Prostaglandin E₂ inhibits vasotocin-induced osmotic water permeability in the frog urinary bladder by EP₁-receptor-mediated activation of NO/cGMP pathway

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Submitted 17 November 2006; accepted in final form 9 March 2007

Prostaglandin E₂ (PGE₂) is one of the most important locally acting factors antagonizing the cellular effect of antidiuretic hormone (ADH) on the increase in water reabsorption in different osmoregulatory epithelia, e.g., mammalian kidney collecting duct (CD) (21, 25, 33) and amphibian urinary bladder (28, 29). In general, the cellular effects of PGE₂ are known to be mediated through ligation of four distinct G-protein-coupled receptors, namely, EP₁, EP₂, EP₃, and EP₄, which have been defined on the basis of their molecular structures, pharmacological profiles, and signal transduction pathway (10, 12, 36). The EP₁ receptor is coupled to the activation of phosphatidylinositol 4,5-biphosphate hydrolysis and mobilization of intracellular Ca²⁺. EP₂ and EP₃ both increase adenylate cyclase activity, whereas EP₃ mainly inhibits it. Mammalian CD are characterized by the greatest diversity of PGE₂-receptor subtypes (10) and demonstrate the highest rate of PGE₂ synthesis among kidney structures (6).

Despite intensive investigation of ADH-PGE₂ interactions in the regulation of water reabsorption (10, 25, 37, 48), the mechanisms underlying the inhibitory effect of PGE₂ on ADH-stimulated osmotic water permeability (OWP) in either mammalian CD or amphibian urinary bladder are not completely understood. Two subtypes of PGE₂ receptor, EP₁ and EP₃, have been suggested to mediate the inhibitory effect of PGE₂ on OWP in mammalian CD. In rabbit cortical CD cells, EP₃ receptor was found to be coupled to pertussis toxin-sensitive inhibition of AVP-stimulated OWP through a decrease in cAMP generation (25, 37). Tamma et al. (44) suggested that the EP₃ receptor contributes to AVP-induced antidiuresis through cAMP-independent activation of the small GTP-binding protein Rho and F-actin formation in rat inner medullary CD cells, which prevents the AVP-induced aquaporin-2 shuttle. The EP₁ receptor is highly expressed primarily in the CD along the mammalian nephron (22, 43) and is mainly associated with inhibition of Na⁺ reabsorption via a Ca²⁺-coupled mechanism (22). However, the inhibitory effect of PGE₂ on the AVP-induced increase of OWP in rabbit and rat CD was reversed by staurosporine, a PKC inhibitor, suggesting the involvement of the EP₁-receptor-mediated activation of phosphatidylinositol 4,5-biphosphate hydrolysis in the regulation of water reabsorption (24, 33). The cellular mechanisms underlying the Ca²⁺-coupled effect of PGE₂ on AVP-stimulated OWP remain unclear.

During the past few years, several studies have addressed the role of nitric oxide (NO) in the regulation of water reabsorption in the mammalian CD. Endogenous NO is produced from conversion of l-arginine to l-citrulline in a reaction catalyzed by NO synthase (NOS). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, and their enzymatic activities are regulated by intracellular Ca²⁺ through...
binding of $Ca^{2+}$ to calmodulin (27). Activity of inducible NOS (iNOS) is $Ca^{2+}$ independent and is regulated predominantly at the transcriptional level (35). All three isoforms of NOS have been detected in the mammalian CD (46). Most interest has been focused on nNOS because it is expressed in the principal cells of the CD system (45) and because its expression is regulated by AVP (30). Garcia et al. (19, 20) found that exogenous NO inhibits AVP-stimulated OWP in perfused rat cortical CD, whereas another group demonstrated that, in rat kidney slices and LLC-PK1 cells, NO promotes the membrane insertion of aquaporin-2 (8). In our previous study (17), in which we used a frog (Rana temporaria L.) urinary bladder as a model of osmoregulatory epithelium, we showed that an NO donor significantly inhibits arginine-vasotocin (AVT)-induced OWP via a cGMP-dependent mechanism.

The aim of the present paper was to test the hypothesis that the EP1-receptor-mediated inhibitory effect of PGE2 on ADH-induced OWP in the frog urinary bladder is attributed to increased generation of NO in epithelial cells. This suggestion was based on the findings that 1) both an NO donor and PGE2 inhibit the AVT-induced increase in OWP, 2) PGE2 is known to release $Ca^{2+}$ from intracellular stores in mammalian CD (24, 33) and toad urinary bladder epithelial cells (18, 3) the activity of constitutive NOS is regulated by $Ca^{2+}$/calmodulin binding (27), and 4) a positive immunostaining with antibody against nNOS was revealed in the frog urinary bladder epithelium (17). To investigate the potential interaction between PGE2 and NO in the regulation of water reabsorption in the frog urinary bladder, we studied the effect of an EP1 agonist, 17-phenyl-trinor-PGE2 (17-ph-PGE2), combined with an EP1 antagonist, SC-19220, and an nNOS inhibitor, 7-nitroindazole (7-NI), on AVT-induced OWP, epithelial cell NOS activity, and cGMP concentration. In addition, we analyzed the expression of NOS isoforms in frog urinary bladder epithelial cells. The results show that the inhibitory effect of PGE2 on AVT-induced OWP in the frog urinary bladder is based at least partly on EP1-receptor-mediated activation of the NO/cGMP pathway.

MATERIALS AND METHODS

Animals. Male frogs (Rana temporaria L.) originating from the northern European part of Russia were housed in a laboratory for 1–2 mo in a hemiaquatic bath at +5°C. All experiments were performed within the period from October to April. All procedures using animals were done in accordance with the European Communities Council Directive (24th November 1986; 86/609/EEC) and were approved by the local Institutional Animal Care and Use Committee.

Osmotic water flow measurement. Urinary bladders were removed from doubly pithed frogs, and paired hemibladders were prepared as sacs. The serosal surface was bathed with aerated Ringer solution (in mM): 111 NaCl, 4.0 KCl, 1.0 NaH2PO4, 4.0 Na2HPO4, and 1.0 CaCl2 (pH 7.6, osmolality of 220 mosmol/kgH2O). The mucosal surface was bathed with the same solution diluted 1:10 with distilled water. The osmotic water flow was determined gravimetrically (5); the results were expressed as microliters of water transported through epithelium per minute and centimeter squared of hemibladder surface, which was estimated from the volume of the sac. Because of high individual variability in basal and AVT-stimulated OWP, all experiments were carried out on paired hemibladders (one served as a control). Control and experimental tissues were exposed to the same concentration of the organic solvent, which did not exceed 0.1% in the incubation medium.

Isolation of epithelial cells. Each hemibladder was washed and filled, as a sac, with solution A containing (in mM) 85 NaCl, 4 KCl, 17.5 NaHCO3, 0.8 KH2PO4, 2 glucose, and 2 EDTA, pH 7.6. The sacs were incubated for 45 min at 23°C in solution A under intense aeration. At the end of this time, the cells sloughed off spontaneously. The mucosal fluid containing the cells was filtered through four pieces of gauze and centrifuged for 10 min at 100 g. Supernatant was removed, and the cells were resuspended in solution B (in mM): 85 NaCl, 4 KCl, 17.5 NaHCO3, 0.8 KH2PO4, 2 glucose, 1.5 CaCl2, and 0.8 MgCl2, pH 7.6. From one hemibladder, it was possible to obtain at least 2 × 10⁶ cells. The percentage of damaged cells was determined by the absorption of Trypan blue (0.2%), and this did not exceed 7–10%.

Measurement of NOS activity in epithelial cell homogenates. Freshly isolated epithelial cells were homogenized on ice in 50 mM Tris·HCl buffer containing (in mM) 1 EDTA, 1 DTT, 5 glucose, and 1 PMSF, as well as 10 mg/ml pepstatin A and 20 mg/ml leupeptin, pH 7.5, at +4°C. The homogenates were centrifuged (12 000 g, 15 min), and the protein concentration of the supernatant was determined by a Coomasie blue protein assay with BSA as a standard. The supernatants were immediately used for NOS activity assay. The aliquots of supernatant (60–100 µg of protein) were incubated with 1.25 mM CaCl2, 1 mM β-NADPH, 10 µM tetrahydroliporibiopterin, 1.25 µg/ml calmodulin, 10 µM FAD, and 0.5 µM L-[3H]arginine (1 µCi/ml, specific activity of 68 Ci/mmol; Amersham) in 50 µl of 50 mM Hepes, pH 7.5, containing 1 mM DTT at 25°C for 1 h. Ca2+-independent activity was measured in samples without CaCl2 and calmodulin in the presence of 1 mM EDTA. The reactions were stopped by adding 250 µl of ice-cold 100 mM HEPES buffer (pH 5.5) containing 25 mM EDTA. To separate L-citrulline from l-arginine, 125 µl of equilibrated Dowex 50Wx8 (Na+ form) resin (Serva) was added to each sample. After repeated vortexing, the tubes were centrifuged (5,000 g, 10 min), and radioactivity of the supernatant was measured by an LKB 1209/1215 Rack-Beta scintillation counter. A sample in which the homogenate was boiled before the assay served as a blank.

Measurement of NOS activity in a suspension of epithelial cells. NOS activity in a suspension of freshly isolated epithelial cells was estimated as a value of accumulation of intracellular L-[3H]citrulline. Cells (1.5–2 × 10⁶ in each sample) were incubated in 150 µl of solution B containing 30 µM L-arginine and 0.5 µl L-[3H]arginine (1 µCi/ml, specific activity of 68 Ci/mmol; Amersham) for 45 min at 25°C. Ca2+-independent NOS activity was measured in the absence of external Ca2+ (solution A was used instead of solution B). NOS inhibitors [1 mM nitro-L-arginine methyl ester (L-NAME) or 100 µM 7-NI] were applied 10 min before L-arginine. At the end of the incubation period, the cells were isolated by centrifugation at +4°C (100 g, 10 min), and the pellet was washed in cold solution B and resuspended in 50 µl of 50 mM Tris·HCl (pH 7.5) containing 4 mM EDTA. The tubes were placed in a boiling water bath for 3 min and centrifuged (11,000 g, 5 min). Supernatant aliquots (10–15 µl) were applied to silica gel TLC plates (Merck) along with L-citrulline and L-arginine standards, and the plates were developed in n-propanol-H2O (1:1, vol/vol). The standards spots were visualized after treatment the silica gel with 0.25% ninhydrine in acetone, and the plates were processed for the determination of radioactivity.

Western blot analysis. Freshly isolated urinary bladder epithelial cells rat cerebellum, and frog brain were homogenized in lysis buffer (20 mM Tris·HCl, pH 6.8, containing 25 mM EDTA, 0.1% Triton X-100, 0.2 mM PMSF, 10 mg/ml pepstatin A, 20 mg/ml leupeptin). Subsequently, the probes were heated to 95°C for 5 min in SDS gel-loading buffer. Samples (80–100 µg protein assessed by results from the Bradford assay) were separated by 7% SDS-PAGE and were transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech). The membranes were incubated overnight at +4°C with a rabbit polyclonal anti-universal NOS antibody (Affinity BioReagents). Finally, goat anti-rabbit IgG conjugated with horseradish peroxidase
was used as a secondary antibody, and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) was used to visualize the signal.

**Measurement of intracellular \( \text{Ca}^{2+} \).** The cells were washed in the solution \( B \), centrifuged, and resuspended in 50 μl of the same solution. Thereafter, the cells were allowed to attach to the coverslips coated with poly-l-lysine for 1 h at 25°C and were then loaded with 10 μg/ml fura 2-AM and 0.08% Pluronic F-127 (both from Molecular Probes) for 1–2 h at 25°C in humidified air in the dark. Coverslips were superfused with solution \( B \) and placed on an inverted microscope (Nikon TMS-100) equipped with a dual-wavelength fluorescence photometry module (InCyt I/P-2; Intracellular Imaging). Fura 2-AM was alternatively excited at 340 and 380 nm, and emission was recorded with an 8-bit RS-170 charge-coupled device cooled camera (Cohu) and measured at a wavelength of 510 nm. The fluorescence ratio [fluorescence at 340 to 380 nm (F340/F380)] was calculated after subtraction of the background. The absolute intracellular \( \text{Ca}^{2+} \) concentrations \( ([\text{Ca}^{2+}]_i) \) were determined with the use of the calibration curve obtained from the set of solutions with known \( \text{Ca}^{2+} \) concentrations.

**Measurement of the cGMP content in epithelial cells.** Freshly isolated epithelial cells \( (1.8–2 \times 10^6 \text{ in each sample}) \) were incubated in 150 μl of solution \( B \) containing 1 mM IBMX, 50 μM zaprinast, 30 μM L-arginine, and 1 μM indomethacin for 25 min at 25°C. IBMX [nonspecific phosphodiesterase (PDE) inhibitor] and zaprinast (cGMP-specific PDE5 inhibitor) were used to inhibit cGMP degradation, whereas indomethacin was required for reducing the level of endogenous prostaglandins. 17-phenyl-trinor-PGE2 or an equivalent amount of ethanol (1%) was added in experimental and control probes, respectively, for various times \( (0.5–3 \text{ min}) \). By itself, ethanol did not change the level of intracellular cGMP. The reaction was stopped by placing the tubes in a boiling water bath for 3 min. The samples were centrifuged, and the supernatants were immediately frozen and stored at \(-20°C \) until the day of assay. The cGMP level was determined with a cGMP-enzyme immunoassay system (Amersham). Results are expressed as femtomoles of cGMP per 10⁶ cells.

**Reagents.** Sulprostone and butaprost were obtained from Cayman Chemical (Ann Arbor, MI), M&B-28767 from Rhone-Poulenc Rorer, SC-19220 from Searle Research and Development (Skokie, IL), and 17-phenyl-PGE2 from Biomol (Hamburg, Germany). All other reagents were purchased from Sigma (St. Louis, MO).

**Statistics.** All data are presented as means ± SE. Where appropriate, Student’s \( t \)-test for paired data was used to assess the significance of differences. A one-way ANOVA was performed to assess statistical significance between unpaired data.

**RESULTS**

The inhibitory effect of PGE₂ on the AVT-induced increase in OWP in the frog urinary bladder is associated with activation of the EP₁ receptor. Figure 1A illustrates the results of a typical experiment on the effect of PGE₂ on the AVT-induced increase in OWP in isolated frog paired hemibladders: PGE₂ \( (10^{-8} \text{ M in this case}) \) significantly reduced the hormonal response. To examine whether the inhibitory action of PGE₂ is mediated through activation of the EP₁ receptor, we studied the effect of the EP₁-receptor agonist 17-phenyl-PGE₂ and sulprostone (EP₁/EP₃ agonist) on the AVT-induced increase in OWP. PGE₂ analogs were added to the serosal bath medium 15 min before AVT; in each pair the effect of AVT plus specific agonist in appropriate concentration \( (10^{-9} \text{ to } 10^{-6} \text{ M}) \) was compared with the effect of AVT alone. Both agonists, as well as PGE₂, caused concentration-dependent inhibition of the AVT-induced increase in OWP, with the maximal effect occurring at \( 10^{-6} \text{ M} \) (Fig. 1B). Pretreatment of isolated hemibladders with SC-19220, a specific EP₁-receptor antagonist, dose-dependently decreased the PGE₂-induced inhibition of the AVT response (Fig. 1C). These results demonstrate that the inhibitory effect of PGE₂
on the AVT-induced increase in OWP in the frog urinary bladder is associated with activation of the EP1 receptor.

EP1 agonist increases [Ca$^{2+}$], in epithelial cells from the frog urinary bladder. To determine whether 17-ph-PGE2 in our model acts via its inherent signaling pathway, i.e., increase in [Ca$^{2+}$], we examined its effect on the fura 2-AM F340/F380 in the fura 2-AM-loaded isolated epithelial cells from frog bladder.

Under basal conditions, spontaneous transients of the F340/F380 were seen occasionally, and these cells were not taken into account. Addition of ethanol (no more than 0.05%, the solvent for 17-ph-PGE2) had no effect on the F340/F380 (data not shown). The proportion of cells in the field of view responding to 17-ph-PGE2 (10$^{-6}$ M) by a rise in [Ca$^{2+}$], varied in different experiments from 5 to 50% of the fluorescing cells. Examples of the typical response to 17-ph-PGE2 are shown in Fig. 2, A and B. In the medium with 1.5 mM Ca$^{2+}$, administration of 17-ph-PGE2 elevated [Ca$^{2+}$], from 71.3 ± 3.7 to 104.4 ± 2.5 nM (n = 132, $P < 0.001$, paired Student’s t-test), whereas without external Ca$^{2+}$, [Ca$^{2+}$], was increased in the presence of agonist from 61.5 ± 4.5 to 83.6 ± 4.9 nM (n = 48, $P < 0.001$, paired Student’s t-test) (Fig. 2C). In the presence of external Ca$^{2+}$, 86% of the responding cells demonstrated a prolonged plateau phase after the ratio peak (Fig. 2A), whereas in other cells the ratio transients after addition of 17-ph-PGE2 rapidly decreased to resting values, even in the presence of the drug. In the absence of external Ca$^{2+}$, all responding cells demonstrated only the peak in the ratio (Fig. 2B). The mean value of the transient maximum time was 28.7 ± 2.0 s (n = 125) in the presence of extracellular Ca$^{2+}$ and 16.6 ± 1.4 s (n = 48) in the absence of Ca$^{2+}$ in the medium.

NOS activity in epithelial cells homogenates depends on Ca$^{2+}$/calmodulin and is reduced under 7-NI, an nNOS inhibitor. To determine the NOS activity in mucosal epithelium of the frog urinary bladder, we measured the rate of formation of L-[3H]citrulline from L-[3H]arginine in the presence of appropriate cofactors (see MATERIALS AND METHODS) in crude homogenates of isolated epithelial cells. It was found that L-citrulline formation was more than fivefold inhibited by the nonspecific NOS inhibitor L-NAME (1 mM), indicating that its formation is mainly due to NOS activity. In Ca$^{2+}$/calmodulin-free incubation medium containing 1 mM EDTA, the NOS activity was markedly reduced (Fig. 3). A significant decrease in L-citrulline production was also found in the presence of 100 μM 7-NI. In these experiments, the effect of the last one was compared with the effect of pure ethanol, which was used as a solvent for 7-NI, at a final concentration of 1% (Fig. 3). These data indicate that frog urinary bladder epithelial cells have the capacity to produce NO. High dependence of NOS activity on Ca$^{2+}$ and sensitivity to 7-NI suggest the presence of nNOS isoform.

Western blot analysis. To characterize NOS isoform(s) expressed in the frog urinary bladder epithelial cells, we analyzed cross-reactivity by Western blotting with a rabbit polyclonal antibody against all three NOS isoforms (anti-universal NOS antibody). In the zone of interest (120–160 kDa), this revealed a single band with an apparent molecular mass of ~155 kDa for urinary bladder epithelial cells, which had the same mobility as NOS from rat cerebellum and frog brain used as a positive control (Fig. 4). According to the size, this band corresponds to nNOS. The use of specific nNOS antibody (BD Biosciences Pharmingen) revealed a weak signal in a 155-kDa

Fig. 2. 17-ph-PGE2 (10$^{-6}$ M) increases intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) in epithelial cells of the frog urinary bladder. A and B: original recordings in the presence of external Ca$^{2+}$ and in Ca$^{2+}$-free medium, respectively. Ordinate, fura 2-AM 340-nm to 380-nm fluorescence ratio (F340/F380). C: mean values ± SE of the effect of 17-ph-PGE2 on [Ca$^{2+}$], in the fura 2-AM-loaded epithelial cells. Open bars, control; gray bars, 17-ph-PGE2. Numbers in parentheses refer to the number of individual cells in which the Ca$^{2+}$ response was measured.

***$P < 0.001$ (paired Student’s t-test).
Fig. 3. Nitric oxide synthase (NOS) activity in crude homogenates of epithelial cells isolated from frog urinary bladder (in counts l-[^3]H)citrulline·mg protein·min⁻¹). Cells were homogenized, and aliquots of supernatant were incubated in HEPES buffer with l-[^3]H]arginine and cofactors (β-NADPH, tetrahydrobiopterin, Ca²⁺/calmodulin, FAD) for 1 h at 25°C. Ca²⁺-independent NOS activity was measured in samples without Ca²⁺ and calmodulin in the presence of 1 mM EDTA. Nitro-l-arginine methyl ester (l-NAME) was used at 1 mM. To estimate the effect of 7-nitroindazole (7-NI; 100 μM) dissolved in ethanol, the appropriate amount of the solvent (1%) was added to the control samples. l-Citrulline was separated by ion-exchange resin. A sample in which the homogenate was boiled before the assay served as a blank, which was subtracted from each value. Values are means ± SE for 5 independent experiments. *P < 0.05 vs. control; **P < 0.05 vs. 1% ethanol (1-way ANOVA).

Fig. 4. Immunoblot analysis demonstrating expression of neuronal NOS (nNOS) in frog urinary bladder epithelial cells. Samples of rat cerebellum and frog brain were lysed, and intracellular citrulline was separated by ion-exchange resin. A protein in epithelial cells, whereas application of an antibody against eNOS (BD Biosciences PharMingen) or against iNOS (Biomol) gave no positive results (data not shown).

7-NI prevents the inhibitory effect of an EP₁ agonist on AVT-induced OWP and increases the hydrosmotic response to AVT. To clarify whether nNOS contributes to the regulation of OWP in the frog urinary bladder, we examined the effect of 7-NI on the AVT-induced hydrosmotic response and on the ability of an EP₁ agonist to inhibit this reaction. Pretreatment of frog urinary bladders with 7-NI (50 μM) led to an increase in the AVT-induced hydrosmotic response (Fig. 5A), suggesting the involvement of nNOS in negative modulation of the response to AVT. Very similar results were obtained if l-NAME (1 mM) was used instead of 7-NI (data not shown). The inhibitory effect of the EP₁ agonist on AVT-induced OWP was markedly reduced in the presence of 7-NI (Fig. 5B). Without the inhibitor, 17-ph-PGE₂ (10⁻⁷ M) decreased the hydrosmotic response to 5 × 10⁻¹⁰ AVT from 2.86 ± 0.53 to 1.51 ± 0.48 μl·min⁻¹·cm⁻² (P < 0.05), whereas in the presence of 50 μM 7-NI from 2.1 ± 0.51 to 1.78 ± 0.39 μl·min⁻¹·cm⁻² (not significant; Fig. 5B). These data suggest that the inhibitory effect of the EP₁ agonist on the AVT-induced increase in OWP depends on activation of nNOS.

PGE₂ stimulates NOS activity in intact epithelial cells. To study a potential interaction between activation of the EP₁ receptor and NOS activity, it was necessary for the intracellular signaling machinery to be undestroyed. To achieve this condition, we measured l-[^3]H)citrulline accumulation, as a measure of intracellular NOS activity, in intact epithelial cells loaded with l-[^3]H]arginine. After loading and incubation with prostanooids, cells were lysed, and intracellular citrulline was separated from cell lysate by TLC. The use of TLC allowed an estimation of small amounts of l-[^3]H)citrulline more precisely compared with the use of ion-exchange resin, which binds no more than 80–90% of l-arginine.

In controls, the amount of intracellular l-[^3]H)citrulline was 3,050 ± 502 counts per minute (cpm)·10⁶ cells⁻¹·45 min⁻¹, whereas in the presence of 1 mM l-NAME it was 1,414 ± 267 cpm·10⁶ cells⁻¹·45 min⁻¹ (P < 0.01, n = 20, one-way ANOVA). The difference between these values corresponds to l-[^3]H)citrulline accumulated because of NOS activity. For this reason, we calculated the level of intracellular l-[^3]H)citrulline in all experiments on intact cells after subtracting the value in the presence of l-NAME, which could be considered to be nonspecific radioactivity.

As in experiments in lysed epithelial cells, NOS activity in intact cells was markedly reduced in the Ca²⁺-free EDTA incubation medium (492 ± 111 vs. 1,636 ± 269 cpm l-[^3]H)citrulline·10⁶ cells⁻¹·45 min⁻¹ in control; P < 0.001, n = 20), indicating a significant predominance of Ca²⁺⁺-dependent isomorfs(s). To study whether PGE₂ regulates NOS activity in epithelial cells, the isolated cells were treated for 20 min with various doses of PGE₂. The data showed that PGE₂ increased NOS activity, with the maximal effect of 152.2 ± 7.7% occurring at a concentration of 10⁻⁹ M (Fig. 6). Elevation of the PGE₂ concentration up to 10⁻⁶ M led to a gradual decrease in the stimulatory effect. At 10⁻⁶ M, PGE₂ had no effect on NOS activity (Fig. 6).

PGE₂ stimulates NOS activity in epithelial cells through the EP₁ receptor. To clarify which receptor subtype(s) mediates the effect of PGE₂ on NOS activity, we examined the ability of different PGE₂-receptor agonists to enhance l-[^3]H)citrulline accumulation in epithelial cells (Fig. 7). Among the agonists used, the stimulatory effect was observed under 17-ph-PGE₂ (an EP₁ agonist) and sulprostone (an EP₁/EP₃ agonist). No effect was seen under M&B-28767 (EP₃ agonist) or butaprost (EP₂ agonist). Moreover, the stimulatory effect of PGE₂ on NOS activity was eliminated in the presence of the EP₁-selective antagonist SC-19220 (Fig. 7). These data demonstrate that PGE₂ stimulates NOS in the frog bladder epithelial cells via the EP₁-receptor subtype.

To examine whether 17-ph-PGE₂ stimulates nNOS activity in epithelial cells, we tested its effect on cells pretreated for 10 min with 7-NI (100 μM). By itself, 7-NI significantly decreased NOS activity: 447 ± 35 vs. 930 ± 64 cpm l-[^3]H)citrulline·10⁶ cells⁻¹·45 min⁻¹ in control (Fig. 8). In the presence
of 7-NI, the ability of 17-ph-PGE2 to stimulate NOS activity was reduced (Fig. 8), suggesting that the EP1 agonist specifically activates nNOS in the frog urinary bladder epithelial cells.

AVT (10^{-9} to 10^{-7} M) and forskolin (50 μM), both cAMP-elevating agents, as well as wortmannin (50 μM), an inhibitor of phosphatidylinositol 3-kinase (PI3K), did not alter the NOS activity (data not shown).

17-ph-PGE2 elevates cGMP concentration in epithelial cells. Because soluble guanylate cyclase is an intracellular receptor for NO, we measured the level of intracellular cGMP in isolated epithelial cells after addition of 17-ph-PGE2 (10^{-6} M). The incubation medium contained IBMX and zaprinast to inhibit cGMP degradation and indomethacin to decrease the production of endogenous prostaglandins. As depicted in Fig. 9, administration of 10^{-6} M 17-ph-PGE2 caused a transient rise in cGMP concentration 30 s after addition. At 1 and 3 min, the cGMP level was equal to the level in unstimulated cells.

DISCUSSION

As in mammalian CD, the ADH-regulated reabsorption of water in amphibian urinary bladder is achieved through cAMP-dependent phosphorylation of intracellular aquaporins and their translocation to the apical plasma membrane of epithelial cells, thereby allowing the water to be transported (23). In our previous paper (17), we have shown that, in the frog urinary bladder, exogenous NO inhibits AVT-induced increase of OWP, acting, at least partly, via activation of cGMP-dependent...
protein kinase. However, it remained unclear 1) whether the urinary bladder epithelial cells are able to produce endogenous NO, 2) whether the OWP of the urinary bladder epithelium depended on NOS activity, and 3) what was the mode of regulation of NO generation in epithelial cells.

The present study demonstrates that epithelial cells of the frog urinary bladder produce NO. The NOS enzymatic activity in both intact and lysed cells was dependent on the presence of Ca\(^{2+}\) and was reduced by 7-NI, an nNOS inhibitor. Immunoblot analysis of epithelial cell lysate with the use of an universal NOS antibody revealed a single protein band that corresponds to nNOS. This antibody is specific to highly conserved NADPH binding domain of all three NOS isoforms (1). Of interest, a synergism in the effects of PGE\(_2\) and Ca\(^{2+}\)-free medium, indicating that the 17-ph-PGE\(_2\)-induced increase in Ca\(^{2+}\) is due to both the release of Ca\(^{2+}\) from intracellular stores and the influx of Ca\(^{2+}\) from the extracellular environment. Together, these results demonstrate that the inhibitory effect of PGE\(_2\) on AVT-induced OWP is mediated by the EP\(_1\) receptor that is coupled to Ca\(^{2+}\) mobilization.

Fig. 8. 7-NI reduces the stimulatory effect of 17-ph-PGE\(_2\) on NOS activity (in cpm L-[\(^{3}\)H]citrulline/10\(^6\) cells/45 min) in epithelial cells of the frog urinary bladder. Freshly isolated cells (1.5–2 \times 10^6 in each sample) were incubated in 150 \(\mu\)l of solution B containing a mixture of 30 \(\mu\)M L-arginine and tracer amounts of L-[\(^{3}\)H]arginine for 45 min at 25°C. 7-NI (100 \(\mu\)M) was added 10 min before L-arginine. 17-ph-PGE\(_2\) (10\(^{-6}\) M) was added during the last 20 min of incubation. Values are means \pm SE; \(n=8–10\) experiments. Indicated statistical significance was determined by 1-way ANOVA.

A role for prostaglandins in the regulation of NO generation has been demonstrated by other authors, primarily at the level of regulation of NOS gene expression. It was shown that PGE\(_2\) stimulates expression of nNOS in the brain and eNOS in cerebral microvessels in newborn pigs via EP\(_3\) receptor (14, 15). In macula densa cells of the mammalian kidney, PGE\(_2\) exerts an inhibitory effect on nNOS expression (38). There is also evidence that, in rat alveolar macrophages, inhibitors of prostaglandin synthesis markedly inhibit the expression of iNOS (1). Of interest, a synergism in the effects of PGE\(_2\) and NO on downregulation of aquaporin-2 expression was described recently in mammalian CD during escape from AVP-induced antidiuresis (32). The exact mechanisms underlying the synergism between PGE\(_2\) and NO remain unclear.

To study the molecular mechanisms providing the interaction between PGE\(_2\) and Ca\(^{2+}\)-dependent NOS activity, we first examined the involvement of the EP\(_1\) receptor in the inhibitory effect of PGE\(_2\) on AVT-induced osmotic water flow and its coupling to Ca\(^{2+}\) mobilization. Because both 17-ph-PGE\(_2\), an EP\(_1\) agonist, and sulprostone, an EP\(_1\)/EP\(_3\) agonist, dose-dependently inhibited AVT-induced OWP, and the inhibitory effect of PGE\(_2\) was significantly reduced in the presence of SC-19220, an EP\(_1\) antagonist, it is possible to conclude that the inhibitory effect of PGE\(_2\) is associated with activation of the EP\(_1\) receptor. This conclusion does not exclude the involvement of EP\(_3\)-receptor activation because M&B-28767, an EP\(_3\) agonist, also inhibits the AVT-induced increase in OWP in R. temporaria urinary bladder (39). The EP\(_3\) receptor has been found in the epithelium of frog R. esculenta skin where it mediates the inhibitory effect of PGE\(_2\) on AVT-induced increase of trans-epithelial Na\(^{+}\) transport (41).

According to our data, the action of EP\(_1\) agonist on epithelial cells is coupled to an increase in [Ca\(^{2+}\)]\(_i\). In most responding cells, the plateau, but not the peak, was absent in Ca\(^{2+}\)-free medium, indicating that the 17-ph-PGE\(_2\)-induced increase in [Ca\(^{2+}\)]\(_i\) is due to both the release of Ca\(^{2+}\) from intracellular stores and the influx of Ca\(^{2+}\) from the extracellular environment. Together, these results demonstrate that the inhibitory effect of PGE\(_2\) on AVT-induced OWP in the frog urinary bladder is mediated by the EP\(_1\) receptor that is coupled to Ca\(^{2+}\) mobilization.

Fig. 9. Time course of cGMP accumulation (in fmol cGMP/10\(^6\) cells) in epithelial cells of the frog urinary bladder in response to 17-ph-PGE\(_2\). Freshly isolated cells (1.8–2 \times 10^6 in each sample) were incubated in 150 \(\mu\)l of solution B containing 1 mM IBMX, 50 \(\mu\)M zaprinast, 30 \(\mu\)M arginine, and 1 \(\mu\)M indomethacin for 25 min at 25°C. 17-ph-PGE\(_2\) (10\(^{-6}\) M) was added at point 0 for various times (0.5–3 min). The reaction was stopped by placing the tubes in a boiling water bath for 3 min. The samples were centrifuged, and the amount of cGMP was determined in the supernatant with a cGMP-enzyme immunoassay system. Values are means \pm SE; \(n=6\) experiments. Indicated statistical significance was determined by 1-way ANOVA.
Administration of PGE₂ (10⁻⁹ to 10⁻⁷ M) caused stimulation of NOS activity in frog urinary bladder epithelial cells. Among different agonists used, only EP₁ (17-ph-PGE₂) or EP₃/PGE₃ (sulprostone) agonists had the ability to stimulate NOS activity. PGE₂ did not increase NOS activity in the presence of SC-19220, an EP₁ antagonist. The inability of butaprost, a cAMP-mobilizing EP₂ agonist, to raise NOS activity indicates that the observed effect of PGE₂ is cAMP independent. Together, these data show that PGE₂ activates NOS through the EP₁ receptor coupled to mobilization of Ca²⁺ concentration. The stimulatory effect of 17-ph-PGE₂ on NOS activity was reduced in the presence of 7-NI, suggesting that nNOS is a downstream target for EP₁-receptor-mediated signaling in the frog urinary bladder epithelial cells.

The dose dependency of the PGE₂ effect on NOS activity indicates that the increase of PGE₂ concentration (up to 10⁻⁶ M) leads to the loss of the stimulatory effect. This observation suggests an involvement of multiple PGE₂-receptor subtypes coupled to different signaling pathways. Indeed, our previous data showed that PGE₂ at high concentrations (10⁻⁷ to 10⁻⁶ M) acting through EP₂ receptor significantly elevates cAMP in the frog urinary bladder epithelial cells (2). In general, cAMP may be a negative regulator of nNOS activity. It has been shown that phosphorylation of purified nNOS by protein kinase A markedly reduces nNOS catalytic activity (13). Because the last one strongly depends on Ca²⁺, cAMP could affect nNOS activity also via modulation of [Ca²⁺]. Such a relationship between cAMP and [Ca²⁺] has been shown in human platelets, where cell-permeable analogs of cAMP, cAMP PDE inhibitors, and PGE₁, cAMP-mobilizing agents (10⁻⁷ to 10⁻⁵ M), suppress thrombin-induced increases in [Ca²⁺], (40, 47). Thus we could speculate that the inability of PGE₂ at 10⁻⁶ M to stimulate NOS activity in the frog urinary bladder epithelial cells is related to the increased level of intracellular cAMP, which by unknown mechanism prevents EP₁-receptor-mediated nNOS activation.

Because 7-NI has a close selectivity for nNOS and eNOS, the activation of eNOS, which is also Ca²⁺/calmodulin dependent, by PGE₂ cannot be completely excluded. It is widely accepted that the major mechanisms responsible for activation of eNOS are PI3K/Akt and cAMP/PKA signaling pathways (7, 16). For example, it is known that PGE₂ stimulates angiogenesis in human umbilical vein endothelial cells by activating eNOS and NO production through a cAMP-dependent protein kinase/P38/PI3K/Akt-dependent pathway (34). However, in our experiments, neither wortmannin, a PI3K inhibitor, nor forskolin, a cAMP-elevating agent, altered total NOS activity in epithelial cells, arguing against the presence of eNOS in the frog bladder epithelial cells. Besides nNOS, no bands were revealed in immunoblots of epithelial cell lysate with anti-universal NOS antibody. Also, as shown in our previous work (17), immunohistochemistry of the frog urinary bladder slices with NOS antibodies revealed marked immunostaining only with nNOS antibody, whereas no reaction was found with eNOS antibody. Thus, using different approaches, we failed to reveal the presence of eNOS in the frog urinary bladder epithelial cells. These data suggest that the inhibitory effect of 7-NI both on the basal and PGE₂-stimulated NOS activity is mainly associated with inhibition of nNOS.

According to our data, pretreatment of the isolated frog urinary bladder with 7-NI led to increased AVT-induced OWP and prevented the ability of an EP₁ agonist to inhibit this reaction, providing evidence for the contribution of nNOS and nNOS-generated NO to regulation of the hydrosmotic response to AVT. However, we could not specify the type of cell of the frog urinary bladder epithelium that contains PGE₂-sensitive NOS. Two morphologically distinct cell types constitute the epithelial layer of frog urinary bladder: granular cells and mitochondria-rich cells (9). Granular cells comprise 80–90% of the epithelial cells and cover most of the apical surface facing the lumen of the bladder. It is well established that ADH-induced cellular modifications (i.e., translocation of aquaporins to apical membrane, osmotic water flow-induced cell swelling) occur exclusively in granular cells (9, 23). By histochemical reaction to NADPH diaphorase activity, which is widely used as an indicator of NOS-containing cells, we have shown that most of the cells in a suspension of epithelial cells isolated from the frog urinary bladder demonstrated NADPH diaphorase staining (3), suggesting that NOS is located in granular cells.

The addition of 17-ph-PGE₂ to epithelial cell suspensions led to a transient increase in intracellular cGMP levels: the peak elevation occurred within the first 30 s, followed by a decrease to the basal level. These experiments were performed in the incubation medium with IBMX, a nonspecific PDE inhibitor, and zaprinast, a specific PDE5 inhibitor. The time course of the cGMP response indicates that 17-ph-PGE₂-induced cGMP appears to be degraded by a cGMP-specific and IBMX- and zaprinast-insensitive PDE. According to accepted classification, there are 11 different families of PDEs described mainly in mammals (4); some of them, such as PDE9, are characterized by a high affinity for cGMP and an insensitivity to IBMX. Further studies are required to examine the nature of the PDE responsible for the breakdown of PGE₂-mobilized cGMP in frog urinary bladder epithelial cells.

The downstream target for EP₁-agonist-induced cGMP remains unclear. As shown previously (17), the effect of cGMP elevated by an NO donor in the frog urinary bladder cells seems to be associated with cGMP-dependent protein kinase activation, since the inhibitory effect of an NO donor on AVT-induced OWP was not accompanied by changes in cAMP levels in epithelial cells and was almost completely reversed by KT-5823, an inhibitor of cGMP-dependent protein kinase. Whether this is true for the EP₁-agonist-dependent pool of cGMP remains to be elucidated.

In summary, our data show that, in the frog urinary bladder, PGE₂ exerts at least two effects mediated by Ca²⁺ concentration-coupled EP₁ receptor: it inhibits AVT-induced OWP and stimulates NOS activity in epithelial cells. Epithelial cells express NOS, and NO apparently generated by NOS attenuates AVT-stimulated OWP, acting in a cGMP-dependent manner. Together, these data demonstrate that the EP₁-receptor-mediated inhibitory effect of PGE₂ on the AVT-induced increase in OWP in the frog urinary bladder is based on activation of the NO/cGMP signaling pathway. These results suggest a novel cross talk between ADH, PGE₂, and NOS in osmoregulatory epithelium that may play an important role in the regulation of water transport.
GRANTS

This work was supported by the Russian Fund of Fundamental Research (05-04-48899-a).

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