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Desensitization of prostacyclin responsiveness in a neuronal hybrid cell line: selective loss of high affinity receptors

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1 The binding of [^{3}H]-iloprost (ZK36374) to NCB-20 membranes revealed a single population of high affinity receptors (K_D = 9.55 nM, B_{max} = 431 fmol mg^{-1} protein) and a low affinity, non-saturable binding component.

2 Desensitization of prostacyclin-responsiveness of NCB-20 cells is induced by culture in the presence of the stable protacyclin analogue carbacyclin. Desensitization is accompanied by an increase in the K_{act} value for prostacyclin (64.1 nM to 175 nM), and a reduction in the prostacyclin-dependent increase in adenylate cyclase activity (41.2 to 15.1 pmol cyclic AMP min^{-1} mg^{-1} protein).

3 Desensitization is not accompanied by changes in the coupling of the catalytic (C) to the regulatory (Ns) subunit of adenylate cyclase. In addition, the physical identity of the receptor molecule (as characterized by its sensitivity to electron bombardment in the beam of a linear accelerator) is not changed by desensitization.

4 Desensitization of prostacyclin-dependent activation of adenylate cyclase may be explained most simply by a loss of prostacyclin receptors. The anomalous increase in the K_{act} (concentration of prostaglandin giving half-maximum enzyme activation) for prostacyclin-stimulated adenylate cyclase was not accompanied by a substantial change in the K_D of [^{3}H]-iloprost binding, and is explained by a loss of spare receptors.

5 Prostacyclin responsiveness in non-dividing cells may be restored after desensitization by prolonged culture (up to 48 h) in the absence of carbacyclin. Resensitization is accompanied by restoration of the high affinity K_{act} value (143 nM to 45.5 nM), and is dependent on *de novo* protein synthesis.

Introduction

Prostacyclin (PGI_2; epoprostenol) is an unstable arachidonic acid metabolite that is synthesized in many tissues including blood vessels (Moncada *et al.*, 1977) and the brain (Abdel-Halim *et al.*, 1980). It is a potent vasodilator (Bunting *et al.*, 1976; Dusting *et al.*, 1977) and an inhibitor of platelet aggregation (Moncada *et al.*, 1976).

The regulation of adenylate cyclase activity by prostacyclin has recently been demonstrated in many tissues including several neuroblastoma cell lines (Ortmann, 1978; Blair *et al.*, 1980; Howlett, 1982). The NCB-20 neuroblastoma hybrid cell line (Minna *et al.*, 1975) was derived by fusion of the N18TG2 mouse neuroblastoma (Minna *et al.*, 1972) with cells of embryonic hamster brain. Cells of this line have a high affinity binding site for prostacyclin (Blair & MacDermot, 1981) that activates adenylate cyclase 10–15 fold (Blair *et al.*, 1980). This prostacyclin receptor has been characterized by radiation inactivation, and is a polypeptide with a molecular weight of 82,800 (Leigh *et al.*, 1984).

Incubation of NCB-20 cells with carbacyclin (a stable prostacyclin analogue) results in desensitization of the prostacyclin-dependent activation of adenylate cyclase (Blair *et al.*, 1982). There is a coincidental loss of prostacyclin receptors from the cell surface. It was suggested that desensitization is also accompanied by an affinity change in the binding of prostacyclin to its receptor, but this interpretation of the results should now be revised.

Previous studies of radioligand binding to the prostacyclin receptor of NCB-20 cells have employed [^{3}H]-11β-prostacyclin (Blair & MacDermot, 1981; Blair *et al.*, 1982). However, this ligand is unstable and exhibits high non-specific binding. In this study, [^{3}H]-iloprost (ZK36374) (Skuballa & Vorbruggen, 1983)

has been employed as a radioligand for the identification of the prostacyclin receptor. This ligand has the advantages that it is stable and has low non-specific binding. Results are presented that extend the investigation into the mechanism(s) of desensitization of prostacyclin responsiveness in these cells.

Methods

Cell culture

Cells of the NCB-20 neuroblastoma somatic hybrid cell line were cultured in Dulbecco's modified Eagle's medium containing either 10% (v/v) newborn calf serum or 5% newborn calf serum and 5% foetal calf serum. The medium was supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. Culture flasks and plates (Falcon) were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. Cells were harvested by agitation in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline, and cell pellets were frozen at − 80°C until required.

Adenylate cyclase assay

NCB-20 cells were suspended in 25 mM Tris-HCl buffer pH 8.5 containing 0.29 M sucrose, and were homogenized on ice with a tightly fitting Dounce homogeniser. Homogenates were used immediately, or were stored at − 80°C until required.

The activation of adenylate cyclase was measured using a modification (Sharma *et al.*, 1975) of the method of Salomon *et al.* (1974). Reaction mixtures of 100 μl contained 50 mM Tris-HCl buffer pH 8.5, 5 mM $MgSO_4$, 20 mM creatine phosphate (disodium salt), 10 international units (iu) creatine kinase, (150 iu mg^{-1} protein; adenosine 5'-triphosphate (ATP): creatine N-phosphotransferase; EC 2.7.3.2), 1 mM adenosine 3':5'-cyclic monophosphate (cyclic AMP), (sodium salt), 4 μM guanosine 5'-triphosphate (GTP), 0.25 mM Ro20-1724 (a phosphodiesterase inhibitor), 1 mM [α^{32}P]-ATP (2–6 μCi, 10–50 Ci $mmol^{-1}$), selected concentrations of prostacyclin, fluoride, or other activator and 0.05–0.46 mg of homogenate protein. Reactions were prepared on ice and then incubated at 30°C for 10 min. Reactions were terminated by the addition of 600 μl of 6.25% (w/v) trichloroacetic acid. To each tube was added 100 μl of [8-^{3}H]-cyclic AMP (approximately 15,000 c.p.m., 26 Ci $mmol^{-1}$), and the reaction tubes were centrifuged for 15 min at 200 *g*. A two step chromatographic procedure was used to separate [α^{32}P]-ATP and [^{32}P]-cyclic AMP. The loss of [^{32}P]-cyclic AMP on the columns was corrected for by comparison with the losses of the [^{3}H]-cyclic AMP standard.

Binding of [^{3}H]-iloprost

Membranes of NCB-20 cells were prepared. Cell homogenates were prepared as described for the adenylate cyclase assay using buffers at pH 7.4. Undisrupted cells and nuclei were pelleted by centrifugation at 500 *g* for 20 min at 4°C. The membranes were then pelleted by centrifugation at 100,000 *g* for 20 min at 4°C, and washed by resuspension in 50 mM Tris-HCl buffer, pH 7.4, and subsequent centrifugation. Finally, membranes were suspended in 50 mM Tris-HCl pH 7.4 and frozen at − 80°C until required.

The binding assay performed was a modification of that of Pert & Snyder (1973). Incubations of 100 μl contained 50 mM Tris-HCl buffer pH 7.4, 10 mM $MgSO_4$, 0.25–0.48 mg membrane or homogenate protein and selected concentrations of [^{3}H]-iloprost (19.5 Ci $mmol^{-1}$). Non-specific binding was determined by inclusion of an excess (4 μM, unless otherwise stated) of carbacyclin (6α-carba-prostaglandin I_2) in parallel incubations. Reactions proceeded at 30°C for 20 min, and were then terminated by the addition of 4 ml of 50 mM Tris-HCl buffer pH 7.4 at 4°C. The membranes were filtered through Whatman GF/C or GF/B glass fibre filters and washed three times with 4 ml volumes of the Tris-HCl buffer at 4°C. The filters were dried for 1 h under an infra-red lamp and then counted in 10 ml Instagel (Packard Instrument Co. Ltd). Data were analysed by an iterative non-linear regression analysis for complex binding (Koeppe & Hamann, 1980).

Radiation inactivation

Radiation inactivation experiments were performed as described previously (Leigh *et al.*, 1984). NCB-20

Table 1 Desensitization of prostaglandin-stimulated adenylate cyclase activity by carbacyclin

Addition to assay	Control		Desensitized	
	δV_{max}	K_{act}	δV_{max}	K_{act}
Prostacyclin	41.2	64.1	15.1	175
Carbacyclin	158	294	89.7	1670
Iloprost	62.9	51.6	22.9	172.4
Prostaglandin E_1	66.4	188	41.2	833

NCB-20 cells were cultured with 1 μM carbacyclin for 16 h. Cells were harvested and the adenylate cyclase activity stimulated by prostacyclin, carbacyclin, prostaglandin E_1 and iloprost were measured in cell homogenates. δV_{max} is the increase in maximum adenylate cyclase activity stimulated by the prostaglandin (pmol cyclic AMP min^{-1} mg^{-1} protein), and K_{act} is the concentration of prostaglandin giving half-maximum enzyme activation (nM).

membranes were prepared as described for [^{3}H]-iloprost binding. Finally, the membranes were resuspended in 25 mM Tris-HCl buffer pH 8.5 containing 0.29 M sucrose, and were divided into 450 μl volumes in stoppered polycarbonate tubes, each containing approximately 1.5 mg protein. Samples were frozen at − 80°C and then lyophilised.

Samples were irradiated at 4°C using the 7 MeV linear accelerator at the MRC Cyclotron Unit, London. Doses were administered additively in triplicate up to 15 Mrad, using a dose rate of 400 Krad min^{-1}. Dosimetry was performed using lithium fluoride thermoluminescence. After irradiation, samples were stored at − 80°C until required, and were then reconstituted with water. The specific binding of 25 nM [^{3}H]-iloprost was measured in duplicate. Surviving binding was plotted on a log scale against the dose administered (Mrad). Data were regressed as a linear function, unweighted and constrained through the origin ($x = 0, y = 100\%$). The molecular weight was calculated from the relationship of Kepner & Macey (1968):

$$\text{molecular weight} = \frac{6.4 \times 10^{11}}{D_{37}}$$

where D_{37} was the dose (rad) giving a decrease in binding to 37% of its original value.

Protein measurements were made following the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Dulbecco's modified Eagle's medium, phosphate-buffered saline and newborn calf serum were supplied by Gibco Europe, Uxbridge. Foetal calf serum was supplied by Flow Laboratories, Rickmansworth. [α^{32}P]-ATP and [8-^{3}H]-cyclic AMP were from Amersham International, Amersham. Aminopterin, hypoxanthine, thymidine, creatine phosphate, creatine kinase, cyclic AMP, GTP and ATP were supplied by Sigma Chemical Co. Ltd, Poole.

The following generous gifts were received: prostacyclin and carbacyclin from the Wellcome Research Laboratories; [^{3}H]-iloprost (5-[(E)-(1S, 5S, 6R, 7R)-7-hydroxy-[7-^{3}H]-6-[(E)-(3S, 4RS)-3-hydroxy-4-methyl-1 octen-6-inyl]-bicyclo [3, 3, 0]octan-3-ylidene] pentanoic acid) from Schering AG, Berlin; Ro20-1724 from Roche Products and NCB-20 cells from Dr M. Nirenberg, NIH, U.S.A.

Results

Desensitization of prostacyclin-stimulated adenylate cyclase activity

NCB-20 cells were incubated with 1 μM carbacyclin for 16 h. The subsequent prostacyclin-, carbacyclin-, prostaglandin E_1- and iloprost-stimulated adenylate cyclase activities were all reduced (Table 1). The activation of adenylate cyclase by carbacyclin was measured at 37°C, whereas the activation of adenylate cyclase by the other prostaglandins was measured at 30°C. For each of the prostaglandins δV_{max} (the maximum increase in adenylate cyclase activity above basal levels stimulated by the prostaglandin) was calculated from the x-intercept of an Eadie Hofstee plot, and the K_{act} value (the concentration of prostaglandin required for half-maximum enzyme activation) was obtained from − 1/slope of this plot. In these experiments the reduction in δV_{max} was accompanied by an increase in the K_{act} value.

In contrast, culture of NCB-20 cells with 10 μM 5-hydroxytryptamine, 10 μM noradrenaline, 10 μM morphine or 10 μM 2-chloroadenosine for 16 h did not alter significantly subsequent prostacyclin-stimulated adenylate cyclase activity. Furthermore, cells that had been cultured for 16 h with 1 μM carbacyclin did not exhibit altered 2-chloroadenosine-stimulated adenylate cyclase activity, or altered noradrenaline- or morphine-inhibited enzyme activity. In none of these experiments was there a change in basal adenylate cyclase activity.

NCB-20 cells were cultured in the absence or

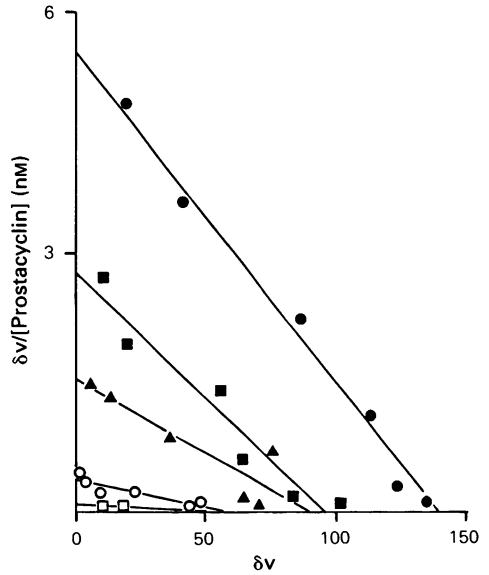

Figure 1 Activation of adenylate cyclase by 4–1000 nM prostacyclin in control cells (●), and cells that had been cultured for 16 h with 0.01 μM (■), 0.10 μM (▲), 1.00 μM (○) or 10 μM (□) carbacyclin. An Eadie-Hofstee plot of the data is presented, where δv is the increase in adenylate cyclase activity stimulated by prostacyclin. Data are means of triplicate determinations. $r = 0.992$, 0.947, 0.872, 0.908 and 0.999, respectively.

presence of carbacyclin at concentrations of 0.01 to 10 μM. Cells were harvested and the activation of adenylate cyclase by 4–1000 nM prostacyclin was measured (Figure 1). The results show that incubation with increasing concentrations of carbacyclin resulted in a sequential decrease in δV_{max} and a sequential increase in K_{act} for prostacyclin-stimulated adenylate cyclase activity.

Binding of [^{3}H]-iloprost to control and desensitized NCB-20 membranes

NCB-20 cells were cultured with or without 1 μM carbacyclin in 0.05% (v/v) ethanol for 16 h. Cells were harvested, and cell membranes were prepared. The specific binding of selected concentrations of [^{3}H]-iloprost to membranes of control (a), and carbacyclin-treated cells (b) was measured (Figure 2). Specific binding was taken as that displaced by 40 μM carbacyclin in parallel incubations. Scatchard plots of the data were non-linear and were analysed as a three parameter model identifying the affinity (K_D) and the maximum binding capacity (B_{max}) of a high affinity specific binding site and a constant (c) defining the low

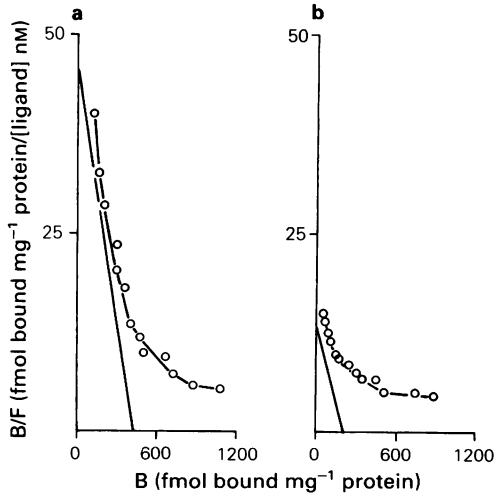

Figure 2 Scatchard plot of the specific binding of 3–200 nM [^{3}H]-iloprost to control NCB-20 membranes (a), and the membranes of cells that had been cultured with 1 μM carbacyclin for 16 h (b). B is fmol [^{3}H]-iloprost bound mg^{-1} protein, and F is the free ligand concentration (nM). Data are means of triplicate determinations, and are analysed as a three parameter model identifying a single high affinity binding site and a non-saturable binding component. The straight line defines the single high affinity binding site, determined by non-linear regression analysis as described in Methods and Results.

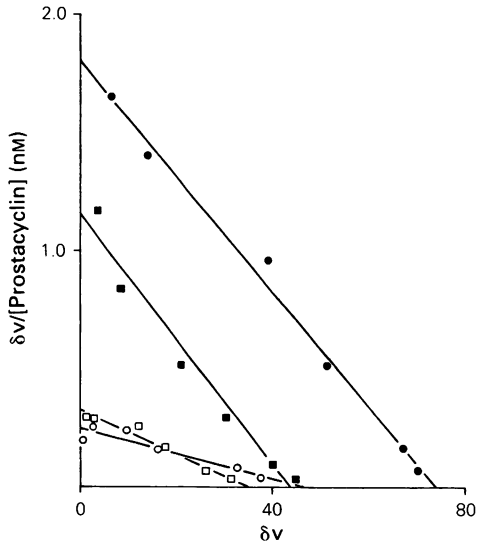

Figure 3 An Eadie-Hofstee plot for the activation of adenylate cyclase by 4–1000 nM prostacyclin in control cells (●), and cells that had been cultured for 16 h with 1 μM carbacyclin (○), 20 μg ml^{-1} cycloheximide (■) or 1 μM carbacyclin plus 20 μg ml^{-1} cycloheximide (□). Data are means of triplicate determinations. $r = 0.997$ (●), 0.994 (○), 0.976 (■) and 0.992 (□), respectively.

affinity non-saturable site. $\delta b = B_{max}/[1 + (K_D/[L])] + C[L]$, where [L] is the ligand concentration.

In control membranes, the high affinity binding site had a K_D (equilibrium dissociation constant) of 9.55 nM, and a B_{max} (maximum binding capacity) of 431 fmol [^{3}H]-iloprost bound mg^{-1} protein. The membranes from desensitized cells had a K_D for [^{3}H]-iloprost binding of 14.8 nM and a B_{max} of 200 fmol bound mg^{-1} protein.

The effect of prolonged culture with carbacyclin and cycloheximide on prostacyclin-stimulated adenylate cyclase activity

NCB-20 cells were cultured for 14 h with either 1 μM carbacyclin, 20 μg ml^{-1} cycloheximide or 1 μM carbacyclin plus 20 μg ml^{-1} cycloheximide. Control cells were cultured with the ethanol and saline carriers. Cells were harvested, and prostacyclin-stimulated adenylate cyclase activity was measured in cell homogenates (Figure 3).

The control cells had a maximum prostacyclin-stimulated increase in adenylate cyclase activity (δV_{max}) of 73.7 pmol cyclic AMP min^{-1} mg^{-1} protein and a K_{act} of 41.0 nM. Cells that had been desensitized by treatment with carbacyclin alone had a δV_{max} of 47.1 pmol cyclic AMP min^{-1} mg^{-1} protein and a K_{act}

of 189 nM. Cycloheximide-treated cells had a δV_{max} of 43.5 pmol cyclic AMP min^{-1} mg^{-1} protein and a K_{act} of 37.8 nM. Cells that had been treated with both carbacyclin and cycloheximide had a δV_{max} of 34.3 pmol cyclic AMP min^{-1} mg^{-1} protein and a K_{act} of 106 nM.

Thus, treatment of cells with carbacyclin or cycloheximide, or a combination of both, resulted in a loss of maximum prostacyclin-stimulated adenylate cyclase activity. Treatment with carbacyclin or a combination of carbacyclin and cycloheximide resulted in an increase in the K_{act} value for prostacyclin-stimulated adenylate cyclase activity. There was, however, no change in K_{act} following treatment of cells with cycloheximide alone.

Activation of adenylate cyclase by fluoride in control and desensitized cells

NCB-20 cells were cultured for 16 h with or without 1 μM carbacyclin. Cells were harvested, and the activation of adenylate cyclase by sodium fluoride was measured in cell homogenates (Figure 4). There was no difference in fluoride-stimulated adenylate cyclase activity in control or carbacyclin-treated cells.

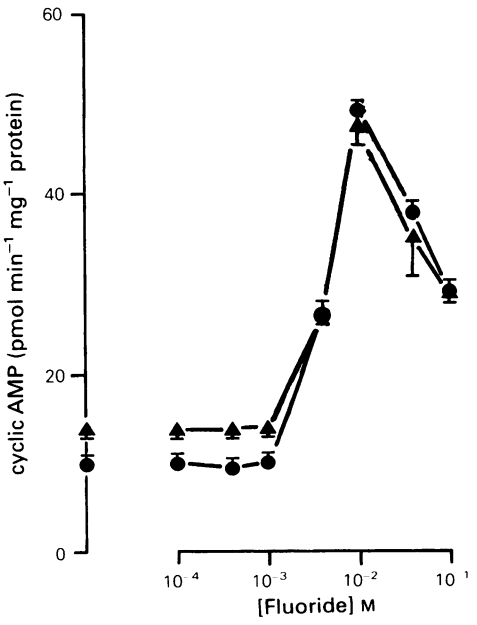

Figure 4 Activation of adenylate cyclase by sodium fluoride in control cells (●) and cells that had been cultured with 1 μM carbacyclin for 16 h (▲). Data are means (vertical lines are s.e.means) of triplicate determinations.

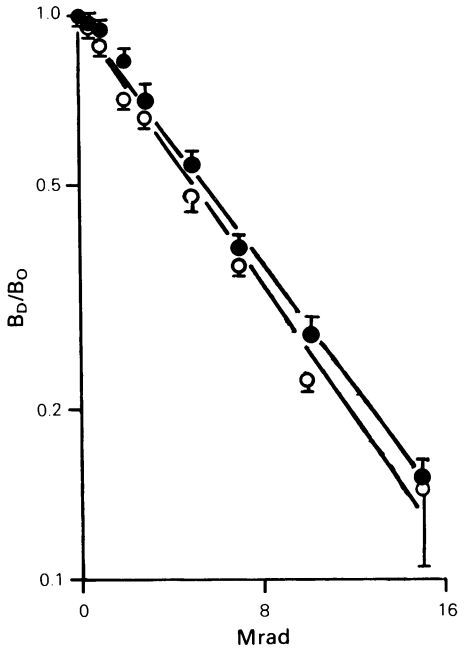

Figure 5 Radiation inactivation of the prostacyclin receptor from control (●) and desensitized cells (○). Desensitized cells had been cultured with 1 μM carbacyclin for 16 h. Membranes were prepared, lyophilised and irradiated in triplicate at 4°C. The surviving specific binding of 25 nM [^{3}H]-iloprost was measured in duplicate. B_D was the surviving specific binding of [^{3}H]-iloprost after a dose, D (Mrad) and BO was the original binding (control = 177.1 ± 0.34 and desensitized = 117.9 ± 3.36 fmol bound mg^{-1} protein). $r = 0.998$ (control) and 0.995 (desensitized). (Data for control cells are taken from Leigh *et al.* (1984)).

Radiation inactivation of the prostacyclin receptor of control and desensitized cells

NCB-20 cells were cultured with or without 1 μM carbacyclin for 16 h. The cells were harvested, and cell membranes prepared. The membranes were lyophilised and irradiated at doses up to 15 Mrad. The molecular weight of the receptor was calculated by measurement of the loss of specific [^{3}H]-iloprost binding (25 nM) with increasing doses of radiation (Figure 5). In three separate experiments the molecular weight of the prostacyclin receptor has been determined previously as 82,800 ± 12,900 (Leigh *et al.*, 1984). The results of one of these experiments is shown in Figure 5. Radiation inactivation of the prostacyclin receptor from desensitized cells is also shown, and a molecular weight of 88,000 was obtained.

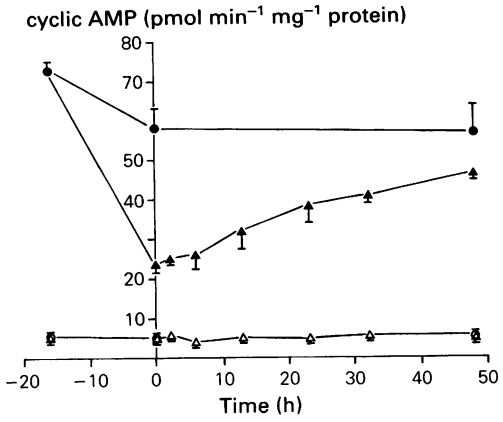

Figure 6 Resensitization of prostacyclin-stimulated adenylate cyclase activity. NCB-20 cells were cultured with 10 μM cytosine arabinofuranoside for 24 h. They were then cultured with (▲, △) or without (●, ○) 1 μM carbacyclin for 16 h. The cells were washed with culture medium and allowed to resensitize for 48 h. Basal (open symbols) and prostacyclin- (4 μM, closed symbols) stimulated adenylate cyclase activity were measured. Values are means (vertical lines are s.e.means) of triplicate determinations.

Resensitization of prostacyclin-stimulated adenylate cyclase activity

Experiments were designed to investigate whether desensitization of prostacyclin-stimulated adenylate cyclase activity was reversible in NCB-20 cells. The cells were cultured for 24 h with 10 μM cytosine arabinofuranoside (to inhibit further cell division) and then for 16 h with 1 μM carbacyclin (in the ethanol carrier). Cells were harvested after this treatment, or were washed three times in prostaglandin-free medium and maintained in culture for up to 48 h. Control cells were treated similarly with cytosine arabinofuranoside and then the ethanol carrier. Cell counts performed on cells before desensitization, and on control and desensitized cells at $t = 48$ h, showed that there was no significant change in cell numbers per plate throughout the 64 h of the experiment ($P > 0.05$ on an unpaired t test). Thus, cytosine arabinofuranoside was effective at this concentration as an inhibitor of cell division. Basal, and prostacyclin-stimulated (4 μM) adenylate cyclase activity were measured in cell homogenates (Figure 6).

After culture of NCB-20 cells for 16 h with carbacyclin, prostacyclin-stimulated adenylate cyclase activity

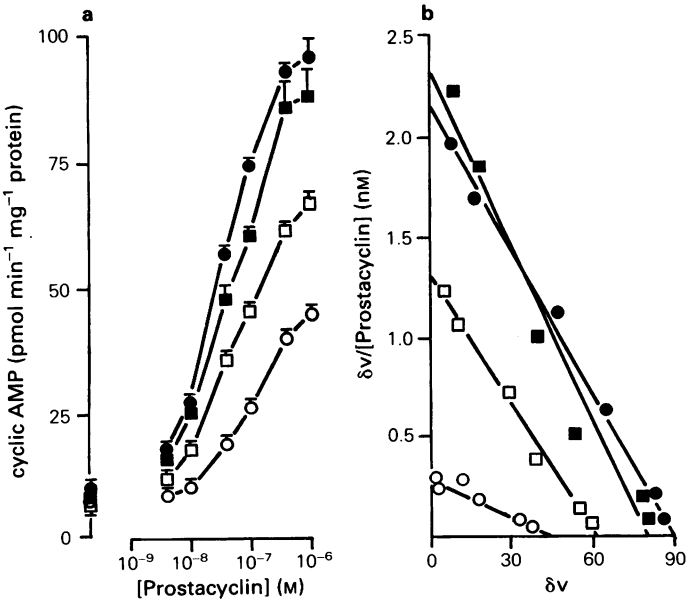

Figure 7 Activation of adenylate cyclase by prostacyclin in desensitized cells (○) and their controls (●), and resensitized cells (□) and their appropriate controls (■). NCB-20 cells were cultured with cytosine arabinofuranoside for 24 h. They were then cultured with 1 μM carbacyclin for a further 16 h and either harvested (desensitized cells) or allowed to resensitize for 48 h (resensitized cells). Prostacyclin-stimulated adenylate cyclase activity was measured (a). Data are means (vertical lines are s.e.means) of triplicate determinations. The data are presented as an Eadie-Hofstee plot (b) where δv is the increase in adenylate cyclase activity stimulated by prostacyclin. $r = 0.992$ (●), 0.980 (■), 0.989 (○) and 0.996 (□), respectively.

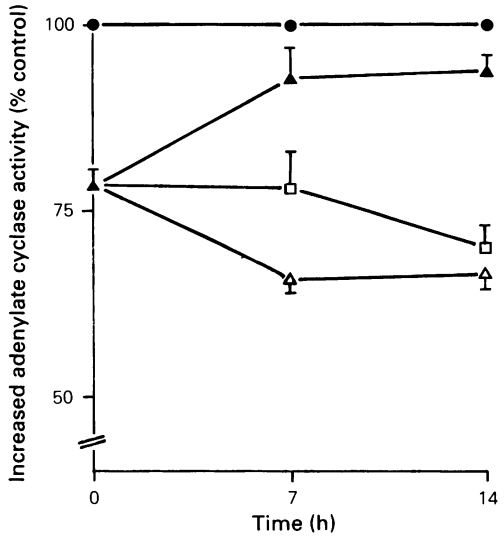

Figure 8 Inhibition of resensitization by cycloheximde and actinomycin D. NCB-20 cells were cultured with 10 μM cytosine arabinofuranoside for 24 h and were then cultured with 1 μM carbacyclin for 16 h. Cells were washed and allowed to resensitize in the absence (▲), or presence of 20 μg ml^{-1} cycloheximide (△) or 16 μM actinomycin D (□). The increase in adenylate cyclase activity stimulated by 4 μM prostacyclin was measured in cell homogenates. Values are means (vertical lines are s.e.means) of triplicate determinations; the results are expressed as a percentage of values obtained in control (non-desensitized) homogenates (118 ± 5.0 pmol cyclic AMP min^{-1} mg^{-1} protein).

had decreased to 41% of control values. Following 48 h culture in prostaglandin-free medium, this value had returned to 82% of control values with a half-time of approximately 18 h.

The activation of adenylate cyclase by prostacyclin in desensitized and resensitized cells was further investigated in cells that had been treated concurrently with those of the previous experiment (Figure 7). Cells were treated with cytosine arabinofuranoside, and were then either cultured with carbacyclin for 16 h before harvesting (desensitized cells), or left for a further 48 h in prostaglandin-free medium (resensitized cells). The appropriate controls were included as in the previous experiment.

In desensitized cells, the δV_{max} for prostacyclin-stimulated adenylate cyclase activity had decreased to 48.8% of control levels (control = 90.1, desensitized = 44.0 pmol cyclic AMP min^{-1} mg^{-1} protein). The K_{act} value had increased from 41.7 nM (control cells) to 143 nM (desensitized cells). In resensitized cells, the δV_{max} value was restored to 75.3% of control values (control = 80.9, resensitized = 60.9 pmol cyclic AMP min^{-1} mg^{-1} protein) and the K_{act} value had decreased almost to that of control cells (control = 34.5 nM, resensitized = 45.5 nM).

Inhibition of resensitization by inhibitors of protein synthesis

NCB-20 cells were cultured for 24 h with 1 μM cytosine arabinofuranoside to terminate cell division. They were then cultured for 16 h with 1 μM carbacyclin in 0.05% ethanol (desensitized cells), or the ethanol carrier alone (control cells). Cells were then washed and either allowed to resensitize for 14 h, or were treated with 20 μg ml^{-1} of cycloheximide (saline carrier) or 16 μM actinomycin D (methanol carrier) ('resensitizing cells' were treated with the methanol and saline carriers alone). The increase in adenylate cyclase activity stimulated by 4 μM prostacyclin was measured in cell homogenates, and was expressed as a percentage of the control ('non-desensitized') values (Figure 8). After 16 h desensitization, prostacyclin-stimulated adenylate cyclase activity had decreased to 78.5% of control values. Following 14 h resensitization, prostacyclin-stimulated adenylate cyclase activity was restored to 93.5% of control values. However, when cells were treated with cycloheximide or actinomycin D after desensitization, prostacyclin-stimulated adenylate cyclase activity never increased above the level of desensitized cells.

Comparison of the rates of loss of prostacyclin receptors and adenylate cyclase activity following treatment of NCB-20 cells with carbacyclin or cycloheximide

NCB-20 cells were cultured in 35 mm culture plates with 20 μg ml^{-1} of cycloheximide or 1 μM carbacyclin for 8 h. The cells were harvested at selected times and were counted in a haemocytometer. Cell homogenates were prepared taking care to ensure maximum recovery of cells, and either basal adenylate cyclase activity or the specific binding of 25 nM [^{3}H]-iloprost was measured. Adenylate cyclase activity and the binding of [^{3}H]-iloprost were expressed per cell rather than per mg cell protein so that the changes in total cellular protein caused by cycloheximide did not affect the results. Each value was expressed as a percentage of control levels, and was plotted on a semi-logarithmic plot against time. The 1st order rate constant for the decrease in adenylate cyclase activity or binding was calculated from $k = 0.693/t_{\frac{1}{2}}$.

Basal adenylate cyclase activity and [^{3}H]-iloprost binding decreased slowly when cells were incubated with cycloheximide ($k = 1.94 \times 10^{-5}$ s^{-1} and 0.92×10^{-5} s^{-1}, respectively). Linear regressions of these plots yielded r values of 0.952 and 0.984 respectively. No significant change was seen in basal aden-

ylate cyclase activity following treatment with carbacyclin, but a rapid loss of [^{3}H]-iloprost binding occurred ($k = 5.08 \times 10^{-5} s^{-1}, r = 0.984$). Thus desensitization of cells with carbacyclin results only in a rapid loss of prostacyclin receptors. In contrast, incubation of cells with cycloheximide results in a slower loss of both the catalytic subunit of adenylate cyclase (as reflected by basal enzyme activity) and the prostacyclin receptor.

Discussion

Agonist specific desensitization of prostacyclin-stimulated adenylate cyclase activity has been demonstrated in human fibroblasts (Gorman & Hopkins, 1980) and platelets, *in vivo* (Sinzinger *et al.*, 1981) and *in vitro* (Miller & Gorman, 1979) and in the NCB-20 cell line (Blair *et al.*, 1982). In an extension of the studies with the NCB-20 cell line, the mechanism of this desensitization has been investigated.

Incubation of NCB-20 cells with the stable prostacyclin analogues, carbacyclin and iloprost, caused a desensitization of prostacyclin-stimulated adenylate cyclase activity. The desensitization was specific, and was limited to agonists that have been shown to bind to the prostacyclin receptor, i.e. prostacyclin, iloprost, carbacyclin and prostaglandin E_1. No change in basal adenylate cyclase activity was seen following desensitization, indicating that desensitization was not caused by a reduction or an alteration in the catalytic subunit of adenylate cyclase. This observation contrasted with a similar study of the desensitization of prostaglandin E_1-stimulated adenylate cyclase activity in the related NG108-15 hybrid cell line (Kenimer & Nirenberg, 1981), where basal adenylate cyclase activity was reduced by culture of the cells with prostaglandin E_1. In addition, incubation of NG108-15 cells with prostaglandin E_1 resulted in a decrease in 2-chloroadenosine-stimulated adenylate cyclase activity (Kenimer & Nirenberg, 1981). This was not observed when NCB-20 cells were incubated with carbacyclin.

The concentration of carbacyclin giving half-maximum desensitization of prostacyclin-stimulated adenylate cyclase activity (from Figure 1) is similar to the K_D for [^{3}H]-iloprost binding, and the concentration of ligand required for half-maximum enzyme activation. This suggests that desensitization is mediated by receptor occupancy.

Desensitized cells did not have altered levels of fluoride-stimulated adenylate cyclase activity. Fluoride has been shown to bind to the regulatory (N) subunit of adenylate cyclase (Northup *et al.*, 1982), cause dissociation of the α and β-subunits, and activation of adenylate cyclase. The observations that fluoride-stimulated adenylate cyclase activity is unchanged in desensitized cells, and that basal adenylate cyclase activity does not change following desensitization would suggest that coupling between the regulatory and catalytic subunits of adenylate cyclase is unchanged during desensitization, and that desensitization is probably due to an alteration of the receptors.

The binding of [^{3}H]-iloprost to both control and desensitized NCB-20 cell membranes yielded non-linear Scatchard plots. This was in contrast to previous studies on NCB-20 cells with [^{3}H]-11β-prostacyclin (Blair & MacDermot, 1981) and [^{3}H]-iloprost (Leigh *et al.*, 1984) where only one binding site for prostacyclin was seen. Non-linear Scatchard plots for binding to the prostacyclin receptor have previously been obtained for guinea-pig lung (MacDermot *et al.*, 1981) and platelets (Shepherd *et al.*, 1983) using [^{3}H]-11β-prostacyclin, and for platelets (Siegl *et al.*, 1979, Schillinger & Prior, 1980, Lombroso *et al.*, 1984) using [^{3}H]-9β-prostacyclin. The lower affinity binding now identified on NCB-20 cell membranes was not previously identified using [^{3}H]-11β-prostacyclin because sufficiently high concentrations of [^{3}H]-prostacyclin were not employed. This limitation in the experimental design was necessitated by high values of non-specific binding at high ligand concentrations, and the relative scarcity of the radio-ligand. Similar studies with [^{3}H]-iloprost in NCB-20 cells have employed only relatively low ligand concentrations (up to 100 nM), and so the second binding site was not identified (Leigh *et al.*, 1984).

Data from the Scatchard plots were analysed as a three parameter model identifying a single high affinity binding site, and a lower affinity binding component which was assumed to be non-saturable. This second binding component had no biological significance, did not appear to be stereospecific (data not presented) and was not saturable at concentrations of ligand which could readily be employed. In only one study has a biological response been shown for the second, low affinity prostacyclin binding component (Lombroso *et al.*, 1984).

Following desensitization, the maximum [^{3}H]-iloprost binding capacity of NCB-20 membranes was reduced, but this was not accompanied by a substantial alteration in the affinity of the remaining high affinity receptors for [^{3}H]-iloprost. In a previous study with the NCB-20 cells (Blair *et al.*, 1982), the reduction in number of binding sites with desensitization appeared to be accompanied by a small reduction in the affinity of the remaining binding sites. However, these data were quite scattered and high concentrations of ligand could not be employed. The results might now be re-evaluated and should be considered to represent a large decrease in the numbers of high affinity receptors without an alteration in the low affinity non-saturable binding component. Thus,

without subtraction of the low affinity binding, there appeared to be an affinity change.

The change in K_{act} of prostacyclin-stimulated adenylate cyclase activity that accompanies desensitization is most readily explained as an apparent affinity change resulting from a reduction in the number of receptors on the cell membrane without an alteration in the number of adenylate cyclase molecules (Homburger *et al.*, 1980). This mechanism can be invoked if the number of receptors per unit area of membrane exceeds the number of adenylate cyclase molecules, in which case there are said to be 'spare' receptors.

The nature of the apparent affinity change was characterized by investigating the reduced responsiveness to prostacyclin resulting from culture of cells with cycloheximide. Cycloheximide treatment resulted in a decrease in maximum prostacyclin-stimulated adenylate cyclase activity without an alteration in the K_{act} value. This indicates that the two processes initiated by treatment of cells with either carbacyclin or cycloheximide were different. On further investigation, it was shown that the rate of loss of prostacyclin receptors and adenylate cyclase proteins resulting from cycloheximide treatment were both slow, and of approximately equal k values. Thus, the relative proportions of these two proteins in the membrane would remain constant following cycloheximide treatment. In contrast, the rate of loss of prostacyclin receptors due to desensitization with carbacyclin was much more rapid, and was not accompanied by a loss of adenylate cyclase molecules. It follows, therefore, that more prostacyclin would be required to occupy the same number of receptors per unit area of membrane. Activation of adenylate cyclase relies on the number of occupied receptors per unit area of membrane and not specifically on the fractional occupancy of the receptors. Thus, an apparent affinity change would accompany a loss of receptors in a receptor-adenylate cyclase system where the receptor number exceeds the number of adenylate cyclase molecules (Homburger *et al.*, 1980).

In NG108-15 cells a reduction in the affinity of the ligand-receptor interaction accompanied desensitization (Kenimer, 1982). An affinity change has also been implicated in the mechanism of desensitization of the β-adrenoceptor-stimulated adenylate cyclase system of turkey erythrocytes (Stadel *et al.*, 1981). However in turkey erythrocytes desensitization of the β-adrenoceptor mediated response is not accompanied by a loss of receptors.

Desensitization of prostacyclin-stimulated adenylate cyclase activity did not involve a change in the target size of the prostacyclin receptor, when measured with the linear accelerator. There is no evidence for a clustering of receptors before internalization, or of desensitization involving a coupling of the receptors to any other components of the adenylate cyclase system. The methodology for radiation inactivation of the prostacyclin receptor has been described previously (Leigh *et al.*, 1984). Membranes were prepared for lyophilisation and irradiation in a 25 mM Tris-HCl buffer, pH 8.5, containing 0.29 M sucrose. Under these conditions the molecular weights obtained for the catalytic and regulatory subunits of adenylate cyclase, and for acetylcholinesterase have been shown to be in good agreement with values found in the literature from both hydrodynamic and radiation inactivation studies (Leigh *et al.*, 1984). Briefly, the affinity of [^{3}H]-iloprost binding and the physical properties of the receptor, as identified by target size analysis, were unchanged during desensitization (Figure 5). The unchanged nature of the receptor during desensitization suggests further the need to invoke the concept of spare receptors to explain the reduced affinity of prostacyclin for adenylate cyclase activation during desensitization. The results may be expressed numerically if the following assumptions are made:

(i) The receptor (R) interacts with the complex (NC) of the adenylate cyclase catalytic subunit (C) and regulatory subunit (N) with a 1:1 stoichiometry, and (y) molecules of NC are activated when an equal number (y) of receptors are occupied.

(ii) The probability of an interaction between any particular NC complex and any receptor molecule is equal.

(iii) The enzyme exists in a 'basal' or 'activated' state, but in no intermediate states, and gradual changes in enzyme activity are mediated by sequential activation of NC complexes.

(iv) The level of enzyme activation is dependent on the number of occupied receptors per unit area of membrane, and not (necessarily) on the fractional occupancy of the receptors with agonist.

The number of occupied receptors (r) is given by $r = [R]_n/[1 + (K_D/L)]$ where $[R]_n$ is the total number (n) of receptors. Clearly at 50% occupancy, $r = [R]_n/2$. If $[R]_n$ exceeds the number of adenylate cyclase molecules (y) by a factor, x, then $[R]_n = xy$.

Further, if $[R]_y$ is the number of receptors equal to y enzyme molecules then $[R]_n = [R]_y x$, or $[R]_y = [R]_n/x$.

Now, 50% enzyme activation is produced when $r = [R]_y/2$

$$\text{(1)} \qquad \text{whence } r = \frac{[R]_n}{2x}$$

In the general case, the concentration of ligand, [L], required to occupy r receptors is given by

$$\text{(2)} \qquad [L] = K_D \frac{r}{[R]_n - r}$$

Thus, to produce 50% enzyme activation (1) may be substituted into (2) and:

$$[L] = K_{act} = \frac{\frac{K_D[R]_n}{2x}}{[R]_n - \frac{[R]_n}{2x}}$$

$$\text{or: } K_{act} = \frac{K_D}{2x - 1} \quad (3)$$

The expression (3) is most certainly a simplification of the events described in Figure 1, but it defines the increase in the K_{act} value that will occur when receptors are lost (and x decreases) during desensitization. It also explains the increase in K_{act} while K_D remains constant. In the experimental results presented, the values of K_{act} for adenylate cyclase activation by prostacyclin in homogenates of non-desensitized cells are consistently greater than the value (18.1 nM) of K_D for [^{3}H]-prostacyclin binding (Blair *et al.*, 1982). The expression $K_{act} = K_D/2x - 1$ would predict the reverse for all values of $x > 1$. It should be noted, however, that the experimental value of K_{act} is dependent on several variables, particularly GTP concentration, in addition to the value of K_D. The binding of GTP to the regulatory subunit (N) during the receptor-mediated activation of adenylate cyclase is accompanied by a decrease in the affinity of the ligand-receptor interaction (Rodbell, 1980). In these calculations no account is taken of GTP concentration, ionic concentrations, etc.

Desensitization of prostacyclin-stimulated adenylate cyclase activity was reversible in non-dividing cells (Figure 6). However, this process was slow, which indicated that it was not due to a simple recycling of receptors that had been internalised during desensitization. Protein synthesis was shown to be required for resensitization (Figure 8). Both actinomycin D and cycloheximide (at concentrations that inhibited [^{3}H]-leucine incorporation into protein in NCB-20 cells) inhibited resensitization. This indicated that both transcription and translation were required for *de novo* protein synthesis during resensitization. Prostacyclin-stimulated adenylate cyclase activity in homogenates of cells that had been allowed to resensitize for several hours revealed that the cells had regained the capacity for the higher affinity enzyme activation (i.e. the K_{act} value was restored to the non-desensitized level). This suggested strongly that resensitization was due to *de novo* receptor synthesis.

In conclusion, the data reveal a reduction in maximum prostacyclin-dependent activation of adenylate cyclase in desensitized cells. The change is accompanied by an increase in the concentration of prostacyclin required for half-maximum enzyme activation. There is no evidence of altered levels of the enzyme adenylate cyclase following desensitization, and the coupling of the catalytic subunit of the enzyme to the regulatory subunit (N) appears unaltered. The presumptive change in the receptor has therefore been investigated extensively. The K_D for [^{3}H]-iloprost binding at equilibrium was unchanged, and the 'target size' of the receptor was also unaltered. These findings suggest strongly that desensitization is not accompanied by aggregation of the receptor molecules within the plasma membrane; neither is there coupling of the receptors to the regulatory (N) subunit to form a stable complex. Desensitization was accompanied by a reduction in maximum [^{3}H]-iloprost binding, and the concept of spare receptors has been invoked to explain the anomalous increase in the K_{act} for prostacyclin-dependent activation of adenylate cyclase without simultaneous alteration in the K_D of [^{3}H]-iloprost binding.

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