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DIRECT EVIDENCE FOR THE PRESENCE OF SELECTIVE BINDING SITES FOR (^{3}H) PROSTAGLANDIN E_2 ON RAT PERITONEAL MACROPHAGES

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A method is presented which provides for a simple and rapid determination of PGE_2 receptors on viable peritoneal macrophages. Incubation of the harvested cells with (^{3}H)PGE_2 revealed specific binding of (^{3}H)PGE_2 by use of the Millipore filter assay system. Maximum binding was attained in the presence of 1 mM EDTA. Specific binding was saturable at 65 fmol/mg protein with an equilibrium dissociation constant (K_d) of 3.2×10^{-8}M. Inhibition of (^{3}H)PGE_2 binding with unlabelled prostaglandins revealed a potency series of $PGE_2 > PGE_2 > PGI_2$. The PGE_2 concentration which displaced 50% of the labelled ligand was 10^{-7}M. Comparable kinetic data were obtained for adenylate cyclase stimulation, since the concentration which showed a halfmaximal stimulation of cAMP production was 2×10^{-7}M of PGE_2. Since PGE_1 and PGI_2 compete with (^{3}H)PGE_2 binding in a non-parallel manner compared to PGE_2 itself, it is proposed that macrophages possess different types of PG receptors.

Prostaglandins (PGs) must bind to the cell to initiate their programmed responses. Indeed, membrane-associated receptors have been identified in several target organs or cells, specific for the various PGs (1-3). PGE_2 has been shown to bind specifically to skin membrane fractions (4) or adrenal medulla (5), and this binding correlated with adenylate cyclase activity.
In response to an inflammatory stimulus, elicited peritoneal macrophages (PMϕ) release a variety of products including PGE_2 and prostacyclin (PGI_2). Moreover, when PMϕ are exposed to PGs, they respond with elevated levels of cAMP (6). In contrast to the general stimulatory effect of PGs on adenylate cyclase, it was found that low concentrations of PGE_2 could inhibit the PGI_2-induced elevation of cAMP in elicited populations of PMϕ (7).
Although there is some evidence for a common PG receptor, including the same binding site for PGE_1 and PGI_2 in platelets (8), no specific receptor binding studies with PGs on PMϕ have been published yet. In order to investigate the

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antagonism of PGE_2 and PGI_2 in terms of stimulation of cAMP formation in PMϕ in more detail, we developed an assay for the measurement of specific binding of (^{3}H)PGE_2 to rat PMϕ and examined various assay conditions.

EXPERIMENTAL PROCEDURES

Materials: (^{3}H)PGE_2 (specific activity 160 Ci/mmol) Amersham BV. PGE_1 and PGE_2 were gifts from Dr. A.J. Vergroesen, Unilever Research Lab., Vlaardingen, The Netherlands. PGI_2-sodium salt, through courtesy of Dr. E. Schillinger, Schering A.G. Berlin, F.R.G. (±)-5E-13,14-didehydro-carbo-prostacyclin (DDH-carbo-PGI_2) from Prof. C.A. Gandolfi (Farmitalia Carlo Erba, Milan, Italy).

Isolation of macrophages and (^{3}H)PGE_2 binding studies: Elicited PMϕ were obtained as described earlier (7).

Aliquots (0.3 ml) of the cell suspension (± 1 mg protein/ml) were added to various concentrations of (^{3}H)PGE_2 dissolved in buffer, in the presence or absence of various concentrations of unlabelled PGs (total assay volume was 360 µl). Assay buffer (pH 7.8) contained NaCl (0.9%); Tris HCl (15 mM); $CaCl_2$ (0.05 mM); KCl (5 mM), $MgSO_4$ (1 mM); glucose (0.1%) and indomethacin (10^{-5}M). The assay mixtures were incubated (4°C) in a metabolic shaker. Steady state binding was reached within 90 min. The incubation was terminated by diluting triplicate samples (100 µl) to 4 ml with icecold buffer in siliconized glass tubes, followed by rapid filtration under reduced pressure through Whatman GF/A glass filters. The glass tubes and filters were then quickly washed successively with four portions of 5 ml incubation buffer. The residue on the dry filters was subsequently extracted with 200 µl Soluene 350 for 16h. Radioactivity (dpm) was determined by liquid scintillation spectrometry (counting efficiency 46%).
cAMP levels were measured by the protein binding method (9). ADP-induced platelet aggregation was measured by the turbidimetric procedure of Born (10) using a Payton aggregation module.

RESULTS

The goal of the study was to develop a methodology that provides evidence that PGE_2 receptor binding could be adequately measured and correlated with adenylate cyclase, using intact PMϕ. In a separate study we will present binding characteristic data which are further confined to high and low affinity sites using a membrane fraction of the cells (Opmeer et al., submitted for publication).

Since PGI_2 was degradated very rapidly at 37°C and virtually no loss of activity could be detected after 90 min of incubation at 4°C, as measured on the inhibiting potency of ADP-induced platelet aggregation, experiments were carried out at 4°C.

Separate tubes were incubated with 10^{-6} to 10^{-5}M unlabelled PGE_2 in addition to the labelled ligand to determine "nonspecific" binding which was substracted from experimental values to yield "specific" binding. Specific binding of

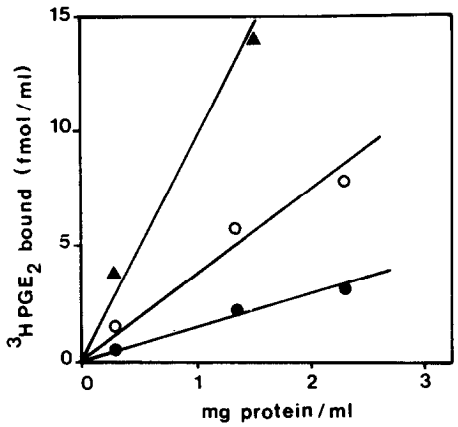

Figure 1. Linearity of (^{3}H)PGE$_2$ binding with protein. Aliquots of the macrophage suspension (protein content between 0.3 and 2.3 mg/ml) were incubated with (^{3}H)PGE$_2$ in concentrations of 1 (●); 3 (o); or 10 nM (▲) in the absence (total-binding) or presence (non-specific binding) of excess (3 μM) unlabelled PGE$_2$ as described in the text. Specific binding is presented as mean values of triplicate determinations from 3 experiments. Lines represent least squares fits of the data points.

various (^{3}H)PGE$_2$ concentrations to PMϕ showed linearity with protein concentration (Fig.1). At the used protein concentrations less than 1% of the total (^{3}H)PGE$_2$ added was bound, so that free ligand concentrations were essentially constant throughout an experiment.

Fig.2 demonstrates the specific binding of (^{3}H)PGE$_2$ to PMϕ as a function of concentration of (^{3}H)PGE$_2$. As the pattern of (^{3}H)PGE$_2$ binding to intact PMϕ

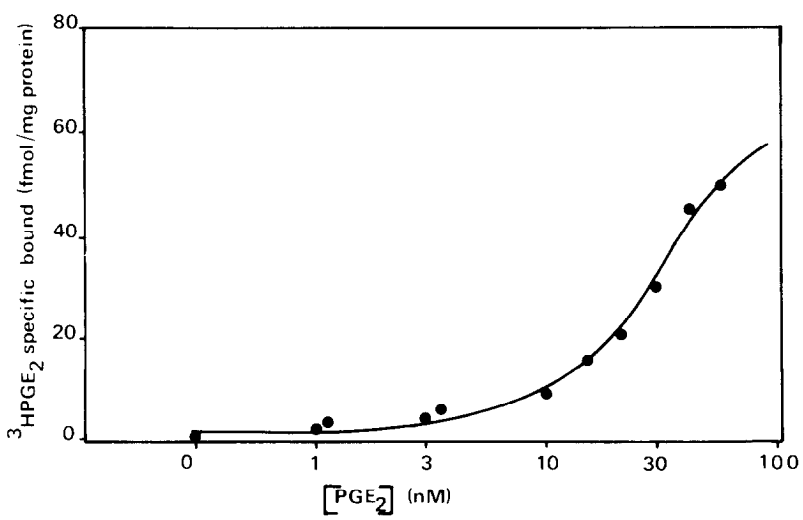

Figure 2. Concentration dependence of (^{3}H)PGE$_2$ specific binding to macrophages. Incubations were carried out as described in the text. Points represent means of 4 experiments.

Table 1

Effect of calcium and chelating agents (EDTA and EGTA) on specific binding of (^{3}H)PGE_2 to macrophages.

Free	Specific bound (^{3}H)PGE_2 (fmol/mg)				
^{3}HPGE_2 (nM)	Ca^{++} (0.05 mM)	Ca^{++} (1 mM)	Ca^{++} (2.5 mM) or EGTA (5 mM)	EDTA (1 mM)	EDTA (5 mM)
1.0	1.8 (6)	1.3 (4)	-	5.7 (4)	-
3.0	4.7 (6)	2.9 (4)	0.0 (3)	17.3 (4)	7.0(3)
10.0	14.0 (3)	-	0.0 (3)	19.9 (6)	9.4(3)

Aliquots of a peritoneal macrophage suspension were incubated (90 min; 4°C) with (^{3}H)PGE_2 at the indicated concentrations in the absence (total binding) or presence (non-specific binding) of excess (3 µM) unlabelled PGE_2. Specific binding was estimated as indicated in the text. Incubation mixtures consisted of normal buffer (containing 1 mM Mg^{++}) with the indicated concentrations of Ca^{++} or chelating agents. Average binding values of (n) determinations are given.

shows, saturation occurred at ± 65 fmol ligand bound/mg protein. At a ligand concentration of approximately 30 nM, one half of the binding sites were occupied. This value gives an estimate of the K_d of (^{3}H)PGE_2 for the binding sites. Accordingly, analysis of the binding data by the method of Lineweaver/ Burk, using 6 different concentrations of (^{3}H)PGE_2 between 0.3 and 11 nM, revealed a K_d value of 3.2×10^{-8}M.

Table 1 shows the effects of the ionic composition of the buffer (various calcium, EDTA and EGTA concentrations) at three different ligand concentrations on specific binding. Higher calcium concentrations compared to the concentration in the standard buffer (0.05 mM) reduced specific binding, while non-specific binding was not affected. EGTA (5 mM) completely abolished specific binding, while EDTA at a concentration of 1 mM increased specific binding at all ligand concentrations tested.

The selectivity of the PG binding sites of PMϕ was studied by measuring the binding of (^{3}H)PGE_2 (5 nM) to the cells in the presence of various concentrations of PGE_1, PGE_2 or PGI_2. From the results shown in Fig.3, it can be concluded that unlabelled PGE_2 was the most effective competitor for the binding of (^{3}H)PGE_2. The order of potency was $PGE_2 > PGE_1 > PGI_2$. Specific binding of PGs to PMϕ itself is not a sufficient criterion for the detection of PG receptors. Therefore, we tested the ability of PGE_2 and a stable analogue

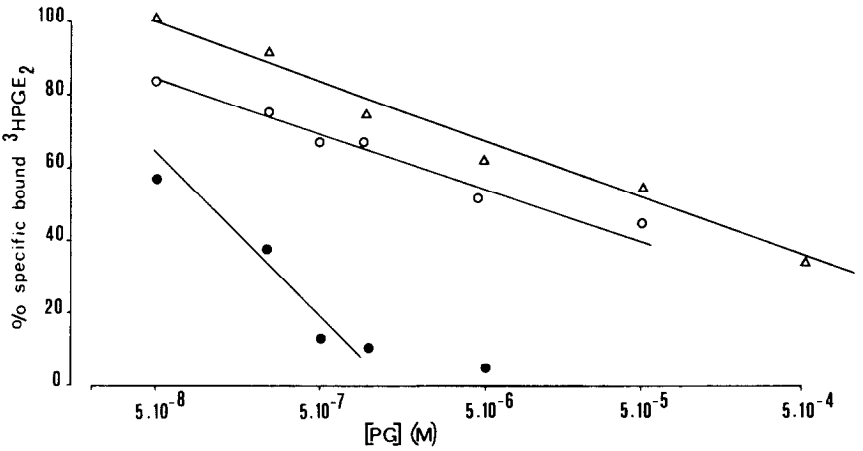

Figure 3. Specificity of (^{3}H)PGE$_2$ binding to macrophages. PGE$_2$ (•), PGE$_1$ (o) and PGI$_2$ (Δ) were included in the binding reaction mixture at the indicated concentrations with 5 nM (^{3}H)PGE$_2$ - The results are expressed as a percentage of total fmol/mg bound (^{3}H)PGE$_2$ in the absence of any competing compound.

of PGI$_2$ to stimulate adenylate cyclase of the cells. Since in this assay system the incubation temperature was 37°C, DDH-carbo PGI$_2$ was used instead of PGI$_2$, which rapidly degraded at this temperature. Fig.4 shows that PGE$_2$ and the PGI$_2$ analogue are able to stimulate adenylate cyclase in a concentration range similar to the concentrations used in the displacement study (Fig.3).

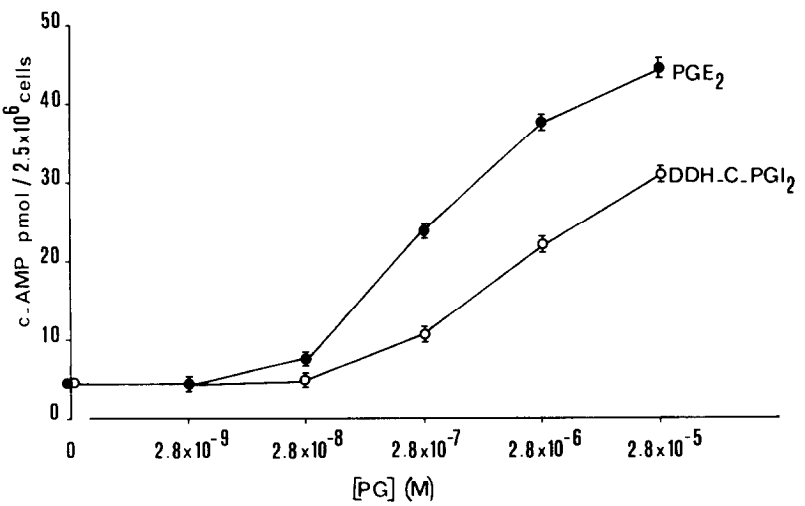

Figure 4. Adenylate cyclase activation of macrophages by PGE$_2$ (•) and DDH-carboPGI$_2$ (o). Incubations (10 min; 37°C) were carried out in the presence of the phosphodiesterase inhibitor IBMX (200 μM). Each point is the average (± SEM) of triplicate determinations.

PGE_2 was approximately 20 times more potent than the PGI_2 analogue as estimated graphically from half maximal stimulation values.

DISCUSSION

Since PGs are fatty acid-like molecules, which can be expected to associate non-specifically with hydrophobic membrane components, regardless of a specific binding capacity, there are apparent problems with PG binding studies (11). Non-specific binding will be dependent on the pH of the assay buffer. At pH 7.8 acceptable specific binding could be detected, which could be further increased up to 61% by including 1 mM of EDTA in the buffer. A further complication to accurate measurements of PG receptor binding would be the occupancy of binding sites by endogenous synthesized PGs. In order to minimize these complications, indomethacin (10^{-5}M) was included in all solutions used. Binding of (^{3}H)PGE_2 was saturable, specific, reversible, dependent on the protein concentration and correlated to a biological response. Therefore it might be concluded that binding sites actually represent receptor binding. Furthermore, specific binding of PGE_2, displacement studies and adenylate cyclase activation agree concerning potency order and effective concentration range. The displacement study reveals that the relative affinities of PGE_2: PGE_1:PGI_2 are 100:1:0.5. The IC_{50} of PGE_2 was 10^{-7} M (Fig.3) and the K_d of adenylate cyclase stimulation (Fig.4) was approximately 2 x 10^{-7} M. The displacement curves for PGE_1 and PGI_2 were not parallel to that of PGE_2, indicating a different type of competition may be involved. Several lines of evidence indicate that PGE_1 and PGI_2 share common binding sites different from PGE_2 binding sites (12,13). Although these studies were carried out with liver membranes and platelets, it is tempting to speculate that PMϕ also possess different types of PG receptors. Evidence for this assumption may be the parallel displacement lines of PGE_1 and PGI_2, while the line of PGE_2 had a different slope. In agreement with the data presented here, are the binding studies with PGE_2 on rat skin membrane fractions with respect to the effects of cations on specific binding (4). The data suggest that ionic forces may contribute to the binding of PGE_2 to its receptors.

In conclusion, viable peritoneal macrophages can be used to investigate PGE_2 receptor binding. In the same system PGE_2 showed to stimulate adenylate cyclase as also reported earlier (7). Binding of (^{3}H)PGE_2 showed to be specific, saturable and selective. Compared to PGE_2, PGE_1 and PGI_2 were poor competitors for (^{3}H)PGE_2 binding and it was suggested that a different type of competition exist for these PGs. Further studies are in progress to elucidate the interaction of PGE_2 and PGI_2 with regard to cAMP production of rat PMϕ at the receptor level in order to study the functional activity of macrophages in inflammatory processes in more detail.

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