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Prostacyclin analogues reduce ADP-ribosylation of the α-subunit of the regulatory G_s-protein and diminish adenosine (A_2) responsiveness of platelets

Robert J. Edwards, John MacDermot & Amanda J. Wilkins

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Ducane Road, London W12 0HS.

1 Prostacyclin and adenosine activate adenylate cyclase in human platelet membranes and inhibit platelet aggregation. Results are presented which show that prolonged incubation of platelets with iloprost (a stable prostacyclin analogue) results in a reduction in the capacity for adenylate cyclase activation by the adenosine analogue 5'-(N-ethyl)-carboxamidoadenosine (NECA), NaF, guanyl-5'-yl imidodiphosphate or GTP. However, iloprost pretreatment resulted in no change in the binding of [^{3}H]-NECA to platelet membranes.

2 These results contrast with those obtained after pretreatment with 2-chloroadenosine which revealed no change in NaF or guanyl-5'-yl imidodiphosphate sensitivity of adenylate cyclase. Pretreatment with 2-chloroadenosine resulted in reduced NECA-dependent adenylate cyclase activation, and loss of [^{3}H]-NECA binding sites.

3 The heterologous desensitization of adenosine A_2-receptors by iloprost is accompanied by a loss (greater than 80%) of a 45 kDa protein from the plasma membrane, as revealed by [^{32}P]-ADP-ribosylation in the presence of cholera toxin.

4 It is proposed that this example of heterologous desensitization is mediated by elimination of $G_{s\alpha}$, a subunit of the stimulatory guanyl nucleotide-binding regulatory protein.

Introduction

During vascular injury, platelets aggregate on damaged endothelial cells that line blood vessel walls. The process is triggered by numerous factors, including exposed collagen on the subendothelial matrix (Wilner *et al.*, 1968), and ADP from damaged cells (Born, 1962). The size of a developing platelet plug is further increased by the pro-aggregatory factors adenosine 5'-pyrophosphate (ADP) (Born, 1962; Born & Kratzer, 1984), thromboxane A_2 and the prostaglandin endoperoxides released from platelets during aggregation (Hamberg *et al.*, 1975). The increase in the size of the platelet aggregate is finally arrested by several anti-aggregatory factors including prostaglandin I_2 (PGI_2) (Moncada *et al.*, 1976) and adenosine (Born *et al.*, 1965) (a product of ATP hydrolysis) released from adjacent endothelial cells during injury. Both PGI_2 (Gorman *et al.*, 1977; Tateson *et al.*, 1977) and adenosine (Haslam & Rosson, 1975; Huttemann *et al.*, 1984) mediate inhibition of platelet aggregation by activation of adenylate cyclase (ATP: pyrophosphate-lyase (cyclizing), EC 4.6.1.1). Desensitization of platelet PGI_2 responsiveness following exposure to PGI_2 receptor agonists has been described previously (Miller & Gorman, 1979), and is mediated by a reduction in the number of receptors on the platelet plasma membrane (Alt *et al.*, 1986). Reduced platelet responsiveness to PGI_2 has also been demonstrated *in vivo* following the therapeutic administration of PGI_2 in vascular disease (Sinzinger *et al.*, 1981).

Diminished responsiveness of a receptor that follows previous exposure to an agonist that occupies the same receptor is termed homologous desensitization. This effect is mediated by a reduction in receptor number, a decrease in agonist affinity or altered coupling between the receptor and G_s or G_i (Clark & Butcher, 1979; Su *et al.*, 1980; Doss *et al.*, 1981; Kassis & Fishman, 1982; Strulovici *et al.*, 1983; Stadel *et al.*, 1983a, b; Insel *et al.*, 1983; Garrity *et al.*, 1983) (the two different guanyl nucleotide-binding regulatory proteins that mediate stimulation or inhibition respectively of adenylate cyclase by their interaction between the receptor and the adenylate cyclase molecule).

[1] Correspondence.

Heterologous desensitization, on the other hand, describes the reduction in responsiveness of one receptor type that accompanies previous occupation by an agonist of a different receptor type. The mechanism(s) involved remains somewhat obscure, but appears to involve changes in G_s, or altered coupling of the G-protein to either the catalytic subunit of adenylate cyclase or to the receptor (Kassis & Fishman, 1982; Garrity *et al.*, 1983). Heterologous desensitization may involve stimulatory or inhibitory receptor-mediated changes in adenylate cyclase activity (Hsia *et al.*, 1985).

The subunit structure of the G-protein involved in the activation of adenylate cyclase (G_s) has been elucidated (Northup *et al.*, 1982; Manning & Gilman, 1983; Gilman, 1984; Hildebrandt *et al.*, 1984; Katada *et al.*, 1984 a, b, c). G_s is a heterotrimer with α (45 kDa), β (35 kDa) and γ (10 kDa) subunits (Gilman, 1984; Hildebrandt *et al.*, 1984). The interaction between the receptor, the catalytic adenylate cyclase subunit and the G_s heterotrimer have been reviewed extensively. In the presence of an agonist, the binding of guanosine 5'-triphosphate (GTP) to $G_{s\alpha}$ leads to the dissociation of $G_{s\alpha}$ from $G_{s\beta\gamma}$ (Northup *et al.*, 1982; Gilman, 1984; Katada *et al.*, 1984c). $G_{s\alpha}$ is then coupled to the adenylate cyclase catalytic subunit and ATP hydrolysis is accelerated (Northup, *et al.*, 1983; Katada, *et al.*, 1984c). Finally, GTP is hydrolysed by the catalytic activity of $G_{s\alpha}$ and the activation of the adenylate cyclase is terminated by the re-association of $G_{s\alpha}$ with $G_{s\beta\gamma}$ (Northup *et al.*, 1983; Gilman, 1984). The high and low affinity states of the receptor for the agonist are also dependent on the G_s protein. The receptor exists in a high affinity state when coupled to G_s (Lefkowitz *et al.*, 1984). In the presence of GTP, the affinity of the ligand-receptor interaction is reduced, probably by dissociation of the receptor-G_s complex (Gilman, 1984; Lefkowitz *et al.*, 1984). The ligand is then released rapidly from the receptor, and the G_s heterotrimer dissociates as described previously to facilitate adenylate cyclase activation.

The present paper describes an investigation into the possibility of heterologous desensitization of adenosine A_2-receptors following occupation of PGI_2 receptors by a stable structural analogue of PGI_2 (iloprost) (Skuballa & Vorbruggen, 1983).

Methods

Platelets

Blood samples (50–150 ml) from human volunteers (aged 19–39 years) were mixed with 3.15% (w/v) trisodium citrate (9 : 1 v/v). Following centrifugation at 500 *g* for 15 min at 18°C, the supernatant PRP (platelet-rich plasma) was removed. In all experiments, HEPES buffer, pH 7.4, was added to a final concentration of 20 mM, and other additions made as described in the text.

Platelet membranes were prepared from PRP at 4°C as follows. The platelets were pelleted by centrifugation at 80,000 *g* for 20 min, and then disrupted in 5 ml 5 mMM Tris-HCl buffer, pH7.4, containing 0.25 mM EDTA, in a tighly fitting Dounce homogeniser. The membranes were centrifuged at 80,000 *g* for 20 min and the pellet washed 2 further times by resuspension in 5 ml of the same Tris-HCl/EDTA buffer. Membranes were finally suspended in an appropriate volume (usually 1.5 ml) of 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 mM EDTA. Samples were then stored at −80°C before measurement of adenylate cyclase activity or the binding of [^{3}H]-NECA.

Adenylate cyclase

Adenylate cyclase activity was measured by a modification (Leigh *et al.*, 1984) of previously described methods (Salomon *et al.*, 1974). Briefly, reaction mixtures of 100 μl contained 50 mM Tris-HCl buffer, pH 8.5, 5 mM magnesium sulphate, 20 mM creatine phosphate, disodium salt, 10 iu of creatine kinase, 150 iu mg^{-1} protein (where one iu will transfer 1.0 μmol of phosphate from phosphocreatine to ADP per min at pH 7.4 and 30°C), 1 mM cyclic AMP, sodium salt, 0.25 mM Ro20-1724 (a phosphodiesterase inhibitor), 1 mM [α-^{32}P]-ATP (2–3 μCi), 4 μM GTP and 30–50 μg of membrane protein. Reactions were incubated at 37°C for 20 min and then terminated by the addition of 800 μl of 6.25% (w/v) trichloroacetic acid. To each tube was added 100 μl of [8-^{3}H]-cyclic AMP (approximately 10,000 c.p.m.), and the reaction mixtures were centrifuged at 4°C for 20 min at 800 *g*. The [α^{32}P]-ATP and [^{32}P]-cyclic AMP were separated by a 2-step chromatographic procedure (Salomon *et al.*, 1974), and the yield of [^{32}P]-cyclic AMP was corrected for losses on the columns by measurement of the recovery of [^{3}H]-cyclic AMP.

[^{3}H]-5'-(N-ethyl)-carboxamidoadenosine binding

The binding of [^{3}H]-NECA to human platelet membranes was performed by a modification (MacDermot & Nirenberg, 1978) of the method of Pert & Snyder (1973). Triplicate reaction volumes of 100 μl contained 50 mM Tris-HCl buffer pH 7.4, human platelet membranes (18–44 μg membrane protein) and [^{3}H]-NECA at concentrations between 5 and 2000 nM. Incubations were performed at 4°C for one hour, and then terminated by the addition of 4 ml ice cold 50 mM Tris-HCl buffer pH 7.4. The membranes were then separated by rapid filtration under reduced pressure through GF/B glass filter discs (25 mm diameter), and washed 3 times with 4 ml of the same buffer. The filters

were then dried under an infra-red lamp for 1 h, and radioactivity counted following the addition of 10 ml Instagel (Packard Instrument Co. Inc.). Specific binding was defined as that displaced by 100 μM NECA in 3 parallel tubes at each [^{3}H]-NECA concentration. The data were analysed by an iterative programme for non-linear regression (Koeppe & Hamann, 1980), and binding was fitted to a 4 parameter model identifying 2 independent binding sites.

ADP-ribosylation of $G_{s\alpha}$

[^{32}P]-ADP-ribosylation of platelet membrane proteins was measured by a modification of methods described previously (Katada & Ui, 1982; Ribeiro-Neto *et al.*, 1985). Reaction mixtures of 110 μl contained 15 mM Tris-HCl buffer pH 7.4, 1 mM ATP, 15 mM glycine, 2.5 mM $MgCl_2$, 10 mM thymidine, 10 μM [^{32}P]-NAD (20 μCi), 10 μg cholera toxin (a subunit, activated as described below), 100 μM GTP, 300 mM potassium phosphate buffer pH 7.0, and 122–158 μg human platelet membrane protein. Incubations were performed at 37°C for 30 min, and the reactions terminated by the addition of 1 ml 20% (w/v) trichloroacetic acid. After 20 min at 0°C, the precipitate was pelleted in a microfuge (10 min at about 14,000 *g*). The supernatants were discarded, and the pellets washed 2 times in 1 ml ether : ethanol (1 : 1 v/v). The membranes were then resuspended in 1 ml ether, pelleted in the microfuge, and dried under a stream of N_2. The pellet was then dissolved in 400 μl of 0.0625 M Tris-HCl buffer, pH 6.8 containing 10% (v/v) glycerol, 1% (w/v) SDS (sodium dodecyl sulphate), 1% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue. The samples were heated in a boiling water bath for 3 min, and then loaded on to a 10% SDS-polyacrylamide gel with a 3.75% polyacrylamide stacking gel. Molecular weights were determined by comparison with standards of bovine serum albumin (68 kDa), alcohol dehydrogenase (41 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), α-chymotrypsin inhibitor (26 kDa) and myoglobin (17 kDa). After completion of electrophoresis, the gel was stained and fixed for 30 min in 0.2% (w/v Coomassie Brilliant Blue R, 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained in 25% (v/v) methanol and 7.5% (v/v) acetic acid until the background was clear. The gel was dried using a Bio-Rad gel drier and examined by autoradiography for 70 h at −80°C using Kodak X-Omatic RP film in a Kodak X-Omatic cassette incorporating regular intensifying screens. The autoradiographic plates were then scanned in a Gelman DCD-16 Scanner with transmitted light at 600 nm.

The cholera toxin (A subunit) was activated after being dissolved in 250 μl H_2O, at a final concentration of 1 μg μl^{-1}. A 10 μl volume of the toxin solution was added to 10 μl 50 mM dithiothreitol. The tube was capped and incubated at 37°C for 30 min.

Statistical analyses involved 2-way analysis of variance or Student's *t* tests (unpaired, 2-tailed) as appropriate.

Materials

[^{3}H]-NECA (5'-(N-ethyl)-carboxamidoadenosine, 31 Ci mmol^{-1}), [8-^{3}H]-adenosine 3' : 5'-cyclic monophosphate ([8-^{3}H]-cyclic AMP; 23.6 Ci mmol^{-1}) and [α-^{32}P]-ATP (40–50 Ci mmol^{-1}) were obtained from Amersham International; [^{32}P]-NAD (23.3 Ci mmol^{-1}) from New England Nuclear; ATP, cyclic AMP, creatine kinase (ATP: creatine N-phospho- transferase; EC 2.7.3.2), creatine phosphate, 5'-(N-ethyl)-carboxamidoadenosine) (NECA), GTP, Gpp(NH)p (guanyl-5'-yl imidodiphosphate), 2-chloroadenosine, 3-isobutyl-1-methylxanthine and cholera toxin (A subunit) were obtained from Sigma Chemical Co. Ltd. Ro20-1724 was a generous gift from Roche Products; iloprost a gift from Schering AG Berlin, 6α-carbaprostaglandin I_2 a gift from the Wellcome Research Laboratories and PGE_1 a gift from Upjohn Co.

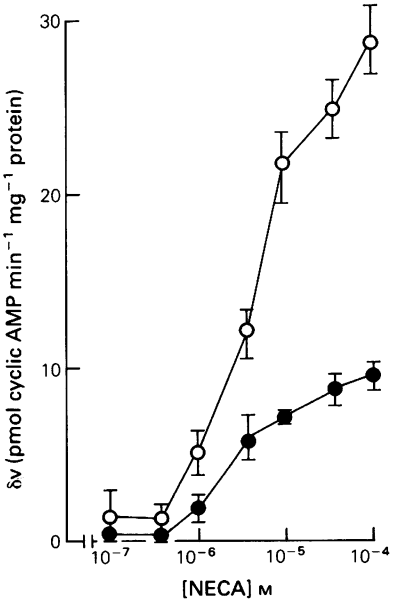

Figure 1 Heterologous desensitization of adenosine A_2 receptors by iloprost. Platelets from one subject were incubated for 24 h in the absence (○) or presence (●) of 10 μM iloprost. Platelet membranes were prepared, and adenylate cyclase activity measured in triplicate in the absence or presence of 5'-(N-ethyl)-carboxamidoadenosine (NECA, 0.1 to 100 μM). Results show mean (vertical lines are s.e.mean) values for the increase in enzyme activity (δv).

Results

In preliminary experiments on human platelet membranes, the activation of adenylate cyclase by 2-chloroadenosine and NECA were compared. The concentrations of these adenosine agonists that mediated half-maximum enzyme activation (K_{act}) were 2.89 μM and 1.69 μM respectively. In other experiments, the inhibitory effect of 3-isobutyl-1-methylxanthine on NECA (10 μM)-stimulated adenylate cyclase activity was measured. The K_i value was 8.7 μM. The value of K_i was determined from the expression $K_i = IC_{50}/[1 + ([agonist]/K_{act})]$, where IC_{50} was the inhibitor concentration (60 μM) producing 50% inhibition, the agonist (NECA) concentration was 10 μM, and $K_{act} = 1.69$ μM (as above).

Platelets in 15–25 ml PRP were incubated at room temperature (18–22°C) for 24 h in 20 mM HEPES buffer pH 7.4 in the absence or presence of 10 μM iloprost. Platelet membranes were prepared, and adenylate cyclase activity measured in the absence or presence of 0.1–100 μM NECA. A typical result is shown in Figure 1 which compares the increase in enzyme activity above the basal levels. Basal enzyme activity in this experiment was reduced from 24.7 ± 1.1 to 12.0 ± 2.6 pmol cyclic AMP min^{-1} mg^{-1} protein. The results (means ± s.e.) from 5 similar experiments were as follows. There was a consistent

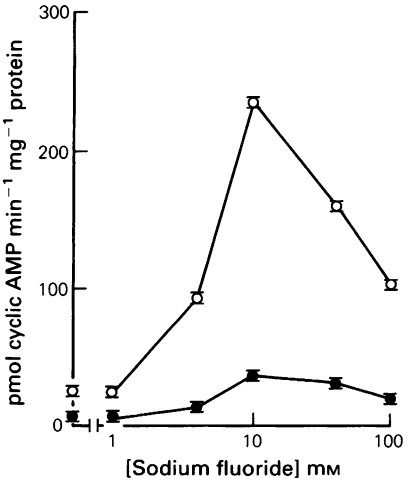

Figure 2 Adenylate cyclase activation by NaF after pretreatment with iloprost. Platelets were incubated for 24 h in the absence (○) or presence (●) of 10 μM iloprost. Platelet membranes were prepared, and adenylate cyclase activity measured in the absence or presence of NaF (1 to 100 mM). Results show mean values (vertical lines are s.e.means, $n = 3$) of enzyme activity.

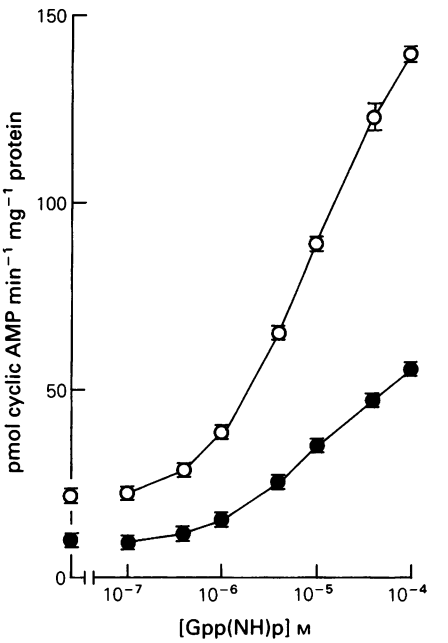

Figure 3 Adenylate cyclase activation by guanyl-5'-yl imidodiphosphate (Gpp(NH)p) after pretreatment with iloprost. Platelets were incubated for 24 h in the absence (○) or presence (●) of 10 μM iloprost. Platelet membranes were prepared, and adenylate cyclase activity measured in the absence or presence of Gpp(NH)p (0.1 to 100 μM). Results show mean values (vertical lines are s.e.means, $n = 3$) of enzyme activity.

and statistically significant (Student's t test) reduction in the basal enzyme activity from 24.9 ± 0.4 to 15.1 ± 2.5 pmol cyclic AMP min^{-1} mg^{-1} protein $P < 0.05$). The maximum enzyme activation produced by NECA was reduced ($P < 0.05$) after iloprost pretreatment (28.8 ± 1.1 to 17.8 ± 3.0 pmol cyclic AMP min^{-1} mg^{-1} protein). The maximum enzyme activation was determined from Eadie-Hofstee plots. There was no significant change in the K_{act} value for NECA-dependent adenylate cyclase activation (2.71 ± 0.23 μM in controls and 4.0 ± 0.74 μM after iloprost pretreatment).

A comparison was made of the increase in adenylate cyclase activity mediated by NaF (1–100 mM) or Gpp(NH)p (0.1–100 μM) in membranes from control platelets, or platelets pretreated for 24 h with 10 μM iloprost. An examination of Figures 2 and 3 shows substantially reduced responses to either agent following pretreatment with iloprost. In a similar experiment (Figure 4), the increase in adenylate cyclase activity mediated by GTP (10 nM–10 μM) was compared in membranes of control or iloprost-pretreated platelets.

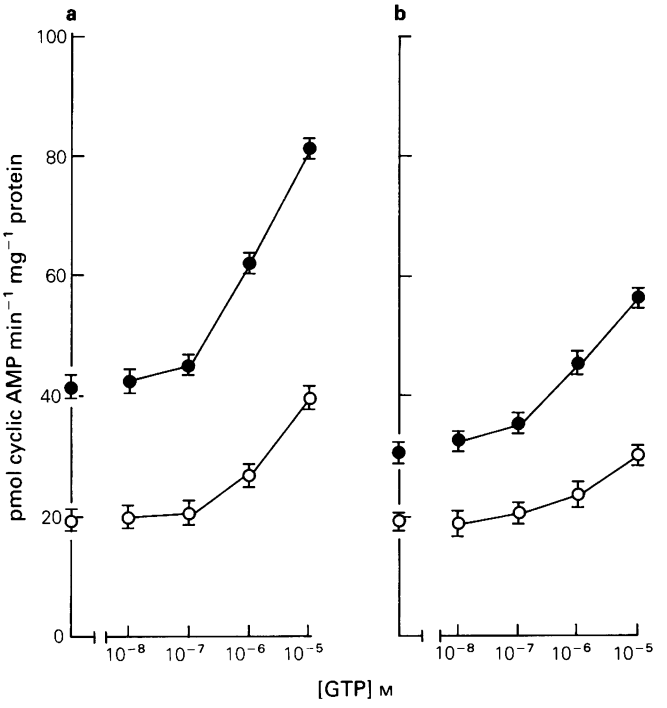

Figure 4 5'-(N-ethyl)-carboxamidoadenosine (NECA)-dependent activation of adenylate cyclase in the absence or presence of GTP, after pretreatment with iloprost. Platelets were incubated for 24 h in the absence (a) or presence (b) of 10 μM iloprost. Platelet membranes were prepared, and adenylate cyclase activity measured in the absence (○) or presence (●) of 10 μM NECA. Enzyme activity was measured under these conditions in the absence or presence of GTP (10 nM to 10 μM). Results show mean values (vertical lines are s.e.means, $n = 3$) of enzyme activity.

In addition, the GTP responsiveness was compared in the absence or presence of 10 μM NECA. Iloprost pretreatment of the platelets resulted in a decreased response to GTP (in the absence or presence of NECA), but, most significantly, the decrease in basal enzyme activity that accompanied iloprost pretreatment was only seen in the presence of GTP.

Pretreatment of platelets with iloprost results in decreased responsiveness of the platelet membranes to iloprost (Alt *et al.*, 1986), NaF (Figure 2), NECA (Figure 1) and in basal enzyme activity. Experiments were performed to establish whether there was a close temporal relationship between these events, or whether, alternatively, they could be shown to occur at different times after the addition of iloprost. The results are shown in Figure 5, which provide compelling evidence that, after the addition of 10 μM iloprost, the decrease in responses to 50 μM NECA, 1 μM iloprost, 10 mM NaF or basal enzyme activity were simultaneous.

In another experiment (data not shown), platelets were pretreated for 24 h with selected concentrations of iloprost between 20 nM and 20 μM. The levels of basal adenylate cyclase activity and NECA- iloprost- or NaF-dependent activation of the enzyme were reduced by very similar amounts at each iloprost concentration. Furthermore, there was no tendency for a reduction in one or other response in the absence of the others. Thus, the different changes that have been observed following a 24 h exposure of platelets to iloprost occur together, and cannot be separated in experiments that vary either the iloprost concentration or the duration of the incubation.

The effect of iloprost pretreatment of human platelets was then compared to pretreatment with 2-chloroadenosine. Platelets in PRP were incubated for 24 h with 50 μM 2-chloroadenosine, and the activation of adenylate cyclase in the resultant platelet membranes by NECA (0.1–100 μM) or iloprost (1 nM–1 μM) was measured. The results are shown in Figure 6, which reveal no changes in basal enzyme activity or iloprost-dependent activation of adenylate cyclase, but a diminished NECA-dependent increase in enzyme activity. In a similar experiment (data not

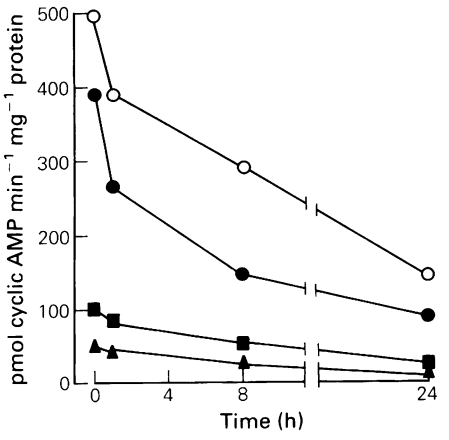

Figure 5 Time-course for iloprost-dependent loss of the capacity to activate adenylate cyclase by iloprost, NaF or 5'-(N-ethyl)-carboxamidoadenosine (NECA). Platelets were incubated in the presence of 10 μM iloprost for the times shown between 0 to 24 h. At selected times, platelet membranes were prepared and adenylate cyclase activity measured in the absence (▲) or presence of 1 μM iloprost (○), 50 μM NECA (■) or 10 mM NaF (●). Results are means of duplicate estimations of enzyme activity. There was no significant loss of enzyme activity in membranes prepared from platelets incubated for 24 h in the absence of iloprost.

presented), pretreatment of platelets with 50 μM 2-chloroadenosine produced no change in NaF- or Gpp(NH)p-dependent activation of adenylate cyclase in the resultant platelet membranes.

The binding of [^{3}H]-NECA to human platelet membranes at concentrations of [^{3}H]-NECA between 5 and 2000 nM is shown in Figure 7 as a Scatchard plot. The high affinity component of the binding isotherm of [^{3}H]-NECA was then identified as the adenosine (A_2) receptor in experiments in which the radiolabelled ligand was displaced with adenosine, or 3-isobutyl-1-methylxanthine. Measurements were made in triplicate of the binding of 60 nM [^{3}H]-NECA in the absence and presence of 400 nM to 100 μM adenosine, or 100 nM to 100 μM 3-isobutyl-1-methylxanthine. The K_i values of the 2 displacing agents were 5.09 and 33.5 μM respectively. The K_i values were determined from the expression $K_i = IC_{50}/[1 + ([^3H]\text{-NECA}/K_D)]$, where IC_{50} was the concentration of the displacing agent producing 50% of the displacement mediated by 10 μM unlabelled NECA, and the K_D value of NECA was 62.2 nM. The Hill interaction coefficients for the displacement of [^{3}H]-NECA by adenosine or 3-isobutyl-1-methylxanthine were 0.889 and 0.736, respectively. A comparison of the binding of [^{3}H]-NECA to the high affinity site after pretreatment with 10 μM iloprost or 50 μM 2-chloroadenosine is shown in Table 1. Iloprost pretreatment resulted in no significant

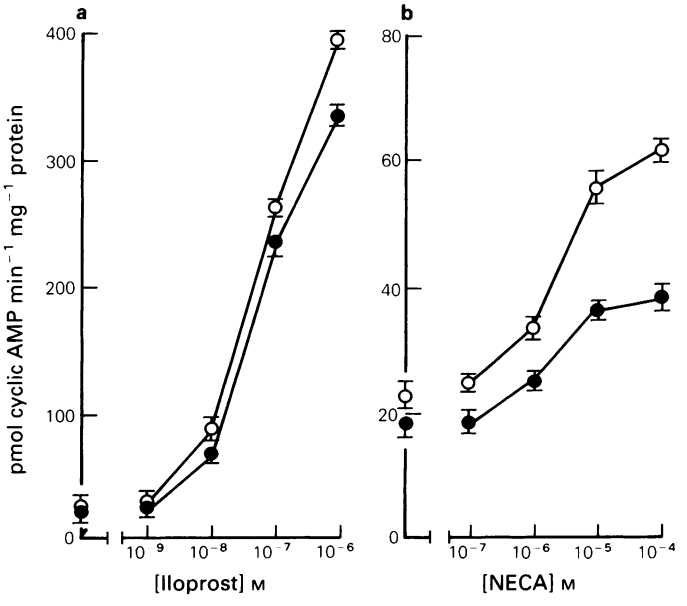

Figure 6 Homologous desensitization of adenosine (A_2) receptors by 2-chloroadenosine. Platelets were incubated for 24 h in the absence (○) or presence (●) of 50 μM 2-chloroadenosine. Platelet membranes were prepared, and adenylate cyclase activity measured in the absence or presence of 1–1000 nM iloprost (a) or 0.1–100 μM 5'-(N-ethyl)-carboxamidoadenosine (NECA) (b). Results show mean values (vertical lines are s.e.means, $n = 3$) of enzyme activity.

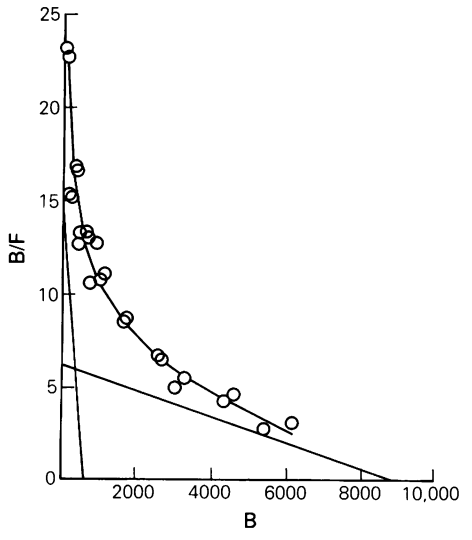

Figure 7 The binding of [^{3}H]-5'-(N-ethyl)-carboxamidoadenosine ([^{3}H]-NECA) to platelet membranes. Platelet membranes were prepared, and specific binding of [^{3}H]-NECA measured at concentrations between 5 and 2000 nM. The data are presented as a Scatchard plot, where B is fmol [^{3}H]-NECA bound mg^{-1} protein, and F is the free concentration of [^{3}H]-NECA (nM). The data have been analysed as described in Methods, and the results show individual measurements of the binding.

change in the [^{3}H]-NECA binding while 2-chloroadenosine pretreatment reduced the maximum binding capacity to the high affinity NECA site from 595 to 245 fmol mg^{-1} protein. There was no accompanying change in the K_D value for the high affinity [^{3}H]-NECA binding. The low affinity [^{3}H]-NECA binding site was unaffected by pretreatment with either iloprost or 2-chloroadenosine.

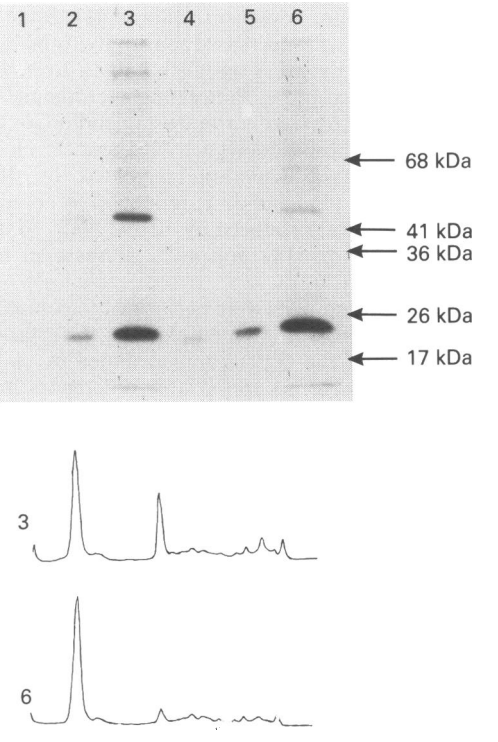

Figure 8 [^{32}P]-ADP ribosylation of platelet membrane proteins after pretreatment with iloprost. Platelets were incubated for 24 h in the absence (lanes 1–3) or presence (lanes 4–6) of 10 μM iloprost. Platelet membranes were prepared, and ADP-ribosylation of membrane proteins performed in the presence of cholera toxin (A subunit). The gels were loaded with (lanes 1 to 6) 3.95, 9.88, 39.5, 3.05, 7.63, 30.5 μg protein. The figure shows the autoradiograph of the six lanes and a densitometer scan of lanes 3 and 6. In a control experiment, there was no significant labelling of platelet membrane proteins in an incubation from which cholera toxin has been omitted.

Table 1 The binding parameters for the association of [^{3}H]-5'-(N-ethyl)-carboxamidoadenosine ([^{3}H]-NECA) and platelet membranes following iloprost or 2-chloroadenosine pretreatment.

Incubation Conditions	B_{max1}	K_{D1}	B_{max2}	K_{D2}
Control	595 ± 3.07	62.2 ± 6.7	9380 ± 506	765 ± 130
Iloprost 10 μM	571 ± 52.5	60.1 ± 8.3	7650 ± 1410	1070 ± 154
2-Chloroadenosine 50 μM	245 ± 13*	57.6 ± 9.6	8040 ± 816	952 ± 153

Platelets were incubated for 24 h in the absence or presence of 10 μM iloprost or 50 μM 2-chloroadenosine. Platelet membranes were prepared, and the specific binding of [^{3}H]-NECA was measured at concentrations between 5 and 2000 nM. The data were analysed as described in Methods, and the results presented are the values of K_D (nM) and B_{max} (fmol mg^{-1} protein) for the 2 independent binding sites. Results show means ± s.e.means (n = 3) of the values for each parameter. Statistical analyses employed 2-way analysis of variance and Student's t tests. *$P < 0.01$.

The heterologous desensitization of adenosine (A_2) responsiveness with decreased sensitivity to NaF was mediated also by other agonists known to activate platelet PGI_2 receptors. Pretreatment of platelets for 24 h with either 6 μM 6α-carbaprostaglandin I_2 or 6 μM PGE_1 resulted, respectively, in a 41% and 48% loss of adenylate cyclase activation mediated by 10 μM NECA, and a 34% and 33% reduction in adenylate cyclase activation produced by 10 mM NaF. In addition there was a 41% and 47% decrease in basal enzyme activity.

Finally, experiments were performed to compare in control or iloprost pretreated platelets the [^{32}P]-ADP ribosylation of the $G_{s\alpha}$ subunit mediated by cholera toxin. There were no appreciable differences in the protein constituents of the platelet membranes as revealed by Coomassie Blue staining of the polyacrylamide gels. However, autoradiography following [^{32}P]-ADP ribosylation showed a 5.5 fold greater ^{32}P-labelling of a 45 kDa species in membranes from control platelets than of those prepared from platelets pretreated with 10 μM iloprost for 24 h (Figure 8).

Discussion

Homologous desensitization of PGI_2 responses has been examined in the NCB-20 neuronal somatic hybrid cell line (Blair *et al.*, 1982; Leigh & MacDermot, 1985).Culture of these cells with stable PGI_2 analogues resulted in decreased PGI_2-dependent activation of adenylate cyclase that is mediated by a loss of membrane receptors for PGI_2. There was no accompanying change in F^- sensitivity of adenylate cyclase, and responses to other surface receptors on the same cell, whether stimulatory (adenosine A_2 or 5-hydroxytryptamine) or inhibitory (morphine or α_2-adrenoceptor) were unaltered. The PGI_2 receptors of the NCB-20 somatic hybrid have been examined by their sensitivity to ionising radiation (Leigh *et al.*, 1984), and the 'target size' of this receptor was unaffected during homologous desensitization (Leigh & MacDermot, 1985).

Desensitization of PGI_2 responses in platelets differs from that in NCB-20 cells in several ways. The reduction in PGI_2-dependent activation of adenylate cyclase that follows prolonged exposure of platelets to PGI_2 receptor agonists is accompanied also by reduced basal enzyme activity (at least when measured in the presence of GTP). Furthermore, there is heterologous desensitization of the response to adenosine mediated by the A_2-receptor. The identity of the adenosine receptor as the A_2 subtype was confirmed by the capacity for adenylate cyclase activation, the potencies of NECA and 2-chloroadenosine, and the K_i value of 3-isobutyl-1-methylxanthine. The displacement of [^{3}H]-NECA binding by 3-isobutyl-1-methylxanthine revealed a K_i value (33.5 μM) substantially greater than that obtained for inhibition of the biological response (8.7 μM). This effect has been observed previously (Hutteman *et al.*, 1984), and is associated with a Hill interaction coefficient of less than 1.0. Figure 7 reveals that [^{3}H]-NECA binds to at least 2 independent sites, and this property undoubtedly contributes to the high value obtained for K_i in the displacement curve of [^{3}H]-NECA binding by 3-isobutyl-1-methylxanthine. It is proposed that the high affinity component of the binding shown in Figure 7 is the adenosine (A_2) receptor that mediates adenylate cyclase activation.

The reduction in sensitivity to NECA that follows prolonged exposure to stable PGI_2 analogues appears to be mediated by changes in the G-protein. There was a reduction in the activation of adenylate cyclase produced by both F^- and Gpp(NH)p, suggesting that $G_{s\alpha}$ was involved in the change in G-protein activity. The possibility that the heterologous desensitization of adenosine A_2 responses was mediated by changes in the activity of the catalytic subunit of adenylate cyclase (with defective or altered coupling to $G_{s\alpha}$), was largely eliminated by the finding that basal enzyme activity in the absence of GTP was unaltered during heterologous desensitization. Thus the initial finding, observed also in other systems (Kenimer & Nirenberg, 1981), that heterologous desensitization is accompanied by a reduction in basal enzyme activity seems true only in the presence of GTP.

Efforts to separate the two events after exposure to iloprost, namely (a) loss of PGI_2 receptors as described previously (Alt *et al.*, 1986), and (b) changes in $G_{s\alpha}$ that mediate heterologous desensitization of adenosine A_2 responses were unsuccessful. Following the addition of iloprost to PRP, both events occurred at the same rate, and furthermore there were no differences in the concentration of iloprost that mediated either effect. The reduction in F^-- and Gpp(NH)p-dependent enzyme activation was shown by [^{32}P]-ADP-ribosylation to be due to a very substantial decrease in the $G_{s\alpha}$ subunit after iloprost pretreatment. A similar reduction in $G_{s\alpha}$ has been observed previously in examples of heterologous desensitization (Garrity *et al.*, 1983), although $C_{s\alpha}$ (as identified by [^{32}P]-ADP ribosylation) may remain unaltered in other systems (Rich *et al.*, 1984). The mechanism of heterologous desensitization has also been shown to involve an increase in $G_{i\alpha}$ after preincubation with a stimulatory agonist (Rich *et al.*, 1984).

The mechanism by which $G_{s\alpha}$ is altered during heterologous desensitization remains obscure, and might reasonably be expected to involve transcriptional regulation of the turnover rate of this protein, since the process in most systems requires several hours. For reasons that are not totally clear, this is particularly slow in platelets *in vitro*. However, in the present experiments, incubations of PRP were perfor-

med at room temperature (18–22°C) to minimize aggregation, and thus the metabolic state of the platelets would be substantially reduced from their normal level at 37°C. However, transcriptional regulation of the synthesis or turnover of $G_{s\alpha}$ is not possible in mature human platelets, which have no nucleus, and the reduced level of $G_{s\alpha}$ presumably relates to its rate of elimination (at least in its original form) from the plasma membrane.

No explanation is provided by these results for the differences in desensitization mediated by agonist occupation of PGI_2 or adenosine A_2 receptors. Activation of both receptor species is followed by loss of the high affinity binding site of that particular agonist. In addition, the present experiments suggests that $G_{s\alpha}$ is lost at the same time, and under the same conditions, as the PGI_2 receptor. Whether both are internalised (and degraded) together following agonist occupation of PGI_2 receptors is unknown.

In conclusion, evidence is provided for a novel interaction between two receptors expressed on human platelet membranes. Both receptors appear to serve the same purpose, namely to limit the extension of the platelet plug following vascular injury. There is now great interest in the therapeutic potential of platelet anti-aggregatory agents in human vascular disease, and there are already reports of desensitization of PGI_2 responses *in vivo* following PGI_2 infusion (Sinzinger *et al.*, 1981). A more thorough appreciation of the mechanism(s) involved may allow further developments in this important clinical area.

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