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Research Article

We examined cell-attached patches on principal cells of primary cultured, rabbit cortical collecting tubules. Under basal conditions, apical 9-pS Cl(-)-selective channels were observed in 9% of patches (11/126), and number of channels times open probability (NP0) was 0.56 +/- 0.21. The channel had a linear current-voltage relationship, reversal potential (Erev) near resting membrane potential, a P0 (0.30-0.70) that was independent of voltage, and complicated kinetics (i.e., bursting) at hyperpolarized potentials. NP0 and channel frequency were increased after 30 min of basolateral exposure to 0.5 microM PGE2 (18/56), 10 microM forskolin (23/36), or 0.5 mM dibutyryl cyclic adenosine monophosphate (cAMP) (25/41). Increases in NP0 appeared to be mediated primarily through an increase in the number of observed channels per patch (N), not changes in P0. After these cAMP-increasing maneuvers, N was inconsistent with a uniform distribution of channels in the apical membrane ($P < 0.001$), but rather the channels appeared to be clustered in pairs. Apical 0.5 microM PGE2 (12/91), apical or basolateral 0.5 microM PGF2 alpha (8/110), or 0.25 microM thapsigargin (releaser of intracellular Ca2+ stores) (7/73) did not increase NP0 or channel frequency. Conclusions: (a) 9-pS Cl- channels provide a conductive pathway for apical membrane Cl- transport across principal cells. (b) Channel activation by basolateral PGE2 is mediated via a cAMP-, but not a Ca(2+)-dependent mechanism. (c) Apical channels […]

Prostaglandin E_2 Activates Clusters of Apical Cl^- Channels in Principal Cells via a Cyclic Adenosine Monophosphate–dependent Pathway

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Abstract

We examined cell-attached patches on principal cells of primary cultured, rabbit cortical collecting tubules. Under basal conditions, apical 9-pS Cl^--selective channels were observed in 9% of patches (11/126), and number of channels times open probability (NP_0) was 0.56±0.21. The channel had a linear current–voltage relationship, reversal potential (E_{rev}) near resting membrane potential, a P_0 (0.30–0.70) that was independent of voltage, and complicated kinetics (i.e., bursting) at hyperpolarized potentials. NP_0 and channel frequency were increased after 30 min of basolateral exposure to 0.5 μM PGE_2 (18/56), 10 μM forskolin (23/36), or 0.5 mM dibutyryl cyclic adenosine monophosphate (cAMP) (25/41). Increases in NP_0 appeared to be mediated primarily through an increase in the number of observed channels per patch (N), not changes in P_0. After these cAMP-increasing maneuvers, N was inconsistent with a uniform distribution of channels in the apical membrane ($P < 0.001$), but rather the channels appeared to be clustered in pairs. Apical 0.5 μM PGE_2 (12/91), apical or basolateral 0.5 μM $PGF_{2\alpha}$ (8/110), or 0.25 μM thapsigargin (releaser of intracellular Ca^{2+} stores) (7/73) did not increase NP_0 or channel frequency. Conclusions: (*a*) 9-pS Cl^- channels provide a conductive pathway for apical membrane Cl^- transport across principal cells. (*b*) Channel activation by basolateral PGE_2 is mediated via a cAMP-, but not a Ca^{2+}-dependent mechanism. (*c*) Apical channels are clustered in pairs. (*d*) With its low baseline frequency and E_{rev} near resting membrane potential, this channel would not contribute significantly to transcellular Cl^- flux under basal conditions. (*e*) However, cAMP-producing agonists (i.e., PGE_2, arginine vasopressin) would increase apical Cl^- transport with the direction determined by the apical membrane potential. (*J. Clin. Invest.* 1994. 93:829–837.) Key words: adenyl cyclase • Cl^- conductance • cortical collecting tubule • eicosanoids • patch clamp

Introduction

The principal cell of the mammalian cortical collecting tubule (CCT)[1] is the primary site for hormonal modulation of NaCl reabsorption. The mechanism for Na^+ reabsorption is known to involve apical Na^+ channels and basolateral Na^+/K^+-ATPase (1), but the mechanism(s) for Cl^- reabsorption across the mammalian CCT has (have) been less well characterized. Recent reviews have concluded that renal Cl^- transport across the mammalian CCT occurs primarily through a voltage-mediated, paracellular shunt pathway, and that a transcellular pathway across principal cells contributes little to transepithelial Cl^- permeability (2–4). Regardless, Hanley et al. (5) have demonstrated that microperfused rabbit CCT can reduce luminal fluid Cl^- concentration to ≤ 4 meq/liter in the presence of mineralocorticoid-stimulated Na^+ reabsorption. Under their experimental conditions, if passive paracellular Cl^- reabsorption were the only pathway present, then the Nernst equation would predict that luminal Cl^- concentration should only be reduced to 10 meq/liter. Other evidence for an apical Cl^- entry pathway comes from intracellular microelectrode studies examining halide ion selectivities of rabbit principal cells (6) and microperfusion studies of ouabain-induced rabbit principal cell swelling (7). Apical exposure of cultured rabbit CCT principal cells to the Cl^- channel blocker, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), results in a small, but significant hyperpolarization consistent with the presence of an apical Cl^- conductance (8). Unfortunately, it is difficult to characterize the apical Cl^- conductance in isolated native CCT preparations, but patch clamp methods allow a way to determine the number and properties of individual apical Cl^- channels. Preliminary reports have documented the existence of low conductance Cl^- channels in the apical membrane of principal cells from primary cultured rabbit CCT, but how they are regulated has received little attention (9).

cAMP is one candidate for the intracellular signaling pathway that regulates apical Cl^- transport in the mammalian CCT. Elevation of intracellular cAMP increases conductive Cl^- reabsorption in isolated rabbit CCT (10). A variety of mechanisms might increase intracellular cAMP in CCT cells, but exposure to physiological concentrations of PGE_2 stimu-

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1. *Abbreviations used in this paper:* CCT, cortical collecting tubule; CFTR, cystic fibrosis transmembrane conductance regulator; db-cAMP, dibutyryl cyclic AMP; DIDS, diisocyanostilbene disulfonic acid; E_{rev}, reversal potential; EP, effector pathway; I-V, current-voltage (relationship); IP_3, inositol triphosphate; NMDG, *N*-methyl-D-glucamine; NP_0, number of channels times the open probability; NPPB, nitrophenylpropylaminobenzoic acid.

lates cAMP accumulation in freshly isolated and cultured rabbit CCT (11–15).

Sodium reabsorption across the mammalian CCT principal cell must be accompanied by either potassium secretion or chloride reabsorption or both. Human studies by Vasavattakul et al. (16) suggest that, under conditions of K^+ depletion, aldosterone can function as a Na^+- and Cl^--retaining hormone, instead of the usual Na^+-reabsorbing and K^+-secreting hormone. We have previously applied patch clamp technology to principal cell apical membranes of rabbit CCT primary cultures to characterize both the amiloride-sensitive, 4-pS Na^+ channel and the Ba^{2+}-sensitive, 13-pS K^+ channel responsible for aldosterone-dependent Na^+ reabsorption and K^+ secretion, respectively (1, 17). In the present study, we examined the role of principal cell apical conductive pathways in Cl^- transport across aldosterone-stimulated rabbit CCT primary cultures. The physiologic roles of PGE_2 and the cAMP-dependent signaling cascade in the regulation of apical Cl^- channels were also examined.

Methods

Preparation of rabbit CCT primary cultures. Renal cortices were separated from New Zealand white rabbit (1–2 kg) kidneys and incubated for 60 min 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 type I collagenase (Sigma Chemical Co., St. Louis, MO), and 4 ml of RK-1 medium (Irvine Scientific, Santa Ana, CA). RK-1 medium contained 50% Ham's F-12 (by volume) and 50% Dulbecco's modified Eagle's medium (high bicarbonate and glucose) supplemented with 1.5 μM aldosterone, 5 mg/liter transferrin, 5 mg/liter insulin, 0.05 μM sodium selenite, and 0.05 μM hydrocortisone, pH 7.4. After high-speed centrifugation in a Percoll density gradient, rabbit CCT fragments in the lower part of the top band were incubated in RK-1 medium at 37°C and gassed with 4% CO_2 in air. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were present for the first 24–48 h. In 4–7 d the culture monolayer reached confluency (1, 17, 18). On days 5–10, patch clamp experiments were performed at 37°C using a model TC-202 temperature controller and model PDMI-2 open perfusion micro-incubator (Medical Systems Corp., Great Neck, NY).

Rabbit CCT fragments were plated at confluent density on permeable, collagen-coated Millipore-CM filters (Millipore Corp., Bedford, MA) attached to the bottoms of small lucite rings (1, 17–20). This preparation allowed patch pipette access to the apical membrane, and control of the apical and basolateral bath compositions. We have previously shown that the transepithelial electrical resistance and transepithelial voltage for this preparation (1) are very close to the measurements made by O'Neil and Sansom (21) in freshly isolated and perfused rabbit CCT. The more abundant principal cells are polygonal in shape, while the minority cell type (< 20% of the cell population) are round and form conglomerates. These minority cells have been identified as intercalated cells by luminal binding of fluorescently-labeled peanut lectin (22). Also, as previously described these two cell types are easily visualized and distinguished morphologically with our Hoffman modulation optics (Modulation Optics, Inc., Greenvale, NY).

Patch clamp recording and analysis. Cell-attached patches were obtained on the principal cells of rabbit CCT primary culture monolayers as previously described (1, 17–20). Patch pipette and extracellular bath solutions consisted of a physiologic saline (in 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. Unitary channel events were measured with a List EPC-7 patch clamp (Medical Systems Corp.), digitized by a DAS 601 pulse code modulator (Dagan Corp., Minneapolis, MN) and recorded on a SL-HF860D video cassette recorder (Sony Corp. of America, Park Ridge, NJ). Data acquisition utilized a 902LPF 8-pole Bessel filter (Frequency Devices Inc., Haverill, MA), TL-2 acquisition hardware and Axotape software (Axon Instruments Inc., Burlingame, CA).

The voltage convention ($-V_{pipet}$) represents the negative applied potential through the cell-attached patch pipette ($-V_{pipet} = 0$ mV represents zero applied potential; i.e., resting apical membrane potential). Values are expressed as the cell interior potential with respect to the patch pipette interior (i.e., negative values = apical hyperpolarization; positive values = apical depolarization). Outward current (cell to pipette) is represented as upward transitions in single channel records.

Analysis of data was performed on a model 486SX computer (Mitsuba Southeast Inc., Norcross, GA) utilizing locally- and commercially-developed software (1, 17–20). As a measure of channel activity, number of channels times the open probability (NP_0) was calculated from the expression:

$$NP_0 = \sum_{n=0}^{N} \frac{n \cdot t_n}{T}$$

T was the total record time, n was the number of channels open and t_n was the record time during which n channels are open. Therefore, NP_0, can be calculated without making assumptions about the total number of channels in a patch or the open probability of a single channel. The number of functional channels (N) in each patch were estimated by observing the number of peaks detected on current-amplitude histograms. The probability that any one channel was open (P_0) was calculated from the expression:

$$P_0 = (\sum_{n=1}^{N} P_n)/N$$

P_n, the probability that n channels are open, was calculated as the amount of time in the open state divided by the total record time for each unitary current level. Summation of P_n's for each level were then divided by N. The assumptions made were that the channels are independent and identical, that n channels were open when the current is between $(n - 1/2)i$ and $(n + 1/2)i$ where i is the unit current, and that estimations of N were correct.

Statistics. NP_0 is reported as mean±1 SD; the number of channels per patch, N, is reported as mean±1 SE. Certain cell-attached patch experiments were conducted in a paired fashion; data from each patch membrane served as the control for an experimental manipulation. In this case, the average change in NP_0 for a group of patches, compared before and after an experimental manipulation, was analyzed using a paired t-test or ANOVA for multiple comparisons (23). The normality of the distribution of channels per patch was tested using a Kolmogorov-Smirnov normality test (24). The goodness of the fit of the model for channel clustering data was determined using a reduced χ^2 test. The effect of the different treatments on the number of channels per patch was compared using a Kruskal-Wallis ANOVA and Dunn's test (24). Statistical analysis was performed using SigmaStat (Jandel Scientific, Corte Madera, CA).

Chemicals. PGE_2 and $PGF_{2\alpha}$, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), forskolin, N^6,2'-*O*-dibutyryladenosine 3',5'-cyclic monophosphate (db-cAMP), and thapsigargin (Calbiochem Corp., La Jolla, CA or Sigma Chemical Co.) were of the highest commercial grade available. NPPB was a gift from SmithKline Beecham Pharmaceuticals, Philadelphia, PA. Appropriate solvent vehicles were added to control baths and by themselves caused no change in Cl^- channel activity.

Results

Apical membranes contain 9-pS, Cl^--selective channels. In cell-attached patches of principal cell apical membranes in rabbit CCT primary cultures, small outward current events were identified at depolarized apical membrane potentials ($-V_{pipet} > 0$ mV), with a reversal potential (E_{rev}) very near resting membrane potential ($-V_{pipet} = 0$ mV) (Fig. 1 *A*).

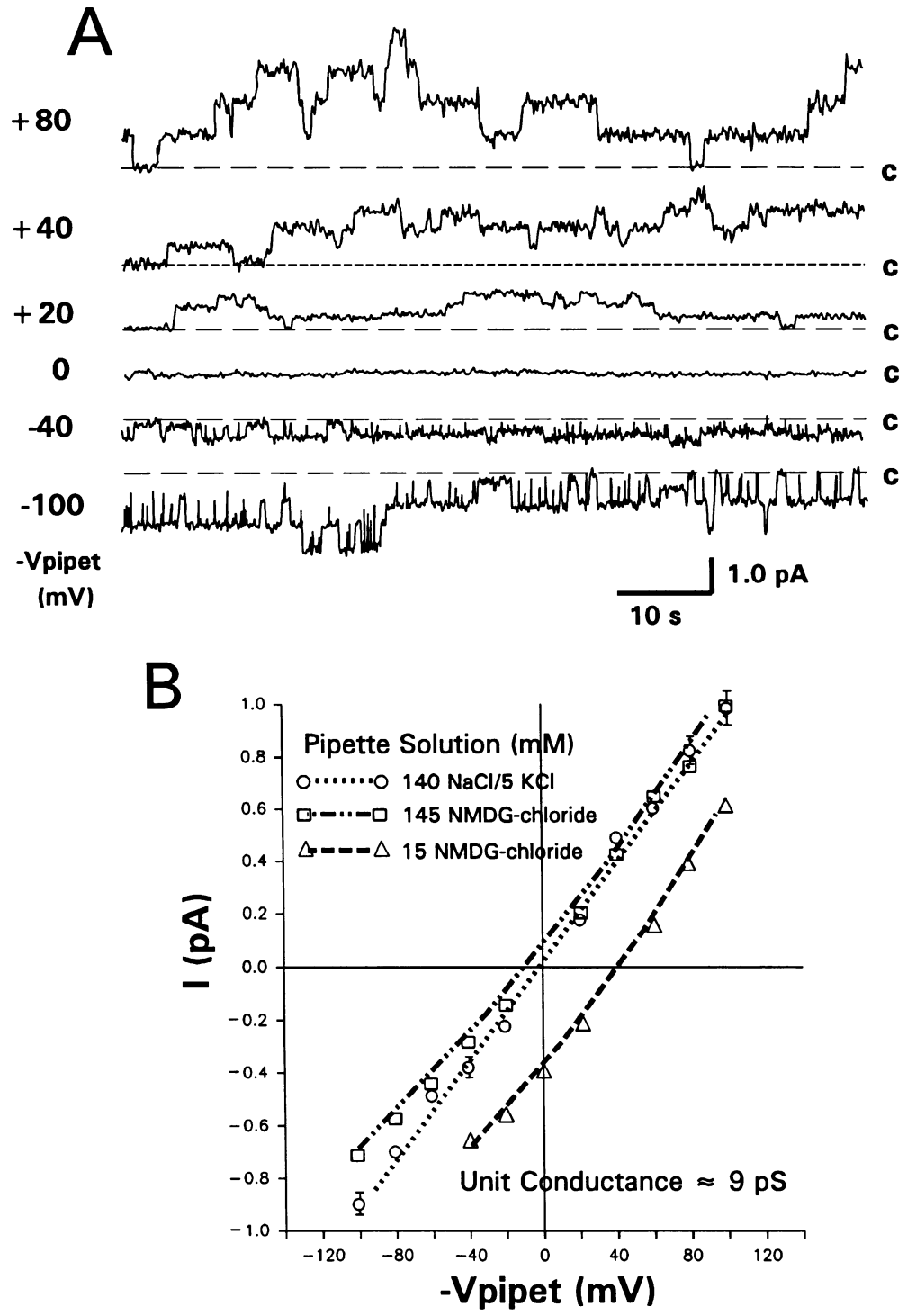

Figure 1. Low-conductance Cl^- channel events in rabbit CCT primary cultures. (*A*) Cell-attached patches of principal cell apical membranes reveal small inward current events at hyperpolarized pipette potentials and an E_{rev} very near resting apical membrane potential ($-V_{pipet} = 0$ mV). Complicated channel kinetics were noted with longer open times at depolarizing potentials and more rapid, "flickery" events at hyperpolarized potentials. Fc = 1 KHz, sampling = 5 KHz, and software filtering = 150 Hz. (*B*) I-V relationship. Mean channel amplitude for cell-attached patches is plotted vs. negative applied potential ($-V_{pipet}$). I-V curve with 140 mM NaCl/5 mM KCl in the pipettes was linear and E_{rev} was $\approx$ 0 mV (○; n = 11). Unit conductance was 9.2±0.8 pS. E_{rev} did not shift when intrapipette cations (Na^+ and K^+) were replaced with 145 mM NMDG chloride (□; n = 4). E_{rev} shifted to more depolarized cell potentials when intrapipette Cl^- was reduced to 15 mM (△; n = 4).

Mean channel amplitude in cell-attached patches was plotted against negative applied potential ($-V_{pipet}$) to create a current-voltage (I-V) relationship (Fig. 1 *B*). The I-V curve with 140 mM NaCl/5 mM KCl in both the pipette and extracellular bath solutions was linear and E_{rev} was $\approx$ 0 mV (○; n = 11). Unitary conductance derived from the slope of the best-fit linear regression line was 9.2±0.8 pS. E_{rev} did not shift when intrapipette cations (Na^+ and K^+) were replaced with 145 mM *N*-methyl-D-glucamine (NMDG) chloride (□; n = 4). However, when intrapipette Cl^- was reduced to 15 mM by partial replacement of NaCl with Na gluconate (△; n = 4), E_{rev} shifted to more depolarized cell potentials ($\approx$ +45 mV). These latter two maneuvers are consistent with a Cl^--selective apical membrane conductance.

Kinetics of the 9-pS Cl^- channel. 9-pS Cl^- channel activity was observed in 11 of 126 cell-attached patches. Mean NP_0 was 0.56±0.21 and the number of functional channels (N) per patch equaled 1.5±0.3 based on current-amplitude histograms (n = 11). In the four patches with only one observable channel level, the mean P_0 was 0.43±0.08. Time-interval histograms derived from the open and closed event durations of these four single-channel patches showed one open and two closed states,

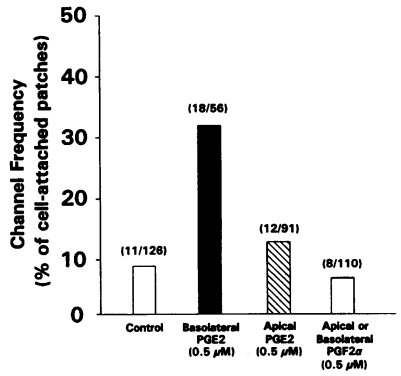

Figure 2. Basolateral PGE_2 pretreatment increases the frequency of 9-pS Cl^- channels. The percentage of apical cell-attached patches containing observable 9-pS Cl^- channel activity was 9% under unstimulated basal conditions (control). Channel frequency increased to 32% after pretreatment with basolateral PGE_2. Apical PGE_2 (n = 12), or either apical or basolateral $PGF_{2\alpha}$ (n = 8) pretreatment had no effect on Cl^- channel frequency or activity.

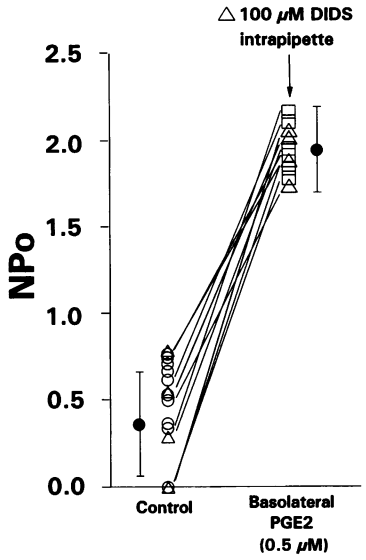

Figure 4. Cl^- channel activation by PGE_2 is DIDS-insensitive. NP_0 is plotted for cell-attached patches under baseline conditions (○; n = 18). After acute exposure to basolateral 0.5 μM PGE_2 for 5 min, channel activity consistently increased (□; n = 9). With 100 μM DIDS in the pipette solution, NP_0 still increased with basolateral PGE_2 (△; n = 4).

respectively. Single-channel recordings demonstrated a complicated kinetic pattern, with rapid, "flickery" open events at hyperpolarized potentials and longer open times noted at depolarizing potentials (Fig. 1 *A*). The mean open time increased from 93±45 ms at $-V_{pipet}$ = −100 mV to 786±106 ms at $-V_{pipet}$ = +100 mV. In contrast, the mean closed times did not vary with applied potential; the shorter closed duration was 12.9±1.0 ms and the longer closed duration was 3.37±1.08 s.

In the 115 out of 126 patches that did not contain basal Cl^- channel activity, we often observed amiloride-sensitive, 4-pS Na^+ channel (n = 72) and Ba^{2+}-sensitive, 13-pS K^+ channel (n = 54) activity. Our previous studies indicate that these latter two channels in principal cell apical membranes are responsible for physiologic, mineralocorticoid-dependent Na^+ reabsorption and K^+ secretion, respectively (1, 17).

9-pS Cl^- channel activation by basolateral PGE_2. Basolateral PGE_2 in micromolar concentrations stimulates cAMP accumulation in freshly isolated and cultured rabbit CCT (12–14) and elevations in intracellular cAMP increase conductive Cl^- reabsorption in isolated rabbit CCT (3, 10). Under basal conditions, 9-pS Cl^- channel activity was observed in only 9% (11/126) of apical, principal cell-attached patches on rabbit CCT primary cultures (Fig. 2). However, after rabbit CCT cultures were pretreated by adding PGE_2 to the basolateral bath (0.5 μM final concentration) for 30 min, channel frequency increased to 32% (18/56) of cell-attached patches. Mean NP_0 was 5.4±1.1 and mean P_0 was 0.63±0.10 after basolateral PGE_2 pretreatment (n = 18). In contrast, 30-min pretreatment with 0.5 μM PGE_2 added to the apical bath (12/91 patches), or 0.5 μM $PGF_{2\alpha}$ added to either the apical (3/47 patches) or basolateral bath (5/63 patches) had no effect on Cl^- channel frequency or activity.

In other experiments, cell-attached patches were established on principal cells before PGE_2 was acutely added to the basolateral bath (0.5 μM final concentration). Fig. 3 depicts the marked increase in single Cl^- channel activity observed after 5 min of exposure to PGE_2. NP_0 was plotted for cell-attached patches under basal conditions (mean NP_0 = 0.36±0.32; ○; n = 18) (Fig. 4). Using patches as their own control, acute exposure to basolateral 0.5 μM PGE_2 for 5 min consistently increased channel activity (mean NP_0 = 1.95±0.27, P_0 = 0.53±0.21; □; n = 9) (Fig. 4). Three of the nine patches had no initial Cl^- channel activity before addition of PGE_2.

9-pS Cl^- channel is NPPB-sensitive and DIDS-insensitive. We also examined the effects of several "chloride channel blockers" on PGE_2-induced, Cl^- channel activity. When 100 μM DIDS was added to the cell-attached patch pipette solution, the baseline 9-pS Cl^- channel activity (mean NP_0 = 0.39±0.33; △; n = 4) was not different from control patches made without intrapipette DIDS (Fig. 4). Subsequent basolateral exposure to 0.5 μM PGE_2 for 5 min still consistently increased NP_0 in each patch despite the presence of DIDS (mean NP_0 = 1.92±0.14; △; n = 4) (Fig. 4).

In contrast, when pipettes were filled with 100 nM NPPB, no baseline 9-pS Cl^- channel activity was observed in cell-attached patches (n = 5). After 5 min of basolateral PGE_2 exposure, Cl^- channel activity (mean NP_0 = 0.11±0.07) was induced in only one of five cell-attached patches and was still less than the baseline activity observed in patches made without intrapipette NPPB.

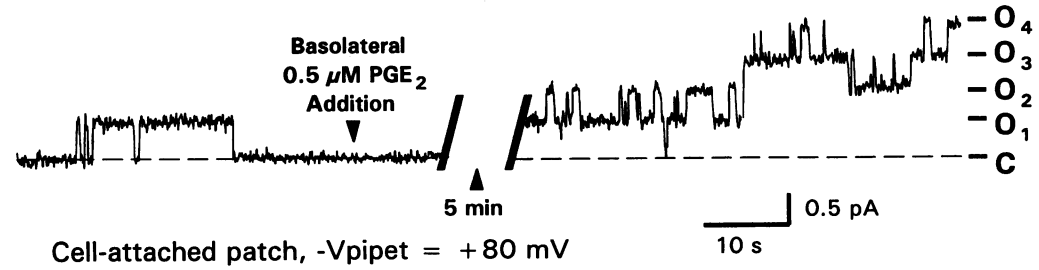

Figure 3. Acute basolateral PGE_2 exposure stimulates 9-pS Cl^- channel activity. After a cell-attached patch was established, PGE_2 was added to basolateral bath (0.5 μM final concentration). After 5 min of exposure a marked increase in channel activity was observed. Fc = 1 KHz, sampling = 5 KHz, software filtering = 100 Hz.

Cl^- channel activation by PGE_2 is mimicked by increases in intracellular cAMP, but Not Ca^{2+}. Isolated rabbit CCT principal cells contain PGE_2 receptors in the basolateral membrane capable of stimulating adenyl cyclase and promoting cAMP generation (12–15). We investigated the role of cAMP in modulating the basolateral PGE_2-induced stimulation of apical, 9-pS Cl^- channels. Cl^- channel frequency increased from 9% of patches made under basal conditions to 64% (23/36 cell-attached patches) after adenyl cyclase activation by basolateral pretreatment with 10 μM forskolin for 30 min (Fig. 5). Mean NP_0 was 6.0±0.8 and mean P_0 was 0.58±0.09 after forskolin pretreatment (n = 23). Similar increases in observed Cl^- channel frequency, 61% (25/41 cell-attached patches), were seen after 30 min of pretreatment with the cell-permeable cAMP analogue, 0.5 mM db-cAMP (Fig. 5). Mean NP_0 was 5.8±2.3 and mean P_0 was 0.72±0.15 after db-cAMP pretreatment (n = 25). After formation of cell-attached patches on untreated cells, we acutely increased intracellular cAMP levels using each patch as its own control. After 30-s basolateral exposure to 0.5 mM db-cAMP, a marked increase in Cl^- channel activity and channel number was observed in single-channel recordings (Fig. 6). Cl^- channel activity was consistently stimulated after acute, 1–5 min of exposure to 10 μM forskolin (mean NP_0 = 2.62±0.11, P_0 = 0.61±0.17; ▽; n = 5) or 0.5 mM db-cAMP (mean NP_0 = 3.55±0.18, P_0 = 0.55±0.14; ◇; n = 6) in the basolateral bath (Fig. 7). 6 of the 11 patches started with no observed Cl^- channel activity before forskolin or db-cAMP exposure.

Isolated and primary cultures of rabbit CCT also possess another basolateral PGE_2 receptor that stimulates phospholipase C, leading to inositol triphosphate (IP_3)-dependent release of intracellular Ca^{2+} stores (13, 14, 18). However, pretreatment with 0.25 μM thapsigargin, an IP_3-independent releaser of intracellular Ca^{2+} stores, for 5 min before patching did not affect baseline 9-pS Cl^- channel frequency (7/73 cell-attached patches) or activity (Fig. 5).

I-V relationship and halide selectivity of stimulated Cl^- channel. Basolateral PGE_2 or maneuvers to increase intracellular cAMP could stimulate the apical 9-pS Cl^- channel directly or indirectly by affecting other apical ion conductances and changing apical membrane electrochemical driving forces.

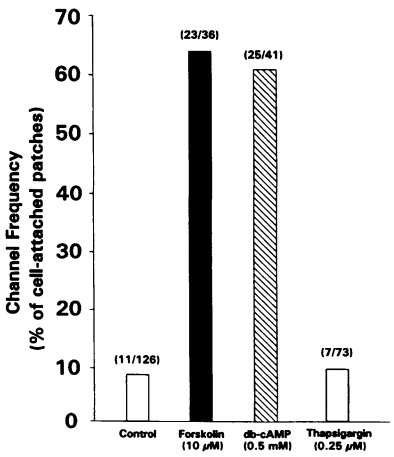

Figure 5. Activation of 9-pS Cl^- channel by basolateral PGE_2 is mimicked by cyclic AMP. Cell-attached patches show an increase in the frequency of channel activity to 64% after basolateral pretreatment with 10 μM forskolin (n = 23) and to 60% with 0.5 mM db-cAMP (n = 25). Exposure to 0.25 μM thapsigargin, an IP_3-independent releaser of $[Ca^{2+}]_i$ stores, did not affect channel frequency in cell-attached patches (n = 7).

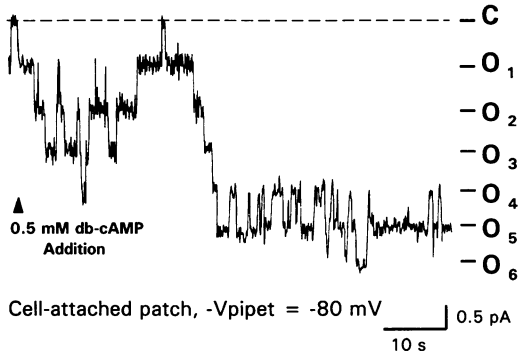

Figure 6. Cl^- channel activation is increased by a cell-permeable cAMP analogue. After cell-attached patch was established, db-cAMP was added to basolateral bath (0.5 mM final concentration). After 30 s of exposure a marked increase in channel activity was observed. Fc = 1 KHz, sampling = 5 KHz, software filtering = 100 Hz.

However, after 30 min of pretreatment with basolateral PGE_2 (○; n = 11), forskolin (□; n = 23) or db-cAMP (△; n = 25) the I-V relationship from cell-attached patches was unchanged, rather than shifted along the voltage axis as would be expected for a change in apical membrane potential (Fig. 8).

We also investigated the halide selectivity for this channel. Rabbit CCT cultures were pretreated with either basolateral PGE_2 (○) or db-cAMP (□) for 30 min before patching. Single-channel conductance was reduced to 1.3±0.8 pS in cell-attached patches when pipettes were filled with 145 mM NMDG-iodide (*dashed line*, n = 5; Fig. 9). Although the I-V curve shifted to the right in the presence of external NMDG-I^-, the exact E_{rev} potential could not be measured since baseline noise became excessive at depolarized potentials ($-V_{pipet} > 0$ mV). Conversely, unitary conductance (8.3±0.5 pS) and E_{rev} with pipettes containing 145 mM NMDG-bromide (*solid line*, n = 4; Fig. 9) was similar to patches made with 145 mM NMDG-chloride containing pipettes (Fig. 1 *B*). This indicates that channel selectivity for Cl^- was approximately equal to Br^-, but greater than I^-.

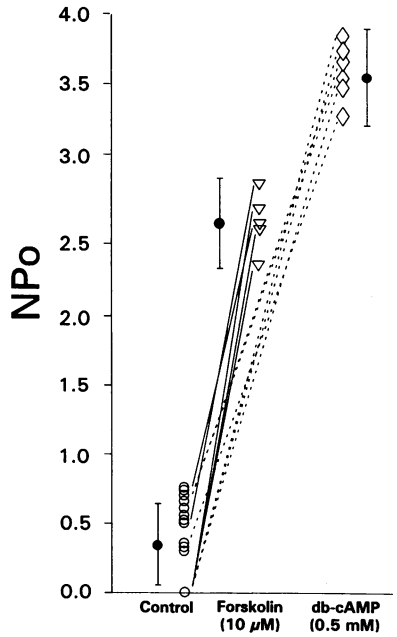

Figure 7. Effects of PGE_2 are mimicked by increases in cyclic AMP. The presence of channel activity was stimulated after 1–5 min of exposure to the adenyl cyclase activator, 10 μM forskolin (▽; n = 5) or the cell-permeable cAMP analogue, 0.5 mM db-cAMP (◇; n = 6).

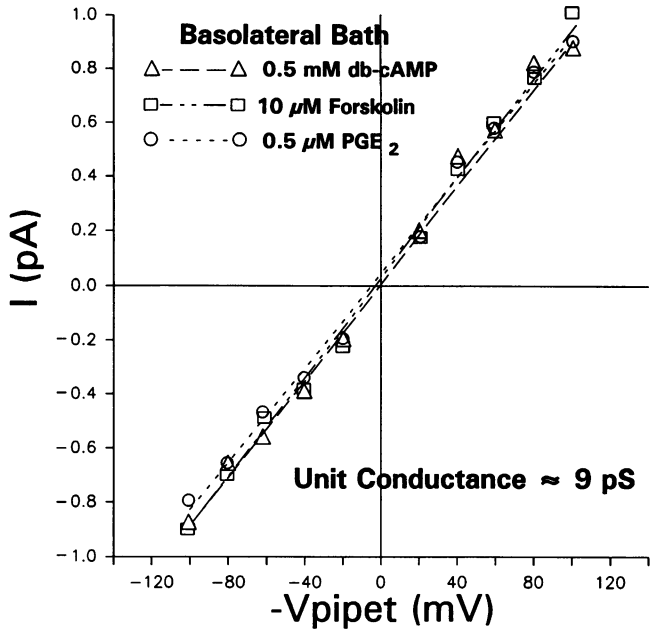

Figure 8. I-V relationship for Cl^- channel after cAMP-induced stimulation. After stimulation with basolateral PGE_2 (○; n = 11), forskolin (□; n = 23) or db-cAMP (△; n = 25) pretreatment, unitary conductance was unchanged and E_{rev} remained near 0 mV.

Apical membrane Cl^- channel density is increased by PGE_2, forskolin or db-cAMP. In 126 untreated patches, we observed 0.127±0.039 channels per patch. However, when rabbit CCT cultures were pretreated with 0.5 μM basolateral PGE_2 for 30 min, the channel density increased to 0.982±0.241 channels per patch (n = 56) which was significantly different than in untreated principal cells ($P < 0.05$). 30 min of pretreatment with 10 μM forskolin or 0.5 mM db-cAMP also increased channel density to 2.028±0.317 channels per patch (n = 36) for forskolin and 1.805±0.307 channels per patch (n = 41) for db-cAMP. The density of channels after forskolin or db-cAMP pretreatment was significantly different from the density in untreated cells ($P < 0.05$), but the density after forskolin pretreatment was not different from that after db-cAMP and the density after db-cAMP was not significantly different from that after PGE_2 ($P > 0.05$ for both). These increases in observed Cl^- channel density could be due to activation of "cryptic" channels already existing in the principal cell apical membrane and/or actual increases in the number of channels in the membrane.

9-pS Cl^- channels are clustered in the principal cell apical membrane. We plotted the distribution of observed 9-pS Cl^- channel levels (i.e., number of channels) per cell-attached patch for untreated cells and for cells pretreated as above with PGE_2, forskolin, or db-cAMP (Fig. 10; *filled bars*). Under all conditions, the histogram of the frequency of observed numbers of channels per patch was not a normal distribution ($P < 0.001$). For instance, in untreated cultures there were 115 patches with no activity and 6 patches with one channel per patch. These numbers would suggest a very low density of channels and therefore, patches with multiple channels should be quite rare. However, we observed 5 patches with two channels per patch: many more than would be expected for a normal distribution. The lack of a normal distribution implies that either the channels did not open independently of one another or that they were grouped within the apical membrane. An examination of the frequency histograms after db-cAMP or forskolin pretreatment suggests the latter hypothesis: that channels were clustered in pairs since patches with an even number of channels were substantially overrepresented.

A simple model of clustering and partial inactivation of apical membrane Cl^- channels predicted the channel densities we observed in all of the histograms (Fig. 10; *unfilled bars*). We assumed that Cl^- channel proteins were present in pairs and that these pairs were uniformly (normally) distributed in the apical membrane, but that there was a certain probability that either member of a pair was completely inactivated. This means that we would still observe odd numbers of functional channels in cell-attached patches if one member of a pair was inactivated and passed no detectable current. Indeed, this model yielded very good fits to our experimental data (untreated, forskolin-treated or db-cAMP-treated cells, $P < 0.05$; PGE_2-treated cells, $P < 0.07$).

Our model predicted that the mean number of actual channels per patch should be higher than the mean number of observed channels per patch since some of the channels would be inactivated. In 126 untreated patches, we observed 0.127±0.039 channels per patch, while the model predicted that there were actually 0.498±0.327 channels per patch with a probability of channel inactivation equal to 0.546. This implied that there should be many patches with no observable channels, and about the same number of patches with one functional channel (with one of the pair inactivated) as with two functional channels. Indeed, we observed 115 patches with no channels, 6 patches with one channel, and 5 patches with two channels. For rabbit CCT cultures pretreated with 0.5 μM

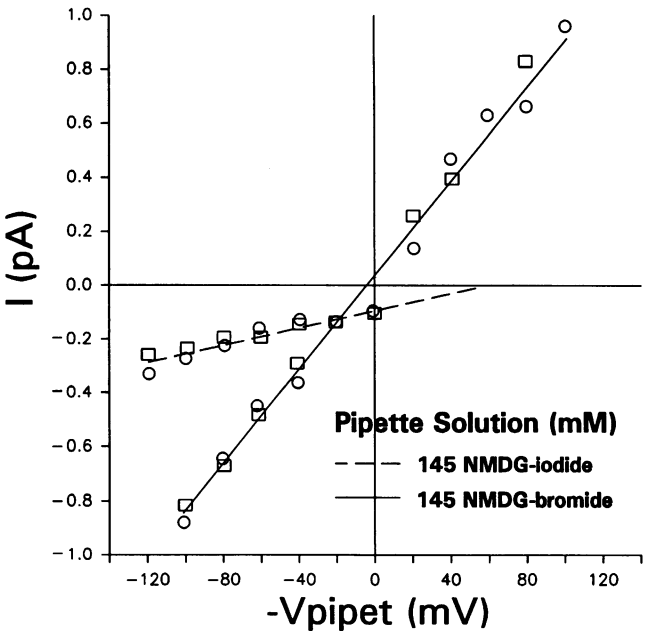

Figure 9. I-V relationship for stimulated Cl^- channels after external halide substitution. Rabbit CCT cultures were pretreated with basolateral PGE_2 (○) or db-cAMP (□) for 30 min before patching. Cell-attached patches with pipettes containing 145 mM NMDG-iodide show reduced inward current amplitude (*dashed line*; n = 5) reflected in a lower unitary conductance (1.3±0.8 pS). The I-V curve and E_{rev} shifted to the right in the presence of external NMDG-I^-. Conversely, unitary conductance (8.3±0.5 pS) and E_{rev} with pipettes containing 145 mM NMDG-bromide (*solid line*; n = 4) was similar to patches made with 145 mM NMDG-chloride containing pipettes (Fig. 1 *B*).

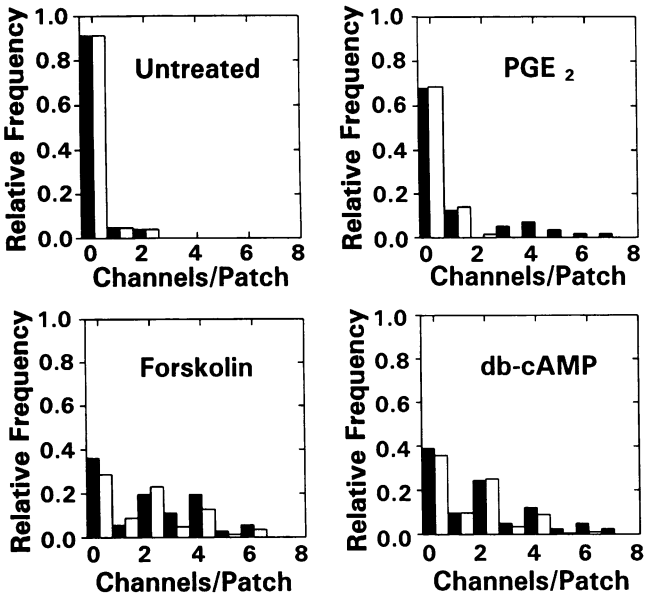

Figure 10. Apical membrane density of Cl^- channels increases with cAMP-induced stimulation. The distribution of the number of channels observed per cell-attached patch is plotted (*filled bars*) under control conditions (labeled "Untreated"), and after 30 min pretreatment with basolateral PGE_2, forskolin, or db-cAMP. The distributions are shifted to higher mean numbers of channels per patch by all three cAMP-producing maneuvers. The distributions cannot be fit by any simple binomial distribution. A model (*open bars*) in which it is assumed that apical Cl^- channels are paired, but that either channel has a certain probability of being completely inactivated predicts the observed data well (see Results).

basolateral PGE_2 for 30 min, the predicted channel protein density was 1.32±0.585 channels per patch with a probability of channel inactivation equal to 0.260. After 30 min of pretreatment with 10 μM forskolin or 0.5 mM db-cAMP, the predicted channel density was 1.31±1.20 channels per patch for forskolin and 0.954±0.985 channels per patch for db-cAMP with probabilities for inactivation of 0.277 and 0.280, respectively. The major difference between the untreated and treated patches was that the probability of channel inactivation was reduced from > 50% to < 30%. The probability of channel inactivation was similar for PGE_2, forskolin, and db-cAMP as might be expected for agents that all increase intracellular cAMP levels.

In addition to altering the probability of inactivation for Cl^- channels already existing within the apical membrane, these cAMP-producing agents also increased the number of channels (active or inactive) present within the membrane. In untreated rabbit CCT cultures, every fourth patch contained a pair of channels (with one of the pair inactivated $\approx$ 50% of the time). In PGE_2-, forskolin-, and db-cAMP-treated cultures, every other patch contained a pair of channels (with one of the pair inactivated $\approx$ 20% of the time).

Discussion

Principal cells of rabbit CCT primary cultures contain apical, 9-pS Cl^--selective channels. In cell-attached patches on principal cell apical membranes of mineralocorticoid-stimulated rabbit CCT primary cultures, we identified low conductance channels with a linear I-V relationship and a E_{rev} near the apical membrane resting potential. This 9-pS channel was open $\approx$ 50% of the time, but was observed in only 9% of patches. Ion substitution experiments confirm that this apical, 9-pS conductance is a highly selective Cl^- channel. Superdock and associates (9) have described a similar apical, 8-pS channel in primary cultures of immunodissected rabbit CCT cells. This 8-pS, Cl^--selective (Cl^-/cations > 20:1) channel also had a nonrectifying I-V curve, a high P_o (0.4–0.7) and was present in 12.5% of apical, excised inside-out patches.

Under unstimulated, basal conditions in the intact cell-attached configuration at 37°C, with physiologic extracellular saline composition and no applied potential we would expect this apical, 9-pS Cl^- channel to conduct little Cl^- current. This may explain earlier results from intracellular microelectrode studies that found no evidence of an apical Cl^- conductance in rabbit CCT principal cells (2).

Basolateral PGE_2 activates the apical 9-pS Cl^- channel via increases in cyclic AMP. Evidence indicates that multiple PGE_2 receptors (EP_1, EP_2, and EP_3) modulating different effector pathways, exist in the basolateral membrane of the mammalian CCT (13, 14, 18). While the actions of PGE_2 on Na^+, Ca^{2+}, and PO_4^{+2} reabsorption in the mammalian CCT have been investigated, little is known about PGE_2's effects on renal Cl^- transport (13, 18).

We found that the frequency of observed 9-pS Cl^- channel activity in cell-attached patches increased from 9% under basal conditions to 32% after pretreatment with 0.5 μM PGE_2 in the basolateral bath for 30 min. In addition, experiments using each patch as its own control showed that acute exposure to basolateral 0.5 μM PGE_2 alone consistently increased NP_o for this channel. The relevance of these observations is that physiologic concentrations of renal cortical PGE_2, the predominant CCT eicosanoid, are $\sim 10^{-6}$ M (11).

Basolateral PGE_2 in micromolar concentrations binds with EP_2 receptors and stimulates adenyl cyclase and cyclic AMP accumulation in freshly isolated and cultured rabbit CCT (12–15). Schuster and his co-worker (3, 10) reported that cAMP-induced Cl^- reabsorption in microperfused rabbit CCT was consistent with stimulation of an apical membrane Cl^- conductance. We found that increasing intracellular cAMP levels by pretreatment with an adenyl cyclase activator, forskolin, or a cell-permeable cAMP analogue, db-cAMP, mimicked the stimulatory effect of PGE_2 and increased 9-pS Cl^- channel frequency in cell-attached patches by $\approx$ 7-fold. Acute exposure to these cAMP-increasing agents also consistently increased NP_o within 5 min.

Isolated and primary cultures of rabbit CCT also possess basolateral EP_1 receptors linked to phospholipase C, which when activated result in IP_3-dependent discharge of intracellular Ca^{2+} stores (13, 18). However, we did not find any effect on 9-pS Cl^- channel activity with exposure to 0.25 μM thapsigargin. In this same rabbit CCT preparation, we have documented that similar exposure to thapsigargin inhibits apical, high-selectivity Na^+ channels through release of intracellular Ca^{2+} stores (18).

Lastly, isolated mammalian CCT also contain basolateral EP_3 receptors which inhibit adenyl cyclase and reduce intracellular cAMP production (12–14). However, Sonnenburg and Smith (12) have shown that this PGE_2-induced inhibition of adenyl cyclase disappears when rabbit CCT cells are cultured.

PGE_2, forskolin, and db-cAMP increase apical Cl^- channel density. Stimulation of total apical membrane Cl^- conductance (G_{Cl}) could occur through changes in apical channel den-

sity (N) and/or open probability (P_o) and/or unitary channel conductance (γ_{Cl}).

$$G_{Cl} = \gamma_{Cl} \cdot NP_0$$

Acute exposure to PGE_2, forskolin, or db-cAMP increased NP_o for the 9-pS Cl^- channel, sometimes within seconds. However, the open probability of channels in patches from untreated and acutely PGE_2-, forskolin-, or db-cAMP-treated cells were not significantly different. After longer pretreatment of rabbit CCT cultures with PGE_2, forskolin, or db-cAMP, the frequency of cell-attached patches with observed 9-pS Cl^- channel activity increased while the open probability again did not change from baseline. This suggests that both acute and extended exposure to elevated intracellular cAMP levels increases 9-pS Cl^- channel activity through an increase in apical membrane channel density, rather than a change in channel kinetics. However the distribution of these channels (i.e., number of channels/patch) in both untreated cells and in cells pretreated with PGE_2, forskolin, or db-cAMP cannot be described by a normal binomial distribution. The actual distributions imply that channels are clustered in pairs since patches with an even number of channels were substantially over-represented in the distributions.

Our model suggests that cAMP-producing agonists have two effects on 9-pS Cl^- channels: one is to switch nonfunctional, "cryptic" channels which already exist in the apical membrane into an active state; and the second is to increase the available number of channels (both active and inactive) present in the apical membrane. The latter effect suggests that new Cl^- channels are being inserted into the apical membrane in clusters. Consistent with a basic mechanism underlying cAMP-induced channel stimulation, the principal cell apical membrane densities for both Na^+ channels and water channels are also increased by cAMP, presumably through fusion of channel-containing submembranous vesicles (25, 26).

In the present study, I-V curves show that the unitary conductance (γ_{Cl}) for this 9-pS Cl^- channel was not altered by pretreatment with PGE_2, forskolin, or db-cAMP. The lack of I-V curve shift and therefore, change in E_{rev} with cAMP-producing maneuvers (i.e., basolateral PGE_2, forskolin or db-cAMP) also indicates that the apical membrane potential remains unchanged after Cl^- channel activation.

Role of apical Cl^- channels in the CCT principal cell. Because the E_{rev} for this 9 pS Cl^- channel is poised at apical resting membrane potential and the I-V relationship is linear, this Cl^- conductance will act to balance potential changes produced by apical Na^+ and K^+ conductances. In states of stimulated Na^+ reabsorption (e.g., extracellular volume depletion, increased mineralocorticoid states, etc.), apical membrane depolarization would result in apical Cl^- reabsorption via the Cl^- channel, maintaining a favorable electrochemical gradient for continued Na^+ reabsorption. Conversely, apical membrane hyperpolarization due to inhibition of Na^+ reabsorption (i.e., amiloride, volume overload, etc.) or stimulation of K^+ secretion (i.e., hyperkalemia) would be expected to promote Cl^- secretion. Favoring Cl^- secretion, the intracellular Cl^- activity for rabbit CCT principal cells is significantly elevated above electrochemical equilibrium (27, 28). Indeed, Wingo (29) has demonstrated active Cl^- secretion in microdissected, microperfused rabbit CCT after the animals were fed a K^+-rich diet and concluded that the rabbit CCT "possesses significant bidirectional Cl flux."

We and other groups have demonstrated that several hormones (i.e., aldosterone, arginine vasopressin and PGE_2) are important to the regulation of apical Na^+ reabsorption and K^+ secretion in the mammalian CCT (18, 20, 30–32). Na^+ reabsorption across the mammalian principal cell must be accompanied by either K^+ secretion or Cl^- reabsorption or both. Halperin and associates (16) have studied both distal nephron K^+ and Cl^- transport, in the presence of stimulated Na^+ reabsorption, in human subjects after extracellular fluid contraction or exogenous mineralocorticoid treatment. After dietary-induced K^+ depletion, Na^+ reabsorption was accompanied by a decrease in chloriuresis instead of the usual mineralocorticoid-induced increase in kaliuresis (16). Under these conditions kaliuresis increased when the urinary concentration of Cl^- was lowered. The authors proposed that the reduction in K^+ secretion "on the low-K^+ diet was due to an increased permeability for Cl^- in the luminal membrane of the CCD." When there is a K^+ deficit, "aldosterone could now be a NaCl-retaining hormone at some times (low intake of NaCl)," instead of the usual Na^+-reabsorbing and K^+-secreting hormone. The apical Cl^- channel we have characterized in this study provides a likely mechanism for this clinically observed process.

Exposure to physiologic concentrations of AVP (picomolar) initially stimulates both apical Na^+ entry and K^+ efflux via increases in principal cell cAMP levels (20, 31, 32). The ensuing increase in intracellular Na^+ secondarily activates basolateral Ca^{2+} entry via the Na^+/Ca^{2+} exchanger. This cAMP-dependent rise in intracellular Ca^{2+} activates apical membrane-bound, Ca^{2+}-dependent protein kinase C and leads to "feedback inhibition" of both apical, high selectivity Na^+ channels and secretory K^+ channels (20, 31, 33, 34). Activation of basolateral PGE_2 receptors in rabbit CCT principal cells also leads to a complex signaling response. PGE_2-induced increases in intracellular cAMP initially results in stimulation of apical Na^+ entry, which is then followed by "feedback inhibition" of apical Na^+ channel activity (31, 34). Direct release of IP_3-sensitive, intracellular Ca^{2+} stores in response to PGE_2 also inhibits apical Na^+ channels (18, 35). As discussed above, Cl^- reabsorption or secretion via the cAMP-sensitive, apical Cl^- channel would serve to balance the complex arginine vasopressin- or PGE_2-induced alterations in principal cell membrane potential that result from these cAMP-dependent effects on apical Na^+ and K^+ conductances.

Comparison of the 9-pS Cl^- channel in principal cells to cystic fibrosis transmembrane conductance regulator (CFTR). CFTR protein is the gene product which is linked to the defect in cAMP-mediated, apical epithelial Cl^- transport observed in cystic fibrosis patients (28, 36–39). Overexpression of the protein in a variety of cell types is associated with the appearance of a cAMP-sensitive Cl^- channel. The CFTR-expressed channel has a low unitary conductance (4.3–10.4 pS), linear or slightly rectified I-V relationship, halide selectivity of $Br^- \approx Cl^- > I^-$, and no voltage-dependence, and is stimulated by cAMP-producing agonists. Inward current events exhibit characteristic "flickery" openings at hyperpolarized potentials. Interestingly the 9-pS Cl^- channel we have observed in principal cells of primary cultured rabbit CCT possesses all of the above characteristics.

Gross and associates (8) found that apical exposure of cultured rabbit CCT principal cells to 100 nM NPPB, a chloride channel blocker, results in a minute, but significant hyperpolarization, consistent with the presence of a small apical Cl^- con-

ductance. We also found that the 9-pS renal Cl^- channel was blocked by 100 nM NPPB, but was insensitive to DIDS. CFTR-expressed Cl^- channels are similarly insensitive to stilbene disulfonates (36–39). However, sensitivity of CFTR-expressed Cl^- channels to NPPB has been variable; some studies showing no response (36) while Hasegawa et al. (38) observed inhibition with 1 mM, but not 0.1 mM NPPB. Also suggesting a possible link between the 9-pS renal Cl^- channel that we have described and CFTR, Breyer and associates (40) using PCR amplification have recently demonstrated the expression of CFTR mRNA in native rabbit CCT cells, rabbit CCT primary cultures, and a transformed rabbit CCT cell line.

Conclusions. In the principal cell apical membrane of rabbit CCT primary cultures we have characterized a 9-pS Cl^--selective channel which is stimulated by basolateral exposure to physiologic concentrations of PGE_2. Channel activation appears to be mediated via a cAMP-dependent signaling pathway and is not dependent on changes in intracellular Ca^{2+}. Frequency distributions of channel density suggest that pretreatment of rabbit CCT cultures with cAMP-producing agonists promotes activation of previously nonfunctional apical channels and apical membrane insertion of Cl^- channels in pairs. This 9-pS Cl^- conductance will act to balance potential changes produced by apical Na^+ and K^+ conductances. Vectorial apical Cl^- transport will be determined by the apical membrane potential (Cl^- secretion with hyperpolarization and Cl^- reabsorption with depolarization).

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