Internalization and down-regulation of the prostacyclin receptor in human platelets

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The internalization of [^{3}H]iloprost, a prostacyclin analogue, was studied in human platelets by binding studies. After incubation with [^{3}H]iloprost at 37 °C, addition of unlabelled ligand at either 37 °C or 4 °C caused dissociation of 74 % and 52 % of the bound ligand respectively, suggesting that a portion had been internalized. The percentage of [^{3}H]iloprost bound at equilibrium to the surface (evaluated by acid treatment) at either 37 °C or 4 °C was markedly different (80 % versus 25 %). Internalization was dependent on time and on the ligand nature and concentration. Energy-depleting agents (dinitrophenol and 2-deoxyglucose) completely inhibited internalization, whereas probenecid (inhibitor of organic anion transporters) did not affect it significantly. Subcellular fractionation indicated that, at 4 °C or in the absence of ligand, most of the receptor was present in membrane fractions (pellet at 27000 or 105000 ***g***), whereas, when platelets were preincubated at 37 °C with iloprost, the receptor was found mainly in the cytosolic fraction. In platelets preincubated with iloprost at 4 °C, two classes of binding sites were present, whereas after preincubation at 37 °C only the lower-affinity sites were detected. After exposure to the agonist, iloprost-induced inhibition of platelet aggregation and activation of adenylate cyclase and cAMP production were significantly lower. Taken together, these data demonstrate that human platelets can internalize a high-affinity binding site for iloprost, presumably the prostacyclin receptor.

INTRODUCTION

Prostacyclin (prostaglandin I_2, PGI_2) is one of the most potent endogenous inhibitors of platelet aggregation [1], and its anti-aggregatory effect is mediated by binding to plasma-membrane receptors and activation of adenylate cyclase [2–5]. The potential therapeutic applications of PGI_2 as an antithrombotic drug have so far been limited by its chemical and biological lability, which make a prolonged intravascular infusion necessary [6]. The latter, in turn, brings about tachiphylaxis to the anti-aggregatory effect of either PGI_2 itself [7] or iloprost, a stable PGI_2 analogue [8]. This phenomenon is mirrored, at a molecular level, by the desensitization of PGI_2-sensitive adenylate cyclase, which occurs in platelets [9–11] as well as in many cultured cells [12–17].

Various molecular mechanisms have been proposed to explain the desensitization phenomenon. Among them, down-regulation and internalization of the receptor have been demonstrated to occur in response to a variety of agents, including PGD_2 [10] and thromboxane [18]. Therefore the aim of the present work was to investigate whether down-regulation and internalization of PGI_2 platelet receptors can occur and to characterize such a process. In order to avoid the problem of the intrinsic lability of prostacyclin, we labelled the receptor with the chemically stable analogue [^{3}H]iloprost, which binds to the PGI_2 receptor [19,20].

MATERIALS AND METHODS

Materials

[^{3}H]Iloprost (14.7 Ci/mmol) and [8-^{14}C]ATP (50–60 Ci/mmol) were obtained from Amersham (Little Chalfont, Bucks., U.K.) and [2,8-^{3}H]cAMP (30–50 Ci/mmol) from Dupont NEN (Boston, MA, U.S.A.); unlabelled iloprost was kindly given by Schering AG (Berlin, Germany). PGI_2, 6-keto-$PGF_{1\alpha}$, probenecid, 2-deoxy-D-glucose (2DG), 2,4-dinitrophenol (DNP), cAMP, ATP, phosphocreatine and creatine phosphokinase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagen was from Mascia Brunelli (Milan, Italy) and hydroxyapatite from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Platelet preparation

Blood from healthy volunteers who had not taken drugs for at least 10 days was collected into plastic tubes containing 3.8 % trisodium citrate (9:1, v/v). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by centrifugation as previously described [21]. Washed platelets were prepared from PRP according to Hallam and Rink [22].

Binding to intact platelets

Binding experiments were performed essentially as previously described [20]. Briefly, unless otherwise specified, conditions were: 10 nM [^{3}H]iloprost, $(3–4) \times 10^8$ platelets in 1 ml of Hepes-buffered saline, pH 7.4, at 4 °C for 30 min. Non-specific binding was measured in the presence of 100 μM iloprost. Incubation was terminated by centrifugation at 8150 ***g***, the pellet was dissolved by addition of 0.5 ml of 1 M NaOH, and the radioactivity was counted in 9 ml of Ultima Gold (Canberra Packard).

Acid-wash procedure

Pellets obtained after binding studies on intact cells were resuspended in 800 μl of ice-cold acidic buffer [0.2 M acetic

Abbreviations used: PG, prostaglandin; DNP, 2,4-dinitrophenol; PRP, platelet rich plasma; PPP, platelet poor plasma; P_{27}, pellet after centrifugation at 27000 ***g***; P_{105}, pellet after centrifugation at 105000 ***g***; S_{105}, supernatant after centrifugation at 105000 ***g***; ANOVA, analysis of variance; 2DG, 2-deoxyglucose.

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acid/0.5 M NaCl/0.2 % (w/v) bovine serum albumin, pH 2.5] for 10 min and centrifuged at 8150 ***g*** for 5 min. The radioactivity was determined both in the resulting pellets and in the supernatants.

Subcellular fractionation

Washed platelets were preincubated with and without 10 nM iloprost at 37 °C or 4 °C for 18 h, centrifuged at 900 ***g*** for 15 min at 4 °C and the resulting pellet was resuspended in 10 mM Tris/HCl, pH 7.4. Cells were disrupted by freezing and thawing (three times) and centrifuged at 27000 ***g*** for 30 min (membrane fraction, P_{27}). The supernatant, centrifuged at 105000 ***g*** for 60 min, yielded a pellet (P_{105}) and a supernatant (S_{105}). On each of these fractions, specific [^{3}H]iloprost binding, specific marker-enzyme activities and protein concentration (Coomassie protein assay reagent, Pierce) were determined.

Binding to subcellular fractions

Binding of 10 nM [^{3}H]iloprost to P_{27} and P_{105} fractions was performed at 4 °C for 30 min in 0.5 ml of 10 mM Tris/HCl, pH 7.4 (0.15 and 0.04 mg of protein/sample respectively) and terminated by filtration on Whatman GF/C fibreglass filters, pretreated with 0.3 % (v/v) polyethyleneimine for 24 h. The filters were washed twice with 3 ml of Tris/HCl, pH 7.4. Binding to the S_{105} fraction was performed by incubating 10 nM [^{3}H]iloprost in 1 ml (0.15 mg of protein/sample) for 30 min. Bound ligand was separated from free by addition of 300 ml of 70 % (v/v) hydroxyapatite and filtration through GF/C filters, according to the method of Forti et al. [23]. Non-specific binding was evaluated as mentioned above.

Platelet aggregation

PRP was incubated at 37 °C for 2 h with or without 100 nM iloprost. At the end of incubation, platelets were centrifuged, resuspended in PPP at a final concentration of $(3–4) \times 10^8$ cells/ml, preincubated for 1 min with the indicated iloprost concentrations and challenged with 6 μg/ml collagen. Aggregation was assessed according to Born [24].

Enzyme activities

(a) Adenylate cyclase was assayed by the method of Salomon et al. [25] both as a plasma-membrane marker (on P_{27}, P_{105} and S_{105}), and in response to iloprost stimulation (on P_{27}). The incubation conditions (8 min at 30 °C) were as previously described [3], with 0.02, 0.06 and 0.03 mg of protein/sample for the three fractions respectively. [8-^{14}C]ATP was the substrate, and [2,8-^{3}H]cAMP was included to correct for the possible loss due to purification and phosphodiesterase activity. cAMP formation in response to iloprost in washed intact platelets was measured by enzyme immunoassay (Biotrak, Amersham) after 8 min at 30 °C.

(b) The activity of the esterases, endoplasmic-reticulum markers, was assayed in the three fractions evaluating the formation of *p*-nitrophenol from *p*-nitrophenyl acetate (0.07, 0.03 and 0.02 mg of protein/sample for P_{27}, P_{105} and S_{105} respectively) [26].

(c) Cytosol marker, lactic dehydrogenase, was quantified with the Merckotest® kit (Merck, Darmstadt, Germany) by evaluating NADH decrease spectrophotometrically (0.004, 0.002 and 0.0001 mg of protein/sample for P_{27}, P_{105} and S_{105} respectively)

Data analysis

Concentration–response curves were analysed by means of the computer program ALLFIT [27], which allows the evaluation of EC_{50} or IC_{50} values, as well as of upper and lower plateaus. The same program was used also to evaluate the statistical significance of the difference between parameters, by means of the F-test on the extra sum of squares, and to create the curves shown.

Equilibrium binding curves were analysed and drawn by means of the computer program LIGAND [28], which allows calculation of the binding parameters (K_d and B_{max}), selection of the best model (1-site versus 2-sites) by means of the F-test on the extra sum of squares, and calculation of the confidence limits of each fitted model.

The data are expressed as mean $\pm$ S.E.M. of independent experiments, except in Figures 1, 3 and 5, where representative experiments are shown, and the starting values, presented as absolute values (i.e. binding at time 0, ratio of bound over total ligand concentration at low [^{3}H]iloprost concentration and basal adenylate cyclase activity) were somewhat different from one another, due to inter-individual variability.

RESULTS

Internalization of [^{3}H]iloprost

Specific binding of 10 nM [^{3}H]iloprost to isolated human platelets attained equilibrium in 15 min at 37 °C and remained constant for up to 2 h (results not shown). At this point, a time-dependent dissociation was induced by addition of excess (0.1 mM) unlabelled iloprost to platelets that were either maintained at 37 °C or shifted to 4 °C. The two plateaus (reached after 30 min) were significantly different ($P < 0.05$): although at 37 °C approx. 74 % of bound [^{3}H]iloprost was dissociated, at 4 °C only 52 % was lost (Figure 1). The remaining 22 % of radioactivity

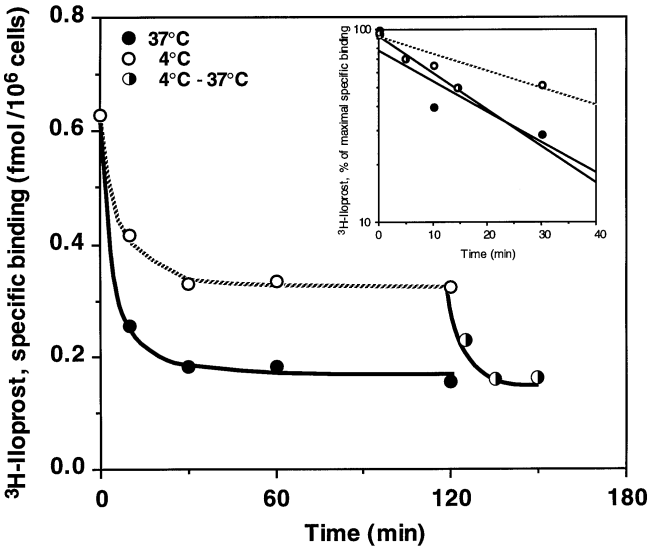

Figure 1 Effect of temperature on the dissociation of [^{3}H]iloprost

Platelets were incubated with 10 nM [^{3}H]iloprost for 2 h at 37 °C. At the end of incubation, one aliquot was maintained at 37 °C (●), whereas the other one was shifted to 4 °C (○); dissociation of bound ligand was induced by addition of 100 μM iloprost. Specifically bound radioactivity was determined over 2 h, after which time the second aliquot of cells was shifted to 37 °C (◑) and dissociation was evaluated over the next 30 min. The inset shows a semilogarithmic plot of the data from the same experiment, the units on the *y*-axis being percentage of maximal specific binding. Data are means of three replicates within one experiment, representative of two others.

Table 1 Evaluation of surface-bound [^{3}H]iloprost by means of acid washes

Platelets were incubated with 10 nM [^{3}H]iloprost for 2 h at either 4 °C or 37 °C, centrifuged and resuspended as described in the Materials and methods section; an aliquot was washed with the acidic buffer (0.2 M acetic acid/0.5 M NaCl /0.2% bovine serum albumin, pH 2.5) and the platelets were pelleted again. The radioactivity was determined both in the resulting pellets and in the supernatants. The percentage of [^{3}H]iloprost removed by acid wash represents the difference between binding to cells that have been washed with acid and the binding to unwashed cells, divided by the binding to unwashed cells. *$P < 0.005$ versus 4 °C.

Conditions		Specifically bound [^{3}H]Iloprost (fmol/10^6 cells)	[^{3}H]Iloprost removed by acid wash (%)
Temp. (°C)	Acid washes		
4	−	0.693 ± 0.074	80
4	+	0.141 ± 0.012	
37	−	0.644 ± 0.032	25*
37	+	0.486 ± 0.024	

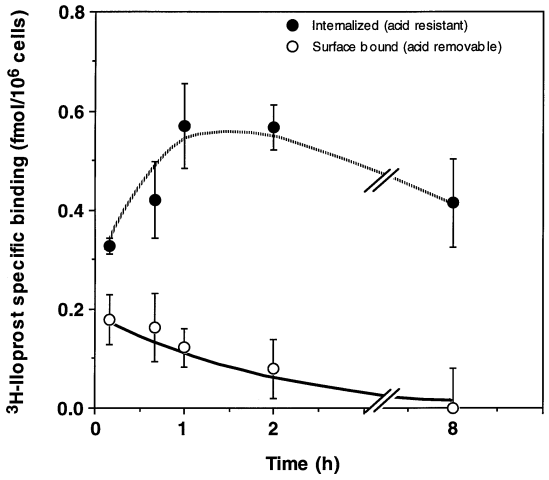

Figure 2 Effect of time on internalization process

Platelets were incubated at 37 °C with 10 nM [^{3}H]iloprost for the indicated times. Platelets were then centrifuged, resuspended, washed with the acidic buffer and pelleted again. The radioactivity was determined both in the resulting pellets (internalized, ●) and in the supernatants (surface-bound, ○). Data are means ± S.E. of three experiments, each performed in triplicate.

dissociated upon shifting the platelets from 4 °C to 37 °C, at a rate very similar to that of cells maintained at 37 °C (inset). This result indicates that [^{3}H]iloprost is internalized at a physiological temperature, and lowering the temperature inhibits recycling of the internalized component to the cell surface, thus preventing dissociation [29]. It should be noticed, however, that dissociation is not complete even at 37 °C, indicating that a further portion of the ligand (26%) is not bound to the cell surface in a reversible way.

Ligand internalization was confirmed through a different experimental approach. Platelets were incubated with [^{3}H]iloprost for 2 h at either 37 °C or 4 °C and an aliquot was subjected to acid treatment to remove surface-bound radioactivity [30]; the superficial (i.e. present in acidic buffer after acid wash), internalized (i.e. cell-associated after acid wash) and total radioactivity were determined. Table 1 indicates that, when incubation took place at 4 °C, 80% of the specifically bound [^{3}H]iloprost could be removed by acid wash, and was therefore present on the cell surface. In contrast, in platelets incubated at 37 °C only 25% of bound iloprost was acid-sensitive. Thus much less [^{3}H]iloprost was present on the cell surface at 37 °C than at 4 °C, indicating internalization of the surface-bound iloprost.

Table 2 Effect of ligand concentration on internalization

Platelets were preincubated with the indicated concentration of unlabelled iloprost for 2 h at either 4 °C or 37 °C. After washing, binding of 700 nM [^{3}H]iloprost was performed at 4 °C. Internalization at 37 °C shows a significant linear correlation with iloprost concentration ($P < 0.05$, one-way ANOVA).

Preincubation conditions		[^{3}H]Iloprost, bound (fmol/10^6 cells)	% of Basal	Amount internalized (fmol/10^6 cells)
Iloprost (M)	Temp. (°C)			
0	4	38.0 ± 7.1		
0	37	32.0 ± 6.4	100 ± 20.0	6.0[a]
10^{-9}	37	29.4 ± 6.2	91.0 ± 19.5	8.6[b]
3×10^{-9}	37	16.6 ± 1.2	51.9 ± 3.9	21.4[b]
10^{-8}	37	11.4 ± 2.3	35.6 ± 7.2	26.6[b]

[a] Calculated from the difference between fmol bound at 4 °C and fmol bound at 37 °C.
[b] Calculated from the difference between fmol bound to control cells and fmol bound to iloprost-pretreated cells, plus fmol internalized under control conditions.

Characteristics of the internalization process

Iloprost internalization was a time-dependent process, as demonstrated by acid treatment of platelets incubated at 37 °C with [^{3}H]iloprost for different time intervals (Figure 2). The acid-resistant (i.e. internalized) specific binding increased for up to (at least) 2 h, and the amount of surface-bound ligand decreased with time.

Internalization was augmented in a concentration-dependent way by the ligand [linear correlation, $P < 0.05$, one-way analysis of variance (ANOVA)]. In fact, when the platelets were pre-incubated at 37 °C with increasing concentrations of unlabelled iloprost (to promote internalization) and then binding to washed platelets was assayed at 4 °C (to prevent further internalization), the amount of [^{3}H]iloprost specifically bound to the cell surface was progressively lowered (Table 2).

Moreover, internalization depends on the nature of the ligand used in the preincubation step. The ability of 10 nM iloprost to induce the internalization process was compared with that of the same concentration of PGI_2 and 6-keto-$PGF_{1\alpha}$, the product of non-enzymic PGI_2 hydrolysis. In order to overcome the problem

Table 3 Effect of ligands on internalization process

Platelets were incubated at either 37 °C or 4 °C with or without the indicated ligands at a concentration of 10 nM. PGI_2 was added every 20 min. After 2 h cells were centrifuged, washed, resuspended and incubated with 10 nM [^{3}H]iloprost at 4 °C for 30 min. Internalized amount is expressed as the difference between specific binding in cells preincubated at 4 °C and 37 °C, divided by the amount bound at 4 °C. In control cells, the amount of [^{3}H]iloprost internalized was 0.33 ± 0.21 fmol/10^6 cells. Data are means of three experiments, except for control ($n = 5$), each performed in triplicate. *$P = 0.05$, **$P < 0.02$ versus control.

Ligand during incubation	[^{3}H]Iloprost, internalized amount (% of specific binding at 4 °C)
Control	15.3 ± 8.1
Iloprost	65.1 ± 13.4**
PGI_2	56.3 ± 20.1*
6-Keto-$PGF_{1\alpha}$	29.2 ± 10.3

Table 4 Effect of metabolic inhibitors on receptor internalization

Platelets were incubated with 10 nM [^{3}H]iloprost at 37 °C and 4 °C for 30 min with or without 45 mM 2DG and 1 mM DNP. At the end of incubation platelets were centrifuged, resuspended, washed with acidic buffer and then pelleted again. The radioactivity was determined both in the resulting pellets and in the supernatants. Results are expressed as the percentage of specifically bound radioactivity present either in pellet or in supernatant, versus total specifically bound radioactivity. Data are means of three experiments, each performed in triplicate. $^*P < 0.02$ versus control at 37 °C.

Preincubation conditions		[^{3}H]Iloprost, specific radioactivity (% of total)	
Treatment	Temp. (°C)	Internalized	Surface bound
Control	37	74.4 ± 11.1	25.6 ± 10.6
2DG + DNP	37	22.8 ± 4.6*	77.2 ± 5.3*
Control	4	26.2 ± 1.9	73.8 ± 0.5
2DG + DNP	4	20.4 ± 1.5	79.6 ± 6.8

Table 5 Distribution of [^{3}H]iloprost and of marker enzymes among subcellular fractions

Platelets were preincubated for 18 h at either 4 °C or 37 °C, with or without 10 nM iloprost, washed and disrupted. P_{27}, P_{105} and S_{105} were assayed for [^{3}H]iloprost-specific binding. The results are expressed as the percentage of specifically bound radioactivity present in each fraction, versus the sum of specific binding to the three fractions. Data are means of three experiments, each performed in triplicate. Distribution of adenylate cyclase, esterase and lactic dehydrogenase is expressed as relative specific activity according to Marcus et al. [44]. Data are means of three experiments, each performed in duplicate. $^*P < 0.02$ versus groups A and D, $^{**}P < 0.001$ versus groups A, C, D (two-way ANOVA). $^{***}P < 0.01$ versus P_{27} and $P < 0.005$ versus P_{105}, $^{****}P < 0.001$ versus P_{27} and P_{105}, $^{*****}P < 0.001$ versus P_{105} and S_{105} (one-way ANOVA).

Preincubation conditions		[^{3}H]Iloprost specific binding (% of total)		
Treatment	Temp. (°C)	P_{27}	P_{105}	S_{105}
A, Control	37	52.8 ± 7.0	42.4 ± 11.90	4.85 ± 4.85
B, Iloprost	37	15.8 ± 2.4**	17.6 ± 1.95	66.70 ± 4.35*
C, Control	4	62.5 ± 2.7	24.6 ± 10.15	12.80 ± 12.83
D, Iloprost	4	60.4 ± 4.1	31.6 ± 12.10	8.1 ± 8.0
	Relative specific activity			
Enzymic assay	P_{27}	P_{105}	S_{105}	
Adenylate cyclase	2.26 ± 0.08 *****	0.77 ± 0.01	0.05 ± 0.04	
Esterase	0.85 ± 0.01	0.63 ± 0.01	1.17 ± 0.04***	
Lactic dehydrogenase	0.09 ± 0.01	0.15 ± 0.06	1.78 ± 0.17 ****	

of PGI_2 lability (half-life 13 min at pH 8 and 37 °C) at least in part, the whole procedure was performed at pH 8 and the same concentration of PGI_2 was repeatedly added every 20 min during the preincubation. Under these conditions, PGI_2 and iloprost were approximately equally effective in inducing the internalization of [^{3}H]iloprost, whereas 6-keto-$PGF_{1\alpha}$ did not elicit any significant effect (Table 3).

Table 4 shows the combined effect of 2DG, a blocker of the entry of sugars into the glycolytic pathway [31], and of DNP, a decoupling agent [32], on the internalization process investigated by means of acid treatment. In this case, the incubation was limited to 30 min, because the presence of these metabolic inhibitors for a longer time affected the platelet viability. Under control conditions at 37 °C, 74.4 % of specifically bound [^{3}H]iloprost could not be removed by acid wash. Inclusion of 2DG and DNP during the incubation drastically reduced the percentage of acid-resistant [^{3}H]iloprost (22.8 %), approximately to the same level observed when the incubation was performed at 4 °C, when internalization should be inhibited. The latter condition was not significantly affected by treatment with the energy depleting agents.

The experimental protocol described for the experiment in Figure 1 was used to investigate whether a transporter was involved in the internalization process: probenecid, an inhibitor of the organic anion transporter [33], did not modify the pattern of the dissociation kinetics at different temperatures (results not shown), suggesting that such a transporter does not play a major role.

Receptor internalization and down-regulation

In order to investigate whether not only the ligand but also the receptor was internalized and therefore down-regulated, two different approaches, i.e. binding to subcellular fractions and binding to platelets that had undergone desensitization, were used. In both cases, preincubation with iloprost was performed for 18 h. Platelets were still viable after this prolonged preincubation, as demonstrated by their ability to respond to pro- and anti-aggregating agents: although the equally effective concentration of collagen was slightly increased (3-fold), iloprost potency was unaffected (see also Figure 4).

With the first approach, platelets were subjected to subcellular fractionation under conditions where internalization was either induced or inhibited, and binding of [^{3}H]iloprost to the different fractions was performed. Table 5 shows that 50–60 % of the specific binding of the total homogenate occurred to the P_{27} fraction when internalization was prevented (preincubation at 4 °C, or at 37 °C without iloprost). In contrast, under conditions previously demonstrated to favour internalization (37 °C with iloprost), only 16 % of the binding occurred to the above-mentioned fraction, versus 67 % to the S_{105} fraction. The P_{27} fraction was enriched in plasma membranes, whereas S_{105} contained mostly the cytosol, although contaminated by endoplasmic reticulum, and P_{105} represented a lighter particulate fraction, possibly microsomal, as indicated by the specific activity of the marker enzymes (Table 5). Thus translocation of the receptor from the plasma membrane to either the cytosol or the microsomal fraction occurs upon prolonged treatment of the platelets with iloprost. A parallel experiment performed after a preincubation with iloprost for only 2 h yielded results with a trend similar to those shown in Table 5, but less clear-cut (results not shown). The decrease in binding observed after preincubation with iloprost at 37 °C could not be due to incomplete removal of the ligand, because preincubation with unlabelled ligand at 4 °C did not induce a significant decrease in binding.

With the second approach, specific binding of [^{3}H]iloprost to the cell surface was assayed at 4 °C in platelets that had been exposed to 10 nM iloprost either at 37 °C or at 4 °C for 18 h (Figure 3). When the preincubation step was performed at low temperature internalization was prevented (Figure 3A), [^{3}H]iloprost binding was biphasic, and indeed non-linear fitting of the data indicated the 2-site model to be significantly better ($P < 0.001$) than the 1-site model (Table 6). In contrast, binding to platelets preincubated with the unlabelled ligand at 37 °C (internalization induced) was best fitted by the model for a single class of sites (Figure 3B), with characteristics very close to those of the lower-affinity class (Table 6). Preincubation of platelets without iloprost, at either temperature, resulted in a situation similar to that of Figure 3(A), i.e. the presence of heterogeneous binding sites (results not shown).

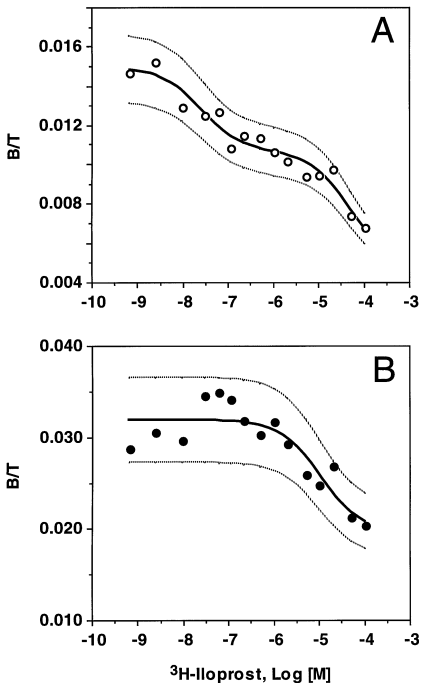

Figure 3 Binding curves for [^{3}H]iloprost in platelets preincubated at 4 °C or 37 °C

PRP was preincubated for 18 h at either 4 °C (**A**) or 37 °C (**B**), with 100 nM iloprost, washed, and assayed for [^{3}H]iloprost-specific binding. [^{3}H]Iloprost concentration was varied between 1 and 10 nM. Higher ligand concentrations were obtained by adding increasing unlabelled iloprost to 10 nM [^{3}H]iloprost (mixed-type curve [45]). B/T: ratio of bound over total ligand concentration. The solid line represents the curve obtained by computerized analysis with the program LIGAND [28] and the broken lines represent the 95% confidence limits of the fitted model. The experiment is representative of three others, each performed in triplicate.

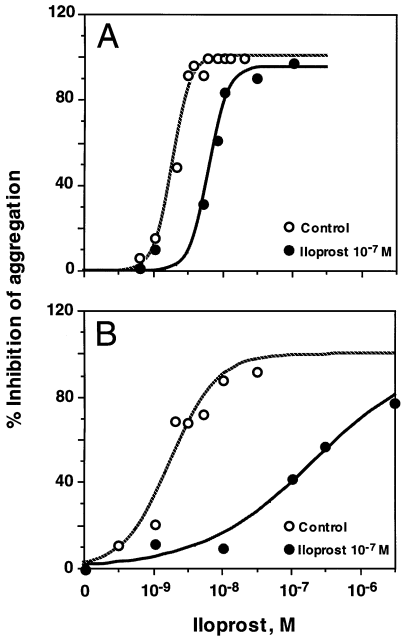

Figure 4 Effect of preincubation with iloprost on iloprost-induced inhibition of platelet aggregation

Platelet-rich plasma was preincubated at 37 °C for either 2 (**A**) or 18 h (**B**) with (●) or without (○) 100 nM iloprost. At the end of the incubation, platelets were centrifuged, washed, resuspended in PPP, preincubated for 1 min with the indicated iloprost concentrations and challenged with 6 μg/ml collagen. Data are means of three experiments, each performed in duplicate.

In vitro desensitization of human platelets

Figure 4 shows that preincubation of human PRP with 100 nM iloprost at 37 °C for 2 h (Figure 4A) resulted in an approx. 3.4-fold reduction in the potency of this ligand as inhibitor of collagen-induced aggregation. Thus the concentrations yielding 50% inhibition of maximal aggregation (IC_{50}) were 1.86 ± 0.11 nM and 6.30 ± 0.41 nM in control and iloprost-treated platelets ($P < 0.001$, as evaluated by computer analysis of the concentration–response curves). When the preincubation with iloprost was prolonged for 18 h (Figure 4B), the apparent difference in potency was 121-fold ($IC_{50} = 1.73 \pm 0.24$ nM and 210 ± 75 nM respectively, $P = 0.005$), although in this case a rigorous comparison is not possible because the two curves have a different slope. It has been suggested that, if spare receptors are present, the apparent decrease in potency upon desensitization might not reflect a true affinity alteration, but rather be secondary to a decrease in receptor number, as one would expect during internalization [12].

The activation of adenylate cyclase by iloprost was evaluated in membranes prepared from platelets preincubated in plasma at 37 °C for either 2 or 18 h with or without 100 nM iloprost. In platelets preincubated for 2 h, the maximal cAMP increase over basal was only modestly lower in pretreated platelets than in controls (291 and 223 pmol/mg of protein per min, for untreated and iloprost-treated membranes respectively).

The pattern was markedly different when iloprost-sensitive adenylate cyclase was assessed in membranes from platelets preincubated for 18 h; the presence of iloprost during the preincubation period induced a marked desensitization of the response (Figure 5; maximal increment 205 and 53 pmol/mg of

Table 6 Parameters of [^{3}H]iloprost binding sites detected after preincubation at different temperatures with iloprost

Platelets were preincubated for 18 h at either 4 °C or 37 °C with 10 nM iloprost, washed, and assayed for [^{3}H]iloprost specific binding at 4 °C for 30 min. CV = coefficient of variation.

Preincubation Temp. (°C)	K_{d1} (M)	CV (%)	K_{d2} (M)	CV (%)	B_{max1} (fmol/10^6 cells)	CV (%)	B_{max2} (fmol/10^6 cells)	CV (%)
4	2.6×10^{-8}	66	1.0×10^{-5}	78	1.1	63	2800	102
37	—	—	4.8×10^{-5}	61	—	—	650	71

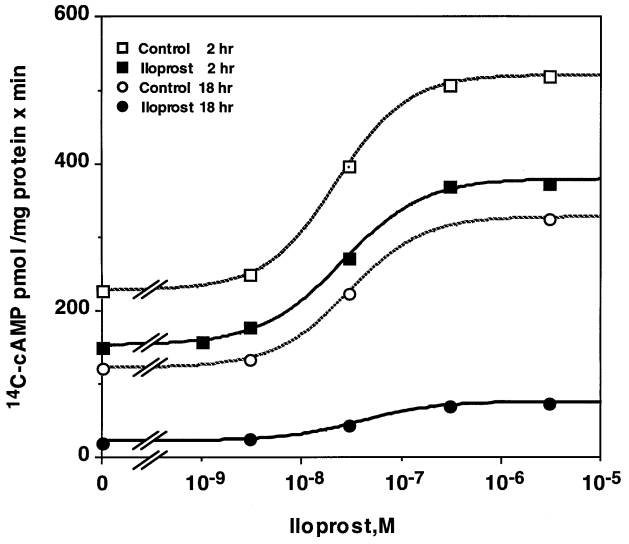

Figure 5 Iloprost-sensitive adenylate cyclase in membranes from platelets preincubated with or without iloprost

Platelet-rich plasma was preincubated for either 2 h (□, ■) or 18 h (○, ●) at 37 °C with (desensitized, solid symbols) or without (control, open symbols) 100 nM iloprost, washed, disrupted, and membranes (pellet at 27 000 ***g***) were prepared. The experiment is representative of two others, each performed in triplicate.

protein per min in control and desensitized platelets respectively, $P < 0.01$).

The changes in the amount of cAMP formed were not due to modifications of the phosphodiesterase activity (results not shown).

The effect of iloprost on cAMP formation was assessed also in intact platelets, by means of enzyme immunoassay. Preincubation of PRP with 100 nM iloprost even for 2 h reduced the subsequent stimulation of cAMP production by iloprost itself (1.30 ± 0.50 and 0.39 ± 0.09 pmol/10^6 cells, $n = 3$, in control and pretreated cells respectively). Such a decrease was more marked after 18 h (1.18 ± 0.52 versus 0.11 ± 0.11, $n = 3$). In these experiments, iloprost concentration during preincubation was 10-fold higher than that used in washed platelets: in fact, the apparent iloprost affinity in PRP is almost 10-fold lower than in washed platelets [20], probably because it binds to plasma proteins.

DISCUSSION

The studies presented here support the concept that prostacyclin receptors in human platelets are internalized and down-regulated. Although previous reports demonstrated desensitization of this receptor, with a consequent loss of platelet responsiveness to PGI_2 and its analogues [9–11], we present here evidence for a shift of binding sites from the cell surface to another subcellular compartment and for the loss of the high-affinity class of sites from the cell surface; such a phenomenon might represent one of the molecular mechanisms underlying desensitization [34].

The internalization of [^{3}H]iloprost, a ligand known to bind to the prostacyclin receptor [19,20], has been demonstrated here by means of two different approaches, i.e. through kinetic studies performed at different temperatures [29] and by stripping the surface-bound ligand with acid washes [30]. Furthermore, the loss of specific binding sites for [^{3}H]iloprost from the surface was demonstrated by binding to different cell fractions and by computer analysis of binding curves in control and desensitized platelets.

Surprisingly, the kinetic studies showed that, when platelets were incubated with [^{3}H]iloprost for 2 h, the dissociation was not complete even at 37 °C (when recycling of the receptors should be allowed), at variance with the complete dissociation which we obtained in PRP incubated with the same ligand for 10 min [20]. Given the complete dissociability after 10 min [20], it is unlikely that the highly lipophilic [^{3}H]iloprost is trapped by the membrane lipids, as suggested for platelet activating factor [35]. Thus the discrepancy might be explained if, upon a more prolonged incubation, the ligand–receptor complex is transferred to a compartment where either ligand dissociation or receptor recycling is prevented.

The kinetic studies and the acid-wash procedure clearly demonstrate that [^{3}H]iloprost is lost from the surface of human platelets, but this is no proof of receptor internalization: in fact, iloprost itself might be taken up by the cells, possibly by a carrier-mediated process [36,37]. However, this is unlikely to take place under our experimental conditions, in view of the fact that probenecid, an inhibitor of transporters for organic anions [33], including eicosanoids [36,37], does not modify the pattern of [^{3}H]iloprost dissociation at different temperatures (results not shown).

On the other hand, the experiment depicted in Figure 3 clearly demonstrates a loss of specific binding sites from the surface upon preincubation with iloprost, ruling out the possibility that only the ligand is internalized. Interestingly, two different classes of binding sites for [^{3}H]iloprost can be identified under conditions where internalization is prevented, and the high-affinity class is totally lost when internalization is allowed. This behaviour is similar to that demonstrated for thromboxane A_2 receptor in platelets [38], but is at variance with the results of Alt et al. [9], who observed only a partial loss of iloprost high-affinity sites: this discrepancy might perhaps be explained by the lower temperature used by these authors during desensitization.

Furthermore, when desensitization was performed for 18 h, we have demonstrated a marked shift of the binding sites from the subcellular fraction P_{27}, enriched in plasma membranes, to fraction S_{105}, composed mainly of cytosol plus endoplasmic reticulum (Table 5). This occurs only upon prolonged preincubation with iloprost at 37 °C, although a small aliquot of the binding sites was found in P_{105} (lighter particulate fraction) and S_{105} even under non-internalizing conditions. This represents further support of the hypothesis of internalization of the ligand–receptor complex, and it is interesting to remember that specific high-affinity binding sites for PGE_1, which binds also to the PGI_2 receptor [3], had been described in the liver cytosol [39].

We have characterized the internalization process and have shown that it is detectable already at 10 min (Figure 2), increases with the time of exposure to the agonist, and is complete after 8 h, when no more radioactivity can be removed from the cell surface. Other results are in agreement with the conclusion that complete internalization requires more than 2 h. First, the desensitization of iloprost-sensitive adenylate cyclase (Figure 5) and cAMP formation was more pronounced after 18 h than at 2 h. Moreover, the redistribution of binding sites between the different subcellular fractions was clearly detectable after 18 h (Table 5), whereas after 2 h the results were not as clear-cut (results not shown). Finally, the shift of the concentration–response curve for the anti-aggregatory effect of iloprost (Figure 4) was more marked after the longer preincubation.

Internalization is increased in a concentration-dependent manner by the presence of iloprost in a nanomolar range (Table 2), but a limited extent of constitutive, i.e. ligand-independent,

internalization was detectable in most experiments (see for instance Tables 3 and 5), similarly to other ligands [40,41]. We have ruled out that the decrease in [^{3}H]iloprost-specific binding to the cell surface upon preincubation with unlabelled ligand is due solely to the latter remaining bound to the receptor. Indeed, when the preincubation is performed at 4 °C, only a minor, not significant, decrease in binding occurs upon treatment with iloprost (see for instance Table 5).

Not only iloprost, but also PGI_2 itself, is able to trigger this process, their effect being roughly similar (Table 3); in contrast, 6-keto-$PGF_{1\alpha}$, which is known to bind with a very low affinity and to be very weak as an inhibitor of platelet aggregation [42], has no significant effect on the loss of surface binding sites.

The internalization of [^{3}H]iloprost is an energy-dependent process, as demonstrated by its inhibition by energy-depleting agents such as 2DG [31], which inhibits the glycolytic pathway, and DNP [32], a decoupling agent (Table 4). Moreover, in various experiments we have shown that the temperature (37 °C versus 4 °C) affects both the kinetics and the subcellular distribution of binding sites, and this is compatible with an energy requirement for the internalization process.

Although we have not addressed the problem of receptor recycling directly, externalization of the receptors is suggested by the dissociation studies shown in Figure 1. Furthermore, a large discrepancy exists between the maximal amount bound to the high-affinity site (1.1 fmol/10^6 cells, Table 6) and the amount internalized at a saturating iloprost concentration (26.6 fmol/10^6 cells, Table 2): the latter exceeds the site capacity by approx. 25-fold, strongly suggesting that each receptor shuttles between the plasma membrane and another compartment, transferring some 25 ligand molecules per receptor molecule during the incubation period [43].

In conclusion, our data indicate that the prostacyclin receptor is internalized and such internalization could subserve desensitization. Indeed, it had already been demonstrated by various laboratories, and is confirmed under our experimental conditions, that on preincubation with agonists, human platelets are desensitized to iloprost, in terms of both inhibition of aggregation and adenylate cyclase activity.

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