The effects of prostaglandin E_2 are thought to be mediated via G protein-coupled plasma membrane receptors, termed EP receptors. However, recent data implied that prostanoids may also act intracellularly. We investigated if the ubiquitous EP_3 and the EP_4 receptors are localized in nuclear membranes. Radioligand binding studies on isolated nuclear membrane fractions of neonatal porcine brain and adult rat liver revealed the presence of EP_3 and EP_4. A perinuclear localization of EP_3 and EP_4 receptors was visualized by indirect immunofluorescence and confocal microscopy in porcine cerebral microvascular endothelial cells and in transfected HEK 293 cells that stably overexpress these receptors. Immunoelectron microscopy clearly revealed EP_3 and EP_4 receptors localization in the nuclear envelope of endothelial cells; this is the first demonstration of the nuclear localization of these receptors. Data also reveal that nuclear EP receptors are functional as they affect transcription of genes such as inducible nitric-oxide synthase and intranuclear calcium transients; this appears to involve pertussis toxin-sensitive G proteins. These results define a possible molecular mechanism of action of nuclear EP_3 receptors.

Prostaglandin E_2 (PGE_2) is one of the most abundant prostanoids in the brain (1) and plays an important role in many cerebral functions, particularly in the newborn (2). PGE_2 also influences mitogenesis (3), promotes growth and metastasis of tumors (4), and stimulates gene transcription (5). To date, the biological actions of PGE_2 have been attributed to result from its interaction with plasma membrane G protein-coupled receptors termed EP, which include EP_1, EP_2, EP_3, and EP_4 subtypes (6). Recent studies have shown that the nuclear membrane contains high levels of cyclooxygenase-1 and -2 and of PGE_2 (7). Possible intracellular sites of action for prostanoids are also suggested by other data. For example, a transporter that mediates the influx of prostanoid has been identified (8). Cytosolic phospholipase A_2 undergoes a calcium-dependent translocation to the nucleus envelope (9), and cyclooxygenase-2 has been shown to translocate to the nucleus in response to certain growth factors (10). It is thus possible that prostanoids may exert some of their effects via intracellular EP receptors, to have a direct nuclear action as recently proposed by Goetzl et al. (11), and Morita et al. (12).

It has generally been assumed that the signal transduction cascades are initiated at the plasma membrane and not the nuclear membranes. However, recent studies have disclosed that the nuclear envelope plays a major role in signal transduction cascades (13, 14). In fact, a novel nuclear lipid metabolism that is a part of unique nuclear signaling cascade termed NEST (nuclear envelope signal transduction) has been hypothesized (15). Both heterotrimERIC and low molecular weight G proteins (15, 16), phospholipase C (13), phospholipase D (15), and adenylate cyclase (17) have been shown to be localized at the nucleus. The nuclear membranes also have distinct inositol cycles (18) and receptors for 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate (13). Altogether these data raise the possibility of the presence of nuclear prostanoid receptors. This inference has recently been suggested for EP_3 (19), but whether or not this single observation is specific for this receptor or applies to other prostanoid receptors, especially of the widely distributed EP_3 and EP_4 subtypes, is unknown.

In the present study, we investigated the possible expression of nuclear EP_3 and EP_4 receptors using human embryonic kidney (HEK) 293 cells, porcine microvascular endothelial cells, newborn pig brain, and adult rat liver. We selected these tissues because many high affinity PGE_2 binding sites have been reported in the plasma membranes of pig brain (20) and rat liver (21). We focused on EP_3 receptors that are most ubiquitous of the four EP subtypes (6) and also examined localization of EP_3 receptors. Our data provide novel evidence for the existence of EP_3 and EP_4 receptors in the nuclear envelope and reveal that these receptors are functional, and their actions appear to involve pertussis toxin (PTX)-sensitive G proteins.

EXPERIMENTAL PROCEDURES

Materials—AH23848B was a gift from Glaxo-Wellcome, UK and M&B 28,767 from Rhone-Poulenc Rorer, UK. The following products were purchased: PGE_2; 17-phenyl trinor PGE_2 (Cayman Chemicals, Ann Arbor, MI); DAB in Eagle’s medium, Geneticin (Life Technologies, Inc., Burlington, ON); [3H]PGE_2 (182 Ci/mmol), 45Ca^2+ (2 mCi/49.5 μg of Ca), and [α-32P]ATP (3000 Ci/mmol) (Amersham Pharmacia Biotech, Mississauga, ON); all other chemicals were from Sigma.

Animals—Newborn pigs (1–3 days old) were killed with intracardiac pentobarbital under halothane anesthesia, and tissues of interest were
removed. Adult Sprague-Dawley male rats (250–300 g) were decapitated and had livers removed.

**Cell Culture**—HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Primary cultures of porcine cerebral endothelial cells from brain microvessels (20) were established as described previously (22).

**Preparation of Subcellular Fractions**—All steps were performed at 4 °C using solutions containing 1.1 mM acetylsalicylic acid, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride and 100 μg/ml soybean trypsin inhibitor. Nuclei were isolated from adult rat liver (23) and newborn porcine brain cortex (24). Endoplasmic reticulum (ER) was isolated as described previously (26). The purification of the cellular fractions was confirmed by the contamination by plasma membranes. Glucose-6-phosphatase (an ER marker) activity in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1M phosphate buffer, pH 7.5 was assayed as a marker for ER (26). Proteins were determined by the method of Lowry et al. (27) using bovine serum albumin as a standard.

**Nuclear Prostaglandin EP3 and EP4 Receptors**

**Immunoblotting of EP Receptors**—Western blotting of EP3, and EP4 receptors was conducted as described (28) on newborn brain nuclear and plasma membrane fractions. After immunoblotting using EP3- or EP4-specific polyclonal rabbit antibodies (29) (1:1000), immunoreactive bands were visualized by chemiluminescence (Amersham Pharmacia Biotech) as per manufacturer instructions.

**Saturation isotherms of specific binding of [3H]PGE2 to membrane fractions from newborn porcine brain cortex and endothelial cells (35), and aliquots (100 μg of protein) were incubated in the presence or absence of 0.1 μM EP, agonist, M&B 28,767, for 60 min at 37 °C in a total volume of 40 μl (per reaction tube) of 10 mM Tris-HCl buffer (pH 8.0) containing 5 mM MgCl2, 300 mM KCl, 0.5 mM each of ATP, CTP, GTP, and UTP, RNase guard (111 units), and DNase (10 units). RNA was extracted as described previously (28). For the isolation of total cytoplasmic RNA, cells were incubated in the presence or absence of test agents for 1 h and washed with ice-cold PBS. Nuclear and total RNA were applied to a nylon membrane using a vacuum filtration apparatus (36).

**RESULTS AND DISCUSSION**

**TABLE I**

| Tissue                  | Plasmalemmembranes | Endoplasmic reticulum | Nuclear membranes |
|-------------------------|--------------------|-----------------------|-------------------|
| **Brain**               |                    |                       |                   |
| B<sub>max</sub>         | 14.8 ± 2           | 12.2 ± 3.1            | 13.2 ± 2          |
| KD<sub>o</sub>          | 8.8 ± 1.5          | 7.2 ± 2.8             | 8.1 ± 1.2         |
| **Liver**               |                    |                       |                   |
| B<sub>max</sub>         | 30.2 ± 4.3         | ND                    | 25.7 ± 3.8        |
| KD<sub>o</sub>          | 6.2 ± 3.7          | ND                    | 7.1 ± 2.2         |

**RESULTS AND DISCUSSION**

**EP<sub>3</sub> and EP<sub>4</sub> Receptor Expression in HEK 293 Cells**—The full-length cDNA fragments corresponding to human EP<sub>3</sub> (30) and EP<sub>4</sub> (31) were cloned separately into the mammalian expression vector pRC-CMV (Invitrogen). HEK 293 cells (2 × 10<sup>5</sup>) were transfected with 2 μg of plasmid DNA and 8 μl of LipofectAMINE in Opti-MEM (Life Technologies, Inc.) according to the manufacturer instructions; Genetin (1 mg/ml) -resistant clones were selected and maintained in Dulbecco’s modified Eagle’s medium medium containing Genetin (0.2 mg/ml).

**Immunocytochemical Detection of EP Receptors**—The immunolocalization of EP receptors in HEK 293 and porcine cerebrovascular endothelial cells was performed by indirect immunofluorescence (32).

**Nuclear Calcium Signals and Uptake**—The uptake of Ca<sup>2+</sup> in isolated nuclei was determined as described previously (14). Briefly, nuclei were resuspended in buffer A (125 mM KCl, 2 mM K<sub>2</sub>HPO<sub>4</sub>, 2.5 mM Hepes, 4 mM MgCl<sub>2</sub>, and 400 mM CaCl<sub>2</sub>, pH 7.0). Ca<sup>2+</sup> (<sup>45</sup>Ca<sup>2+</sup>) was added, and samples were incubated in the presence or absence of test agents at 37 °C for different time periods. The reaction was terminated with ice-cold buffer containing 50 mM Tris-HCl and 150 mM KCl (pH 7.0), filtered under vacuum on glass fiber filters (GF/P, Whatman). The radioactivity on filters was counted on a beta-counter (Beckman LS 7500). The radioactive precursor was defined as the radioactivity at a given time minus the radioactivity at time zero.

**Effects of test agents on calcium transients**—Fluorescence in iso-
ability of specific anti-EP<sub>3</sub> and anti-EP<sub>4</sub> receptor antibodies (29) led us to focus our investigation on the cellular localization of EP<sub>3</sub> and EP<sub>4</sub> receptors, and the remaining studies concentrated on these two receptor subtypes.

**Expression of EP<sub>3</sub> and EP<sub>4</sub> Immunoreactive Protein in Newborn Brain Subcellular Fractions**—Immunoblot analysis revealed immunoreactive bands in plasma and nuclear fractions of similar molecular masses (EP<sub>3</sub>, 60 kDa; EP<sub>4</sub>, 63 kDa) (Fig. 2).

**Indirect Immunofluorescence of EP<sub>3</sub> and EP<sub>4</sub> Receptors in Porcine Cerebral Vessel Endothelial Cells**—Because cerebral microvessels contain a number of high affinity PGE<sub>2</sub> binding sites (20), primary cultures of newborn pig brain microvascular endothelial cells were used to study the intracellular distribution of EP<sub>3</sub> and EP<sub>4</sub> receptors by confocal microscopy. No fluorescence was detected in the absence of the primary antibodies (Fig. 3a). Immunoreactivity for both receptor subtypes was detected in the plasma membrane (Figs. 3b and 4a), in the cytoplasm, and at the nucleus (Figs. 3c and 4b). EP<sub>3</sub> specific fluorescence in the nuclear envelope appeared as a perinuclear halo (Fig. 3c); the latter was more prominent than that of EP<sub>4</sub> receptors (Fig. 4b). The cells were stained with DiOC<sub>6</sub> (3) to identify intracellular membranes, mainly ER (Figs. 3d and 4c). Merging the images from EP<sub>3</sub> or EP<sub>4</sub> specific red immunofluorescence with DiOC<sub>6</sub> (3) green staining revealed that EP<sub>3</sub> receptors colocalized on intracellular membranes as indicated by the bright yellow-orange fluorescence (Fig. 3e and 4d); however, the stains did not fully converge, suggesting distinct sites particularly evident in the perinuclear region. Cells were also stained with a nuclear stain, Sytox Green (Fig. 3g). A transverse section (Z-section) of this image superimposed with that of the EP<sub>3</sub> immunoreactive staining (Fig. 3h) in the same cell revealed that the immunoreactivity was perinuclear and not intranuclear (Fig. 3i); similar results were obtained using the EP<sub>4</sub> antibody (data not shown). No immunofluorescence was detected when the antibodies were preincubated with their cognate peptides (Figs. 3f and 4e). EP<sub>3</sub> and EP<sub>4</sub> immunoreactivity in co-localization with DiOC<sub>6</sub> (3) staining apparently in the nucleoli of a few endothelial cells (Figs. 3, c–e and 4, b–d) was not consistently observed (Fig. 3i) and remains unexplained at this point.

**Indirect Immunofluorescence of EP<sub>3</sub> and EP<sub>4</sub> Receptors in**

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**FIG. 1.** Competitive displacement of [3H]PGE<sub>2</sub> binding to plasma and nuclear membrane from newborn pig brain and adult rat liver by prostaglandins and analogs. Shown are brain plasma membrane (a), nuclear membrane (b), liver plasma membrane (c), and nuclear membrane (d). Membranes were incubated with 10 nM [3H]PGE<sub>2</sub> at 37°C for 30 min in the presence or absence of 25 μM unlabeled PGE<sub>2</sub> to determine 100% specific binding. ■, PGE<sub>2</sub>; □, AH6809 (EP<sub>1</sub> antagonist); ●, butaprost (EP<sub>2</sub> agonist); ○, M&B 28,767 (EP<sub>4</sub> agonist); ▼, AH2384B (EP<sub>4</sub> antagonist). Each data point is the mean ± S.E. of four experiments performed in duplicate.

**FIG. 2.** Immunoblot of EP<sub>3</sub> and EP<sub>4</sub> receptor proteins (see arrows) in plasma membrane (P) and nuclear membrane (N) fractions from newborn pig brain. Top and bottom arrows point to EP<sub>3</sub> and EP<sub>4</sub> bands, respectively; only one band was detected in the range of interest (50–65 kDa).

**FIG. 3.** Confocal microscopy of porcine cerebral microvascular endothelial cells immunofluorescently stained for EP<sub>3</sub> receptors. Cells were subjected to indirect immunofluorescent staining using affinity purified rabbit anti-peptide EP<sub>3</sub> antibodies followed by Texas Red-conjugated anti-rabbit IgG. a, Texas Red-conjugated anti-rabbit IgG alone; b, anti-EP<sub>3</sub> antibody (data not shown). No immunofluorescence was detected when the antibodies were preincubated with their cognate peptides (Figs. 3f and 4e). EP<sub>3</sub> and EP<sub>4</sub> immunoreactivity in co-localization with DiOC<sub>6</sub> (3) staining apparently in the nucleoli of a few endothelial cells (Figs. 3, c–e and 4, b–d) was not consistently observed (Fig. 3i) and remains unexplained at this point.
HEK 293 Cells—To assess whether this perinuclear distribution of EP₃ and EP₄ receptors applies generally to cells, the localization of these receptors was studied after transfection of cDNA for EP₃ and EP₄ into HEK 293 cells that do not normally express prostanoid receptors (39); ectopically expressed EP receptors in HEK 293 cells bind PGE₂ and are functional (39, 40). Immunoreactivity for EP₃ and EP₄ receptors was seen on the plasma membrane (Fig. 5, c and g) and perinuclear area, which are in proximity to each other in the transfected HEK 293 cells (Fig. 5d, h), which are relatively small and contain limited cytoplasm compared with endothelial cells. As expected, no immunofluorescence was detected in the wild-type cells (Fig. 5a) or after preincubation of the antibodies with their cognate peptide epitopes (Fig. 5 f and j).

Immunogold Labeling of EP₃ and EP₄ Receptors—Thus far, indirect immunofluorescence studies revealed a perinuclear localization of EP₃ and EP₄ receptors. To distinguish the nuclear envelope, immunoelectron microscopy of porcine cerebrovascular endothelial cells was performed and confirmed that EP₃ and EP₄ immunoreactivity was indeed at the nuclear envelope (Fig. 6, c and f). As expected, these receptors were detected on plasma membranes (Fig. 6 b and e) and Golgi vesicles (Fig. 6d). No immunogold staining was observed in the absence of the primary antibodies (Fig. 6a) or in the nuclei of cells (data not shown). EP₃ and EP₄ nuclear envelope immunogold staining was detected in the majority of cells observed (95% of cells, over 100 cells observed in each case).

Effects of Stimulation of Porcine Cerebrovascular Endothelial Cells Nuclear EP Receptors on iNOS Gene Transcription—Recent studies have shown that endogenous PGE₂ has a stimulatory effect on inducible nitric-oxide synthase (iNOS) (41, 42). We tested whether the stimulation of nuclear EP receptors by prostaglandin analogs may affect iNOS transcription, as determined by dot hybridization of RNA studies. Stimulation of intact nuclei isolated from primary cultures of porcine brain endothelial cells with EP₃ receptor agonist M&B 28,767 (0.1 mM) increased transcription of iNOS (Fig. 7a) to a greater extent than after stimulation of whole cells.

Effects of Stimulation of Rat Liver Nuclear EP Receptors on Ca²⁺ Transients—The nuclear envelope contains distinct nuclear calcium pools that play crucial roles in major nuclear
functions including gene transcription (13). The amplitude and
duration of calcium signals have been shown to control differen-
tial activation of transcription factors (43). In addition, Ca^{2+}
can activate iNOS independent of protein kinases C and A (44).
We tested whether stimulation of nuclear EP_3 receptors with
prostaglandin analogs could affect calcium concentrations in
isolated nuclei of liver; stimulation of EP_3 receptors could not
be performed because of lack of availability of specific agonists.
Application of M&B 28,767 (1 μM, an EP_3-selective agonist) to
intact isolated nuclei caused rapid nuclear uptake of ^45Ca^{2+}
(Fig. 7b). In addition, this EP_3 agonist produced a dose-depen-
dent increase in rat liver nuclear calcium as determined by
fura-2/AM, a fluorescent dye which localizes in the nuclear
envelope space (45) (Fig. 7c); M&B 28,767 (1 μM) was nearly as
effective as the nonelective EP agonist 16,16-dimethyl PGE_2
(1 μM).

We determined whether the nuclear calcium uptake evoked
by the EP_3 agonist M&B 28,767 was dependent on G proteins.
EP_3 couples to G_i or G_o (46), which are known to affect Ca^{2+}
mobilization (46, 47); such G proteins are detected in rat liver
nuclei (38). Because PTX causes these G proteins to lose their
ability to associate with receptors, we tested the effects of PTX
on M&B 28,767-induced Ca^{2+} transients. Pretreatment of iso-
lated nuclei with PTX markedly attenuated the stimulatory
effect of M&B 28,767 (1 μM) on intranuclear calcium levels,
suggesting the involvement of a PTX-sensitive G protein in
mediating the effects of nuclear EP_3 receptors (Fig. 7d). In
contrast, M&B 28,767 did not inhibit forskolin-stimulated
CAMP formation (data not shown). These findings are consist-
ent with coupling of nuclear EP_3 receptors to G proteins which
may directly control Ca^{2+} channels independently of second
messengers, as mostly reported for G_i (47, 48).

In conclusion, the data presented provides the first clear
evidence for the presence of the G protein-coupled receptors,
EP_3 and EP_4 at nuclear membranes of native tissues as well
as primary and transfected cells. Furthermore, these receptors
seem to be functional as revealed by increased iNOS transcrip-
tion and nuclear calcium by EP_3 agonist, M&B 28,767, which
also appears to involve PTX-sensitive G proteins. The plasma
and nuclear membrane EP_3, as well as EP_4 receptors appear
to be related because they had similar molecular weights, binding
kinetics, ligand binding properties, and immunoreactivity.
Also, the comparable distribution of ectopically expressed EP_3,
and EP_4 receptors in HEK 293 cells suggested that the plasma
and nuclear membrane EP receptors may be alike. Radioligand
binding studies have identified the presence of two other
classes of G protein-coupled receptors at the nuclear mem-
brane, the muscarinic acetylcholine (49) and angiotensin II
receptors, AT_1 (23); but the AT_1 receptor can be detected in the
nucleus only after stimulation by angiotensin II (50). Other
prostanoids, namely PGD_2, its metabolite PGJ_2, and PGI_2
can activate the peroxisome proliferator-activated receptors
(PPARs) that are members of the nuclear receptor superfamily,
but PPARs are not responsive to PGE_2 (51, 52). However, the
presence of EP_1 receptors at the nuclear membranes has re-
cently been suggested (19) albeit its mechanism of action is not
clear.

In the newborn brain and cerebral microvasculature, PGE_2
receptors and associated functions at the plasma membrane
are down-regulated (2, 20). On the other hand, PGE₂ plays a role in neuroprotection by acting on EP₃ and perhaps EP₄ (53). PGE₂ also increases the expression of nitric-oxide synthase via stimulation of EP₃ receptors in the neonate (54). In addition, the perinuclear cyclooxygenase-1 and -2 (7) can produce PGE₂ also increases the expression of nitric-oxide synthase via stimulation of EP₃ receptors in the neonate (54). In addition, the perinuclear cyclooxygenase-1 and -2 (7) can produce prostanooids that can act at the nuclear level (11, 12) and modulate transcription of genes, as had been speculated for iNOS (41). The present discovery of nuclear EP₃ and EP₄ receptors proposes new avenues for the intracellular actions of prostanooids, which may also explain certain effects of PGE₂ especially when plasma membrane EP receptors are barely detectable. Further studies are needed to clarify the detailed signal transduction mechanisms involved in this action of prostaglandins via nuclear EP receptors.

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