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Human platelet β_2-adrenoceptors: agonist-induced internalisation and down-regulation in intact cells

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1 The effect of isoprenaline (10 μM at 37°C for 30 min) pretreatment on [^{125}I]-(−)-pindolol ([^{125}I]-(−)-Pin) binding to β_2-adrenoceptors on intact human platelets has been examined.

2 By use of saturation analysis, maximal binding capacity (B_{max}) of [^{125}I]-(−)-Pin binding in control and treated cells was assessed in the presence of 1 μM (−)-propranolol or 1 μM (±)-CGP 12177 which were taken to represent total or cell surface β-adrenoceptors respectively. Assay incubations were performed at 37°C and 4°C, the latter to prevent recycling of internalised receptors.

3 Isoprenaline treatment resulted in an identical, highly significant, loss of binding sites ($\simeq$ 25%) defined by (−)-propranolol at both assay temperatures as compared to control cells. Binding sites identified in the presence of (±)-CGP 12177 were reduced to a much greater extent ($\simeq$ 70%), but this was only seen when assays were performed at 4°C.

4 Agonist-induced changes in receptor numbers were concentration-dependent with half maximal receptor loss occurring at an isoprenaline concentration of approximately 2×10^{-8} M. These effects were inhibited by the presence of a β-adrenoceptor antagonist and absent if agonist pretreatment was performed at 4°C.

5 Recovery experiments showed that the isoprenaline-induced reduction in total receptor number defined by (−)-propranolol was irreversible whereas the reduction in cell surface receptors defined by (±)-CGP 12177 was rapidly reversible (<40 min).

6 These data suggest that isoprenaline treatment of intact human platelets causes redistribution of β_2-adrenoceptors. A proportion are sequestered away from the cell surface (internalised), becoming inaccessible to the hydrophilic ligand (±)-CGP 12177. A smaller proportion defined by (−)-propranolol are apparently totally lost from the cell (down regulated).

Introduction

Agonist-induced desensitization appears to be a general homeostatic mechanism by which target cells modulate responsiveness to agonists acting at cell surface receptors. Cells that possess β-adrenoceptors become refractory when they are continuously exposed to β-adrenoceptor agonists and in many cases this reduced responsiveness is agonist specific (homologous). Two different mechanisms have been proposed to be involved in this process; firstly a fast reversible loss of the agonist-induced adenylate cyclase activity (uncoupling) and secondly a slow decrease in the total number of receptors, ie 'down regulation' of the receptors (Su *et al.*, 1980; Green *et al.*, 1981). The earlier loss in response correlates with an apparent decrease in receptor affinity for agonists, an event that has recently been proposed to represent loss of cell surface receptors perhaps to distinct intracellular sites (Hertel *et al.*, 1983). Receptor down regulation, however, may sometimes involve enzymatic breakdown through lysosomes with resultant loss of total cell receptors (Chuang, 1982). Thus a current model of desensitization is that receptor internalisation or sequestration precedes and sometimes initiates receptor loss. However, it is still unclear how uncoupling, internalisation and receptor loss are linked (see reviews Harden, 1983; Sibley & Lefkowitz, 1985).

Although many studies of β-adrenoceptor desensitization and redistribution have been performed on cultured cell lines, few studies have examined normal tissues and little is known about the molecular events that accompany desensitization in human cells. Internalisation of cell surface β-adrenoceptors following agonist incubation has been recently demonstrated in

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human lymphocytes (De Blasi *et al.*, 1985), in which the receptor is relatively well coupled to adenylate cyclase. Coupling to adenylate cyclase as a pre-requisite for the process of internalisation is disputed, as experiments with cyc$^-$ S49 lymphoma cells (which lack functional Ns) have shown similar agonist-induced sequestration of β-adrenoceptors away from the cell surface (Mahan *et al.*, 1985). We and others (Cook *et al.*, 1985; Wang & Brodde, 1985) have previously established the presence of a homogeneous population of β_2-adrenoceptors on human platelet membranes which are poorly (if at all) coupled to adenylate cyclase. In the present study we examine agonist modulation of β_2-adrenoceptors in this human anucleate cell. In previous intact cell studies (Staehelin & Hertel, 1983; Staehelin *et al.*, 1983) it has been observed that the hydrophilic β-antagonist (±)-CGP 12177 (4-(3-tertiary-butylamino-2-hydroxy-propoxy)-benzimidazol-2-one hydrochloride), is relatively membrane impermanent at low concentrations and binds selectively to cell surface receptors. On the other hand relatively lipophilic compounds such as (−)-propranolol distribute internally and identify intracellular as well as cell surface receptors. In the experiments to be described we have employed this differential permeability effect on the binding of [^{125}I]-(−)-pindolol ([^{125}I]-(−)-Pin) to β-adrenoceptors on intact human platelets, in order to study the events occurring following agonist incubation.

Methods

Platelet preparation

Blood was drawn from healthy normal volunteers with a 19 gauge needle into 0.17 vol of acid-citrate dextrose (10 mM citrate) as anticoagulant. After centrifugation of blood at 700 *g* for 5 min, the platelet rich plasma (PRP) was removed and incubated at 37°C for 30 min (unless otherwise stated) in the presence (treated cells) or absence (control cells) of 10 μM isoprenaline (unless otherwise stated). Platelet suspensions were essentially prepared as previously described (Cook *et al.*, 1985) but with all manipulations being performed at 2–4°C. The PRP was diluted 1:1 with Tris isosaline pH 6.5 (50 mM Tris-HCl, 100 mM NaCl, 5.0 mM EDTA, 0.2 mM sodium metabisulphite) and centrifuged at 450 *g* for 20 min. The cells were washed once in 50 vol of Tris isosaline pH 6.5 before final resuspension in 'whole cell' assay buffer (Tris isosaline pH 7.8) at a concentration of $5-9 \times 10^8$ cells per ml.

Platelet membranes were prepared by centrifugation of platelet suspension at 27000 *g* at 4°C for 10 min. The pellet was resuspended in ice cold-lysing buffer (5 mM EDTA, 5 mM Tris-HCl, pH 7.2) and homogenised using an Ultraturrax homogeniser. The resulting membrane suspension was centrifuged at 27000 *g* for 10 min, washed twice and finally resuspended in 'whole cell' assay buffer at a final concentration of 1.5–3.0 mg membrane protein per ml. By use of this method, all platelet preparations were virtually free of white blood cell and erythrocyte contamination as assessed by means of a Model S-Plus IV Coulter cell counter and visual counting. Protein was determined by the method of Lowry *et al.* (1951). All experiments were carried out using freshly prepared platelets.

Binding assay

Platelets were incubated to equilibrium with [^{125}I]-(−)-Pin and appropriate concentrations of drugs in a final volume of 500 μl assay buffer for 40 min at 37°C or 5 h at 4°C. Assays were terminated by the addition of 10 ml ice cold assay buffer diluted 1:10 with water. Tubes were kept on ice for 30 min before rapid vacuum filtration. This incubation with cold hypotonic buffer substantially lowered non-specific binding without reducing specific binding and thus significantly improved the total binding: non-specific binding ratio as has been reported by others (Staehelin *et al.*, 1983). Filters were washed with 3×10 ml ice cold buffer (25 mM Tris HCl, 154 mM NaCl, pH 7.8) and radioactivity bound to the filters was determined directly in a Packard Auto Gamma 500C counter at an efficiency of around 75%. [^{125}I]-(−)-Pin bound never exceeded 10% of the total radioactivity added. Non-specific binding of [^{125}I]-(−)-Pin was defined in the presence of the lipophilic non selective antagonist (−)-propranolol (1 μM) or the relatively hydrophilic antagonist (±)-CGP 12177 (1 μM) as appropriate. These concentrations of antagonists were determined from displacement experiments of [^{125}I]-(−)-Pin binding to intact platelets. Specific binding, defined as total minus non-specific binding at both temperatures was around 70–80% of total binding at the concentration of [^{125}I]-(−)-Pin (30–40 pM) used in the competition experiments.

Platelet membranes were assayed as previously described (Cook *et al.*, 1985) in a final volume of 250 μl 'whole cell' assay buffer and incubated for 40 min at 37°C or 5 h at 4°C.

Data analysis

All experiments were conducted a minimum of three times in duplicate. Equilibrium dissociation constants (K_D) and binding site maxima (B_{max}) were determined by Scatchard analysis. Results are reported as the mean ± s.e.mean for each variable, statistical significance was established by Student's t test for comparison of paired means, a P value < 0.05 was considered significant. Data from competition experiments were analysed by non linear least squares

curve fitting techniques. The competition curves were initially analysed by the four parameter logistic model (Allfit) to obtain slope factors (pseudo Hill coefficients) and IC_{50} values (concentration producing 50% displacement of specific [^{125}I]-(−)-Pin binding). All the curves for each group of experiments were examined simultaneously to enable parameter equivalence between individual curves to be tested directly. The value of the slope factors were also tested for significant deviation from unity using a partial F test (DeLean *et al.*, 1978).

Iodination of (−)-pindolol

(−)-Pindolol was iodinated to a theoretical specific activity of 2175 Ci mmol^{-1} according to the method of Barovsky & Brooker (1980). [^{125}I]-(−)-Pin was only used within 30 days of iodination.

Materials

Na^{125}I (carrier-free) was obtained from Amersham International, England. (−)-Isoprenaline bitartrate, (−)-adrenaline bitartrate and (−)-noradrenaline bitartrate were obtained from Sigma Chemical Company. The following drugs were kindly donated by the indicated companies: (−)-pindolol (Sandoz), (+)- and (−)-propranolol and (±)-ICI 118,551 (ICI Pharmaceuticals), CGP 20712A and (±)-CGP 12177 (Ciba-Geigy), guanyl-5'-imidodiphosphate (Gpp-(NH)p) was obtained from Boehringer Mannheim.

Results

In these experiments we have employed the differential permeability effect of (±)-CGP 12177 and (−)-propranolol. We have taken [^{125}I]-(−)-Pin binding in the presence of 1 μM (−)-propranolol to represent total cell β-adrenoceptor number; [^{125}I]-(−)-Pin binding in the presence of 1 μM (±)-CGP 12177 to represent cell surface receptors; and the difference between them to represent internalised receptors that are no longer accessible to (±)-CGP 12177 (see Staehelin *et al.*, 1983; Staehelin & Hertel, 1983).

Pharmacological characterization of [^{125}I]-(−)-pindolol binding sites on intact human platelets (control cells) at 4°C and 37°C

Specific binding of [^{125}I]-(−)-Pin to intact human platelets at 4°C and 37°C defined as the binding displaceable by 1 μM (±)-CGP 12117 or 1 μM(−)-propranolol was saturable (Figure 1). The maximum number of binding sites (B_{max} fmol mg^{-1} protein) assessed by Scatchard analysis were not significantly different whether defined in the presence of (−)-propranolol or (±)-CGP 12177 at either temperature (1.21 ± 0.13 and 1.17 ± 0.12 at 37°C; 1.23 ± 0.06 and 1.24 ± 0.09 at 4°C respectively). In all cases the data produced linear Scatchard plots with Hill slope values not significantly different from unity, indicative of a homogeneous population of binding sites. The dissociation constant (K_D) for [^{125}I]-(−)-Pin was slightly lower at 4°C than at 37°C whether defined in the presence of either (−)-propranolol (16.17 ± 1.49 and 22.62 ± 0.13 [pM] respectively) or (±)-CGP 12177 (17.21 ± 1.3 and 22.58 ± 1.04 [pM] respectively).

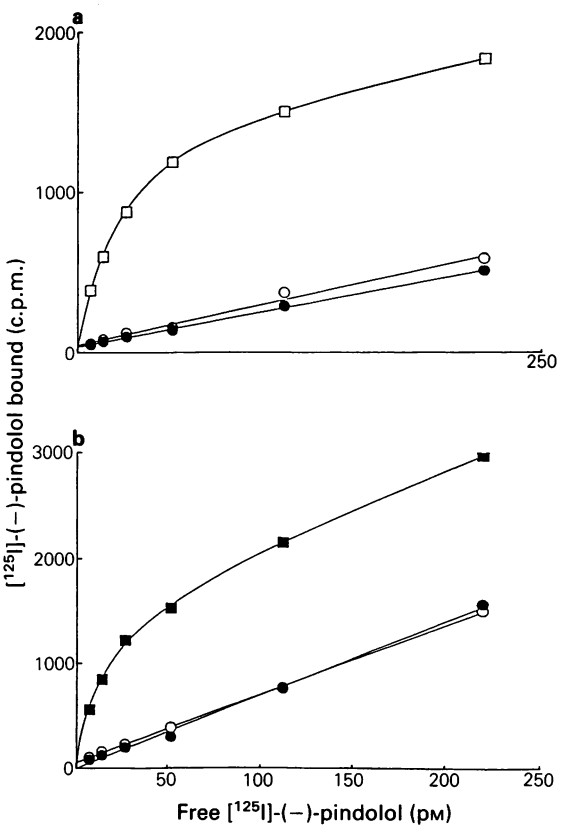

Figure 1 [^{125}I]-(−)-pindolol binding to intact human platelets at 37°C (a) and 4°C (b). Saturation data shown are from a single representative experiment. This figure illustrates raw untransformed data of total binding (□, ■) and non-specific binding in the presence of 1 μM (±)-CGP 12177 (○) or 1 μM (−)-propranolol (●) at 37°C (a) and 4°C (b).

The binding of [^{125}I]-(−)-Pin to intact human platelets was indicative of an interaction with a β-adrenoceptor. Data presented in Table 1 indicate that the binding was stereoselective with the (−)-isomer of propranolol being more potent than the (+)-isomer by approximately two orders of magnitude. The

Table 1 Overall IC_{50} values and Hill slope factors (nH) for β-adrenoceptor agonist and antagonist displacement of [^{125}I]-(−)-pindolol binding to intact human platelets

	IC_{50} [M]* (nH)			
	4°C		*37°C*	
(−)-Propranolol	$3.02 \pm 0.82 \times 10^{-10}$	(0.94)	$3.83 \pm 0.38 \times 10^{-10}$	(0.93)
(+)-Propranolol	$4.3 \pm 0.14 \times 10^{-8}$	(1.08)	$2.51 \pm 0.08 \times 10^{-8}$	(0.95)
(±)-CGP 12177	$6.16 \pm 1.05 \times 10^{-10}$	(0.93)	$5.3 \pm 0.09 \times 10^{-10}$	(0.99)
CGP 20172A	$1.8 \pm 0.42 \times 10^{-5}$	(1.04)	$4.29 \pm 0.3 \times 10^{-6}$	(0.95)
ICI 118,551	$6.6 \pm 0.4 \times 10^{-10}$	(0.99)	$7.87 \pm 0.78 \times 10^{-10}$	(0.88)
(−)-Isoprenaline	$1.74 \pm 0.37 \times 10^{-7}$	(0.73**)	$4.0 \pm 0.2 \times 10^{-7}$	(0.59**)
(−)-Adrenaline	$6.07 \pm 1.21 \times 10^{-7}$	(0.75**)	$2.2 \pm 0.11 \times 10^{-6}$	(0.52**)
(−)-Noradrenaline	$1.35 \pm 0.34 \times 10^{-5}$	(0.92)	$4.3 \pm 0.92 \times 10^{-5}$	(0.87**)

*The values given are mean ± s.e.mean of at least three separate experiments performed in duplicate and were derived from computer-assisted (Allfit) simultaneous curve-fitting analysis of individual curves as described in the text.
**Indicate slope is significantly different from one ($P < 0.01$).

overall affinities of the non-selective β-adrenoceptor antagonists: (+)-propranolol, (−)-propranolol and (±)-CGP 12177 and the selective β-adrenoceptor antagonists: ICI 118,551 (β_2 selective) and CGP 20172A (β_1 selective) were virtually identical at 37°C and 4°C. These antagonists generated steep displacement curves with Hill slope factors not significantly different from unity ($P > 0.1$, Table 1) at both temperatures which suggest binding to a single class of site with binding governed by law of mass action. In addition, the selective β-adrenoceptor antagonists exhibited affinities for the binding site similar to those previously reported in human platelet membranes (Cook *et al.*, 1985) and at the β_2 subtype in other mammalian tissues (Dickinson & Nahorski 1981; Dooley *et al.*, 1986).

The displacement of [^{125}I]-(−)-Pin binding to intact human platelets by adrenoceptor agonists was also indicative of a β-adrenoceptor (Table 1) and the hierarchy of potency displayed (−)-isoprenaline > (−)-adrenaline > (−)-noradrenaline is typical of the β_2 subtype at both temperatures.

Distribution of β-adrenoceptors following agonist treatment of intact human platelets

Human platelets were incubated for 30 min at 37°C in the presence (treated cells) or absence (control cells) of 10 μM isoprenaline, washed at 4°C and assayed for [^{125}I]-(−)-Pin binding (as described in methods section) at both physiological temperature (37°C) and at a low temperature (4°C). Incubations performed at a low temperature have been shown to block the receptor recycling process (Hertel & Staehelin, 1983).

Isoprenaline treatment resulted in a significant (25% approximately) decrease in the number of [^{125}I]-(−)-Pin sites labelled at 37°C when compared with control cells (Figure 2). This decrease in the number of binding sites was identical whether defined in the presence of 1 μM (−)-propranolol or 1 μM (±)-CGP 12177 (Table 2) suggesting that when assayed at 37°C all the remaining receptors in the treated cells were on the cell surface.

[^{125}I]-(−)-Pin binding to treated cells performed at 4°C showed a similar loss of binding sites as defined by 1 μM (−)-propranolol, but a much greater loss of binding (approximately 70%) as defined by 1 μM (±)-CGP 12177, when compared with control cells (Figure 2, Table 2). This difference between 1 μM (±)-CGP 12177 and 1 μM (−)-propranolol displaceable binding at 4°C indicates that isoprenaline treatment not only induces an irreversible reduction (down-regulation) in total receptor number but also an additional sequestration of cell surface receptors. Thus 40–50% of the receptors remaining following agonist treatment although still labelled by (−)-propranolol and [^{125}I]-(−)-Pin are no longer accessible to 1 μM (±)-CGP 12177, possibly due to removal from the immediate cell surface.

To determine whether these receptor changes were distinguishable over a wide concentration range of displacing agent or only at high saturating concentrations (1 μM), competition experiments were performed with the antagonists (−)-propranolol and (±)-CGP 12177, and the hydrophilic β-agonist (−)-isoprenaline in treated cells at 37°C and 4°C. In assay incubations performed at 37°C (−)-propranolol, (±)-CGP 12177 and (−)-isoprenaline all competed with [^{125}I]-(−)-Pin binding on treated platelets to the same extent displacing approximately 85% of total radioligand bound (Figure 3). The overall affinities $3.65 \pm 0.35 \times 10^{-10}$ M, $2.19 \pm 0.2 \times 10^{-9}$ M, $9.33 \pm 1.25 \times 10^{-7}$ M for (−)-propranolol, (±)-CGP 12177 and (−)-isoprenaline respectively were in excellent agreement with those previously shown in control cells at 37°C (Table 1) although (−)-isopren-

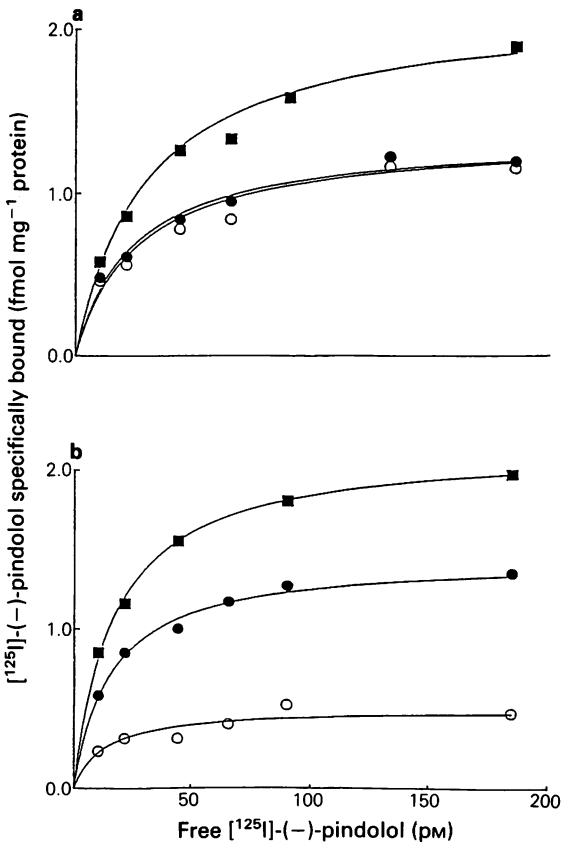

Figure 2 [^{125}I]-(−)-pindolol binding to control and agonist-treated cells. Control and (−)-isoprenaline-treated (10 μM for 30 min at 37°C) cells were prepared and binding assays performed at 37°C (a) or 4°C (b) as described in the methods section. For control cells (squares) non-specific binding (NSB) was defined in the presence of 1 μM (−)-propranolol (■). For treated cells (circles) NSB was defined in the presence of either 1 μM (−)-propranolol (●) or 1 μM (±)-CGP 12177 (○). The data shown are from a single representative experiment performed in duplicate.

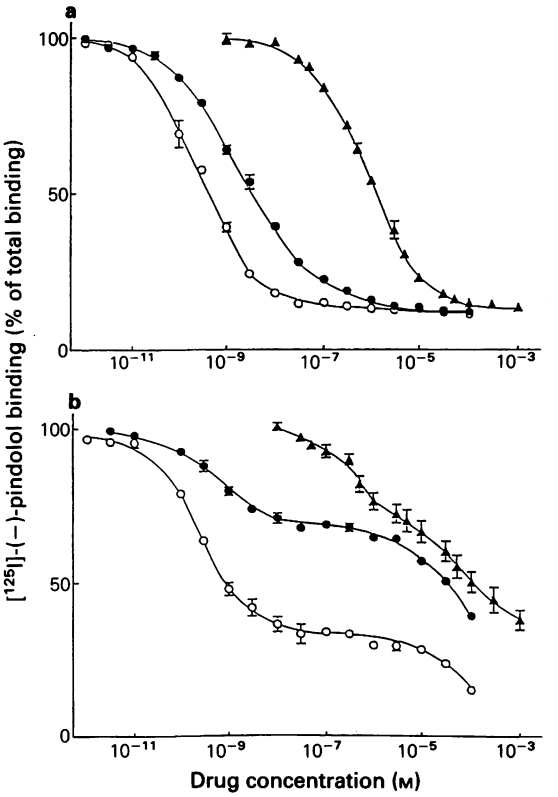

Figure 3 Competition for [^{125}I]-(−)-pindolol ([^{125}I]-(−)-Pin) binding sites on isoprenaline-treated intact human platelets at 37°C and 4°C by the lipophilic antagonist (−)-propranolol (○), the hydrophilic antagonist (±)-CGP 12177 (●) and the hydrophilic β agonist (−)-isoprenaline (▲). Intact human platelets were incubated with 10 μM (−)-isoprenaline for 30 min at 37°C, washed at 4°C and assayed for [^{125}I]-(−)-Pin binding at 37°C (a) or 4°C (b) as described in the methods section. Data are plotted as % of [^{125}I]-(−)-Pin bound at the indicated concentrations of competitive agents and represent the mean of at least three separate experiments performed in duplicate; s.e.mean shown by vertical lines.

aline and (±)-CGP 12177 exhibited slightly lower affinities following agonist treatment.

In binding assays performed at 4°C, however, these three agents produced markedly different competition binding isotherms in the treated cells. Thus (−)-propranolol inhibited approximately 70% of total [^{125}I]-(−)-Pin binding at saturating concentrations (<1 μM) with further displacement of binding, probably from non-specific sites, occurring at higher (10–100 μM) concentrations. In contrast, (±)-CGP 12177 even at concentrations of 100 μM did not inhibit binding to the same extent as (−)-propranolol and at saturating concentrations of 1 μM occupied only 35% of total sites identified by [^{125}I]-(−)-Pin binding. Isoprenaline exhibited similar inhibition of [^{125}I]-(−)-Pin binding (≃35%) at concentrations of up to 10 μM). Increasing the concentration caused further inhibition of binding, however, even at very high concentrations (1 mM) isoprenaline did not inhibit [^{125}I]-(−)-Pin binding to the same extent as (−)-propranolol.

Concentration-dependence of (−)-isoprenaline effects in intact platelets

Treatment of intact human platelets with (−)-isopren-

Table 2 Apparent equilibrium dissociation constants (K_D) and receptor density (B_{max}) of [^{125}I]-(−)pindolol binding to control and isoprenaline-treated intact human platelets

Temperature	*Cells*	*Agent used to define non-specific binding*	B_{max} (*fmol mg^{-1}*)	K_D (pM)
37°C	Control	1 μM (−)-Propranolol	1.72 ± 0.16	17.61 ± 2.94
	Treated	1 μM (−)-Propranolol*	1.27 ± 0.07	18.82 ± 4.02
	Treated	1 μM (±)-CGP 12177*	1.24 ± 0.05	20.36 ± 4.42
4°C	Control	1 μM (−)-Propranolol	2.05 ± 0.14	13.28 ± 1.0
	Treated	1 μM (−)-Propranolol**	1.46 ± 0.12	12.83 ± 1.04
	Treated	1 μM (±)-CGP 12177**†	0.556 ± 0.06	13.96 ± 0.32

Dissociation constants and number of binding sites were determined at 4°C and 37°C in control and (−)-isoprenaline-treated (10 μM for 30 min at 37°C) cells simultaneously. Specific binding was assessed as described in the text in the presence of 1 μM (−)-propranolol for control cells, and in the presence of 1 μM (−)-propranolol or 1 μM (±)-CGP 12177 for treated cells. Data are expressed as mean ± s.e.mean (n = 3). Results were analysed using Student's t test for paired data.

*$P < 0.05$ by one tailed paired t test analysis compared to control cells at 37°C.

**$P < 0.05$ by one tailed paired t test analysis compared to control cells at 4°C.

†$P < 0.05$ by one tailed paired t test analysis compared to treated cells defined in the presence of 1 μM (−)-propranolol.

aline induced a concentration-dependent loss of both total and cell surface receptors (Figure 4). The effect although evident at a concentration of 10^{-8} M (−)-isoprenaline was only statistically significant with concentrations of 10^{-7} M and above. Maximal effect was apparent with 10^{-5} M isoprenaline. Although the extent of cell surface receptor loss was significantly greater than total receptor loss from 10^{-7}–10^{-5} M isoprenaline, half-maximal receptor loss occurred at around $1-3 \times 10^{-8}$ M (−)-isoprenaline for both processes. Isoprenaline-induced changes in receptor numbers were inhibited by the presence of a non-selective β-antagonist (sotalol) and absent if agonist treatment in PRP was performed at 4°C (data not shown).

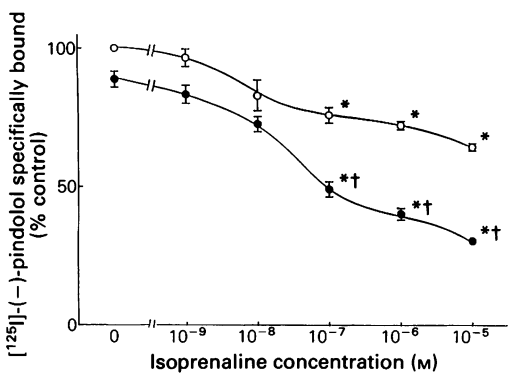

Figure 4 Concentration-dependence of (−)-isoprenaline induced internalisation and down-regulation of intact human platelet β-adrenoceptors. Intact human platelets were incubated with (treated cells) or without (control cells) the indicated concentrations of (−)-isoprenaline for 30 min at 37°C, washed at 4°C and assayed for [^{125}I]-(−)-pindolol ([^{125}I]-(−)-Pin) binding at 4°C. Specific binding was defined in the presence of 1 μM (−)-propranolol (○) to assess total β-adrenoceptor number and 1 μM (±)-CGP 12177 (●) to assess cell surface receptor number. Data points represent the mean of at least three separate experiments performed in duplicate; s.e.mean shown by vertical lines. Results were analysed using Student's t test for paired data.

*$P < 0.05$ by one tailed paired t test analysis to control cells.

†$P < 0.05$ by one tailed paired t test analysis to treated cells defined in the presence of 1 μM (−)-propranolol.

Effect of (−)-isoprenaline treatment of intact human platelets on subsequent [^{125}I]-(−)-Pindolol binding to platelet membranes

The above results obtained with intact platelets indicate that β-adrenoceptor sequestration and down-regulation occur when platelets are incubated with the β-agonist (−)-isoprenaline. As no significant change in the apparent K_D of pindolol was observed at either 37°C or 4°C (Table 2) it is unlikely that the loss of sites after incubation with isoprenaline is attributable to free agonist retained during the washing procedure and interfering with subsequent binding assay. However, in order to establish whether or not retained receptor bound (−)-isoprenaline is responsible for the loss of [^{125}I]-(−)-Pin observed with intact platelets, a series of experiments were performed on human platelet membranes.

We and others have previously shown that the addition of Gpp(NH)p (a non-hydrolysable analogue of GTP) or Na^+ decreases the affinity of [^{125}I]-(−)-Pin binding sites for (−)-isoprenaline in human platelet membranes (Cook *et al.*, 1985; Wang & Brodde, 1985). In our hands the effect of Gpp (NH)p on agonist affinity at the β_2-adrenoceptor in platelet membranes

Table 3 Apparent equilibrium dissociation constants (K_D) and receptor density (B_{max}) of [^{125}I]-(−)-pindolol binding to human platelet membranes prepared from control or isoprenaline-treated cells

	Control	*Treated*	*Treated* + 100 μM Gpp(NH)p	*Treated* + 100 mM NaCl
B_{max} (fmol mg^{-1})	3.65 ± 0.16	2.25 ± 0.06*	2.4 ± 0.09*	2.34 ± 0.05*
K_D (pM)	16.91 ± 0.71	26.1 ± 2.76	22.74 ± 2.9	22.46 ± 2.0

Human platelets were incubated with (treated) or without (control) 10 μM (−)-isoprenaline at 37°C for 30 min, washed at 4°C and membranes prepared and assayed as described in the methods section.
The results shown are the mean ± s.e.mean of at least three separate experiments performed in duplicate. Results were analysed using Student's *t* test for paired data.
*$P < 0.05$ by one tailed paired *t* test analysis compared to control membranes.

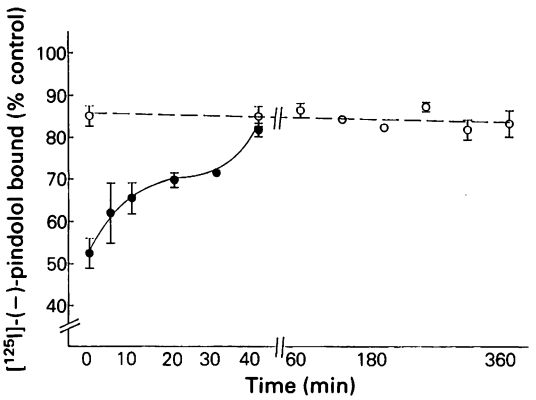

Figure 5 Time course of intact human platelet β-adrenoceptor reappearance following (−)-isoprenaline treatment. Intact human platelets were incubated with (treated cells) or without (control cells) 0.1 μM (−)-isoprenaline for 30 min at 37°C and then recovery of down-regulated and 'internalised' receptors evaluated for up to 6 h as described in the text. Time intervals represent periods of further incubation in fresh buffer following initial agonist treatment. [^{125}I]-(−)-pindolol binding assays were performed at 4°C using 1 μM (−)-propranolol (○) and 1 μM CGP 12177 (●) as described in the methods section. Data points are shown relative to control (100%) and represent the mean of at least three separate experiments performed in duplicate; s.e.mean shown by vertical lines.

is minimal due to the poor coupling efficiency (Cook *et al.*, 1985). However, addition of NaCl induces a marked shift to the right of the isoprenaline competition binding curves in control membranes (Cook *et al.*, 1985) which is quantitatively the same in membranes prepared from platelets following agonist treatment (unpublished observations). In the light of these findings we reasoned that if the decreased [^{125}I]-(−)-Pin binding demonstrated above following preincubation of intact platelets with (−)-isoprenaline was the result of competition by retained (−)-isoprenaline, then lowering the affinity of the receptor for the (−)-isoprenaline by the addition of Gpp(NH)p or Na^+ in membranes prepared from those cells should reverse this effect. If, on the other hand, true down-regulation had occurred the reduced [^{125}I]-(−)-Pin binding would persist.

As shown in Table 3 in the presence of Gpp(NH)p or Na^+ there was no significant difference in the maximal binding capacity (B_{max}) or the apparent equilibrium dissociation constant (K_D) observed in membranes prepared from treated cells.

Reappearance of cell surface β-adrenoceptors following (−)-isoprenaline treatment

Intact human platelets were incubated with 0.1 μM (−)-isoprenaline at 37°C for 30 min (as described in methods section). Agonist incubation was terminated by centrifugation and washing of the platelets at 4°C. Cells were then resuspended in fresh agonist free buffer and maintained at 37°C for up to 6 h. At various time intervals, platelet aliquots were removed and [^{125}I]-(−)-Pin binding (using a saturating concentration of radioligand, 150 [pM]) performed at 4°C in the presence of (−)-propranolol or (±)-CGP 12177 to define total and cell surface receptor number respectively as in previous experiments.

The receptors which were down-regulated (defined by (−)-propranolol) by agonist treatment did not reappear even after prolonged (> 6 h) incubation in agonist-free medium at 37°C. Conversely, the recovery of cell surface receptors defined by (±)-CGP 12177 was rapid such that by 40 min there was no significant difference in [^{125}I]-(−)-Pin binding defined by (±)-CGP 12177 or (−)-propranolol.

Discussion

In the present study we have examined the binding of [^{125}I]-(−)-Pin at 4°C and 37°C to β-adrenoceptors on intact human platelets. The results of this study are

similar to those previously performed in platelet membranes (Cook *et al.*, 1985) and indicate that the platelet β-adrenoceptor is purely of the β_2 subtype and exhibits identical pharmacological characteristics to β_2-adrenoceptors found in other mammalian tissues (Rugg *et al.*, 1978; Dickinson & Nahorski, 1981). In addition using intact human platelets we have examined agonist-mediated internalisation or sequestration and down-regulation of these β_2-adrenoceptors.

Results of saturation binding experiments show that preincubation of intact human platelets with (−)-isoprenaline leads to a dramatic reduction in total β-adrenoceptor number and the redistribution or sequestration of a large fraction of the remaining cell surface receptors into a cellular environment not accessible to the hydrophilic antagonist (±)-CGP 12177. This selective loss of cell surface receptors can only be detected when the agonist-induced state of the receptor is preserved by cooling during receptor determination. No detectable difference in cell surface and total receptor number in treated cells is apparent when the receptor assay is performed at physiological temperature (37°C) probably due to rapid recycling of the 'internalised' receptors to the surface during the assay incubation period as has been suggested by others (Hertel & Staehelin, 1983) and as indicated in the receptor recovery experiments in this study.

The results of competition binding experiments performed at 4°C and 37°C in treated cells with the antagonists (−)-propranolol and (±)-CGP 12177 and the β agonist (−)-isoprenaline support the observations made above. They show that if binding assays are performed at 37°C in treated cells, all the remaining receptors not only exhibit the pharmacological characteristics of β-adrenoceptors but are also equally accessible to (−)-propranolol, (±)-CGP 12177 and (−)-isoprenaline. Conversely at 4°C, the receptors that remain following agonist treatment are not equally accessible to these agents. (−)-Propranolol has access to a far greater number of receptors than the hydrophilic compounds (±)-CGP 12177 and (−)-isoprenaline, which even at very high concentrations can only displace [^{125}I]-(−)-Pin binding from cell surface and a proportion of the 'internalised' receptors.

The concentration-dependence for (−)-isoprenaline induced internalisation and down-regulation of β-adrenoceptors on intact human platelets was similar to that observed in other cell types (Staehelin & Simons, 1983) with half maximal receptor loss occurring at the same (−)-isoprenaline concentration for both processes. Isoprenaline induced changes in total and cell surface β-adrenoceptor numbers were inhibited by the presence of the non-selective β-antagonist sotalol and absent if agonist treatment was performed at 4°C.

As we found no change in the apparent K_D of pindolol following agonist treatment it is unlikely that the decrease in [^{125}I]-(−)-Pin binding observed at either 37°C or 4°C in treated cells is due to interference by high affinity binding of isoprenaline retained during the washing procedure. To establish whether retained isoprenaline actually explained the decreased [^{125}I]-(−)-Pin binding, we examined membranes prepared from control and agonist-treated cells and found that under conditions designed to decrease the affinity of (−)-isoprenaline (addition of Gpp(NH)p/Na^+) there was no change in the maximal binding capacity or the apparent equilibrium dissociation constant in membranes prepared from treated cells. These results support the hypothesis that the reduction in total [^{125}I]-(−)-Pin binding sites observed following (−)-isoprenaline treatment represents true down-regulation and is not a consequence of retained agonist.

Recent reports (Hertel *et al.*, 1983; Toews *et al.*, 1986) have shown that [^{125}I]-(−)-Pin whilst able to label all available receptors (surface and intracellular) at 37°C, exhibited limited accessibility to internalised receptors at 4°C. Other studies have thus assumed that [^{125}I]-(−)-Pin labels both surface and intracellular receptors at 37°C and only surface receptors at 4°C (Linden *et al.*, 1984). This temperature-dependent differential permeability of [^{125}I]-(−)-Pin may vary with tissue involved and length of assay incubation. Thus, careful examination of the kinetics of [^{125}I]-(−)-Pin binding at 4°C in agonist-treated human astrocytoma cells shows substantially less ligand bound following 1 h incubation compared with control but virtually identical binding after 5 h (Toews *et al.*, 1986). Our results suggest that since the number of total receptors measured was not influenced by temperature of incubation in control or treated cells even after 5 h incubation at 4°C, [^{125}I]-(−)-Pin is not restricted to the immediate cell surface at 4°C in the intact human platelet. The recovery experiments show that as with other cells (Hertel & Staehelin, 1983), if agonist-treated human platelets are not maintained at a low temperature (4°C) following agonist incubations then rapid recycling of the 'sequestered' receptors defined by (±)-CGP 12177 occurs and that the difference in the number of receptors defined by (±)-CGP 12177 at 37°C and 4°C is not due to temperature modulated changes in the permeability of this hydrophilic compound.

In the light of these observations we examined receptor distribution in lysates from control and agonist-treated human platelets on non-linear sucrose density gradients employing the method of Kassis & Sullivan (1986). Two fractions were examined, a heavy membrane fraction (which co-sedimented adenylate cyclase activity) and a light density fraction. Our results show that following agonist treatment there

was a significant (40–45%) decrease in the number of receptors associated with the heavy plasma membrane fraction but due to the low number of receptors involved, accurate determinations of receptor numbers in the light density fraction proved impossible (unpublished observations).

In our hands, the β-adrenoceptor of the human platelet does not appear to be coupled to adenylate cyclase. Thus we have previously shown that isoprenaline displacement curves performed in human platelet membranes exhibited a steep slope factor and were not influenced by the addition of guanine nucleotide (Cook *et al.*, 1985). More recently we have also failed to show any isoprenaline stimulation of cyclic AMP accumulation in whole platelets free from white cell contamination. In addition, experiments performed to explore the ability of forskolin to potentiate agonist stimulation of cyclic AMP accumulation in platelets showed no synergistic interaction with (−)-isoprenaline (up to 10^{-4} M), whilst as shown by others (Siegl *et al.*, 1982) in the presence of a threshold concentration of prostaglandin E_1 (PGE_1) the same concentration of forskolin produced a 15 fold increase in [cyclic AMP] (unpublished observations). This is in contrast with previously reported data from others (Jakobs *et al.*, 1978; Wang & Brodde, 1985) in which isoprenaline was shown to produce a small stimulation ($\simeq$ 1.5 fold) of adenylate cyclase activity. The possibility must be entertained that these differences relate to the purity of the platelet preparations employed, as minimal contamination by well coupled mononuclear leukocytes could explain the spurious appearance of a 'weakly coupled' β_2-adrenoceptor in the platelets.

In the present study we have shown that isoprenaline treatment of intact human platelets results in a rapid and reversible internalisation of β-adrenoceptors present. In addition, a rapid (< 30 min) down-regulation of the total receptor number occurs which is apparently not reversible despite prolonged incubation in agonist-free medium. The agonist-induced effects on receptor redistribution we have observed in the absence of β-adrenoceptor stimulation of cyclic AMP are similar to those previously found in some cultured cell studies. The cyc^- variant of the S49 lymphoma cell line lacks the α_s subunit of the stimulatory coupling protein N_s and hence hormone-sensitive adenylate cyclase activity. Using this model, it has been demonstrated that sequestration of the β-adrenoceptor occurs in cyc^- cells (Mahan *et al.*, 1985) following agonist treatment. However, in contrast to results obtained above in platelets, agonist-induced down-regulation (i.e. loss of receptors) either does not occur or is blunted in these cells (Shear *et al.*, 1976; Su *et al.*, 1980; Mahan *et al.*, 1985).

In conclusion, the present study demonstrates agonist regulation of β_2-adrenoceptors on a human cell which appear to be very weakly coupled (if at all) to adenylate cyclase, and thus provides new information on the receptor changes that occur following agonist interactions with β-adrenoceptors on intact human cells. The rapid regulation of these receptors in the presence of an agonist suggests they may have a functional role which as yet remains undefined and probably involves another biochemical effector system to adenylate cyclase.

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