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Segregation of discrete $G_{s\alpha}$-mediated responses that accompany homologous or heterologous desensitization in two related somatic hybrids

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1 Prostacyclin and adenosine A_2 receptors activate adenylate cyclase in the neuroblastoma hybrid cell lines NG108-15 and NCB-20. Prolonged exposure of NG108-15 cells to iloprost (a stable analogue of prostacyclin) results in a subsequent reduction in the capacity for adenylate cyclase activation by iloprost, the adenosine analogue 5'-(N-ethyl)-carboxamidoadenosine (NECA) or NaF. In contrast prolonged exposure of NCB-20 cells to iloprost results only in the loss of iloprost responsiveness.

2 Iloprost pretreatment of NG108-15 cells also magnified the morphine-dependent inhibition of iloprost-stimulated adenylate cyclase activity from 36 to 48%. This change was not due to lower iloprost stimulation following desensitization, since the % inhibition of adenylate cyclase activity by morphine in control cells was constant irrespective of enzyme activity.

3 These heterologous effects observed in NG108-15 cells following iloprost pretreatment may involve changes in the $G_{s\alpha}$ protein, since there was a reduction of about 30% in the cholera toxin-induced [^{32}P]-ADP-ribosylation of a 45 kDa protein from cell membranes (corresponding to the extent of loss of NECA or NaF responsiveness). A similar reduction was not observed in NCB-20 cells.

4 These results indicate that iloprost pretreatment induces different forms of desensitization in NG108-15 and NCB-20 cell lines. The heterologous desensitization in the former may, like the human platelet, involve a functional loss of $G_{s\alpha}$ from the cell membrane. Changes in the activity of $G_{s\alpha}$ may also account for the heterologous effects on receptors that mediate inhibition of adenylate cyclase.

Introduction

Prostacyclin (PGI_2) is a naturally occurring derivative of the prostaglandin endoperoxides which mediates the inhibition of platelet aggregation (Moncada *et al.*, 1976). Other substances such as adenosine are also able to inhibit platelet aggregation (Born *et al.*, 1965). Both PGI_2 and adenosine interact with specific receptor sites on the platelet (PGI_2 and A_2 receptors respectively) and inhibit aggregation by activation of adenylate cyclase, thus raising intracellular levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Haslam & Rosson, 1975; Gorman *et al.*, 1977; Tateson *et al.*, 1977; Huttemann *et al.*, 1984).

Both adenosine and PGI_2 analogues have therapeutic potential as 'anti-platelet' agents. In clinical trials, however, it became evident that the use of PGI_2 agonists might be problematic since PGI_2 infusion was followed by a desensitization of platelet PGI_2 responsiveness (Sinzinger *et al.*, 1981). Desensitization *in vitro* of PGI_2 responsiveness has also been widely demonstrated in platelets (Miller & Gorman, 1979; MacDermot, 1986; Edwards *et al.*, 1987; Jaschonek *et al.*, 1988), human fibroblasts (Gorman & Hopkins, 1980) and NCB-20 neuroblastoma hybrid cells (Blair *et al.*, 1982; Leigh & MacDermot, 1985). However, the characteristics of desensitization produced by prolonged activation of PGI_2 receptors are not the same in different cell types. In platelets, prolonged PGI_2 receptor activation leads to heterologous loss of responsiveness to adenylate cyclase-stimulating agents (Edwards *et al.*, 1987; Jaschonek *et al.*, 1988), whereas in NCB-20 cells the desensitization is homologous, affecting only subsequent responses to PGI_2 receptor stimulation (Leigh & MacDermot, 1985). These differences in desensitization probably relate to the different consequences of prolonged PGI_2 receptor activation upon the transmembrane signalling pathway (receptor, G-protein and adenylate cyclase) in each tissue (Gilman, 1984).

Since in practice platelets are a somewhat inconvenient preparation in which to perform long-term manipulations, we decided to use clonal cell lines as a more robust preparation to investigate in greater depth the mechanisms underlying PGI_2 receptor-mediated desensitization. In the present study we have identified two related neuroblastoma somatic hybrid cell lines which express receptors for both PGI_2 and adenosine, stimulation of which activates adenylate cyclase. The effects are described of prolonged incubation of these cells with iloprost, a stable structural analogue of PGI_2 (Skuballa & Vorbruggen, 1983), on the subsequent responsiveness of these cells to agents that activate or inhibit adenylate cyclase.

Methods

Cell culture

Cells of the NG108-15 and NCB-20 neuroblastoma somatic hybrid cell lines were both derived by fusions of the 6-thioguanine-resistant clone of N18TG2 mouse neuroblastoma. Fusions were made with (a) C6BU-1, a 5-bromodeoxyuridine-resistant clone of rat glioma to yield NG108-15, or (b) foetal Chinese hamster brain cells to yield NCB-20 (further details and references to these cells are given in MacDermot *et al.*, 1979). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum and supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. Culture flasks (80 cm^2) were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. For iloprost pretreatment, this medium was replaced for 17 h with one containing only DMEM, to which iloprost was added (final concentration = 10 μM). Control cells were treated similarly except that iloprost was not included. Cells were harvested by agitation in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline, and then washed three times in warm DMEM. The resultant pellets were frozen at −80°C until

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required. For each experiment, control and iloprost pretreated cells were always grown and harvested in parallel.

Adenylate cyclase

Adenylate cyclase activity was measured as described previously (Salomon *et al.*, 1974) with some modifications (Edwards *et al.*, 1987). Cell pellets were thawed and disrupted in a glass Dounce homogenizer using a homogenization buffer (25 mM Tris-HCl, 0.29 M sucrose, pH 7.4). Reaction mixtures of 100 μl contained 50 mM Tris-HCl pH 7.4, 5 mM magnesium chloride, 20 mM creatine phosphate disodium salt, 10 iu creatine kinase, 1 mM cyclic AMP sodium salt, 0.25 mM Ro20-1724 as a phosphodiesterase inhibitor, 1 mM [α-^{32}P]-ATP (2 μCi), 1 μM GTP and 200–400 μg of membrane protein. Reactions were incubated at 37°C for 15 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 (about 20 000 c.p.m.) and the reaction mixtures centrifuged at 4°C for 20 min at 800 *g*. The [^{32}P]-ATP and [^{32}P]-cyclic AMP were separated by a two 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]-iloprost binding

[^{3}H]-iloprost binding was assayed in whole homogenates of control and desensitized cells. Cells were homogenized as before using 50 mM Tris-HCl, pH 7.4 containing 5 mM magnesium chloride. The homogenates (0.1–0.6 mg protein per sample) were incubated in a final volume of 250 μl of 50 mM Tris-HCl pH 7.4, 5 mM magnesium chloride containing 0.3–100 nM [^{3}H]-iloprost in the absence or presence of 10 μM unlabelled iloprost to determine non-specific binding. Samples were incubated for 90 min at 4°C and then filtered onto Whatman GF1B glass fibre filters and washed with 3 × 3.5 ml ice-cold buffer using a Brandel Cell Harvester. Scintillation fluid was added to the filters and they were left overnight before counting.

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

[^{32}P]-ADP ribosylation of cell membranes was measured as described previously (Edwards *et al.*, 1987) and based on earlier methods (Katada & Ui, 1982; Ribeiro-Neto *et al.*, 1985). Cell pellets were prepared as described above, and after thawing were homogenized in homogenization buffer, centrifuged at 500 *g* for 10 min and the supernatant retained. This was then microfuged for 6 min, and the resultant pellet resuspended in 100 μl of 50 mM Tris-HCl, pH 7.4. Reaction mixtures (volume = 100 μl) contained 30 μl of the membrane suspension (100–400 μg protein), 1 mM ATP, 15 mM glycine, 2.5 mM magnesium chloride, 10 mM thymidine, 100 μM GTP, 90 mM potassium phosphate buffer pH 7.0, 10 μM [^{32}P]-nicotinamide adenine dinucleotide (NAD 20 μCi) and 10 μg cholera toxin (A subunit, activated as described below). 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 4°C, the precipitate was pelleted by centrifugation (10 000 *g* for 10 min). The supernatants were discarded and the pellets washed once in 1 ml ether:ethanol (1:1 v/v), and once in 1 ml ether, then dried under a stream of N_2. The pellet was then dissolved in 200 μl of 0.0625 M Tris-HCl buffer pH 6.8, containing 10% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue. The samples were heated in a boiling water bath for 5 min and then loaded onto a 10% SDS-polyacrylamide gel with a 3.75% polyacrylamide stacking gel. Molecular weights were estimated by comparison with protein standards in a Dalton Mark 7 kit (Sigma). 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 48 h at −80°C using Kodak X-Omatic RP film in a Kodak X-Omatic cassette. The autoradiographic plates were then scanned in a Schoeffel SD3000 Spectrodensitometer with SDC300 Density Computer with reflected light at 550 nm. From the resulting scan obtained by pen recorder, $G_{s\alpha}$ peaks were cut out and weighed. To account for differences in the protein content of samples added to each well, the peak produced by the protein front (see Figure 9) was also cut out and weighed. In each experiment, the size of the $G_{s\alpha}$ peak was normalized by expressing it as a percentage of the respective protein front peak.

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 of 50 mM dithiothreitol. The tube was capped and incubated at 37°C for 30 min.

The dose-response data for adenylate cyclase assays were analysed with a non-linear iterative curve fitting programme (Statgraphics, Statistical Graphics Corp., U.S.A.) according to a simple 2 parameter model that identifies a single receptor species, and gives V_{max} and EC_{50}. [^{3}H]-iloprost binding data were analysed by iterative non-linear regression analysis (Graph Pad).

Statistical analyses involved Student's *t* tests (unpaired, two-tailed) as appropriate.

Materials

[8-^{3}H]-adenosine 3′:5′-cyclic monophosphate (23.6 Ci mmol^{-1}), [^{32}P]-ATP (40–50 Ci mmol^{-1}) and [^{3}H]-iloprost (14.7 Ci mmol^{-1}) were obtained from Amersham International; [^{32}P]-NAD (23.3 Ci mmol^{-1}) from New England Nuclear. Ro20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) was a kind gift from Roche products and iloprost a generous gift from Schering AG Berlin. All other chemicals and drugs were obtained from Sigma Chemical Co. Ltd. or BDH Chemicals Ltd.

Results

In homogenates prepared from either NG108-15 or NCB-20 cells, iloprost, 5′-(N-ethyl)-carboxamidoadenosine (NECA) and NaF all produced concentration-dependent increases in adenylate cyclase activity (Figures 1–3). The EC_{50}s for adenylate cyclase activation by these agents in NG108-15 homogenates were 24 nM for iloprost, 420 nM for NECA and around 4 mM for NaF. Similar values were obtained in NCB-20 homogenates, the EC_{50}s being 28 nM for iloprost, 990 nM for NECA and again around 4 mM for NaF. Concentrations of NaF greater than 10 mM tended to reduce enzyme activity in both cell lines (Figure 3). Furthermore, iloprost produced around a 10 fold increase in adenylate cyclase activity in both cell lines, whereas NECA produced a smaller 2 to 4 fold increase (Figures 1 and 2).

Preincubation of cells with 10 μM iloprost for 17 h reduced basal adenylate cyclase activity in NG108-15 but not NCB-20 cells. In NG108-15 cells iloprost pretreatment reduced basal adenylate cyclase activity by 28% from 8.81 ± 0.78 to 6.34 ± 0.52 pmol cyclic AMP min^{-1} mg^{-1} protein, $n = 8$, $P < 0.05$. In NCB-20 cells iloprost pretreatment did not significantly affect basal adenylate cyclase activity, which was 4.58 ± 0.26 and 3.93 ± 0.40 pmol cyclic AMP min^{-1} mg^{-1} protein in control and iloprost pretreated cells respectively, $n = 8$. In both NG108-15 and NCB-20 cells, iloprost pretreatment greatly reduced the subsequent ability of iloprost to activate adenylate cyclase (Figure 1). This involved both a reduction in the maximum response to iloprost by about 70%, and an increase in EC_{50} by 6 fold and 4 fold in NG108-15 and

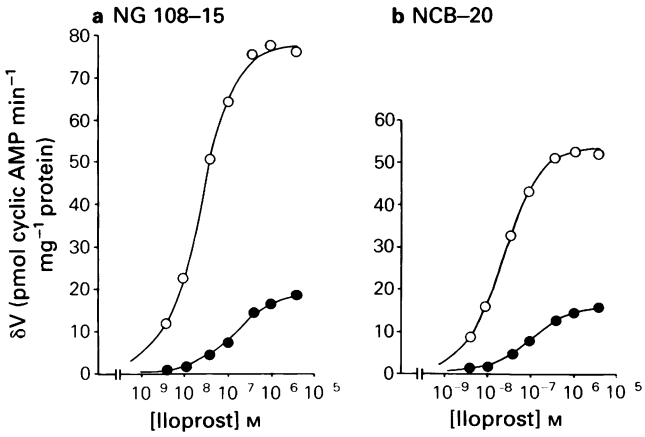

Figure 1 Activation of adenylate cyclase by iloprost in (a) NG108-15 and (b) NCB-20 cell homogenates prepared from control cells (○), and cells that had been cultured for 17 h with 10 μM iloprost (●). The increase in adenylate cyclase activity over basal is expressed as δV. The graphs give data from a single experiment, whereas the following data represent the mean values ± s.e.mean of three different experiments: NG108-15 cells, control EC_{50} = 24 ± 2 nM, iloprost desensitized EC_{50} = 144 ± 14 nM*, control δV_{max} = 103 ± 12 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, iloprost desensitized δV_{max} = 33 ± 8 pmol cyclic AMP $min^{-1} mg^{-1}$ protein*; NCB-20 cells, control EC_{50} = 28 ± 3 nM, iloprost desensitized EC_{50} = 119 ± 17 nM*, control δV_{max} = 69 ± 8 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, iloprost desensitized δV_{max} = 19 ± 3 pmol cyclic AMP $min^{-1} mg^{-1}$ protein*. δV_{max} indicates the maximum increase in adenylate cyclase over basal. In both cell lines, iloprost pretreatment reduced the δV_{max} and increased the EC_{50} of iloprost (*$P < 0.05$, Student's t test).

NCB-20 cells respectively (Figure 1). In NG108-15 cells iloprost pretreatment also reduced the NECA and NaF dependent increases in adenylate cyclase activity by around 30% (Figures 2 and 3). Similar decreases were not observed in NCB-20 cells. In the case of NECA-activated adenylate

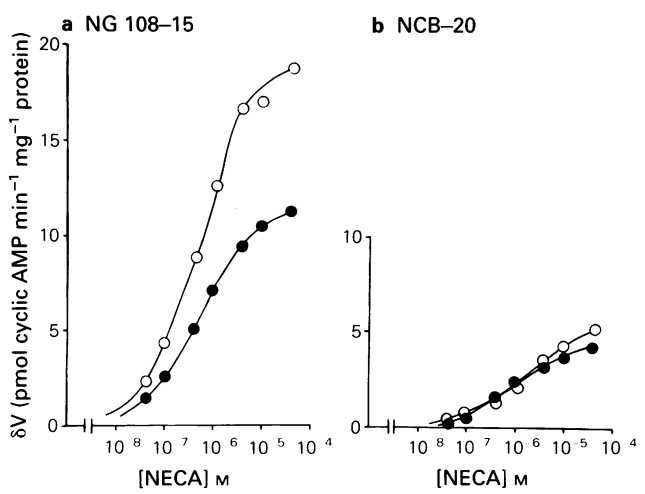

Figure 2 Activation of adenylate cyclase by 5'-(N-ethyl)-carboxamidoadenosine (NECA) in (a) NG108-15 and (b) NCB-20 cell homogenates prepared from control cells (○), and cells that had been cultured for 17 h with 10 μM iloprost (●). Details as in legend to Figure 1. The graphs give data from a single experiment whereas the following data represent the mean values ± s.e.mean of three different experiments: NG108-15 cells, control EC_{50} = 420 ± 10 nM, iloprost desensitized EC_{50} = 310 ± 80 nM, control δV_{max} = 26 ± 1 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, iloprost desensitized δV_{max} = 17 ± 1 pmol cyclic AMP $min^{-1} mg^{-1}$ protein *; NCB-20 cells, control EC_{50} = 990 ± 170 nM, iloprost desensitized EC_{50} = 570 ± 110 nM, control δV_{max} = 7 ± 1 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, iloprost desensitized δV_{max} = 7 ± 1 pmol cyclic AMP $min^{-1} mg^{-1}$ protein. In neither cell line did iloprost pretreatment alter the EC_{50} of NECA but it reduced δV_{max} for NECA in NG108-15 cells (*$P < 0.05$, Student's t test).

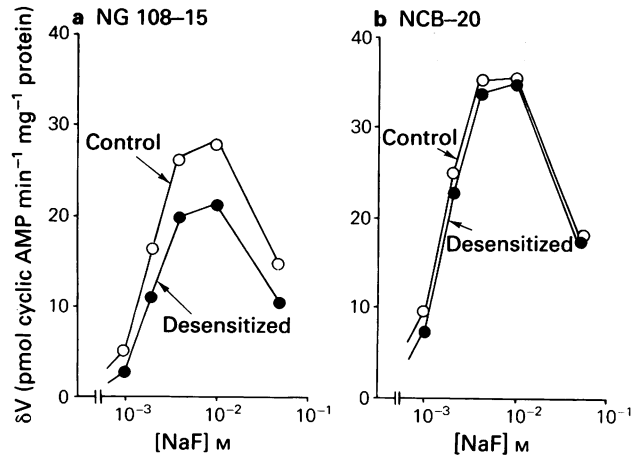

Figure 3 Activation of adenylate cyclase by NaF in (a) NG108-15 and (b) NCB-20 cell homogenates prepared from control cells (○), and cells that had been cultured for 17 h with 10 μM iloprost (●). Details as in the legend to Figure 1. In six separate experiments using a single (10 mM) concentration of NaF, iloprost pretreatment reduced δV by 28.7 ± 6.5% in NG108-15 cell homogenates, but only by 7.1 ± 3.3% in NCB-20 cell homogenates.

cyclase in NG108-15 cells, iloprost pretreatment reduced maximal enzyme activation without affecting the EC_{50} value of NECA (Figure 2).

Iloprost pretreatment of either cell line reduced the 'fold-stimulation' by iloprost of adenylate cyclase over basal activity (Figure 4). However, even in NG108-15 cells, iloprost pretreatment had no effect on the 'fold-stimulation' due to NECA or NaF.

Ligand binding experiments were performed to investigate the effects of iloprost pretreatment on the subsequent binding of [^{3}H]-iloprost to membrane preparations of the cell lines. We did not attempt to obtain full saturation curves for [^{3}H]-iloprost, as it is very difficult to obtain sufficient protein from the cultured cells. However, it has previously been shown (Leigh & MacDermot, 1985) that the binding of [^{3}H]-iloprost (D) to untreated NCB-20 cell membranes can be best described by a single population of high affinity sites (affinity,

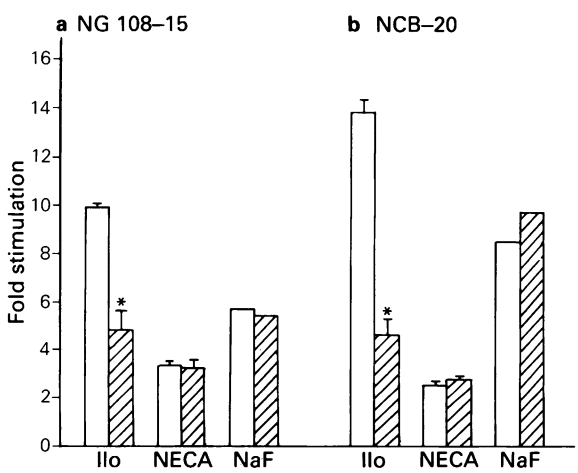

Figure 4 'Fold-stimulation' of adenylate cyclase over basal by different agents in (a) NG108-15 and (b) NCB-20 cell homogenates prepared from control cells (open columns) and cells that had been cultured for 17 h with 10 μM iloprost (hatched columns). 'Fold-stimulation' is defined as the maximum level of adenylate cyclase activation in the presence of drug divided by the level of adenylate cyclase activation in the absence of drug. Values are means ± s.e.mean from 3 separate experiments each, except for NaF which is displayed as the mean of 2 experiments. 'Fold-stimulation' was reduced only for iloprost (Ilo) following iloprost pretreatment (*$P < 0.05$, Student's t test). NECA, 5'-(N-ethyl)-carboxamidoadenosine.

K_D = 10 nM) with the addition of a low affinity, apparently non-saturable component of binding (C), which leads to significant deviation of the [^{3}H]-iloprost binding curve from a simple Langmuir isotherm at concentrations of [^{3}H]-iloprost above ~50 nM. In the present study iloprost displaceable [^{3}H]-iloprost binding to homogenates of untreated NG108-15 and NCB-20 cells could also be described by this model (goodness of fit assessed by actual distance: $r^2 > 0.95$).

$$\text{amount bound} = B_{max}\frac{[D]}{K_D + [D]} + C[D]$$

which yielded estimates of K_D in the range 5–15 nM, none of which differed significantly from 10 nM.

The iloprost displaceable binding of 4–6 concentrations of [^{3}H]-iloprost (0.3–100 nM) to homogenates of control and iloprost-pretreated NG108-15 and NCB-20 cells was measured as described in Methods (Figure 5). Iloprost pretreatment consistently resulted in a decrease in subsequent [^{3}H]-iloprost binding at all concentrations in the range 1–30 nM. This decrease was 77.6 ± 8.6% (mean ± s.d., n = 12) in NG108-15 cells and 67.6 ± 10.5% (mean ± s.d., n = 11) in NCB-20 cells. In all cases, the data could be described by the above two-component model with K_D constrained to be the same in control and treated preparations ($r^2 > 0.95$), suggesting that the iloprost pretreatment does not affect [^{3}H]-iloprost binding affinity. However, it was not possible to obtain independent estimates of [^{3}H]-iloprost affinity in homogenates of iloprost-treated cells, as the number of binding sites was so low. The B_{max} values obtained for [^{3}H]-iloprost binding to the high affinity sites in homogenates from untreated cells were 72.4 ± 11.3 fmol mg^{-1} protein in NG108-15 cells and 102.1 ± 26.8 fmol mg^{-1} protein in NCB-20 cells (mean ± s.e.mean, n = 6). The mean decrease in B_{max} of the high affinity site was 83.3 ± 2.0% in NG108-15 cells and 67.5 ± 12.7% in NCB-20 cells (n = 4). Iloprost pretreatment had no effect on the low affinity component of binding in either cell line; the value obtained for this component of binding was consistently 0.5 fmol mg^{-1} protein nM^{-1} in both cell lines.

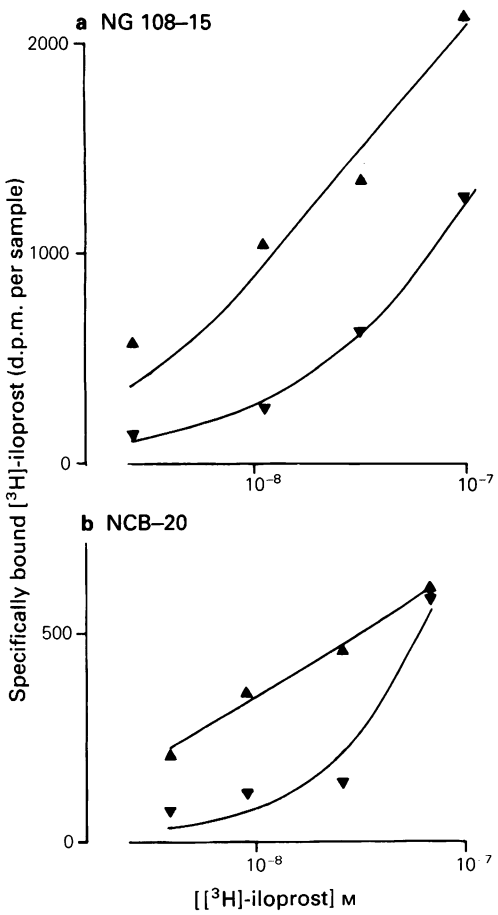

Figure 5 Specific binding of [^{3}H]-iloprost to (a) NG108-15 and (b) NCB-20 homogenates prepared from control cells (▲), and cells that had been cultured for 17 h with 10 μM iloprost (▼). The points on the graph are mean values from single representative experiments. Data are expressed as d.p.m. per sample and are not corrected for protein concentration. The solid lines represent the best fit of the two component model, amount bound = B_{max} ([D]/K_D + [D]) + C[D] to the data (see text), where K has been constrained to be the same in control and treated preparations. In NG108-15, K = 10 nM, B_{max}(control) = 1750 ± 304 d.p.m. per sample, B_{max}(treated) = 394 ± 67 d.p.m. per sample, C(control) = 5 ± 4 d.p.m. per sample nM^{-1} and C(treated) = 9 ± 1 d.p.m. per sample nM^{-1}. In NCB-20 homogenates, K = 5 nM, B_{max}(control) = 495 ± 11 d.p.m. per sample, B_{max}(treated) = 9 ± 88 d.p.m. per sample, C(control) = 2 ± 2 d.p.m. per sample nM^{-1}, C(treated) = 8 ± 2 d.p.m. per sample nM^{-1}.

Morphine produced a concentration-dependent inhibition of basal and iloprost-stimulated adenylate cyclase activity in NG108-15 cells (Figure 6). These effects were abolished in the presence of 100 μM of the opiate receptor antagonist naloxone (data not shown). In iloprost-pretreated NG108-15 cells, the maximum extent to which morphine could inhibit iloprost stimulated adenylate cyclase activity was increased by about one-third, with no change in the IC_{50} of morphine (Figure 6). The extent to which morphine could inhibit basal adenylate cyclase activity also appeared to be increased following iloprost pretreatment, but in this series of experiments the effect did not reach statistical significance (Figure 6). It is unlikely that the increase in the inhibitory capacity of morphine following iloprost pretreatment is due to the reduced iloprost stimulation, since in another experiment the percentage by which a given concentration of morphine inhibited iloprost-activated adenylate cyclase in NG108-15 cells was constant, and unrelated to initial enzyme activity (Figure 7).

Finally, experiments were performed to compare in control or iloprost pretreated cells the [^{32}P]-ADP-ribosylation of $G_{s\alpha}$ catalysed by cholera toxin. Autoradiography following ADP-ribosylation revealed reduced ^{32}P labelling of a 45 kDa protein in NG108-15 cell membranes after iloprost pretreatment when compared to control (Figure 8), and densitometer scanning of the autoradiographs revealed this to be a 31 ± 7% (mean ± s.e.mean from 4 experiments) decrease. However, it should be noted that the autoradiographic ADP-ribosylation analysis is only semi-quantitative, and relies upon the assumption that iloprost treatment does not change the labelling of the 'peak front' protein (see Methods). In two experiments, no similar change was observed in NCB-20 cells preincubated with iloprost (Figure 9), and in a single experiment pretreatment of NG108-15 cells with 100 μM NECA for 17 h also did not affect the ADP-ribosylation of $G_{s\alpha}$ (Figure 8). In two of the four experiments, two ^{32}P labelled bands were observed close to the 45 kDa marker (e.g. Figure 8), although we were unable to determine accurately their constituent molecular weights. The overall changes in cell function following iloprost pretreatment are collated in Table 1.

Table 1 Effects of iloprost desensitization on transmembrane signalling in NG108-15 and NCB-20 cells

	NG108-15	*NCB-20*
Iloprost activated adenylate cyclase	−68%	−73%
[^{3}H]-iloprost binding	−83%	−67%
Basal adenylate cyclase	−28%	−14%
NECA activated adenylate cyclase	−35%	0
NaF activated adenylate cyclase	−28%	−7%
$G_{s\alpha}$ (by ADP-ribosylation)	−31%	+4%
Morphine inhibited adenylate cyclase	+31%	

The values refer to % changes in function taken from the experiments described in the text. Decreased function is denoted by − and increased function by +, 0 refers to no change. The small decrease in basal adenylate cyclase in NCB-20 cells following iloprost pretreatment did not reach statistical significance.

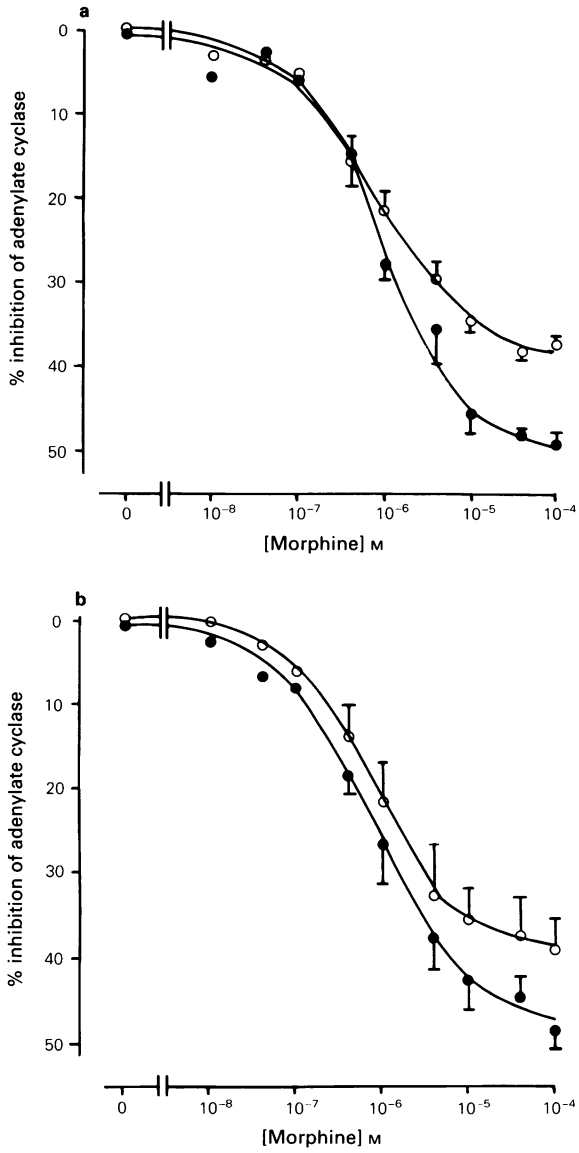

Figure 6 Inhibition of adenylate cyclase activity by morphine in NG108-15 cell homogenates prepared from control cells (○), and cells that had been cultured for 17 h with 10 μM iloprost (●). (a) Morphine inhibition of 1 μM iloprost-activated adenylate cyclase activity, whereas (b) shows morphine inhibition of basal adenylate cyclase activity. The points on the graph show mean values from three separate experiments, the data being normalized to give % inhibition of adenylate cyclase; vertical lines indicate s.e.mean. Mean parameters ± s.e.mean from these data were as follows: inhibition of iloprost-activated adenylate cyclase, control IC_{50} = 696 ± 233 nM, iloprost desensitized IC_{50} = 914 ± 218 nM, control maximum % inhibition = 36.7 ± 1.0%, iloprost desensitized maximum % inhibition = 48.0 ± 1.5%*; inhibition of basal adenylate cyclase, control IC_{50} = 1004 ± 502 nM, iloprost desensitized IC_{50} = 722 ± 199 nM, control maximum % inhibition = 38.6 ± 4.1%, iloprost desensitized maximum % inhibition = 46.1 ± 2.4%. Thus iloprost pretreatment did not affect the potency with which morphine inhibited adenylate cyclase. However, the extent to which morphine could inhibit iloprost-activated adenylate cyclase activity was increased following iloprost (*$P < 0.05$ by Student's t test).

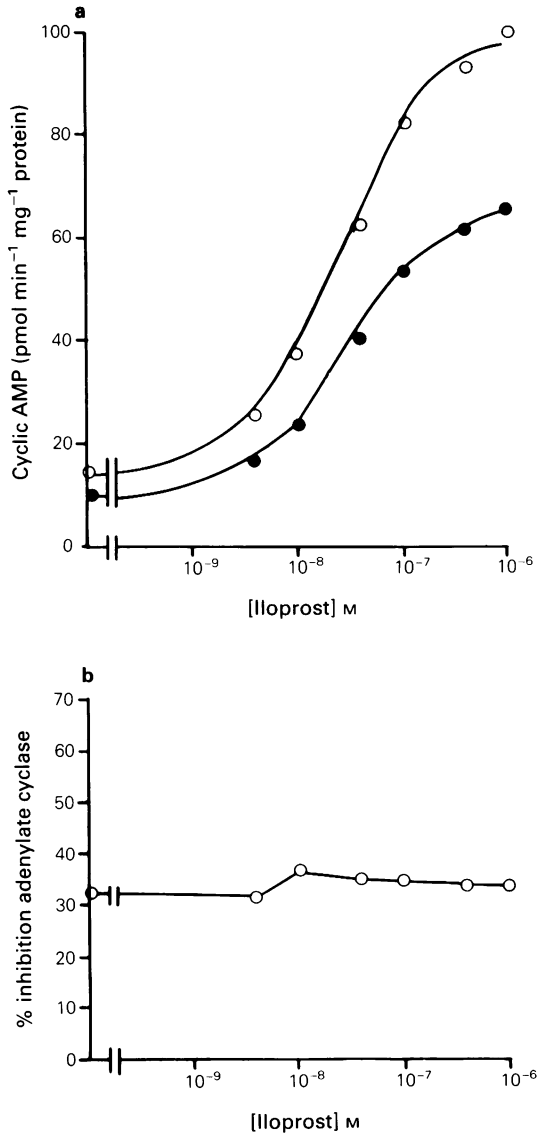

Figure 7 Inhibition of iloprost-activated adenylate cyclase by 10 μM morphine in a cell homogenate of control NG108-15 cells. (a) The activation of adenylate cyclase by increasing concentrations of iloprost in the absence (○) or presence (●) of 10 μM morphine. (b) Data transformed from (a) to show the % inhibition of adenylate cyclase by morphine at different iloprost concentrations. As can be seen, the extent of morphine inhibition was independent of adenylate cyclase activation by iloprost. The graphs contain data from a single experiment, each determination being done in triplicate. This experiment was repeated on one other occasion with the same result.

Discussion

Receptors such as PGI_2 and adenosine A_2 receptors, which mediate activation of adenylate cyclase, are coupled to the effector enzyme by the G_s protein. The G_s protein is a heterotrimer composed of α (45 kDa), β (35 kDa) and γ (10 kDa) subunits, although α and β subunits of other molecular weights have also been reported (for recent reviews see Gilman, 1987; Spiegel, 1987; Neer & Clapham, 1988). In the presence of agonist, the binding of guanosine 5'-triphosphate (GTP) to $G_{s\alpha}$ leads to its dissociation from $G_{s\beta\gamma}$. The $G_{s\alpha}$ GTP complex then binds to, and activates the adenylate cyclase enzyme. Finally, the GTP bound to $G_{s\alpha}$ is hydrolysed to GDP, and $G_{s\alpha}$ and $G_{s\beta\gamma}$ reassociate to terminate the activation of adenylate cyclase. Due to the apparent complexity of this system, desensitization of receptor responsiveness can occur in more than one way. Diminished responsiveness of a receptor that follows previous exposure to an agonist that occupies the same receptor is termed homologous desensitization. Homologous or 'agonist specific' desensitization is considered to involve mechanisms such as loss of receptor number, decrease in agonist affinity, or altered coupling between receptor and G protein (e.g. Clark & Butcher, 1979; Kassis & Fishman, 1982; Leigh & MacDermot, 1985; for review see Harden, 1983). More recently homologous desensitization of the β-adrenoceptor in some tissues has been shown to proceed by an agonist-induced phosphorylation of the β-adrenoceptor by

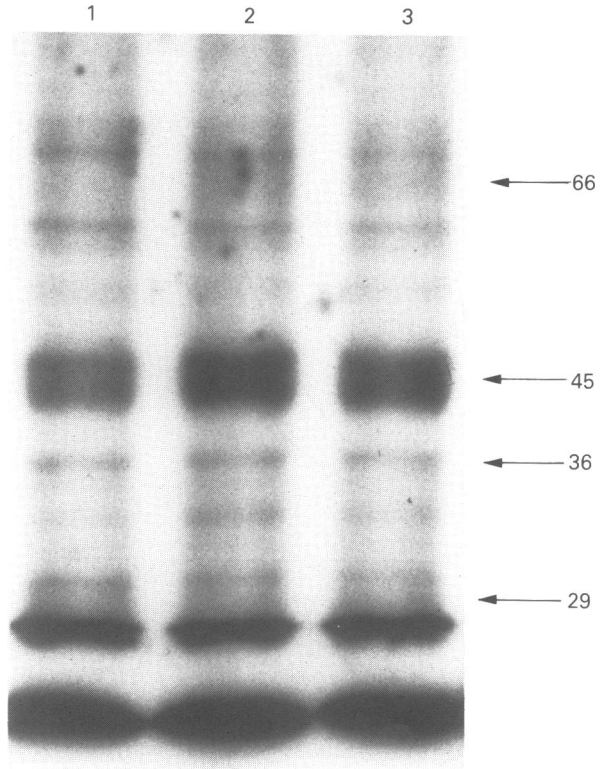

Figure 8 Autoradiograph of cholera toxin induced [^{32}P]-ADP-ribosylation of NG108-15 cell membrane proteins. Cells were incubated for 17 h with 10 μM iloprost (Lane 1), no drug (Lane 2), or 100 μM 5'-(N-ethyl)-carboxamidoadenosine (Lane 3) before membranes were prepared and ADP ribosylation carried out in the presence of [^{32}P]-nicotinamide adenine dinucleotide and cholera toxin. Iloprost pretreatment reduced the ^{32}P labelling of the protein bands around 45 kDa. As described in the results, two ADP-ribosylated protein bands are discernible close to 45 kDa in this experiment.

a specific kinase enzyme, which only phosphorylates the agonist-occupied receptor (Benovic *et al.*, 1986; for reviews see Sibley *et al.*, 1987 and Huganir & Greengard, 1987). On the other hand, heterologous desensitization describes the reduction in responsiveness of one receptor type that accompanies previous occupation by an agonist of a different receptor type. The mechanisms underlying heterologous or 'agonist non-specific' desensitization are less well characterized, but may well involve some modification of the G proteins, or their coupling to receptors or effector enzymes (Kassis & Fishman, 1982; Garrity *et al.*, 1983; Hsia *et al.*, 1985; Edwards *et al.*, 1987; Jaschonek *et al.*, 1988). The increased intracellular cyclic AMP probably mediates these changes for G_s coupled receptors, thus accounting for the non-specific nature of this type of desensitization (Harden, 1983). In the present study, we have investigated the characteristics of desensitization produced by the prostacyclin analogue, iloprost, in two closely related neuroblastoma hybrid cell lines. These cells were chosen because, like the human platelet, both express receptors for PGI_2 and adenosine which activate adenylate cyclase.

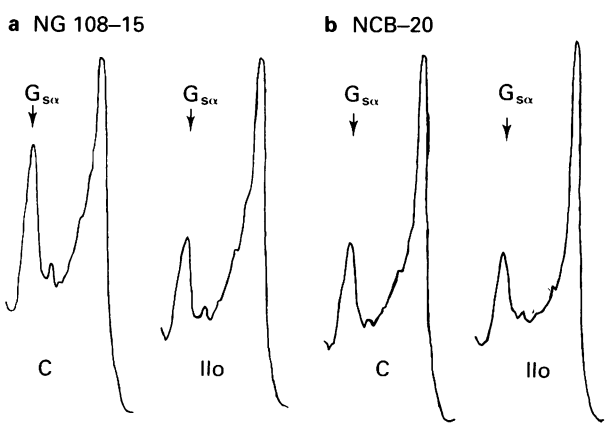

Figure 9 Densitometer scans of four lanes from an autogradiograph following cholera toxin induced [^{32}P]-ADP ribosylation of (a) NG108-15 and (b) NCB-20 cell membrane proteins in the absence (C) or presence (Ilo) of 17 h pretreatment with 10 μM iloprost. The peak to the right of each scan corresponded to the protein front at the base of each lane, whereas the peak to the left in each case corresponded to a 45 kDa protein and is identified as $G_{s\alpha}$. Iloprost pretreatment of NG108-15 cells produced a decrease in $G_{s\alpha}$ (almost 40% in this experiment), whilst having no effect on $G_{s\alpha}$ in NCB-20 cells.

Prolonged incubation of both NG108-15 and NCB-20 cells with iloprost reduced the subsequent ability of iloprost to activate adenylate cyclase. There was a large reduction in enzyme activation by iloprost, and an increase in the EC_{50} of iloprost in both cell lines. Similar results have been obtained following pretreatment of NCB-20 cells with prostacyclin agonists (Leigh & MacDermot, 1985), and we now describe a similar phenomenon in NG108-15 cells. Furthermore, in both cell lines [^{3}H]-iloprost binding was decreased by iloprost pretreatment, indicating reduced receptor numbers. It has previously been shown that the increase in EC_{50} for iloprost-activated adenylate cyclase following iloprost pretreatment of NCB-20 cells is due to the loss of spare receptors with no change in receptor affinity (Leigh & MacDermot, 1985; MacDermot, 1986). Our results here confirm this finding, since we also observed a decrease in prostacyclin receptor number and apparently no change in the affinity of the ligand-receptor interaction. The desensitization of iloprost-activated adenylate cyclase in NG108-15 and NCB-20 cells appears therefore to proceed by a similar mechanism, but the molecular events involved remain obscure at present. However, by analogy with the homologous loss of β-adrenoceptor responsiveness in other tissues, possibilities include agonist-induced receptor phosphorylation followed by internalization and/or down-regulation (Sibley *et al.*, 1987).

The two cell lines showed completely different patterns of responsiveness with regard to basal and NECA or NaF activated adenylate cyclase following iloprost pretreatment. In NG108-15 cells, iloprost pretreatment produced about a 30% reduction in basal adenylate cyclase activity, as well as in NECA and NaF activated enzyme activity. A similar heterologous loss of responsiveness has been demonstrated following PGE_1 pretreatment of NG108-15 cells (Kenimer & Nirenberg, 1981) and iloprost pretreatment of human platelets (Edwards *et al.*, 1987; Jaschonek *et al.*, 1988). The heterologous loss of NECA or NaF responsiveness in NG108-15 cells was much less than the homologous loss of iloprost responsiveness, whereas the reduction in basal as well as NECA and NaF stimulated adenylate cyclase activity was very similar, suggesting a common mechanism for the heterologous effects. In marked contrast to the findings in NG108-15 cells, iloprost pretreatment of NCB-20 cells did not affect basal, or NECA and NaF activated adenylate cyclase activity. Thus, even though both cell lines share a common parent, the mouse N18TG2 neuroblastoma, and both express to a large extent the same receptors, iloprost-induced heterologous desensitization of adenylate cyclase-linked receptors is not seen in NCB-20 cells.

Interestingly, the differences between the two cell lines were not apparent when 'fold stimulation' over basal was taken into account. In both NG108-15 and NCB-20 cells, 'fold stimulation' produced by iloprost was reduced by iloprost pretreatment, whereas 'fold stimulation' produced by NECA and NaF was unchanged. This is not surprising for NCB-20 cells where no heterologous effects were apparent. However, in NG108-15 cells, although there were reductions in basal, NECA and NaF stimulated adenylate cyclase activities, they were all reduced proportionately, and thus no change in 'fold stimulation' was observed. This finding illustrates an inter-

esting difference between homologous and heterologous desensitization in these neuroblastoma cell lines, and may indicate the mechanism(s) involved. It seems that the loss of iloprost responsiveness following iloprost pretreatment (i.e. the reduction in maximal enzyme activation and 'fold stimulation') is due to a large reduction in prostacyclin receptor number in both cell lines. On the other hand, heterologous loss of sensitivity to NECA or NaF (with no change in 'fold stimulation') in NG108-15 cells pretreated with iloprost suggests a different mechanism perhaps involving the G_s protein.

This interpretation of the results is further elaborated by the following considerations. First, there was an iloprost-induced reduction in NaF-activated adenylate cyclase activity in NG108-15 cells. Since NaF activates adenylate cyclase by causing dissociation of the G_s heterotrimer to $G_{s\alpha}$ and $G_{s\beta\gamma}$ subunits, then the reduction in NaF activation of adenylate cyclase must be due to an effect at or beyond the level of the G_s protein. Secondly, the reduction in NECA-activated adenylate cyclase activity following iloprost pretreatment of NG108-15 cells was not accompanied by reduced 'fold stimulation'. This suggests that iloprost pretreatment of this cell line does not affect the adenosine A_2 receptor or its ability to couple to the G_s protein, but rather that the relative activity of G_s has changed. (We have attempted to characterize [^{3}H]-NECA binding to NG108-15 membranes to investigate this hypothesis, but have shown that the ligand predominantly labels a site that is not the A_2 receptor in these cells; Keen *et al.*, unpublished observations). Thirdly, in experiments to assess the abundance of the $G_{s\alpha}$ protein using cholera toxin, we observed the reduced [^{32}P]-ADP-ribosylation of a 45 kDa protein from NG108-15 cells pretreated with iloprost. A similar decrease was not evident in NCB-20 cells, and therefore a functional loss of $G_{s\alpha}$ from the NG108-15 cell membrane probably accounts for heterologous desensitization in this cell line. Reductions in $G_{s\alpha}$ have been observed in other cases of heterologous desensitization of G_s coupled receptors (Kassis & Fishman, 1982; Garrity *et al.*, 1983; Edwards *et al.*, 1987; Jaschonek *et al.*, 1988). Other mechanisms are, however, possible to account for some types of heterologous desensitization, such as an increase in G_i (Rich *et al.*, 1984). The means by which a functional loss of $G_{s\alpha}$ occurs upon desensitization is not known but possibilities include reduced transcription of $G_{s\alpha}$, covalent modification of the polypeptide (Jacquemin *et al.*, 1986), or the physical loss of $G_{s\alpha}$ from the cell membrane.

Some comment is also required about the protein bands labelled in the presence of cholera toxin and [^{32}P]-NAD. In some experiments using NG108-15 cells, two labelled bands could be observed close to the 45 kDa marker protein (Figure 8), although we were unable to deduce accurately the molecular weights of these bands. Cholera toxin-induced ADP-ribosylation of 45 and 42 kDa proteins has previously been observed in NG108-15 cell membranes (Milligan & McKenzie, 1988), so we may be observing the same phenomenon here. One intriguing possibility is that these two proteins represent different forms of $G_{s\alpha}$ from mouse and rat, sequencing of which has indicated small differences which might account for the variation in molecular weight (Sullivan *et al.*, 1986; Itoh *et al.*, 1986). However, some caution is required before accepting this interpretation, as $G_{s\alpha}$ does not align accurately on polyacrylamide gels according to its molecular weight(s) defined by cDNA sequence (for review see Gilman, 1987).

The potential involvement of cyclic AMP-dependent protein kinase in the functional loss of $G_{s\alpha}$ remains unresolved by these experiments. Since pretreatment of NG108-15 cells with NECA produces only homologous desensitization (Keen *et al.*, unpublished observations), it would appear that raised intracellular cyclic AMP is not responsible for heterologous desensitization in this case. However, not only does iloprost produce a much greater activation of adenylate cyclase in these cells than NECA, but we also do not know the relative time-courses of desensitization for these two agents. Thus, on the available evidence the involvement of cyclic AMP remains equivocal, and we are at present investigating these possibilities using non-hydrolysable cyclic AMP analogues, as well as different conditions for iloprost and NECA desensitization.

These experiments revealed an increased capacity of morphine to inhibit iloprost-activated adenylate cyclase in NG108-15 cells following iloprost pretreatment. Since the inhibitory capacity of morphine is unrelated to initial adenylate cyclase activity (see also Hsia *et al.*, 1984), then it appears that the enhanced inhibition caused by morphine is due to a real adaptive change in the cells rather than an artifact of the experimental protocol. However, these results were somewhat unexpected, since in human fibroblasts the ability of oxotremorine, a muscarinic agonist, to inhibit prostaglandin E_1 (PGE_1)-activated cyclic AMP accumulation was greatly reduced following PGE_1 pretreatment (Hsia *et al.*, 1985). These differences probably relate to qualitative differences between cell lines. The mechanism underlying the increased inhibitory capacity of morphine is unclear, but could involve an increase in the number of opiate receptors or G_i. In the present experiments the inhibitory capacity of morphine was increased to the same extent as the loss of NECA and NaF responsiveness following iloprost pretreatment of NG108-15 cells, suggesting that these effects may in some way be linked. If the level of adenylate cyclase activity in a cell is the vectorial sum of activated G_s and G_i proteins, then an agonist-induced loss of $G_{s\alpha}$ could lead to a related increase in G_i activity so accounting for the effects above. Pretreatment of NG108-15 cells with pertussis toxin, which inactivates amongst others the G_i protein, leads to an increase in basal adenylate cyclase activity (Hsia *et al.*, 1984). Thus, the level of adenylate cyclase activity in these cells may bear a direct relationship to the functional levels of G_s and G_i. It would also have been of interest to monitor the inhibition of adenylate cyclase by morphine following iloprost pretreatment of NCB-20 cells, but in a number of experiments the inhibition was small and we were thus unable to quantify modification of the response.

In conclusion, prolonged activation of prostacyclin receptors leads to different forms of desensitization of adenylate cyclase coupled receptors in NG108-15 and NCB-20 cells. The heterologous loss of responsiveness in NG108-15 cells probably relates to the functional loss of $G_{s\alpha}$ in these cells, and in this respect the characteristics of desensitization resemble those for human platelets following iloprost pretreatment. Furthermore, prolonged activation of prostacyclin receptors in this cell line alters the characteristics of adenylate cyclase inhibition by opiate receptors. These cell lines therefore provide convenient systems with which to study further the mechanisms of receptor desensitization, with particular reference to receptors considered important in the regulation of platelet function. It will be of particular interest to us to investigate why prolonged agonist occupation of a single receptor type leads to segregation of $G_{s\alpha}$-mediated responses that accompany homologous or heterologous desensitization in two closely related somatic hybrids.

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References

BENOVIC, J.L., STRASSER, R.H., CARON, M.G. & LEFKOWITZ, R.J. (1986). β-adrenergic receptor kinase: Identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 2797–2801.

BLAIR, I.A., LEIGH, P.J. & MACDERMOT, J. (1982). Desensitization of prostacyclin receptors in a neuronal hybrid cell line. *Br. J. Pharmacol.*, **77**, 121–127.

BORN, G.V.R., HASLAM, R.J., GOLDMAN, M. & LOWE, R.D. (1965).

Comparative effectiveness of adenosine analogues as inhibitors of blood-platelet aggregation and as vasodilators in man. *Nature*, **205**, 678–680.

CLARK, R.B. & BUTCHER, R.W. (1979). Desensitization of adenylate cyclase in cultured fibroblasts with prostaglandin E_1 and epinephrine. *J. Biol. Chem.*, **254**, 9373–9378.

EDWARDS, R.J., MACDERMOT, J. & WILKINS, A.J. (1987). Prostacyclin analogues reduce ADP-ribosylation of the α-subunit of the regulatory G_s-protein and diminish adenosine (A_2) responsiveness of platelets. *Br. J. Pharmacol.*, **90**, 501–510.

GARRITY, M.J., ANDREASEN, T.J., STORM, D.R. & ROBERTSON, R.P. (1983). Prostaglandin E-induced heterologous desensitization of hepatic adenylate cyclase. Consequences on the guanyl nucleotide regulatory complex. *J. Biol. Chem.* **258**, 8692–8697.

GILMAN, A.G. (1984). G-proteins and dual control of adenylate cyclase. *Cell*, **36**, 577–579.

GILMAN, A.G. (1987). G-proteins: transducers of receptor-generated signals. *Ann. Rev. Biochem.*, **56**, 615–649.

GORMAN, R.R., BUNTING, S. & MILLER, O.V. (1977). Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins*, **13**, 377–388.

GORMAN, R.R. & HOPKINS, N.K. (1980). Agonist-specific desensitization of PGI_2-stimulated cyclic AMP accumulation by PGE_{11} in human foreskin fibroblasts. *Prostaglandins*, **19**, 2–16.

HARDEN, T.K. (1983). Agonist-induced desensitization of the -adrenergic receptor-linked adenylate cyclase. *Pharmacol. Rev.*, **35**, 5–32.

HASLAM, R.J. & ROSSON, G.M. (1975). Effects of adenosine on levels of adenosine cyclic 3′,5′-monophosphate in human blood platelets in relation to adenosine incorporation and platelet aggregation. *Molec. Pharmacol.*, **11**, 528–544.

HSIA, J.A., MOSS, J., HEWLETT, E.L. & VAUGHAN, M. (1984). Requirement for both choleragen and pertussis toxin to obtain maximal activation of adenylate cyclase in cultured cells. *Biochem. Biophys. Res. Commun.*, **119**, 1068–1074.

HSIA, J.A., HEWLETT, E.L. & MOSS, J. (1985). Heterologous desensitization of adenylate cyclase with prostaglandin E_1 alters sensitivity to inhibitory as well as stimulatory agonists. *J. Biol. Chem.*, **260**, 4922–4926.

HUGANIR, R.L. & GREENGARD, P. (1987). Regulation of receptor function by protein phosphorylation. *Trends Pharmacol. Sci.*, **8**, 472–477.

HUTTEMAN, E., UKENA, D., LENSCHOW, V. & SCHWABE, U. (1984). Ra adenosine receptors in human platelets. Characterization by 5′-N-ethylcarboxamido [^{3}H] adenosine binding in relation to adenylate cyclase activity. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325**, 226–233.

ITOH, H., KOZASA, T., NAGATA, S., NAKAMURA, S., KATADA, T., UI, M., IWAI, S., OHTSUKA, E., KAWASAKI, H., SUZUKI, K. & KAZIRO, Y. (1986). Molecular cloning and sequence determination of cDNAs for α subunits of the guanine nucleotide-binding proteins G_s, G_i and G_o from rat brain. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 3776–3780.

JACQUEMIN, C., THIBOUT, H., LAMBERT, B. & CORREZE, C. (1986). Endogenous ADP-ribosylation of G_s subunit and autonomous regulation of adenylate cyclase. *Nature*, **323**, 182–184.

JASCHONEK, K., FAUL, C., SCHMIDT, H. & RENN, W. (1988). Desensitization of platelets to iloprost. Loss of specific binding sites and heterologous desensitization of adenylate cyclase. *Eur. J. Pharmacol.*, **147**, 187–196.

KASSIS, S. & FISHMAN, P.H. (1982). Different mechanisms of desensitization of adenylate cyclase by isoproterenol and prostaglandin E_1 in human fibroblasts. Role of regulatory components in desensitization. *J. Biol. Chem.*, **257**, 5312–5318.

KATADA, T. & UI, M. (1982). ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. *J. Biol. Chem.*, **257**, 7210–7216.

KENIMER, J.G. & NIRENBERG, M. (1981). Desensitization of adenylate cyclase to prostaglandin E_1 or 2-chloroadenosine. *Molec. Pharmacol.*, **20**, 585–591.

LEIGH, P.J. & MACDERMOT, J. (1985). Desensitization of prostacyclin responsiveness in a neuronal hybrid cell line: selective loss of high affinity receptors. *Br. J. Pharmacol.*, **85**, 237–247.

MACDERMOT, J. (1986). Desensitization of prostacyclin responsiveness in platelets. Apparent differences in the mechanism *in vitro* or *in vivo*. *Biochem. Pharmacol.*, **35**, 2645–2649.

MACDERMOT, J., HIGASHIDA, H., WILSON, S.P., MATZUSAWA, H., MINNA, J. & NIRENBERG, M. (1979). Adenylate cyclase and acetylcholine release regulated by separate serotonin receptors of somatic hybrid cells. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 1135–1139.

MILLER, O.V. & GORMAN, R.R. (1979). Evidence for distinct prostaglandin I_2 and D_2 receptors in human platelets. *J. Pharmacol. Exp. Ther.*, **210**, 134–140.

MILLIGAN, G. & MCKENZIE, F.R. (1988). Opioid peptides promote cholera-toxin-catalysed ADP-ribosylation of the inhibitory guanine-nucleotide binding protein (G_i) in membranes of neuroblastoma X glioma hybrid cells. *Biochem. J.*, **252**, 369–373.

MONCADA, S., GRYGLEWSKI, R., BUNTING, S. & VANE, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263**, 663–665.

NEER, E.J. & CLAPHAM, D.E. (1988). Roles of G protein subunits in transmembrane signalling. *Nature*, **333**, 129–134.

RIBEIRO-NETO, F.A.P., MATTERA, R., HILDEBRANDT, J.D., CODINA, J., FIELD, J.B., BIRNBAUMER, L. & SEKURA, R.D. (1985). ADP-ribosylation of membrane components by pertussis and cholera toxin. In *Methods in Enzymology*. ed. Birnbaumer, L. & O'Malley, B.W., Vol. 109, pp. 566–572. London: Academic Press.

RICH, K.A., CODINA, J., FLOYD, G., SEKURA, R., HILDEBRANDT, J.D. & IYENGER, R. (1984). Glucagon-induced heterologous desensitization of the MDCK cell adenylyl cyclase. Increases in the apparent levels of the inhibitory regulator (N_i). *J. Biol. Chem.*, **259**, 7893–7901.

SALOMON, Y., LONDOS, C. & RODBELL, M. (1974). A highly sensitive adenylate cyclase assay. *Anal. Biochem.*, **58**, 541–548.

SIBLEY, D.R., BENOVIC, J.L., CARON, M.G. & LEFKOWITZ, R.J. (1987). Regulation of transmembrane signalling by receptor phosphorylation. *Cell*, **48**, 913–922.

SINZINGER, H., SILBERBAUER, K., HORSCH, A.K. & GALL, A. (1981). Decreased sensitivity of human platelets to PGI_2 during long-term intraarterial prostacyclin infusion in patients with peripheral vascular disease-rebound phenomenon? *Prostaglandins*, **21**, 49–51.

SKUBALLA, W. & VORBRUGGEN, H. (1983). Synthesis of iloprost (ZK 36 374): a chemically stable and biologically potent prostacyclin analog. In *Advances in Prostaglandin Thromboxane and Leukotriene Research*. ed. Samuelsson, B., Paoletti, R. & Ramwell, P.W., Vol. 11, pp. 299–305. New York: Raven Press.

SPIEGEL, A.M. (1987). Signal transduction by guanine nucleotide binding proteins. *Mol. Cell. Endocrinol.*, **49**, 1–16.

SULLIVAN, K.A., LIAO, Y., ALBORZI, A., BEIDERMAN, B., CHANG, G., MASTERS, S.B., LEVINSON, A.D. & BOURNE, H.R. (1986). Inhibitory and stimulatory G proteins of adenylate cyclase: cDNA and amino acid sequences of the α chains. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 6687–6691.

TATESON, J.E., MONCADA, S. & VANE, J.R. (1977). Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins*, **13**, 389–397.

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