Inhibition of Apical Na^+ Channels in Rabbit Cortical Collecting Tubules by Basolateral Prostaglandin E_2 Is Modulated by Protein Kinase C

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Abstract

We used the cell-attached patch clamp technique to investigate the interaction of exogenous prostaglandins (PG), intracellular $[Ca^{2+}]_i$, and protein kinase C (PKC) on the high selectivity, 4 pS Na^+ channel found in the principal cell apical membrane of rabbit cortical collecting tubule (CCT) cultures grown on collagen supports with 1.5 μM aldosterone. Application of 0.5 μM PGE_2 to the basolateral membrane decreased mean NP_o (number of channels times the open probability) for apical Na^+ channels by 46.5% ($n = 9$). There was no consistent change in NP_o after apical 0.5 μM PGE_2 ($n = 12$) or after apical or basolateral 0.5 μM $PGF_{2\alpha}$ ($n = 8$). Release of $[Ca^{2+}]_i$ stores with 0.25 μM thapsigargin ($n = 7$), or activation of apical membrane PKC with apical 0.1 μM 4β-phorbol-12-myristate-13-acetate ($n = 5$) or 10 μM 1-oleyl-2-acetylglycerol ($n = 4$) also decreased NP_o. Depletion of $[Ca^{2+}]_i$ stores (0.25 μM thapsigargin pretreatment) ($n = 7$) or inhibition of apical PKC (100 μM D-sphingosine pretreatment) ($n = 8$) abolished the inhibitory effects of basolateral PGE_2. Conclusions: (*a*) apical Na^+ transport in rabbit CCT principal cells is modulated by basolateral PGE_2; (*b*) the mechanism involves release of IP_3-sensitive, $[Ca^{2+}]_i$ stores; and (*c*) Ca^{2+}-dependent activation of apical membrane PKC, which then inhibits apical Na^+ channels. (*J. Clin. Invest.* 1992. 90:1328–1334.) Key words: intracellular Ca^{2+} • patch clamp • sphingosine • phorbol esters • principal cell

Introduction

Mammalian cortical collecting tubule (CCT)[1] principal cells are responsible for mineralocorticoid-dependent Na^+ reabsorption and, as such, represent the primary point for discretionary control of total body Na^+ balance (1–4). At a molecular level, this transepithelial Na^+ transport is regulated by altering the permeability of Na^+ channel proteins located in the principal cell apical membrane (5, 6).

The mechanisms for controlling Na^+ reabsorption can be thought of as a threefold hierarchy. The first level involves circulating hormones interacting with specific receptors at the basolateral (serosal) cell surface. The second level involves intracellular second messenger cascades that transduce the hormonal signal from the basolateral to the apical (luminal) membrane, where the primary control of Na^+ transport occurs. The third level involves molecular events confined to the local microenvironment of the apical membrane itself. Apical membrane-bound regulatory elements activated by cytosolic second messengers then directly modulate high selectivity Na^+ channel molecules.

Several groups have demonstrated that basolateral PGE_2 exposure (first level or hormonal control) inhibits Na^+ reabsorption in microperfused rabbit CCT (7–11). Studies by Marver (12) and Stokes (13) suggest that inhibition of Na^+ transport in rabbit CCT by basolateral PGE_2 occurs at the apical Na^+ entry step (i.e., Na^+ channel), rather than at the basolateral Na^+ exit step (Na^+, K^+-ATPase). Hebert et al. have shown that this inhibitory response to exogenous PGE_2 is dependent on the release of intracellular Ca^{2+} stores (second level or second messenger control) (11). Activation of the Ca^{2+}-dependent enzyme, protein kinase C (PKC) also inhibits sodium absorption in isolated rabbit CCT (12, 14). Previously, we have shown that feedback-inhibition of single, high selectivity Na^+ channel activity in amphibian distal nephron cells (A6) is mimicked by activation of PKC in the apical membrane, but not the basolateral membrane, and is reversed by PKC inhibition (third level control or local microenvironmental control) (15).

We have recently characterized sodium-permeable channels present in the principal cell apical membranes of rabbit CCT grown in primary culture (3). The low conductance (4 pS) channel has all the physiologic properties predicted from studies of macroscopic Na^+ absorption in microperfused rabbit CCT, namely high Na^+ selectivity, mineralocorticoid-dependence, amiloride-sensitivity, and long mean open times (seconds). The present study examines inhibition of Na^+ reabsorption in the rabbit CCT by exogenous PGE_2 at the level of the high selectivity Na^+ channel using patch clamp methodology and rabbit CCT primary cultures. We also investigate the role of intracellular Ca^{2+} and protein kinase C in PGE_2-induced inhibition of principal cell Na^+ channels.

Methods

Preparation of rabbit cortical collecting tubule primary cultures. As previously described, kidneys were dissected from New Zealand white rabbits (1–2 kg) in ice-cold Hepes-buffered saline solution containing (mM): 118 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.25 $MgSO_4$, 1.18 KH_2PO_4, 10

A preliminary report of this work was presented at the American Society of Nephrology Annual Meeting, 17–20 November 1991 Baltimore, MD.

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Received for publication 13 January 1992 and in revised form 3 April 1992.

1. *Abbreviations used in this paper:* CCT, cortical collecting tubule; IP_3, inositol triphosphate; OAG, 1-oleyl-2-acetylglycerol; PKC, protein kinase C; PMA, 4β-phorbol-12-myristate-13-acetate.

J. Clin. Invest.

0021-9738/92/10/1328/07 $2.00
Volume 90, October 1992, 1328–1334

glucose, 20 Na cyclamate, and 5 Hepes at pH 7.4 (3, 16–18). The renal cortex was separated and incubated for 60 minutes at 37°C (equilibrated with 4% CO_2 in air) in a solution containing: 4 ml of Hepes-buffered saline, 0.25 ml 10% BSA, 6.2 mg of collagenase (type I; Sigma Chemical Co., St. Louis, MO), and 4 ml of RK-1 medium. RK-1 medium contains 50% Ham's F-12 (by volume) and 50% DME (high bicarbonate and glucose) supplemented with 5 mg/liter transferrin, 5 mg/liter insulin, 0.05 μM sodium selenite, and 0.05 μM hydrocortisone, pH 7.4. The cell mixture was then washed three times by suspension in RK-1 media and pelleting at 150 *g* for 1.5 min. CCT fragments were separated by one high speed centrifugation (15,000–30,000 *g*) in an isotonic mixture of 50% Percoll and saline.

CCT fragments, contained in the lower part of the Percoll F1 separation band, were plated at confluent density on permeable, glutaraldehyde-fixed collagen films attached to the bottoms of small lucite rings (3, 15, 17, 18). This sided preparation allows patch pipette access to the apical membrane, and separate control of the apical and basolateral bath compositions (Fig. 1). Cultures were incubated in RK-1 medium with 1.5 μM aldosterone (Sigma Chemical Co.) at 37°C and gassed with 4% CO_2 in air. Penicillin (100 U/ml) and streptomycin (100 μg/ml) was present for the first 24–48 h. In 4–7 d, the culture monolayer reached confluency. Patch voltage clamp experiments were performed on days 7–14.

Patch clamp recording and analysis. Polygonal shaped principal cells of rabbit CCT primary culture monolayers were visualized with Hoffman modulation optics (Modulation Optics Inc., Greenvale, NY) mounted on a Nikon Diaphot-TMD inverted microscope (16). Patch pipettes were fabricated and cell-attached patches with gigaohm seal resistances (10–20 GΩ) were obtained on principal cell apical membranes, as previously described (3, 17, 18). Unitary channel current events were obtained using an List-Medical EPC-7 patch clamp (Medical Systems Corp., Greenvale, NY). Events were processed by a Digital Data Adaptor (DAS 601; Dagan Corp., Minneapolis, MN), recorded on a video cassette recorder (SL-HF860D; Sony Corp. of America, Park Ridge, NJ), and digitized using digitizing hardware (Scientific Solutions Inc., Solon, OH) and an 80286 computer (200 μs/point; low-pass filtered at 1 kHz). Analysis of data was performed on an IBM PC/AT using locally and commercially developed software (19).

All experiments were conducted at 37°C with a TC-202 temperature controller and PDMI-2 open perfusion micro-incubator (Medical Systems Corp.). Patch pipettes contained a physiologic saline solution (mM): 140 NaCl (final NaCl concentration after titration to pH 7.4 with NaOH), 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 Hepes. The basic apical and basolateral bath compositions were the same as the patch pipette solution above.

When channels were too numerous to clearly distinguish the individual channel current levels by histogram, the amplitude of single channel events were measured from the actual patch clamp recordings. The total number of functional channels (N) in the patch were preliminarily estimated by observing the number of peaks detected on current amplitude histograms. As a measure of high selectivity Na^+ channel activity, NP_o (number of channels times the open probability) was calculated (19).

$$NP_o = \sum_{n=0}^{N} \frac{n \cdot t_n}{T}$$

T is the total record time, *n* is the number of channels open, and t_n is the record time during which *n* channels are open. Therefore, NP_o can be calculated without making assumptions about the total number of channels in a patch or the open probability of a single channel.

PGE_2, $PGF_{2\alpha}$, thapsigargin (Calbiochem Corp., San Diego, CA), 4β-phorbol-12-myristate-13-acetate (PMA), 1-oleyl-2-acetylglycerol (OAG), 4α-phorbol, and D-sphingosine (Sigma Chemical Co.) were of the highest commercial grade available. Appropriate solvent vehicles (dimethyl sulfoxide or ethanol) were added to control baths, and by themselves caused no change in Na^+ channel activity.

Experiments were conducted in a paired fashion with each cell-attached patch membrane acting as its own control. Data is reported as mean values±SD.

Results

High selectivity 4-pS Na^+ channels were identified in apical cell-attached patches of principal cells of rabbit CCT primary cultures (3). This channel was distinguished from other Na^+ permeable channels (i.e., low selectivity, 9 pS; and nonselective, 30 pS) by its lower unitary conductance, nonlinear current-voltage (I-V) relationship, longer mean open times and inward current (pipette to cell) at all applied potentials, $V_{app} < +80$ mV (cell interior with respect to pipette interior) (3). In previous patch clamp studies of high selectivity Na^+ channels in rabbit and rat CCT, and amphibian distal nephron cells (A6), large variability in baseline channel activity was observed between different patches (3, 15, 20). Because of this, each cell-attached patch was used as its own control when proposed regulatory factors were added to the apical bath (outside the patch pipette) or basolateral bath (Fig. 1).

Exogenous, basolateral prostaglandin E_2 inhibits apical, high selectivity, Na^+ channels. In the cell-attached patch configuration, baseline channel activity was recorded at resting cell membrane potential (applied pipette potential, $V_{app} = 0$ mV). The basolateral bath solution was then replaced with the same solution containing PGE_2 (final concentration 0.5 μM). Within 1 min of exposure, the fraction of time that Na^+ channels spent in the open state decreased (Fig. 2 *A*). Fig. 2 *B* plots channel activity (measured as the product of the number of channels times the open probability, designated NP_o) for the 3-min "control" recording period immediately before and the 3-min recording period occurring after 1 min of basolateral PGE_2 application for nine separate cell-attached patch clamp experiments. A consistent decrease in NP_o was observed in all patches with mean NP_o falling from 0.49±0.22 to 0.26±0.18

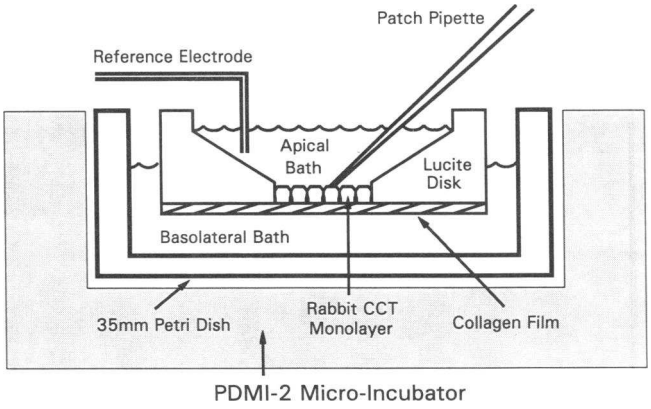

Figure 1. Bath exchange chamber. This schematic depicts a rabbit CCT primary culture monolayer grown on a collagen film attached to the bottom of a plastic ring. The petri dish, containing the plastic ring, is seated in a PDMI-2 Microincubator (see Methods) to maintain bath temperature at 37°C. Apical and basolateral baths are separated by the collagen film and ring. Apical bath exchanges were conducted outside the patch pipette.

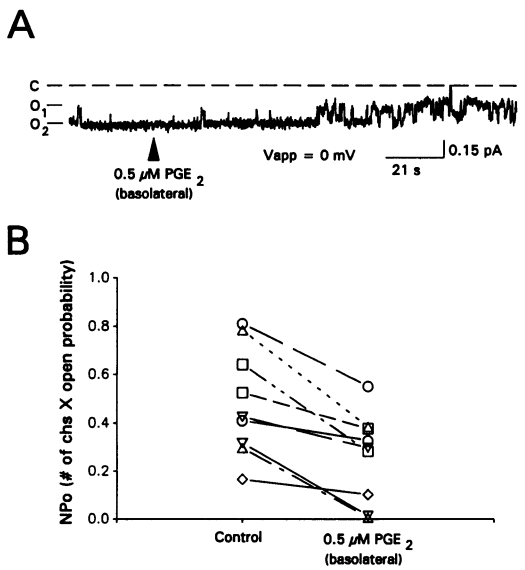

Figure 2. Effect of basolateral PGE_2 on high selectivity Na^+ channels. (*A*) Cell-attached patch recording depicts inward Na^+ current (pipette to cell) as downward deflections. This 3-min trace shows at least two Na^+ channels open under baseline conditions. Within 1 min of basolateral membrane exposure to 0.5 μM PGE_2 (*arrowhead*), a marked decrease in open channel events can be seen. Horizontal bars mark zero current level (*C*, closed state) and open channel current levels (*O*, open state). V_{app} = 0 mV, represents no applied pipette voltage to the patch membrane; i.e., resting membrane potential. Traces are recorded at a corner frequency of 1 kHz, sampled at 5 kHz (200 μs/point), and low pass filtered at 450 Hz. (*B*) Na^+ channel activity, measured as NP_o (number of channels times the open probability) is depicted before and after basolateral PGE_2 exposure for nine separate cell-attached patches at V_{app} = 0 mV. Control NP_o was calculated for the 3-min recording period just before PGE_2 exposure (mean NP_o = 0.49±0.22). NP_o's were then calculated for the 3-min recording period starting 1 min after basolateral membrane exposure to 0.5 μM PGE_2 (mean NP_o = 0.26±0.18). Symbols connected by lines represent relative change in channel activity for the same cell-attached patch (mean NP_o decrease = 46.5%).

(46.5% decrease) (n = 9). In contrast, comparable Na^+ channel inhibition was not observed when 0.5 μM PGE_2 was applied to the apical membrane outside the cell-attached patch pipette (n = 12) (Fig. 3). The change in NP_o after basolateral application of PGE_2 appeared specific, since neither apical (n = 4) nor basolateral (n = 4) exposure to 0.5 μM $PGF_{2\alpha}$ resulted in channel inhibition (data not shown).

PGE_2-induced Na^+ channel inhibition is dependent on release of intracellular Ca^{2+} stores. Hebert et al. have shown that basolateral PGE_2 inhibits Na^+ absorption in microperfused rabbit CCT by increasing intracellular Ca^{2+} via release from inositol triphosphate (IP_3)-dependent stores (11). Thapsigargin is a cell permeable tumor promotor that rapidly releases Ca^{2+} from intracellular pools without hydrolysis of inositol polyphosphates (21). Basolateral bath application of 0.25 μM thapsigargin inhibited Na^+ channel activity in single channel recordings within 1 min (Fig. 4 *A*) and decreased NP_o in 7/7 cell-attached patches (Fig. 4 *B*). Mean NP_o fell from 0.44±0.16 to 0.26±0.13 (40.2% decrease).

Rabbit CCT primary cultures were then pretreated with 0.25 μM thapsigargin for 30 min to deplete $[Ca^{2+}]_i$ stores. Thastrup et al. have shown that a similar pretreatment protocol abolishes intracellular Ca^{2+} transients elicited by IP_3 or GTP (21). Baseline mean NP_o for cell-attached patches in the presence of thapsigargin alone (0.38±0.11; n = 7) was not significantly different from control channel activity in principal cells grown without pretreatment (mean NP_o = 0.45±0.19; n = 55) (Figs. 2–4, 6, and 7). However, under these conditions, basolateral application of 0.5 μM PGE_2 no longer inhibited Na^+ channel activity suggesting an important modulatory role for intracellular Ca^{2+} (n = 7) (Fig. 5 *A* and *B*).

Ca^{2+}-dependent PKC mediates Na^+ channel inhibition by basolateral PGE_2. Despite the apparent involvement of intracellular Ca^{2+}, we have previously shown that Na^+ channel activity in principal cells of this rabbit CCT preparation is not inhibited when the cytoplasmic surface of excised inside-out patches are directly exposed to increasing free Ca^{2+} concentrations (10^{-9}–10^{-5} M) (3). One possible alternative mediator of an indirect effect of cytoplasmic Ca^{2+} on apical Na^+ permeability is Ca^{2+}-induced activation of calcium/phospholipid-dependent PKC (15, 22). PKC activation by phorbol esters does inhibit Na^+ reabsorption in rabbit CCT at a whole tissue level and in rat CCT at a single channel level (14, 23).

PKC was activated by applying 0.1 μM phorbol ester, 4β-phorbol 12-myristate 13-acetate (n = 5), or 10 μM synthetic diacylglycerol, 1-oleyl-2-acetylglycerol (n = 4) to the apical surface of rabbit CCT primary cultures. Mean NP_o for high selectivity Na^+ channels decreased by 44.1% (Fig. 6 *A* and *B*). However, basolateral application of 0.1 μM PMA (n = 10) or 10 μM OAG (n = 8) produced inconsistent effects on Na^+ channel activity (Fig. 7). In addition, exposure of the apical membrane to 0.1 μM inactive phorbol ester, 4α-phorbol, did not affect Na^+ channel activity (n = 4) (data not shown).

To determine if the basolateral effect of PGE_2 was mediated by apical PKC, we established cell-attached apical patches on primary cultures pretreated with 100 μM PKC antagonist, D-sphingosine, in the apical bath for 30 min (24). Baseline mean NP_o in the presence of D-sphingosine alone (0.41±0.13; n = 8) was not significantly different from control channel activity in principal cells grown without pretreatment (mean NP_o = 0.45±0.19; n = 55) (Figs. 2–4, 6, and 7). Subsequent exposure to 0.5 μM PGE_2 on the basolateral surface resulted in no consistent reduction in Na^+ channel activity (n = 8) (Fig. 8 *A* and *B*).

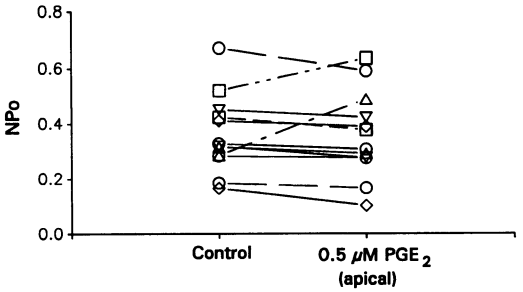

Figure 3. Effect of apical PGE_2 on high selectivity Na^+ channels. Na^+ channel activity, measured as NP_o is depicted before and after apical 0.5 μM PGE_2 exposure for 12 separate cell-attached patches. Channel inhibition, similar in magnitude to basolateral PGE_2 exposure, was not observed. Mean NP_o did not change (0.36±0.14 vs. 0.36±0.16).

Discussion

We have previously characterized Na^+-permeable channels in principal cell apical membranes of rabbit CCT primary cultures (16) chronically exposed to aldosterone (1.5 μM) and grown on permeable collagen supports (3, 17). The most frequently observed Na^+ channel (69% of successful patches) is mineralocorticoid-dependent and sensitive to amiloride. It has a low unitary conductance (4 pS), rectifying current-voltage (I-V) relationship, high Na^+ to K^+ permeability ratio (P_{Na}/P_K > 19:1), long mean open times (2.1±0.5 sec), and inward current (pipette to cell) at all applied potentials, V_{app} < +80 mV (cell interior with respect to pipette interior). At resting membrane potential, mean P_o was 0.38±0.17, and the mean number of observed channels was 2.4±0.7.

Several groups have demonstrated that basolateral PGE_2 inhibits Na^+ absorption in microperfused rabbit CCT (7–11). The present study is the first to use patch clamp methods to investigate at a single channel level the mechanism for inhibition of distal nephron Na^+ transport by exogenous PGE_2.

Apical membrane Na^+ channels are inhibited by basolateral membrane PGE_2 exposure. Exogenous application of 0.5 μM PGE_2 to the basolateral membrane bath resulted in a 46.5% inhibition of apical Na^+ channel activity (NP_o) in rabbit CCT primary cultures. In vitro perfusion studies of rabbit CCT show inhibition of lumen to bath ^{22}Na flux (J_{Na}) ranging from 20 to 80% with peritubular bath PGE_2 exposure (7–11). This large variability likely reflects the different experimental protocols used in these studies, such as the presence or absence of deoxycorticosterone, arginine vasopressin, (AVP), and meclofenate. We examined rabbit CCT cultures that had been chronically exposed to aldosterone, but not exogenous AVP or cyclooygenase inhibitors.

In addition, these previous microperfusion studies used PGE_2 concentrations ranging from 0.1 to 10 μM. PGE_2 in the

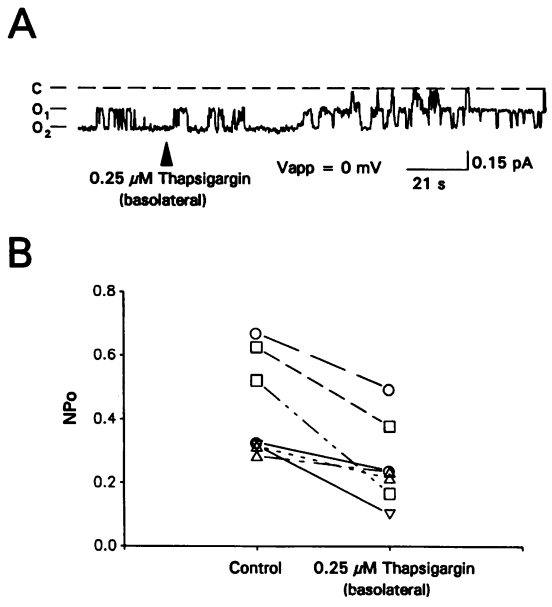

Figure 4. Effect of intracellular Ca^{2+} release on high selectivity Na^+ channels. (*A*) Cell-attached patch depicts the decrease in open channel activity observed after acute exposure to 0.25 μM thapsigargin in the basolateral bath. (*B*) Na^+ channel activity, measured as NP_o, is depicted before and after basolateral thapsigargin exposure for seven separate cell-attached patches. Relative channel inhibition, similar to basolateral PGE_2 exposure, was observed. Mean NP_o fell from 0.44±0.16 to 0.26±0.13 (40.2% decrease).

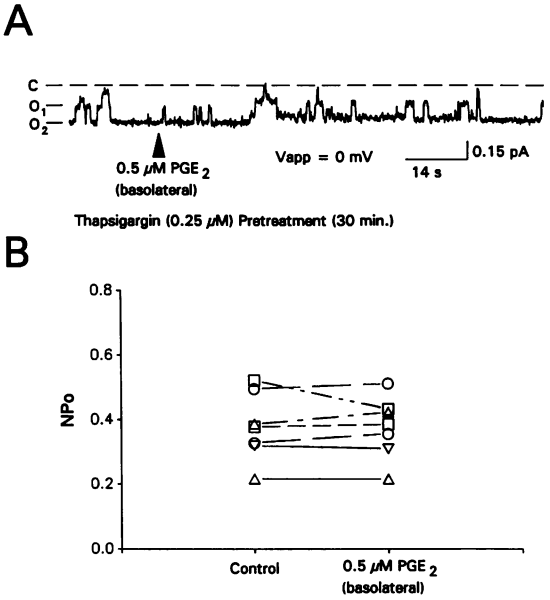

Figure 5. Effect of basolateral PGE_2 after depletion of intracellular Ca^{2+} stores. Primary cultures were pretreated with 0.25 μM thapsigargin for 30 min. (*A*) Cell-attached patch reflects no PGE_2-induced channel inhibition after $[Ca^{2+}]_i$ pool depletion. (*B*) Under these conditions, mean NP_o did not change in seven cell-attached patches with PGE_2 exposure (0.38±0.11 vs. 0.36±0.09).

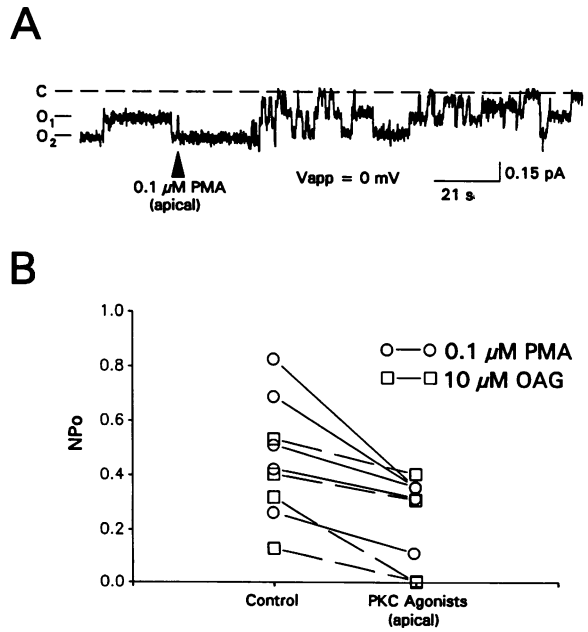

Figure 6. Effect of apical membrane exposure to PKC agonists. (*A*) Cell-attached patch reveals increased channel closures after apical application of 0.1 μM PMA. (*B*) Mean NP_o decreased from 0.45±0.22 to 0.25±0.16 (44.1% decrease) after PKC activation by apical application of either 0.1 μM PMA or 10 μM OAG.

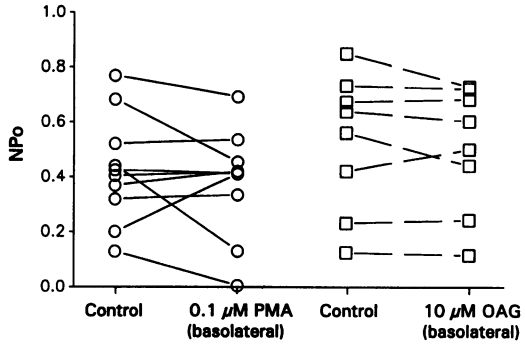

Figure 7. Effect of basolateral membrane exposure to PKC agonists. Minimal change in mean NP_o was observed after PKC activation by basolateral application of 0.1 μM PMA (0.43±0.20 to 0.38±0.19) or 10 μM OAG (0.53±0.25 vs. 0.50±0.23).

10–100 μM range causes 50% inhibition of rabbit cortical and medullary collecting tubule Na^+,K^+-ATPase activity (12, 25). However, since these latter concentrations are orders of magnitude higher than the PGE_2 concentration (0.5 μM) that inhibited the high selectivity Na^+ channel, we believe that our results largely reflect inhibition of apical Na^+ entry rather than the basolateral Na^+ exit step. The physiologic range of PGE_2 concentrations in the renal cortex is near 1 μM (26). Marver (12) demonstrated that inhibition of basolateral Na^+,K^+-ATPase activity by 10 μM PGE_2 occurs secondarily to a primary decrease in luminal Na^+ entry. PGE_2-induced decreases in Na^+,K^+-ATPase activity were abolished if apical Na^+ entry was maintained with monensin (a Na^+ ionophore). Stokes (13) also concluded that inhibition of Na^+ transport by PGE_2 occurs at the apical Na^+ entry step in isolated rabbit CCT, since PGE_2 increases lumen to bath K^+ flux similar to the effects of blocking sodium channels with amiloride, instead of decreasing K^+ flux as seen when Na^+, K^+-ATPase activity is blocked with ouabain.

We also observed a sidedness to the inhibitory responses of exogenous PGE_2. The decrease in apical, Na^+ channel NP_o was larger and more consistent when applied to the basolateral rather than the apical membrane of rabbit CCT cultures. Transepithelial potential difference decreases when PGE_2 is added to the peritubular but not luminal bath in isolated rabbit CCT (7, 8, 26). These observations are consistent with recent evidence, suggesting that multiple receptors for PGE_2 exist in the rabbit CCT basolateral membrane (11, 27, 28).

Release of intracellular Ca^{2+} stores by PGE_2 indirectly modulates Na^+ channel inhibition. It is well established that changes in intracellular Ca^{2+} modulate Na^+ transport in tight epithelia, including mammalian CCT (11, 15, 20, 22, 29, 30). PGE_2 but not $PGF_{2\alpha}$ stimulates a prompt and transient increase in cytosolic Ca^{2+} in rabbit CCT cells that is independent of extracellular Ca^{2+} (25, 29). This mobilization of $[Ca^{2+}]_i$ stores appears to be caused by inositolpolyphosphate hydrolysis resulting from basolateral PGE_2 receptor coupling to phospholipase C. The $K_{0.5}$ of PGE_2 for this process is 0.6 μM, similar to the concentration we applied (0.5 μM).

In our rabbit CCT cultures, the onset of Na^+ channel inhibition after 0.5-μM PGE_2 exposure occurred within 60 s, while in isolated rabbit CCT the peak elevation in $[Ca^{2+}]_i$ after 0.1 μM PGE_2 occurs within a similar time course (100 sec) (11). In contrast, $PGF_{2\alpha}$ exposure had no effect on high selectivity Na^+ channels. We have demonstrated that release of IP_3-sensitive $[Ca^{2+}]_i$ stores by acute thapsigargin exposure also inhibits apical Na^+ channel activity, while previous depletion of the $[Ca^{2+}]_i$ pool with thapsigargin pretreatment attenuates or eliminates the inhibitory effect of PGE_2. Pretreatment with thapsigargin alone did not affect baseline Na^+ channel activity in cell-attached patches, suggesting that neither thapsigargin itself nor earlier transient elevations in intracellular Ca^{2+} are responsible for the attenuation of basolateral PGE_2 effects seen with $[Ca^{2+}]_i$ depletion. Others have shown that an inhibitor of $[Ca^{2+}]_i$ pool discharge (TMB-8) in combination with low bath Ca^{2+} blocks the ability of PGE_2 to produce a sustained rise in $[Ca^{2+}]_i$ and reverses PGE_2-induced inhibition of J_{Na} in isolated rabbit CCT (11).

While elevations in $[Ca^{2+}]_i$ reduce apical Na^+ conductance (22, 29), several groups, including our own, have shown no direct affect on apical Na^+ channel activity when excised mammalian CCT principal cell patches are exposed to increasing cytoplasmic Ca^{2+} concentrations (3, 22). These results imply that PGE_2-induced elevation of $[Ca^{2+}]_i$ acts indirectly by modulating a secondary cascade of intracellular events, which, in turn, directly inhibits the apical Na^+ channel.

Basolateral PGE_2-induced inhibition of Na^+ channels is modulated by apical, Ca^{2+}-dependent PKC. Ca^{2+}/phospholipid-dependent PKC is a likely candidate for mediating the indirect effect of cytoplasmic Ca^{2+} on apical Na^+ permeability. Raising epithelial cell $[Ca^{2+}]_i$ with carbachol or the Ca^{2+} ionophore, A23187, results in translocation of PKC from the cytosolic to apical membrane fraction within 1 min (29, 31). Exposure of rabbit CCT primary cultures to the phorbol ester, PMA or synthetic diacylglycerol, OAG also promotes translocation

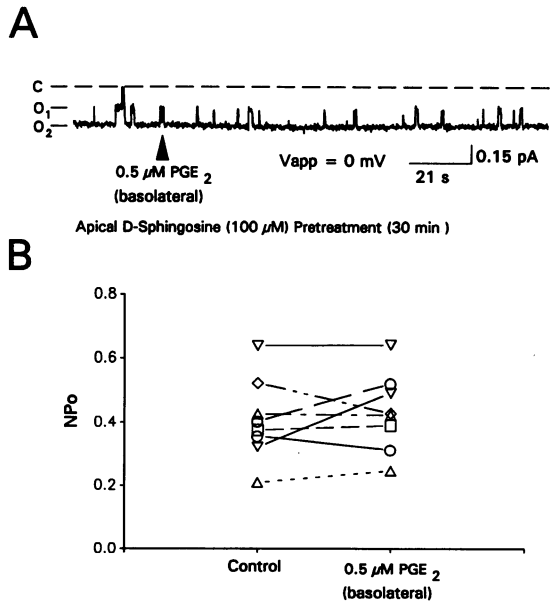

Figure 8. Effect of basolateral PGE_2 after apical exposure to PKC antagonist. (*A*) Single channel recording shows no inhibition of channel activity by PGE_2 in the presence of the PKC inhibitor, D-sphingosine in the apical bath. (*B*) Mean NP_o did not decrease with PGE_2 exposure after apical membrane PKC inhibition (0.41±0.13 vs. 0.43±0.12).

of PKC from the soluble to membrane fraction within minutes (32).

We have shown that apical exposure of rabbit CCT principal cells to PKC agonists (PMA or OAG) results in apical Na^+ channel inhibition. D-sphingosine is an endogenous PKC antagonist that competitively interacts with diacylglycerol and Ca^{2+}, and affects the regulatory domain of PKC (24). Apical pretreatment with this PKC inhibitor prevented the inhibitory effects of basolateral PGE_2 on the apical Na^+ channel. Exposure to D-sphingosine alone did not stimulate channel activity when compared to control patches. Since all experiments were performed under conditions (i.e., permeable supports; mineralocorticoids) that promote maximal apical sodium transport, we might not expect additional Na^+ channel stimulation by PKC inhibition. We have not examined the effects of D-sphingosine on rabbit CCT cultures grown in the absence of aldosterone.

In contrast to apical application, basolateral PMA or OAG yielded conflicting responses with much less consistent or dramatic channel inhibition. This sidedness (apical $\gg$ basolateral) of Na^+ transport inhibition by PMA and OAG has also been noted by ourselves and others in A6 distal nephron cells (15, 33). We propose that basolateral membrane-associated PKC, generated from PGE_2 receptor-mediated activation of phospholipase C, does not directly mediate apical Na^+ channel inhibition. Instead, inositol 1,4,5-triphosphate (IP_3)-mediated release of intracellular Ca^{2+}, the other limb of PGE_2-induced PLC activation, causes translocation of PKC to the apical membrane which then directly modulates apical Na^+ channel inhibition. These observations may explain the observation that basolateral exposure to PKC antagonists (staurosporine or H-7) alone inhibits rabbit CCT J_{Na} (11). Basolateral PKC is also known to feedback and inhibit the PGE_2 receptor (25).

Numerous examples of ion channels modulated by phosphorylation exist (15, 34). PKC activation by phorbol esters inhibits sodium transport in microperfused rabbit CCT (14), and Marver (12) has demonstrated that PGE_2-induced inhibition of apical Na^+ permeability in rabbit CCT occurs through stimulation of PKC. In the latter study, basolateral Na^+,K^+-ATPase activity falls secondary to decreased luminal Na^+ entry. PGE_2- and phorbol ester–induced decreases in Na^+,K^+-ATPase activity were reversed by staurosporine (a PKC inhibitor) or monensin (a Na^+ ionophore). We have previously shown that apical exposure to PMA or OAG decreases single Na^+ channel activity in A6 distal nephron cells, which is reversed by another PKC inhibitor, sphingosine. Cell-attached patches of freshly dissected rat CCT also show a decrease in open Na^+ channel density following treatment with phorbol esters (22).

Conclusion. Modulation of the high selectivity 4-pS Na^+ channel in rabbit cortical collecting tubules involves a complex system constituting a three-level hierarchy of regulation: First, interaction of circulating hormone (PGE_2) with principal cell basolateral membrane receptors stimulates basolateral effectors (phospholipase C); second, activation of intracellular signalling cascades (IP_3-sensitive Ca^{2+} pool release) transduce basolateral membrane signals to the apical membrane; and finally, the third level of regulation occurs at a molecular level within the apical membrane and involves second messenger (PKC) interaction with apical membrane-associated molecules including the Na^+ channel protein itself.

Acknowledgments

The authors are indebted to Christine L. Webster for her skillful technical assistance in the preparation of the rabbit cortical collecting tubule primary cultures.

Brian N. Ling was supported by Veterans Administration Merit Review Award and National Institutes of Health (NIH) Biomedical Research Support grant S07 RR05364. Douglas C. Eaton was supported by NIH Grant DK-37963.

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