Biochemical Pharmacology, Vol. 30, No. 15, pp. 2041–2044, 1981.
Printed in Great Britain.

0006-2952/81/152041-04 $02.00/0

COMMENTARY

PROSTACYCLIN RECEPTORS OF A NEURONAL HYBRID CELL LINE

DIVALENT CATIONS AND LIGAND–RECEPTOR COUPLING

JOHN MACDERMOT* and IAN A. BLAIR

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

and

TERRY M. CRESP

Department of Chemistry, University College London, London WC1H 0AJ, U.K.

(*Received* 24 *February* 1981)

Prostaglandins (PG's) E_2, $F_{2\alpha}$ [1] and D_2 [2] are synthesised in homogenates or slices of mammalian brain. The role of these compounds in brain has not been established, although the effects of PGE_2 on peripheral noradrenergic transmission in the autonomic nervous system suggests that these compounds might possibly have a role as modulators of synaptic events. PGE_2 inhibits transmitter release from both cortical slices [3] and peripheral nerves [4]. This subject has been reviewed extensively [5].

Prostacyclin [6] (PGI_2) is an unstable derivative of the PG endoperoxides, and is hydrolysed rapidly to 6-oxo-$PGF_{1\alpha}$. 6-Oxo-$PGF_{1\alpha}$ has been identified in cerebrospinal fluid [7], and its presence there suggests that PGI_2 is synthesised in brain, as no other biosynthetic route to 6-oxo-$PGF_{1\alpha}$ is known. The role of PGI_2 in the central nervous system is unknown. However, inhibitory effects of PGI_2 have been demonstrated on noradrenergic transmission in the autonomic nervous system [8], suggesting that PGI_2 might have a central role as a regulator of synaptic transmission.

Investigation of PG's and their putative synaptic functions in the central nervous system is impeded by contamination of tissue homogenates, brain slices and even synaptosomes by cellular debris derived from non-neuronal sources. Unlike the classical neurotransmitters, PG's are synthesised widely in most body tissues, in particular small blood vessels. Arterioles of the systemic vascular tree synthesise [6] and are sensitive to [9] many prostaglandins. Thus to determine which of the PG's in a brain homogenate are derived from any particular cell type (in this case neuronal) is a formidable undertaking. The identification and characterisation of neuronal PGI_2 receptors is subject to the same limitations. Cellular debris derived from vascular and erythropoietic sources is rich in PG receptors, and may mask putative neuronal receptors. Despite these reservations, pilot studies by our group (and many others) have shown only low levels of PGI_2-dependent activation (about 10 per cent) of adenylate cyclase in brain homogenates. This finding might readily be interpreted as due to contamination of the preparation with PGI_2-sensitive debris from blood vessels. Alternatively, these results would be consistent with expression of PGI_2 receptors in only a small proportion of central neurons.

In recent years, several highly differentiated neuronal cell lines have been identified. By a variety of simple manipulations, the genome of transformed cell lines may be altered, with subsequent expression of more highly differentiated functions. Somatic hybridisation has yielded numerous clones, the cells of which are differentiated to the point at which functionally active synapses between cells are observed in culture [10, 11]. We have recently reported studies which were performed on one such cell line (NCB-20) [12], which was derived by Sendai virus-induced fusion of the N18TG2 mouse neuroblastoma, resistant to 6-thioguanine, and brain cells of foetal Chinese hamster (18 days *in utero*). Cells of this line synthesise acetylcholine, they are electrically excitable, and transmitter release is coupled to membrane depolarisation [11]. In co-culture with myotubes derived from rat hind limb they form stable synapses [11]. Cells of this line also synthesise PGI_2, which has been identified as its stable hydrolysis product (6-oxo-$PGF_{1\alpha}$) [13]. In our pursuit of putative neuronal PGI_2 receptors, we have examined the NCB-20 cell line as a model of differentiated mammalian nerves. The system is free from the non-neuronal tissues already referred to.

PGI_2 receptors have been identified on cells of the NCB-20 hybrid [14, 15]. These receptors are coupled to adenylate cyclase [ATP pyrophosphate-lyase (cyclising); EC 4.6.1.1] and maximum receptor activation results in a 10–15 fold increase in enzyme activity [15]. Receptor-mediated activation of adenylate cyclase is not confined to PGI_2 and the concentrations for half-maximum activation (K_{act}) were 18 nM (PGI_2), 290 nM (PGE_1) and 4.3 μM (6β-PGI_1). Analysis of these results demonstrated a non-cooperative mechanism of interaction between these three PG's and their receptor molecules. Eadie–Hofstee plots of the activation of adenylate cyclase by these PG's were linear, thus identifying

* Author to whom correspondence should be addressed.

a single affinity, and probably a single receptor species for each PG.

The activation of adenylate cyclase by 6β-PGI_1 (a stable analogue of PGI_2) is dependent on the presence of guanosine 5′-triphosphate (GTP). The concentration of GTP that produced a half-maximum increase in enzyme activity at a single (10 μM) 6β-PGI_1 concentration was 0.3 μM.

This approximates closely to the concentrations of GTP required for the same process in several neurotransmitter systems [16, 17]. The requirement for GTP in the 6β-PGI_1-dependent activation of adenylate cyclase suggests a receptor–enzyme coupling mechanism similar to that observed in other hormonal [18, 19] and neurotransmitter systems [16, 17].

Cell surface PGI_2 receptors may be identified by radio-ligand binding, and we have synthesised 11β-^{3}H–PGI_2 [20] to delineate the binding characteristics. The binding assay was based on a modification [21] of the method of Pert and Snyder [22]. Specific binding of ^{3}H–PGI_2 was defined as that displaced by 10 μM PGI_2 or 70 μM PGE_1, as both ligands have been shown to bind competitively to a single receptor species in these cells [14]. Specific binding to a washed membrane preparation of NCB-20 cells was saturable and revealed a single affinity species. The equilibrium dissociation constant (K_d) was 16.6 nM. The maximum binding capacity was 1280 fmole ^{3}H–PGI_2/mg membrane protein, which corresponded to a receptor density of 2.57×10^5 PGI_2 receptors on each cell. Analysis of the binding kinetics of ^{3}H–PGI_2 at 20° revealed a second order rate constant (k_{+1}) of $2.26 \times 10^5\ M^{-1}\ sec^{-1}$ for the forward reaction. Dissociation of the ligand–receptor complex obeyed 1st order kinetics, and the rate constant (k_{-1}) was $3.85 \times 10^{-3}\ sec^{-1}$. The true dissociation constant (k_{-1}/k_{+1}) was calculated to be 17 nM.

The maximum increase in adenylate cyclase activity produced by PGI_2 and PGE_1 was similar which suggested that the responses of these PG's might be mediated by a single receptor population. This was confirmed by the demonstration that PGE_1 inhibits the binding of ^{3}H–PGI_2 competitively. The inhibitory effects of other PG's on ^{3}H–PGI_2 binding revealed a rank order of potency similar to that observed in a comparison of the same PG's as activators of the NCB-20 adenylate cyclase (Table 1). For each PG, the K_i value for inhibition of ^{3}H–PGI_2 binding was less than the K_{act} value for activation of adenylate cyclase. The reduced affinity of the ligand for its receptor under the conditions of the adenylate cyclase assay may be explained by the presence of GTP. Coupling of the GTP-binding protein (N) with the receptor results in a reduction in ligand–receptor affinity if the N-protein is itself coupled to GTP [23].

The detailed relationships of PG structure and affinity for the PGI_2 receptor on these cells have been discussed extensively elsewhere [14, 15], and only those of relevance to the present discussion are highlighted. The vinyl ether moiety of PGI_2 is critical, and hydrolysis results in total loss of activity (cf. 6-oxo-$PGF_{1\alpha}$). Reduction of the 5,6-double bond of PGI_2 to form 6β-PGI_1 results in significant loss of activity. Additional structure–activity relationships of PG's were determined on the readily available E, F and D-series PG's, rather than on the preferable (but unavailable) prostacylcin analogues. The affinity of 5,6-*trans*-PGE_2 was greater than that of the *cis* isomer. This may be explained as follows. The Z-configuration of the 5,6-double bond of PGI_2 more closely resembles the *trans* configuration of 5,6-*trans*-PGE_2 than the *cis* configuration of the natural isomer (PGE_2).

One interesting anomaly is that reduction of the 5,6-*trans* double bond of 5,6-*trans*-PGE_2 to form PGE_1 increased significantly the binding to the membrane receptor. The finding that PGE_1 and PGI_2 bind competitively to the same receptor suggests that the anomalous affinities ($PGI_2 > 6\beta$-PGI_1 whereas 5,6-*trans*-$PGE_2 < PGE_1$) are not explained by the presence of multiple receptor types. It has been proposed [15] that the α-side chain of PGE_1 takes up a conformation that is different from PGE_2 and more closely resembles PGI_2.

Table 1. The relative potencies of PG's that inhibit binding of ^{3}H–PGI_2 to NCB-20 membrane receptors, and activate adenylate cyclase*

Prostaglandin	^{3}H–PGI_2 binding K_i (nM)	Adenylate cyclase activation K_{act} (nM)
PGI_2	16.6	24.6
PGE_1	137	415
13,14-dihydro-PGE_1	315	668
8-*iso*-PGE_1	735	2200
5,6-*trans*-PGE_2	735	2590
17-phenyl-PGE_2	4370	—
6β-PGI_1	7350	5000
13,14-dihydro-PGE_2	8410	60,000
PGE_2	13,370	31,500
13,14-dihydro-15-oxo-PGE_2	>100,000	>100,000
15-*epi*-PGE_2	>100,000	—
$PGF_{1\alpha}$	>100,000	>100,000
6-oxo-$PGF_{1\alpha}$	>100,000	>100,000
$PGF_{2\alpha}$	>100,000	>100,000
PGD_1	>100,000	>100,000

* Data from Refs [14, 15].

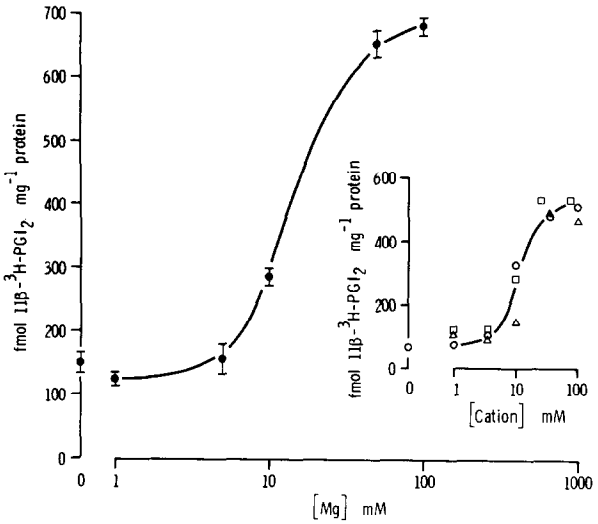

Fig. 1. The binding of ^{3}H–PGI$_2$ to membranes of NCB-20 somatic hybrid cells. Results show the Mg^{2+}-dependent increase in specific ^{3}H–PGI$_2$ binding in the presence of 10 mM EDTA. Data points are the means (± S.E.M.) of triplicate determinations of binding in the presence of 30 nM ^{3}H–PGI$_2$. The inset shows a similar increase in ^{3}H–PGI$_2$ binding in the presence of Ca^{2+} (○), Ba^{2+} (□) or Sr^{2+} (△) ions (data extracted from Ref. 24).

The 15-hydroxyl group is an absolute requirement for high affinity binding (cf. PGE_2 and 15-oxo-PGE_2; 13,14-dihydro-PGE_2 and 15-oxo-13,14-dihydro-PGE_2). Furthermore the absolute configuration at C15 is critical, as the activity of the natural isomer (15S-OH) is lost in 15-*epi*-PGE_2 (15R-OH).

Divalent cations regulate the binding of PGI_2 to its membrane receptor. Magnesium ions increase the binding of ^{3}H–PGI_2 (Fig. 1) [24]. In the absence of added Mg^{2+} ions and in the presence of 10 mM EDTA, there was a low but detectable level of ^{3}H–PGI_2 binding. Ba^{2+}, Ca^{2+} or Sr^{2+} ions resulted in similar increases in ^{3}H–PGI_2 binding (Fig. 1, inset). A comparison of two concentration curves of ^{3}H–PGI_2 in the presence of 1 or 50 mM $MgSO_4$ revealed that the Mg^{2+}-dependent increase in ^{3}H–PGI_2 binding is produced by an increase in the affinity of the ligand-receptor interaction (K_d values of 57.4 and 21.9 nM), with no accompanying change in the maximum binding capacity.

This result compares with similar Mg^{2+}-dependent increases in ligand affinity of α- [25–27] and β-adrenergic agonists [28, 29]. The mechanism of the affinity change remains obscure, but suggested sites of action of the divalent cation are on the complex of the receptor with the GTP binding protein (*N*) [29], or on the adenylate cyclase molecule [28]. We have investigated an alternative mechanism, namely a change in conformation of the ligand induced by an interaction with the cation. These experiments were prompted by the lack of specificity for any particular divalent cation in the increase in ^{3}H–PGI_2 binding, which suggested that the Mg^{2+}-dependent change in ligand affinity was probably not initiated by binding of the cation to a specific receptor site on one or other of the molecules involved in receptor-mediated activation of adenylate cyclase.

Proton magnetic resonance spectroscopy has been used extensively for monitoring conformational changes in complex molecules. We have employed this technique to determine whether Mg^{2+} can induce conformational changes in PGI_2. Deuteromethanol was used as solvent. Like the lipid bilayer it is more hydrophobic than water but still maintains magnesium salts in solution. The ^{1}H-n.m.r. spectrum (200 MHz) of PGI_2 in deuteromethanol was identical to that described previously [30]. A portion of this spectrum is shown (Fig. 2) in the presence of a 50-fold excess of anhydrous $MgCl_2$ (A), or in the absence of $MgCl_2$ (B). The spectrum shows resonances that have been assigned previously to protons (H) at carbon atoms C_5, C_9, C_{11} and C_{15}. An upfield shift of H_5 and downfield shift of H_{15} was observed in the presence of Mg^{2+} ions, with no corresponding shifts of H_9 or H_{11}. These results are consistent with formation of a complex between Mg^{2+} and the PGI_2 molecule. Changes in the chemical shifts of protons remote from C_1 (H_5 and H_{15}) suggest that the conformation of PGI_2 is changed by this interaction. No Mg^{2+}-dependent changes in the ^{1}H-n.m.r. spectrum were observed in an aqueous (D_2O) solvent.

Some of the structural requirements of PGI_2 for high affinity occupation of its receptor have been discussed previously, and the significance of the 5,6-double bond and the 15S-OH group of PGI_2 were highlighted. Clearly the cation-dependent shifts in the ^{1}H–n.m.r. spectrum occurred at sites that are critical for high affinity binding, and we propose that

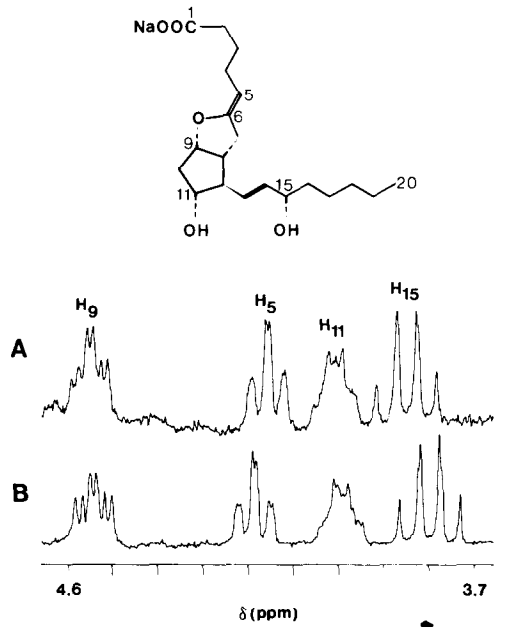

Fig. 2. ^{1}H–n.m.r. spectrum of PGI_2 (Na salt). The spectra were obtained from a solution of PGI_2 (5.3 mM) in D_4-methanol. The spectra were obtained on a Varian XL200 spectrometer at 200 MHz, with the temperature maintained at 22°. Chemical shifts are relative to the internal standard tetramethylsilane ($\delta = 0$). Results were obtained in the presence (A) of 300 mM $MgCl_2$ or absence (B) of $MgCl_2$.

PGI_2 may exist in two (or more) conformational states with different K_d values in the ligand–receptor interaction. The functionally critical substituents, the vinyl ether moiety and the 15S-hydroxyl group, are adjacent to protons that undergo Mg^{2+}-dependent changes in their electronic environment. We suggest that these substituents allow formation of a complex between the divalent cation and prostacyclin, and that the altered geometry of PGI_2 satisfies the geometric constraints of the PGI_2 receptor.

This model provides an explanation for altered receptor sensitivity that occurs in the hydrophobic environment of the lipid bilayer in the presence of magnesium. An intriguing possibility may be considered, namely that divalent cations, coupled to an agonist, might have an additional role within the receptor site to initiate the evoked biological response.

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