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$P2Y_2$ receptor-stimulated release of prostaglandin E_2 by rat inner medullary collecting duct preparations

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Welch, Brett D., Noel G. Carlson, Huihui Shi, Leslie Myatt, and Bellamkonda K. Kishore. $P2Y_2$ receptor-stimulated release of prostaglandin E_2 by rat inner medullary collecting duct preparations. *Am J Physiol Renal Physiol* 285: F711–F721, 2003. First published June 10, 2003; 10.1152/ajprenal.00096.2003.—Extracellular nucleotides, acting through the $P2Y_2$ receptor and the associated phosphoinositide-Ca^{2+} signaling pathway, inhibit AVP-stimulated osmotic water permeability in rat inner medullary collecting duct (IMCD). Because a rise in intracellular Ca^{2+} is frequently associated with enhanced arachidonic acid metabolism, we examined the effect of activation of the $P2Y_2$ receptor on release of PGE_2 in freshly prepared rat IMCD suspensions. Unstimulated IMCD released moderate, but significant, amounts of PGE_2, which were more sensitive to cyclooxygenase (COX)-2 than COX-1 inhibition. Agonist activation of $P2Y_2$ receptor by adenosine 5′-*O*-(3-thiotriphosphate) enhanced release of PGE_2 from IMCD in a time- and concentration-dependent fashion. Purinergic-stimulated release of PGE_2 was completely blocked by nonspecific COX inhibitors (flurbiprofen and 2-acetoxyphenylhept-2-ynyl sulfide). Differential COX inhibition studies revealed that purinergic-stimulated release of PGE_2 was more sensitive to a COX-1-specific inhibitor (valeroyl salicylate) than a COX-2-specific inhibitor (NS-398). Thus purinergic stimulation resulted in significantly more release of PGE_2 in the presence of COX-2 inhibitor than COX-1 inhibitor. If it is assumed that increased release of PGE_2 is related to its increased production, our results suggest that purinergic stimulation of IMCD results in enhanced production and release of PGE_2 in a COX-1-dependent fashion. Because PGE_2 is known to affect transport of water, salt, and urea in IMCD, interaction of the purinergic system with the prostanoid system in IMCD can modulate handling of water, salt, and urea by IMCD and, thus, may constitute an AVP-independent regulatory mechanism.

cyclooxygenases; arachidonic acid; extracellular nucleotides; arginine vasopressin; purinergic receptor

THE MEDULLARY COLLECTING DUCT plays an important role in the regulation of body water, sodium, and acid-base balance and in the recycling of urea. It is a complex tubular segment that responds to stimulation by a variety of hormones or autacoids or paracrine agents, such as AVP, α- and β-adrenergic agonists, adenosine, atrial natriuretic peptide, bradykinin, endothelin, epidermal growth factor, muscarinic cholinergic agents, and PGE_2. These agents act through a variety of signaling pathways or systems, such as adenylyl cyclase, guanylyl cyclase, phospholipases A_2 and C, and the associated protein kinases (40). These diverse signaling pathways interact with each other in modulation of the overall function of the inner medullary collecting duct (IMCD). Central to this interaction is the mutually inhibitory relation between the activation of adenylyl cyclase and phospholipases. Thus increased cellular cAMP content impairs activation of phospholipases A_2 and C, and, conversely, stimulation of phospholipase C impairs AVP-stimulated adenylyl cyclase activity via activation of protein kinase C (40).

Extracellular nucleotides bind to specific subtypes of P2 purinergic receptors on cell membranes and elicit a wide variety of biological responses in several tissues. P2 receptors are classified into two families: the iontropic P2X and the metabotropic P2Y receptors. The former are extracellular nucleotide-activated membrane channels that allow a variety of ions and/or small molecules to enter the cells (31, 32). The P2Y receptors are G protein-coupled receptors that mostly act through the phosphoinositide signaling pathway (10, 42). Recent experimental studies have unraveled the role of extracellular nucleotides and autocrine and/or paracrine purinergic signaling in the regulation of glomerular, microvascular, and epithelial functions of the kidney in health and disease (3, 19, 27, 37, 38).

Using a pharmacological approach of measuring the agonist-stimulated rise in intracellular calcium responses, Ecelbarger et al. (12) identified and characterized the presence of the $P2Y_2$ (previously known as P2u) receptor in rat IMCD. Kishore et al. (23) showed that extracellular nucleotides (ATP/UTP) inhibit the AVP-stimulated osmotic water permeability of in vitro microperfused rat IMCD, thus establishing a physio-

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logical role for the $P2Y_2$ receptor in IMCD. Subsequently, Kishore et al. (24) demonstrated by molecular approaches that $P2Y_2$ receptor mRNA and protein are expressed in rat IMCD. Immunocytochemical localization studies revealed the expression of $P2Y_2$ receptor protein on apical and basal domains of collecting duct cells, as well as on thin limbs and vascular elements (24).

A rise in intracellular calcium, such as that induced by agonist stimulation of the $P2Y_2$ receptor in the medullary collecting duct, is known to be frequently associated with enhanced arachidonic acid metabolism. It has been demonstrated that, in several types of tissues or cells (e.g., guinea pig ileum and uterus, bovine aortic smooth muscle cells, rabbit heart and tracheal epithelial cells, isolated rat dura mater, thymic epithelial cells and astrocytes, porcine or human endothelial cells, and mouse peritoneal macrophages), agonist activation of P2Y receptors resulted in stimulation of arachidonic acid metabolism and production of prostanoids. In most of these tissues or cells, especially those of nonendothelial nature, the predominant prostanoid produced was PGE_2 (1, 2, 9, 11, 14, 16, 20, 28, 33, 34, 39, 44).

Experiments conducted on Madin-Darby canine kidney-D1 cells also revealed that agonist stimulation of the $P2Y_2$ receptor results in activation of cytosolic phospholipase A_2 ($cPLA_2$) and release of arachidonic acid in a protein kinase C- and MAPK-dependent fashion (43). However, to the best of our knowledge, no studies are available on the synthesis and release of prostanoids by renal medullary collecting duct cells after purinergic stimulation. Initial experiments conducted by Ecelbarger et al. (12) demonstrated that when terminal IMCD segments of rat were exposed to indomethacin, a nonspecific inhibitor of cyclooxygenases (COX), for 5 min and then challenged with ATP, the signals displayed a reduced intracellular calcium peak. This finding suggests that, in rat IMCD, purinergic activation stimulates arachidonic acid metabolism and enhanced production of COX products, which apparently potentiate the intracellular calcium response. On the basis of these observations, we hypothesized that COX products of arachidonic acid metabolism, such as PGE_2, are formed and released as a result of agonist activation of the $P2Y_2$ receptor in rat IMCD. To test this hypothesis, we conducted studies on freshly prepared rat IMCD suspensions and examined whether the basal and adensoine 5′-*O*-(3-thiotriphosphate) (ATPγS)-stimulated release of PGE_2 was affected by nonspecific inhibitors of COX or COX-1- or COX-2-specific inhibitors.

MATERIALS AND METHODS

Experimental Animals

The animal experiments were conducted according to the protocol approved by the Institutional Animal Care and Use Committee of the Veterans Administration Salt Lake City Health Care System. Specific pathogen-free male Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed two or three per cage in the Veterinary Medical Unit of the Veterans Administration Salt Lake City Health Care System, which is an American Association for Accreditation of Laboratory Animal Care-accredited and US Department of Agriculture-approved animal facility. The rats were maintained in pathogen-free state and fed ad libitum a commercial rodent diet and had free access to drinking water. The rats were acclimated to the housing conditions for ≥5 days before the experiments were conducted. The rats weighed 220–400 g (mean 292 g) at the time of euthanasia.

Agents

ATPγS (95.7% purity) and arachidonyltrifluoromethyl ketone (AACOCF3, 97% purity) were purchased from Calbiochem-Novabiochem (La Jolla, CA), UTP (97% purity) and DMSO (99.9% purity) from Sigma Chemical (St. Louis, MO), 2-acetoxyphenylhept-2-ynyl sulfide (APHS, 98% purity), 2-fluoro-α-methyl-(1,1′-biphenyl)-4-acetic acid (flurbiprofen, 99% purity), 2-[(1-oxopentyl)oxy]-benzoic acid (valeroyl salicylate, 99% purity), and *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398, 99% purity) from Cayman Chemical (Ann Arbor, MI), collagenase B from Roche Molecular Biochemicals (Indianapolis, IN), and bovine testes hyaluronidase from Worthington Biochemical (Lakewood, NJ). All other chemicals used were of the highest purity available.

Preparation of Fractions Enriched in IMCD

Fractions enriched in IMCD were prepared from rat kidney inner medullae essentially as described previously (24, 26). Briefly, rats were euthanized by pentobarbital sodium overdose, and both kidneys were removed rapidly. The kidneys were chilled in ice-cold phosphate-buffered saline, and the inner medullae or papillae were dissected on ice and transferred to an isotonic HEPES-buffered physiological solution of the following composition (in mM): 135 NaCl, 0.5 KCl, 0.1 Na_2HPO_4, 0.3 sodium acetate, 0.12 $NaSO_4$, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 5 HEPES, and 5.5 D-glucose (pH 7.4, 311 ± 9 mosmol/kgH_2O). This solution was oxygenated by bubbling in 95% O_2-5% CO_2. For each experiment, depending on body weight of the rats, renal papillae from 6–10 rats were pooled to obtain a sufficient quantity of the IMCD preparation for 36 incubations. The pooled papillae were minced with a razor blade and digested at 37°C with collagenase B (3 mg/ml) and hyaluronidase (600 U/ml) in the same HEPES-buffered physiological solution for ~40–50 min with continuous oxygenation. Halfway through the digestion process, DNase I (GIBCO-BRL, Gaithersburg, MD) was added to the digestion mixture to a final concentration of 1 U/ml to digest stray DNA released from broken cells. The digestion mixture was intermittently aspirated into and pushed through a glass Pasteur pipette to disperse the tubules into a uniform suspension. After digestion of the papillary tissue to a uniform suspension, the IMCD fraction was separated from the non-IMCD elements (thin limbs and vasculature) by sedimentation by low-speed centrifugation and repeated washings. The final pellet was suspended in the oxygenated HEPES-buffered physiological solution to a protein concentration of ~1 mg/ml and kept on ice for ~30 min to allow for recovery of IMCD cells from stress. This preparation consisted of mostly IMCD segments or sheets of IMCD cells and very few other non-IMCD elements. Representative IMCD fractions were assessed for enrichment and viability by immunoblotting for collecting duct-specific water channel protein aquaporin-2 (AQP2) and by binding of ethidium homodimer-1 to the cellular DNA of dead cells, respectively.

Incubation of IMCD Preparations

Fractions enriched in IMCD were incubated with or without the addition of inhibitors of COX and/or nucleotides (ATPγS or UTP), and the amount of PGE_2 released from the cells was assayed. Briefly, the IMCD suspensions prepared and kept on ice for 30 min for recovery from stress as described above were aliquoted into 1.5-ml plastic microtubes kept on ice. Nucleotide stock solutions (10× final incubation concentration) were prepared in the same oxygenated HEPES-buffered physiological solution used for the preparation of IMCD suspensions. Stock solutions of the various inhibitors were dissolved in DMSO for preparation at a very high concentration (several millimolar, depending on the solubility), aliquoted, and frozen at −20°C. These stock solutions were diluted freshly before use with the HEPES-buffered physiological solution to give 10× final incubation concentrations. These dilutions resulted in 0.05–0.3% of DMSO in the final incubations. The aliquots of IMCD suspensions were warmed to 37°C for 5 min on heat blocks before the agents were added. When nucleotides alone were used without any inhibitors, the incubations were started immediately after the 5-min warm-up period and lasted for 10 min unless otherwise specified. When the inhibitors were used, the IMCD, after the 5-min warm-up period, were preincubated with the inhibitors for 5 or 15 min, depending on the type of the inhibitor, and incubated for another 10 min after the addition of nucleotides. To control incubations that did not contain any inhibitor and/or nucleotide, equal volumes of the vehicle (incubation buffer) were added, so that all the incubations had a final volume of 200 μl. All incubations were carried out in triplicate. To stop the reactions, chilled HEPES-buffered physiological solution (200 μl) was added and the tubes were kept on ice for a few minutes. The tubes were centrifuged at 8,000 *g* for 10 min in a cold room (4°C), and 350 μl of the supernatant from each tube were transferred to a fresh tube, frozen, and stored at −80°C until assayed for the PGE_2 content. The pellets with the remaining 50 μl of the incubation buffer were frozen at −20°C for protein assay.

Assay of PGE_2

PGE_2 content in the supernatants from the incubations described above was determined according to the instructions of the manufacturer using the PGE_2 enzyme-linked immunoassay (EIA) kit-monoclonal (catalog no. 514010, Cayman Chemical). The absorbance of the product was read spectrophotometrically at 405 nm in a microplate reader (Molecular Devices, Menlo Park, CA). According to the manufacturer, this assay system has a specificity of 100% to PGE_2 and PGE_2 ethanolamide and 43 and 18.7% to PGE_3 and PGE_1, respectively. Other prostanoids have specificities of 0.01–1%. For our assays, the frozen supernatants were thawed on ice and diluted with EIA buffer to yield final dilutions of 1:200 and 1:400 with respect to the original incubation. The assays were run on 50 μl of these diluted samples. The raw data from the plate reader were stored in a computer and analyzed using Soft MaxPro software (Molecular Devices). Protein pellets were thawed at room temperature, and cellular proteins were precipitated and delipidated by addition of methanol. After the separation of methanol by centrifugation and drying, the protein pellets were dissolved in 0.05 N NaOH. Aliquots of the clear solutions thus obtained were assayed for the protein content by Coomassie Plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The concentrations of PGE_2 in the incubations were normalized with the corresponding protein contents and expressed as nanograms of PGE_2 released per milligram of protein.

Immunoblotting for AQP2 Protein

Representative samples of whole inner medullae (collagenase- and hyaluronidase-digested tissue), IMCD-enriched fraction (pellet after low-speed centrifugations), and non-IMCD fractions (pooled supernatants from the low-speed centrifugations) were assessed for the AQP2 protein content by immunoblotting. Briefly, the cellular elements in these fractions were sedimented by centrifugation and washed free of soluble proteins. The final pellets were suspended in homogenization buffer containing protease inhibitors, homogenized, assayed for protein content, solubilized in Laemmli buffer, and immunoblotted for AQP2 protein according to the standard procedures established in our laboratory (13, 24).

Cell Viability Assay

The effect of various inhibitors and DMSO on the viability of IMCD was assessed using the ethidium homodimer-1 dead cell stain as outlined by the manufacturer (Molecular Probes). Only dead or dying cells with damaged (30) membranes are stained by this fluorescent DNA stain, which has been used as an indicator of viability of cultured neurons (8) with the methodology described below for IMCD suspensions. IMCD preparations suspended in the HEPES-buffered physiological solution with or without the added agents were incubated with 2 μM ethidium homodimer-1 for 60 min at room temperature. A positive control for cell staining was run in parallel using 1% Triton X-100 to permeate cells for staining. The incubated suspensions were examined under a fluorescent microscope using filters for Texas red dye (excitation at 495 nm and emission at 635 nm). The samples were coded so that the investigator performing the microscopic examination did not know the identities of the agents being examined. Images were captured with a digital imaging system using Image-Pro Plus (Media Cybernetics, Silver Spring, MD), and the images were grouped using Adobe Photoshop (Adobe Systems, San Jose, CA).

Statistical Analysis

Values are means ± SE. Unless otherwise stated, data were analyzed by analysis of variance followed by assessment of differences between the means of the groups by Tukey-Kramer's multiple comparison test or Bonferroni's multiple comparison test. $P < 0.05$ was considered significant.

RESULTS

Characterization of IMCD Preparation

Efficacy of cell separation technique. Representative samples from an IMCD preparation were assessed for enrichment of collecting ducts using the collecting duct-specific marker protein AQP2 water channel. Renal medullary suspension (whole IM) was fractionated into collecting duct-enriched (IMCD) and non-collecting duct (non-IMCD) fractions. Figure 1 shows an immunoblot prepared from SDS-polyacrylamide gels loaded with an equal amount of protein from each of these fractions and probed with AQP2 antibody. The IMCD fraction was enriched severalfold in the collecting duct-specific AQP2 protein compared with the starting material (whole IM; Fig. 1, *left* and *middle*

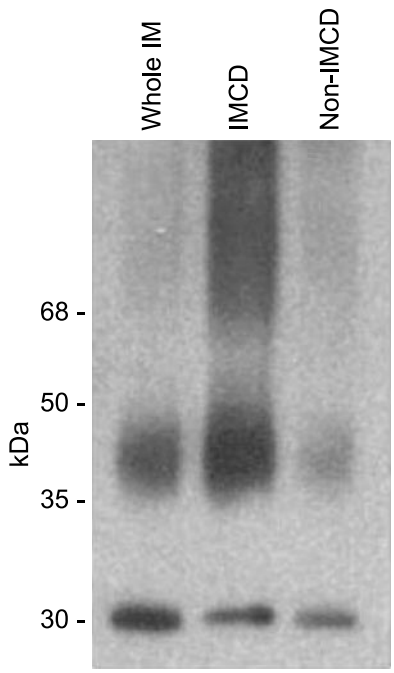

Fig. 1. Assessment of inner medullary collecting duct (IMCD) fractions for enrichment of the collecting duct-specific water channel protein aquaporin-2 (AQP2) by immunoblotting. Cellular elements in samples of whole inner medullary digest (whole IM), IMCD-enriched fractions (pellets from low-speed centrifugations), and non-IMCD fractions (pooled supernatants from low-speed centrifugations) were collected by centrifugation and washed free of soluble proteins. Equal amounts (5 μg) of solubilized proteins from each fraction were immunoblotted and probed with a peptide-derived polyclonal antibody to rat AQP2 protein (24). Secondary antibody was donkey anti-rabbit IgG conjugated to horseradish peroxidase. Sites of antigen-antibody interactions were visualized using chemiluminescence reaction and captured on a light-sensitive imaging film. The 29-kDa band corresponds to the nonglycosylated form; streaks that extend from 35 kDa and above represent various species of glycosylated forms of AQP2 protein.

lanes). Conversely, the non-IMCD fraction showed very little contamination of collecting duct cells (Fig. 1, *right lane*). This demonstrates the efficacy of the cell separation technique.

Cell viability. We assessed the viability of the cellular elements in the IMCD preparations using ethidium homodimer-1, a DNA stain that will stain only dead or dying cells (see MATERIALS AND METHODS). Representative examples from one such assay are shown in Fig. 2. Control preparations (vehicle) contained very few cells stained with ethidium homodimer-1, indicating that most of the cells in the preparation were intact and viable. When DMSO was added to a final concentration of 0.3%, the proportion of cells that were stained with ethidium homodimer-1 was comparable to that in vehicle controls. On the other hand, permeabilization of cells with 1% Triton X-100 resulted in staining of all cellular elements with ethidium homodimer. This established that the cellular elements in IMCD suspensions were viable and remained viable in the presence of DMSO, the solvent used to prepare the various inhibitors used in this study. A similar cell viability assay was performed using various inhibitors, dissolved in DMSO, at their maximum concentrations used in this study. These inhibitors also did not show any significant effect on cell viability compared with the vehicle controls (data not shown), which indicates that any of the inhibitory effects on prostanoid biosynthesis were not due to loss of cells. The dead cells in incubations with these agents, similar to the vehicle controls, were mostly single cells or clumps of a few cells, but not intact tubular segments.

Efficiency of PGE$_2$ Detection

PGE$_2$ accumulation in the IMCD preparations was assessed using a commercially available EIA kit. In our hands and in our system, this assay had an intra-assay coefficient of variation of 8% as assessed by determinations on two dilutions of six control incubations of a single IMCD preparation. Thus this 8% coefficient of variation represents the variation due to incubations also, in addition to the inherent variations in the assay technique. Figure 3 shows the pooled data from the standard curves that were run on different days. The day-to-day coefficient of variation was 5–15% in the linear range of the standard curve (20–90% binding of tracer; Fig. 3).

Time Course of ATPγS-Stimulated PGE$_2$ Release by IMCD

Figure 4 shows the time course of PGE$_2$ release by IMCD after stimulation with 100 μM ATPγS or no stimulation (vehicle controls). Even unstimulated IMCD released moderate, but significant, amounts of PGE$_2$ into the medium. Significant amounts of PGE$_2$ were released under unstimulated conditions for up to 30 min, after which no significant increases were observed up to 60 min. Thus there was apparently no significant increase in the amount of PGE$_2$ released between 30 and 60 min under unstimulated conditions. When the IMCD were stimulated with 100 μM ATPγS, release of PGE$_2$ was enhanced at all time points. The differences between the unstimulated and stimulated release became significant as early as 10 min. Furthermore, in contrast to the unstimulated cells, the stimulated release in Fig. 4 continued to show an increase when the cells were incubated for up to 60 min. In experiments with longer incubations, the stimulated release of PGE$_2$ continued to increase approximately twofold between 60 and 120 min. Under those conditions, the unstimulated release of PGE$_2$ also showed a twofold increase between 60 and 120 min, without a significant increase between 30 and 60 min (data not shown). Comparable results were obtained when the time course experiments were conducted using a different P2Y$_2$ receptor agonist, UTP (100 μM), instead of ATPγS (data not shown).

Effect of Different Concentrations of ATPγS on PGE$_2$ Release by IMCD

The ATPγS concentration-response curve for the release of PGE2 by IMCD is shown in Fig. 5. Because the time course response showed significant increases in

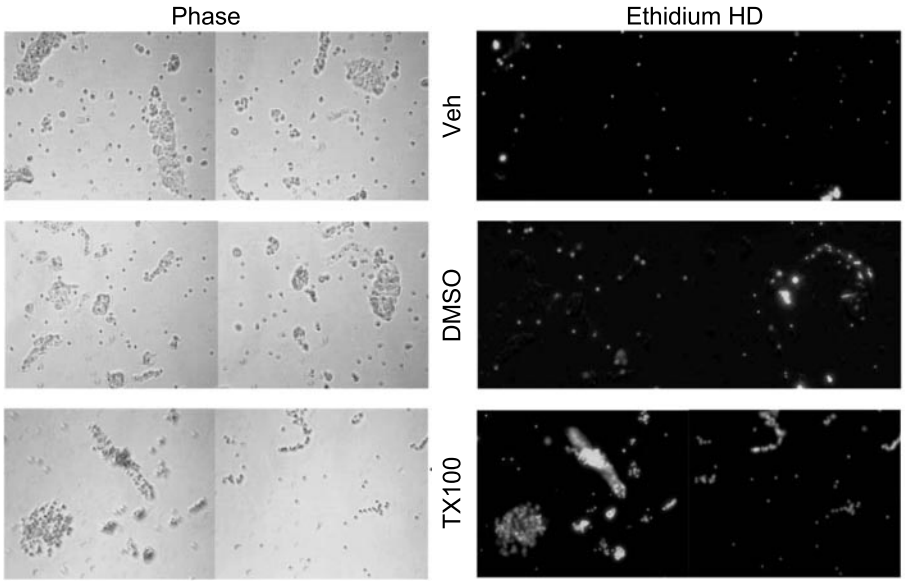

Fig. 2. Cell viability assay using ethidium homodimer-1 (ethidium HD). IMCD suspensions containing no added agents [vehicle control (Veh)] or 0.3% DMSO (solvent used to dissolve inhibitors) or 1% Triton X-100 (positive control for cell death) were incubated in a 24-well microplate with 2 μM ethidium homodimer-1, a fluorescent DNA stain, for 60 min at room temperature. At the end of the incubation period, suspensions were examined under a fluorescent microscope (excitation at 495 nm, emission at 635 nm), and images were captured digitally. *Left*: living and dead cellular elements in the suspension as seen under phase contrast mode; *right*: fluorescence of dead or dying cells in the corresponding fields, inasmuch as ethidium homodimer-1 is not permeable to intact living cells.

the release of PGE_2 as early as 10 min after stimulation of IMCD with ATPγS, the concentration-response curve was assessed at 10 min after stimulation. There was a rapid and linear increase in the release of PGE_2 from IMCD after stimulation with ATPγS at concentrations up to 25 μM. The amount of PGE_2 released became significant at 25 μM, and no further significant increase in PGE_2 release was observed up to 100 μM ATPγS.

Effect of Various COX Inhibitors on PGE$_2$ Release by Unstimulated IMCD

Figure 6 shows the effect of various COX inhibitors on release of PGE_2 by unstimulated IMCD incubated at 37°C for 5 or 15 min. The nonspecific COX inhibitors flurbiprofen and APHS caused a significant decrease in the release of PGE_2 by unstimulated IMCD. The COX-2-specific inhibitor NS-398 also caused a comparable level of decrease in the release of PGE_2, even at 10 μM. Increasing the concentration of NS-398 to 30 μM did not result in a further decrease in the amount of PGE_2

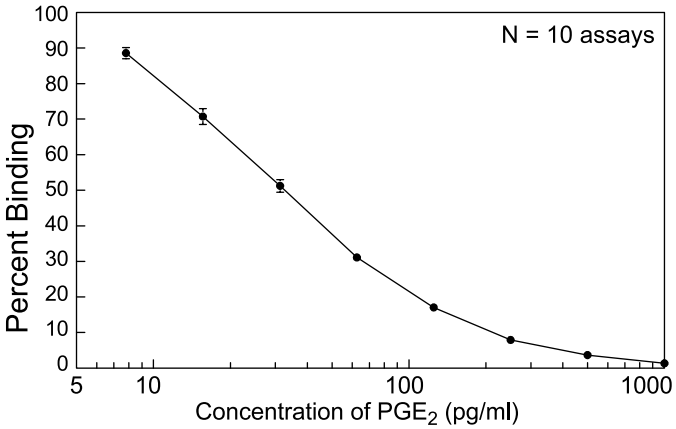

Fig. 3. Standard curve for determination of PGE_2 by enzyme-linked immunoassay (EIA). PGE_2 standards were run in each assay plate. Values obtained for percent binding were plotted against PGE_2 concentrations in standards. Values are means ± SE for 10 assays, with each standard run in duplicate. Thus the standard curve represents day-to-day variation in assays that were run to generate data presented in this study. On the basis of the standard curve, day-to-day coefficient of variation is 5–15% in the linear range of the curve (20–90% binding of tracer).

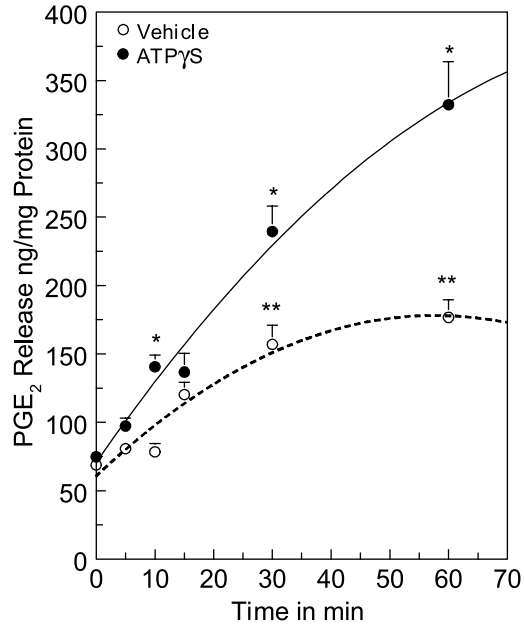

Fig. 4. Time course of release of PGE_2 by IMCD preparations under unstimulated conditions (vehicle) or after stimulation with 100 μM adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS). IMCD preparations, suspended in oxygenated HEPES-buffered physiological solution, were warmed to 37°C, and vehicle (incubation buffer) or freshly prepared ATPγS solution was added to obtain a final concentration of 100 μM in the incubation. Incubations were continued for 10–60 min. Values are means ± SE of triplicate incubations. *Significantly different from corresponding unstimulated value ($P < 0.05$ or better); **significantly different from unstimulated value at 0, 5, and 10 min ($P < 0.01$ or better). Tukey-Kramer's multiple comparisons test was used for all comparisons.

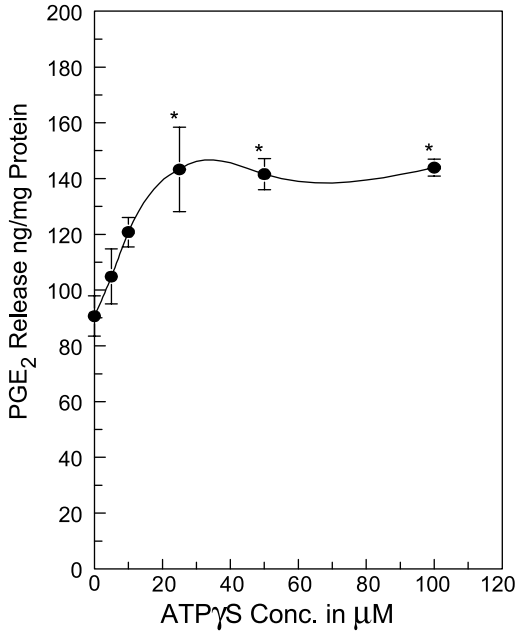

Fig. 5. ATPγS concentration (Conc)-response curve for release of PGE_2 by IMCD preparations. IMCD preparations, suspended in oxygenated HEPES-buffered physiological solution, were warmed to 37°C and then challenged with 0–100 μM ATPγS for 10 min at 37°C. Values are means ± SE of triplicate incubations. *$P < 0.01$ vs. 0 μM ATPγS. Tukey-Kramer's multiple comparisons test was used for all comparisons.

released. On the other hand, 30 μM valeroyl salicylate, a COX-1-specific inhibitor, did not cause a significant decrease in the amount of PGE_2 released. Only at the 10-fold increase in the concentration of valeroyl salicylate to 300 μM did the amount of PGE_2 released significantly decrease compared with the control values. Therefore, all the COX inhibitors tested decreased the amount of unstimulated PGE_2 release. The amounts of PGE_2 shown in Fig. 6 that correspond to the highest concentrations of the COX inhibitors do not actually represent the amount of PGE_2 release that is resistant or insensitive to COX inhibition. Rather, these bars represent the amount of PGE_2 in the preparation before addition of the inhibitor. Because the procedure for preparation of IMCD suspensions is a lengthy one, cells may release PGE_2 and other substances into the medium during the process. Therefore, the ability of various inhibitors to prevent the release of PGE_2 after their addition to the incubation is represented as the difference between 100% and the lowest values. The minor (but not significant) differences in maximal inhibition between flurbiprofen and APHS incubations may likely represent differences in the ability of these inhibitors to reach effective concentrations in the cell and/or initial rate of COX inactivation. Figure 6 also shows the lack of effect of the DMSO solvent (0.08%) on PGE_2 release by unstimulated IMCD preparations.

Effect of Nonspecific COX Inhibition on ATPγS-Stimulated PGE$_2$ Release by IMCD

We used 50 μM ATPγS and 10 min of incubation in all the following experiments with COX inhibitors, on the basis of the time course (Fig. 4) and the concentration-response curve (Fig. 5), which showed that 10 min of stimulation with 25–50 μM ATPγS caused optimal amounts of PGE_2 release. Figure 7 shows the inhibitory effect of flurbiprofen, a nonselective competitive COX inhibitor (5), on the ATPγS (50 μM)-stimulated release of PGE_2 by IMCD. Flurbiprofen at 30 or 300 μM completely inhibited the stimulated release of PGE_2 from IMCD. We also tested the effect of APHS, a potent covalent inhibitor of COX-1 and COX-2 that is

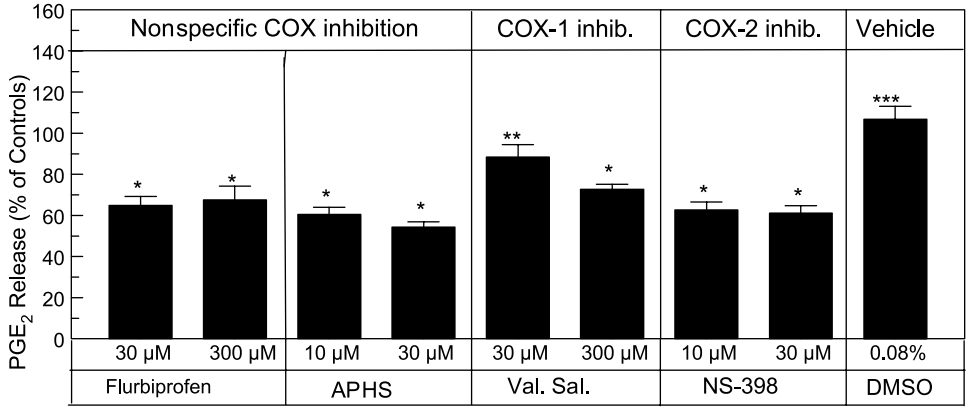

Fig. 6. Effect of various inhibitors of cylcooxygenases (COX) or DMSO on release of PGE_2 by IMCD under basal conditions. IMCD preparations, suspended in oxygenated HEPES-buffered physiological solution, were warmed to 37°C and then challenged with nonspecific COX inhibitors [flurbiprofen or 2-acetoxyphenylhept-2-ynyl sulfide (APHS)], COX-1-specific inhibitor [valeroyl salicylate (Val Sal)], COX-2-specific inhibitor (NS-398), or DMSO. All incubations, except valeroyl salicylate, were carried out for 5 min. Valeroyl salicylate was incubated for 15 min. PGE_2 release was assayed, normalized to protein concentration, and expressed as percentage of respective vehicle control value. Data were generated from 5 different assays, in which each agent was run in triplicate incubations. For valeroyl salicylate and NS-398, data from 2 different assays were pooled. Thus values are means ± SE of 3 or 6 incubations. *Significantly different from corresponding control value ($P < 0.05$ or better). **Significantly different from 300 μM valeroyl salicylate, but not from corresponding control value. ***Not significantly different from corresponding control value. Tukey-Kramer's multiple comparisons test was used for all comparisons.

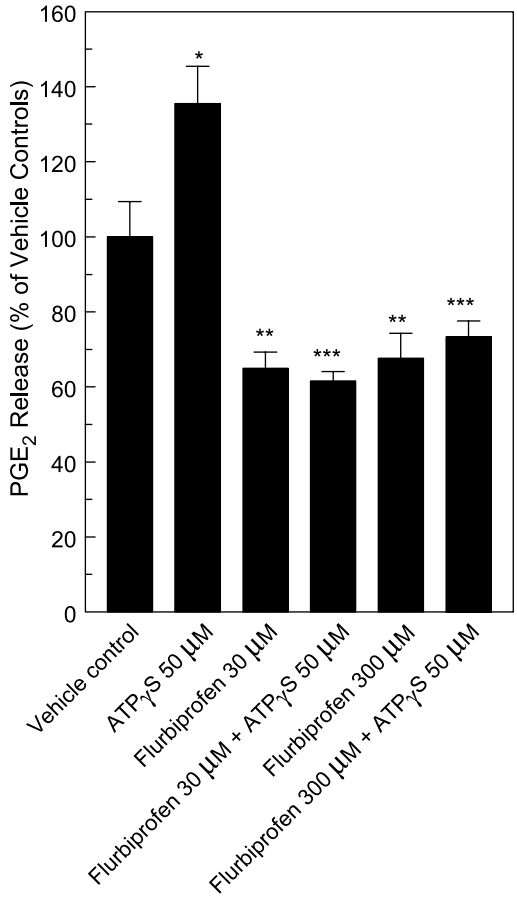

Fig. 7. Effect of nonspecific COX inhibition by flurbiprofen on ATPγS-stimulated release of PGE_2 by IMCD. IMCD preparations, suspended in oxygenated HEPES-buffered physiological solution, were warmed to 37°C and then preincubated for 5 min with or without addition of flurbiprofen to a final concentration of 30 or 300 μM. After preincubation, ATPγS was added to some incubations to a final concentration of 50 μM, and incubation was continued for 10 min at 37°C. PGE_2 release was determined by EIA and normalized to protein content. Values are means ± SE of triplicate incubations. Results are expressed as percentage of mean values in vehicle controls (105 ng PGE_2/mg protein). *$P < 0.01$ vs. vehicle control. **$P < 0.05$ vs. vehicle control. ***Not significantly different from respective flurbiprofen-alone group. Tukey-Kramer's multiple comparisons test was used for all comparisons.

similar to aspirin (22), on the release of PGE_2 by IMCD. As shown in Fig. 8, 10 or 30 μM APHS completely inhibited the ATPγS (50 μM)-stimulated release of PGE_2 from IMCD.

Effect of COX-1 Inhibition on ATPγS-Stimulated PGE_2 Release by IMCD

Figure 9 shows the effect of valeroyl salicylate, a selective, irreversible inhibitor of COX-1 (6), on ATPγS (50 μM)-stimulated PGE_2 release from IMCD. Lower concentrations of valeroyl salicylate (30 μM) produced little inhibition of the ATPγS-stimulated PGE_2 release. However, 300 μM valeroyl salicylate completely inhibited the ATPγS-stimulated release of PGE_2 from IMCD.

Effect of COX-2 Inhibition on ATPγS-Stimulated PGE_2 Release by IMCD

Figure 10 shows the effect of NS-398, a selective competitive inhibitor of COX-2 (5), on ATPγS (50 μM)-stimulated PGE_2 release by IMCD. The stimulated release of PGE_2 was not inhibited by 10 and 30 μM NS-398. Although NS-398 lowered the basal release of PGE_2 to ~60% of the vehicle control (Fig. 10, cf. 10 and 30 μM NS-398 with vehicle control), the relative amount of ATPγS-stimulated PGE_2 release in the presence of 10 and 30 μM NS-398 was ~1.35-fold greater than the unstimulated amount (Fig. 10, cf. 10 μM NS-398 with 10 μM NS-398 + 50 μM ATPγS and 30 μM NS-398 with 30 μM NS-398 + 50 μM ATPγS). This relative increase in ATPγS-stimulated PGE_2 release in the presence of NS-398 was comparable to the 1.34-fold

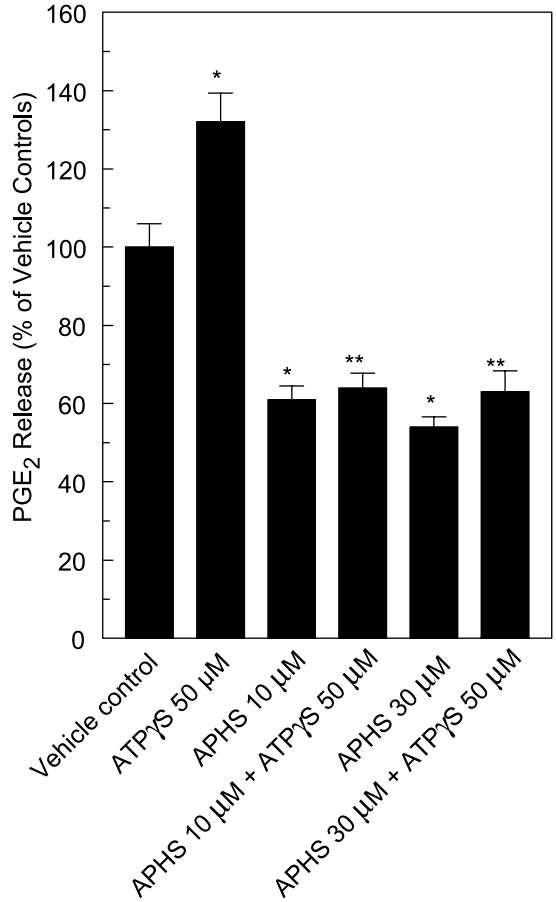

Fig. 8. Effect of nonspecific COX inhibition by APHS on ATPγS-stimulated release of PGE_2 by IMCD. IMCD preparations, suspended in oxygenated HEPES-buffered physiological solution, were warmed to 37°C and then preincubated with or without addition of APHS to a final concentration of 10 or 30 μM. After preincubation, ATPγS was added to some incubations to a final concentration of 50 μM, and incubation was continued for 10 min at 37°C. PGE_2 release was determined by EIA and normalized to protein content. Results are expressed as percentage of mean values in vehicle controls (185 ng PGE_2/mg protein). Values are means ± SE of triplicate incubations. *$P < 0.001$ vs. vehicle control. **Not significantly different from respective APHS-alone incubation. Tukey-Kramer's multiple comparisons test was used for all comparisons.

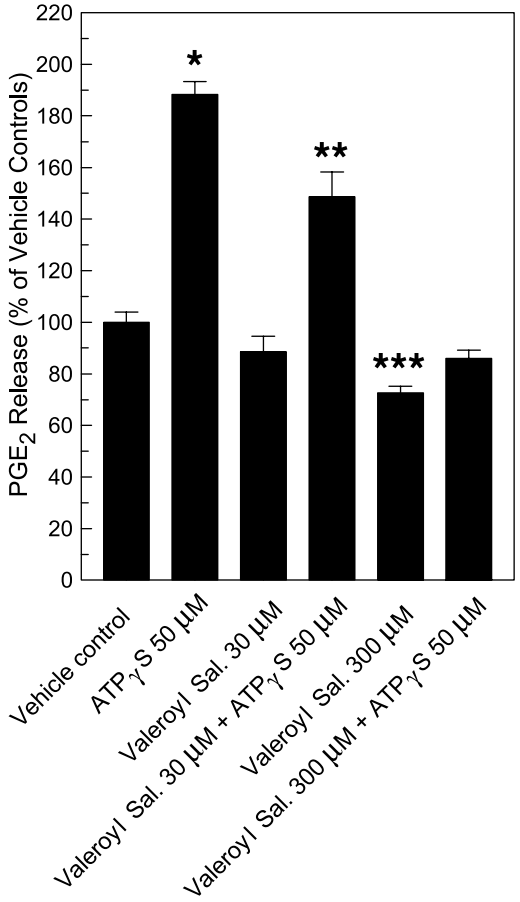

Fig. 9. Effect of COX-1 inhibition by valeroyl salicylate (Valeroyl Sal) on ATPγS-stimulated release of PGE_2 by IMCD. IMCD preparations, suspended in oxygenated HEPES-buffered physiological solution, were warmed to 37°C and then preincubated for 15 min with or without addition of valeroyl salicylate to a final concentration of 30 or 300 μM. After preincubation, ATPγS was added to some incubations to a final concentration of 50 μM, and incubations were continued for 10 min at 37°C. PGE_2 release was determined by EIA, normalized to protein content, and expressed as percentage of mean values obtained for vehicle controls. Values are means ± SE of pooled data from 2 different experiments, where each incubation was run in triplicate. *$P < 0.001$ vs. vehicle control. **$P < 0.001$ vs. ATPγS alone or 30 μM valeroyl salicylate. ***$P < 0.01$ vs. vehicle control and not significantly different from 300 μM valeroyl salicylate + ATPγS. Bonferroni's multiple comparisons test was used for all comparisons.

increase observed without NS-398 (Fig. 10, cf. vehicle control with 50 μM ATPγS).

Comparative Stimulatory Effect of ATPγS on PGE_2 Release by IMCD in the Presence of COX-1 or COX-2 Inhibition

A direct comparison of the inhibition of ATPγS-stimulated PGE_2 release by 300 μM valeroyl salicylate (a COX-1 inhibitor) or 30 μM NS-398 (a COX-2 inhibitor) is presented in Fig. 11. As shown in Fig. 11, ATPγS could stimulate significantly more PGE_2 release in the presence of COX-2 inhibition than in the presence of COX-1 inhibition.

Effect of Inhibition of cPLA$_2$ on ATPγS-Stimulated PGE_2 Release by IMCD

The availability of arachidonic acid is a rate-limiting factor for the synthesis of prostanoids by COX, and cPLA$_2$ is considered to be a major player in the kidney for the release of arachidonic acid from membrane phospholipids (7). Hence, we examined the role of cPLA$_2$ in the ATPγS-stimulated PGE_2 release of PGE_2 by the IMCD. Under our experimental conditions, 30 μM AACOCF3, a cPLA$_2$-specific inhibitor, did not have an effect on ATPγS-stimulated PGE_2 release by IMCD preparations (data not shown).

DISCUSSION

We demonstrated that *1*) unstimulated IMCD release moderate, but significant, amounts of PGE_2, which are more sensitive to COX-2 than COX-1 inhi-

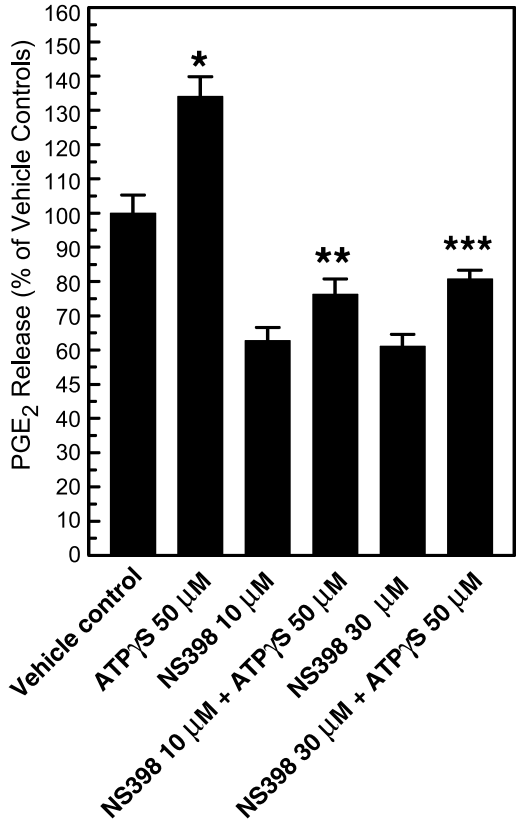

Fig. 10. Effect of COX-2 inhibition by NS-398 on ATPγS-stimulated release of PGE_2 by IMCD. IMCD preparations, suspended in oxygenated HEPES-buffered physiological solution, were warmed to 37°C and then preincubated for 5 min with or without addition of NS-398 to a final concentration of 10 or 30 μM. After preincubation, ATPγS was added to some incubations to a final concentration of 50 μM, and incubations were continued for 10 min at 37°C. Release of PGE_2 was determined by EIA, normalized to protein content, and expressed as percentage of mean values obtained for vehicle controls. Except for 30 μM NS-398 and 30 μM NS-398 + 50 μM ATPγS, values are means ± SE of 2 experiments, where incubations were run in triplicate. Data for 30 μM NS-398 and 30 μM NS-398 + 50 μM ATPγS are from 1 experiment with triplicate incubations. *$P < 0.001$ vs. vehicle control. **$P < 0.05$ vs. 10 μM NS-398 alone. ***$P < 0.05$ vs. 30 μM NS-398 alone. Bonferroni's multiple comparisons test was used for all comparisons.

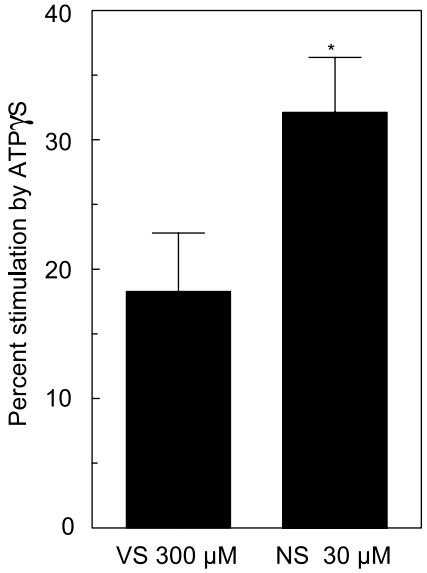

Fig. 11. Comparative stimulatory effect of ATPγS on release of PGE$_2$ from IMCD preparations in the presence of a COX-1 inhibitor [300 μM valeroyl salicylate (VS)] or a COX-2 inhibitor [30 μM NS-398 (NS)]. Data are from experiments shown in Figs. 9 and 10. Ability of ATPγS to overcome the effect of COX inhibitors was calculated as percent stimulation of PGE$_2$ release over and above values obtained in the presence of the corresponding inhibitor alone. Values are means ± SE of 6 (300 μM valeroyl salicylate) and 3 (30 μM NS-398) incubations. *Median and mean values are significantly different from those of 300 μM valeroyl salicylate as assessed by Mann-Whitney's test and unpaired *t*-test with Welch correction, respectively.

bition, *2*) activation of the P2Y$_2$ receptor by the agonist ATPγS results in enhanced release of PGE$_2$ from IMCD in a time- and concentration-dependent fashion, *3*) purinergic-stimulated release of PGE$_2$ by IMCD is blocked by nonspecific COX inhibition, and *4*) purinergic-stimulated release of PGE$_2$ by IMCD is more sensitive to COX-1- than COX-2-specific inhibition. If it is assumed that increased release of PGE$_2$ by IMCD is related to its increased production, our results suggest that purinergic stimulation of IMCD causes an enhanced production and release of PGE$_2$, which appear to be mediated by COX-1.

We used a model of freshly prepared IMCD fractions from collagenase- and hyaluronidase-digested rat inner medullae. This preparation is a well-characterized model for the study of hormonal response of IMCD. We validated the purity and viability of the IMCD preparation by demonstrating the enrichment of collecting duct-specific water channel protein (AQP2) and by visually assessing the proportion of dead cells by ethidium homodimer staining, respectively. Because earlier studies have shown that PGE$_2$ synthesis in IMCD is sensitive to increasing osmolality of the medium (21), we carried out all our experiments at ~300 mosmol/kgH_2O to exclude variability due to osmolality of the medium. We also demonstrated that DMSO, the solvent used for various inhibitors, and the inhibitors at the concentrations used in this study do not have a significant effect on the viability of the IMCD cells. The commercial EIA kit used for determination of PGE$_2$ has a high degree of reproducibility in our hands, with acceptable intra-assay and day-to-day coefficients of variation in the linear range of standard curve.

In our experiments, we used the specific agonists ATPγS and UTP to examine the effect of P2Y$_2$ receptor activation in IMCD on the release of PGE$_2$. To the best of our knowledge, the P2Y$_2$ receptor is the only purinergic receptor with well-characterized expression and functional significance in rat IMCD (12, 23, 24). Attempts to identify other purinergic receptors by Ecelbarger et al. (12) did not reveal an effect of stimulation with ADP (a P2Y$_1$ agonist), 2-methylthio-ATP (a P2Y$_1$ agonist), and α,β-methylene-ATP (a P2X agonist) on the intracellular calcium rise in rat IMCD. Our in vitro microperfusion experiments also confirmed that rat IMCD does not respond to ADP (23). These studies apparently rule out the possibility that P2Y$_1$ and P2X receptors are expressed in rat IMCD. Therefore, we focused our research efforts on understanding the cellular and molecular mechanisms of P2Y$_2$ receptor-mediated effects in rat IMCD.

ATPγS was the major agonist that we used to stimulate the P2Y$_2$ receptor because of its nonhydrolyzable nature, which prevents the formation of adenosine, which can interact with adenosine A$_1$ receptors in IMCD. We have also observed similar results using other P2Y$_2$ agonists (data not shown here). In preliminary experiments using the same model of freshly prepared IMCD fractions where RIA was used for the measurement of PGE$_2$, we observed that the agonists ATP and UTP (100 μM), but not the nonagonist ADP, stimulated comparable amounts of PGE$_2$ release after 10 min of incubation at 37°C. Those preliminary studies also showed that carbachol, a muscarinic cholinergic agonist, used as a positive control for intracellular calcium rise, had a similar effect on the production of PGE$_2$ by IMCD (unpublished data).

The time course experiments clearly demonstrated that the P2Y$_2$ agonist-stimulated release of PGE$_2$ was significantly higher than the unstimulated basal release from IMCD. Concentration-response experiments showed that the PGE$_2$ release reaches a plateau at 25 μM ATPγS, with 50% of the increase at 10 μM. This finding is consistent with the earlier observations by Ecelbarger et al. (12), who showed that 10 μM ATP increased intracellular calcium by ~50% of that observed at 100 μM. In vitro microperfusion experiments conducted by us (23) also showed that the decrease in AVP-stimulated osmotic water permeability in the presence of 100 μM ATP is not much different from that caused by 10 μM ATP, thus indicating that higher concentrations of agonists do not have any added effect.

Our experiments showed that the basal or unstimulated release of PGE$_2$ by IMCD was apparently dependent on the activity of COX-2, rather than COX-1, inasmuch as it was more sensitive to the COX-2-specific inhibition. Although there is no evidence in the literature that IMCD cells express COX-2 protein, a recent study using a combination of RT-PCR analyses

of microdissected renal tubular segments and immunocytochemistry on tissue sections indicated that COX-2 mRNA, but not protein, was present in rat IMCD (41). However, these investigators could not exclude the possibility that the observed results for COX-2 mRNA were due to small amounts of residual medullary interstitial cells that remained attached to the IMCD in their preparations (41). Because medullary interstitial cells are known to express abundant amounts of COX-2 mRNA and protein (17), even a few of these cells in the IMCD preparations could result in detectable amounts of COX-2 transcripts. The same could also be said for assays for COX-2 activity with IMCD preparations, where small amounts of medullary interstitial cells could yield detectable activity. Hence, the COX-2-dependent release of PGE_2 that we observed under basal conditions was possibly due to small amounts of medullary interstitial cells that remained attached tightly to the IMCD preparations. Conversely, it may be due to a yet to be identified activity of COX-2 in IMCD cells in our experimental conditions. This issue needs further investigation, and we did not address these aspects in this study.

Because the ATPγS-stimulated PGE_2 release by IMCD was completely suppressed by two different types of nonspecific COX inhibitors, namely, the competitive and covalent inhibitors flurbiprofen and APHS, respectively, the activity of COX is required for the $P2Y_2$ receptor-mediated release of PGE_2 in IMCD. On the other hand, our studies with differential COX inhibitors revealed that, unlike the basal or unstimulated production, the ATPγS-stimulated PGE_2 release is dependent on COX-1, rather than COX-2, activity. These observations are consistent with the documented expression of the $P2Y_2$ receptor and COX-1 in IMCD cells (24, 41). In our immunocytochemical studies using a peptide-derived polyclonal antibody specific to the $P2Y_2$ receptor, we could not detect $P2Y_2$ receptor protein in medullary interstitial cells (24; unpublished observations). Hence, even if present in our IMCD preparations, the interstitial cells may not respond to stimulation by ATPγS.

The availability of arachidonic acid is the rate-limiting step in the synthesis of prostanoids by COX in many tissues (18). However, our attempts to examine the effect of inhibition of $cPLA_2$ activity on ATPγS-stimulated PGE_2 release using the specific inhibitor AACOCF3 were not successful. This may be due to the difficulties in attaining effective intracellular concentrations of AACOCF3 under our experimental conditions. It is also possible that the release of arachidonic acid by IMCD after $P2Y_2$ receptor activation is more complex and may likely involve phospholipases other than $cPLA_2$. This aspect needs further investigation; hence, it was not probed further in the present series of experiments.

Finally, from our experiments where we could inhibit the release of PGE_2 by the IMCD, it appears that the "release" is due to de novo synthesis, and not "secretion" of premade and stored PGE_2. The inhibitors that we used here are known to inhibit the activities of COX and are not known to inhibit release or secretion of PGE_2 from cells. Furthermore, available evidence shows that the release of PGE_2 from cells, including collecting duct principal cells, is dependent on specific prostaglandin transporters (PGTs) in the cell membranes (4). Several PGTs have been cloned and characterized. These are broadly expressed in COX-positive cells and are coordinately regulated by COX. However, there is some evidence in Madin-Darby canine kidney cells that the PGTs are exocytotically inserted into the collecting duct apical membrane, where they could control the concentration of luminal prostaglandins (14). The various factors that may modulate the release of prostaglandins, such as the exocytotic insertion of PGTs into the cell membrane, are poorly understood. Further studies in this area of research will definitely shed new light in the near future on the paracrine regulation of collecting duct function.

In conclusion, our study has important physiological significance with regard to the role of purinergic regulation of medullary collecting duct function beyond the direct modulation of AVP-stimulated water permeability. Because PGE_2 is known to affect the transport of water, salt, and urea in IMCD (29, 35, 36), the production and release of PGE_2 after purinergic stimulation can indirectly influence handling of water, salt, and urea by the medullary collecting duct. Thus the interaction between the purinergic and prostanoid systems in IMCD, expounded here, further emphasizes the complex nature of the AVP-independent regulatory mechanisms that determine the overall function of IMCD in the renal concentration mechanism.

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DISCLOSURES

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