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DIVALENT CATIONS INCREASE [^{3}H]-PROSTACYCLIN BINDING TO MEMBRANES OF NEURONAL SOMATIC HYBRID CELLS

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1 [^{3}H]-prostacyclin binding to membranes of a highly differentiated neuronal somatic hybrid is increased equally in the presence of Ca^{2+}, Ba^{2+}, Mg^{2+} or Sr^{2+} ions.

2 Analysis of the binding of [^{3}H]-prostacyclin in the presence of low (1 mM) or high (50 mM) Mg^{2+} ion concentrations has revealed a cation-dependent increase in ligand receptor affinity (K_d' values = 57.4 nM and 21.9 nM).

3 The increase in [^{3}H]-prostacyclin binding due to divalent cations is not accompanied by an alteration in the maximum binding capacity of the membranes.

Introduction

Prostacyclin (PGI_2) is derived from the prostaglandin endoperoxides (Moncada, Gryglewski, Bunting & Vane, 1976). Neuronal PGI_2 receptors have been identified (Blair, Hensby & MacDermot, 1980) on the highly differentiated NCB-20 somatic hybrid (Minna, Yavelow & Coon, 1975), which is derived from a mouse neuroblastoma and brain of foetal Chinese hamster. Cells of this line synthesize acetylcholine, they are electrically excitable, and are competent to form stable, physiologically functional synapses with myotubes in co-culture (MacDermot, Higashida, Wilson, Matsuzawa, Minna & Nirenberg, 1979). PGI_2 receptors of this cell line are coupled with adenylate cyclase [ATP pyrophosphate-lyase (cyclising); EC 4.6.1.1], and increase enzyme activity about 15 fold (Blair *et al.*, 1980). The activation of adenylate cyclase by PGI_2 is dependent on the presence of guanosine 5′-triphosphate (GTP) (Blair *et al.*, 1980), which suggests that the receptor–enzyme coupling mechanism is similar to that observed in other neurotransmitter (Sabol & Nirenberg, 1979; MacDermot, 1979) and hormonal systems (Lad, Welton & Rodbell, 1977; Levitzki, 1977).

Neuronal PGI_2 receptors of the NCB-20 cell line have also been identified directly by the binding of [^{3}H]-PGI_2 to NCB-20 cell membranes (Blair & MacDermot, 1981). Kinetic and steady-state analyses of [^{3}H]-PGI_2 binding, and PGI_2-dependent activation of adenylate cyclase, have revealed a single PGI_2 receptor population, with a non-cooperative, bimolecular interaction between ligand and receptor. The rank order of potency of many prostaglandins was unchanged whether determined by a comparison as activators of adenylate cyclase, or inhibitors of [^{3}H]-PGI_2 binding.

In preliminary experiments to establish conditions for the binding of [^{3}H]-PGI_2 to NCB-20 membranes, a requirement for Mg^{2+} ions was identified, with the result that 10 mM magnesium sulphate was added to each incubation (Blair & MacDermot, 1981). In the present paper, results are presented which illustrate the effects of selected divalent cations on the affinity of the PGI_2-receptor interaction.

Methods

Cell culture

NCB-20 hybrid cells were cultured as described previously (MacDermot *et al.*, 1979), and binding studies performed on a washed membrane preparation (Blair & MacDermot, 1981). Whole cells from 15 flasks (75 cm^2) were homogenized at 4°C in 15 ml 25 mM Tris-HCl buffer pH 8.5, containing 0.32 M sucrose by 20 strokes of a tightly fitting Dounce homogenizer. Undisrupted cells and nuclei were pelleted by centrifugation at 500 g for 10 min at 4°C. The pellet was discarded, and the supernatant centrifuged at 100,000 g for 20 min at 4°C. The pellet was then washed three times at 4°C in 50 ml 50 mM Tris-HCl buffer, pH 8.5, by resuspension followed by centrifugation at 100,000 g for 20 min. The pellet was finally suspended in 10 ml 50 mM Tris-HCl buffer pH 8.5, divided into 1 ml aliquots, and stored at −80°C.

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

The synthesis of [11β-^{3}H]-PGI_2 (Blair, Hensby & MacDermot, 1981) yielded a radioligand with a speci-

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fic activity of 8 Ci/mmol (1 Ci = 3.7×10^{10} Bq). The binding assay was performed in incubations of 100 μl by a modification (Blair & MacDermot, 1981) of techniques described previously (Pert & Snyder, 1973; MacDermot & Nirenberg, 1978). Incubations contained 50 mM Tris-HCl pH 8.5, [^{3}H]-PGI_2 and divalent cations at selected concentrations, and 80–150 μg of membrane protein. Triplicate reaction mixtures were incubated at 20°C for 20 min, and the reaction terminated by the addition of 4 ml of 50 mM Tris-HCl buffer pH 8.5 at 4°C. The membranes were then filtered rapidly under reduced pressure through Whatman GF/B glass filter discs (24 mm diam.). The filtered membranes were then washed 3 times with 4 ml of 50 mM Tris-HCl buffer pH 8.5 at 4°C. The filtration procedure was completed within 20 s. The radioactivity on the filter disc was counted in 10 ml Insta-Gel (Packard Instrument Co. Inc.). Specific binding was defined as that displaced by 10 μM PGI_2 (unlabelled) or 70 μM PGE_1 in triplicate parallel incubations, as both ligands have been shown to compete for a single receptor species in these cells (Blair & MacDermot, 1981). Under the conditions described, the assay has been shown to be linear within the range of 50–1050 μg of membrane protein. The protein content of membrane preparations was determined by a modification of the method of Lowry, Rosebrough, Farr & Randall (1951).

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Results

The specific binding of [^{3}H]-PGI_2 (30 nM) to NCB-20 membranes in the presence of 10 mM ethylenediamine tetraacetic acid (EDTA) is shown in Figure 1a. Additions of increasing concentrations of magnesium sulphate increased bindings from 150 ± 10 fmol/mg protein (means ± s.e. means of triplicate determinations) to a maximum of 680 ± 14 fmol/mg protein, with a half-maximum increase in binding at about 16 mM $MgSO_4$. At this concentration, the Mg^{2+} ion concentration was 6 mM in excess of the EDTA concentration. Similar results to those presented for Mg^{2+} ions were obtained in other experiments with $CaCl_2$, $Ba(NO_3)_2$ or $SrCl_2$. The effects of group 1A ions on [^{3}H]-PGI_2 binding were also investigated. In the presence of 10 mM EDTA, both 100 mM Na^+ or K^+ ions (with chloride counter ion) produced only 20–25% of the increase in [^{3}H]-PGI_2 binding observed in the presence of the group 2A cations. The effect of La^{3+} ions on [^{3}H]-PGI_2 binding was investigated to elucidate further the site of action of the required cation, as calcium transport across numerous membranes is inhibited by La^{3+} ions. Addition of La^{3+} ions (with chloride counter ion) in the presence of 10 mM EDTA resulted in an increase in [^{3}H]-PGI_2 binding, similar to that produced by Mg^{2+} ions. However, responses to concentrations of La^{3+} above 10 mM were not investigated because of the limitation of its solubility under the conditions of the assay.

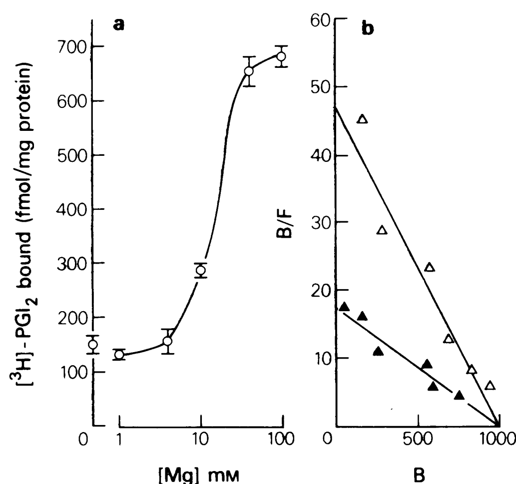

Figure 1 The binding of [^{3}H]-prostacyclin ([^{3}H]-PGI_2) to membranes of the NCB-20 somatic hybrid. Results in (a) show the Mg^{2+}-dependent increase in specific [^{3}H]-PGI_2 binding in the presence of 10 mM EDTA. Data points are the means (vertical lines indicate s.e. means) of triplicate determinations of binding in the presence of 30 nM [^{3}H]-PGI_2. The results in (b) show a Scatchard plot of two [^{3}H]-PGI_2 concentration curves (4–150 nM) in the presence of 1 mM (▲) or 50 mM (△) $MgSO_4$. Data points show the means of triplicate determinations. B is fmol [^{3}H]-PGI_2 bound per mg membrane protein and F is the free ligand concentration (nM).

The mechanism of increased PGI_2 binding in the presence of divalent cations has been investigated. A Scatchard plot of two concentration curves (4–150 nM) of specific [^{3}H]-PGI_2 binding in the presence of 1 mM or 50 mM $MgSO_4$ (no EDTA) is shown in Figure 1b. The maximum binding capacity of [^{3}H]-PGI_2 remained unchanged (1014 and 1030 fmol/mg protein in the presence of 1 mM or 50 mM $MgSO_4$ respectively). However, the affinity of [^{3}H]-PGI_2 for its membrane receptor was greater in the presence of 50 mM $MgSO_4$ (K_d = 21.9 nM) than in the presence of 1 mM $MgSO_4$ (K_d = 57.4 nM).

The possibility that these results might be explained by the chelating properties of Tris-HCl buffer (50 mM) used in the assay was considered. If [^{3}H]-PGI_2 formed a complex with the Tris base, and [^{3}H]-PGI_2 was displaced from Tris by divalent cations, a similar result to those obtained would be predicted. This possibility was excluded as follows.

Two [^{3}H]-PGI_2 concentration curves (5–100 nM) were performed in the presence of 10 mM $MgSO_4$ (no EDTA) and 5 mM or 50 mM Tris-HCl buffer. The affinity of specific [^{3}H]-PGI_2 binding in each curve (K_d = 20 nM) was identical, suggesting that the observed changes in ligand affinity with changes in the divalent cation concentration were independent of any proposed chelating properties of the Tris base.

Discussion

The binding of PGI_2 to its high affinity neuronal receptor on the NCB-20 cells is regulated by changes in cation concentration that are within or near the physiological concentration of extracellular calcium. The facilitation of [^{3}H]-PGI_2 binding by Mg^{2+} ions is mediated by an increase in ligand affinity. Similar effects were observed in the presence of Sr^{2+}, Ca^{2+}, Ba^{2+} or La^{3+}. A requirement for divalent cations in the binding of both α- (Tsai & Lefkowitz, 1978; Rouot, U'Prichard & Snyder, 1980; U'Prichard & Snyder, 1980) and β-adrenoceptor agonists (Williams, Mullikin & Lefkowitz, 1978; Bird & Maguire, 1978) has been described. There was no divalent cation requirement for binding of the corresponding antagonists, and a comparison of selected divalent cations revealed differences in their abilities to increase agonist binding. The mechanism of cation-dependent changes in ligand-receptor affinities remains obscure, although possible interaction sites on the complex of the receptor-GTP binding protein (Bird & Maguire, 1978) or on the adenylate cyclase molecule (Williams *et al.*, 1978) have been proposed. The results here show no differences among a variety of cations for their ability to increase [^{3}H]-PGI_2 binding, which suggests that the mechanism of the affinity change may be different from that of α- and β-adrenoceptor agonists.

Studies are in progress to elucidate further the mechanism of the cation-dependent increase in affinity of PGI_2 binding. The possibility has been considered that cations may alter the conformation of PGI_2 in solution with a secondary change in the affinity for the PGI_2 receptor. In preliminary experiments (unpublished results), divalent cation-dependent changes in the chemical shifts of protons adjacent to regions of the PGI_2 molecule previously identified as critical for high affinity binding (Blair & MacDermot, 1981) have been demonstrated by ^{1}H-nuclear magnetic resonance spectroscopy.

In conclusion, regulation of PGI_2 binding by divalent cations is dependent on an increase in the ligand-receptor affinity. The similarity of this response with other group 2A cations suggests that the process may not be dependent on the binding of the cation to a specific receptor on any one of the protein molecules involved in the receptor-mediated activation of adenylate cyclase. An alternative mechanism that involves complex formation between the cation and PGI_2 is currently under investigation.

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