Endogenously produced prostanoids stimulate calcium reabsorption in the rabbit cortical collecting system

Jürgen van Baal*, Maarten D. de Jong*, Freek J. Zijlstra†, Peter H. G. M. Willems‡ and René J. M. Bindels*

Departments of *Cell Physiology and †Biochemistry, University of Nijmegen, PO Box 9101, 6500 HB Nijmegen and ‡Department of Pharmacology, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands

1. The influence of endogenously produced prostanoids on active transepithelial Ca2+ transport and cAMP formation was investigated in immunodissected rabbit kidney connecting and cortical collecting tubule cells grown to confluency on permeable supports.

2. The cyclo-oxygenase inhibitor indomethacin dose-dependently (IC50 = 18 nM) reduced the net apical-to-basolateral Ca2+ transport by 57%. Inhibition was reversed in medium obtained from monolayers incubated in the absence of indomethacin.

3. HPLC analysis following incubation with 14C-labelled arachidonic acid revealed the presence of a wide variety of radiolabelled prostanoids in both the apical and basolateral media. These findings are compatible with the endogenous production and subsequent release of stimulatory prostanoids.

4. The inhibitory action of indomethacin was reversed by the addition of the prostanoids PGE1, PGE2 and PGA2, but not PGD2, PGF2α, the stable PGI2 analogue cicaprost or the thromboxane A2 mimetic U-46619. PGE2 stimulated transepithelial Ca2+ transport dose-dependently (EC50 = 3 nM), irrespective of the compartment to which it was added. The stimulatory effect of PGE2 was paralleled by increased cAMP formation, suggesting the apical and basolateral presence of stimulatory prostanoid receptors EP2 and/or EP4.

5. Sulprostone, an analogue selective for EP1 and EP3 receptors, inhibited transepithelial Ca2+ transport in indomethacin-treated monolayers only when applied basolaterally, suggesting the exclusive presence of inhibitory EP receptors on the basolateral membrane.

6. The percentage by which parathyroid hormone and arginine vasopressin increased both transepithelial Ca2+ transport and cAMP formation was dramatically increased in indomethacin-inhibited cells as compared with control cells, demonstrating that indomethacin unmasks the actions of these hormones to their full extent.

The cyclo-oxygenase enzyme system is the major pathway for arachidonic acid metabolism in the kidney and is responsible for the biosynthesis of prostanoids, including PGD2, PGE2, PGF2α, PGI2 and thromboxane A2 (Bonvalet et al., 1994). Both by their direct action and by their modulation of the action of other hormones, prostanoids participate in the regulation of renal vascular tone, mesangial and glomerular functions, and the handling of salt and water (Badr & Jacobson, 1991). Prostanoids are locally produced and exert their effect in a paracrine or autocrine fashion. Until now, only the effects of the predominant prostanoid, PGE2, have been characterized in greater detail at the cellular and molecular level. For instance, four distinct E-prostanoid (EP) receptor subtypes, designated EP1, EP2, EP3 and EP4, have recently been defined (Coleman et al., 1994). Three of these receptors (EP1, EP3 and EP4) are highly expressed along the nephron where they are involved in the regulation of a wide variety of functions (Sugimoto et al. 1992; Sugimoto, Namba, Shigemoto, Negishi, Ichikawa & Narumiya, 1994; Breyer et al. 1994).

The importance of prostanoids in urinary excretion is exemplified by their role as negative feedback modulators of the action of arginine vasopressin (AVP) on the kidney. Ample studies have focussed on the collecting duct as the main site of cyclooxygenase activity in the kidney and as a target tissue for vasopressin-regulated Na+ and water transport (Bonvalet et al., 1987; Coleman et al. 1994). In this nephron segment, PGE2 affects transport processes via at least three distinct mechanisms. First, PGE2 interacts with a basolateral EP3 receptor coupled to a pertussis toxin (PTX)-sensitive G protein to inhibit adenyl cyclase-catalysed cAMP formation resulting in the inhibition of...

In contrast to the well-documented actions of PGE2 on Na+ and water reabsorption, to date little is known about the effects of PGEa and other prostanoids on active Ca2+ reabsorption. Active Ca2+ reabsorption takes place primarily in the distal convoluted tubule, connecting tubule (CNT) and cortical collecting duct (CCD), where it determines the final urinary excretion of Ca2+ (Bindels, Hartog, Van Os & Bindels, 1991; Costanzo & Windhager, 1992; Timmermans & Van Os, 1991; Costanzo & Windhager, 1992; Bindels, 1993; Friedman & Gesek, 1995). In recent years, a model has been developed that allows the study of hormone-responsive transepithelial Ca2+ transport. The model, consisting of a primary culture of immunodissected rabbit CNT and CCD cells grown to confluency on a permeable support, has been shown to retain several characteristics of the original epithelium, including hormone-responsive transepithelial Ca2+ transport, high cytosolic calbindin-D28k levels and benzamil-sensitive Na+ transport (Bindels et al., 1991; Bindels, Hartog, Abrahamse & Van Os, 1994; Bindels, Van Baal, De Slegte & Willems, 1995; Koster, Hartog, Van Os & Bindels, 1996). In the present study, this model has been used to test whether endogenously produced prostanoids modulate basal and/or hormone-stimulated Ca2+ reabsorption. The data presented demonstrate that these primary cultures synthesize and demonstrate that these primary cultures synthesize and release a broad range of prostanoids and that these prostanoids exert an overall stimulatory effect on transepithelial Ca2+ transport.

A preliminary report of this study has been presented in abstract form at the 1995 Annual Meeting of the American Society of Nephrology (Van Baal, Zijlstra, Willems & Bindels, 1995).

**METHODS**

**Materials**

Collagenase A and hyaluronidase were obtained from Boehringer Mannheim and pertussis toxin (PTX) was from Research Biochemicals International. U-46019 was purchased from Biomol (Plymouth Meeting, PA, USA) and 3H-labelled arachidonate acid and 3H-labelled cAMP (succinyltyrosine ester) were from Amersham. Sulprostone and cicaprost were kindly donated by Dr P. McDonald (Schering AG, Berlin, Germany). The monoclonal mouse anti-cAMP antibody was purchased from Dr J. C. Stoof (Free University, Amsterdam, Netherlands). All other chemicals and hormones were obtained from Sigma. Ethanol stock solutions of prostanoids (1 mM) and indomethacin (5 mM) were stored at -20 °C. For experiments, the prostanoids were diluted in the appropriate buffer.

**Culture of rabbit cortical collecting system cells**

Rabbit kidney connecting tubule (CNT) and cortical collecting duct (CCD) cells were immunodissected and set in primary culture on permeable filters (0.33 cm²; Costar, Cambridge, MA, USA), as described previously (Bindels et al., 1991). New Zealand White rabbits weighing ~0.5 kg were killed by cervical dislocation. The kidneys were removed under sterile conditions. A thin layer of superficial cortical tissue was carefully dissected and finely minced with a razor blade. The minced tissue was incubated for 30 min at 37 °C in 10 ml 0.2% (w/v) collagenase A and 0.1% (w/v) hyaluronidase in Krebs-Henseleit buffer (KHB; composition (mM): 128 NaCl, 5 KCl, 1 MgSO4, 1 CaCl2, 2 NaH2PO4, 10 glucose, 10 sodium acetate, 4 L-lactate, 1 L-alanine and 20 Hepes (adjusted to pH 7.40 with Tris)). The tissue was pelleted at 200 g for 5 min, resuspended in 20 ml of 10 mM EDTA in KHB and subsequently incubated at 37 °C for 10 min. Finally, 40 ml KHB was added to the dispersed cell suspension, sieved through nylon filters (100 and 45 μm) and centrifuged at 200 g for 5 min. The resulting cell pellet was resuspended in 10 ml hybridoma medium containing the monoclonal antibody R209 and incubated for 60 min on ice. This antibody is of the immunoglobulin M (IgM) subclass and recognizes specifically a cell-surface antigen shared by rabbit connecting tubules and collecting ducts. After three washings with KHB containing 5% (v/v) fetal calf serum (FCS), the cells were resuspended in the same medium. Subsequently, 5 ml of cell suspension was added to each of six goat anti-mouse IgM-coated bacterial dishes. After 15 min at room temperature (20 °C), the dishes were washed carefully 6 times with KHB containing 5% (v/v) FCS and the adherent cells were scraped off the dishes with a plastic scraper. The immunoscelleted cells of the collecting system were seeded at a high density of 1 x 106 cells cm⁻² on permeable filters (Poretics, CA, USA) and cultured for 10 days in 100 x mixture of non-essential amino acids (Gibco), 5 μg/ml insulin, 5 μg ml⁻¹ transferrin, 60 nM hydrocortisone, 70 ng ml⁻¹ PGE2, 50 nM Na2SeO3 and 5 μM triiodothyronine, equilibrated with 5% CO2-95% O2 at 37 °C. Confluency of the monolayers was routinely checked by determining transepithelial potential difference and resistance with two ‘chopstick’-like electrodes connected to a Millicell-ERS meter (Millipore). Cell monolayers reached confluency at day 3 and were used between 5 and 8 days after seeding. During this last period the number of cells, expressed as micrograms of protein per filter, remained constant and amounted to 74 ± 2 μg filter⁻¹ (n = 10).

**Measurement of transepithelial Ca2+ transport**

Transepithelial Ca2+ transport was measured as described previously (Bindels et al., 1991). Briefly, confluent monolayers were washed three times and pre-incubated in a physiological salt solution (PSS) containing (mM): 140 NaCl, 2 KCl, 1 KH2PO4, 1 MgCl2, 1 CaCl2, 5 glucose, 5 1-L-alanine and 10 Hepes (adjusted to pH 7.40 with Tris) for 15 min at 37 °C. Subsequently, the monolayers were incubated in PSS for another 90 min to measure transepithelial Ca2+ transport. Drugs and hormones were added to the apical and/or basolateral compartment as indicated in the text. At the end of the incubation period, 25 μl samples were removed in triplicate from the apical compartment and assayed for Ca2+ with a colorimetric test kit (Boehringer). Ca2+ transport is expressed in nanomoles per hour per centimetre squared.
Measurement of intracellular cAMP

In order to assess the effects of hormones on adenylyl cyclase activity, the rate of cyclic AMP formation was measured in the presence of the inhibitor of cyclic nucleotide phosphodiesterase activity, 3-isobutyl-1-methylxanthine (IBMX). Confluent monolayers were pre-incubated in PSS containing 1 mM IBMX for 15 min at 37 °C. Subsequently, hormones were added to the apical and/or basolateral compartment and the monolayers were incubated in PSS/IBMX for another 15 min. At 15 min, both the apical and basolateral media were discarded and filters excised. Reactions were terminated by rapidly transferring the filters to Eppendorf microtubes containing 50 μl of 0.2 m HCl. After neutralization with 50 μl of 0.2 m NaOH, the samples were rapidly frozen in liquid nitrogen. The extracts were diluted with an appropriate volume of sodium acetate buffer (pH 6.3) containing 50 mM sodium acetate, 0.05% (w/v) bovine serum albumin and 0.02% (w/v) NaN₃ and the cAMP content was determined by radioimmunoassay essentially as described by Morel & Butlen (1990). In order to enhance the sensitivity of the assay, cyclic nucleotides were acetylated by adding 5 μl of acetic anhydride and triethylamine (1:2, v/v) to each 100 μl sample. After 30 min, 200 μl of a mixture of 125I-labeled cAMP (~0.5 μCi) and anti-cAMP antibody (1 μg ml⁻¹) was added. After overnight incubation at 4 °C, the antigen–antibody complex was precipitated by adding 1 ml of a mixture of 30% (w/v) polyethylene glycol and 5% (w/v) ovalbumin, followed by centrifugation at 2600 g for 30 min at 4 °C. Supernatants were discarded and the pellets counted by gamma spectrometry. Results are expressed as picomoles of cAMP per filter per minute.

Identification of synthesized and released eicosanoids

Confluent monolayers were incubated in the apical presence of 7 μCi 14C-labelled arachidonic acid (58 mCi nmol⁻¹) for 24 h at 37 °C. After 24 h, media from the apical and basolateral compartments were collected separately and centrifuged at 2800 g for 2 min. The eicosanoids were isolated by chromatography using SEP PAK C18 cartridges and separated by reverse-phase HPLC chromatography as described previously (Zijlstra & Vincent, 1984).

Statistics

Results are given as means ± s.e.m. Overall statistical significance was determined by analysis of variance (ANOVA), and in the case of significance individual groups were compared by contrast analysis according to Scheffé. P values of less than 0.05 were considered significant. Half-maximal stimulatory concentrations and maximal transport values were calculated by means of a non-linear regression computer program (Kaleidograph, Synergy Software, Reading, PA, USA).

RESULTS

Effect of indomethacin on transepithelial Ca²⁺ transport

In a static incubation of 90 min, rabbit cortical collecting system cells, grown to confluency on permeable supports, exhibited a net apical-to-basolateral Ca flux of 95 ± 3 nmol h⁻¹ cm⁻² (n = 16). In order to investigate the influence of endogenously produced prostanooids on basal Ca²⁺ transport, the monolayers were pre-incubated in the

Figure 1. Inhibitory effect of indomethacin on Ca⁺⁺ transport

A, confluent monolayers of rabbit cortical collecting system cells were pre-incubated in the presence of 5 μM indomethacin for the indicated periods of time at 37 °C. The cells were washed and subsequently incubated with 5 μM indomethacin for a further 90 min. At the end of the incubation period, apical medium was collected to determine the amount of Ca²⁺ transported across the monolayer. The data presented are the means ± s.e.m. of 16 filters. B, monolayers were pre-incubated in the presence of the indicated concentrations of indomethacin for 15 min at 37 °C, washed and subsequently incubated with the indicated concentrations of indomethacin for a further 90 min. At the end of the incubation period, apical medium was collected to determine the amount of Ca²⁺ transported across the monolayer. The data presented are the means ± s.e.m. of 6 filters. * Significantly different from control values.
Figure 2. Release of Ca\textsuperscript{2+} transport-stimulating factors

Cells were incubated with or without 5 µM indomethacin for 90 min at 37 °C. Subsequently, the apical and basolateral media of each filter cup were collected, mixed and applied to both compartments of a new set of monolayers pretreated with 5 µM indomethacin for 15 min. In the case of medium harvested from monolayers incubated without indomethacin, this drug (5 µM) was added to the collected medium prior to addition to the new set of monolayers. As a reference, indomethacin-pretreated monolayers (5 µM for 15 min) were incubated with fresh medium containing 5 µM indomethacin. After an incubation period of 90 min the amount of Ca\textsuperscript{2+} transported across the monolayers was determined. The data presented are the means ± s.e.m. of 6 filters. * Significantly different from control values.

Figure 3. Production and release of eicosanoids

Cells were incubated in the presence of 7 µCi \textsuperscript{14}C-labelled arachidonic acid in the apical compartment for 24 h at 37 °C. After 24 h, both the apical and basolateral media were subjected to reverse-phase HPLC. □, apical medium; ■, basolateral medium. Abbreviations are: 12-HETE, 12-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; LTB\textsubscript{4}, leukotriene B\textsubscript{4}; epi-LTs, 6-trans-leukotrienes B\textsubscript{4} and 12-epi,6-trans-leukotriene B\textsubscript{4}; SP-LTs, sulphidopeptide-leukotrienes; TXB\textsubscript{2}, thromboxane B\textsubscript{2}; 6k-PGF\textsubscript{1α}, 6-keto-PGF\textsubscript{1α}. Data presented are from a representative experiment.
presence of the specific inhibitor of cyclo-oxygenase activity, indomethacin (5 μM), for 15 min. Subsequently, the monolayers were incubated in the presence of indomethacin for another 90 min and Ca^{2+} transport was measured. Indomethacin significantly (P < 0.05) reduced Ca^{2+} transport to 43 ± 2% of the untreated control value (Fig. 1A). Extension of the pre-incubation period to 24 h did not lead to a further reduction, suggesting that cyclo-oxygenase activity is already effectively blocked within the first 15 min of indomethacin treatment. Moreover, this observation demonstrates that the absence of endogenous produced prostanoids does not affect the viability of the monolayer.

The above finding that indomethacin markedly reduced Ca^{2+} transport by 66 ± 6% (P < 0.05) on Ca^{2+} transport measured in the absence of any added stimulus and therefore hitherto referred to as basal Ca^{2+} transport (Bindels et al. 1991, 1992, 1995; Koster et al. 1996), suggests that endogenously produced prostanoids significantly contribute to this so-called basal Ca^{2+} transport. In order to investigate a possible autostimulatory pathway in more detail, monolayers were incubated in either the absence or presence of indomethacin for 90 min. At the end of incubation, media from both the apical and basolateral compartment were collected, mixed and added to a new set of monolayers pretreated with 5 μM indomethacin for 15 min.

Endogenous production and release of prostanoids

The above finding that indomethacin markedly reduced Ca^{2+} transport measured in the absence of any added stimulus and therefore hitherto referred to as basal Ca^{2+} transport (Bindels et al. 1991, 1992, 1995; Koster et al. 1996), suggests that endogenously produced prostanoids significantly contribute to this so-called basal Ca^{2+} transport. In order to investigate a possible autostimulatory pathway in more detail, monolayers were incubated in either the absence or presence of indomethacin for 90 min. At the end of incubation, media from both the apical and basolateral compartment were collected, mixed and added to a new set of monolayers pretreated with 5 μM indomethacin for 15 min. Transepithelial Ca^{2+} transport was then measured for 90 min in the presence of indomethacin. The data presented in Fig. 2 show that media obtained from monolayers incubated in the presence of indomethacin stimulated Ca^{2+} transport by 66 ± 6% (P < 0.05), whereas media obtained from monolayers incubated in the presence of indomethacin did not significantly (P > 0.8) affect the Ca^{2+} transport rate. In order to identify the putative endogenously produced cyclo-oxygenase metabolites in the control medium, monolayers were labelled with ³⁴Cl-labelled arachidonic acid for 24 h at 37 °C. Subsequent HPLC analysis revealed the presence of a broad spectrum of radioactive eicosanoids in both the apical and basolateral media (Fig. 3). PGE₂, PGF₂α, PGD₂ and 6-keto-PGF₁α were the major prostanoids synthesized and released by rabbit cortical collecting system cells in vitro.

Effect of exogenous prostanoids on Ca^{2+} transport and cAMP formation

Addition of PGE₁ (1 μM), PGE₂ (1 μM) or PGA₂ (1 μM) to indomethacin-treated monolayers resulted in a significant (P < 0.05) increase in transepithelial Ca^{2+} transport (Fig. 4). In contrast, no effect was observed with PGD₂ (1 μM), PGF₂α (1 μM), the stable prostacyclin analogue cicaprost (1 μM) (Coleman et al. 1994), or the thromboxane A₂ mimetic U-46619 (1 μM) (Coleman et al. 1994). Figure 5A shows that the stimulatory effect of PGE₂ was dose dependent (EC₅₀ = 3 nM) and that maximal stimulation was obtained at an EC₅₀ of 0.4 μM. Both potency and efficacy were independent of the size of application. Moreover, addition of a maximal concentration of PGE₂ (0.1 μM) to both sites did not lead to a further stimulation of Ca^{2+} transport (201 ± 13, 165 ± 14 and 222 ± 14% of basal transport, for stimulation from apical, basolateral and both sites, respectively; N = 11, P > 0.7). The stimulatory action of PGF₁α was paralleled by a dose-dependent increase in the rate of cAMP formation (Fig. 5B). Similar to PGE₂, PGA₂ stimulated transepithelial Ca^{2+} transport dose dependently (EC₅₀ = 81 nM): the EC₅₀ was 3 μM. The stimulatory effect of PGA₂ was accompanied by increased cAMP formation. In order to obtain functional evidence for the presence of EP₁ and/or EP₃ receptors, the effect of sulprostone, a stable acylsulphonamide analogue of PGE₂, with selectivity for both the EP₁ and EP₃ receptors (Coleman, Kennedy & Sheldrick, 1987), was tested.

**Figure 4. Influence of prostanoids on Ca^{2+} transport**

Cells pretreated with 5 μM indomethacin for 15 min at 37 °C were incubated in the presence of the indicated prostanoids (1 μM) in either the apical (□) or basolateral (■) compartment for a further 90 min. At the end of the incubation period, apical medium was collected to determine the amount of Ca^{2+} transported across the monolayer. The data presented are the means ± S.E.M. of 5 filters. In each experiment the transport value obtained with unstimulated monolayers (45 ± 4 nmol h⁻¹ cm⁻²; N = 5) is set at 100%, to which all other values are related. *Significantly different from control levels.
Figure 5. Dose–response curve for the stimulatory effect of PGE$_2$ on Ca$^{2+}$ transport and cAMP formation

A, cells pre-incubated with 5 μM indomethacin for 15 min at 37 °C were incubated in the presence of the indicated concentrations of PGE$_2$ in the apical (○) or basolateral compartment (●) for a further 90 min. At the end of the incubation period, apical medium was collected to determine the amount of Ca$^{2+}$ transported across the monolayer. The data presented are the means ± s.e.m. of 6 filters. B, cells pre-incubated in the presence of 1 mM IBMX and 5 μM indomethacin for 15 min at 37 °C were incubated in the presence of the indicated concentrations of PGE$_2$ in either the apical (○) or basolateral compartment (●) for a further 15 min. At the end of the incubation period, filters were collected for cAMP measurement.

Sulprostone (0·1 μM) significantly ($P < 0·05$) decreased Ca$^{2+}$ transport from 49 ± 2 to 28 ± 3 nmol h$^{-1}$ cm$^{-2}$ in indomethacin-treated monolayers. Inhibition was only observed when sulprostone was added to the basolateral compartment. Similarly, sulprostone significantly inhibited the stimulatory effect of the inhibitor of cyclic nucleotide phosphodiesterase activity IBMX (1 mM) in indomethacin-treated monolayers (Fig. 6). Interestingly, the inhibitory effect of sulprostone was completely reversed in monolayers pretreated with pertussis toxin (PTX, 170 ng ml$^{-1}$) for 24 h.

Figure 6. Effect of pertussis toxin on sulprostone-inhibited Ca$^{2+}$ transport

Cells grown to confluency on permeable supports were pre-incubated in the presence or absence of 170 ng ml$^{-1}$ pertussis toxin for 24 h at 37 °C. Subsequently, the monolayers were pretreated with 5 μM indomethacin for 15 min at 37 °C followed by incubation with 1 mM IBMX in the absence (□) or presence of 0·1 μM sulprostone (○) in the basolateral compartment for another 90 min. At the end of the incubation period, apical medium was collected to determine the amount of Ca$^{2+}$ transported across the monolayer. IBMX significantly ($P < 0·05$) stimulated Ca$^{2+}$ transport from 48 ± 2 to 105 ± 3 nmol h$^{-1}$ cm$^{-2}$ ($n = 4$) in indomethacin-treated monolayers. The data presented are the means ± s.e.m. of 4 filters. * Significantly different from control values.
Prostaglandin-stimulated Ca\textsuperscript{2+} reabsorption

Figure 7. Dose–response curves for the stimulatory effects of AVP and PTH on transepithelial Ca\textsuperscript{2+} transport

Cells pre-incubated in the absence (○) or presence of 5 μM indomethacin (●) for 15 min at 37 °C were subsequently stimulated with the indicated concentrations of AVP (A) or PTH (B) added to the basolateral compartment for a further 90 min. At the end of the incubation period, apical medium was collected to determine the amount of Ca\textsuperscript{2+} transported across the monolayer. The data presented are the means ± s.e.m. of 8 filters. * Significantly different from the corresponding control value.

Effect of endogenous prostanoids on the stimulatory actions of arginine vasopressin and parathyroid hormone

The effect of arginine vasopressin (AVP) and parathyroid hormone (PTH) on Ca\textsuperscript{2+} transport was investigated in the absence and presence of endogenously produced prostanoids. Figure 7 shows that AVP and PTH stimulated Ca\textsuperscript{2+} transport dose dependently in both control and indomethacin-treated monolayers. For both hormones the EC\textsubscript{50} value did not differ significantly between control and indomethacin-treated monolayers (EC\textsubscript{50} values of 0.4 nM and 0.3 nM in the case of AVP and 0.9 nM and 1 nM in the case of PTH for control and indomethacin-treated monolayers, respectively). Moreover, for both hormones the maximal transport value was not significantly different between control and indomethacin-treated cells. However, the percentage increase over basal was dramatically enhanced in indomethacin-treated monolayers due to the fact that the baseline was lowered by this treatment. The effect of indomethacin on the rate of AVP-induced cAMP formation was determined in the presence of IBMX. Both in the absence and presence of indomethacin, AVP stimulated cAMP accumulation dose dependently (Fig. 8). At hormone concentrations above 1 nM, the rate of cAMP formation was significantly (P < 0.05) higher in indomethacin-treated monolayers as compared with untreated control cells. Similar effects were obtained with PTH (data not shown). No difference in basal cAMP production was detected between control and indomethacin-treated cells.

Figure 8. Dose–response curve for the stimulatory effect of AVP on cAMP accumulation

Cells pre-incubated with 1 mM IBMX in either the absence (○) or presence of 5 μM indomethacin (●) for 15 min at 37 °C were stimulated with the indicated concentrations of AVP added to the basolateral compartment for a further 15 min. At the end of the incubation period, filters were collected for cAMP measurement. The data presented are the means ± s.e.m. of 5 filters. * Significantly different from the corresponding control value.
DISCUSSION
Endogenous production and release of prostanoids
The important finding of the present study is that primary cultures of the rabbit cortical collecting system synthesize and release a broad range of prostanoids, which exert an overall stimulatory effect on transepithelial Ca\textsuperscript{2+} transport. The present findings concerning the mechanism of action of prostanoids are schematically summarized in Fig. 9. Evidence for the involvement of endogenously produced prostanoids in active Ca\textsuperscript{2+} transport, up to now regarded as being basal (Bindels et al. 1991, 1994, 1995; Koster et al. 1996), comes from the following observations: (i) indomethacin markedly reduced Ca\textsuperscript{2+} transport in the absence of any added stimulus; (ii) medium from non-treated control cells contained stimulatory factors that were absent in medium from indomethacin-treated cells; (iii) HPLC analysis of the medium following incubation with \textsuperscript{14}C-labelled arachidonic acid revealed the presence of a broad range of radiolabelled prostanoids; and (iv) addition of some exogenous prostanoids effectively restored Ca\textsuperscript{2+} transport in indomethacin-treated cells. Although the present study shows that the primary cultures produce and release a broad spectrum of prostanoids, including PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, PGD\textsubscript{2}, thromboxane and 6-keto-PGF\textsubscript{1α}, it is tempting to speculate that PGE\textsubscript{2} is the major prostanoid responsible for increased Ca\textsuperscript{2+} transport measured in the absence of indomethacin, since the other two stimulatory prostanoids, namely PGE\textsubscript{1} and PGA\textsubscript{2\theta}, were not detected in the medium. PGF\textsubscript{2\alpha}, PGD\textsubscript{2}, U-46619 and eicaprosta, a metabolite of 6-keto-PGF\textsubscript{1α}, did not affect transepithelial Ca\textsuperscript{2+} transport and are, therefore, not likely to be involved in the regulation of this transport process. Our findings are in agreement with earlier studies reporting that native and cultured cells of connecting and cortical collecting tubules synthesize and release a variety of prostanoids (Garcia-Perez & Smith, 1984; Schlondoff, Satriano & Schwartz, 1985; Farman, Pradelles & Bonvalet, 1987; Noland et al. 1992) and that PGE\textsubscript{2} is a potent regulator of renal transport processes (Iino & Imai, 1978; Hébert, Jacobson & Breyer, 1990; Hébert et al. 1993, 1995; Ando & Asano, 1995; Sakairi, Jacobson, Noland & Breyer, 1995).

Figure 9. Schematic model for the regulation of transepithelial Ca\textsuperscript{2+} transport by prostanoids
For explanation see text. Metabolism of arachidonic acid is responsible for the biosynthesis of prostanoids, including PGE\textsubscript{1}, PGE\textsubscript{2}, PGD\textsubscript{2}, PGF\textsubscript{2\alpha}, PGI\textsubscript{2} and TXA\textsubscript{2}. This synthesis can be inhibited with the cyclooxygenase inhibitor indomethacin. EP\textsubscript{2} and/or EP\textsubscript{4} receptors couple to G\textsubscript{2} proteins to increase the adenyl cyclase (AC)-catalysed formation of cAMP, which stimulates the process of transepithelial Ca\textsuperscript{2+} transport. EP\textsubscript{3} receptors couple to G\textsubscript{1} proteins to inhibit the AC-catalysed formation of cAMP. The intracellular Ca\textsuperscript{2+} binding protein, calbindin-D\textsubscript{28k} (CaBP) facilitates transepithelial Ca\textsuperscript{2+} transport.
Apical and basolateral EP<sub>2</sub> and/or EP<sub>4</sub> receptors stimulate Ca<sup>2+</sup> transport

The stimulatory effect of PGE<sub>2</sub> on active Ca<sup>2+</sup> transport was paralleled by an increase in adenylyl cyclase activity. This observation, suggesting that cAMP plays a mediatory role in hormone-stimulated Ca<sup>2+</sup> transport, is in line with earlier findings (Bindels et al., 1991, 1995; Costanzo & Windhager, 1992; Bindels, 1993). A stimulatory role for cAMP in active Ca<sup>2+</sup> transport is further supported by the present finding that inhibition of cyclic nucleotide phosphodiesterase activity by IBMX results in a marked increase in active Ca<sup>2+</sup> transport. Thus far, two prostanoid receptors that couple to adenylyl cyclase in a stimulatory fashion have been identified, i.e. the EP<sub>2</sub> and EP<sub>4</sub> receptors (Coleman et al., 1994). Evidence that cultured rabbit cortical collecting duct cells lack a functional EP<sub>2</sub> receptor comes from the recent observation that butaprost, a selective agonist for this receptor subtype, failed to increase the cellular cAMP level in these cells (Sakairi et al., 1995). Together with the present finding that the EP<sub>2</sub> and EP<sub>4</sub> receptor agonist sulprostone did not mimic the stimulatory effect of PGE<sub>2</sub>, this suggests that PGE<sub>2</sub> interacts with EP<sub>4</sub> receptors to stimulate transepithelial Ca<sup>2+</sup> transport in cultured cortical collecting system cells. The stimulatory effect of PGE<sub>2</sub> on Ca<sup>2+</sup> transport and cAMP generation occurred irrespective of the compartment to which the hormone was added, suggesting that EP<sub>1</sub> receptors are present on both the apical and basolateral membrane. A similar distribution of EP<sub>4</sub> receptors has been reported with respect to PGE<sub>2</sub>-induced stimulation of Na<sup>+</sup> and water transport in cortical collecting duct (Hebert et al., 1990; Ando & Asano, 1995; Sakairi et al., 1995).

Like PGE<sub>2</sub>, PGA<sub>4</sub> increased Ca<sup>2+</sup> transport dose dependently and in a cAMP-dependent fashion when administered to either the apical or basolateral compartment. PGA<sub>4</sub>, however, was less potent in that its EC<sub>50</sub> was found to be 28-fold higher than that of PGE<sub>2</sub>. Although this observation suggests that PGA<sub>4</sub> exerts its stimulatory effect through a specific PGA<sub>4</sub> receptor, it is also possible that PGA<sub>4</sub> acts through the cross-activation of the EP<sub>4</sub> receptor. The latter possibility is supported by the observation of PGA<sub>4</sub> being 10-fold less potent than PGE<sub>2</sub> in inhibiting <sup>3</sup>H-labelled PGE<sub>2</sub> binding to rat whole kidney membranes (Eriksson, Larsson & Andersson, 1990).

Notably, inhibition of active Ca<sup>2+</sup> transport by indomethacin was not accompanied by a detectable decrease in the rate of cAMP accumulation. Similarly, PGE<sub>2</sub>, at a concentration of 10 nM, restored Ca<sup>2+</sup> transport to pre-inhibitory values without detectably increasing the rate of cAMP accumulation. This observation suggests that the stimulatory effect of PGE<sub>2</sub> at concentrations at or below 10 nM is paralleled by possibly local increases in cAMP production too small to be detected. Alternatively, in addition to cAMP, another second messenger might be involved in PGE<sub>2</sub>-stimulated Ca<sup>2+</sup> transport.

Basolateral EP<sub>3</sub> receptor inhibits basal and hormone-stimulated Ca<sup>2+</sup> transport

Sulprostone, an EP receptor agonist with selectivity for the EP<sub>3</sub> and EP<sub>4</sub> receptors, suppressed both indomethacin-inhibited and cAMP-stimulated transepithelial Ca<sup>2+</sup> transport. However, inhibition was observed only when sulprostone was added to the basolateral compartment, suggesting that EP<sub>3</sub> and/or EP<sub>4</sub> receptors are exclusively present on the basolateral membrane. This observation is in agreement with recent findings that sulprostone inhibits AVP-stimulated Na<sup>+</sup> and water transport when applied to the basolateral, but not to the luminal, surface of microperfused rabbit cortical collecting ducts (Sakairi et al., 1995). The finding that PTX completely reversed the inhibitory action of sulprostone on IBMX-stimulated Ca<sup>2+</sup> transport indicates that this analogue acts via the G<sub>i</sub>-protein-coupled EP<sub>3</sub> receptor, rather than via the G<sub>i</sub>-protein-coupled EP<sub>1</sub> receptor (Coleman et al., 1994). This is supported by the observation that ciasprostat, a prostacyclin analogue which acts as an agonist on EP<sub>3</sub> receptors (Coleman et al., 1994), did not affect Ca<sup>2+</sup> transport in indomethacin-treated monolayers. Our finding is in agreement with recent observations that sulprostone inhibits hormonal activation of adenylyl cyclase through a PTX-sensitive G-protein in rabbit cortical collecting duct (Hébert, 1994; Hébert et al., 1995). Interestingly, in this nephron segment PGE<sub>2</sub> was found to couple to both apical EP<sub>2</sub> and EP<sub>4</sub> receptors to inhibit transepithelial Na<sup>+</sup> transport and AVP-stimulated water flow (Hébert et al., 1990; Chabardes, Montegut, Zhou & Siaume-Perez, 1990). The present finding that PGE<sub>2</sub> stimulates Ca<sup>2+</sup> transport to the same extent irrespective of the compartment to which it is added suggests that the signal of inhibitory basolateral EP<sub>3</sub> receptors is completely overruled by that of stimulatory EP<sub>1</sub> receptors.

AVP and PTH increased active Ca<sup>2+</sup> transport to the same extent in control and indomethacin-treated monolayers. This observation demonstrates that the stimulatory effects of the endogenously produced prostanoids and the exogenously added hormones are not additive. The most likely explanation is that both the prostanoids and the above hormones use the same intracellular messenger, namely cAMP, to stimulate Ca<sup>2+</sup> transport. Conversely, at the level of hormone-induced cAMP accumulation indomethacin significantly enhanced the maximal effect of the hormones. This finding further substantiates the presence of a basolateral EP receptor inhibiting hormone-induced adenylyl cyclase activity (Hébert, 1994; Hébert et al., 1995). From the observation that AVP stimulated both Ca<sup>2+</sup> transport and cAMP accumulation with the same potency in control and indomethacin-treated cells it can be concluded that the endogenously produced prostanoids do not influence the sensitivity of the cultured cortical collecting system cells for the hormone. Finally, as a result of the marked decrease in basal Ca<sup>2+</sup> transport in indomethacin-treated cells, the factor by which AVP and PTH stimulated
active Ca\textsuperscript{2+} transport was dramatically increased, thus unmasking the stimulatory effects of both hormones to their full extent.

In general, the effect of prostanooids on renal Ca\textsuperscript{2+} handling in vivo is unclear and conflicting since both stimulation and inhibition of Ca\textsuperscript{2+} reabsorption by these hormones has been reported (Holt & Lechene, 1981; Roman, Skelton & Lechene, 1984). Our studies demonstrate that prostaglandins stimulate basal calcium reabsorption via activation of EP\textsubscript{2} and/or EP\textsubscript{4} receptors, but inhibit cAMP-stimulated calcium reabsorption via activation of EP\textsubscript{3} receptors. This could be in line with the in vivo findings, since depending on the hormonal status of the animal, calcium reabsorption is low or high and prostaglandins could stimulate or inhibit calcium reabsorption accordingly. In the present study, the endogenous prostaglandin production certainly explains the high basal calcium transport levels. At present, however, it is premature to conclude that the endogenous prostaglandin production in vivo is involved in resting calcium reabsorption.

In conclusion, the data obtained with primary cultures of rabbit cortical collecting system cells points to a possible physiological role of endogenously produced prostanooids, in particular PGE\textsubscript{2}, in the regulation of Ca\textsuperscript{2+} reabsorption in this nephron segment.


Koster, H. P. G., Hartog, A., Van Os, C. H. & Bindels, R. J. M. (1996). Inhibition of Na\textsuperscript{+} and Ca\textsuperscript{2+} reabsorption by PGE\textsubscript{2} purinoceptors requires protein kinase C but not Ca\textsuperscript{2+} signaling. American Journal of Physiology 270, F63-80.
Prostaglandin-stimulated Ca\(^{2+}\) reabsorption


Acknowledgements
The authors thank Dr C. H. Van Os for helpful discussions. The research of Dr P. H. G. M. Willems has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

Author’s email address
J. van Baal: J.vanBaal@cellys.kun.nl

Received 25 April 1996; accepted 29 July 1996.