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Radioligand Binding Analysis of Receptor Subtypes in Two FP Receptor Preparations that Exhibit Different Functional Rank Orders of Potency in Response to Prostaglandins

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ABSTRACT

The rat colon and Swiss 3T3 cells have been proposed as FP receptor preparations. However, the rank orders of potency for contraction of the rat colon and Ca^{++} signaling in Swiss 3T3 cells were found to be disparate. Although both appeared to be FP receptor preparations in that $PGF_{2\alpha}$ and FP receptor selective analogs were the most potent agonists, the potency ranking for other PGs and their analogs differed markedly. This presented two alternative major hypotheses for interpreting these data: (1) Swiss 3T3 cells and the rat colon possess different FP receptor subtypes and (2) the rat colon contains a heterogeneous population of prostanoid receptors. To further characterize prostanoid receptor populations in these two preparations, radioligand binding studies were performed with ^{3}H-PGE_2 and ^{3}H-17-phenyl-$PGF_{2\alpha}$. The rank order of potency for inhibition of ^{3}H-PGE_2 binding in the rat colon was consistent with EP_3 receptor pharmacology. Thus, MB 28767, sulprostone and PGE_2 were potent inhibitors, whereas $PGF_{2\alpha}$, PGD_2 and other analogs were substantially less potent. The rank order of potency for inhibition of ^{3}H-17-phenyl-$PGF_{2\alpha}$ binding in the rat colon was consistent with the presence of an FP receptor. Thus, the potency rank order for the natural PGs was $PGF_{2\alpha} > PGD_2 > PGE_2$ and among the synthetic analogs only $PGF_{2\alpha}$ analogs were potent competitors. In Swiss 3T3 cells an identical rank order of potency for eliciting a Ca^{++} transient signal and inhibition of ^{3}H-17-phenyl-$PGF_{2\alpha}$ binding was obtained. Since the rank order of potency in radioligand binding competition studies with ^{3}H-17-phenyl-$PGF_{2\alpha}$ in the Swiss 3T3 cells and the rat colon were similar, the data suggested the presence of an FP receptor in the rat colon. The results are consistent with the presence of both EP_3 and FP receptors in the rat colon.

The biological actions of prostanoids have been described as bewildering and awesome (Kennedy *et al.*, 1982). This is reflected by the substantially different approaches that have been adopted for receptor classification (Gardiner and Collier, 1980; Coleman *et al.*, 1982; Muallem *et al.*, 1989). Most researchers have avoided devising expansive nomenclatures and restricted their interpretations to descriptive terminology, presumably to avoid adding to the confusion. However, the contention that distinct receptor subtypes exist for each of the natural prostanoids has become increasingly accepted in recent years and only one classification for prostanoid receptors (Coleman *et al.*, 1981, 1982) is now widely employed. Separate receptors for prostaglandins D_2, E_2 and $F_{2\alpha}$ (PGD_2, PGE_2 and $PGF_{2\alpha}$) were originally suggested from studies comparing their rank order of agonist potency (Jones, 1976; Welburn and Jones, 1978). Identification of additional smooth muscle preparations with particular sensitivity to only one prostanoid allowed rank order of potency comparisons to be extended to include prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) to provide a basis for a comprehensive classification (Coleman *et al.*, 1982, 1984). The current working classification was set on a firmer foundation by the activity of several TxA_2 antagonists and two antagonists which selectively blocked the myotropic effects of PGE_2 (Coleman *et al.*, 1981, 1982, 1987). In the absence of antagonists, other receptor subtypes were proposed according to the selective agonist properties of certain synthetic prostaglandin analogs (Coleman *et al.*, 1982). The nomenclature for prostanoid receptor subtypes with particular sensitivity to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TxA_2 mimetics provided the designations DP-, EP-, FP-, IP- and TP-, respectively. Further subdivisions exist and four EP receptor subtypes have now been described (Coleman *et al.*, 1987, 1994; Lawrence and Jones, 1989). Thus, the EP_2 and EP_4 receptors mediate smooth muscle relaxation and the two distinct EP-receptor subtypes that stimulate smooth muscle contraction are designated EP_1 and EP_3 according to their susceptibility to antagonism by AH 6809. The current working classification is also supported by the recent identification of a potent and selective DP receptor antagonist BW A868C (Giles *et al.*, 1989; Trist *et al.*, 1989). Finally, the molecular structure of the TxA_2 receptor, the EP_1 receptor, the EP_2 receptor, the EP_3 receptor and

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ABBREVIATIONS: PG, prostaglandins; TX, thromboxane.

the FP receptor have been reported recently (Hirata *et al.*, 1991; Narumiya *et al.*, 1993; Honda *et al.*, 1993; Funk *et al.*, 1993; Sugimoto *et al.*, 1994). In spite of such recent progress, the potential for further subtypes of the major prostanoid receptor classes cannot yet be dismissed, as indicated by the recent proposal of a fourth EP receptor subtype (Coleman *et al.*, 1994).

In comparing the functional responses in two FP receptor preparations, Ca^{++} signaling in Swiss 3T3 cells (Woodward and Lawrence, 1994) and contraction of the rat colon (Eglen and Whiting, 1988), we found that the agonist potency rank order differed considerably between the two preparations. In particular, the potency of PGE_2 and EP_3 receptor agonists in eliciting contraction of the rat colon was much higher than for Ca^{++} signaling in Swiss 3T3 cells and reported data on FP receptors associated with the dog and cat iris sphincter muscles (Coleman *et al.*, 1982; 1984; Krauss *et al.*, 1993). Because EP_3 agonists were less potent than $PGF_{2\alpha}$ and selective FP receptor agonists in the rat colon but were also less potent than in typical EP_3 receptor preparations, the possibility that the rat colon and Swiss 3T3 cells contain different subtypes of FP receptor was presented. To further investigate the rat colon receptor associated with prostanoid-induced contractions, we performed radioligand binding studies on receptor-enriched plasma membrane fractions prepared from the rat colon using ^{3}H-PGE_2 and the selective FP-receptor agonist ^{3}H-17-phenyl-$PGF_{2\alpha}$ (Miller *et al.*, 1975; Woodward *et al.*, 1989). These were compared with radioligand binding and Ca^{++} signaling in Swiss 3T3 cells, the Ca^{++} signaling being used as a functional marker.

Experimental Procedures

Female Sprague-Dawley rats weighing 150 to 200 g were sacrificed by CO_2 inhalation. Each experiment involved the surgical removal of approximately 2 cm of proximal colon.

Rat Colon

Functional studies. Prostanoid activity on rat colon isolated smooth muscle preparations was recorded isometrically with a Grass polygraph model 79E. Sections (1.5 cm) of proximal ascending rat colon were suspended under 1 × *g* tension in 10-ml jacketed organ baths (Radnoti, Monrovia, CA) containing Krebs' physiological solution (NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2$, 1.9 mM; $NaHCO_3$, 25 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.18 mM; glucose, 11.7 mM). The Krebs' solution was adjusted to pH 7.4 by aeration with a mixture of 95% O_2–5% CO_2 and the temperature was maintained at 37°C. Indomethacin (1 μM) was added to prevent production of endogenous prostaglandins. Tissues were allowed to equilibrate for approximately 1 hr with periodic washing performed during the equilibration period. After equilibration, a noncumulative dose-response curve for the standard $PGF_{2\alpha}$ (10 nM–10 μM) was performed. The tissues were washed between each dose. A noncumulative dose-response curve was then performed with the selected compound. A maximal dose of $PGF_{2\alpha}$ (1 μM) was given between each dose-response curve and at the end of the experiment. A rest period of approximately 30 min was allowed between each dose. Contractile responses were calculated as a percent of the maximal contraction obtained with $PGF_{2\alpha}$.

Radioligand binding. Each radioligand binding study involved 20 sections of rat proximal colon. After removing excess fat and connective tissue, each colon was cut open and the epithelium removed by gentle rubbing with a Q tip.

The tissues were divided equally into four tubes containing homogenization buffer (0.25 M sucrose, 5 mM TRIS, 1 μM indomethacin, adjusted to pH 7.4 with HCl). The tissues were homogenized with a polytron homogenizer for 3 sec at setting 7. The homogenate was centrifuged at 400 × *g* for 3 min. After careful removal of the fat layer from the top, the supernatant fluid was removed with a transfer pipette and saved. The residue was resuspended in 25 ml of buffer, rehomogenized and centrifuged again as above. The pooled supernatant was filtered through a triple layer of gauze and the filtrate was centrifuged at 177,000 × *g* in a Beckman XL-90 ultracentrifuge for 40 min.

PG binding site-enriched plasma membrane fractions were prepared based on a procedure involving two-step discontinuous gradients (Beinborn *et al.*, 1988; Woodward and Lawrence, 1994). The 177,000 × *g* pellet was suspended in homogenization buffer and layered on top of a 4-ml cushion of buffer containing variable concentrations of sucrose (0.771, 0.842, 0.914, 1.06, 1.28, 1.60). The sucrose cushion that provided the highest level of specific binding for each radiolabeled ligand was ultimately selected for further studies. The two-step discontinuous gradient preparation was centrifuged at 112,700 × *g* for 2 hr with a Beckman SW 28 rotor. The bands at the interface of each cushion were carefully aspirated, suspended in TRIS buffer (50 mM, pH 7.4), and centrifuged at 304,000 × *g* for 40 min. The final pellet was washed once with TRIS buffer and stored at −70°C.

Radioligand binding assays were performed on the final 304,000 × *g* pellet. The pellet was suspended in TRIS buffer with the aid of sonication. All binding assays were performed in triplicate in a final volume of 200 μl for 1 hr. Preliminary studies were performed to optimize the assay conditions. These examined the influence of pH and various cations. Final conditions for ^{3}H-PGE_2 binding were provided by 50 mM TRIS buffer at pH 7.4 containing 100 mM NaCl. Final conditions for ^{3}H-17-phenyl-$PGF_{2\alpha}$ binding were provided by 50 mM TRIS buffer at pH 5.75 containing 2.5 mM $MgCl_2$. The final protein concentration in each tube was approximately 50 μg/ml for the ^{3}H-PGE_2 studies and 100 μg/ml for the ^{3}H-17-phenyl-$PGF_{2\alpha}$. The need to use a higher protein content for the ^{3}H-17-phenyl-$PGF_{2\alpha}$ studies was necessitated by its lower specific activity compared to ^{3}H-PGE_2. Nonspecific binding was determined by using 10 μM respective unlabeled ligand. Binding reactions were started by adding membrane protein. The reactions were terminated by addition of 4 ml of ice-cold TRIS buffer and rapid filtration through Whatman GF/B filters using a Brandel cell harvester. The filters were washed three times with ice-cold TRIS buffer and allowed to dry 1 hr in an oven. The filters were placed in scintillation vials and 5 ml of Beckman "ready protein" was added. Radioactivity was counted in a Beckman LS 3801 counter. All saturation and competition assays were performed in triplicate and three replicates of each assay were obtained by use of different plasma membrane preparations.

Studies involving graded concentrations of ^{3}H-PGE_2 and ^{3}H-17-phenyl-$PGF_{2\alpha}$ were limited to the established selectivity range of these agents and Scatchard and Hill analyses were performed after correction for dilution of the specific activity. Competition studies involved 10-fold incremental increases in unlabeled ligand concentration *vs.* fixed 5 nM concentrations of both radiolabeled ligands. All data were analyzed with the EBDA/LIGAND program (McPherson, 1985). Data obtained from saturation studies are given with standard deviations in parentheses. Protein concentrations were determined by Bradford assay.

Swiss 3T3 Cells

Ca^{++} signaling. Mouse Swiss 3T3 fibroblasts were plated in culture flasks and were fed Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM L-glutamine and 0.05 mg/ml gentacin (all purchased from Gibco, Grand Island, NY). Cell cultures were maintained in a humidified atmosphere of 95% air, 5% CO_2 and grown to confluency.

Cells were removed from the culture flasks by approximately 1-min treatment with trypsin 0.05%, 0.52 mM EDTA (Gibco, Grand Island, NY) at 37°C. Proteolytic activity was arrested by adding 5 ml

of 10% fetal bovine serum in DMEM. The cells were washed consecutively in Hank's BSS and medium containing 140 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES; TRIS, 5 mM glucose, 5 mM Na-pyruvate, 0.1% bovine serum albumin at pH 7.4 (Yamaguchi *et al.* 1988); centrifugation for the washes was performed for 15 min at 200 × *g* at room temperature. Cells were counted, resuspended in the medium above and incubated with 2 μM Fura 2/acetoxymethyl ester in a shaking water bath for 30 min at 37°C. The cells were subsequently washed in medium as above and resuspended at a concentration of 2 × 10^6 cells/ml. Aliquots of 0.5 ml cell suspension were then added to autocap microtubes to provide 10^6 cells per experimental determination of intracellular Ca^{++} concentration ($[Ca^{++}]_i$).

Fluorescence was measured in a Perkin-Elmer LS-5 fluorescence spectrophotometer at excitation and emission wavelengths of 340 and 492 nm, respectively, with both slits at 10 nm. For each experimental determination 10^6 cells were washed (200 × *g* for 5 min) and suspended in a 3-ml cuvette with buffer containing 120 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 20 mM HEPES, 1 mg/ml glucose, and 1 mg/ml Na pyruvate. Stirring was achieved by a paddle stirrer mounted overhead with the temperature maintained at 37°C. Calibration of the Fura 2 signal was as previously described for UMR-106 cells (Yamaguchi *et al.*, 1988). The cells were lysed with digitonin (10 μl in 100 mg/ml DMSO) to obtain f_{max}. EGTA (100 mM) and sufficient 10 N NaOH to adjust the pH to 8.5 were then added successively to obtain f_{min}.

Radioligand binding. Mouse Swiss 3T3 fibroblasts were grown in culture flasks and were removed by scraping. The cells were suspended in 10% FBS (fetal bovine serum)/DMEM (Dulbecco's modified Eagle's Medium) and centrifuged at 400 × *g* for 10 min. The pellet was resuspended in ice-cold buffer containing 0.25 M sucrose, 5 mM TRIS, 1 μM indomethacin at pH 7.4 and centrifuged as above. Plasma membrane preparation enriched with PG binding sites were prepared as described for the rat colon. Optimal specific binding for ^{3}H-17-phenyl-$PGF_{2\alpha}$ in Swiss 3T3 cell plasma membranes was obtained by use of a 1.06 sucrose cushion and 3 mM $CaCl_2$ at pH 7.4.

Materials

The following materials were used: PGD_2, $PGF_{2\alpha}$, PGE_2, PGJ_2, PGI_2, U-46619 (9,11-dideoxy-9α,11α-methanoepoxy-$PGF_{2\alpha}$), 17-phenyl-17,18,19-trinor prostaglandin $F_{2\alpha}$ (17-phenyl-$PGF_{2\alpha}$) and 11-deoxy-PGE_1 were purchased from Cayman Chemical (Ann Arbor, MI). 15α-Hydroxy-9-oxo-16-phenoxy-17,18,19,20-tetranor prost 13-*trans*-enoic acid (MB 28767) was a gift from Rhone-Poulenc (Dagenham, UK); 16-*m*-trifluoromethyl phenoxy-17,18,19,20-tetranor prostaglandin $F_{2\alpha}$ (fluprostenol) was purchased from Pittman-Moore (Berkhamsted, UK); 11,15-dihydroxy-9-oxo-16-phenoxy-17,18,19,20-tetranor-5,13 prostadienoic acid-methyl sulfonylamide (sulprostone) was a gift from Berlex (Cedar Knolls, NJ); 5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl) hydantoin (BW 245 C) was a gift from Burroughs Wellcome (Beckenham, UK); AH-6809 (6-isopropoxy-9-oxoanthene-2-carboxylic acid), AH 13205 (±*trans*-2-[4-(1-hydroxyhexyl) phenyl]-5-oxycyclopentaneheptonoic acid) and GR 63799X were gifts from Glaxo (Ware, UK). All agents were dissolved in 50 mM TRIS buffer containing 0.01% (w/v) polysorbate 80 as a cosolvent; each assay tube or bath contained 0.001% polysorbate 80 as the final concentration.

Radiolabeled prostaglandin E_2 (PGE_2 [5,6,8,11,12,14,15-^{3}H(N)]-specific activity 200 Ci/mmol) and radiolabeled prostaglandin $F_{2\alpha}$ ($PGE_{2\alpha}$ [5,6,8,9,11,12,14,15-^{3}H(N)]-specific activity 200 Ci/mmol) were purchased from New England Nuclear (Wilmington, DE). The ethanol/water solvent was evaporated under N_2 before dissolution in 50 mM TRIS buffer-0.01% polysorbate 80 as above. Radiolabeled 17-phenyl trinor ($PGF_{2\alpha}$ [16,16,17,17^{3}H(N)]-specific activity 85 Ci/mmol) was purchased from Amersham (Arlington, IL). Ethanol solvent was evaporated from each aliquot before preparation of solution in 50 mM TRIS. All cell culture supplies were purchased from Gibco (Grand Island, NY).

Results

Rat Colon Contraction

The contractile response of the isolated rat colon to natural PGs and their analogs is depicted in figure 1. $PGF_{2\alpha}$ was the most potent natural PG, but was only about 10-fold more potent than PGE_2 and 30-fold more potent than PGD_2 (fig. 1A). PGI_2 and PGJ_2 were both >100 less potent than $PGF_{2\alpha}$ (fig. 1A). Although the FP receptor agonists fluprostenol and 17-phenyl-$PGF_{2\alpha}$ were the most potent PG analogs examined, the EP_3 agonists GR 63799X, sulprostone and MB 28767 also exhibited substantial activity (fig. 1B). 11-Deoxy-PGE_1 and the thromboxane A_2 (TxA_2) mimetic U-46619 were less active, the EP_2 agonist AH 13205 was only weakly active and the DP agonist BW 245C was inactive at concentrations up to 10 μM. The potency rank order of the natural PGs and their analogs is given as follows with EC_{50} values (nM) in parentheses. Fluprostenol (6) > 17-phenyl $PGF_{2\alpha}$ (11) ≥ $PGF_{2\alpha}$ (13) > sulprostone (69) > GR 63799 X (91) > PGE_2 (148) > MB 28767 (302) > PGD_2 (395) > 11-deoxy PGE_1 (706) > U-46619 (2018) > PGI_2 (4508) > PGJ_2 ≃ AH 13205 > BW 245C (inactive).

Rat Colon

^{3}H-PGE_2 binding. Experiments to determine appropriate conditions for ^{3}H-PGE_2 binding assays first involved a series of two-step sucrose gradients. Low density sucrose cushions retained more binding sites. The following values were obtained for specific binding of 5 nM ^{3}H-PGE_2 at each sucrose concentration: 0.771 M = 26%, 0.914 M = 27%, 1.06 M = 9%,

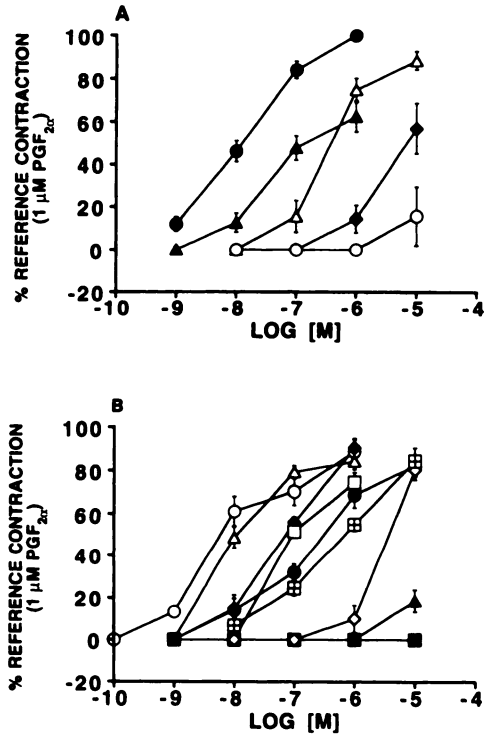

Fig. 1. Contractile effect of PGs and their analogs on the rat colon. Panel A depicts the effects of $PGF_{2\alpha}$ (●), PGD_2 (△), PGE_2 (▲), PGI_2 (◆), PGJ_2 (○). Panel B depicts the effects sulprostone (◆), GR 63799X (□), MB 28767 (●), fluprostenol (○), 17-phenyl $PGF_{2\alpha}$ (△), U-46619 (◇), BW 245C (■), AH 13205 (▲), 11-deoxy-PGE_1 (⊠). Points represent mean values of % maximal $PGF_{2\alpha}$ response ± S.E.M., *n* = 4.

1.28 M = 11%, 1.60 M = 5%. Divalent cations (Ca^{++} and Mg^{++}) did not appreciably improve the level of specific binding obtained using a 0.914 M sucrose cushion. The presence of 100 mM NaCl elevated specific binding from 28% to 57%.

The data obtained from three separate studies involving a 0.25 to 100 nM dose range of ^3H-PGE_2 were then subjected to Scatchard analysis. The dose-range of the radiolabeled ligands was not extended to 1 μM because competition studies indicated that nonselective binding occurred at such high concentrations. A K_d value of 11.2 (±3.9) nM and B_{max} of 240 (±53.6) fmol/mg protein were obtained. The data were consistent with a one-site model and did not fit a two-site model. All binding data values in parentheses are standard deviations. This was confirmed by Hill plot analysis that resulted in Hill coefficient of 1.06 (±0.08). The Scatchard plot of the pooled data are graphically depicted in figure 2A. ^3H-PGE_2-specific binding was inhibited by natural PGs (fig. 3A) with the following rank order of potency $PGE_2 > PGF_{2\alpha} > PGD_2$.

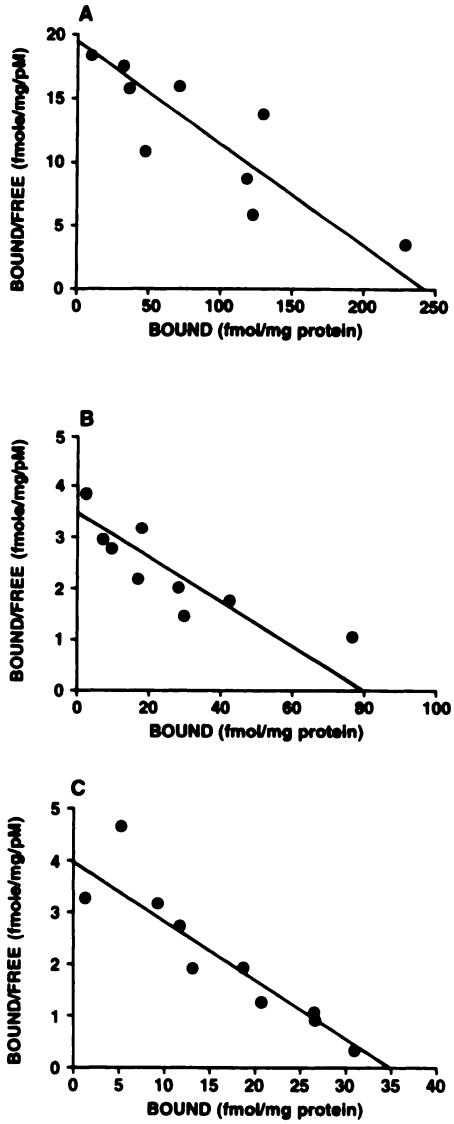

Fig. 2. Scatchard analysis of (A), ^3H-PGE_2 binding to membranes prepared from rat colon (B) ^3H-17-phenyl $PGF_{2\alpha}$ binding to membranes prepared from rat colon (C) ^3H-17-phenyl-$PGF_{2\alpha}$ to membranes prepared from Swiss 3T3 cells.

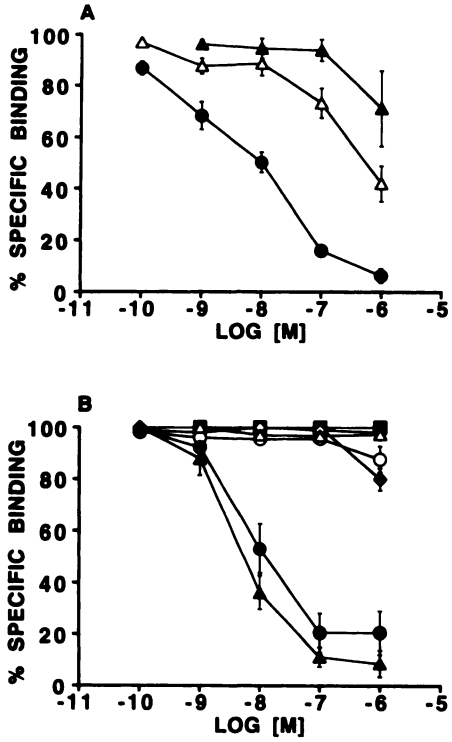

Fig. 3. Inhibition of ^3H-PGE_2 specific binding to membranes from the rat colon. Panel A shows the inhibitory effects of $PGF_{2\alpha}$ (△), PGD_2 (▲), PGE_2 (●). Panel B depicts the effects of sulprostone (▲), MB 28767 (●), fluprostenol (○), 17-phenyl $PGF_{2\alpha}$ (□), U-46619 (△), BW 245C (■), AH 13205 (◆), AH 6809 (△). Points represent mean values ± S.E.M., n = 3, except for PGE_2 where n = 8.

Sulprostone and MB 28767 were potent inhibitors of ^3H-PGE_2 binding. AH 13205, 17-phenyl-$PGF_{2\alpha}$, and fluprostenol at 1 μM concentrations competed for about 20% of the binding sites, AH 6809, BW 245C and U-46619 had no effect (fig. 3B).

17-Phenyl-$PGF_{2\alpha}$ binding. The binding conditions were optimized for ^3H-17-phenyl-$PGF_{2\alpha}$ after some preliminary experiments with ^3H-$PGF_{2\alpha}$. These were performed to conserve the ^3H-17-phenyl-$PGF_{2\alpha}$ supply which was custom made. The specific binding obtained with 6.1 nM ^3H-$PGF_{2\alpha}$ for sucrose cushions of various densities in the presence and absence of cations is summarized in table 1. These studies were conducted at pH 5.75 because specific binding at pH 7.4 did not exceed 15% under any conditions. A higher level of specific binding was readily obtained with ^3H-17-phenyl-$PGF_{2\alpha}$. In the presence of 2.5 mM $MnCl_2$ and at pH 5.75, the following percent specific binding was obtained for each su-

TABLE 1

Specific binding obtained with 6.1 nM 3H-$PGF_{2\alpha}$ in rat colon plasma membrane preparations with sucrose cushions of different densities.

Values are percent specific binding for a single experimental determination performed in triplicate.

Cations	% Specific 3H-$PGF_{2\alpha}$ Binding at Different Sucrose Densities (M)			
	0.771	0.840	9.914	1.06
	11	20	2	0
Ca^{++} (3.0 mM)	0	16	14	8
Mn^{++} (2.5 mM)	9	25	11	13

crose density: 0.771 M = 61%; 0.84 M = 63%, 0.914 M = 49%; 1.06 M = 45%. A study at pH 7.4 using a 0.84 M sucrose cushion and 2.5 mM $MnCl_2$ resulted in 56% specific binding. The procedure was not refined further because >60% specific binding for ^{3}H-17-phenyl-$PGF_{2\alpha}$ was obtained using a 0.84 M sucrose cushion, with assay buffer containing 2.5 mM $MnCl_2$ at pH 5.75.

Scatchard analysis of ^{3}H-17-phenyl-$PGF_{2\alpha}$ binding over a 0.25 to 100 nM range was derived from the pooled data of three separate saturation experiments (fig. 2B) and from determining the mean ± standard deviation from analysis of the three separate experiments. The data conformed to a one-site model and did not fit a two-site model. This was confirmed by Hill plot analysis where a coefficient of 1.05 (±0.11) was obtained. A K_d value of 24.3 (±8.3) nM and B_{max} of 92.3 (±15.4) fmol/mg protein were obtained. ^{3}H-17-phenyl-$PGF_{2\alpha}$ binding was potently inhibited by $PGF_{2\alpha}$, but PGD_2 and PGE_2 were less active (fig. 4A). The effects of prostanoid analogs on ^{3}H-17-phenyl-$PGF_{2\alpha}$ binding is depicted in fig. 4B. The FP receptor agonists fluprostenol and 17-phenyl-$PGF_{2\alpha}$ were potent inhibitors, sulprostone and MB 28767 afforded substantially less active and at a 1 μM <50% inhibition was achieved. AH 13205, U-46619, AH 6809, and BW 245C exhibited little or no competitive activity.

Swiss 3T3 Cells

Ca^{++} signal. The effects of PGs and their analogs on intracellular [Ca^{++}] in Swiss 3T3 cells is depicted in figure 5. $PGF_{2\alpha}$ was at least 10 times more potent than PGD_2 and more than 100 times more potent than PGE_2 (fig. 5). The FP

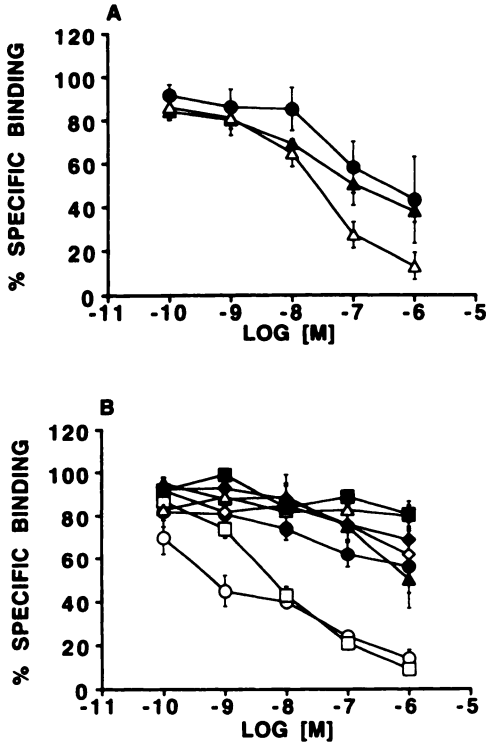

Fig. 4. Inhibition of ^{3}H-17-phenyl-$PGF_{2\alpha}$ specific binding to membranes from the rat colon. Panel A shows the inhibitory effects of $PGF_{2\alpha}$ (△), PGD_2 (▲), PGE_2 (●). Panel B depicts the effects of sulprostone (▲), MB 28767 (●), fluprostenol (○), 17-phenyl-$PGF_{2\alpha}$ (□), U-46619 (◇), BW 245C (■), AH 13205 (◆), AH 6809 (△). Points represent mean values ± S.E.M., n = 3 except for 17-phenyl-$PGF_{2\alpha}$ where n = 9.

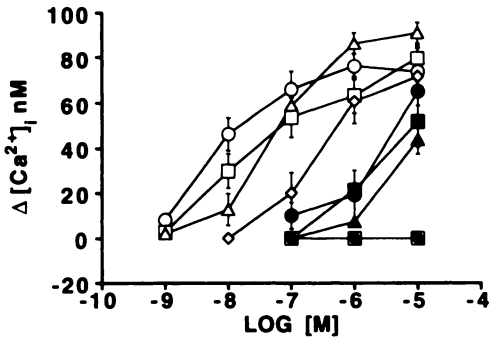

Fig. 5. Effects of natural prostaglandins and synthetic prostaglandin analogs on intracellular [Ca^{++}] in Swiss 3T3 cells. Symbols are as follows: $PGF_{2\alpha}$ (△), PGD_2 (◇), PGE_2 (●), sulprostone (▲), MB 28767 (■), fluprostenol (□), 17-phenyl-$PGF_{2\alpha}$ (○), AH 13205 (◆), 11-deoxy PGE1 (□). Points represent mean values ± S.E.M., n = 3–4.

receptor selective agonists fluprostenol and 17-phenyl-$PGF_{2\alpha}$ were substantially more active than MB 28767 or sulprostone. 11-Deoxy-PGE_1, and AH 13205 did not elicit a response over the dose range tested (fig. 5).

^{3}H-17-phenyl $PGF_{2\alpha}$ binding. Before commencing radioligand binding studies with ^{3}H-17-phenyl-$PGF_{2\alpha}$, preliminary experiments were performed with ^{3}H-$PGF_{2\alpha}$ to assist in optimizing the assay conditions. The ^{3}H-$PGF_{2\alpha}$ specific binding obtained by using sucrose cushions of graded density was as follows: 0.84 M = 24%; 0.914 M = 20%; 1.06 M = 27.8%; 1.22 M = 18%; 1.5 M = 21.5%. A 1.06 M sucrose cushion was selected for studying the influence of cations on specific binding. In a separate study the presence of 3 mM $CaCl_2$ increased specific binding from 38% to 58%. ^{3}H-$PGF_{2\alpha}$ binding was increased to 51% by 100 mM NaCl.

The level of specific binding obtained with ^{3}H-17-phenyl-$PGF_{2\alpha}$ was again substantially greater than for ^{3}H-$PGF_{2\alpha}$. In a study that used a 1.06 M sucrose cushion and 3 mM $CaCl_2$, 87% specific binding was obtained for ^{3}H-17-phenyl-$PGF_{2\alpha}$ in a Swiss 3T3 cell plasma membrane preparation. An acidic pH did not, however, improve the level of specific binding but caused a small reduction.

Scatchard analysis of ^{3}H-17-phenyl-$PGF_{2\alpha}$ binding was performed on the separate experiments and on combined data from two saturation experiments as depicted in figure 2C. A K_d value of 8.1 (±5.6) nM and a B_{max} of 35 (±5.4) fmol/mg protein were obtained. The data conformed to a one-site model and did not fit a two-site model. A one-site model was confirmed by Hill plot analysis and a value of 1.01 (±0.048) was obtained for the Hill coefficient.

Inhibition of ^{3}H-17-phenyl-$PGF_{2\alpha}$ specific binding (fig. 6) followed an essentially similar pattern of activity to that observed for Ca^{++} signaling. Unlabeled 17-phenyl-$PGF_{2\alpha}$ and fluprostenol were potent inhibitors followed by $PGF_{2\alpha}$. As in the Ca^{++} signaling studies, PGE_2 and its analogs MB 28767 and sulprostone were about 100-fold less potent than $PGF_{2\alpha}$ and its analogs, and PGD_2 was intermediate between these two sets of compounds. The EP_2 receptor agonist AH 13205 did not compete effectively with ^{3}H-17-phenyl-$PGF_{2\alpha}$ for its binding sites.

Discussion

The complexity of PG-induced effects has been a barrier to understanding the underlying pharmacology for almost three

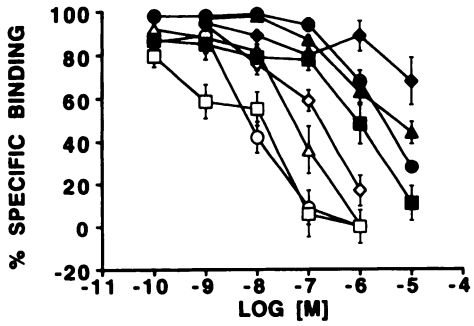

Fig. 6. Inhibition of ^{3}H-17-phenyl-PGF$_{2\alpha}$ specific binding to membranes from Swiss 3T3 cells by natural prostaglandins and synthetic prostaglandin analogs: symbols are as follows: PGF$_{2\alpha}$ (△), PGD$_2$ (◇), PGE$_2$ (●), sulprostone (▲), MB 28767 (■), fluprostenol (□), 17-phenyl-PGF$_{2\alpha}$ (○), AH 13205 (◆). Points represent mean values ± S.E.M., n = 3.

decades. The currently accepted classification for prostanoid receptors (Coleman *et al.*, 1982, 1984), although providing a real advance in understanding these receptors, does not adequately address some of the more complex phenomena. The contractile response of the rat colon provides such a case in which the potency rank order for natural PGs and their selective agonists markedly differs from any receptor subtype characterized by either traditional pharmacological approaches or cloning and expression of cDNA. Thus, in the rat colon, PGF$_{2\alpha}$ was the most potent natural PG but the rank order of potency PGF$_{2\alpha}$ > PGE$_2$ > PGD$_2$ was inconsistent with the PGF$_{2\alpha}$ > PGD$_2$ > PGE$_2$ rank order reported in FP receptor preparations (Coleman, 1993; Krauss *et al.*, 1993). Furthermore sulprostone, GR 63799X and MB 28767 were, respectively, only about 5, 7, and 23 times less potent than PGF$_{2\alpha}$ in the rat colon. This was not only inconsistent with an FP receptor preparation but also did not correlate with the high EP$_3$ receptor selectivity and potency of these PGE$_2$ analogs (Coleman *et al.*, 1982; Bunce *et al.*, 1991). The EC$_{50}$ values obtained for sulprostone and MB 28767 in the rat colon, however, did differ greatly from those at the Swiss 3T3 cell FP receptor where no activity was apparent until a 1 μM dose was attained. 11-Deoxy-PGE$_1$ exhibited contractile activity in the rat colon but did not stimulate the FP receptor in Swiss 3T3 cells at a 10 μM concentration, which further indicated a difference in responsiveness of these two preparations.

Radioligand binding studies with tritiated PGE$_2$ and 17-phenyl-PGF$_{2\alpha}$ provided results that supported the co-existence of EP$_3$ and FP receptors as independent entities in the rat colon. The rank order of potency for inhibition of ^{3}H-PGE$_2$ binding essentially correlated with relative potencies for functional activity in EP$_3$ receptor preparations (Coleman *et al.*, 1987; Ohia and Jumblatt, 1990; Hennies *et al.*, 1992). Moreover, the potency rank order for competition at ^{3}H-PGE$_2$ binding sites in the rat colon was similar to results obtained from binding studies with the cloned EP$_3$ receptor (Sugimoto *et al.*, 1992) and binding studies in cells and tissues containing functional EP$_3$-like receptors (Watanabe *et al.*, 1986; Sonnenberg *et al.*, 1990; Hennies *et al.*, 1992). The K_d value obtained for PGE$_2$ in this study was about half that reported for the canine renal outer medulla (Watanabe *et al.*, 1986) but two to four times higher than the K_d reported for the cloned mouse EP$_3$ receptor (Sugimoto *et al.*, 1992) and in EP$_3$-like receptors in cortical collecting tubule cells and gastric mucosa (Sonnenberg *et al.*, 1990; Tomoi *et al.*, 1990; Hennies *et al.*, 1992). PGE$_2$ was, however, more potent in eliciting second messenger or functional responses in these preparations (Coleman *et al.*, 1987; Hennies *et al.*, 1992; Sugimoto *et al.*, 1992) than in the contracting rat colon. Presumably, stimulus-response coupling associated with the EP$_3$ receptor in the rat colon is less efficient compared with most other biological systems.

In the absence of studies in which the FP receptor has been defined extensively by direct comparison of functional and radioligand binding studies in a single system, studies in Swiss 3T3 cells provided a useful reference framework for interpreting the results of 17-phenyl-PGF$_{2\alpha}$ binding experiments in the rat colon. The rank order of potency for inhibition of ^{3}H-17-phenyl-PGF$_{2\alpha}$ binding in plasma membrane preparations from the rat colon and Swiss 3T3 cells was similar: fluprostenol ≥ 17-phenyl-PGF$_{2\alpha}$ > PGF$_{2\alpha}$ > PGD$_2$ > PGE$_2$ ≥ MB 28767, sulprostone > AH 13205 (inactive at 1 μM). These competition studies support the separate identity of an FP receptor in the rat colon. Scatchard and Hill analyses and competition studies confirmed that PGE$_2$ and 17-phenyl-PGF$_{2\alpha}$ bind to individual single sites over a 0.25 to 100 nM dose range.

The FP receptor is described by the current classification for prostanoid receptors as having particular sensitivity to PGF$_{2\alpha}$. The stimulatory properties of PGD$_2$ and PGE$_2$ in preparations with particular sensitivity to PGF$_{2\alpha}$ has been ascribed to interaction with a single (FP) receptor (Coleman *et al.*, 1982, 1984). The results that we obtained by directly comparing Ca^{++} second messenger signaling and radioligand binding in Swiss 3T3 cells supports the FP receptor definition as proposed in the current classification (Coleman *et al.*, 1982, 1984). This definition is further underscored by our ability to identify the FP receptor in a tissue such as the rat colon, where the potency rank order for the contraction evoked by PGD$_2$, PGE$_2$, PGF$_{2\alpha}$ and their analogs provided no definitive indication of the underlying receptor pharmacology.

The functional response of the rat colon to PGD$_2$ probably involves stimulation of the FP receptor and not an EP receptor. No DP receptor involvement is indicated by the inactivity of the selective agonist BW 245C (Giles and Leff, 1988). A lack of DP receptor involvement in contractile responses of the rat colon is not surprising because this particular receptor is coupled positively to adenylate cyclase (Trist *et al.*, 1989). Although not indicated by these present studies in the rat colon, the possibility of an alternative subtype of DP receptor cannot be dismissed because PGD$_2$ can elicit certain effects such as increased DNA synthesis (Tsashita *et al.*, 1992), eosinophil chemotaxis and conjunctival goblet cell discharge (Woodward *et al.*, 1990), which do not occur in response to BW 245C. In these responses the signal transduction process does not seem to involve cAMP formation and involvement of those prostanoid receptors proposed in the current classification is difficult to envisage. Interestingly, PGJ$_2$ has been found to mimic certain effects of PGD$_2$ which would not involve cAMP as the second messenger (Narumiya and Toda, 1985; Woodward *et al.*, 1990). However, the very weak activity of PGJ$_2$ in contracting the rat colon argues against the involvement of such a PGD$_2$-sensitive receptor in this tissue.

In summary, our studies support the presence of both FP

and EP_3 receptors in the rat colon. Stimulation of both receptors results in contraction, although PGE_2 and selective EP_3 agonists were less potent myotropic agents in the rat colon compared with their activity in other preparations purported to contain the EP_3 receptor. These studies underscore the importance of radioligand binding for satisfactory characterization of receptors involved in functional responses to prostanoids. Based solely on functional rank order and the moderate potency of EP_3 agonists in the rat colon compared with other tissues, erroneous interpretations postulating subdivision of the FP receptor may have ensued.

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