In the article “Absence of tumor necrosis factor mediates this abnormality and the associated lethality, as viable 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 with relA-deficient mice,” should be removed.

In Table 2, page 2997, line 2, right column, the genotype of the offspring which currently reads “21 TNFy relAy” should be “21 TNFy relA1y.”

In Table 1, page 2995, line 1 of the title, the second sentence, “Results of matings of TNFy mice with 2 relAy mice,” should be “Results of matings of TNFy/ mice with 2 relA/ mice.”

The labeling of the gel in Fig. 1O was erroneously moved down and to the right. The corrected figure and its legend are shown below.

**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.

**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. 1O should be corrected as follows:

**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 parafollicular 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 (A), α-melanocyte-stimulating hormone (B), calcitonin (H and I), or parathyroid hormone (K) with hematoxylin counterstaining. (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, Guillermima Almazan, Alfredo Ribeir-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. 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.)

**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.)
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/P, 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 E2 receptors

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

*Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada H3G 1Y6; †Department of Pediatrics and 2Hôpital Sainte Justine, Montreal, QC Canada H3T 1C5; ‡Biotechnology Research Institute, Montreal, QC Canada H4P 2R2; §Merck Frosst Labs, Pointe-Clair, QC Canada H9R 4P9; and ¶Department of Ophthalmology, Wayne State University, Detroit, MI 48201

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: PGE2 and 17-phenyltrinor PGE2 (Cayman Chemicals, Ann Arbor, MI); DMEM, HSFM, and geneticin (GIBCO/BRL); fetal calf serum, and goat serum (Jackson ImmunoResearch Laboratories); and radiolabeled prostaglandins and nucleotides (Amersham). 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 acetyl salicylic acid, 1 mM benzamidine, 0.2 mM phenylmethylsulfonylfluoride, and 100 μg/ml soybean trypsin inhibitor. 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) was also 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 [3H]PGE2 and [3H]PGD2 to membranes, time course of association and dissociation experiments, and displacement of [3H]PGE2 by receptor isoform-specific ligands were performed as reported (4). Receptor densities (Bmax), affinity (Kd), and association (k) and dissociation (k) constants were determined from saturation isotherms (Prism, GRAPHPAD; LIGAND; ref. 18).

EP, Receptor Expression in Swiss 3T3 Cells. The human EP3 receptor cDNA [HindIII–XbaI fragment (19)] was cloned into the mammalian expression vector pRC-cytomegalovirus (pRC-CMV; Invitrogen). This EP1/pRC-CMV plasmid was

ABSTRACT Prostaglandin E2 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 EP1 was also found in fibroblast Swiss 3T3 cells stably overexpressing EP1 and in human embryonic kidney 293 (Epstein–Barr virus-encoded nuclear antigen) cells expressing EP1 fused to green fluorescent protein. High-resolution immunostaining of EP1 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 modulate 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 E2 (PGE2), a major brain prostaglandin, has been implicated in various cerebral functions during development (2). PGE2 acts on prostaglandin E receptors EP1, EP2, EP3, and EP4; EPs are found in most tissues and are abundant in brain (cortex) endothelial cells and in situ in brain (cortex) endothelial cells and neurons. Stimulation these nuclear receptors modulate nuclear calcium and gene transcription.

Edited by Robert J. Lefkowitz, Duke University Medical Center, Durham, NC, and approved October 9, 1998 (received for review June 10, 1998)


This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: PG, prostaglandin; EP, PG receptors; ER, endoplasmic reticulum; GFP, green fluorescent protein; EBNA, Epstein–Barr virus-encoded nuclear antigen; HEK, human embryonic kidney.

**To whom reprint requests should be addressed at: Hôpital St. Justine, 3175 Côte St. Catherine; Montreal, QC Canada H3T 1C5. e-mail: chemtobs@ere.umontreal.ca.
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′-dihexyloxacarbocyanine iodide [DiOC(3)], and nuclei were stained with either 4′,6-diamidino-2-phenylindole (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–XhoI 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 and a XhoI 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 XhoI. Transient transfections were done for 6 h in 6-well plates seeded at 10^5 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 OptiMem. The nuclei were stained with either 4′,6-diamidino-2-phenylindole (DAPI), sytox green, or propidium iodide as per instructions of the manufacturer (Molecular Probes).

**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 (k_a) and dissociation (k_d) 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 5′ 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 expressed PGD2 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-phenyltrinitro 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; 11 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-filteration 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).

**Table 1. Maximum specific binding on cell fractions**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PG</th>
<th>PM</th>
<th>ER</th>
<th>NM</th>
<th>CYT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterus</td>
<td>PGE2 58 ± 4</td>
<td>37 ± 4</td>
<td>23 ± 3</td>
<td>ud</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PGD2 36 ± 3</td>
<td>2 ± 0.4</td>
<td>ud</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>PGE2 13 ± 3</td>
<td>13 ± 1</td>
<td>12 ± 3</td>
<td>ud</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PGD2 21 ± 1</td>
<td>ud</td>
<td>15 ± 1</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

Values (B_max 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 ± 3.2, 23.1 ± 2.2, and 19.2 ± 1.4 nmol of PO₄ 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 [³H]PGE₂ by receptor subtype ligands, AH6809 (EP₁ antagonist), butaprost (EP₂-selective agonist), M&B 28,767 (EP₃-selective agonist), and AH23848B (EP₄ antagonist) revealed the presence of all EP subtypes in both plasma-membrane and nuclear-membrane fractions of myometrium, albeit EP₃ was the most abundant (50%; Fig. 1a and b). In plasma-membrane fractions of newborn brain, EP₃ accounted for all EP receptors (Fig. 1c), whereas in nuclear membranes, EP₃ comprised 45% of total EPs, and the balance was evenly distributed among EP₁, EP₂, and EP₄ (Fig. 1d).

Intracellular Localization of EP₁ in HEK 293(EBNA) Cells. There are many isoforms of EP₃ (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 [³H]PGE₂ by EP₁ antagonist AH6809 on nuclear fractions of two distinct tissues, brain and myometrium (Fig. 1b and d), and the availability of specific anti-EP₁ antibodies (22) led us to focus our investigation on the cellular localization of EP₁ receptors. In HEK 293(EBNA) cells, which do not naturally express EP₁ receptors (32) and subsequently are stably transfected with human EP₁ cDNA (33), EP₁ immunoreactivity was distributed in the cytoplasm and perinuclear areas (Fig. 2c); no immunoreactivity was detected in the wild-type cells (Fig. 2a), which confirms specificity of the EP₁ antibodies.

Intracellular Localization of EP₁ by Indirect Immunofluorescence. On murine fibroblast Swiss 3T3 cells overexpressing the EP₁ receptor, EP₁-specific immunofluorescence was distributed in the cytoplasm (Fig. 4a and b), although an intensified halo surrounding the nucleus was seen in transfected cells (Fig. 4b). Confocal microscopy of these EP₁-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. 3a and b; Fig. 4c and d). EP₁ antibody and intracellular membrane stains were partially superimposed, as revealed by the yellow and orange speckles (Figs. 3c and 4c). Confocal microscopic images were also obtained for EP₁-overexpressing cells stained with the nuclear stain, Sytox green (Fig. 4g). Superimposition of EP₁ immunofluorescence (Fig. 4f) with nuclear staining (Sytox green; Fig. 4g) disclosed colocalization (bright yellow nucleus) of EP₁ immunoreactivity in the nuclear/perinuclear region (Fig. 4h); a Z-section of these cells indicated that EP₁-specific fluorescence was limited to perinuclear and surrounding regions, but was not found within the nucleus (Fig. 4i).

Distribution of EP₁ 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 β₂-adrenergic receptors (34). The protein fusions to either β₂-adrenergic receptors (34) or to human EP₁ (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 EP₁-GFP construct, and the intracellular localization of the fusion protein was visualized. Nuclear (optical microscopy) and more specifically perinuclear (confocal microscopy) localization of EP₁–GFP protein was observed in transfected cells (Fig. 5a and c); in cells transfected

![Fig. 1](image1.png)

**Fig. 1.** Competitive displacement of [³H]PGE₂ 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 [³H]PGE₂ and increasing concentrations of prostaglandins and analogs. ○, PGF₂α; ■, AH6809; ●, Butaprost; ●, M&B 28,767; ▲, AH23848B. Each point is mean ± SE of three experiments, in duplicate.

![Fig. 2](image2.png)

**Fig. 2.** EP₁ immunofluorescence of ectopically expressed human EP₁ receptor in HEK293(EBNA) cells. (a) Cells transfected with vector alone; note the absence of fluorescence. (b) Nuclear stain (DAPI) of cells from a. (c) An EP₁-overexpressing clone; note the perinuclear halo. (d) Nuclear stain (DAPI) of cells from c.

![Fig. 3](image3.png)

**Fig. 3.** Confocal microscopic images of EP₁ immunostaining in porcine cerebrovascular endothelial cells. (a) Anti-EP₁ antibody and Texas red–conjugated IgG. (b) DIOC₆(3); intracellular membranes (mostly endoplasmic reticulum) stain. (c) Superimposed images of a and b. (d) Anti-EP₁ in the presence of cognate peptide (10 μg/ml) and nuclear stain (propidium iodide); note the absence of immunostaining.
with GFP alone, fluorescence was diffusely distributed in the cytoplasm (Fig. 5a). In contrast, b2-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-

**Fig. 4.** EP1 immunofluorescence of ectopically expressed EP1 receptor in murine Swiss 3T3 cells: (a) Cells transfected with vector alone. (b) EP1-overexpressing clone; note the intense perinuclear halo. Confocal microscopic images of EP1 overexpressing cells. (c) Anti-EP1 antibody and Texas red-conjugated IgG. (d) DiOC6(3), intracellular membranes (mostly ER) stain. (c) Superimposed images of c and d. (f) Anti-EP1 antibody and Texas red-conjugated IgG. (g) Sytox green nucleus stain. (h) Superimposed images of f and g. (i) Z section of h.

**Fig. 5.** GFP protein localization in transfected HEK293(EBNA) cells. a and b are optical fluorescent microscopic images and c and d are confocal images. (a) GFP alone; note the diffuse staining pattern. (b and c) EP1–GFP fusion protein; perinuclear halo at higher confocal magnification (c). (d) Nuclear stain (propidium iodide) of cells from b.

**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.)
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 EP₁-specific immunostaining in the plasma membrane (Fig. 6 b and c), the nuclear envelope (Fig. 6 c and f), the Golgi apparatus (Fig. 6g), and vesicles (not shown). No immunostaining was detected in the ER.

To verify that the localization of EP₁ in the nuclear envelope was not only a newborn and/or an in vitro characteristic, sections from adult rat cerebral cortex were examined. EP₁ immunostaining was seen in inner and outer nuclear membranes of endothelial cells (Fig. 7a) and neurons (Fig. 7b).

Effects of Nuclear EP₁ 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 Ca²⁺-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 EP₁ are capable of eliciting a function such as affecting calcium entry directly into isolated nuclei. As shown (Fig. 8 a and b), 17-phenyltrinor PGE₂ (EP₁ agonist) caused a concentration-dependent increase in myometrial intranuclear calcium concentration; this effect was abrogated by EP₁ antagonist AH6809.

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 PGE₂ (39). We tested whether the stimulation of nuclear EP₁ with prostaglandin analogs affects c-fos transcription. Dot hybridization of RNA revealed that exposure of nuclei from Swiss 3T3 cells to EP₁ agonist 17-phenyltrinor PGE₂ (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 EP₁ (Fig. 8 c 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 EP₁ 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 AT₁ 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 EP3 receptor (19). So far, other than the EP3 variant lacking all C-terminal tail (41) and thus undetectable with our antibody (22), no other EP3 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 EP receptors 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.

We thank H. Fernandez and M. Ballak for their technical assistance and A. Mcleod and S. Grant for sharing their expertise. M.B. is a recipient of the Doctoral Research Award from the Medical Research Council of Canada (MRC). This study was supported by grants from the MRC.