Br. J. Pharmac. (1982), **77**, 121–127

DESENSITIZATION OF PROSTACYCLIN RECEPTORS IN A NEURONAL HYBRID CELL LINE

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1 Prostacyclin and its stable analogue, carbacyclin, bind competitively to a single population of receptors, and activate adenylate cyclase of the NCB-20 neuronal somatic cell hybrid (K_{act} = 40.1 nM and 96.1 nM respectively).

2 Culture of NCB-20 cells in the presence of 1 μM carbacyclin for 4 to 16 h results in a progressive decrease in the prostacyclin-dependent activation of adenylate cyclase in cell homogenates with an increase at 16 h of the K_{act} from 64.1 nM to 174.0 nM and decrease in the maximum adenylate cyclase activation from 41.2 to 15.1 pmol cyclic AMP min^{-1} mg^{-1} protein.

3 The prediction that the apparent decrease in affinity in the prostacyclin-dependent activation of adenylate cyclase was secondary to a reduction in receptor numbers was tested directly by measuring binding of [^{3}H]-prostacyclin to membranes of cells exposed to carbacyclin for 16 h. This showed an actual decrease in affinity of the prostacyclin-receptor interaction, as well as a decrease in the total receptor numbers. Thus prolonged exposure of NCB-20 cells to carbacyclin caused reductions in both receptor numbers and affinity, reflected by measurements both of binding and adenylate cyclase activation.

Introduction

Prostacyclin (PGI_2) is an unstable prostaglandin endoperoxide metabolite. The biological responses of tissues to PGI_2 are mediated by activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1], and the molecular events that accompany PGI_2-dependent activation of adenylate cyclase have been studied extensively in a cloned hybrid cell line (NCB-20) derived from a mouse neuroblastoma and embryonic hamster brain (Blair, Hensby & MacDermot, 1980; Blair & MacDermot, 1981; Blair, Cresp & MacDermot, 1981a; MacDermot, Blair & Cresp, 1981b). PGI_2 binds to specific membrane receptors of this cell line (Blair & MacDermot, 1981), and increases adenylate cyclase activity 10 to 15 fold (Blair *et al.*, 1980). PGI_2-dependent activation of adenylate cyclase requires guanosine triphosphate (GTP) (Blair *et al.*, 1980), which suggests that coupling of the receptor to the enzyme is similar to other examples of receptor-mediated activation of adenylate cyclase (Rodbell, 1980). The binding of PGI_2 to its membrane receptor is dependent on divalent cations (Blair *et al.*, 1981a), and a novel mechanism involving a cation-dependent change in the conformation of the PGI_2 molecule has been proposed (MacDermot *et al.*, 1981b). The altered geometry that accompanies the formation of a cation-PGI_2 complex in solution may satisfy the geometric constraints of the PGI_2 receptor. In this paper we have investigated whether PGI_2 receptors of the NCB-20 neuronal somatic hybrid become desensitized after prolonged exposure to the stable PGI_2-receptor agonist, carbacyclin (Aristoff, 1981). The affinity of carbacyclin approximates closely to that of PGI_2 (Whittle, Moncada, Whiting & Vane, 1980; Whittle, Steel & Boughton-Smith, 1980), but the substitution of a methylene group for the vinyl ether oxygen renders the molecule stable to hydrolysis at neutral pH.

Methods

Cell culture

The NCB-20 hybrid cell line (Minna, Yavelow & Coon, 1975) was derived by fusion of a 6-thioguanine-resistant mouse neuroblastoma cell line, N18TG2 (Minna, Glazer & Nirenberg, 1972) and brain cells of embryonic Chinese hamster (18 days *in utero*). Cells were cultured in Dulbecco's modified Eagle medium (Gibco Bio-Cult Ltd) with 10% (v/v) new-born calf serum (Gibco Bio-Cult Ltd), 1 μM aminopterin (Sigma UK Ltd), 100 μM hypoxanthine (Sigma UK Ltd) and 16 μM thymidine (Sigma UK Ltd). Culture flasks and dishes (Falcon Plastics) were maintained in a humidified atmosphere of 90% air and 10% CO_2. Cells were harvested in Dulbecco's phosphate-buffered saline (no Ca^{2+} or Mg^{2+} ions).

0007-1188/82/090121-07 $01.00

Adenylate cyclase assay

Harvested cells were suspended in 25 mM Tris-HCl buffer pH 8.5 containing 0.29 M sucrose, and homogenized by 20 strokes of a tightly fitting Dounce homogenizer at 4°C (2 ml buffer per 75 cm^2 flask of cells). The homogenates were frozen, and stored at −80°C. Before assay, they were thawed, dispersed by a further 10 strokes of the Dounce homogenizer, and stored at 4°C for less than 10 min.

Method C of Salomon, Londos & Rodbell (1974) was modified according to Sharma, Nirenberg & Klee, 1975. Reaction mixtures of 100 μl contained 50 mM Tris-HCl buffer pH 8.5; 5 mM magnesium sulphate; 87 mM sucrose; 20 mM creatine phosphate, disodium salt (Sigma UK Ltd); 10 international units creatine kinase, 150 iu/mg protein (ATP:creatine N-phosphotransferase; EC 2.7.3.2) from Sigma UK Ltd; 1 mM adenosine 3'5'-monophosphate (cyclic AMP), sodium salt (Sigma UK Ltd); 0.25 mM Ro20-1724 (a phosphodiesterase inhibitor, Roche Products Ltd); 0.25 mM ethanol; 1 mM [α^{32}P]-ATP (2–3 μCi, Amersham International Ltd; 1Ci = 3.7×10^{10} Bq) and 0.071 to 0.275 mg of homogenate protein. Reaction mixtures were incubated at 30°C for 10 min, and the reaction was then terminated by the addition of 800 μl 6.25% (w/v) trichloroacetic acid. After the addition of 100 μl [8-^{3}H]-cyclic AMP (30 Ci/mmol, about 10,000 ct/min per reaction mixture, Amersham International Ltd), the reaction mixtures were centrifuged at 2000 *g* for 10 min. The [^{32}P]-cyclic AMP was separated from the [α^{32}P]-ATP by a 2 step chromatographic procedure (Salomon *et al.*, 1974), and corrected for losses by estimation of the recovered [^{3}H]-cyclic AMP. The production of [^{32}P]-cyclic AMP was proportional to protein concentration within the range 50 to 300 μg of homogenate protein per reaction mixture, and [^{32}P]-cyclic AMP synthesis increased linearly with time for 30 min.

Synthesis of [11β-^{3}H]-prostacyclin

[11β-^{3}H]-PGI_2 was synthesized (Blair *et al.*, 1981b) from prostaglandin D_2 (PGD_2). The method employed reduction of PGD_2 with sodium [^{3}H]-borohydride (32 Ci/mmol, Amersham International Ltd) to yield [11β-^{3}H]-$PGF_{2\alpha}$. The synthesis of [11β-^{3}H]-PGI_2 from [11β-^{3}H]-$PGF_{2\alpha}$ followed the method of Nicolaou, Barnette, Gasic, Magolda & Sipio (1977). [11β-^{3}H]-PGI_2 was identified by hydrolysis to [11β-^{3}H]-6-oxo-$PGF_{1\alpha}$ which was compared to authentic 6-oxo-$PGF_{1\alpha}$ by gas chromatography-mass spectrometry. The biological activity of the newly synthesized [11β-^{3}H]-PGI_2 was compared with authentic PGI_2 by adenylate cyclase activation in cell homogenates of the NCB-20 hybrid (Blair *et al.*, 1981b; Blair & MacDermot, 1981).

Binding of [^{3}H]-prostacyclin to membranes of NCB-20 cells

Cell homogenates were prepared as described for measurement of adenylate cyclase activity. Undisrupted cells and nuclei were than pelleted by centrifugation at 500 *g* at 4°C for 10 min. The supernatant was centrifuged at 100,000 *g* for 20 min at 4°C, and the pellet washed 3 times by resuspension in 50 mM Tris-HCl pH 8.5, followed by centrifugation at 100,000 *g* for 20 min. The pellet was finally suspended in 50 mM Tris-HCl buffer pH 8.5 (0.25 ml per 75 cm^2 flask of cells) and stored at −80°C. The protein concentrations of the cell homogenates and the washed membrane preparations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

The binding assay employed a modification of techniques described previously (Pert & Snyder, 1973). Reaction mixtures of 100 μl contain 50 mM Tris-HCl buffer pH 8.5; 10 mM magnesium sulphate; [^{3}H]-PGI_2 at selected concentrations (8 Ci/mmol); and 160–175 μg protein of washed membranes of NCB-20 hybrid cells. The reaction mixtures were prepared at 4°C in an ice bath, and then incubated for 15 min at 20°C.

The incubations were terminated by the addition of 4 ml ice-cold 50 mM Tris-HCl buffer pH 8.5 (washing buffer), followed by filtration through Whatman GF/C glass filter discs. The filters were then washed 3 times with 4 ml ice-cold washing buffer, and dried for 1 h under an infra-red lamp. The filters were then suspended in 10 ml Insta-Gel (Packard Instrument Co. Inc.). Counting was performed in a Packard Liquid Scintillation Spectrometer. Specific binding was taken to be that displaced by 10 μM unlabelled PGI_2 in parallel incubations.

The following generous gifts were received: PGI_2 and carbacyclin from the Wellcome Research Laboratories; PGD_2 from Upjohn Co.; Ro20-1724 from Roche Products Ltd; the NCB-20 hybrid cells from Dr Marshall Nirenberg, National Institutes of Health, USA.

The statistical analyses performed were unpaired, two tailed Student's *t* tests.

Results

Carbacyclin was compared with PGI_2 as an activator of adenylate cyclase. Basal adenylate cyclase activity (4.3 ± 0.2 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, mean ± s.e.mean) was increased more than 12 fold, with similar activities observed in the presence of saturating concentrations of PGI_2 or carbacyclin (Figure 1a). The data are presented as an Eadie-Hofstee plot (Figure 1b), which reveals that enzyme activation is mediated by interactions between the

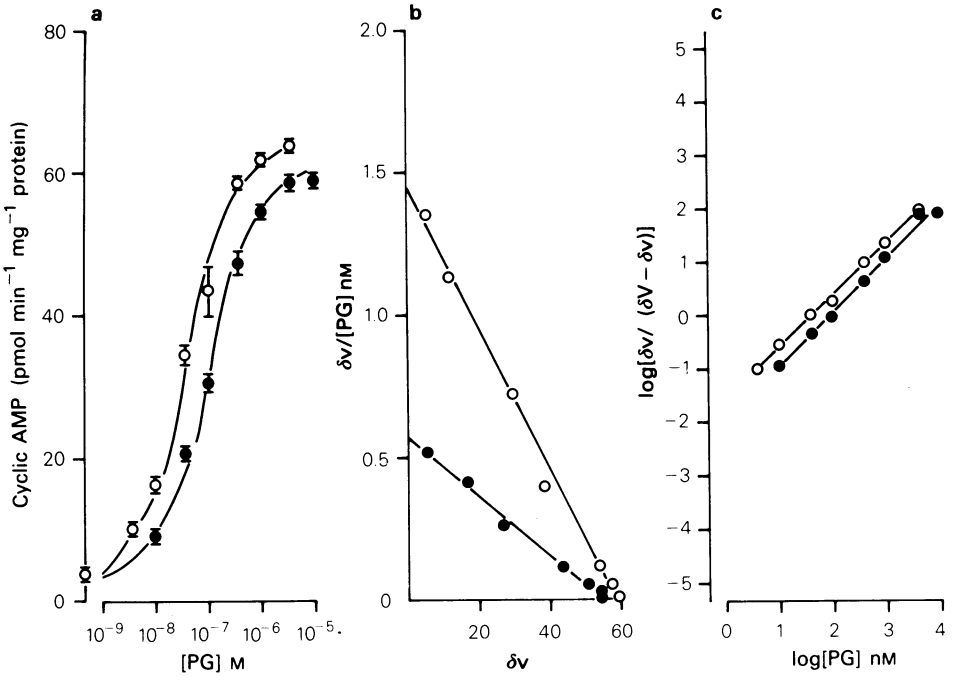

Figure 1 Activation of adenylate cyclase by prostacyclin (○) and carbacyclin (●) in a homogenate of NCB-20 cells (a). Results show means (vertical lines indicate s.e.means) of triplicate determinations. The same data are presented as Eadie-Hofstee (b) and Hill (c) plots, where δv is the increase in enzyme activity at any particular prostaglandin concentration, and δV is the maximum increase (determined from the abscissa intercept in the Eadie-Hofstee plot).

ligands and a single population of receptors, as shown by the linear plots for both PGI_2 ($r = 0.99$) and carbacyclin ($r = 0.99$). The concentration of PGI_2 producing half-maximum adenylate cyclase activation (K_{act}) is given by 1/slope (40.1 nM), which compared with a K_{act} value of 96.1 nM for carbacyclin. There was no difference in the maximum increase in adenylate cyclase activity above the basal level produced by saturating concentrations of PGI_2 (58.9 pmol min^{-1} mg^{-1} protein) or carbacyclin (54.9 pmol min^{-1} mg^{-1} protein). In a Hill plot (Figure 1c), the interaction coefficient (n) is given by the slope, and was 1.0 for PGI_2 and 0.99 for carbacyclin. The results suggest a non-cooperative interaction between PGI_2 or carbacyclin and their receptor molecules.

PGI_2 and carbacyclin bind competitively to the same receptor in the NCB-20 membranes. This was shown by comparing [^{3}H]-PGI_2 binding at concentrations between 10nM and 100 nM in the absence or presence of 50 nM or 0.2 μM carbacyclin. The data (not shown) were consistent with a simple competitive model for the inhibition of [^{3}H]-PGI_2 binding by carbacyclin ($K_i = 36.1$ nM).

The possibility that altered responsiveness of NCB-20 cells to PGI_2 might accompany prolonged exposure to carbacyclin was investigated. Cells were plated in replicate dishes and maintained in culture until judged to be greater than 80% confluent across the dish. At an arbitrary zero time point, carbacyclin in ethanol was added to half of the dishes (final concentration = 1 μM), and the ethanol carrier to the others. The cells from individual dishes were harvested at 4 hourly intervals as shown in Figure 2. The medium was aspirated, and the cells washed with 2 ml of culture medium, which was aspirated after 1.5 min. The cells were removed in 2 ml Dulbecco's phosphate-buffered saline (lacking Ca^{2+} or Mg^{2+} ions), pelleted by centrifugation at 150 g for 3 min and stored at −80°C for subsequent measurement of adenylate cyclase activity. This was measured in duplicate in individual homogenates in the presence or absence of 4 μM PGI_2.

The results in Figure 2 show that the basal adenylate cyclase activity of NCB-20 homogenates was unchanged by prolonged culture of the cells in carbacyclin. Furthermore there was no evidence of contamination of the homogenates by carbacyclin carried over from the culture dishes, as these homogenates did not show increased basal adenylate cyclase activity when compared to controls. In contrast, PGI_2-stimulated adenylate cyclase activity of the

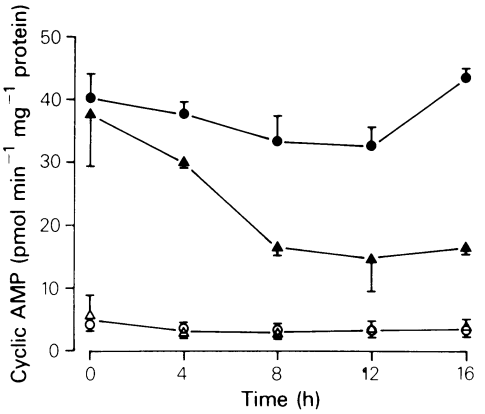

Figure 2 Basal and prostacyclin-stimulated adenylate cyclase activity of NCB-20 homogenates from cells cultured in the absence or presence of carbacyclin. Cells were plated in 35 mm (diameter) dishes and cultured for selected time periods in the absence (circles) or presence (triangles) of 1 μM carbacyclin. All dishes contained 0.05% v/v ethanol. Adenylate cyclase activity was determined in duplicate in homogenates of these cells in the absence (○, △) or presence (●, ▲) of 4 μM prostacyclin. Results show means (vertical lines indicate s.e.means) of triplicate plates at each time point.

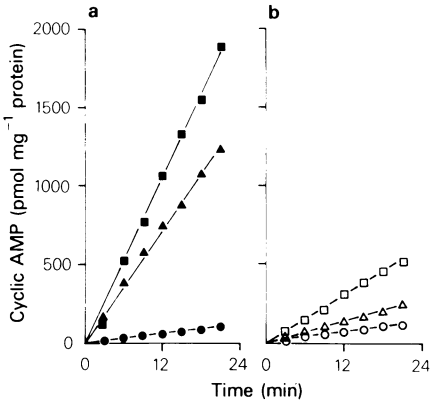

Figure 3 Adenylate cyclase activity of NCB-20 homogenates, from cells cultured in the absence or presence of 1 μM carbacyclin. NCB-20 cells were cultured in 75 cm^2 flasks in the absence (closed symbols) or presence (open symbols) of 1 μM carbacyclin for 16 h. All flasks contained 0.05% ethanol. Accumulation of cyclic AMP is shown as a function of time in control (a) and desensitized (b) cell homogenates. Enzyme activity was measured in the absence (●, ○) or presence of 100 nM (▲, △) or 1 μM (■, □) prostacyclin.

NCB-20 homogenates decreased significantly ($P<0.001$) in those cells exposed to 1 μM carbacyclin for 16 h (16.9 ± 1.0 pmol cyclic AMP min^{-1} mg^{-1} protein) when compared to controls (43.4 ± 1.6 pmol cyclic AMP min^{-1} mg^{-1} protein).

The diminished responsiveness to PGI_2 of NCB-20 homogenates prepared from cells cultured for 16 h in 1 μM carbacyclin is illustrated further in Figure 3. Single incubations of 1 ml were used in the adenylate cyclase assay, and at the times shown 100 μl was removed and added to 800 μl trichloroacetic acid (6.25% w/v). The adenylate cyclase assay was in every other respect similar to that described in the Methods section. The accumulation of cyclic AMP with time in the absence or presence of 100 nM or 1 μM PGI_2 is shown in Figure 3. The enzyme activity of the control homogenate in the presence of PGI_2 was much greater than that prepared from cells cultured in carbacyclin. Once again there was no difference in the basal adenylate cyclase activity of the two homogenates.

The desensitization of the adenylate cyclase response to PGI_2 in cells cultured in carbacyclin was examined further by a comparison of [^{3}H]-PGI_2 binding and the activation of adenylate cyclase at various PGI_2 concentrations in control homogenates and homogenates of cells cultured in carbacyclin (Figure 4).

An Eadie-Hofstee plot of the PGI_2-dependent activation of adenylate cyclase in homogenates of NCB-20 cells cultured for 16 h in the absence or presence of 1 μM carbacyclin is shown in Figure 4a. This shows one of three similar experiments. In homogenates of control cells, the K_{act} value was 64.1 ± 1.6 nM compared with 174.0 ± 9.2 nM in homogenates of carbacyclin treated cells ($P<0.001$). Further, the maximum increase in adenylate cyclase activity produced by PGI_2 was greater ($P<0.001$) in homogenates of control cells (41.2 ± 1.0 pmol cyclic AMP min^{-1} mg^{-1} protein) than homogenates of carbacyclin-treated cells (15.1 ± 2.6 pmol cyclic AMP min^{-1} mg^{-1} protein). The correlation coefficients for the data presented in Figure 4a were 0.99 and 0.89 for control and desensitized cell homogenates respectively, showing that both control and desensitized PGI_2 receptors are single affinity populations, although their K_{act} values differ widely.

The binding of [^{3}H]-PGI_2 was also compared in homogenates of control and desensitized NCB-20 cells (Figure 4b). The equilibrium dissociation constant of [^{3}H]-PGI_2 binding to control membranes (K_d = 18.1 nM) was smaller than that to membranes of carbacyclin-treated cells (K_d = 50.0 nM), suggesting that a decrease in affinity accompanied desensitization. The correlation coefficients (r) for control and desensitized cell membranes were 0.98 and 0.67 respectively. There was, in addition, a reduction in the maximum binding capacity of the membranes from carbacyclin-treated cells (542.5 fmol mg^{-1}

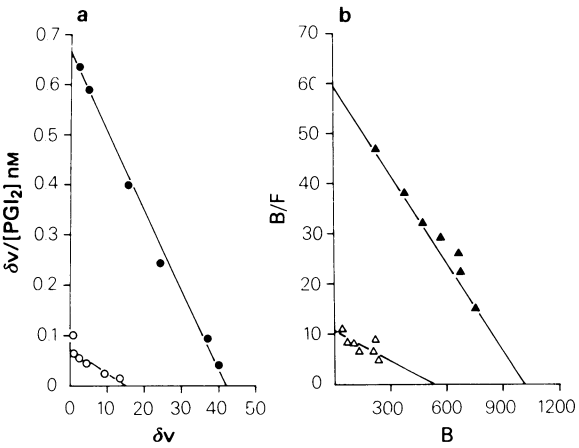

Figure 4 Prostacyclin responsiveness and [^{3}H]-prostacyclin binding to cell homogenates of control and carbacyclin-desensitized NCB-20 cells. The results in (a) are typical of 3 similar experiments. Adenylate cyclase activity was determined in homogenates of cells cultured in the absence (●) or presence (○) of 1 μM carbacyclin for 16 h. All cells were cultured in 75 cm^2 flasks which contained 0.05% ethanol. Points show means of triplicate determinations of enzyme activity in the presence of increasing prostacyclin concentrations, and are presented as an Eadie-Hofstee plot. The results in (b) show Scatchard plots for the binding of [^{3}H]-PGI_2 to membranes of cells cultured in the absence (▲) or presence (△) of 1 μM carbacyclin for 16 h. Membranes were prepared in each case from cells pooled from three 75 cm^2 flasks. All cells were cultured in the presence of 0.05% ethanol, and data points show means of triplicate determinations. The units of the bound ligand (B) are fmol [^{3}H]-PGI_2 bound mg^{-1} protein, and of the free ligand concentration (F) are nM.

protein) when compared with controls (1076.0 fmol mg^{-1} protein).

A comparison was also made of the carbacyclin-dependent activation of adenylate cyclase in NCB-20 homogenates of control cells and cells cultured in 1 μM carbacyclin. The results were essentially the same as those presented in Figure 4a. There was a 42% reduction in receptor numbers of desensitized cells, and the K_{act} value was increased from 30.4 nM to 2.14 μM.

Discussion

Adenylate cyclase of NCB-20 cells is activated by PGI_2 or its stable analogue, carbacyclin. Carbacyclin has a lower affinity suggesting that the vinyl ether of PGI_2 facilitates high affinity binding to its membrane receptor. This confirms previous structure-activity studies (Blair *et al.*, 1980; Blair & MacDermot, 1981; MacDermot *et al.*, 1981b). Both PGI_2 and carbacyclin bind to a single population of receptors, and the Eadie-Hofstee and Hill analyses of the adenylate cyclase activation suggest a simple non-cooperative, bimolecular coupling of ligand and receptor. Such a model is supported by binding studies of [^{3}H]-PGI_2 to NCB-20 membranes (Blair & MacDermot, 1981). This cell line differs from other PGI_2-sensitive tissues, such as platelets (Siegl, Smith, Silver, Nicolaou & Ahern, 1979) or pulmonary blood vessels (MacDermot, Barnes, Waddell, Dollery & Blair, 1981) in which complex binding kinetics are observed with two or more independent binding sites. However the PGI_2-dependent activation of adenylate cyclase in platelets (Gorman, Bunting & Miller, 1977; Tateson, Moncada & Vane, 1977) and pulmonary vascular homogenates (MacDermot & Barnes, 1980) obeys simple mass-action kinetics, and is mediated by the higher affinity PGI_2 receptor in each tissue.

In the present study, carbacyclin rather than PGI_2 was used as a desensitizing agent because of its greater stability at physiological pH. Prolonged exposure of these neuronal hybrid cells to carbacyclin at a concentration (1 μM) near to that required for receptor saturation resulted in a substantial (61%) reduction in the maximum increase in adenylate cyclase activity produced by PGI_2. The half-time for this response was 5–6 h. There was no significant alteration in basal enzyme activity.

The diminished responsiveness of NCB-20 homogenates to PGI_2 was further examined by comparison of the PGI_2-dependent activation of adenylate cyclase in the absence or presence of two PGI_2 concentrations (Figure 3). The accumulation of cyclic AMP with time was linear, which justified the analysis of the PGI_2 concentration curves by Eadie-Hofstee transformations (Figure 1b and Figure 4a). There was no change in basal adenylate cyclase activity (given by the slope), but the PGI_2-responsiveness was markedly reduced (72.7% at 1 μM PGI_2, and 80.0% at 100 nM PGI_2) in desensitized cell homogenates.

Comparison of adenylate cyclase activity in homogenates of control and desensitized cells in the presence of increasing PGI_2 concentrations (Figure 4a) revealed a decrease not only in the maximum activation of the enzyme above the basal level but also in affinity. The apparent decrease in affinity (from K_{act} = 64.1 nM to 174 nM) might reflect a true alteration in the equilibrium dissociation constant of the ligand-receptor interaction. Alternatively there might be an apparent rather than a real affinity change, caused by a significant decrease in the receptor numbers in a membrane with high receptor density (Homburger, Lucas, Cantau, Barabe, Penit & Bockaert, 1980). Activation of adenylate cyclase is

related to the absolute number of occupied receptors per unit area of membrane. A decrease in total receptor numbers (with no affinity change) results in a decrease in occupied receptors at any particular ligand concentration, although the fractional occupancy remains unchanged. Thus the responsiveness of control cells to a ligand at any selected concentration is greater than that of desensitized cells, which results in an apparent affinity change due entirely to a change in receptor numbers.

This question was further investigated by measurement of [^{3}H]-PGI_2 binding to membranes of control and desensitized cells. The latter showed reduced affinity, as well as a decrease in receptor numbers (Figure 4b). The scatter of the data points for the binding of [^{3}H]-PGI_2 to membranes from desensitized cells was great when compared to the binding to control membranes, and was due to a significant reduction in specific binding with no change in the non-specific binding. Despite this, the data suggest strongly that there is a real reduction in the affinity of the desensitized PGI_2 receptors for their ligand.

These results are presented as an investigation of the pharmacological events that initiate desensitization of PGI_2 receptors. Diminished responsiveness to PGI_2 has been described previously in platelets after prolonged *in vivo* administration of PGI_2 (Sinzinger, Silberbauer, Horsch & Gall, 1981). Other prostaglandin-dependent responses may in some circumstances be desensitized. Platelet adenylate cyclase activity is increased by PGD_2, and *in vitro* incubation of platelets with PGD_2 resulted in a 45% decrease in PGD_2-dependent activation of enzyme activity after 2 h (Cooper, Schafer, Puchalsky & Handin, 1979). Under similar conditions, the PGE_1-dependent activation of adenylate cyclase is also decreased by prior incubation with PGD_2, with no change in basal adenylate cyclase activity. Other prostaglandin-sensitive tissues demonstrate diminished or desensitized responses after prolonged agonist exposure, including PGE_2 responsiveness of guinea-pig macrophages (Remold-O'Donnell, 1974), human astrocytoma cells (Su, Cubedda & Perkins, 1976) and human synoviocytes (Newcombe, Ciosek, Ishikawa & Fahey, 1975). Desensitized PGE_1 responses have been demonstrated in frog erythrocytes (Lefkowitz, Mullikin, Wood, Gore & Mukherjee, 1977), neuroblastoma × glioma hybrid cells (Kenimer & Nirenberg, 1981) and human synoviocytes (Newcombe *et al.*, 1975).

In conclusion, diminished PGI_2-dependent activation of adenylate cyclase has been demonstrated in homogenates of NCB-20 hybrid cells cultured for 16 h in 1 μM carbacyclin. The half-time for the reduction in the PGI_2 responsiveness was 5 to 6 h. Desensitization was accompanied by an increase in the concentration of PGI_2 required for half-maximum adenylate cyclase activation (K_{act}), and also a decrease in the maximum enzyme activation. The binding of [^{3}H]-PGI_2 to washed membranes of desensitized and control cells revealed a decrease in the PGI_2 receptor numbers, and a decrease in affinity of the PGI_2-receptor interaction. The results suggest that the increase in the K_{act} value for PGI_2 observed in the desensitized cells cannot be attributed solely to a reduction in the total number of receptors, but rather reflects a true affinity change in the remaining receptor population.

This work was supported by the Wellcome Trust.

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(Received February 24, 1982.
Revised April 8, 1982.)