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INHIBITION OF PROSTAGLANDIN E_2 SYNTHESIS BY A BLOCKER OF EPITHELIAL CHLORIDE CHANNELS

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Arginine-vasopressin (AVP) elicits a variety of responses in cultured rat mesangial cells, among them stimulation of prostaglandin biosynthesis and activation of Cl^- channels. AVP produced an 11-fold increase over basal levels in prostaglandin E_2 release from cultured mesangial cells. This response was completely inhibited by 25μM indomethacin and 82±5% inhibited by 25μM 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) which is a potent blocker of epithelial Cl^- channels. The IC_{50} for NPPB inhibition of prostaglandin E_2 release was 8μM. Indomethacin and NPPB at 25μM also inhibited AVP-stimulated cellular accumulation of prostaglandin E_2 by 98% and 79±7% respectively. The inhibitory effect of NPPB was not due to interference with the cellular response to AVP since at 50μM it did not block AVP-stimulated release of arachidonate metabolites from cells metabolically labeled with [^{3}H]-arachidonic acid. It is suggested that NPPB inhibition of prostaglandin E_2 synthesis is at the cyclooxygenase level on the basis of its structural similarity to the fenamic acid type of cyclooxygenase inhibitors.

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Mesangial cells are contractile cells of the renal glomerulus which resemble smooth muscle cells in their morphology and response to vasoactive stimuli (1). Arginine-vasopressin (AVP) has been shown to elicit a variety of responses in cultured rat mesangial cells such as contraction, a rise in cytosolic calcium levels and polyphosphoinositide hydrolysis, as well as prostaglandin biosynthesis (13,15,17) and depolarization due to Cl- channel activation (11,14). In a study of the functional role of the Cl- channels in mesangial cells using Cl-channel blockers, their effect on prostaglandin biosynthesis was examined. The compound 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) has been shown to be a potent inhibitor of Cl- channels in the thick ascending loop of Henle of the mammalian nephron (21). It has also been found to inhibit epithelial Cl- channels in colon cells (10), rectal gland (8), trachea (19), kidney cortex (12) and red outer medulla (2). In this report it is shown that NPPB is a relatively potent blocker of prostaglandin E_2 biosynthesis in mesangial cells. Since NPPB is structurally related to the fenamate type of cyclooxygenase inhibitors, it is suggested that it acts at the same site. This should be taken into consideration when evaluating the physiological effects of this and other Cl-channel inhibitors.

MATERIALS AND METHODS

Tissue culture medium and serum were obtained from Gibco (Burlington, Ontario). [^{3}H]-arachidonic acid (100 Ci/mmol) and PGE_2 radioimmunoassay kits were obtained from New England Nuclear (Boston, MA). Arginine-vasopressin, probenecid, indomethacin, fatty-acid-free bovine serum albumin and reduced glutathione were obtained from Sigma Chem. Co. (St. Louis, MO). 5-nitro-2-(3-phenylpropylamino)-benzoic acid, (NPPB), was a generous gift from Prof. R. Greger, Albert-Ludwigs Universitat, Freiburg, FRG. The compound IAA-94/5, (±)-[(2-cyclopentyl-6,7-dichloro-2-methyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy] acetic acid, (22), was obtained from Biological and Chemical Transport Systems (New York). All inhibitors were kept as 20mM stock solutions in dimethyl sulfoxide.

Cell Culture. Cultures of renal glomerular mesangial cells were derived from collagenase-treated glomeruli isolated from young Sprague-Dawley rats and were characterized as previously described (9). Cloned populations derived from single colonies were selected for their continued responsiveness to arginine-vasopressin. Cells used in this study were between passages 30 to 40, and were maintained in Corning 75 cm^2 flasks in Dulbecco's modified Eagle's media supplemented with 17% fetal bovine serum at 37°C with 5% CO_2.

Determination of [^{3}H]-arachidonate release. Mesangial cells were plated at approximately 200,000 cells per well in Corning 6 well plates (well diameter 30mm). After 24 hrs. 0.5μCi of [^{3}H]-arachidonate was added to each well and the

cells were cultured for an additional 24 hrs. The cells were homogeneously confluent at this stage. Experiments were initiated by washing each well five times with 2 ml of buffer containing in mM: 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 10 HEPES, pH 7.4 (Buffer A) and 5 mg/ml defatted bovine serum albumin, prewarmed to 37°C, followed by the addition of 1 ml of Buffer A. After 4 min. at 37°C the buffer was removed and saved for determination of basal [^{3}H]-arachidonate release, and replaced with fresh 1 ml of Buffer A prewarmed to 37°C. Inhibitors were then added, incubation was continued for 2 min, followed by addition of 400 nM AVP (from 20 μM stock in H_2O). After 2 min at 37°C all of the buffer was removed, mixed with 10 ml Aquasol 2 scintillation solution (New England Nuclear, Boston, MA) and counted in a scintillation counter. The remaining cell-associated [^{3}H] was extracted by solubilization in 1 ml Buffer A containing 1% sodium dodecyl sulfate.

Determination of PGE_2 synthesis. Mesangial cells were cultured to confluence in 6 well (30 mm well diameter) Corning plates. The cells were prepared for experiments by 2 washes with 2 ml Buffer A prewarmed to 37°C and incubated for 60 min at 37°C in 2 ml Buffer A supplemented with 10 mM D-glucose and 2 mM reduced glutathione. Experiments were initiated by replacing the buffer with 2 ml of fresh Buffer A, 10 mM D-glucose, 2 mM glutathione, followed by the addition of test inhibitors. After 2 min 0.5 ml samples were removed for determination of basal PGE_2 release. At 4 min 400 nM AVP was added and incubation at 37°C was continued for an additional 5 min. The supernatants were then collected for determination of PGE_2 release and kept on ice until assayed (within less than 3 hrs.).

Samples for determination of cellular PGE_2 content were obtained after removal of all of the cell supernatant by solubilizing the cells in 1 ml ice cold Buffer A containing 2 mM glutathione, 20 μM indomethacin and 0.2% Triton X-100 detergent.

PGE_2 determination. PGE_2 levels in cell extracts and supernatants were measured by direct radioimmunoassay using New England Nuclear Prostaglandin E_2 [^{125}I] RIA Kit, after dilutions of 1:20 and 1:50 respectively. The inhibitors at the concentrations used showed no interference with the radioimmunoassay. Triton X-100 at 0.2% used for cell solubilization had a small but negligible effect on the radioimmunoassay after 1:20 dilution. While the levels of PGE_2 synthetized varied with the cell density these were consistent within each experiment.

RESULTS AND DISCUSSION

In the course of studies of the effects of Cl- channel blockers on mesangial cell function, their effects on PGE_2 production were examined. The addition of AVP to cultured rat mesangial cells resulted in a greater than ten-fold increase in the PGE_2 concentration in the medium within 5 min from a basal level of 169±45 pg/well to 1910±551 pg/well (Fig. 1). This response was completely blocked by 25 μM indomethacin. The Cl- channel blocker NPPB inhibited PGE_2 release by

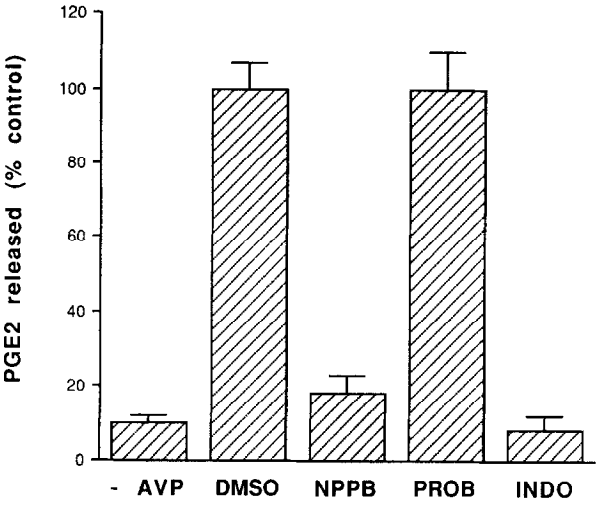

Fig. 1. Effect of inhibitors on AVP-stimulated PGE_2 release from mesangial cells. Cells were preincubated with inhibitors for 4 min. at 37°C, followed by addition of 400 nM AVP and further incubation for 5 min. at 37°C. PGE_2 levels in the supernatant were determined as described in Materials and Methods. - AVP: unstimulated control, no additions; DMSO: vehicle solvent control, 0.25% v/v dimethylsulfoxide and AVP added; Indo: 25 μM indomethacin and AVP added; NPPB: 25 μM NPPB and AVP added; Prob: 100 μM probenecid and AVP added. Results are expressed as percent of PGE_2 released relative to AVP-stimulated control with no additions. Results from 4 separate experiments, ±S.D.

82±5% at 25 μM. On the other hand, 100 μM probenecid, an inhibitor of organic anion transport (7) did not reduce PGE_2 release.

AVP-stimulated accumulation of PGE_2 within cells was also determined. As shown in Fig. 2, μM NPPB inhibited the cellular PGE_2 increase by 79±7% while indomethacin at the same concentration produced virtually complete (98%) inhibition. The basal cellular PGE_2 content was 119±22 pg/well and increased to 2142±467 pg/well 5 min after addition of AVP. While the total amount of cellular PGE_2 accumulated was probably overestimated here due to the adsorption of PGE_2 to the culture plastic and its subsequent extraction by Triton X-100, the inhibitory effect of NPPB is apparent nonetheless.

The concentration dependence of NPPB inhibition of PGE_2 release was examined. As shown in Fig. 3, inhibition was detectable at 1.25 μM NPPB, and the concentration required for 50% reduction of PGE_2 release was 8 μM. Therefore in the assay system used here NPPB is a relatively potent PGE_2 synthesis antagonist.

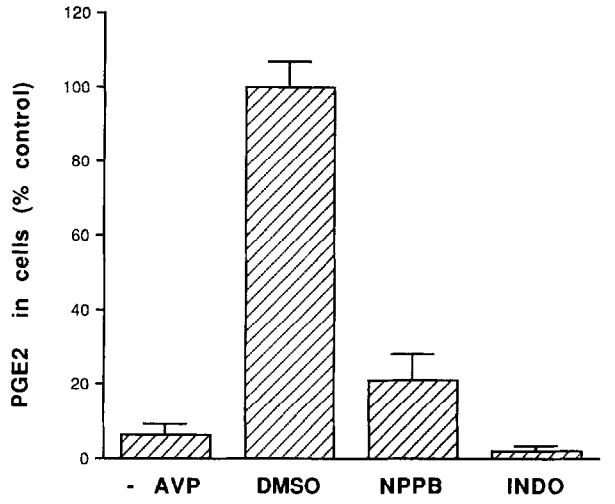

Fig. 2. Effect of inhibitors on AVP-stimulated PGE_2 accumulation in mesangial cells. Cells were preincubated with inhibitors for 4 min at 37°C, followed by addition of 400 nM AVP and further incubation for 5 min at 37°C. Intracellular PGE_2 was determined as described in Materials and Methods. - AVP: unstimulated control, no additions; DMSO: vehicle solvent control, 0.25% v/v dimethylsulfoxide and AVP added; NPPB: 25μM NPPB and AVP added; Indo: 25 μM indomethacin and AVP added. Results are expressed as per cent of cellular PGE_2 accumulation relative to AVP-stimulated control with no additions. Results from 4 separate experiments, ±S.D.

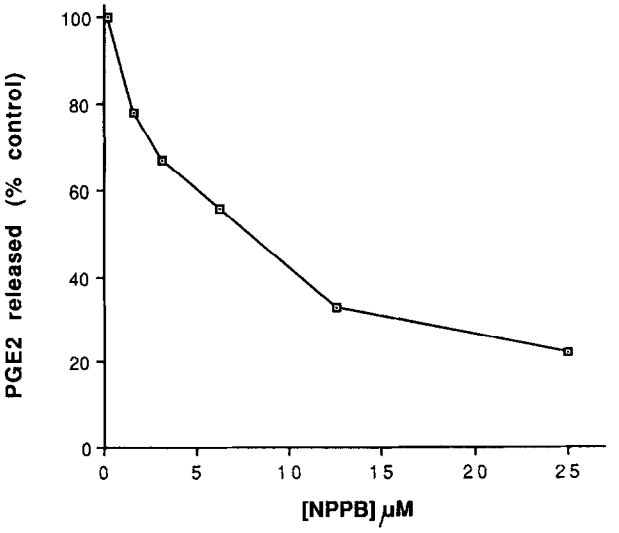

Fig. 3. Effect of NPPB on PGE_2 release: concentration dependence. Cells were preincubated with various concentrations of NPPB for 4 min at 37°C, followed by addition of 400nM AVP and further incubation for 5 mins at 37°C. PGE_2 in the cell supernatants was determined as before. Results are expressed as percent of PGE_2 released relative to AVP-stimulated control with no additions.

Several other inhibitors of anion transport were examined for their effects on PGE_2 release by mesangial cells. No inhibition was found with 100 μM 4,4'-diisothiocyanostilbene-4,4'-disulfonic acid (DIDS), an inhibitor of anion exchange in a variety of systems (3) or 100 μM ethacrynic acid, an inhibitor of Na^+, K^+, 2Cl- co-transport (6,7). However, due to the likelihood of the covalent reaction of these agents with reduced glutathione which was present in the medium, their lack of effect is not conclusive. An additional compound tested was (±) -[(2-cyclopentyl-6,7-dichloro-2-methyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy] acetic acid, (IAA-94/5), which has been shown to inhibit (i) bicarbonate-stimulated cell swelling in cat cerebral cortex (4), (ii) $LiCO_3$- influx into human erythrocytes (6) and (iii) chloride channels in bovine kidney cortical membrane vesicles (12). At 25 μM this compound produced 45±9% inhibition of PGE_2 release and 40±8% inhibition of cellular PGE_2 accumulation in 5 min. Thus it is less potent than NPPB. However, this finding raises the possibility that the anti-edema action of IAA-94/5 could in part be due to inhibition of prostaglandin synthesis.

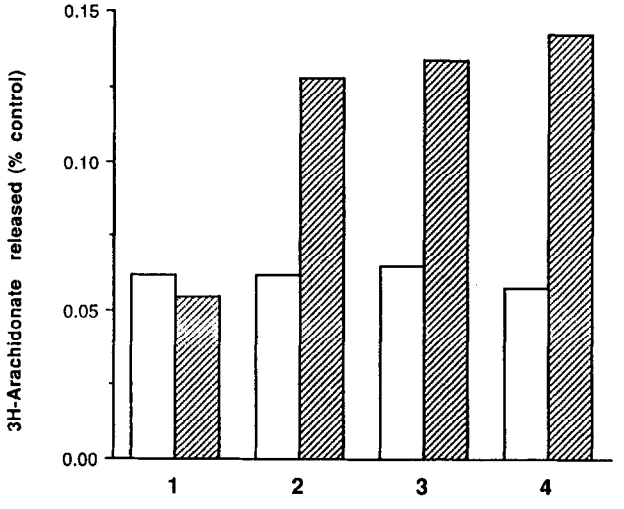

Fig. 4. Effect of NPPB on AVP-stimulated release of [^{3}H]-arachidonate products. Cells were cultured for 24 hrs with 0.5 μCi [^{3}H]-arachidonic acid. After removal of unincorporated label, the cells were preincubated with or without NPPB for 2 min at 37°C and samples were removed for determination of radioactivity (white columns). Then 400 nM AVP was added and after 2 min at 37°C samples of the cell supernatants were removed for determination of radioactivity (stippled columns). 1: unstimulated control, no AVP added; 2: control, only AVP added; 3: vehicle solvent control, 0.5% v/v dimethylsulfoxide and AVP added; 4: 50 μM NPPB and AVP added. Data are average of 3 separate experiments.

In order to test the possibility that the inhibitory effect of NPPB was due to interference with some aspect of AVP stimulation of free fatty acid release, mesangial cells were labeled with [^{3}H]-arachidonic acid for 24 hrs and release of [^{3}H] upon AVP stimulation was measured. The average [^{3}H]-arachidonate incorporation was 403,300±27,600 cpm/well, of which 0.06%/2 min was released into the medium in the absence of AVP and 0.13%/2 min was released in the presence of AVP. This 2-fold increase was not affected by preincubation with 50 μM NPPB, a concentration sufficient for virtually complete abolition of PGE_2 release. The release of [^{3}H]-label from the cells is attributable to the activation of phospholipase C and phospholipase A_2 mediated pathways for arachidonate release, which have been identified in glomerular mesangial cells (13,16). Since PGE_2 has been found to account for less than 20% of the [^{3}H]-labeled arachidonic acid products released from rat mesangial cells (18), inhibition of PGE_2 synthesis alone would not be expected to affect significantly the overall levels of released label. This result indicates therefore that NPPAB inhibits PGE_2 synthesis at some step subsequent to the release of arachidonic acid. (See Fig. 4.)

The NPPB molecule (Fig. 5) bears a partial structural resemblance to some cyclooxygenase inhibitors, particularly the fenamic acid group (5). While the precise site of action of NPPB affecting PGE_2 synthesis has not been determined, cyclooxygenase is a likely target.

However, unlike NPPB, the cyclooxygenase inhibitors aspirin, indomethacin, flufenamic and mefenamic acid have been shown to be ineffective as blockers of the Cl- channel in the thick ascending loop of Henle of mammalian kidney (21). It is of interest that NPPB, beside being a potent blocker of epithelial Cl- channels with an IC_{50} varying between 10^{-4} and 10^{-7} M depending on the channel subtype and assay method (2,12,19,21), also blocks anion exchange in human erythrocytes with an IC_{50} of approx. $3x10^{-5}$ M (Dr. Z.I. Cabantchik, personal communication) and organic anion transport in kidney proximal tubules (20) and cultured proximal tubule cells (Sessa and Breuer, unpublished results). Conceivably NPPB contains structural features which are recognized by anion binding sites on a variety of proteins.

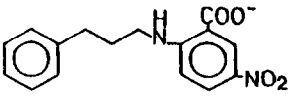

Fig. 5. The chemical structure of NPPB.

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