Investigative Ophthalmology & Visual Science, Vol. 31, No. 6, June 1990

Prostaglandin E_2 Binding Sites in Bovine Iris–Ciliary Body

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In the eye, prostaglandins (PGs), in particular PGE_2 and $PGF_{2\alpha}$, may induce vasodilation, disruption of the blood–aqueous barrier, and biphasic effects on intraocular pressure, depending on the species. The initial event leading to many of these physiologic responses is the interaction between the PG and a receptor. We have explored the specificity and selectivity of PGE_2 receptors in bovine iris–ciliary body (ICB) membrane preparations. Pigment-free bovine ICB membranes were prepared by high-speed sucrose density-gradient centrifugation. Membranes were incubated with 1 nM ^3H-PGE_2 in the presence or absence of varying concentrations of unlabeled PGE_2 or $F_{2\alpha}$. Binding of ^3H-PGE_2 to membranes at 37°C increased linearly with protein concentration, and binding reached equilibrium in 30 min. Specific PGE_2 binding represented 80% of total ^3H-PGE_2 binding. Studies with unlabeled PGE_2 or $F_{2\alpha}$, as competing ligands, showed a dose-dependent inhibition of ^3H-PGE_2 specific binding. The IC_{50} for unlabeled PGE_2 and $F_{2\alpha}$ was 3 and 379 nM, respectively, which suggests a 100-fold greater selectivity of the binding sites for PGE_2 over $F_{2\alpha}$. Scatchard analysis of saturation data revealed a mean K_d value of 13.3 nM with a B_{max} of 156 fmoles bound/mg protein. The general linearity of our Scatchard plots tends to suggests a single class of binding sites for PGE_2, although more than a single binding site could be present. These results indicate that binding sites selective for PGE_2 exist in the bovine ICB. Invest Ophthalmol Vis Sci 31:1109–1113, 1990

Numerous studies over the last two decades have reported the formation and pathophysiologic actions of arachidonic acid (AA) metabolites in ocular and other tissues. Depending on the species and on the type and dose, AA metabolites affect vascular permeability including the blood–aqueous barrier; cause miosis; increase or decrease intraocular pressure; and induce leukocyte infiltration.[1–8]

In view of the multiple effects of prostaglandins (PGs) in the eye, we initiated studies to characterize PG receptors by ligand-binding assay in ocular tissue. Information about the functional aspects of PGs and the relevant receptors in the eye is sparse. Only two studies, one by Kennedy et al,[9] examining cats and dogs, and one by Dong and Jones,[10] examining bullock, have identified PG receptors in iris-sphincter muscles. The iris-sphincter muscle of cats and dogs contained predominantly prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (FP type) receptors and that of bullock, prostaglandin E_2 (PGE_2) (EP type) receptors, according to the proposed classification system for prostanoid receptors.[9,11] Kennedy et al[9] compared the rank order of potency of PGE_2, $PGF_{2\alpha}$, and other naturally occurring prostanoids, as well as a TXA_2 receptor antagonist, U-46619, in their in vitro iris-sphincter muscle preparation, and demonstrated the receptor-mediated nature of iridial responsiveness to prostanoids. In vivo, a pharmacologic study by Bito[4] revealed that $PGF_{2\alpha}$ is miotic in cats. Therefore, there appears to be a strong correlation between the PG-selective receptors and the response of the iris-sphincter muscle.

In this study, we report the characteristics of PGE_2 receptors in the bovine iris–ciliary body (ICB). Parallel studies using rat kidney medulla were performed as a positive control; PGE-type receptors have been characterized previously in this tissue.[12,13]

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Supported by USPHS Research Grant No. EY-06918, an unrestricted grant from Research to Prevent Blindness, Inc., and The Kentucky Lions Eye Foundation.

Submitted for publication: July 21, 1989; accepted September 28, 1989.

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Materials and Methods

Buffer

A 50 mM sodium phosphate buffer (pH 7.6) was used for membrane preparation and for binding assays. The buffer contained: trypsin inhibitor (10 mg/dl), phenyl-methyl-sulfonyl fluoride (8.7 mg/dl), flurbiprofen (0.375 mg/dl), bovine serum albumin (200 mg/dl), and sodium chloride (100 mM). (All chemicals were purchased from Sigma, St. Louis, MO.)

Membrane Preparation

Membrane preparation was carried out at 0–4°C on ice. Bovine eyes were obtained fresh on ice from a local slaughterhouse. The cornea was excised from each eye and then the ICB carefully removed with forceps and placed in homogenization tubes (5 ICB per tube) containing 3 ml buffer. Kidney tissue was recovered (in accordance with the ARVO Resolution on the Use of Animals in Research) from rats after sacrifice by intraperitoneal pentobarbital injection (60 mg/kg). The cortex was dissected free and the medulla was processed as described below. Bovine and rat tissue was homogenized separately using a Polytron tissue homogenizer (3 10-sec bursts at 70% of maximum setting). The homogenates then were filtered through cheesecloth with further buffer washings into precooled ultracentrifuge tubes.

The tubes were centrifuged in a Beckman L8-M ultracentrifuge for 60 min at 120,000 *g*. Supernatants were discarded, and the pellets were minced and then placed in glass scintillation vials containing 2 M sucrose and glass beads. The vials were alternately hand shaken and recooled for a 12-min period to free tissue membranes from the highly compact pellet formed after the first centrifugation. The glass bead procedure was found to be more effective at dispersing the pellet than the use of a manual homogenizer. The suspension was then added to fresh ultracentrifuge tubes and gently overlayed with buffer solution. The tubes were centrifuged for 90 min at 120,000 *g*. The resulting turbid membrane layer formed at the sucrose–buffer interface was then recovered with a Pasteur pipette and transferred to fresh ultracentrifuge tubes for a final centrifugation for 60 min at 120,000 *g*. The resulting pellet was resuspended in buffer to the appropriate protein concentration for binding assays (150–400 μg/ml). Protein content was determined according to the method of Lowry et al[14] using bovine serum albumin as a standard.

Radioligand Binding Assay

All binding assays were performed in buffer at 37°C in a shaking waterbath (120 strokes/min). First, 150–400 μg membrane protein was incubated, in triplicate, in a total volume of 625 μl buffer containing 1 nM ^{3}H-PGE_2 in the presence or absence of various concentrations of unlabeled PGE_2 or $PGF_{2\alpha}$. At the end of incubation periods, free unbound ligand was separated by rapid filtration under vacuum through filters (0.45 μm, type HA; Millipore) using a filtration manifold.

Blanks containing 1 nM ^{3}H-PGE_2 in 625 μl buffer were also filtered as above to determine nonspecific binding by the filters. The membrane–ligand complex retained by the filter was washed twice with 2 ml ice-cold buffer. The filtration and washing were completed within 10 sec. The radioactivity in the filters was counted in a liquid scintillation counter (Model LS-3801; Beckman). Specific binding was calculated as the difference between the total and nonspecific binding.

For time-course studies, bovine and rat membrane were incubated for selected times up to 60 min. In dissociation studies, all tubes were incubated for an initial 30-min period in 1 nM ^{3}H-PGE_2 in the presence or absence of 1 μM unlabeled PGE_2. At this point, 1 μM unlabeled PGE_2 was added to tubes which had been incubated in 1 nM ^{3}H-PGE_2 alone. The incubation period for measuring ^{3}H-PGE_2 dissociation ranged from 2 through 60 min when the incubation was terminated by rapid filtration. Competition studies were performed at 30 min for bovine ICB membranes.

For Scatchard analysis of bovine ICB, the concentration of ^{3}H-PGE_2 ligand was varied around the IC_{50} (3 nM), the concentration of unlabeled PGE_2 required to displace 50% of ^{3}H-PGE_2 binding. Concentrations of unlabeled PGE_2 ranging from 0 to 30 nM were employed. Filter blanks were employed at each concentration.

^{3}H-PGE_2 was purchased from Amersham Corporation (Arlington Heights, IL), and unlabeled PGE_2 and $PGF_{2\alpha}$ from Cayman Chemicals (Ann Arbor, MI). All remaining compounds were purchased from Sigma (St. Louis, MO).

Data Analysis

Data were analyzed and graphically represented by the computer programs EBDA (Biosoft) and Sigma Plot (Jandel Scientific). EBDA performs the transformations necessary to analyze association, competition, and Scatchard data. Sigma Plot graphically represents the data obtained from the EBDA program.

Results

Kinetics of ^{3}H-PGE_2 Binding

Specific binding of ^{3}H-PGE_2 to membrane preparations of bovine ICB and rat kidney medulla was rapid, as shown in Figure 1. Binding was linear with time for the first 15 min and reached equilibrium at 30 min in both preparations. The amount of ^{3}H-PGE_2 bound to specific sites in bovine ICB was 28 fmoles/mg protein, and the amount in rat kidney was 59 fmoles/mg protein. Based on the results of this experiment, subsequent studies were performed at 30 min, the time at which binding reached equilibrium. In bovine ICB (Fig. 2), the dissociation of bound ^{3}H-PGE_2 from its binding sites upon addition of unlabeled PGE_2 at various periods was rapid; within 10 min, 78% of the specifically bound ^{3}H-PGE_2 disso-

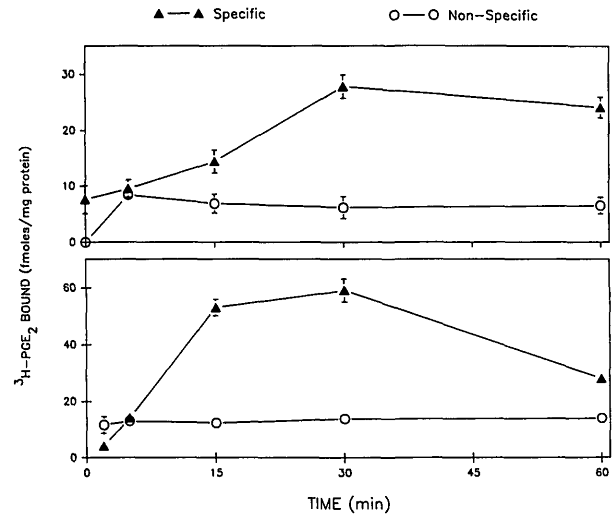

Fig. 1. Time course of specific and nonspecific ^{3}H-PGE$_2$ binding to membrane preparations of bovine ICB (*top*) and rat kidney medulla (*bottom*). Membranes were incubated with 1 nM ^{3}H-PGE$_2$ in the presence or absence of a 1000-fold excess of unlabeled PGE$_2$ at 37.5°C. Each point represents the mean and standard deviation of triplicate determinations.

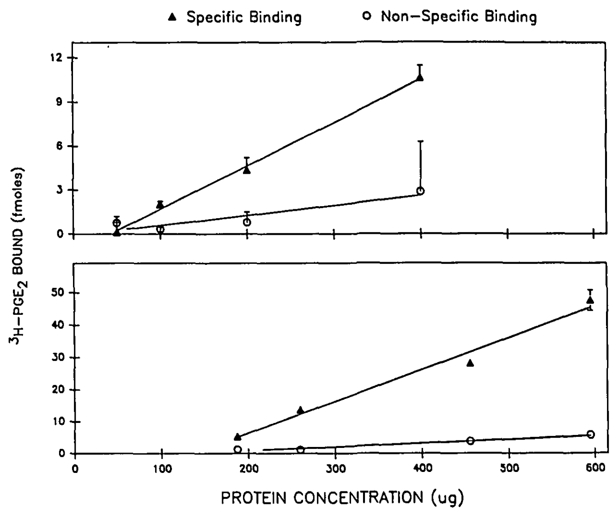

Fig. 3. Specific and nonspecific binding of ^{3}H-PGE$_2$ to bovine ICB (*top*) and rat kidney medulla membranes (*bottom*) with increasing concentration of membrane protein. Membranes were incubated with 1 nM ^{3}H-PGE$_2$ at 37.5°C for 30 min in the presence or absence of a 1000-fold excess of unlabeled PGE$_2$. Each point represents the mean and standard deviation of triplicate determinations.

ciated from the binding sites. The remaining radioligand appeared to be undissociable, at least up to the 60-min time period.

The specific binding, as expected, increased linearly with increasing concentration of membrane protein in both bovine ICB (Fig. 3 *top*) and rat kidney medulla (Fig. 3 *bottom*) preparations. These data suggest that the concentration of radiolabeled PGE$_2$ (1 nM) was far in excess of the binding sites available in the amount of membrane protein (150–400 μg) used.

Saturation Studies

To determine the dissociation constant (K_d) and the maximum number of binding sites (B_{max}), saturation studies were performed. Samples were incubated in 3 nM ^{3}H-PGE$_2$ and concentrations of unlabeled PGE$_2$ ranging from 0 to 30 nM. The results from a typical experiment are shown in Figure 4. Specific binding increased with increasing PGE$_2$ concentration and appeared to reach saturation above 30 nM PGE$_2$. Scatchard analysis of saturation data revealed

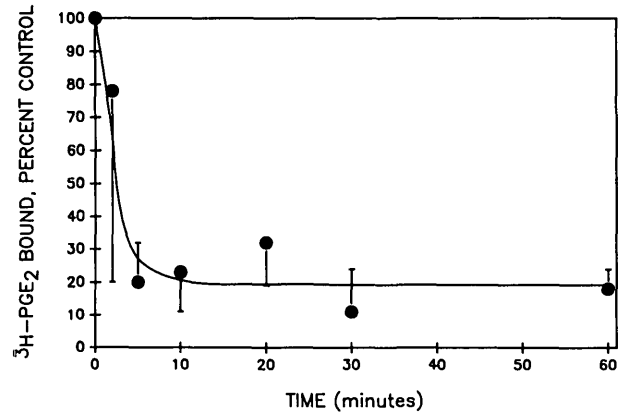

Fig. 2. Representative time course of dissociation of specifically bound ^{3}H-PGE$_2$ to bovine ICB membranes. Membranes in triplicate were incubated for 30 min with 1 nM ^{3}H-PGE$_2$. After 30 min, 1 μM unlabeled PGE$_2$ was added to tubes containing 1 nM ^{3}H-PGE$_2$ alone. This is the zero time in the dissociation curve. The percentage of specifically bound ^{3}H-PGE$_2$ remaining, at intervals through 60 min, is represented. The error bars represent the standard deviation from the mean (n = 3).

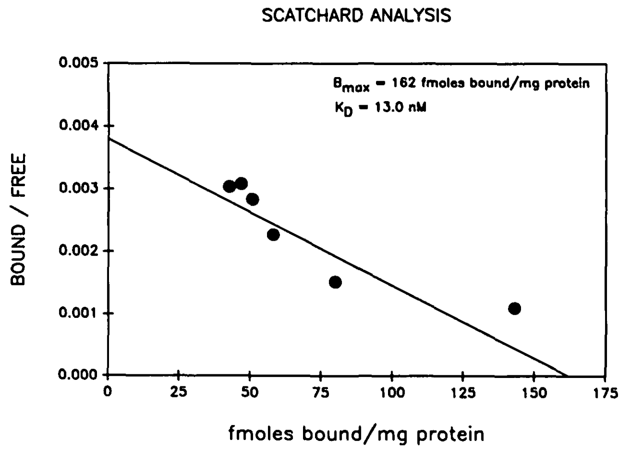

Fig. 4. Representative Scatchard analysis of ^{3}H-PGE$_2$-specific binding sites for determination of dissociation constant and maximum number of binding sites per milligram of membrane protein. Samples were incubated in 3 nM ^{3}H-PGE$_2$ and concentrations of unlabeled PGE$_2$, ranging from 0 to 30 nM (n = 3 for each point represented).

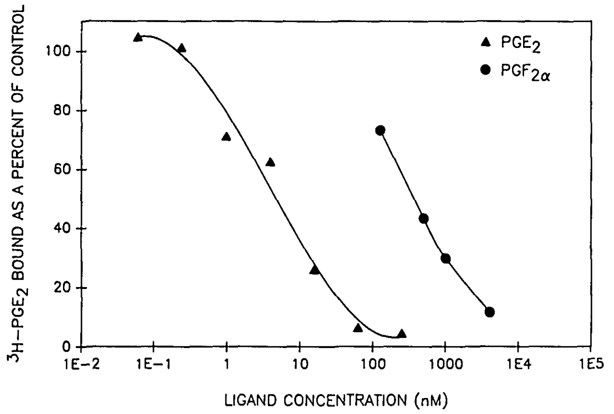

Fig. 5. Competitive inhibition of ^3H-PGE_2-specific binding to bovine ICB membranes by unlabeled PGE_2 and $F_{2\alpha}$. Incubations were performed for 30 min at 37.5°C in triplicate in the presence or absence of various concentrations of unlabeled ligands. 100% specific control binding is defined as the amount of bound ^3H-PGE_2 displaced in the presence of 1 μM unlabeled PGE_2 (n = 6 for each PGE_2 and n = 3 for each $PFG_{2\alpha}$ point represented).

a mean K_d value of 13.3 nM with a B_{max} of 156 fmoles bound/mg protein. The general linearity of our Scatchard plots tends to suggests a single class of binding sites for PGE_2, although more than a single binding site could be present.

Competitive Displacement of Bound ^3H-PGE_2

Competition studies with unlabeled PGE_2 or $PGF_{2\alpha}$ were performed to examine the selectivity of ^3H-PGE_2 binding sites. Competition curves are shown in Figure 5. Unlabeled PGE_2 displaced ^3H-PGE_2 bound to bovine ICB membrane sites in a dose-dependent manner, with a K_I of 3 nM. $PGF_{2\alpha}$ competed for ^3H-PGE_2 binding sites also in a dose-dependent manner, but a had a high K_I, 379 nM. Thus, the affinity of $PGF_{2\alpha}$ for ^3H-PGE_2 binding sites was more than 100-fold less than that of PGE_2. These results indicate that the binding sites are specific for PGE_2 receptors. In kidney medulla preparations, the K_I for PGE_2 (data not shown) was 5 nM, which is in close agreement with that of bovine ICB membranes.

Discussion

In the current study, we demonstrated specific binding sites for ^3H-PGE_2 in bovine ICB and rat kidney medulla membrane preparations. The binding is saturable and dissociable with time. Specific ^3H-PGE_2 binding reached a stable steady state in both membrane preparations, whereas the nonspecific membrane binding did not reach equilibrium. These kinetics strongly suggest that the binding sites in bovine ICB and rat kidney medulla membranes are specific for PGE_2.

The K_I of PGE_2 is 3 nM and 5 nM in bovine ICB and rat kidney medulla, respectively. The similarity of these K_I values suggests that PGE_2 binding sites in two diverse species and tissues represent similar populations of PGE_2 receptors. The fentomoles of PGE_2 bound per milligram protein is lower in bovine ICB than in rat kidney medulla, an observation that may have a physiologic basis. Some tissues may contain greater numbers or density of receptors to facilitate a larger response.

In competition studies, $PGF_{2\alpha}$ was much less effective than PGE_2 at displacing labeled PGE_2 from the binding sites, suggesting a degree of selectivity for PGE_2 binding in both bovine ICB and rat kidney medulla. Furthermore, the relative competition ratio (RCR) was similar to that observed in other tissues, including bovine corpus lutea,[15] for which the IC_{50} for PGF_2 was more than 100-fold higher than that for PGE_2.

The specific binding of an endogenous ligand to the membranes does not necessarily imply that the binding sites actually are receptors. An important criterion for classifying binding sites as receptors is to demonstrate a correlation between binding affinity in vitro and pharmacologic potency in vivo.[16] A good correlation has been reported between the concentration of PGEs required for half-maximal stimulation of adenylate cyclase activity and progesterone synthesis in bovine corpora lutea[17] and mouse ovary,[18] respectively, and the apparent K_d of their PGE receptor types. The time interval required for binding to reach equilibrium in our studies with bovine iris compares favorably to the time period observed between intraocular administration of nanogram quantities of PGE_2 and subsequent alteration of the blood–aqueous barrier and intraocular pressure in rabbits.[7,19,20]

The data presented in this study have demonstrated that this ICB membrane preparation exhibits classic pharmacologic binding parameters commonly associated with prostanoid receptors. Studies relating binding kinetics to physiologic responses in ocular tissues will allow a firmer correlation to be drawn between the binding sites observed in this study and the biologic actions of prostaglandins.

Key words: prostaglandins, binding sites, bovine, iris–ciliary body, receptors

Acknowledgments

The authors wish to thank Cecilia Wroblewski for manuscript preparation and Lori Rhodes for her excellent technical assistance.

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