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PROSTACYCLIN-DEPENDENT ACTIVATION OF ADENYLATE CYCLASE IN A NEURONAL SOMATIC CELL HYBRID: PROSTANOID STRUCTURE-ACTIVITY RELATIONSHIPS

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1 Prostacyclin activates adenylate cyclase of the NCB-20 neuronal hybrid cell line.

2 There is a guanosine 5'-triphosphate requirement for the activation of adenylate cyclase by 5,6β-dihydroprostacyclin (a stable analogue of prostacyclin).

3 Steady-state kinetic analysis of the activation of adenylate cyclase by 5,6β-dihydroprostacyclin suggests a simple non-cooperative bimolecular interaction between the ligand and a single receptor population.

4 Structure-activity relationships of selected prostanoids elucidated certain functional requirements for activation of adenylate cyclase.

Introduction

Prostaglandin E_1 (PGE_1) increases adenylate cyclase (ATP: pyrophosphate-lyase (cyclizing); EC 4.6.1.1) activity in rat brain homogenates (Abdulla & McFarlane, 1972; Collier & Roy, 1974; Duffy & Powell, 1975), many neuroblastoma (Brunton, Wiklund, Van Arsdale & Gilman, 1976) and two neuronal hybrid cell lines (Sharma, Nirenberg & Klee, 1975; MacDermot, Higashida, Wilson, Matsuzawa, Minna & Nirenberg, 1979). A regulatory function of E-prostaglandins at nerve terminals has been proposed (Hedqvist, 1977), with inhibition of noradrenaline release from peripheral autonomic nerves.

Prostacyclin (PGI_2), a relatively unstable derivative of prostaglandin endoperoxides, has been identified (Moncada, Gryglewski, Bunting & Vane, 1976), and shown to be 30 to 40 times more potent than PGE_1 as an activator of adenylate cyclase and inhibitor of platelet aggregation (Gorman, Bunting & Miller, 1977; Tateson, Moncada & Vane, 1977). It has been proposed that the functionally important prostanoid mediating inhibition of platelet aggregation is prostacyclin (Moncada & Vane, 1979). A postsynaptic prostacyclin-dependent inhibition of noradrenergic responses has recently been demonstrated in rabbit kidney (Hedqvist, 1979), although, at present, it is not clear whether the presynaptic E-prostaglandin or postsynaptic PGI_2 response has the greater physiological role as regulator of noradrenergic transmission.

The regulation of adenylate cyclase activity by PGI_2 was examined in a neuronal cell line to establish whether prostacyclin is an activator of neuronal as well as platelet adenylate cyclase. A cloned neuronal cell line was chosen for these studies to eliminate the possibility of contamination with vascular, haemopoietic or other prostacyclin-sensitive tissues, which might predictably occur with extracts of mammalian brain. The NCB-20 mouse neuroblastoma × brain of foetal Chinese hamster somatic cell hybrid (Minna, Yavelow & Coon, 1975) was selected as both parent cells were of neuronal origin and the hybrid expresses numerous functions of differentiated nerve cells (MacDermot *et al.*, 1979). The NCB-20 hybrid cells synthesize and release acetylcholine. They are electrically excitable, and form stable synapses with mouse myotubes when grown in co-culture.

Structure-activity relationships for numerous prostanoids have been examined (Nicolaou, Barnette & Magolda, 1979). We have extended these observations to include a comparison of the relative potencies of selected prostanoids in this system. Some of the functionally critical groups required for activation of prostacyclin receptors, which mediate activation of adenylate cyclase, have been established.

Methods

Cell culture

The NCB-20 hybrid cell line (Minna *et al.*, 1975) was derived by Sendai virus-induced fusion of the N18TG2 mouse neuroblastoma clone (Minna, Glazer & Nirenberg, 1972), resistant to 6-thioguanine, and

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brain cells of foetal Chinese hamster (18 days *in utero*). The cells were cultured and homogenates prepared for assay of adenylate cyclase activity as described previously (MacDermot *et al.*, 1979). A washed particulate preparation of these cells was prepared (MacDermot, 1979) for experiments in which the guanosine 5'-triphosphate (GTP)-requirement for activation of adenylate cyclase by prostacyclin was demonstrated.

Adenylate cyclase assay

Enzyme activity was determined by a modification (Sharma *et al.*, 1975) of method C of Salomon, Londos & Rodbell (1974). Each 100 μl reaction mixture contained 50 mM Tris-HCl pH 7.4; 5 mM magnesium chloride; 87 mM sucrose; 20 mM creatine phosphate, disodium salt (Sigma); 10 International units creatine kinase, 150 iu/mg protein (ATP: creatine *N*-phosphotransferase, EC 2.7.3.2) from Sigma; 1 mM cyclic adenosine 3'-5'-monophosphate (cyclic AMP), sodium salt (Sigma); 0.25 mM Ro20-1724 (a phosphodiesterase inhibitor, Roche Products Ltd); 0.5% ethanol; 1 mM [α^{32}P]ATP (3 μCi, Radiochemical Centre, Amersham; 1 Ci = 3.7×10^{10} Bq); and 100 to 200 μg of homogenate protein. Homogenates were stored at −80°C. They were thawed and maintained at 4°C in an ice bath for no longer than 10 min before incubation. Reaction mixtures were incubated for 12 min at 37°C unless otherwise stated. The production of [^{32}P]-cyclic AMP was proportional to protein concentration within the range 50 to 250 μg of homogenate protein per reaction mixture; similarly [^{32}P]-cyclic AMP synthesis increased linearly for 30 min.

Modified adenylate cyclase assay

The activation of adenylate cyclase by PGI_2 was determined using Tris-HCl buffer at pH 8.5, in order to minimize the spontaneous hydrolysis of PGI_2. In addition the reaction time was reduced to 9 min and performed at 30°C.

15(S)hydroxyprostaglandin dehydrogenase (EC 1.1.1.141) and Δ13 prostaglandin reductase (EC unclassified) assays

Homogenates of NCB-20 hybrid cells were assayed for these enzyme activities under the conditions of the adenylate cyclase assay, by previously described methods (Hensby, 1975).

The following generous gifts were received: Ro20-1724 from Roche Products Ltd; 6β-PGI_1 and PGI_2 from Wellcome Research Laboratories; other prostanoids from Upjohn Co.; the NCB-20 hybrid cell line from Dr Marshall Nirenberg, National Institutes of Health (U.S.A.).

Guanosine 5'-triphosphate (GTP) was obtained from the Sigma Chemical Company, London, Ltd.

Results

The activation of adenylate cyclase by PGI_2, PGE_1 and 6β-PGI_1 (5,6β-dihydroprostacyclin) in whole homogenates of NCB-20 hybrid cells is shown in Figure 1a. The concentrations for half-maximum activation (K_{act}) were 18 nM, 290 nM and 4.3 μM respectively. The results for the three prostanoids may not be identical with respect to the maximum levels of enzyme activity, since activation of adenylate cyclase by PGI_2 was determined at pH 8.5 and 30°C, rather than pH 7.4 and 37°C. In each case, the Hill plot is shown in Figure 1b, from which the interaction coefficients (n) were found to be 1.0, suggesting independent, non-cooperative interactions between the prostanoids and their receptor molecules.

PGI_2 is hydrolysed to 6-oxo-$PGF_{1\alpha}$, and the latter was found to have no stimulatory effect on adenylate cyclase activity at concentrations up to 10 μM.

The stability of 6β-PGI_1 (Johnson, Lincoln, Thompson, Nidy, Mizsak & Axen, 1977; Whittle, Boughton-Smith, Moncada & Vane, 1978) allows for steady-state kinetic analysis that would not be possible for the PGI_2-dependent activation of adenylate cyclase. Enzyme activity was found to be stable for 20 min in the absence or presence of two sub-saturating 6β-PGI_1 concentrations (1 μM and 10 μM), as shown in Figure 2a. Further evidence for the stability of 6β-PGI_1 under the assay conditions was obtained by examination of NCB-20 cell homogenates for either of the enzymes likely to be involved with its metabolism, namely 15(S)hydroxyprostaglandin dehydrogenase and Δ13 prostaglandin reductase. Neither enzyme activity was found with [11β^{3}H]-$PGF_{2\alpha}$ as substrate. The [11β^{3}H]-$PGF_{2\alpha}$ was synthesized (Hensby, unpublished results) from PGD_2 by reduction with NaB^3H_4 (Radiochemical Centre, Amersham).

The saturable increase in adenylate cyclase activity with increasing 6β-PGI_1 concentrations is shown in Figure 2b, and the linear Eadie-Hofstee plot (inset) suggests a simple bimolecular interaction between 6β-PGI_1 and a single receptor population.

The synthesis of [^{32}P]-cyclic AMP as a function of time in the absence or presence of 10 μM GTP is shown in Figure 3. In the absence of GTP, 6β-PGI_1 produced little activation of adenylate cyclase, whereas in the presence of GTP, a much greater enzyme activation was observed. The concentration-dependent requirement for GTP in the activation of adenylate cyclase by 6β-$PG1_1$ is shown in Figure 4. The concentration of GTP producing half-maximum

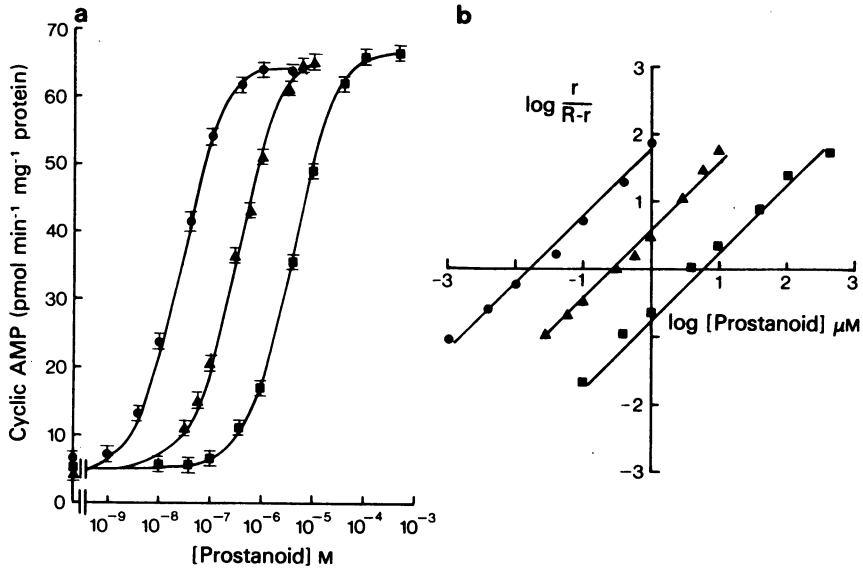

Figure 1 Prostanoid-dependent activation of adenylate cyclase in a whole homogenate of NCB-20 cells. Results in (a) show the means (vertical lines indicate s.e. means) of triplicate determinations of adenylate cyclase activity in the presence of increasing concentration of prostacyclin (PGI_2, ●), prostaglandin E_1, (PGE_1, ▲) and 6β-PGI_1(■). The same data are presented in (b) as Hill plots, where r is the increase in enzyme activity above basal levels at each concentration of prostanoid, and R is the maximum activation produced by each prostanoid.

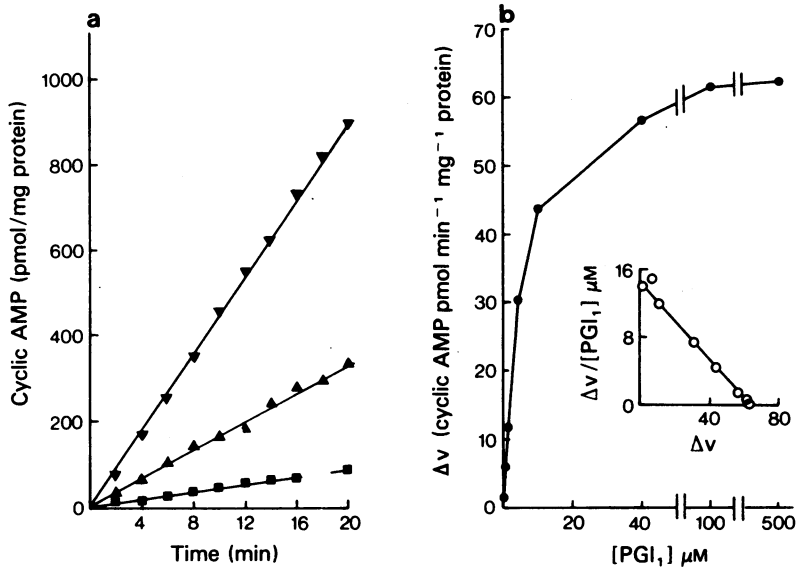

Figure 2 Activation of adenylate cyclase by 6β-PGI_1 in a whole homogenate of NCB-20 cells. Results in (a) show the cyclic AMP formed/mg protein as a function of time in the absence (■) or presence of 1.0 μM (▲) or 10 μM (▼) 6β-PGI_1. Results in (b) were taken from Figure 1a and show the increase in adenylate cyclase activity above the basal level (5.2 pmol cyclic AMP min^{-1} mg^{-1} protein) at several concentrations of 6β-PGI_1. The inset shows an Eadie-Hofstee plot of the same data, where Δv is the increase in enzyme activity at any particular 6β-PGI_1 concentration.

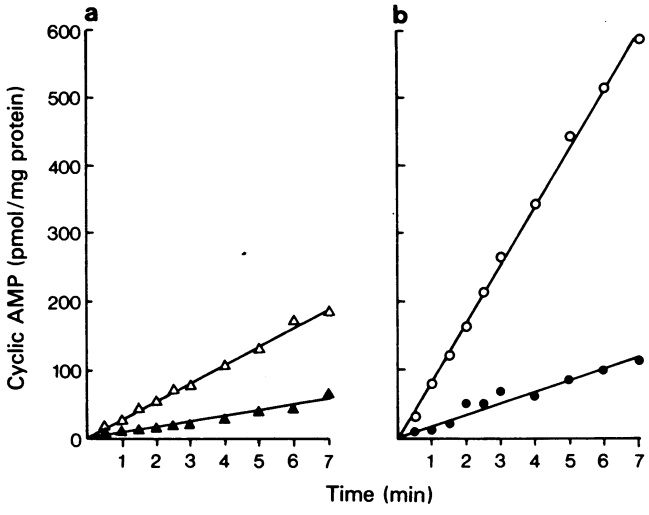

Figure 3 Guanosine 5'-triphosphate (GTP) requirement for activation of adenylate cyclase by 6β-PGI_1 in a washed particulate preparation of NCB-20 cells. Results show the accumulation of cyclic AMP/mg protein with time in the absence (a) or presence (b) of 10 μM GTP. Assays were performed in each case in the absence (▲●) or presence (△ ○) of 10 μM 6β-PGI_1.

activation of adenylate cyclase in the presence of 10 μM 6β-PGI_1 was 0.3 μM.

From Figures 3 and 4, it is apparent that there is a small, but significant 6β-PGI_1-dependent activation of adenylate cyclase in the absence of GTP. This effect was not eliminated by repeated washing of the particulate fraction of the NCB-20 hybrid cells, and is believed to be due to a significant contamination with GTP of the ATP used as substrate in the adenylate cyclase assay. An impurity of 1 part in 10,000 would explain these results.

A comparison of the activation of adenylate cyclase in whole homogenates of NCB-20 hybrid cells by numerous prostanoids is shown in Table 1. The maximum increases in enzyme activity produced by saturating concentrations of each prostanoid tested were similar. However, saturating concentrations of prostanoids which activate adenylate cyclase at very high concentrations ($K_{act} > 10$ μM) were not always obtained due to the limitation of their solubilities in 0.5% ethanol. The potencies (K_{act} values) and maximum responses obtained for the different compounds were determined from Eadie-Hofstee plots.

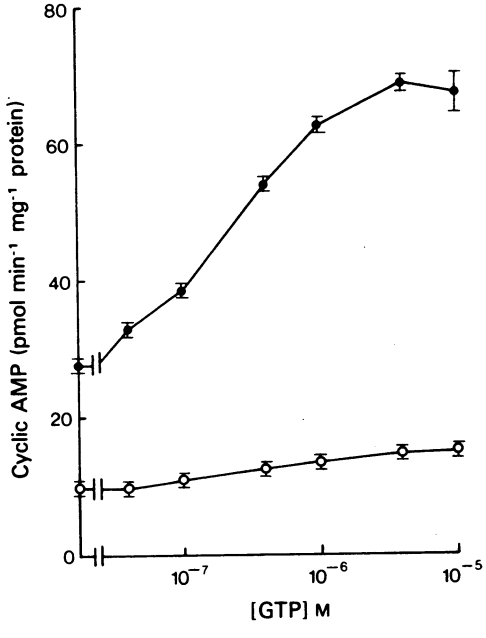

Figure 4 Guanosine 5'-triphosphate (GTP) requirement for activation of adenylate cyclase by 6β-PGI_1 in a washed particulate fraction of NCB-20 cells. Results show the means (vertical lines indicate s.e. means) of triplicate determinations of adenylate cyclase activity at increasing GTP concentrations, in the absence (○) or presence (●) of 10 μM 6β-PGI_1.

Discussion

Maximum activation of receptors with specificity for PGI_2 results in a significant (>10 fold) activation of adenylate cyclase. The results suggest a similar activation of enzyme activity by 6β-PGI_1 and the E-pros-

Table 1 Relative potencies of prostanoids mediating activation of adenylate cyclase of NCB-20 hybrid cells

Prostanoid	K_{act} (nM)	*Equipotent molar ratio*
PGI_2	24.6	1
PGE_1	415	17
13,14-dihydro-PGE_1	668	27
8-*iso*-PGE_1	2200	89
5,6-*trans*-PGE_2	2590	105
6β-PGI_1	5000	203
PGE_2	31,500	1280
13,14-dihydro-PGE_2	60,000	2440
15-oxo-PGE_2	>100,000	>4000
15-oxo-13,14-dihydro-PGE_2	>100,000	>4000
$PGF_{1\alpha}$	>100,000	>4000
6-oxo-$PGF_{1\alpha}$	>100,000	>4000
6,15-dioxo-$PGF_{1\alpha}$	>100,000	>4000
6.15-dioxo-13,14-dihydro-$PGF_{1\alpha}$	>100,000	>4000
$PGF_{1\beta}$	>100,000	>4000
$PGF_{2\alpha}$	>100,000	>4000
PGD_1	>100,000	>4000

Results show the concentrations of selected prostanoids that were required to produce half-maximum activation (K_{act}) of adenylate cyclase in NCB-20 cell homogenates. Results were obtained from Eadie-Hofstee plots. The K_{act} values of each prostanoid are compared with that of prostacyclin (PGI_2). K_{act} values for PGI_2, PGE_1 and 6β-PGI_1 were determined from different experiments from those presented in Figure 1.

taglandins, although the K_{act} values differed greatly. The stable hydrolysis product of PGI_2 is 6-oxo-$PGF_{1\alpha}$ (Johnson, Morton, Kinner, Gorman, McGuire, Sun, Whittaker, Bunting, Salmon, Moncada & Vane, 1976) and this produced little or no activation of adenylate cyclase. Similar negative results have been obtained when 6-oxo-$PGF_{1\alpha}$ was tested as an inhibitor of platelet aggregation (Tateson *et al.*, 1977), gastric acid secretion (Whittle *et al.*, 1978) and noradrenergic transmission (Hedqvist, 1979).

Recent data (Hedqvist, 1979) have suggested that inhibition of peripheral noradrenergic effects by PGI_2 is mediated postsynaptically. Results presented here suggest for the first time that PGI_2 may have a direct effect on neuronal function, by activation of a population of cell surface receptors which regulate cyclic AMP synthesis.

A steady-state kinetic analysis of the activation of adenylate cyclase by 6β-PGI_1, suggests that the increase in enzyme activity is mediated by an independent, non-cooperative, bimolecular interaction between 6β-PGI_1 and a single receptor population. The activation of adenylate cyclase by 6β-PGI_1 is in agreement with the findings of Lad, Welton & Rodbell (1977) and Livitzki (1977) for a receptor mediated interaction, involving the coupling of GTP to the receptor-enzyme complex. The concentration of GTP required for half-maximum activation of adenylate cyclase in the presence of a subsaturating concentration of the receptor agonist (6β-PGI_1) was within the range observed in other systems (Sabol & Nirenberg, 1979; MacDermot, 1979).

The hydrolysis of prostacyclin to 6-oxo-$PGF_{1\alpha}$ resulted in almost total loss of adenylate cyclase activity. The receptor thus appears to be sensitive to changes in the prostanoid molecule around the enol ether bridge. A further illustration of this effect is apparent with 6β-PGI_1 which is 203 times less active than prostacyclin itself. In order to determine other requirements for activation of adenylate cyclase, the more readily available E, F and D prostaglandins were studied.

Prostacyclin differs from 5,6-*trans*-PGE_2 only in the substitution at C-6 and C-9; it possesses a similar oxidation state. The spatial requirements of the Z-5,6 double bond in prostacyclin are fulfilled by a *trans* double bond in 5,6-*trans*-PGE_2, and the α side chain should then be free to take up a similar conformation. 5,6-*trans*-PGE_2 is 105 times less potent than PGI_2 as an activator of adenylate cyclase, but exhibits a two fold increase in potency compared with 6β-PGI_1. The configuration of the double bond is crucial, since the 5,6-*cis*-isomer, PGE_2, is 12 times less active than the *trans* isomer. PGE_2 was sufficiently potent as an activator of adenylate cyclase that the effect of modifications within the molecule could be established.

The 13,14-*trans*-double bond in the ω side chain does not appear to be a requirement for activity, since

13,14-dihydro PGE_2 showed only a two fold diminution of activity compared with PGE_2. The 15,S hydroxyl group is however essential, as 15-oxo-PGE_2 is inactive. A similar loss of activity was seen in 13,14-dihydro-15-oxo-PGE_2, confirming the requirement for a 15,S hydroxyl group.

Reduction of the 5,6 double bond of PGI_2 to form 6β-PGI_1 resulted in a loss of activity, which contrasted with the effect of a similar reduction of 5,6-*trans*-PGE_2 to form PGE_1. The absence of a 5,6-double bond in PGE_1 caused a six fold increase in activity, when compared with 5,6-*trans*-PGE_2. PGE_1 is only 17 times less active than prostacyclin. There are at least two possible explanations. The hybrid cells could have an additional PGE_1 receptor, or the extra degrees of freedom available in the PGE_1 molecule could allow it to take up a conformation different from that of the parent PGE_2, but more closely resembling PGI_2. It is noteworthy that PGE_1 is twelve times more active than the prostacyclin analogue 6β-PGI_1. Reduction of the ω double bond has little effect on potency, 13,14-dihydro PGE_1 being only half as active as PGE_1. A similar result to that obtained in the PGE_2 series. The configuration of the α side chain also appears to be of relatively minor importance, since 8 *iso*-PGE_1 is only five times less active than the parent PGE_1.

The relative potencies of the prostaglandins of the E series prompted an examination of those of the F series. It became apparent however that all the F-prostaglandins have low specificity for the prostacyclin receptor of the NCB-20 hybrid. Examination of the activity of PGD_1 demonstrated almost total loss of activity when compared with PGI_2, which suggests a critical requirement for the 11α-hydroxyl function.

In conclusion, receptors mediating activation of adenylate cyclase by prostacyclin are expressed in a neuronal hybrid cell line. The coupling of these receptors to the adenylate cyclase enzyme is similar to that in numerous transmitter and hormonal systems, and some of the functionally critical substitutions of prostanoic acid (the parent compound) have been identified.

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