Prostaglandin E\textsubscript{2} inhibits vasotocin-induced osmotic water permeability in the frog urinary bladder by EP\textsubscript{1}-receptor-mediated activation of NO/cGMP pathway

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Bachtueva V, Fock E, Lavrova E, Nikolaeva S, Gambaryan S, Parnova R. Prostaglandin E\textsubscript{2} inhibits vasotocin-induced osmotic water permeability in the frog urinary bladder by EP\textsubscript{1}-receptor-mediated activation of NO/cGMP pathway. Am J Physiol Regul Integr Comp Physiol 293: R528–R537, 2007. First published March 15, 2007. First published March 15, 2007. doi:10.1152/ajpregu.00811.2006.—PGE\textsubscript{2} is a well-known inhibitor of the antidiuretic hormone-induced increase of osmotic water permeability (OWP) in different osmoregulatory epithelia; however, the mechanisms underlying this effect of PGE\textsubscript{2} are not completely understood. Here, we report that, in the frog Rana temporaria urinary bladder, EP\textsubscript{1}-receptor-mediated inhibition of arginine-vasotocin (AVT)-induced OWP by PGE\textsubscript{2} is attributed to increased generation of nitric oxide (NO) in epithelial cells. It was shown that the inhibitory effect of 17-phenyl-trinor-PGE\textsubscript{2} (17-ph-PGE\textsubscript{2}), an EP\textsubscript{1} agonist, on AVT-OWP was significantly reduced in the presence of 7-nitroindazole (7-NI), a neuronal NO synthase (nNOS) inhibitor. NO synthase (NOS) activity in both lysed and intact epithelial cells measured as a rate of conversion of L-\textsuperscript{[3H]}arginine to L-\textsuperscript{[3H]}citrulline was Ca\textsuperscript{2+} dependent and inhibited by 7-NI. PGE\textsubscript{2} and 17-ph-PGE\textsubscript{2}, but not M&B-28767 (EP\textsubscript{1} agonist) or butaprost (EP\textsubscript{2} agonist), stimulated NOS activity in epithelial cells. The above effect of PGE\textsubscript{2} was abolished in the presence of SC-19220, an EP\textsubscript{1} antagonist. 7-NI reduced the stimulatory effect of 17-ph-PGE\textsubscript{2} on NOS activity. 17-ph-PGE\textsubscript{2} increased intracellular Ca\textsuperscript{2+} concentration and cGMP in epithelial cells. Western blot analysis revealed an nNOS expression in epithelial cells. The above effect of PGE\textsubscript{2} was abolished by SC-19220, an EP\textsubscript{1} antagonist. 7-NI reduced the stimulatory effect of 17-ph-PGE\textsubscript{2} on NOS activity. 17-ph-PGE\textsubscript{2} increased intracellular Ca\textsuperscript{2+} concentration and cGMP in epithelial cells. Western blot analysis revealed an nNOS expression in epithelial cells. These results show that the inhibitory effect of PGE\textsubscript{2} on AVT-induced OWP in the frog urinary bladder is based at least partly on EP\textsubscript{1}-receptor-mediated activation of the NO/cGMP pathway, suggesting a novel cross talk between AVT, PGE\textsubscript{2}, and nNOS that may be important in the regulation of water transport.

Amphibian urinary bladder; nitric oxide; neuronal nitric oxide synthase

Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) 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 PGE\textsubscript{2} are known to be mediated through ligation of four distinct G-protein-coupled receptors, namely, EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4}, which have been defined on the basis of their molecular structures, pharmacological profiles, and signal transduction pathway (10, 12, 36). The EP\textsubscript{1} receptor is coupled to the activation of phosphatidylinositol 4,5-biphosphate hydrolysis and mobilization of intracellular Ca\textsuperscript{2+}. EP\textsubscript{2} and EP\textsubscript{4} both increase adenylate cyclase activity, whereas EP\textsubscript{3} mainly inhibits it. Mammalian CD are characterized by the greatest diversity of PGE\textsubscript{2}–receptor subtypes (10) and demonstrate the highest rate of PGE\textsubscript{2} synthesis among kidney structures (6).

Despite intensive investigation of ADH-PGE\textsubscript{2} interactions in the regulation of water reabsorption (10, 25, 37, 48), the mechanisms underlying the inhibitory effect of PGE\textsubscript{2} on ADH-stimulated osmotic water permeability (OWP) in either mammalian CD or amphibian urinary bladder are not completely understood. Two subtypes of PGE\textsubscript{2} receptor, EP\textsubscript{1} and EP\textsubscript{3}, have been suggested to mediate the inhibitory effect of PGE\textsubscript{2} on AVP-induced OWP in mammalian CD. In rabbit cortical CD cells, EP\textsubscript{3} receptor was found to be coupled to peritussis toxin-sensitive inhibition of AVP-stimulated OWP through a decrease in cAMP generation (25, 37). Tamma et al. (44) suggested that the EP\textsubscript{3} 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 EP\textsubscript{1} receptor is highly expressed primarily in the CD along the mammalian nephron (22, 43) and is mainly associated with inhibition of Na\textsuperscript{+} reabsorption via a Ca\textsuperscript{2+} -coupled mechanism (22). However, the inhibitory effect of PGE\textsubscript{2} on the AVP-induced increase of OWP in rabbit and rat CD was reversed by staurosporine, a PKC inhibitor, suggesting the involvement of the EP\textsubscript{1}-receptor-mediated activation of phosphatidylinositol 4,5-biphosphate hydrolysis in the regulation of water reabsorption (24, 33). The cellular mechanisms underlying the Ca\textsuperscript{2+} -coupled effect of PGE\textsubscript{2} 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 Ca\textsuperscript{2+} through the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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 insertion of aquaporin-2 (8).

The aim of the present paper was to test the hypothesis that the EP1-receptor-mediated inhibitory effect of PGE\(_2\) 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 PGE\(_2\) inhibit the AVT-induced increase in OWP, 2) PGE\(_2\) is known to release Ca\(^{2+}\) from intracellular stores in mammalian CD (24, 33) and toad urinary bladder epithelial cells (18, 3), and 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 PGE\(_2\) and NO in the regulation of water reabsorption in the frog urinary bladder, we studied the effect of an EP1 agonist, 17-phenyl-trinor-PGE\(_2\) (17-ph-PGE\(_2\)), 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 PGE\(_2\) 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 pithe 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 Na\(_2\)HPO\(_4\), 4.0 Na,HPO\(_4\), and 1.0 CaCl\(_2\) (pH 7.6, osmolality of 220 mmol/kgH\(_2\)O). 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 NaHCO\(_3\), 0.8 KH\(_2\)PO\(_4\), 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 NaHCO\(_3\), 0.8 KH\(_2\)PO\(_4\), 2 glucose, 1.5 CaCl\(_2\), and 0.8 MgCl\(_2\), pH 7.6. From one hemibladder, it was possible to obtain at least 2 × 10\(^6\) 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 Coomassie 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 CaCl\(_2\), 1 mM NADPH, 10 μM tetrahydrobiopterin, 1.25 μg/ml calmodulin, 10 μM FAD, and 0.5 μl l-[\(^3\)H]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. Ca\(^{2+}\)-independent activity was measured in samples without CaCl\(_2\) 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-[\(^3\)H]citrulline. Cells (1.5–2 × 10\(^6\) in each sample) were incubated in 150 μl of solution B containing 30 μM l-arginine and 0.5 μl l-[\(^3\)H]arginine (1 μCi/ml, specific activity of 68 Ci/mmol; Amersham) for 45 min at 25°C. Ca\(^{2+}\)-independent NOS activity was measured in the absence of external Ca\(^{2+}\) (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 0-propanol-n-propanol-H\(_2\)O (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 ml Tris-HCl, pH 6.8, containing 25 ml EDTA, 0.1% Triton X-100, 0.2 ml 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 Bio-tech). 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

**AJP-Regul Integr Comp Physiol • VOL 293 • JULY 2007 • www.ajpregu.org**
was used as a secondary antibody, and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) was used to visualize the signal.

**Measurement of intracellular 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 Ca^{2+} concentrations ([Ca^{2+}]) were determined with the use of the calibration curve obtained from the set of solutions with known Ca^{2+} concentrations.

**Measurement of the cGMP content in epithelial cells.** Freshly isolated epithelial cells (1.8 – 2 × 10^6 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-ph-PGE2 or an equivalent amount of ethanol (1%) was added in experimental and control probes, respectively, for various times (0.5–3 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 10^6 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-ph-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 IA 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} 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 antagonist 17-ph-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} to 10^{-6} 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} 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-PGE\(_2\) 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-PGE\(_2\)) had no effect on the F340/F380 (data not shown). The proportion of cells in the field of view responding to 17-ph-PGE\(_2\) (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-PGE\(_2\) are shown in Fig. 2, A and B. In the medium with 1.5 mM Ca\(^{2+}\), administration of 17-ph-PGE\(_2\) 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-PGE\(_2\) 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-[\(^{3}\)H]citrulline from L-[\(^{3}\)H]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 an antibody against all three NOS isoforms (anti-universal NOS antibody). In the zone of interest (120 – 160 kDa), this revealed a weak signal in a 155-kDa band 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 isoform.
Fig. 3. Nitric oxide synthase (NOS) activity in crude homogenates of epithelial cells isolated from frog urinary bladder (in counts L-[3H]citrulline·mg protein·min⁻¹). Cells were homogenized, and aliquots of supernatant were incubated in HEPES buffer with L-[3H]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-NATE) 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 (BD Biosciences Pharmingen) or against iNOS (Biomol) gave no positive results (data not shown).

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-[3H]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-[3H]citrulline·10⁶cells⁻¹·45 min⁻¹ in control (Fig. 8). In the presence of 7-NI, PGE₂ had no effect on NOS activity (Fig. 6).
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 the 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 anti-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 

\[ \text{PGE}_2 \text{ ACTIVATES nNOS} \]

![Fig. 8](image)

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 (1.3–2 × 10\(^6\) in each sample) were incubated in 150 µl of solution B containing a mixture of 30 µM L-arginine and tracer amounts of L-[\(^{3}\)H]arginine for 45 min at 25°C. 7-NI (100 µM) 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 ± SE; n = 8–10 experiments. Indicated statistical significance was determined by 1-way ANOVA.

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\(^{2+}\)]\(_j\). 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\(^{2+}\)]\(_j\) 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.
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 (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 Ca^{2+} concentration. The stimulatory effect of 17-ph-PGE2 on NOS activity was reduced in the presence of 7-NI, suggesting that NOS 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 Ca^{2+}, cAMP could affect nNOS activity also via modulation of [Ca^{2+}]. Such a relationship between cAMP and [Ca^{2+}] 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 [Ca^{2+}] (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 NOS activation.

Because 7-NI has a close selectivity for nNOS and eNOS, the activation of eNOS, which is also Ca^{2+}/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/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 osmotic 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 Ca^{2+} concentration-coupled EP1 receptor: it inhibits AVT-induced OWP and stimulates NOS activity in epithelial cells. Epithelial cells express nNOS, and NO apparently generated by nNOS 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 nNOS in osmoregulatory epithelium that may play an important role in the regulation of water transport.
Garcia NH, Pomposiello SI, Garvin JL. R536 PGE2 ACTIVATES nNOS


