Immunology. In the article “Absence of tumor necrosis factor resuces RelA-deficient mice from embryonic lethality” by Takahiro S. Doi, Michael W. Marino, Toshitada Takahashi, Yoshinori Yoshida, Teruyo Sakakura, Lloyd J. Old, and Yuichi Obata, which appeared in number 6, March 16, 1999, of Proc. Natl. Acad. Sci. USA (96, 2994–2999), the following corrections should be noted:

In Table 1, page 2995, line 1 of the title, the second sentence, “Results of matings of TNF+/+ or TNF−/− mice with relA+/+ mice,” should be removed.

In Table 2, page 2997, line 2, right column, the genotype of the male parent which currently reads “TNF+/−relA+/−” should read “TNF−/−relA+/−.”

In Table 2, page 2997, line 3, right-most column, the genotype of the offspring which currently reads “−−+/−−relA+/−” should read “TNF+/−relA+/+.”

In line 2 of Discussion, page 2997, the sentence that reads “TNF mediates this abnormality and the associated lethality, as viable RelA-deficient progeny without liver damage result from matings of TNF-deficient RelA-heterozygous mice with RelA-deficient mice” should read “TNF mediates this abnormality and the associated lethality, as viable RelA-deficient progeny without liver damage result from matings of TNF-deficient RelA-heterozygous mice.”

Medical Sciences. In the article “RB-mediated suppression of spontaneous multiple neuroendocrine neoplasia and lung metastases in Rb+/− mice” by Alexander Yu. Nikitin, María I. Juárez-Pérez, Song Li, Leaf Huang, and Wen-Hwa Lee, which appeared in number 7, March 30, 1999, of Proc. Natl. Acad. Sci. USA (96, 3916–3921), the following correction should be noted. The labeling of the gel in Fig. 10 was erroneously moved down and to the right. The corrected figure and its legend are shown below.

Immunology. In the article “Ubiquitin-dependent degradation of 1xGlu is mediated by a ubiquitin ligase Skp1/Cull1/F-box protein FWD1” by Shigetsugu Hatakeyama, Masatoshi Kitagawa, Keiko Nakayama, Michiko Shirane, Masaki Matsumoto, Kimihiko Hattori, Hideaki Higashi, Hiroyasu Nakano, Ko Okumura, Kazunori Onoé, Robert A. Good, and Kei-ichi Nakayama, which appeared in number 7, March 30, 1999, of Proc. Natl. Acad. Sci. USA (96, 3859–3863), due to printer’s error, the following changes should be noted. On page 3859 in the abstract, text, and footnote, the word “Cull 1” should be “Cull 1.”

Biophysics. In the article “High-resolution NMR of encapsulated proteins dissolved in low-viscosity fluids” by A. Joshua Wand, Mark R. Ehrhardt, and Peter F. Flynn, which appeared in number 26, December 22, 1998, of Proc. Natl. Acad. Sci. USA (95, 15299–15302), the term “high pressure NMR” was incorrectly printed as “HPLC NMR,” due to a printer’s error.

**FIG. 1.** Multiple neuroendocrine neoplasia in Rb+/− mice. (A and B) Gross compound pituitary tumor on P394. The tumor consists of two histologically distinct components that substitute for the pituitary anterior (AL) and intermediate (IL) lobes, which are demarcated by remnants of Rathke’s cleft (arrows). AL tumor cells contain α-GSU (A) and are positioned loosely around sinusoid-like vessels. IL tumor cells contain α-melanocyte-stimulating hormone (B) and form poorly vascularized epithelioid fields with central necrotic and hemorrhagic areas. (C and D) EAP in the anterior pituitary lobe on P90 before (C) and after (D) microdissection for genotype analysis by the PCR. (E) C-cell carcinoma on P340 showing the typical arrangement of polygonal tumor cells in solid nests and rough hyalinized collagen with calcification (arrow). (F and G) C-cell EAP (arrow) on P60 before (F) and after (G) microdissection. The atypical cells show a paraffocallicular location. (H) Medullary thyroid carcinoma invading surrounding tissues on P469. Accumulation of calcitonin (brown color) is evident in the cytoplasm of tumor cells (arrow). CR, cartilage. (I) Calcitonin-containing metastatic cells in the lung on P463. The metastatic cells exhibit intraalveolar spreading (arrows). (J) A well-vascularized parathyroid tumor (PG) and a neighboring solid C-cell tumor of the thyroid gland (TG) on P370. (K) Parathyroid hormone expression in parathyroid tumor cells. (L) Phaeochromocytoma of the adrenal medulla (AM) compressing the adrenal cortex (AC). (M and N) EAP in the adrenal medulla (AM) on P60 before (M) and after (N) microdissection. The arrow indicates multiple apoptotic figures. Staining: avidin-biotin-peroxidase immunostaining for α-GSU (4), α-melanocyte-stimulating hormone (B), calcitonin (H and I), or parathyroid hormone (K) with hematoxylin counterstaining; (C–G, J, and L–N) hematoxylin-eosin staining. [Bar: 160 μm (A and B), 40 μm (C, D, and I), 110 μm (E), 60 μm (F and G), 50 μm (H), 150 μm (J), and 20 μm (K), 390 μm (L), and 70 μm (M and N).] (O) Absence of the wild-type Rb allele (151-bp PCR product) in gross tumors (T; lanes 3, 4, 7, 10, 11, 17, and 18) and EAPs (E; lanes 1, 2, 6, 8, 9, 14, and 16) of the pituitary anterior lobe (AL, lanes 1–4), the parathyroid gland (PG, lanes 6 and 7), thyroid C cells (TG, lanes 8–11), lung metastases (L, lanes 12 and 13), or adrenal medulla (lanes 14 and 16–18). N, Rb+/− normal tissue (lanes 5 and 15). Nondenaturing 12% polyacrylamide gel stained with silver. The 236-bp band corresponds to the mutant Rb allele (11).
Pharmacology. In the article “Nuclear localization of prostaglandin E2 receptors” by Mousumi Bhattacharya, Krishna G. Peri, Guillermina Almazan, Alfredo Ribeiro-da-Silva, Hitoshi Shichi, Yves Durocher, Mark Abramovitz, Xin Hou, Daya R. Varma, and Sylvain Chemtob, which appeared in number 26, December 22, 1998, of Proc. Natl. Acad. Sci. USA (95, 15792–15797), the paragraph on page 15793, left column, should read as follows (change indicated in italic type): “Indirect Immunofluorescence of EP1 Receptors. For examining the immunolocalization of EP1 receptors, immunocytochemistry was performed as described (21) on Swiss 3T3, HEK293 (EBNA), or endothelial cells with rabbit anti-EP1 antibodies (22) and FITC-conjugated or Texas red-conjugated anti-rabbit IgG (Bio/Can Scientific, Mississauga, ON), diluted 1:50.” In addition, because Figs. 6 and 7 were printed with markedly poor quality, they and their legends are reproduced below.

**Fig. 6.** Immunoperoxidase and immunogold localization of EP1 in porcine endothelial cells detected by electron microscopy (arrows). (a) Immunoperoxidase-IgG alone; note absence of immunostaining when primary antibody is omitted. (b) A low magnification showing immunostaining in plasma membrane and nuclear envelope. (c) A higher magnification showing immunostaining in the nuclear envelope. (d) Immunogold-IgG alone; note absence of immunostaining. Specific immunostaining can be observed in the plasma membrane in e, nuclear envelope in f, and Golgi apparatus in g. (Bar = 0.5 μm, except in b = 2 μm.)

**Fig. 7.** Immunoperoxidase localization of EP1 in adult rat brain cortex by electron microscopy (arrows). Specific immunostaining observed in plasma membrane and inner and outer nuclear membranes of capillary endothelial cell (a) and nuclear membranes of neurons (b). Note the luminal space of capillary on right in a. (Bars = 0.5 μm.)
Microbiology. In the article “Molecular and biophysical characterization of TT virus: Evidence for a new virus family infecting humans” by Isa K. Mushahwar, James C. Erker, A. Scott Muerhoff, Thomas P. Leary, John N. Simons, Larry G. Birkenmeyer, Michelle L. Chalmers, Tami J. Pilot-Matias, and Suresh M. Desai, which appeared in number 6, March 16, 1999, of Proc. Natl. Acad. Sci. USA (96, 3177–3182), the following correction should be noted. The last author’s name has been misspelled, and it should be amended as Suresh M. Desai and not Suresh M. Dexai.

Physiology. In the article “Parathyroid hormone leads to the lysosomal degradation of the renal type II Na/Pi cotransporter” by Markus F. Pfister, Isabelle Ruf, Gerti Stange, Urs Ziegler, Eleanor Lederer, Jürg Biber, and Heini Murer, which appeared in number 4, February 17, 1998, of Proc. Natl. Acad. Sci. USA (95, 1909–1914), the authors wish to note that the Acknowledgment section should have included the following grant information: “Dr. Lederer is an employee of and her work is supported by a grant from the U.S. Department of Veterans Affairs.”
Nuclear localization of prostaglandin E$_2$ receptors

MOUSUMI BHATTACHARYA*, KRISHNA G. PERI†, GUILLERMINA ALMAZAN*, ALFREDO RIBEIRO-DA-SILVA*, HITOSHI SHICHI‡, YVES DUROCHER§, MARK ABRAMOVITZ§, XIN HOU†, DAYA R. VARMA*, and SYLVAIN CHEMTOB†**

ABSTRACT Prostaglandin E$_2$ receptors (EP) were detected by radioligand binding in nuclear fractions isolated from porcine brain and myometrium. Intracellular localization by immunocytofluorescence revealed perinuclear localization of EPs in porcine cerebral microvascular endothelial cells. Nuclear association of EP$_1$ was also found in fibroblast Swiss 3T3 cells stably overexpressing EP$_1$ and in human embryonic kidney 293 (Epstein–Barr virus-encoded nuclear antigen) cells expressing EP$_1$ fused to green fluorescent protein. High-resolution immunostaining of EP$_1$ revealed their presence in the nuclear envelope of isolated (cultured) endothelial cells and in situ in brain (cortex) endothelial cells and neurons. Stimulation of these nuclear receptors modulates nuclear calcium and gene transcription.

Prostanoids are present in all mammalian tissues and exert a wide variety of actions via G protein-coupled receptors (1). Prostaglandin E$_2$ (PGE$_2$), a major brain prostanoid, has been implicated in various cerebral functions during development (2). PGE$_2$ acts on prostaglandin E receptors EP$_1$, EP$_2$, and EP$_3$; EP$_4$s are found in most tissues and are abundant in the uterus (3) and brain (4). High levels of perinatal prostaglandins arising mostly from cyclooxygenase-2 (5) were established in the uterus (3) and brain (4). High levels of perinatal prostaglandins arising mostly from cyclooxygenase-2 (5) were shown to result in down-regulation of plasma membrane EPs and to reduce their functional responses to barely detectable levels in the newborn neural and neuropoietic tissue (2, 4); however, the actions of PGE$_2$ in the preservation of neural function (6) and in gene transcription (7) appeared to be unaffected. Based on the predominant localization of cyclooxygenase-2 in the perinuclear envelope, it was suggested that prostanoids could act at or near their site of synthesis (8). Furthermore, a transporter that facilitates the inward movement of prostanoids recently has been cloned and characterized (9). These observations suggest possible intracellular sites of action for prostanoids. The presence of other G protein-coupled receptors on nuclei has been suggested for muscarinic (10) and angiotensin (11) receptors. Also, PGI$_2$, its metabolite PGI$_2$O, and PGE$_2$, but not PGE$_2$ or PGI$_2$, can activate the peroxisome proliferator-activated receptors, which are members of the nuclear receptor superfamily, which includes steroid hormones (12, 13).

These observations favor the possibility that some effects of PGE$_2$ may be mediated by EPs other than those found in the plasma membrane. We searched for the presence of nuclear EPs in a variety of cells and tissues from various species by using several experimental approaches. Our results provide evidence for the ultrastructural localization of a G protein-coupled receptor such as EP$_1$ in the nuclear envelope of cells in culture and in situ in brain cortex; data also reveal that these receptors are functional.

MATERIALS AND METHODS

Cell Culture. Murine Swiss 3T3 cells (American Type Culture Collection) were cultured in DMEM with 10% fetal calf serum. Human embryonic kidney (HEK293) cells (Epstein–Barr virus-encoded nuclear antigen, EBNA) (Invitrogen) were grown in hybridoma serum-free medium (HSFM) with 1% bovine calf serum. Primary cultures of porcine cerebral endothelial cells from brain microvessels (4) were established as described (14).

Materials. AH6809 and AH23848B were gifts from Glaxo Wellcome, Stevenage, U.K., M&B 28,767 was a gift from Rhone-Poulenc Rorer, Dagenham, U.K., and Butaprost was a gift from Miles. The following products were purchased: PGE$_2$ and 17-phenyltrinor PGE$_2$ (Cayman Chemicals, Ann Arbor, MI); DMEM, HSFM, and geneticin (GIBCO/BRL); fetal calf serum, and goat serum (Jackson ImmunoResearch Laboratories). Other chemicals were from Sigma.

Animals. Newborn pigs (1–3 days old) were killed with pentobarbital (i.c.) and tissues were removed. Tissues from adult pigs were obtained from a local abattoir.

Preparation of Subcellular Fractions. All solutions contained 1.1 mM acetylsalicylic acid, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 100 μg/ml soybean trypsin inhibitor. The details for cell fractionation methods were described (11, 15, 16). Nuclei and nuclear envelopes were isolated from porcine adult myometrium (11) and newborn brain cortex (15), and endoplasmic reticulum (ER) also was isolated (16). The purity of fractions was verified by enrichment of marker enzymes 5'-nucleotidase for plasma membrane (11) and glucose-6-phosphatase for ER (17). Protein content was determined by Bio-Rad assay.

Radioligand Binding to Subcellular Fractions from Brain and Myometrium. Saturation binding of [H]$^{1}$PGE$_2$ and [H]$^{1}$PGD$_2$ to membranes, time course of association and dissociation experiments, and displacement of [H]$^{1}$PGE$_2$ by receptor isoform-specific ligands were performed as reported (4). Receptor densities (B$_{max}$), affinity (K$_D$), and association (k$_a$) and dissociation (k$_d$) constants were determined from saturation isotherms (Prism, GRAPHPAD; LIGAND; ref. 18).

EP, Receptor Expression in Swiss 3T3 Cells. The human EP$_1$ receptor cDNA [HindIII–XbaI fragment (19)] was cloned into the mammalian expression vector pRC-cytomegalovirus (pRC-CMV; Invitrogen). This EP$_1$/pRC-CMV plasmid was

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transfected into Swiss 3T3 cells by using the calcium phosphate method (20), and genetin (1 mg/ml)-resistant clones were selected. A clone was selected that expressed 2- to 3-fold more EP1 protein than cells transfected with vector alone (as judged by Western blotting; data not shown) and routinely cultured with genetin.

Indirect Immunofluorescence of EP1. For examining the immunolocalization of EP1 receptors, immunocytochemistry was performed as described (21) on Swiss 3T3, HEK293 (EBNA), or endothelial cells with rabbit anti-EP2 antibodies (22) and fluorescein isothiocyanate (FITC)-conjugated or Texas red-conjugated anti-rabbit IgG (Bio/Can Scientific, Montreal) diluted 1:50. As a negative control, primary antibody was omitted or used with its cognate peptide (22). Intracellular membranes, mostly ER, were stained by using 3,3′-dihexylocarbocyanine iodide [DiOC3(3)], and nuclei were stained with either 4′,6-diamidino-2-phenylinodole (DAPI), sytox green, or propidium iodide as per instructions of the manufacturer (Molecular Probes).

Expression of EP1–Green Fluorescent Protein (GFP) Fusion Protein in HEK293(EBNA) Cells. A BamHI–Xhol cDNA fragment encoding the full-length EP1 (19) was cut at position 1,174 with FspI, resulting in the deletion of the 10 C-terminal amino acids (EP1ΔCt). The cDNA encoding the GFP S65T (23) was obtained by PCR amplification using primers containing an in-frame EcoRV site at a Xhol site at the 5′ and 3′ ends, respectively. GFP was ligated in-frame to the C terminus of EP1ΔCt in pcDNA3 expression vector (Invitrogen) linearized with BamHI and Xhol. Transient transfections were done for 6 h in 6-well plates seeded at 10⁵ cells per well in 1.0 ml of OptiMem and transfected with 0.2 ml of OptiMem containing 750 ng of plasmid and 7.5 μl of lipofectamine. An equal volume of HSFM containing 2% bovine calf serum was added, and cells were incubated overnight. Cells were fixed with acetone/methanol (1:1) and viewed by using confocal microscopy.

Electron Microscopic Detection of EP1. Pre-embedding immunoperoxidase and immunogold staining methods (24, 25) were used for localization of EP1 in cells and tissues. Porcine-brain endothelial cells were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde, permeabilized with 0.2% Triton X-100 for 15 min at room temperature, and incubated with anti-EP1 antibodies (1:50) overnight at 4°C. The immunoperoxidase reaction was performed by using a VectaStain ABC kit (Vector Laboratories) as per the manufacturer’s instructions, and the diaminobenzidine reaction product was intensified (25). Ultrathin sections were examined with a transmission electron microscope (Philips 410; Eindhoven, The Netherlands). For immunogold staining (24), cells were fixed and incubated with anti-EP1 antibodies (1:25), followed by incubation overnight with an IgG conjugated to 1-nm gold particles diluted 1:50 (Amersham), followed by silver intensification with a silver enhancement kit (Amersham).

For detection of nuclear EP1, in vivo (25), adult rats (Sprague–Dawley) were perfused with 4% paraformaldehyde and 0.2% glutaraldehyde, and brain cortices were subjected to rapid freeze/thawing to improve penetration of immunoreagents before Vibratome 1000 (Technical Products International, St. Louis) sectioning. Permeabilization was further enhanced by treating sections with 50% ethanol for 30 min before incubating with anti-EP1 antibodies (diluted 1:10) for 48 hr at 4°C. Subsequent steps of the technique are described above.

Nuclear Calcium Measurements. Nuclear calcium was measured as described (14, 26) with some modifications. Isolated myometrium nuclei were resuspended in buffer (125 mM KCl/2 mM K₂HPO₄/25 mM Hepes/4 mM MgCl₂/0.4 mM CaCl₂, pH 7.0) and preloaded with 7.5 μM fura-2-acetoxymethyl for 45 min at 4°C. The nuclei were washed and stimulated (∼2 × 10⁶ nuclei per ml) with 17-phenyltrinor PGE₂ with and without AH6809 (10 μM). The intranuclear calcium concentration was measured with a spectrofluorometer (LS 50, Perkin–Elmer). Calibration of fluorescent signal was determined (14).

Dot Hybridization of RNA. Nuclei were isolated from Swiss 3T3 cells (27). Nuclei (100 μg of protein) were incubated with or without EP1 agonist, 17-phenyltrinor PGE₂ (0.1 μM) in a total volume of 40 μl for 60 min at 37°C in a 10 mM Tris-HCl buffer (pH 8.0) containing 5 mM MgCl₂/300 mM KCl/0.5 mM each ATP, CTP, GTP, UTP; 111 units of RNase guard; and 10 units of DNase per reaction tube. RNA was extracted (5). For the isolation of total cytoplasmic RNA, cells were incubated with or without test agents for 1 hr and washed with ice-cold PBS. Nuclear and total RNA were applied to a nylon membrane by using a vacuum-filtration apparatus (28). 32P-labeled cDNA probes for murine c-fos (29) and β-actin (Ambion) were prepared by using an oligolabeling kit (Pharmacia); unincorporated nucleotides were removed by G-25 column chromatography. Membranes were hybridized to the radiolabeled probes and washed (28). The bands were visualized and quantified by using PhosphorImaging (Molecular Dynamics).

RESULTS AND DISCUSSION

[3H]PGE₂ Binding to Nuclear Membranes from Porcine Brain and Myometrium. [3H]PGE₂ binding was performed on subcellular fractions of homogenates from porcine newborn brain cortex and adult myometrium. Fraction 1 contained plasma membranes, fraction 2 contained cytosol, fraction 3 contained ER, and fraction 4 contained nuclei, nuclear membranes, and some ER. Specific [3H]PGE₂ binding reached equilibrium within 20 min and remained stable for another 25–30 min and was saturable and reversible as described (5). Association (k₁) and dissociation (k₂) constants for plasma-membrane fractions were 0.15 ± 0.04 min⁻¹ and 0.03 ± 0.01 min⁻¹, respectively, and for nuclear fractions were 0.09 ± 0.03 min⁻¹ and 0.04 ± 0.01 min⁻¹, respectively. The affinity constants for PGE₂ binding were comparable in myometrium (6.3 ± 1.4 nM and 8.7 ± 2.6 nM for plasma-membrane and nuclear fractions, respectively) and newborn pig brain (9 ± 1 nM and 8.5 ± 1.2 nM for plasma-membrane and nuclear fractions, respectively). Maximum specific PGE₂ binding was highest in the plasma membrane and undetectable in cytosol of adult myometrium (Table 1). In newborn brain, PGE₂ binding was comparable in fractions 1, 3, and 4. PGE₂ binding in nuclear fraction is not the result of contamination by plasma membranes, as indicated by negligible S' nucleotidase (a plasma membrane marker) activity (220 ± 13.7 and 7.0 ± 0.2 units per mg of protein in plasma-membrane and nuclear fractions, respectively). PGE₂ binding to nuclear envelope and to intact nuclei was comparable; nuclear extracts devoid of nuclear membranes did not bind PGE₂ (data not shown).

Unlike the distribution of PGE₂ binding in various fractions derived from both brain and myometrial tissue, PGD₂ specific binding was minimal-to-undetectable in fraction 3 (ER) derived from both brain and myometrium, whereas nuclear membrane (fraction 4) from brain but not from myometrium displayed PGD₂ binding (Table 1). As expected, ER contiguously:

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PG</th>
<th>PM</th>
<th>ER</th>
<th>NM</th>
<th>CYT</th>
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<tbody>
<tr>
<td>Uterus</td>
<td>PGE₂</td>
<td>58 ± 4</td>
<td>37 ± 4</td>
<td>23 ± 3</td>
<td>ud</td>
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<tr>
<td></td>
<td>PGD₂</td>
<td>36 ± 3</td>
<td>2 ± 0.4</td>
<td>ud</td>
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<tr>
<td>Brain</td>
<td>PGE₂</td>
<td>13 ± 3</td>
<td>13 ± 1</td>
<td>12 ± 3</td>
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<tr>
<td></td>
<td>PGD₂</td>
<td>21 ± 1</td>
<td>ud</td>
<td>15 ± 1</td>
<td>nd</td>
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Values (Bₘₐₓ in fmol/mg protein) are the mean ± SEM of three experiments performed in duplicate. PM, plasma membrane; NM, nuclear membrane; CYT, cytosol; ud, undetectable (<0.1 fmol/mg protein); nd, not determined.
ous with the outer nuclear membrane (30) was found in fraction 4, as indicated by glucose-6-phosphatase (ER marker) specific activity (9.5 \pm 3.2, 23.1 \pm 2.2, and 19.2 \pm 1.4 \text{nmmol of PO}_4 \text{ released per mg of protein in fractions 1, 3, and 4, respectively). Hence, prostaglandin binding detected in nuclear membranes cannot be simply caused by contamination by the ER.

Displacement of [\text{3H}]PGE\text{2} by receptor subtype ligands, AH6809 (EP\text{1} antagonist), butaprost (EP\text{2}-selective agonist), M&B 28,767 (EP\text{3}-selective agonist), and AH23848B (EP\text{4} antagonist) revealed the presence of all EP subtypes in both plasma-membrane and nuclear-membrane fractions of myometrium, albeit EP\text{3} was the most abundant (50%; Fig. 1\text{a} and \text{b}). In plasma-membrane fractions of newborn brain, EP\text{3} accounted for all EP receptors (Fig. 1\text{c}), whereas in nuclear membranes, EP\text{3} comprised 45\% of total EPs, and the balance was evenly distributed among EP\text{1}, EP\text{2}, and EP\text{4} (Fig. 1\text{d}).

**Intracellular Localization of EP\text{1} in HEK 293(EBNA) Cells.**

There are many isoforms of EP\text{3} (31); because specific antibodies or selective pharmacological ligands for these isoforms are not available, we did not conduct further studies of this receptor. On the other hand, the clear displacement of bound [\text{3H}]PGE\text{2} by EP\text{1} antagonist AH6809 on nuclear fractions of two distinct tissues, brain and myometrium (Fig. 1\text{b} and \text{d}), and the availability of specific anti-EP\text{1} antibodies (22) led us to focus our investigation on the cellular localization of EP\text{1} receptors. In HEK 293(EBNA) cells, which do not naturally express EP\text{1} receptors (32) and subsequently are stably transfected with human EP1–cDNA (33), EP\text{1} immunoreactivity was distributed in the cytoplasm and perinuclear areas (Fig. 2\text{c}); no immunoreactivity was detected in the wild-type cells (Fig. 2\text{a}), which confirms specificity of the EP\text{1} antibodies.

**Competing ligands [log M]**

Fig. 1. Competitive displacement of [\text{3H}]PGE\text{2} binding to subcellular fractions from porcine adult myometrium and newborn brain by prostaglandins and analogs: fractions 1 (a) and 4 (b) from myometrium and fractions 1 (c) and 4 (d) from brain. Fraction 1 contained plasma membrane and fraction 4 contained nuclear membrane. Membranes were incubated with 10 nM [\text{3H}]PGE\text{2} and increasing concentrations of prostaglandins and analogs: ○, PGE\text{2}; ■, AH6809; ◆, Butaprost; ●, M&B 28,767; ▲, AH23848B. Each point is mean ± SE of three experiments, in duplicate.

**Intracellular Localization of EP\text{1} by Indirect Immunofluorescence.**

On murine fibroblast Swiss 3T3 cells overexpressing the EP\text{1} receptor, EP\text{1}-specific immunofluorescence was distributed in the cytoplasm (Fig. 4\text{a} and \text{b}), although an intensified halo surrounding the nucleus was seen in transfected cells (Fig. 4\text{b}). Confocal microscopy of these EP\text{1}-overexpressing 3T3 cells and also of cerebral microvascular endothelial cells (primary culture) revealed a distribution of the receptor throughout the cytoplasm concentrated in the perinuclear region (Fig. 3\text{a} and \text{b}; Fig. 4\text{c} and \text{d}). EP\text{1} antibody and intracellular membrane stains were partially superimposed, as revealed by the yellow and orange speckles (Figs. 3\text{c} and 4\text{e}). Confocal microscopic images were also obtained for EP\text{1}-overexpressing cells stained with the nuclear stain, Sytox green (Fig. 4\text{g}). Superimposition of EP\text{1} immunofluorescence (Fig. 4\text{f}) with nuclear staining (Sytox green; Fig. 4\text{g}) disclosed colocalization (bright yellow nucleus) of EP\text{1} immunoreactivity in the nuclear/perinuclear region (Fig. 4\text{h}); a Z-section of these cells indicated that EP\text{1}-specific fluorescence was limited to perinuclear and surrounding regions, but was not found within the nucleus (Fig. 4\text{i}).

**Distribution of EP\text{1} Fused to GFP.**

GFP from the jellyfish *Aequorea victoria* has been used as a fluorescent tag to study the intracellular localization of G protein-coupled receptors such as the \beta2-adrenergic receptors (34). The protein fusions to either \beta2-adrenergic receptors (34) or to human EP\text{1} (Y.D., unpublished results) did not interfere with receptor functions such as ligand binding, G-protein coupling, or activation of second messengers. HEK 293(EBNA) cells were transiently transfected with an EP\text{1}-GFP construct, and the intracellular localization of the fusion protein was visualized. Nuclear (optical microscopy) and more specifically perinuclear (confocal microscopy) localization of EP\text{1}-GFP protein was observed in transfected cells (Fig. 5\text{a} and \text{c}); in cells transfected
with GFP alone, fluorescence was diffusely distributed in the cytoplasm (Fig. 5a). In contrast, β2-adrenergic receptor tagged to GFP was shown to exhibit primarily plasma-membrane labeling without perinuclear localization (34).

**Detection of EP1 Immunoreactivity by Electron Microscopy.** Radioligand binding, immunofluorescence identification of native EP1 protein in endothelial and fibroblast cells, cloned EP1 expressed in fibroblasts, and use of EP1–GFP fusion protein revealed a prominent localization of EP1 receptor in the perinuclear area. To discern the nuclear envelope, high-resolution immunoperoxidase and immunogold localization of EP1 in porcine endothelial cells detected by electron microscopy (arrows). (a) Immunoperoxidase-IgG alone; note absence of immunostaining when primary antibody is omitted. (b) A low magnification showing immunostaining in plasma membrane and nuclear envelope. (c) A higher magnification showing immunostaining in the nuclear envelope. (d) Immunogold-IgG alone; note absence of immunostaining. Specific immunostaining can be observed in the plasma membrane in e, nuclear envelope in f, and Golgi apparatus in g. (Bar = 0.5 μm, except in b = 2 μm.)
resolution studies of cultured newborn pig cerebral microvessel endothelial cells and of adult rat brain cortex were done. In cultured endothelial cells, the immunoperoxidase and immunogold methods revealed EP<sub>1</sub>-specific immunostaining in the plasma membrane (Fig. 6b and c), the nuclear envelope (Fig. 6c and f), the Golgi apparatus (Fig. 6g), and vesicles (not shown). No immunostaining was detected in the ER.

To verify that the localization of EP<sub>1</sub> in the nuclear envelope was not only a newborn and/or an in vitro characteristic, sections from adult rat cerebral cortex were examined. EP<sub>1</sub> immunostaining was seen in inner and outer nuclear membranes of endothelial cells (Fig. 7a) and neurons (Fig. 7b).

Effects of Nuclear EP<sub>1</sub> Stimulation on Modulation of Nuclear Calcium and Gene Transcription. Nuclear membranes contain a variety of intermediate factors involved in EP-mediated signal transduction, such as G proteins (35), calcium channels, and Ca<sup>2+</sup>-ATPase (36). The nuclear envelope serves as a pool for calcium and has been proposed to regulate nuclear calcium signals (37). We therefore tested whether nuclear EP<sub>1</sub> are capable of eliciting a function such as affecting calcium entry directly into isolated nuclei. As shown (Fig. 8a and b), 17-phenyltrinor PGE<sub>2</sub> (17-P) and AH6809 (EP<sub>1</sub> antagonist, 10 μM) on calcium levels in isolated myometrial nuclei loaded with fura-2 AM (a and b). (a) Typical tracings; arrow shows time of compound or vehicle administration; 17-P (1 μM). (b) Peak increases in intranuclear calcium concentrations [[Ca<sup>2+</sup>]<sub>n</sub>] after addition of 17-P (filled bars) or 17-P and AH6809 (10 μM; open bars). Effects of 17-phenyltrinor PGE<sub>2</sub> (0.1 μM) on c-fos transcription in native and EP<sub>1</sub> over-expressing Swiss 3T3 cells as measured by dot blot hybridization of RNA. (c) Dot blot; Cont, control without drug. β-actin dot blot is of parent cells; its intensity was unaffected by EP<sub>1</sub> cDNA transfection. (d) Densitometry of dot blots; c-fos RNA abundance corrected for β-actin RNA is expressed as percentage of native untreated controls (open bar). Data are means ± SE of three experiments. *, P < 0.05 compared with the corresponding control.

Calcium has been implicated to play an important role in nuclear functions, including the regulation of gene transcription (37) such as c-fos (38), which also has been shown to be stimulated by PGE<sub>2</sub> (39). We tested whether the stimulation of nuclear EP<sub>1</sub> with prostaglandin analogs affects c-fos transcription. Dot hybridization of RNA revealed that exposure of nuclei from Swiss 3T3 cells to EP<sub>1</sub> agonist 17-phenyltrinor PGE<sub>2</sub> (0.1 μM) increased transcription of c-fos to a greater extent than that observed after stimulation of whole cells; this effect was augmented in cells overexpressing EP<sub>1</sub> (Fig. 8c and d) and was abolished by AH6809 (10 μM; not shown).

In conclusion, the present study provides evidence for the existence of the prostaglandin receptor EP<sub>1</sub> in the perinuclear region from various species by numerous techniques and most notably in the nuclear envelope in cell lines and in vivo in tissues, as revealed by high-resolution studies. This report shows an ultrastructural localization of a functional G protein-coupled receptor in the nuclear envelope. The presence of nuclear AT<sub>1</sub> receptors that contain a nuclear localization sequence has been detected by radioligand binding (11) and immunoreactivity, mostly after pharmacological stimulation.
with angiotensin II (40). A nuclear localization sequence was not found in EP1 receptor (19). So far, other than the EP1 variant lacking all C-terminal tail (41) and thus undetectable with our antibody (22), no other EP1 have been identified (42). The similarity in the kinetics of PGE2 binding and antibody recognition of EP1 receptors in both plasma and nuclear membranes, matching localization of ectopically expressed EP1 receptor and EP1−GFP fusion protein, as well as pharmacological characteristics and comparable molecular weights [as determined by immunoblotting, which showed a single 65-kDa EP1 immunoreactive band (data not shown)] indicates that the receptors in both cellular compartments are of similar identity. Finally, the presence of nuclear EPs provides an explanation for actions of PGE2 when plasma membrane EPs are barely detectable (4, 6, 7). In addition, consistent with the localization of cyclooxygenase-2 in the perinuclear region (8), it is conceivable that locally generated prostaglandins can activate nuclear EPs that can in turn modulate gene transcription, as recently speculated (43); details of transduction mechanisms remain to be elucidated in this action of prostaglandins via nuclear EP receptors.

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