Prostaglandin E₂ EP2 and EP4 receptor activation mediates cAMP-dependent hyperpolarization and exocytosis of renin in juxtaglomerular cells

Ulla G. Friis,1 Jane Stubbe,1 Torben R. Uhrenholt,1 Per Svenningsen,1 Rolf M. Nüising,2 Ole Skott,1 and Boye L. Jensen1

1Department of Physiology and Pharmacology, University of Southern Denmark, Odense, Denmark; and 2Institute of Clinical Pharmacology, Johann Wolfgang Goethe-University, Frankfurt am Main, Germany

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Frisi, Ulla G., Jane Stubbe, Torben R. Uhrenholt, Per Svenningsen, Rolf M. Nüising, Ole Skott, and Boye L. Jensen. Prostaglandin E₂ EP2 and EP4 receptor activation mediates cAMP-dependent hyperpolarization and exocytosis of renin in juxtaglomerular cells. Am J Physiol Renal Physiol 289: F989–F997, 2005. First published June 28, 2005; doi:10.1152/ajprenal.00201.2005.—PGE₂ and PG₁₂ stimulate renin secretion and cAMP accumulation in juxtaglomerular granular (JG) cells. We addressed, at the single-cell level, the receptor subtypes and intracellular transduction mechanisms involved. Patch clamp was used to determine cell capacitance (Cm), current, and membrane voltage in response to PGE₂, EP2 and EP4 receptor agonists, and an IP receptor agonist. PGE₂ (0.1 μmol/l) increased Cm significantly, and the increase was abolished by intracellular application of the protein kinase A antagonist Rp-8-CPT-cAMPS. EP2-selective ligands butaprost (1 μmol/l), AE1–259-01 (1 mmol/l), EP4-selective agonist AE1–329 (1 mmol/l), and IP agonist iloprost (1 μmol/l) significantly increased Cm mediated by PKA. The EP4 antagonist AE3–208 (10 mmol/l) blocked the effect of EP4 agonist but did not alter the response to PGE₂. Application of both EP4 antagonist and EP2-antagonist AH-6809 abolished the effects of PGE₂ on Cm and current. EP2 and EP4 ligands stimulated cAMP formation in JG cells. PGE₂ rapidly stimulated renin secretion from superfused JG cells and diminished the membrane-adjacent granule pool as determined by confocal microscopy. The membrane potential hyperpolarized significantly after PGE₂, butaprost, AE1–329 and AE1–259 and outward current was augmented in a PKA-dependent fashion. PGE₂-stimulated outward current, but not Cm change, was abolished by the BKCa channel inhibitoriberiotoxin (300 mmol/l). EP2 and EP4 mRNA was detected in sampled JG cells, and the preglomerular and glomerular vasculature was immuno-positive for EP4. Thus IP, EP2, and EP4 receptors are associated with JG cells, and their activation leads to rapid PKA-mediated exocytotic fusion and release of renin granules.

cyclooxygenase; capacitance; kidney; current; potential

RENN IS STORED AND RELEASED by juxtaglomerular granular (JG) cells in the distal part of the afferent glomerular arterioles. The activity of the circulating renin-angiotensin-aldosterone system is determined predominantly by the rate of renin secretion from the JG-cells. Cyclooxygenase (COX) activity supports basal renin release in vivo (32). The increase in renin expression and secretion seen in response to a low epithelial NaCl transport rate across the thick ascending limb of Henle’s loop (TALH) is significantly attenuated by COX blockers, in both experimental animals and humans (10, 22, 31, 40, 41). Expression of the inducible COX enzyme COX-2 correlates inversely with NaCl transport across TALH, and COX-2 activity generates predominantly PGE₂ at this site (5, 9, 29, 41, 42). Compared with the detailed insight into the regulation of prostaglandin synthesis by the epithelium, relatively less is known about the downstream stimulus-secretion mechanism through which prostaglandins promote renin secretion.

We have shown that PGE₂ and PG₁₂ lead to a parallel accumulation of cAMP and stimulation of renin secretion in primary cell cultures enriched in JG cells (18). This finding suggested that the stimulation of renin by PGE₂ and PG₁₂ was through interaction with receptors on JG cells and that it was mediated by cAMP. Intracellular delivery of cAMP in single JG cells elicits exocytosis of renin that depends on PKA activity (11). cAMP-mediated exocytosis of renin is associated with increased outward current through potassium channels (BKCa-ZERO variant) and hyperpolarization (12). PGE₂ activates BKCa channels in vascular myocytes (45), but whether such electrophysiological events and PKA activity underlie the stimulatory effect of prostaglandins on intracellular signaling in JG-cells has not been determined.

The actions of PG₁₂ and PGE₂ are mediated by G protein-coupled prostanooid receptors, IP and EP1–EP4 (7). IP, EP2, and EP4 receptors are encoded by different genes but converge on a Gs protein and can activate adenylyl cyclase with subsequent increased formation of cAMP (3, 7, 27, 33). The EP4 receptor has altered renovascular sensitivity and renin response to PGE₂ (16, 20, 26, 30, 37, 44). EP2 knockout mice display normal plasma renin activity (6, 39), but isolated kidneys have altered renovascular sensitivity and renin response to PGE₂ infusion (34). Thus both EP2 and EP4 receptors contribute to PGE₂-mediated stimulatory effects on renin at integrated levels, but it is not clear whether this is due to direct effects of both receptors on JG cells or indirect effects related to tubular transport of NaCl. In particular, it is not known whether EP2 and EP4 colocalize in single JG cells. The aim of the present study was to examine the transduction mechanisms initiated by PGE₂ and PG₁₂ at the single-JG cell level and to determine the contribution of EP2 and EP4 receptors to PGE₂-mediated stimulatory effects on renin secretion.

MATERIALS AND METHODS

Rat JG cells. JG cells were isolated from rat renal cortex as described (11). For patch clamp, cells were transferred to coverslips in

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RPMI-1640 medium. Superfusion of JG cells and measurement of renin were done as described (11, 25).

Male Sprague-Dawley rats (60–80 g) on a standard diet were used. Animal care was approved and carried out in accordance with institutional and American Physiological Society principles and guidelines on the care and use of laboratory animals.

**Patch-clamp experiments.** The patch-clamp experiments were performed as described (11, 12) with an internal solution (in mmol/l): 135 K-glutamate, 10 NaCl, 10 KCl, 1 MgCl2, 10 HEPES-NaOH, 0.5 Mg-ATP, and 0.3 Na2-GTP; osmolality was 303 mosmol/kgH2O, pH 7.07, with or without Rp-8-CPT-cAMPS (Rp-cAMPS; 5 μmol/l). The external solution was (in mmol/l) 100 HEPES-NaOH, 140 NaCl, 2.8 KCl, 1 MgCl2, 2 CaCl2, 11 glucose, and 10 sucrose; osmolality was ~300 mosmol/kgH2O (range 296–314 mosmol/kgH2O), pH 7.25, with or without agonists/antagonists.

**RT-PCR analysis of EP receptors in sampled JG cells.** Single JG cells were sampled with patch pipettes, and total RNA was isolated and used as a template for RT-PCR analysis (11, 20). DNA oligonucleotide primer sequences for rat EP2 and EP4 receptors were as published (20).

**Immunohistochemical and immunofluorescent labeling.** Fixation, paraffin-embedding, and sectioning of rat kidneys and tissue-labeling procedures for EP4 and EP2 immunoreactive proteins were as described (38). Immunofluorescent labeling of isolated JG cells for renin were done as described (11, 25).

**Statistics.** All values are given as means ± SE. Paired Student's *t*-tests were used to estimate statistical significant difference from zero in experiments where *Cm* was measured. The change in *Cm* was calculated as the difference (in %) in *Cm* (at *t* = 0 min and *t* = 10 min). ANOVA was used to determine whether there was statistical significance among several groups of data. A post hoc test was performed by Student's unpaired *t*-test. *P* < 0.05 was considered statistically significant.

**RESULTS**

Effect of PGE2, EP subtype-, and IP-specific agonists on cell *Cm* in single JG cells. Granular cells were accepted for measurements of *Cm* if they displayed the typical current-voltage relationship (11, 12). Cells selected with these criteria contain active renin and renin mRNA (11). The whole cell configuration was obtained in 61 JG cells from 36 preparations. The cells had an average *Cm* of 1.94 ± 0.08 pF (n = 61). After the whole cell configuration was established, a current-voltage relationship was recorded and then individual cells were superfused with agonists, and *Cm* was followed with time. PGE2 (0.1 μmol/l) significantly increased *Cm* (Fig. 1A, *n* = 4). Dialysis with the PKA antagonist Rp-cAMPS (5 μmol/l) abolished the PGE2-mediated effects on *Cm* (Fig. 1A, *n* = 4). The EP2-specific ligand butaprost (1 μmol/l) significantly increased *Cm* (Fig. 1B, *n* = 4), and in a separate series Rp-cAMPS significantly inhibited the effect of butaprost on *Cm* (Fig. 1B, *n* = 4). The potent EP2-specific agonist AE1–259 also increased *Cm* significantly (1 nmol/l, Fig. 1C, *n* = 4), and this increase also depended on intact PKA activity (Fig. 1C, *n* = 4). The EP4 receptor-specific ligand AE1–329 significantly increased *Cm* (1 nmol/l, Fig. 1D, *n* = 4). The AE1–329-mediated increase in *Cm* was significantly inhibited by intracellular Rp-cAMPS (Fig. 1D, *n* = 4). Iloprost, an IP receptor agonist (1 μmol/l), also enhanced *Cm* significantly (11.5 ± 2%, *n* = 4, *P* = 0.006, not shown). We did not detect any difference in the lag time before *Cm* began to increase in response to the different EP agonists (measurements started 1 min after addition of agonist: PGE2: 138 ± 45 s; butaprost, 98 ± 52 s; AE1–329, 135 ± 17 s; AE1–259, 120 ± 12 s; all *n* = 4; the average of the entire series was 137 ± 29 s). For iloprost, the lag time was 208 ± 29 s.

**Effect of PGE2 and IP-, EP2-, and EP4-specific ligands on whole cell current and membrane potential in single JG cells.** PGE2 (0.1 μmol/l) significantly increased outward current, which was associated with hyperpolarization (Fig. 2A, *n* = 4). This response was abolished by dialysis of the cells with the PKA antagonist Rp-cAMPS (Fig. 2A, right, *n* = 4). The EP2-specific agonists butaprost and AE1–259 led to significant increases in outward current and hyperpolarization, also in a PKA-sensitive manner (Fig. 2B, both *n* = 4). Similar cellular responses were recorded in response to the EP4 agonist AE1–329 (1 nmol/l, *n* = 4 and with blocker *n* = 4, Fig. 2B). An IP agonist, iloprost, significantly enhanced outward current (94 ± 25%, *P* = 0.001) and hyperpolarized cell potential (−10 ± 4.7 mV, *P* = 0.04; not shown, *n* = 6).

**Effect of EP receptor antagonists and K channel blockers on the electrophysiological response to PGE2 in JG cells.** The effect of PGE2 was tested in the presence of an EP4 receptor-selective antagonist, AE3–208 (21). AE3–208 alone (10 nmol/l) did not change current and *Cm* and subsequent addition of the EP4 agonist AE1–329 (1 nmol/l) had no effect on currents, membrane potential (*Vm*, 6.8 ± 7 mV, not significantly), or *Cm* (*Fig.* 3, C–IV, *n* = 4) showing effective blockade of the EP4 receptor. In the presence of the EP4 blocker, PGE2 still induced a significant hyperpolarization (−20.3 ± 2.0 mV, *P* = 0.004, *n* = 3), augmented outward current (Fig. 3E), and increased *Cm* (Fig. 3, A and C–II, *n* = 4). In JG cells pretreated with both AE3–208 and an established EP1-EP2 antagonist, AH-6809, PGE2 had no significant effect on *Cm* and current (Fig. 3, B, C–III, and F) or *Vma* (2.8 ± 4.6 mV, NS) (*n* = 4).

PGE2-induced outward current (0.1 μmol/l, Fig. 2A, Fig. 3, D and G) was reversibly inhibited (79.3 ± 2.5%) by the BKCa blocker ibotrexatoxin (300 nmol/l) (Fig. 3, D and G, *n* = 4).

Hyperpolarization was significantly reduced to −8.0 ± 2.3 mV, but not fully prevented, after ibotrexatoxin. In a separate series, ibotrexatoxin (300 nmol/l) did not modify the increase in *Cm* in response to PGE2 (Fig. 3, C–I, *n* = 4).
response to PGE2 with and without intracellular RpcAMPs.

Woolf plots yielded EC50 values of 1.5 ± 0.3 nmol/l for butaprost and 29 ± 4 nmol/l for AE1–259, and 1.6 ± 0.3 nmol/l (AE1–329), which are in accordance with reported values for these agonists from heterologous expression systems (36).

Forskolin significantly increased cAMP from 355 to 5,901 ± 283 fmol/well in the butaprost-series (n = 5) and from 160 ± 11 to 5,255 ± 942 fmol/well in the AE-compound series (n = 5).

Rapid effects of PGE2 on renin secretion. To validate that Cm changes reflected release of renin, we measured renin release from superfused JG cells at 30-s intervals. PGE2 (10 µmol/l) elicited a significant, transient increase in secretion rate 150 s after addition of PGE2 (Fig. 5A). Moreover, renin granules were visualized by laser confocal renin immunofluorescence. In control JG cells, renin immunofluorescence displayed a distinct radial heterogeneity with confluent areas in the juxtanuclear area and smaller separate granular structures more distant from the nucleus adjacent to the plasma membrane (Fig. 5, Ba–Be). Three-dimensional reconstruction of optical sections showed that peripheral labeling corresponded to limited spheres, probably mature secretory granules (see supplementary data at http://ajprenal.physiology.org/cgi/content/full/00201.2004/DC1). The perinuclear labeling was associated with large confluent structures compatible with renin-containing parts of endoplasmic reticulum and Golgi cisterns. The nucleus was distinct from these cisterns as evi-

Effect of EP receptor-specific ligands on cAMP formation. EP2-specific ligands (butaprost, AE1–259) and EP4-specific AE1–329 increased cAMP formation significantly in a dose-dependent fashion in primary JG cell cultures (Fig. 4). Hanes-Woolf plots yielded EC50 values of 1.5 ± 1 µmol/l for butaprost, 29 ± 4 nmol/l for AE1–259, and 1.6 ± 0.3 nmol/l (AE1–329), which are in accordance with reported values for these agonists from heterologous expression systems (36).

Fig. 1. Effect of PGE2 and EP receptor-specific ligands on cell capacitance changes (Cm) in single juxtaglomerular granular (JG) cells. A: effect of PGE2 (arrow, −; 0.1 µmol/l) on Cm (n = 4) and effect of intracellular dialysis with a protein kinase A inhibitor, Rp-8-CPT-cAMPs (RpcAMPS; +; 5 µmol/l, n = 4) on PGE2-induced changes in Cm (left). Right: average changes in Cm in response to PGE2 with and without intracellular RpcAMPS ± SE. *P ≤ 0.05 at t = 0 vs. t = 10 min. **P ≤ 0.05 PGE2 with and without RpcAMPS. B: conditions are as in A, but cells were superfused with EP2-specific butaprost (1 µmol/l) without (filled bar) and with (open bar) intracellular RpcAMPS. C: conditions are as in A, but cells were superfused with EP2-specific ONO-AE1–259-01 (1 nmol/l) without (filled bar) and with intracellular RpcAMPS (open bar). D: conditions are as in A, but JG cells were superfused with EP4-specific ligand ONO-AE1–329 (1 nmol/l) with and without RpcAMPS. These cells were also used for membrane current and potential measurements, and data are shown in Fig. 2.

Fig. 2. Effect of PGE2 and EP receptor-specific ligands on whole cell current and membrane potential in single JG-cells. A: control average current-voltage (I–V) relationship was measured immediately after the whole cell configuration was obtained (○). The measurement was repeated 10 min after addition of PGE2 (●). Membrane potential was determined as 0-current potential before and after superfusion with EP2-specific agonists. The average change is shown (open bar, n = 4). **P ≤ 0.05, PGE2 with and without RpcAMPS. B: membrane potential was determined as 0-current potential before and after superfusion with EP4-specific agonists and after superfusion of the cell with EP2-specific ligands with and without RpcAMPS. The average change is shown (open bar, n = 4). **P ≤ 0.05, PGE2 with and without RpcAMPS.
enced by labeling with DAPI (Fig. 5, Bk–Bm). The peripheral granules were significantly diminished after PGE2 stimulation for 20 min before fixation (Fig. 5, Bf–Bj), whereas the perinuclear labeling largely remained (see supplementary data with 3D-reconstructed prestimulated JG cell at the URL noted above).

Expression of EP2 and EP4 mRNAs in single sampled JG cells and distribution of EP2 and EP4 receptor immunoreactive protein. Amplification of cDNA from single, sampled JG cells by PCR yielded products for EP2 and EP4 with the expected molecular size and only in the presence of RT and cDNA (Fig. 6A). Immunostaining with the EP2-specific antibody yielded labeling that was associated with descending limbs of Henle’s loop in the medulla (not shown) and distal convoluted tubules in the cortex (Fig. 6B, top left). Labeling was observed predominantly in the basolateral aspect of the epithelial cells, as in human kidney (Fig. 6B, top) (38). In serial sections, there was no apparent overlap in the labeling pattern for EP2 and renin, which was observed in the distal afferent arteriole (Fig. 6B, top).

Labeling of kidney sections with EP4-specific antibody showed that immunoreactivity was associated with all segments of the preglomerular vasculature including afferent arterioles and glomeruli (Fig. 6B, middle and bottom). Intraglomerular labeling was observed along the endothelial cells of glomerular capillaries and in perivascular cells, most likely visceral podocytes (Fig. 6B). EP4 immunostaining was detected in media smooth muscle cells, including the juxtaglomerular end of afferent arterioles, and, of note, also in endothelium (Fig. 6B).

DISCUSSION

The present data showed that PGE2 EP2 and EP4 receptors and the PGI2 IP receptor were associated with single JG cells. Selective activation of each EP receptor subtype led to rapid cAMP formation and a BKCa channel-mediated increase in outward current, hyperpolarization, and an increase in \( C_m \). The PGE2-mediated increase in \( C_m \) and hyperpolarization depended on protein kinase A and was abolished only by combined application of EP2 and EP4 receptor antagonists. The PGE2-induced increase in \( C_m \) was associated with rapid release of active renin and a decreased size of the membrane-adjacent renin granule pool. Thus the data indicate that EP2 and EP4...
receptors are coexpressed in single JG cells and that they contribute redundantly to exocytosis of renin in response to PGE2.

EP4 receptors have been detected consistently in glomeruli, vasculature, and distal nephron segments (4, 16, 26, 30, 35, 37). The present immunostaining data confirmed the vascular and glomerular expression. EP2 receptors have been found in kidney vessels and glomeruli in some studies (16, 20, 44) and not in others (30, 37). We observed EP2 immunoreactivity associated with the basolateral surface of descending limb cells of Henle’s loop. In a previous study, EP2 mRNA was observed in this segment by analysis of microdissected segments (20), which indicates that the EP2 antibody was specific. The fact that EP2 immunoreactivity was not observed in the vasculature whereas EP2 transcripts were detected in sampled JG cells could be due to a lower sensitivity of immunostaining compared with PCR, because data at the functional level suggest that EP2 receptors contribute significantly to PGE2-mediated depressor effects on renal and systemic hemodynamics (1, 17, 23, 34, 44). EP2 binding sites are present in vascular smooth muscle from several vascular beds (24, 43). EP2 is by far the least abundant EP receptor subtype in kidney and vasculature in comparative assays (19, 20, 44). Together, the data indicate functionally significant, although low, EP2 expression in JG cells.

Compared with wild-type mice, EP2 receptor null mice exhibit no difference in basal renin parameters and renin increases similarly after various stimuli (1, 6, 23, 39). In isolated, perfused kidneys from mice with loss-of-function mutations in each of the four EP receptors, both EP2 and EP4 receptors contributed significantly to PGE2-mediated acute effects on renin secretion (and vascular resistance) (34). In the isolated perfused kidney, effects of PGE2 on renin secretion could be either direct on JG cells or indirect through effects on tubular transport of NaCl. Our data suggest that PGE2 interacts with EP2 and EP4 receptors associated with JG cell surface membranes. A recent study reported that PG12, and not PGE2, was the dominant prostanoid to control renin release in vivo and in vitro (15). The present data confirmed at the single-cell level that an IP agonist enhances Cm and whole cell current. The discrepancy on the role of PGE2 could be caused by the fact that Fujino et al. (15) determined total renin activity in cells and supernatant of JG cell cultures after prolonged incubation with agonists. This parameter probably reflects renin synthesis/degradation more than secretion.

We observed that all patch-clamped JG cells were sensitive to either EP ligand and that double blockade of both EP2 and EP4 receptors was necessary to abolish the cellular responses to PGE2. This indicates that both receptors were coexpressed in the same cells. If so, why are there two cAMP-coupled EP receptors associated with JG cells and do they contribute differentially to stimulus-secretion coupling? We did not detect qualitative differences in acute electrophysiological responses (Vn, I, Cm) between PGE2 and EP2- or EP4-specific ligands. A cooperative action of EP2 and EP4 receptors is involved in the transduction of PGE2 signals in other cell types (36), but it appears not to be a prerequisite for the acute effects on renin secretion. Distinguishing features of the EP4 receptor could play a role during prolonged exposure to PGE2 and comprise ligand-mediated desensitization (2, 8, 28), activation of PI3-K/ERK/MAP kinase pathways (13, 14), and a greater susceptibility to metabolic inactivation of PGE2 (28). If EP4 internalization occurs in JG cells, a restricted stable EP2 expression could potentially serve to sustain PGE2 sensitivity.

EP2 and EP4 receptor activation elicited hyperpolarization mediated by cAMP-dependent interaction with the BKCa channel (12). When hyperpolarization was prevented, the PGE2-mediated Cm increase was intact. Thus hyperpolarization is not necessary for exocytosis of renin. In vivo, different and opposing stimuli influence JG cell membrane potential. In this setting, the hyperpolarizing impact of K channel activation...
Fig. 6. Localization of EP2 and EP4 receptor in JG cells and kidney sections. A: PCR amplification of cDNA from single, sampled JG cells for EP2 and EP4 receptor. Negative controls were omission of reverse transcriptase (0-RT) and water instead of cDNA. Size marker is φX174DNA/HaeIII fragments. B: immunohistochemical and immunofluorescent labeling of rat kidney sections for EP2 receptor and renin (top) and EP4 receptor (middle and bottom). In adjacent sections, EP2 and renin immunoreactivity did not colocalize (top). EP4 labeling was associated with preglomerular arterioles and glomeruli (left) and intrarenal arteries (right). EP4 labeling was observed in media smooth muscle and endothelium (right). Bars = 50 μm.

Fig. 5. A: effect of PGE2 on renin secretion from superfused JG cells. Left: result from a single experiment. Right: average renin release rate immediately before change of superfusate and 150 s after the change in superfusate ± SE (n = 5 in each series). *P ≤ 0.05. B: effect of PGE2 on renin granule morphology in JG cells. Single JG cells were labeled with anti-renin antibody that was visualized with a fluorescence-coupled secondary antibody. The photos display 2-dimensional projections of 3-dimensional reconstructed cells (see supplementary data at http://ajprenal.physiology.org/cgi/content/full/00201.2004/DC1). α–e: Untreated control cells. f–j: Cells pretreated with PGE2 for 20 min before fixation. The Bluefire pseudocolor intensity scale was used and is shown at right (a–j). No filtering or intensity changes were made post hoc. The depicted cells were from 4 different preparations (n = 6 experiments). k–m: Immunofluorescence labeling for renin (red) in resting JG cells where the nucleus was visualized (blue). Bars = 8 μm.
may have a permissive role in stimulus-secretion coupling by stabilizing membrane potential in a range that prevents activation of voltage-gated calcium channels. At depolarized potentials, cAMP loses its ability to increase \( C_m \) dependent on voltage-dependent calcium channels (12).

In summary, the present findings demonstrate coexpression of EP2 and EP4 receptors at the level of the single JG cell. The rapid EP2 and EP4 transduction mechanism in JG cells is associated with enhanced production of cAMP, activation of PKA, PKA-mediated membrane hyperpolarization, and an increase in \( C_m \).

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