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# DOWN-REGULATION OF SURFACE BETA-ADRENOCEPTORS ON INTACT HUMAN MONONUCLEAR LEUKOCYTES

## TIME-COURSE AND ISOPROTERENOL CONCENTRATION DEPENDENCE

DAGNY SANDNES, INGUN GJERDE, MAGNE REFSNES and STEN JACOBSEN
Department of Pharmacology, University of Oslo, P.O. Box 1057 Blindern, 0316 Oslo 3, Norway

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**Abstract**—Incubation of human mononuclear leukocytes (MNL) *in vitro* with isoproterenol resulted in a rapid loss of surface beta-adrenoceptors, determined by radioligand binding at 4°. Isoproterenol concentrations in the range of 10 nM to 100 μM resulted in significant down-regulation of beta-adrenoceptors. At a concentration of 1 μM isoproterenol, the time-dependent loss of surface beta-adrenoceptors closely paralleled the loss in isoproterenol-stimulated adenylate cyclase activity. If receptor number in intact cells was determined at 32°, hardly any loss in receptor number was observed, due to reversal of down-regulation during the incubation period. When beta-adrenoceptor number in broken cell preparations was determined by [$^{125}$I]cyanopindolol binding at 37° no significant loss was observed, even after 2 hr of isoproterenol treatment, while [$^{3}$H]CGP-12177 binding resulted in a similar reduction in binding sites as in intact cells. Reversal of loss in surface beta-adrenoceptors was rapid after 1 hr pretreatment with isoproterenol, but followed a biphasic time course after 4 hr pretreatment, with an initial rapid return of about 40% of the down-regulated receptors, followed by a slow, gradual reappearance of receptors. The results indicate that catecholamine exposure leads to a rapid sequestration of MNL surface beta-adrenoceptors away from the cell surface, to a compartment where they are inaccessible to the hydrophilic ligand [$^{3}$H]CGP-12177 as well as to the lipophilic ligand [$^{125}$I]cyanopindolol at 4°. Up to 2 hr of isoproterenol treatment does not lead to any breakdown of sequestered beta-adrenoceptors, as they are still recognized by [$^{125}$I]cyanopindolol binding in broken cell preparations.

The presence of beta-adrenoceptors on human mononuclear leukocytes (MNL) is well established [1, 2]. These cells have been extensively used for studies of receptor changes occurring *in vivo* during drug treatment, or adaptive changes occurring physiologically or during disease states (for review see ref. 2). *In vitro* regulation of MNL beta-adrenoceptors during catecholamine exposure has been less well characterized, although Krall and coworkers have performed extensive studies of the regulation of adenylate cyclase activity [3–5]. These workers have also done one study of receptor changes in parallel with changes in cyclic AMP production [4]. However, as they used the lipophilic radioligand [$^{3}$H]-dihydroalprenolol to characterize the receptors and binding studies were performed at room temperature, the possibility of codetermination of sequestered receptors cannot be excluded. Recently, two other groups of investigators published data [6, 7] on beta-adrenoceptor changes in intact MNL following agonist treatment. By using the lipophilic ligand [$^{3}$H]dihydroalprenolol and the hydrophilic ligand [$^{3}$H]CGP-12177* [8] these investigators demonstrated that beta-adrenoceptors were rapidly redistributed into a cellular compartment to which hydrophilic compounds have limited access, while lipophilic compounds, such as the agonist zinterol, competed for [$^{3}$H]dihydroalprenolol binding to both sequestered and surface receptors [6]. Using [$^{3}$H]CGP-12177, the early time course of receptor disappearance from and reappearance to the cell surface was investigated [7]. The present study, using [$^{125}$I]cyanopindolol ([$^{125}$I]CYP) and [$^{3}$H]CGP-12177 confirm and extend their findings.

* Abbreviations used: cyclic AMP, cyclic adenosine 3′,5′-monophosphate; [$^{125}$I]CYP, [$^{125}$I]cyanopindolol; [$^{3}$H]-CGP-12177, (±)[$^{3}$H](4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazolo-2-on hydrochloride; $PGE_1$, prostaglandin $E_1$.

### MATERIALS AND METHODS

*Materials.* (−)[$^{125}$I]cyanopindolol ([$^{125}$I]CYP) (2200 Ci/mmol) and cyclic [2,8-$^{3}$H]AMP (52 Ci/mmol) were from New England Nuclear, Dreieich, F.R.G. (±)[$^{3}$H]CGP-12177 (41 Ci/mmol) and [α-$^{32}$P]ATP (triethylammonium salt, 25 Ci/mmol) were from the Radiochemical Centre, Amersham, Buckinghamshire, U.K. (−)Propranolol hydrochloride was a gift from Imperial Chemical Industries Ltd., London, U.K. (−)Isoproterenol bitartrate, ascorbic acid, ATP, GTP, cyclic AMP, bovine serum albumin (fraction V), 3-isobutyl-1-methyl-xanthine (IBMX), bovine γ-globulin, pyruvate kinase and $PGE_1$, were purchased from Sigma Chemical Co., St. Louis, MO. Forskolin was from Behring Diagnostics, La Jolla, CA, and phosphoenolpyruvate was from Boehringer, Mannheim, F.R.G. Dowex-50 (AG50W-X4, 200–400 mesh) was from Bio-Rad Labs, Richmond,

CA. Lymphoprep® and seronorm were purchased from Nyegaard & Co, Oslo, Norway. Polyethylene glycol was from Koch-Light Ltd, Haverhill, Suffolk, U.K. Instagel was purchased from Packard Instruments, Groningen, The Netherlands. All other chemicals were of analytical grade.

*Buffer.* A modified Krebs Ringer phosphate buffer was used: NaCl 122 mM, KCl 4.9 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.3 mM, $Na_2HPO_4$ 15.9 mM, D-glucose 10 mM, pH was adjusted to 7.4.

*Isolation and preparation of MNL.* Human mononuclear leukocytes (MNL) were isolated according to Bøyum [9] from 160 ml blood, obtained from healthy donors (27 males, 34 females, mean age 22.3 ± 0.7 years, range 18–47). Blood was mixed with $Na_2EDTA$ to obtain a final concentration of 3 mM and diluted 1:2 with 0.9% (w/v) NaCl. This mixture (40 ml), layered over Lymphoprep (10 ml), was centrifuged at 450 *g* for 20 min in a Heraeus Christ centrifuge (Varifuge st) at room temperature. The layer containing MNL was withdrawn and recentrifuged at 600 *g* for 5 min. The cells were washed twice in buffer (600 *g*, 5 min), resuspended in buffer and counted in a Bürker chamber. Viability, determined by the trypan blue exclusion test, was 95–98%.

*Desensitization of MNL.* Cells (5–10 × $10^6$ cells/ml) were incubated in a final volume of 10 ml with isoproterenol (1 μM final concentration, unless otherwise indicated) in a gyratory incubator at 32° for the indicated time periods. In order to inhibit oxidation of isoproterenol, 10 mM ascorbic acid was included. Control cells were similarly treated with 10 mM ascorbic acid. The incubation was stopped by the addition of 25 ml ice-cold buffer, and the following steps were performed at 4°. Cells were centrifuged in a Heraeus Christ (Varifuge K) centrifuge for 10 min at 600 *g* and washed twice with 25 ml buffer. Finally, cells were resuspended at a density of 0.5–1 × $10^6$ cells/ml and used for radioligand binding studies or centrifuged before broken cell preparations were made.

*Broken cell preparations.* Cells (0.5–1 × $10^6$ cells/ml) were lysed in ice-cold 5 mM Tris/HCl, pH 7.5, with 0.4 mM $MgCl_2$ and 3 mM KCl for 2 min at 4°. The buffer was then adjusted to 50 mM Tris/HCl, pH 7.5, 4 mM $MgCl_2$ and 30 mM KCl. For adenylate cyclase assays, aliquots were immediately frozen in liquid $N_2$ and stored at −80° for not more than a week before assay. For binding studies, the lysate was homogenized with 3 strokes of a motor-driven Teflon pestle and centrifuged twice in a Sorvall RC2-B centrifuge at 30,000 *g* for 15 min at 4°. The pellet was frozen in liquid $N_2$ and stored at −80° for less than 2 weeks.

*Adenylate cyclase assays.* The frozen lysates were thawed and incubated for 15 min at 30° in 100 μl of a mixture containing 0.2 mM of [α-$^{32}$P]ATP (approximately $10^6$ counts/min/tube), 1 mM cAMP, 4 mM $MgCl_2$, 30 mM KCl, 0.1 mM EDTA, 50 mM Tris/HCl pH 7.5, 0.2 mM IBMX, 1.5 I.U. pyruvate kinase, 2 mM phosphoenolpyruvate, 0.1% bovine serum albumin, and lysate equivalent to 50–75 μg protein. The reaction was terminated by heating at 95° for 2 min. [$^3$H]cyclic AMP (approximately 5000 counts/min/tube) was added for recovery determination, the tubes were centrifuged (2000 *g* for 5 min), and the labelled cyclic AMP in the supernatants was purified on a Dowex-50/alumina double column as described by Salomon *et al.* [10], and counted in a Packard Tri-Carb Scintillation spectrometer, model 3300.

*Radioligand binding to intact MNL.* For time and concentration curves, one-point determinations of [$^{125}$I]CYP binding were done. Cells (0.5–1 × $10^6$) were incubated in a total volume of 500 μl with 40 pM [$^{125}$I]CYP in the absence (total binding) and presence of 0.3 μM (−)propranolol (nonspecific binding) for 24 hr in a gyratory incubator in the cold room (4°). When the incubation was completed, viability was checked, the tubes were shaken on Whirlmix, and 2 ml of ice-cold incubation buffer was added. The mixture was poured over Whatman GF/C filters, the tubes rinsed once with 2 ml buffer, and the filters were rapidly washed twice with 12.5 ml ice-cold buffer. The filters were transferred to polypropylene tubes and radioactivity was determined in a Packard Auto-Gamma Scintillation Spectrometer Model 5110 at a counting efficiency of 75%. Specific binding was determined as the difference between total and nonspecific binding. The concentration of [$^{125}$I]CYP used was approximately five times the $K_d$, ensuring a saturation of about 90%.

For determination of receptor binding by Scatchard analysis [11], cells (1–2 × $10^6$) were incubated in a total volume of 500 μl with concentrations of [$^3$H]CGP-12177 ranging from 0.1 nM to 20 nM at 4° for 24 hr. The procedure following incubation was similar to the one described above except that filters were washed once with 10 ml ice-cold buffer. Filters were transferred to counting vials containing 0.5 ml distilled water, and 10 ml scintillation liquid (Instagel) was added. The vials were shaken for 20 min before radioactivity was determined in a Packard Tri-Carb Scintillation Spectrometer Model 3300 at a counting efficiency of 30%. Viability of cells was reduced by the prolonged incubations at 4°, but always to a similar extent in control and isoproterenol-treated cells. Only experiments where viability exceeded 85% were included.

*Radioligand binding to broken cell preparations.* The particulate fraction was thawed by adding 6 ml ice-cold incubation buffer (10 mM Tris/HCl pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA) and leaving on ice for about 1 hr before resuspension. Particulate protein (25–50 μg) was incubated in a gyratory incubator for 120 min at 37° with increasing concentrations of [$^{125}$I]CYP (3 pM–1.5 nM) in a total volume of 500 μl. The reaction was stopped by transferring the tubes to an ice-bath and further processing by polyethylene glycol precipitation as described by Iyengar *et al.* [12]. Control experiments were performed to verify that similar results were obtained with polyethylene glycol precipitation and the filtration method.

*Protein determinations.* Protein was determined according to the procedure of Lowry *et al.* [13] using bovine serum albumin or seronorm as standard.

*Analysis of data.* Data from Scatchard plots were analyzed on a DEC 1099 computer by the program "Ligand" [14]. The best fit of the data to a one-site or a two-site model was evaluated by the *F* statistics

test (incorporated in the program), which is based on the residual sum of squares. Paired data were analyzed by Student's *t*-test.

## RESULTS

### *Effect of isoproterenol concentration on down-regulation of surface beta-adrenoceptors on intact cells*

*In vitro* down-regulation of beta-adrenoceptor number on intact MNL has previously been investigated by Krall *et al.* [4]. However, these investigators utilized the lipophilic radioligand [$^3$H]dihydroalprenolol, and incubations were performed at room temperature. As [$^3$H]dihydroalprenolol does not distinguish between surface receptors and sequestered receptors [6, 8, 15–16], and the elevated temperature may lead to reversal of down-regulation, we have investigated beta-adrenoceptor number at 4°. In accord with other investigators [16, 17] we have found that at this temperature [$^{125}$I]CYP binds only to surface receptors [18].

In Fig. 1, the isoproterenol concentration dependence of surface beta-adrenoceptor down-regulation is shown. It can be seen that significant down-regulation was observed at concentrations of isoproterenol equal to or exceeding 10 nM, when the incubation time was 30 min. This is similar to the findings of Krall *et al.* [4], although our experimental conditions seem to lead to a higher degree of down-regulation. In the following experiments, 1 μM isoproterenol was used for down-regulation.

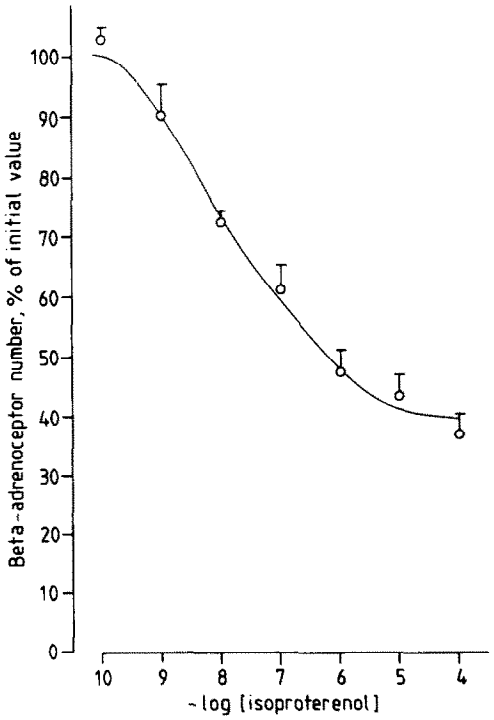


Fig. 1. Dependence of isoproterenol concentration for down-regulation of surface beta-adrenoceptors on intact MNL. Cells (5–10 × 10$^6$/ml) were incubated with increasing concentrations of isoproterenol in the presence of 10 mM ascorbic acid or with 10 mM ascorbic acid (control) for 30 min at 32°. After washing the cells as described in Materials and Methods, beta-adrenoceptor number was determined by [$^{125}$I]CYP binding (40 pM) in the absence (total binding) and presence (nonspecific binding) of 0.3 μM (−)propranolol at 4° for 24 hr. Specific binding was determined as the difference between total and nonspecific binding. 100% represents binding in control cells, and amounts to 2.00 ± 0.17 fmol/10$^6$ cells. Results are presented as mean ± SE of six experiments.

### *Time course of down-regulation of surface beta-adrenoceptor number in intact cells compared to adenylate cyclase activity in cell lysates*

In Fig. 2, the time course of the loss in surface beta-adrenoceptors determined by [$^{125}$I]CYP binding at 4° was compared to the isoproterenol-stimulated adenylate cyclase activity in cell lysates prepared in parallel from the same donors. The down-regulation of surface beta-adrenoceptors and the desensitization of adenylate cyclase activity appeared to follow a similar time course. A similar time course of beta-adrenoceptor number down-regulation was found in a separate series of experiments when [$^3$H]CGP-12177 was used as radioligand (data not shown). Although there was a slight, variable, time-dependent loss of both basal and stimulated adenylate cyclase activity, as previously observed by other investigators [19], there was no effect of the isoproterenol treatment *per se* on $PGE_1$—or forskolin-stimulated activity (Table 1). The loss in isoproterenol-stimulated activity was due to a loss in the maximum level of stimulation, rather than a change in $K_{act}$ (data not shown).

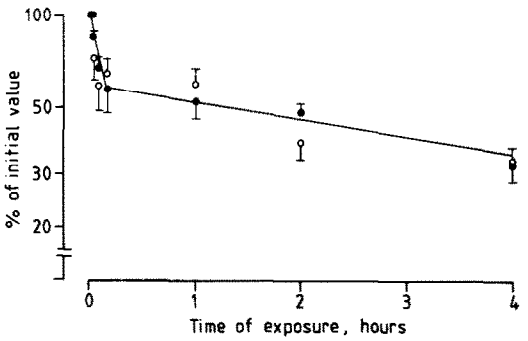


Fig. 2. Time course of down-regulation of surface beta-adrenoceptors on intact MNL and desensitization of isoproterenol-stimulated adenylate cyclase activity determined in cell lysates. Cells (5–10 × 10$^6$/ml) were incubated at 32° with 1 μM isoproterenol in the presence of 10 mM ascorbic acid. Control cells were incubated with 10 mM ascorbic acid. At the times indicated, cells were washed as described in Materials and Methods, and [$^{125}$I]CYP binding (40 pM) in the presence and absence of 0.3 μM propranolol was determined. In parallel, lysates were prepared for adenylate cyclase activity as described in Materials and Methods. Isoproterenol-stimulated adenylate cyclase activity (10 μM isoproterenol + 100 μM GTP, ○–○–○), is shown (basal values, determined in the presence of 100 μM GTP, were subtracted). 100% adenylate cyclase activity represents the mean of activity from control lysates at $t$ = 0 and $t$ = 120 min and was 18.9 ± 7.8 pmol cyclic AMP/mg/min (N = 4). Similarly, 100% receptor binding, (●–●–●) represents the mean of receptor number determined at $t$ = 0 and $t$ = 120 min, and was 2.24 ± 0.56 fmol/10$^6$ cells (N = 4). Results are presented as mean ± SE of three to four experiments.

When Scatchard analysis [11] of total [$^3$H]CGP-12177 binding to intact cells was performed, a loss in the number of receptors, rather than a change in radioligand affinity was found (Table 2, Fig. 3). We have also determined total receptor number in control and desensitized cells by [$^{125}$I]CYP binding, using procedures described in [18]. When nonspecific binding, determined in the presence of 0.3 $\mu$M (−)propranolol was subtracted, we found a down-regulation of 36% after 10 min of isoproterenol exposure (control: 2.82 ± 0.21 fmol/10$^6$ cells, $K_d$ = 17.5 ± 8.3 pM, isoproterenol-treated: 1.80 ± 0.58 fmol/10$^6$ cells, $K_d$ = 29.1 ± 8.6 pM, (mean ± SE, $N$ = 3)). In a separate series of experiments, total [$^{125}$I]CYP binding was determined over a greater concentration range and decomposed by computer analysis [14]. A similar degree of down-regulation was found (Fig. 4). The number of binding sites decreased from 4.71 ± 0.28 fmol/10$^6$ cells to 3.00 ± 0.44 fmol/10$^6$ cells after 10 min of isoproterenol exposure, while the affinity of [$^{125}$I]CYP was 5.3 ± 2.0 pM in control cells and 8.4 ± 0.3 pM in isoproterenol treated cells (mean ± SE, N = 3). It can be seen, both from the time curve in Fig. 2 and from Table 2, that the down-regulation of beta-adrenoceptor number initially followed a rapid time course ($t_{\frac{1}{2}}$ approximately 5 min). After 10 min, an additional phase of slow gradual loss of receptors was seen ($t_{\frac{1}{2}}$ aproximately 5 hr). In some experiments, binding experiments with [$^3$H]-CGP-12177 in intact cells were performed at 32° for 2 hr after the cells had been desensitized. In these experiments, receptor number returned almost to control level during the incubation period (Table 2).

*Determination of beta-adrenoceptor number in broken cell preparations*

In a separate series of experiments, beta-adrenoceptor number was determined both on intact cells and in particulate preparations from the same cells. On intact cells, beta-adrenoceptor number was determined at one concentration of [$^{125}$I]CYP as described in Materials and Methods. A similar degree of down-regulation as in previous experiments was found (Table 3). When beta-adrenoceptor number was determined by Scatchard analysis [11] of [$^{125}$I]CYP binding to broken cell preparations, no significant loss of beta-adrenoceptors was found (Fig. 5, Table 3). When [$^3$H]CGP-12177 binding was determined in the same type of preparation, however, a similar degree of down-regulation was observed as in intact cells (Table 3).

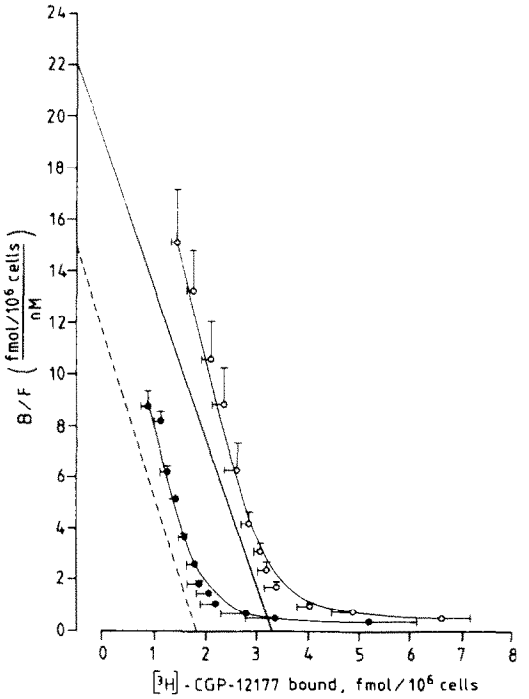


Fig. 3. Scatchard plot [11] of total [$^3$H]-CGP-12177 binding to intact MNL. Cells (5–10 × 10$^6$/ml) were incubated with 1 $\mu$M isoproterenol in the presence of 10 mM ascorbic acid at 32° for 5 min (●–●–●). Control cells (○–○–○) were incubated in the presence of 10 mM ascorbic acid. After washing, cells were incubated with increasing concentrations of [$^3$H]CGP-12177 for 24 hr at 4°, as described in methods. Total binding was decomposed by computer analysis [14] as described in Materials and Methods. Specific binding is indicated for isoproterenol-treated (– – –) and control cells (———). Nonspecific binding (not shown) was non-saturable, parallel to the abscissa, 0.19 ± 0.09 fmol/10$^6$ cells·nM in control cells and 0.17 ± 0.08 fmol/10$^6$ cells·nM in isoproterenol-treated cells. Results are presented as mean ± SE of four experiments.

Table 1. Adenylate cyclase activity in lysates from control and isoproterenol-treated cells

| Time of pretreatment | GTP (100 $\mu$M) | Adenylate cyclase activity (pmol/mg/min) GTP (100 $\mu$M) + isoproterenol (10 $\mu$M) | GTP (100 $\mu$M) + $PGE_1$ (28 $\mu$M) | Forskolin (100 $\mu$M) |
|---|---|---|---|---|
| Control, $t$ = 0 (N = 3) | 42.3 ± 12.2 | 64.1 ± 22.0 | 210.5 ± 81.0 | 443.7 ± 156.4 |
| Control, 2 hr (N = 3) | 43.5 ± 12.6 | 59.9 ± 16.0 | 181.3 ± 64.8 | 362.5 ± 138.4 |
| Isoproterenol, 2 hr (N = 3) | 46.0 ± 12.8 | 52.4 ± 14.2 | 192.3 ± 74.6 | 398.0 ± 164.4 |
| Control, $t$ = 0 (N = 4) | 26.1 ± 2.9 | 36.6 ± 4.1 | 148.6 ± 20.8 | |
| Control, 4 hr (N = 4) | 20.2 ± 3.5 | 36.9 ± 1.6 | 94.5 ± 20.5 | |
| Isoproterenol, 4 hr (N = 4) | 21.1 ± 4.6 | 25.4 ± 5.4 | 96.0 ± 23.5 | |

Cells (5–10 × 10$^6$ ml) were pretreated at 32° for the indicated time with 10 mM ascorbic acid (control) or 1 $\mu$M isoproterenol and 10 mM ascorbic acid (isoproterenol), washed and lysed as described in methods. Adenylate cyclase activity was determined as described in Materials and Methods. Results are presented as mean ± SE.

Table 2. Binding parameters of [$^3$H]-CGP-12177 binding to intact cells

| Time of isoproterenol treatment | | $B_{max}$ (fmol/$10^6$ cells) | % Change | $K_d$ (nM) |
|---|---|---|---|---|
| 2 min | Control | 2.53 ± 0.23 | −37.3 | 0.102 ± 0.022 |
| | Isoproterenol | 1.70 ± 0.09 | | 0.134 ± 0.038 |
| 5 min | Control | 2.96 ± 0.22 | −39.5 | 0.107 ± 0.011 |
| | Isoproterenol | 1.79 ± 0.13 | | 0.105 ± 0.008 |
| 10 min | Control | 2.25 ± 0.53 | −53.8 | 0.075 ± 0.005 |
| | Isoproterenol | 1.04 ± 0.33 | | 0.079 ± 0.009 |
| 60 min | Control | 1.68 ± 0.24 | −57.7 | 0.079 ± 0.010 |
| | Isoproterenol | 0.71 ± 0.14 | | 0.133 ± 0.048 |
| 120 min | Control | 3.27 ± 0.39 | −59.9 | 0.092 ± 0.020 |
| | Isoproterenol | 1.31 ± 0.07 | | 0.100 ± 0.019 |
| 10 min* | Control | 1.95 ± 0.61 | −13.8 | 0.077 ± 0.006 |
| | Isoproterenol | 1.68 ± 0.52 | | 0.122 ± 0.022 |
| 60 min* | Control | 1.46 ± 0.14 | −15.8 | 0.079 ± 0.015 |
| | Isoproterenol | 1.23 ± 0.16 | | 0.125 ± 0.011 |

Cells (5–10 × $10^6$/ml) were incubated with 10 mM ascorbic acid (control) or 1 μM isoproterenol in the presence of 10 mM ascorbic acid (isoproterenol) at 32° for the indicated time, washed, and incubated with [$^3$H]CGP-12177 as described in Materials and Methods. Total binding was decomposed by computer analysis [14] into specific binding and nonsaturable, nonspecific binding. $B_{max}$: number of specific binding sites, expressed as fmol/$10^6$ cells. $K_d$: Dissociation constant of [$^3$H]CGP-12177 binding to receptors, expressed in nM. Results are presented as mean ± SE of 4 experiments unless otherwise indicated.

* In these experiments, radioligand binding was determined by incubation at 32° for 2 hr.

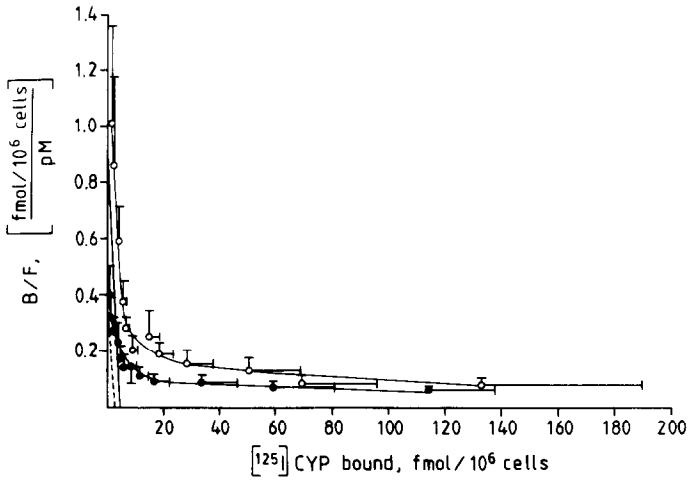


Fig. 4. Scatchard plot [11] of total [$^{125}$I]CYP binding to intact MNL. Cells (5–10 × $10^6$/ml) were incubated with 1 μM isoproterenol in the presence of 10 mM ascorbic acid at 32° for 10 min (●–●–●). Control cells (○–○–○) were incubated in the presence of 10 mM ascorbic acid. After washing, cells were incubated with increasing concentrations of [$^{125}$I]CYP ranging from 1.5 pM to 1.5 nM for 24 hr at 4°. Total binding was decomposed by computer analysis [14] into high affinity binding ($K_d$ 5–8 pM) representing receptor binding and low affinity binding ($K_d$ approximately 1 nM) representing nonspecific binding. Receptor binding is indicated for isoproterenol-treated (– – –) and control cells (———). Results are presented as mean ± SE of three experiments.

Table 3. Down-regulation of beta-adrenoceptor number in intact cells and particulate preparations from the same cells

| Time of pretreatment | | $B_{max}$ (fmol/$10^6$ cells) | % Change | $B_{max}$ (fmol/mg) |
|---|---|---|---|---|
| 5 min (N = 5) | Control | 3.22 ± 0.55 | −42.2 | 45.8 ± 9.4 |
| | Isoproterenol | 1.86 ± 0.30 | | 38.8 ± 9.6 |
| 10 min (N = 5) | Control | 3.31 ± 0.17 | −49.5 | 61.8 ± 4.1 |
| | Isoproterenol | 1.67 ± 0.19 | | 47.7 ± 4.0 |
| 60 min (N = 7) | Control | 3.20 ± 0.45 | −45.3 | 45.2 ± 6.2 |
| | Isoproterenol | 1.75 ± 0.26 | | 46.7 ± 7.2 |
| 120 min (N = 5) | Control | 3.17 ± 0.54 | −52.7 | 57.3 ± 5.7 |
| | Isoproterenol | 1.50 ± 0.21 | | 51.7 ± 6.4 |
| 60 min (N = 3) | Control | 4.59, 3.99* | −61.8 | 56.1 ± 8.2† |
| | Isoproterenol | 1.64, 1.63* | | 23.9 ± 3.3† |

Cells (5–10 × $10^6$/ml) were pretreated with 10 mM ascorbic acid (control) or 1 μM isoproterenol in the presence of 10 mM ascorbic acid at 32° for the indicated time periods. After washing, [$^{125}$I]CYP binding was determined in intact cells at 4° or in particulate preparations at 37°, as described in Materials and Methods. Results are presented as mean ± SE.

$B_{max}$: number of radioligand binding sites, expressed as fmol/$10^6$ cells or fmol/mg particulate protein.

* Binding in intact cells was only determined in two experiments, results of the individual experiments are presented.

† [$^3$H]CGP-12177 binding determined in particulate preparations.

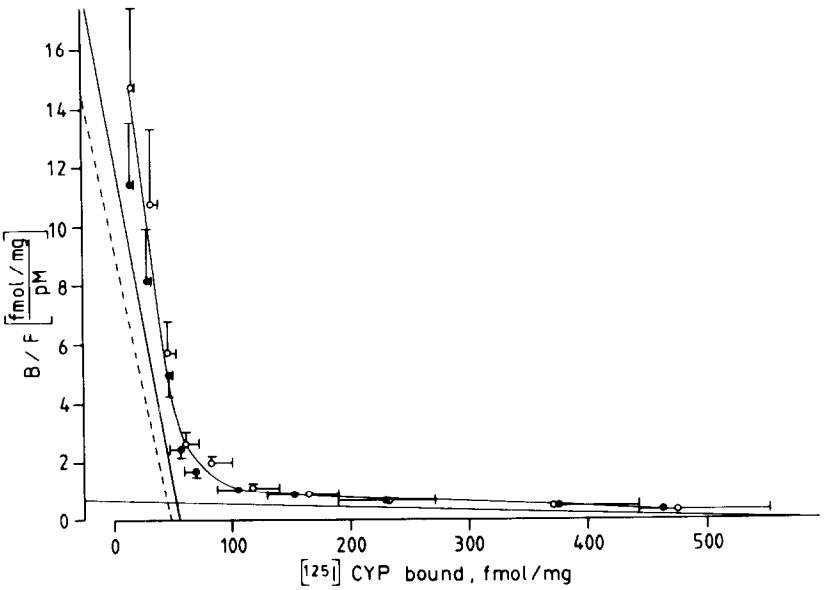


Fig. 5. Scatchard plot of total [$^{125}$I]CYP binding to particulate preparations from MNL. Cells (5–10 × $10^6$/ml) were incubated with 1 μM isoproterenol in the presence of 10 mM ascorbic acid (●–●–●) or ascorbic acid alone (control) (○–○–○) for 60 min at 32°. After washing, particulate preparations were prepared and [$^{125}$I]CYP binding was determined at 37° as described in Methods. Total binding was decomposed by computer analysis [14] into high affinity binding ($K_d$ approximately 3 pM) representing receptor binding, and low affinity binding ($K_d$ approximately 1 nM), representing non-specific binding. In some experiments an additional non-saturable component of non-specific binding was found. Receptor binding is indicated both for control (———) and isoproterenol-treated cells (– – –) while low affinity binding is indicated for control only. Results are presented as mean ± SE of seven experiments.

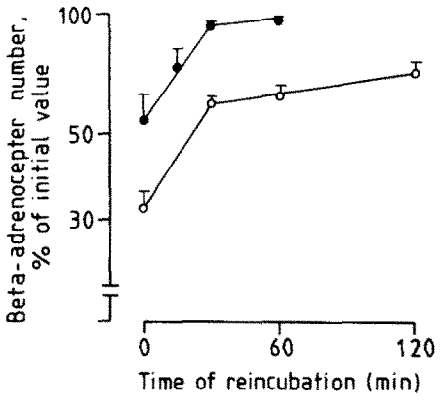


Fig. 6. Time course of reversal of down-regulation of surface beta-adrenoceptors. Cells (5–10 × $10^6$/ml) were incubated with 1 μM isoproterenol in the presence of 10 mM ascorbic acid at 32° for 1 hr (●–●–●, N = 3) or 4 hr (○–○–○, N = 4) before washing and reincubation at 32° (start at $t = 0$). At the indicated time points, cells were cooled to 4° and [$^{125}$I]CYP binding (40 pM) was determined as described in Materials and Methods. 100% represents the binding in control cells incubated with 10 mM ascorbic acid for 1 hr (2.32 ± 0.45 fmol/$10^6$ cells, N = 3) or 4 hr (2.68 ± 0.19 fmol/$10^6$ cells, N = 4) respectively. Control cells were also carried through the washing and additional incubation period to ensure that there was no adverse effect of the prolonged incubations on receptor number. Results are presented as mean ± SE.

*Reversibility of beta-adrenoceptor down-regulation in intact cells*

As we found no down-regulation of beta-adrenoceptor number in MNL particulate preparations, and rapid reversal of down-regulation during incubation of intact cells with [$^3$H]CGP-12177 at 32°, we investigated the time course of reversal of receptor loss. Cells were preincubated with 1 μM isoproterenol at 32° for 1 hr or 4 hr, washed at 4° and reincubated at 32°. At the times indicated, cells were cooled to 4° and binding studies were performed. The time course of receptor reappearance is shown in Fig. 6. After 1 hr of desensitization, the beta-adrenoceptor number returned to the original level within 1 hr ($t_{\frac{1}{2}}$ approximately 30 min). Even after 4 hr exposure to isoproterenol, the receptor number was returned to approximately 80% of control level after 2 hr incubation in the absence of isoproterenol. However, the time course was clearly biphasic. Initially, about 40% of the down-regulated receptors returned with a $t_{\frac{1}{2}}$ of about 30 min. Then a slower phase of recovery was observed, with a $t_{\frac{1}{2}}$ (assuming no further change in rate) of about 5 hr.

## DISCUSSION

The concentration dependence of beta-adrenoceptor down-regulation by isoproterenol in intact MNL was previously investigated by Krall *et al.* [4]. A decrease in [$^3$H]-dihydroalprenolol binding occurred at isoproterenol concentrations ranging from 10 nM to 100 μM, when the exposure period was 30 min. This is very similar to the concentration dependence demonstrated in Fig. 1. Although we observed a slight down-regulation at 1 nM isoproterenol, this did not occur in all experiments, and thus was not judged as significant. No dose–response curve was presented by Krall *et al.* but, judging from their Fig. 4, the extent of down-regulation determined by our experimental conditions is greater.

The loss in beta-adrenoceptors and isoproterenol-stimulated adenylate cyclase activity seem to be closely coupled, as demonstrated by our data in Fig. 2. The initial, rapid, time course of loss of beta-adrenoceptors is similar to the results of DeBlasi *et al.* [6, 7], who found a maximal reduction (approximately 50%) in surface beta-adrenoceptor number and isoproterenol-stimulated cyclic AMP production by 5 min, with no further decrease up to 40 min of incubation. The time course in Fig. 2 is clearly biphasic, with an initial rapid loss of surface beta-adrenoceptors and isoproterenol-stimulated adenylate cyclase activity up to 10 min ($t_{\frac{1}{2}}$ approximately 5 min), followed by a more gradual decline the next 4 hr with a $t_{\frac{1}{2}}$ of about 5 hr. The second, slow phase of beta-adrenoceptor loss was not observed by DeBlasi *et al.* [6, 7]. Other differences in methodology apart from the incubation temperature (37° vs 32° during isoproterenol treatment), may contribute to the different observations.

The isoproterenol-stimulated adenylate cyclase activity, although quite low in our preparation, is comparable to results reported by other investigators [21, 22], with an average increase over basal activity (determined in the presence of GTP) of about 50%. Variable levels of cyclic AMP in intact MNL [19, 24, 25] as well as variable adenylate cyclase activities in broken cell preparations from human MNLs have been reported [20–23]. This is partly due to the temperature during cell isolation [24], and partly due to a time- and temperature-dependent decline of basal [19, 25] as well as hormone-stimulated levels [19]. Clearly, more work is necessary to find optimal conditions for conserving adenylate cyclase activity in human MNL preparations. Due to individual variation, the standard errors of adenylate cyclase activity in Table 1 are rather great, but comparable to published values [20–22]. Although there was some loss of $PGE_1$- and forskolin-stimulated adenylate cyclase activity with time, the levels were comparable in control and isoproterenol-treated cells (Table 1) indicating that the isoproterenol-treatment *per se* did not lead to changes in these activities. Thus, it appears that isoproterenol-induced desensitization of MNL adenylate cyclase is homologous. This was also found by other investigators [6, 25, 26] when measuring hormone-stimulated cyclic AMP accumulation in MNL, while Tuck *et al.* [3] reported that isoproterenol induced heterologous desensitization to $PGE_2$.

The degree of down-regulation of surface beta-adrenoceptors on MNL, whether it was determined by [$^{125}$I]CYP binding or by [$^3$H]CGP-12177 binding appeared to be highly reproducible (Tables 2 and 3). There was a loss in the number of beta-adrenoceptors, with no change in the affinity of either radioligand (Figs 3 and 4, Table 2). In some experiments, [$^3$H]CGP-12177 binding to down-regulated cells has been determined in parallel to [$^{125}$I]CYP binding, and we have found excellent agreement in

receptor number determined with the two radioligands. During the preparation of this manuscript, data were published [28] indicating that [$^{125}$I]CYP binding to intact MNL at 4° identifies both surface and sequestered beta-adrenoceptors in isoproterenol-treated cells. In isoproterenol-treated cells, total number of beta-adrenoceptors, determined in the presence of 1 $\mu$M (−)propranolol was unchanged, while the affinity of [$^{125}$I]CYP to the receptors decreased. There is no obvious reason for this discrepancy in results, although there are significant differences in methodology, the most important perhaps being the post-incubation hypotonic treatment to reduce nonspecific binding, that was used by Motulsky *et al.* [28].

When [$^{3}$H]CGP-12177 binding was determined in intact cells at 32° after isoproterenol pretreatment, the receptor number returned almost to control level within the 2 hr incubation period (Table 2). When broken cell preparations were prepared from isoproterenol pretreated cells, no significant loss of [$^{125}$I]CYP binding sites was observed, while [$^{3}$H]CGP-12177 binding experiments revealed a similar degree of down-regulation as in intact cells (Fig. 5, Table 3). These findings, in addition to the rapid reversal of down-regulation, at least after 1 hr of isoproterenol pretreatment (Fig. 6), strongly indicate that isoproterenol treatment of MNL leads to a sequestration or internalization of beta-adrenoceptors to a compartment where they are not reached by the hydrophilic ligand [$^{3}$H]CGP-12177 at any temperature and by [$^{125}$I]CYP at 4°. This is similar to findings in a number of different cell types [6, 7, 15–17, 29–31] (for review see ref. 32).

The time curve of reappearance of beta-adrenoceptors after 4 hr of isoproterenol exposure was clearly biphasic. Initially, about 40% of the receptors returned at a similar rate as after 1 hr pretreatment ($t_{\frac{1}{2}}$ approximately 30 min. Then the process slowed appreciably down, and the return of receptors was not complete by 2 hr. This could be explained by degradation of receptors in lysosomes, necessitating synthesis of new receptors [32]. However, as we did not follow the process further and did not investigate the protein synthesis dependence of the reappearance of beta-adrenoceptors, the nature of the slow phase of the reappearance process is at present uncertain. Several investigators have recently questioned whether the initial phase of receptor sequestration is due to internalization [16, 34]. Receptors may initially be redistributed to a plasma membrane compartment where they are inaccessible to hydrophilic ligands and certain lipophilic ligands at low temperature [16]. Thus, the two phases of receptor reappearance may be due to reappearance from two compartments, one in the plasma membrane, and one in intracellular vesicles, and none of them dependent on protein synthesis. Further experiments are necessary to clarify this issue.

In conclusion, in the present study we have demonstrated that human MNL beta-adrenoceptors are rapidly sequestered away from the cell surface after isoproterenol exposure. The loss in surface beta-adrenoceptors is paralleled by a loss in isoproterenol-stimulated adenylate cyclase activity. The sequestered receptors are readily recognized in a particulate preparation, and rapidly reappear on the cell surface after short-time treatment with agonist, while more prolonged treatment after an initial rapid return of part of the receptors, leads to a slow phase of receptor reappearance.

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