. This is illustrated by the modest rightward shift of the isoproterenol dose-response curve shown in panel A. However, when LD10 cells were pre-exposed to a concentration of 1 μM isoproterenol, the rightward shift of the isoproterenol dose-response curve was accompanied by a significant decrease in the maximal isoproterenol-stimulated adenylyl cyclase activity. Indeed, as shown in panel B, the agonist-stimulated adenylyl cyclase V_{max} was reduced by more than 30%. The desensitizing effect of a direct activator of protein kinase A, dibutyryl-cAMP (Bt_2cAMP), was then assessed. As seen in Fig. 2, and much like what is observed following treatment with 10 nM isoproterenol, treatment of LD10 cells with 1 mM Bt_2cAMP, for 10 min, slightly decreased the potency of isoproterenol to stimulate the adenylyl cyclase activity without affecting its efficacy. The clearly distinct patterns of desensitization evoked by stimuli believed to stimulate protein kinase A only (10 nM isoproterenol and 1 mM Bt_2cAMP) and that believed to also involve β-adrenoceptor kinase confirmed what had been previously reported (Hausdorff et al., 1989; Lohse et al., 1990; Clark et al., 1989). Very similar results were also obtained using L cells transfected with pBC12BI β_2-adrenoceptor and constitutively expressing a relatively high number of receptor (LC10 expressing approx. 1.0 pmol/mg of membrane protein; data not shown).

The effects of the same desensitizing stimuli was then assessed in the low β_2-adrenoceptor expressing LD1 cells. As shown in Fig. 3, pretreatment for 10 min with either 10 nM or 1 μM isoproterenol led to a dramatic reduction in the efficacy of the agonist to maximally stimulate the adenylyl cyclase. In fact pre-stimulation with as little as 1 nM isoproterenol led to a desensitization pattern mainly characterized by a reduction in agonist efficacy. This contrasted with the results obtained in cells expressing a higher number of receptor (LD10 and LC10). The desensitization patterns evoked by 1 μM and 10 nM isoproterenol were thus further characterized in LD1 cells. As shown in Fig. 4, no qualitative difference in the desensitization patterns evoked by nanomolar and micromolar concentrations of isoproterenol could be observed in these cells. Both treatment led to a time-dependent reduction of the isoproterenol efficacy to maximally stimulate adenylyl cyclase activity. Although the desensitization appeared to be slightly faster in cells pre-treated with the micromolar agonist concentration, the increase in the EC_{50} was more sustained following treatment with 10 nM isoproterenol.

To assess if the desensitization pattern observed following the 10 nM isoproterenol pretreatment could be mimicked by direct activation of protein kinase A, LD1 cells were pre-treated with 1 mM Bt_2cAMP for 15 min. This pretreatment led to a 30% decrease in the maximal isoproterenol-stimulated adenylyl cyclase activity which was accompanied by a rightward shift of the isoproterenol dose-response curve (Fig. 5). The desensitization pattern is virtually identical to that observed in cells pre-treated with 10 nM isoproterenol. These observations therefore support the notion that the desensitization induced by nanomolar concentrations of isoproterenol could be entirely protein kinase A-mediated.

Table 2
Effects of the pretreatment with isoproterenol or Bt_2cAMP on adenylyl cyclase activity

Treatment	EC_{50} (nM)	Basal	Isoproterenol (maximal)	Forskolin (100 μM)
LD1 cells (220 ± 10 fmol / mg of membrane proteins)				
Control	210 ± 70	0.3 ± 0.3	20.1 ± 0.7	40 ± 8
Isoproterenol 1 μM	270 ± 90	0.1 ± 0.1	8.4 ± 1.0 [a]	45 ± 10
Isoproterenol 10 nM	370 ± 140	0.2 ± 0.1	16.3 ± 0.6 [a]	39 ± 7
Bt_2cAMP 1 mM	330 ± 60	0.3 ± 0.1	16.3 ± 0.8 [a]	36 ± 4
LC1 cells (148 ± 27 fmol / mg of membrane proteins)				
Control	250 ± 50	3.6 ± 0.6	22.9 ± 2.2	37 ± 4
Isoproterenol 1 μM	200 ± 60	4.2 ± 1.7	11.4 ± 3.9 [a]	44 ± 23
Isoproterenol 10 nM	770 ± 130 [a]	2.1 ± 0.5	16.2 ± 1.9 [a]	34 ± 7
Bt_2cAMP 1 mM	555 ± 275 [a]	2.8 ± 0.5	18.3 ± 2.7	34 ± 15
Butyric acid 1 mM	140 ± 10	2.7 ± 1.5	23.5 ± 2.8	34 ± 4

All values are pmol/min per mg membrane proteins. LD1 or LC1 cells were pretreated with the indicated agents for 10 min at 37°C. Adenylyl cyclase activity was measured on membrane preparations derived from these cells. Results are expressed as the means ± S.E.M. of 3–7 independent experiments.
[a] $P < 0.05$ vs. control condition.

To rule out the possibility that the desensitization pattern evoked in LD1 cells by nanomolar isoproterenol concentration reflects a trivial specific feature of this particular cellular clone rather than a general property of cells expressing a low number of receptor, the desensitization pattern was characterized in yet another cell line, the LC1. The LC1 cell line is a clonal isolate derived from L cells transfected with the pBC12BI β_2-adrenoceptor vector and expressing approx. 0.150 pmol of human β_2-adrenoceptor per mg of membrane protein. The adenylyl cyclase activity parameters obtained for LC1 and LD1 cells upon 10 min desensitization with 1 μM isoproterenol, 10 nM isoproterenol and 1 mM Bt_2cAMP are compiled in Table 2. The three desensitizing treatments led to consistent reduction in the maximal isoproterenol-stimulated adenylyl cyclase activity without significantly affecting the ability of forskolin to activate the enzyme in the two cell lines thus suggesting a receptor-specific desensitization. No change in receptor density was observed following any of the treatments excluding the contribution of receptor down-regulation to the desensitization observed. In an effort to confirm that the reduction in the maximal stimulated adenylyl cyclase activity induced by the treatment with 10 nM isoproterenol truly results from a protein kinase A-promoted uncoupling and not sequestration of the receptor, the sequestration pattern was studied in LC1 cells. As shown in Fig. 6, treatment with 10 nM isoproterenol did not increase the proportion of sequestered receptor above the level observed in untreated cells. In contrast, treatment with 1 μM isoproterenol promoted a significant increase in sequestration.

To control that the effect of Bt_2cAMP did not result from a non-specific action of the butyric acid itself, cells were treated for 10 min with 1 mM butyric acid. As shown in Table 2, this treatment had no effect on the isoproterenol- or forskolin-stimulated adenylyl cyclase activity

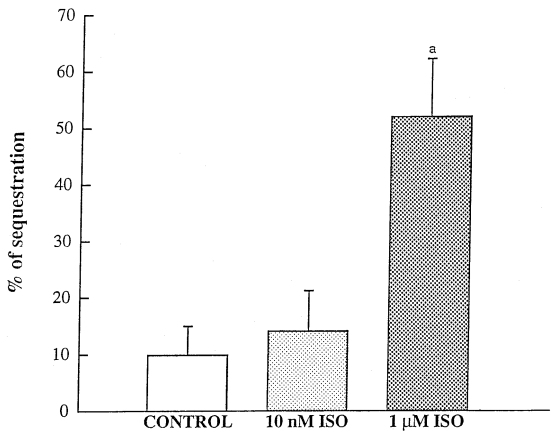

Fig. 6. Agonist-induced sequestration. LC1 cells were incubated in the absence (control) or presence of 10 nM and 1 μM for 15 min at 37°C. The level of sequestration was assessed by whole-cell binding and expressed as percentage of total receptor number. Data represent the mean ± S.E.M. of seven independent experiments. [a] $P < 0.05$.

Table 3
Effects of desensitization with 1 μM isoproterenol and 1 mM Bt_2cAMP on the β_2-adrenoceptor binding properties

	% K_H (2.4 ± 10 nM)	% K_L (180 ± 90 nM)
Control	57 ± 7	43 ± 6
Isoproterenol 1 μM	40 ± 4 [a]	60 ± 4
Bt_2cAMP	39 ± 8 [a]	61 ± 7

The normalized data from the control experiments ($n = 5$) were analyzed simultaneously to yield estimates of K_H, K_L, and the relative number of total receptors associated with each affinity. For each of the other two conditions ($n = 3$), the normalized data were fitted simultaneously assuming the affinities obtained from the control experiments to yield estimates of the proportion of receptors associated with each affinity. In each case, if both the affinities and the binding capacities were fixed to the values obtained from control experiments, a significant increase in the sum of squares was observed ($P < 0.01$). Further details pertaining to the analyses can be found in Section 2.
[a] $P < 0.05$ vs. control condition.

confirming that the effects observed resulted from protein kinase A activation.

3.3. Effects of isoproterenol- and Bt_2cAMP-induced desensitization on the agonist-binding properties of the β_2-adrenoceptor

Isoproterenol competition curves of [^{125}I]cyanopindolol binding in membranes derived from LD1 cells were biphasic and best resolved by a two-affinity-state model using iterative non-linear least-square fitting of the non-transformed data (De Lean et al., 1980). These correspond to the guanine nucleotide-sensitive high-affinity state and the low-affinity state of the receptor for agonist. The capacity of the β_2-adrenoceptor to form the guanine-nucleotide-sensitive high-affinity state is believed to reflect the ability of the receptor to productively interact with G_s. As shown in Table 3, 57% of the receptor present in LD1 cells were found to be in the high-affinity state under control conditions. Pretreatment with either isoproterenol or Bt_2cAMP significantly reduced the proportion of sites in the high-affinity state consistent with a partial uncoupling of the β_2-adrenoceptor from G_s. When bindings were conducted in the presence of 100 μM Gpp(NH)p the displacement curves were best fitted to a single low-affinity site.

4. Discussion

Previous studies have suggested that desensitizing conditions which lead to the activation of protein kinase A result in a reduction of the potency of the agonist to stimulate the adenylyl cyclase without affecting the maximal receptor-mediated stimulation (Hausdorff et al., 1989; Clark et al., 1987). In the present study, we confirm that exposure of L cells expressing a high number of β_2-adren-

oceptor to nanomolar concentrations of isoproterenol or to a cAMP analogue indeed leads to such a pattern of desensitization. However, in L cells expressing a lower number of receptor the changes in β-adrenergic-stimulated adenylyl cyclase activity induced by 10 nM or 1 μM isoproterenol are similar. In fact, both treatments lead to a decrease in the maximal production of cAMP upon subsequent stimulation. A decrease in agonist efficacy was also observed following preincubation of these cells with 1 nM isoproterenol, strongly suggesting that protein kinase A activation alone (Lohse et al., 1990) can lead to such a desensitization pattern in L cells. Moreover, direct activation of protein kinase A by Bt_2cAMP produced a similar desensitization phenotype thus supporting the notion that protein kinase A-phosphorylation of the β_2-adrenoceptor is sufficient to cause a decrease in the maximal isoproterenol-stimulated adenylyl cyclase activity. These observations do not represent idiosyncrasies of a single cell line since they were confirmed in different L cell isolates transfected with either pMAM*neo* β_2-adrenoceptor (LD1, LD10) or pBC12BI β_2-adrenoceptor (LC1, LC10). The absence of agonist-promoted sequestration following treatment with 10 nM isoproterenol clearly indicates that the decrease in maximal response induced by this desensitizing stimulus did not involve sequestration and most likely reflects functional uncoupling resulting from phosphorylation.

The difference in desensitization profiles between cells expressing 0.22 and 2.7 pmol of β_2-adrenoceptor/mg of protein recalls similar observations following receptor alkylation which have been attributed to spare receptors (Furchgott and Bursztyn, 1967). According to the concept of spare receptors, in a system where the number of receptor significantly exceeds that required to maximally activate an effector, incrementally decreasing the number of receptors would be expected first to decrease the potency of an agonist to stimulate the enzyme and subsequently to decrease the maximal effect of the agonist (Hausdorff et al., 1989; Barber, 1986; Bouvier et al., 1989). In cells lacking spare receptors any decrease in receptor number leads to a proportional decrease in maximal stimulation. The effects of treating cells with increasing concentrations of isoproterenol thus resemble the effects of increasing receptor alkylation; L cells expressing 2.7 and 0.22 pmol β_2-adrenoceptor/mg of protein are affected in a manner analogous to systems with and without spare receptors, respectively. Still, absolute receptor density cannot be used to predict whether or not protein kinase A-mediated desensitization will lead to loss of maximal responsiveness since this will most likely vary from one cell line to the next. Indeed, a decreased potency with no change in the efficacy of epinephrine to stimulate the adenylyl cyclase (Clark et al., 1987) has been observed in S49 cells which express a relatively small number of receptor (0.11–0.38 pmol/mg) (Proll et al., 1992; Kassis and Fishman, 1984). The desensitization pattern observed probably reflects more the receptor/G_s/adenylyl cyclase ratio existing in a given cell line than the absolute number of receptors. The isoforms of G_s subunits and adenylyl cyclase expressed could also be important. Nevertheless, our study clearly indicates that, in a given cell type, the pattern of desensitization is influenced by the number of receptor and that, at least in L cells, low level of stimulation and Bt_2cAMP pretreatment each can induce a reduction in maximal β_2-adrenoceptor responsiveness. It therefore follows that the ability of a stimulus to promote such a desensitization pattern does not necessarily indicate the participation of β-adrenoceptor kinase and β-arrestin.

In their study formalizing the relationship between receptor number and agonist efficacy, Whaley et al. (1994) predicted that the loss of coupling efficiency that is induced by desensitization would first manifest itself as an increase in EC_{50} in cells expressing high receptor density while both a reduction in V_{max} and an increase in EC_{50} would be seen in cells expressing low receptor level. Our study clearly shows that their prediction was exact. Indeed, it demonstrates that the level of receptor expression can have a dramatic effect on the amplitude of desensitization observed in a given system and reinforces the idea that receptor density most be taken into consideration before an accurate assessment of the desensitization can be made.

In cells expressing the β_2-adrenoceptor to a low level (LD1 and LC1), the extent of uncoupling provided by protein kinase A phosphorylation is sufficient to promote a reduction in the agonist-stimulated adenylyl cyclase V_{max} which is comparable to that observed in conditions involving phosphorylation by β-adrenoceptor kinase. The agonist binding data presented are consistent with such a notion. Indeed, the desensitization induced by either 1 μM isoproterenol or by 1 mM Bt_2cAMP was accompanied by similar reductions in the proportion of β_2-adrenoceptor exhibiting high affinity for agonist. However, in cells expressing the β_2-adrenoceptor in excess (LD10, LC10) further uncoupling, most likely reflecting the exclusive interaction of β-arrestin with the β-adrenoceptor kinase-phosphorylated receptor (Pitcher et al., 1992), is required to promote a reduction in V_{max}. In addition to further uncoupling the β_2-adrenoceptor from G_s, β-arrestin may also act to maintain the desensitized state of the receptor, by preventing dephosphorylation, as does arrestin for the rhodopsin receptor (Palczewski et al., 1989). Finally, although it has been proposed that sequestration plays little role in the desensitization promoted by micromolar level of isoproterenol (Lohse et al., 1990), one cannot exclude its contribution to the desensitization observed following such stimulation as a significant agonist-promoted sequestration occurs under these conditions (Fig. 6).

The following equation: $EC_{50}\, r/K_d - EC_{50} = k_{-1}/k_1$ has been proposed as a way to accurately calculate and compare the coupling efficiency of receptors (Whaley et al., 1994). In this equation, 'K_d' is the dissociation constant of the agonist tested, 'r' is the number of receptor

expressed, 'k_{-1}' is determined by the endogenous GTPase activity and 'k_1' represents the rate constant for the agonist-mediated activation of the adenylyl cyclase. Since k_{-1} can be assimilated to a constant in a given cell type, the ratio k_{-1}/k_1 can be used as a comparative estimate of the receptor coupling efficiency. Although this equation can certainly be used, to assess the loss of coupling efficiency in a system in which only the EC_{50} is affected (e.g. the protein kinase A-mediated desensitization in LD10 and LC10 cells), it is certainly not appropriate in desensitizing conditions leading to a reduction in V_{max}. This is particularly evident when considering the desensitization pattern evoked by a micromolar concentration of isoproterenol in LD1 cells. Any mathematical assessment of the loss of coupling efficiency in such a system requires that both EC_{50} and V_{max} be considered. The details of such a mathematical treatment have been previously reported (Lohse, 1990).

In summary, the results reported here show that, in L cells expressing a relatively low number of receptor, protein kinase A-mediated phosphorylation of the β_2-adrenoceptor is sufficient to lead to a decrease in the agonist-stimulated adenylyl cyclase V_{max}. In contrast, in cells expressing a higher number of receptor, levels of stimulation which would lead to phosphorylation by β-adrenoceptor kinase and binding of β-arrestin are required to produce such a pattern of desensitization. It therefore follows that the extent of desensitization that can be mediated by protein kinase A is highly dependent on the level of receptor expression.

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