A Cl^- Channel Activated by Parathyroid Hormone in Rabbit Renal Proximal Tubule Cells

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Abstract

Previous data suggested an active Cl^- conductance in the renal proximal convoluted tubule, although single channel conductance and regulation were not found. We have investigated the presence and regulation of the Cl^- channel in proximal convoluted tubules by patch clamp analysis. The current-voltage relationship of whole cells with 130 mM NaCl in the pipette was nonlinear. The addition of 1-34 PTH (10^{-8} M), forskolin, or cAMP significantly increased whole cell Cl^- conductance. We found a single Cl^- channel in excised apical membranes possessing conductance of 33 picosiemens (pS) at positive and 22.5 pS at negative potential, which was blocked by 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (10^{-4} M) and was selective to Cl^- (Cl/Na = 10). The channel was activated by prolonged membrane depolarization, by a catalytic subunit of protein kinase A (PKA), or by purified kinase C (PKC), but not by Ca^{2+} (1 μM) inside the membrane. During cell-attached patch clamping, the channel was similarly activated by PTH, phorbol ester, or dibutyryl cAMP in a dose-dependent manner. To investigate second messenger contributions to the PTH-action, the PTH-evoked channels were modified further by the subsequent addition of several blockers of the second messengers. This suggested that PKA and PKC were involved in Cl^- channel activation. We therefore conclude that renal proximal convoluted tubule cells possess an apical Cl^- channel activated by PTH via the PKA and PKC pathways. (*J. Clin. Invest.* 1991. 88:735–742.) Key words: anion channel • epithelia • protein kinase A • protein kinase C

Introduction

The physiologic function of many epithelia depends on the reabsorption or secretion of fluid. In most epithelia, fluid movement is closely coupled to the transport of Na^+ and Cl^- ions. In fluid-reabsorbing renal proximal tubule epithelia, the energy needed for the net transport of salt is provided by Na/K-ATPase, located in the basolateral membrane. Chloride is believed to be taken up by the cell across the apical membrane by a co- or countertransport pathway, and its exit may be mediated by anion-selective channels in the basolateral membrane. On the other hand, fluid-secreting epithelia possess an apical Cl^- channel to secrete this anion (1). Although the presence of the basolateral Cl^- route has been suggested in many fluid-reabsorbing epithelia, available data by patch clamp analysis have suggested the absence of an anion selective channel in the basolateral membrane of rabbit proximal convoluted tubules (PCT)[1] (2). Rather, a Cl^- channel is found in the basolateral membrane of renal distal tubules (3) and in a number of cultured epithelia on their apical membrane (4–8).

The regulation of ion channels and their location are important in their physiologic function. PTH inhibits fluid transport and HCO_3^- absorption in this nephron segment (9). Most of the inhibitory effects seem to be due to suppression of the Cl^-/base exchanger with that of the parallel Na^+/H^+ antiporter (10). It is possible, however, that parathyroid hormone may also modify an apical anion channel, and inhibits its Cl^- reabsorption.

In general, peptide hormones bind to their membrane receptors and induce various second messengers. PTH is known to induce cAMP, a rise in intracellular free calcium and diacylglyceride leading to the activation of protein kinase C (PKC) (11). The modifications of ion channels by these second messengers have been well demonstrated. For instance, activation of the Cl^- channel by protein kinase A (PKA) via cAMP and by PKC via diacylglyceride-induced membrane phosphorylation has been reported in airway epithelia (12, 13). Thus PTH may similarly modify a Cl^- channel in PCT. Based on these assumptions, we herein explored the presence of an apical Cl^- channel in renal proximal convoluted tubule cells and the regulation of this channel by these second messengers of parathyroid hormone.

Methods

Culture. The procedure for culture of isolated tubules has been described previously (14). New Zealand White male rabbits (wt 1.0–1.5 kg) were used in this study. Nephron segments were isolated by dissection without collagenase from fresh renal slices on an ice cold platform. The instruments, tissues, and solute were aseptic or sterile. Three to five pieces of proximal convoluted tubules (S_1,S_2 segment) were transported and implanted, using a 5-μl pipette, into a 4-well multidish (Nunc, Roskilde, Denmark). The tubules were incubated at 37°C for 15 min to achieve attachment. Serum-supplemented medium composed of DME:HAMF12 (1:1) with 10 mM Hepes, 5 mM glutamine, 10% FCS, and antibiotics (20 mg/dl penicillin and 15 mg/dl streptomycin) were added next. They were incubated in a humidified 5% CO_2/air

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1. *Abbreviations used in this paper:* DiC8, dioctanoylglycerol; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; Fr, fraction: H-8, *N*-2-(methylamino)-ethyl-5-isoquinoline-sulfonamide; H-9, *N*-(2-aminoethyl)-5-isoquinolinesulfonamide; ML-9, 1-(5-chloronaphthalene-1-sulfonoyl)-1H-hexahydroazepine; PCT, proximal convoluted tubules; PKA, protein kinase A; PKC, protein kinase C.

incubator at 37°C until outgrowth of the epithelial cells was achieved. Proliferation of the cells was maintained in hormone-enriched media that included the antibiotics, 5 μg/ml human transferrin, 5×10^{-8} M hydrocortisone, and 5 μg/ml insulin in DME:HAMF12 media. 2–3 wk after implantation, the cells were subcultured into a dish (35 mm; Corning Glass Works, Corning, NY). Proliferation was again maintained in hormonally defined media for a week and then used for patch clamp recordings. Since proximal cells may be electrically coupled to each other, we isolated single cells for the measurement of whole cell current. For this purpose, we treated the monolayer with 0.1 mM EGTA and protease (0.1 mg/ml) to obtain isolated cells. They were washed with media containing 10% FCS and then incubated for 2 h to enhance attachment to the dish. In cell-attached and excised patch testing, we patched an apical membrane of the monolayer, and sometimes briefly treated it with the above solution for enhancement of giga-seal.

Patch clamp experiments. Patch clamp recordings were carried out according to the method described in previous papers (15, 16). Patch pipettes were made from hematocrit capillaries (1.5 mm OD, 1.2 mm i.d.; Terumo Corp., Tokyo, Japan) and fire polished. The usual pipette resistances were 5–10 MΩ. Connection to the amplifier headstage and the bath electrode was made with a Ag-AgCl pellet. All electrical potential differences were expressed as reference to the bath. Currents were recorded at room temperature (20–24°C) with an EPC-7 (List-Electronic, West Germany) or a patch clamp amplifier (SEN 2200; Nihon Koden, Inc., Tokyo, Japan) and stored on a recorder (DAT-200; Sony, Tokyo, Japan). Records were sampled at 2,000 points/s and analyzed by computer (MBC AX and Axon ver. 5.5.1; Sanyo). Current-voltage relations were plotted to determine the reversal potential for current. The junctional potential evoked by changes in bath composition was calculated using the Henderson approximation of the Nernst-Planck equation. Relative ion permeabilities were calculated from the Goldman-Hodgkin-Katz voltage equation after correction for junctional potentials. nPo was determined from the summation of the open probability of each level of the channel activity in the patch. Mean nPo was determined from 10-s recordings initiated at the given time or after the activation of the channel, where n refers to the number of the channels in the patch membrane. Representative data of the currents were filtered at 1 kHz and plotted by an X-Y recorder.

The cells were bathed in a filtered solution of 125 mM NaCl (or CsCl), 1.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 10 mM Hepes (pH 7.4, external solution). The pipette contained a filtered solution of 130 mM NaCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM EGTA, 1 mM ATP, and 10 mM Hepes (pCa 8.0, pH 7.2 by Tris base, internal solution). For excised and cell-attached patch clamps, the NaCl in some experiments was substituted with CsCl in both the internal and the external solutions. $CaCl_2$ was changed to 3 mM with the internal solution to yield 500 nM free calcium. Reagents dissolved in an appropriate internal or external solution were added by another glass pipette located near the patch clamped cell.

Purification of protein kinase C. Purification of PKC was performed by affinity chromatography as described previously (17) with minor modifications. Briefly, the renal cortex from New Zealand White rabbits was minced and homogenized by 10 strokes of a Potter-Elvejem Tefron homogenizer at 1,500 rpm. The homogenate was centrifuged at 10,000 *g*. The supernatant contained the PKC activity. The coupling of cyanogen bromide-activated Sepharose 4B to *N*-(2-aminoethyl)-5-isoquinolinesulfonamide (H-9) was performed as described previously (17).[2] The resin was washed with distilled water, followed by 200 ml of 25 mM Tris-HCl (pH 7.0) containing 2 mM EGTA, 0.001% leupepsin, and 50 mM 2-mercaptoethanol (buffer A). We made another H-9–free resin as a reference. The supernatant from the above containing the PKC activity was applied to the H-9–Sepharose column (3 × 0.6 cm) equilibrated with buffer A containing 0.1 M NaCl. The elution buffer contained L-arginine with a linear gradient concentration (0–1.5 M) and flowed at a rate of 15 ml/h. 2-ml fractions (Fr) were collected, and Fr 25–35 were saved as the PKC-rich fractions, as found previously (17). The test substances, pooled PKC-poor fractions (Fr 0–20), pooled PKC-rich fractions, and a reference fraction (Fr 25–35 through H-9–free column) were applied to a DEAE column (20 × 1.5 cm; Jasco Inc., Tokyo, Japan), and we found a single peak in the PKC-rich fraction alone by high performance chromatography at flow rate of 1 ml/min buffer A. PKC activity was assayed by measuring ^{32}P incorporation from [^{32}P]ATP into lysine-rich histone from calf thymus (type IIIS, Sigma Chemical Co., St. Louis, MO) in the presence of Ca^{2+}, phosphatidylserine, and diolein as reported by others (18). The reaction mixture (0.25 ml) was composed of 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM $CaCl_2$, 0.3 mg/ml histone, 7 μM labeled [^{32}P]ATP, 40 μg/ml phosphatidylserine, and 2 μg/ml diolein. The mean calculated phosphorylation by the PKC-rich fraction in the presence of 500 nM Ca^{2+} and 1 nM dioctanoylglycerol (DiC8) was 127 pmol/ml per 5 min, compared to that by the reference fraction (n = 4). When using a nominally Ca^{2+}-free reaction mixture, the phosphorylation by the PKC-rich fraction was not different than the reference of PKC-poor fractions.

Materials and statistics. Rp-cAMPS, a membrane-permeable, specific competitive blocker of protein kinase A (19), was a gift from Sandoz Pharmaceuticals Corp., East Hanover, NJ (code SaH 62-440). 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydroazepine (ML-9), and H-9 are from Molecular Probes, Inc., Eugene, OR. *N*-2-(methylamino)-ethyl-5-isoquinoline-sulfonamide (H-8) and forskolin are from Hoechst Calbiochem, Japan. Cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The other chemicals were purchased from Sigma Chemical Co. 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and forskolin were dissolved in alcohol, and phorbol 12-myristate 13-acetate (PMA), ML-9, and DiC8 were dissolved in DMSO. The final concentrations of these solutions were 0.1%. No vehicles alone affected the current in a preliminary test. Data were expressed as the mean±SE. Student's t test was applied for unpaired results, and a P value of < 0.05 was considered significant.

Results

Whole cell recordings. We used Ca^{2+} and K^+-free solutions with NaCl on both sides of the cells. In this setting we could determine the current-voltage relationship, outwardly rectifying property (Fig. 1). The subsequent addition of PTH (10^{-7} M) to the bath stimulated inward currents, while the holding potential was −30 mV or hyperpolarized from 0 to −30 mV (Fig. 1, *inset*). Similar results were obtained, when forskolin (5×10^{-5} M) or dibutyryl-cAMP (10^{-4} M) was added to the bath. 10–30 s elapsed between PTH addition and the stimulation, and 30–120 s elapsed between forskolin addition and the stimulation. This suggests that there is not only a diffusion delay to the cell but also some time required for channel activation. Changes of the Cl^- conductance were examined at the hyperpolarized potential from 0 to −30 mV. When the reversal potential of Cl^- was zero, PTH (310±10 vs. 502±22 picosiemens (pS), n = 6, $P < 0.01$), forskolin (310±12 vs. 482±12 pS, n = 6, $P < 0.01$), and dibutyryl-cAMP (318±8 vs. 468±18, n = 4, $P < 0.05$) all significantly increased Cl^- conductance of a single round cell (diameter, 10–20 μm).

Anion selectivity was suggested from an inhibitory effect of

2. The coupling of cyanogen bromide-activated Sepharose 4B to H-9 was as described in detail. H-9 (40 μmol) was dissolved in 6 ml of 50 mM borate buffer (pH 8.0) containing 0.5 M NaCl and added to 3 ml of settled cyanogen bromide-activated Sepharose 4B. After a 2-h incubation at 30°C, the gel was washed with 30 ml of the borate buffer and was resuspended in 6 ml of 1.0 M ethanolamine (pH 8.0). The mixture was again incubated overnight at room temperature.

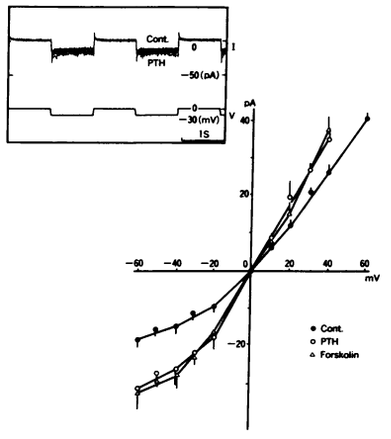

Figure 1. Influences of PTH and forskolin on whole cell Cl^- current. The pipette and bath contained a NaCl solution (see Methods). The current-applied voltage relationship of six individual determinations in control (•), after 10^{-8} M of PTH-treated (○), and after 5×10^{-5} forskolin-treated (dT) cells are illustrated. Inset shows a typical recording for the measurement of Cl^- conductance. Current (*upper*) and voltage (*lower*) of four recordings obtained by a 0 to −30 mV step at 1 Hz in the control and PTH-treated cells were plotted on the same trace. The magnitude of the individual current level was measured by eye. Since currents at 0 mV were not shifted by PTH, we defined 0 mV as ECl and thus Cl^- conductance as current magnitude/30 mV.

DIDS on Cl^- conductance in a few preliminary experiments. Almost all (~ 85%) of the current at the holding potential of −30 mV was blocked by the reagent (10^{-4} M). Cation-anion selectivity of this conductance was estimated by changing the NaCl concentration of the bath solution. The shift of the current-voltage relationship suggested a P_{Na}/P_{Cl} of ~ 0.1 (n = 4).

Excised inside-out patch clamp. The cells were bathed in the internal solution for the recordings. Membrane patches in cell-attached held at positive voltage showed poor (< 10%) channel activities for up to 15 min. Depolarization by holding potentials of −60 mV or greater elicited channel activity in no more than 40% of attached cells. The activation was, however, required for at least 2 min. The channel evoked by the depolarization remained active after membrane patches were excised to form the inside-out recording configuration. The characteristics of this activation were similar to the previous observations reported in several fluid-secreting epithelia, although the reason for the activation is unknown. The current-voltage relation of a single Cl^- channel is shown in Fig. 2 *A*, together with typical records from an inside-out membrane patch. The channel was outwardly rectified; outward conductance (33.0±1.5 pS, n = 6) was larger than inward conductance (22.5±2 pS, n = 6) when both membrane sides were bathed in 130 mM CsCl.

The P_{Cl}/P_{Na} ratio was estimated by decreasing the Na concentration in the internal bath solution from 130 to 40 mM with the pipette of the external 125 mM NaCl solution held constant (Fig. 2 *B*). The reversal potential shifted to −18±0.3 mV. Using the Goldman-Hodgkin-Katz equation for each patch, the P_{Cl}/P_{Na} was 10.3±0.4 (n = 4). The permeability for large anions was determined by replacing the internal NaCl with 40 mM NaCl and 90 mM sodium succinate or gluconate. The reversal potentials were −24±2.2 mV for gluconate and −22±3.2 mV for succinate (Fig. 2 *C*). Thus these large anions did not permeate well through the channel ($P_{Cl}/P_{\text{large anion}} > 15$). The selectivity of Cl^- channel to these ions is similar to the previous report (6). Addition of DIDS (10^{-4} M) from inside the membrane completely blocked the currents between the potential of −60 to +60 mV (Fig. 2 *D*). Since application of the reagent blocked the *trans*- and *cis*-current, DIDS might permeate through the membrane and blunt the activation of the channel from both sides.

Kinetic analysis of the channel evoked by depolarization was performed in eight determinations (Fig. 3). During the test period, a random 10-s sampling was stored in the computer. During this time the open-close lifetime was not dependent upon the timing of activation (data not shown). The open probability versus membrane voltage (negative value of an applied voltage) correlated, suggesting that the channel may be a voltage-dependent type. Open and close lifetime histograms of these channels were analyzed (n = 7). Open lifetime histogram could be fitted to one exponential function. The more negative the membrane potential, the longer the open lifetime. On the other hand, the close lifetime histogram was better approximated by two exponential functions. The fast component of

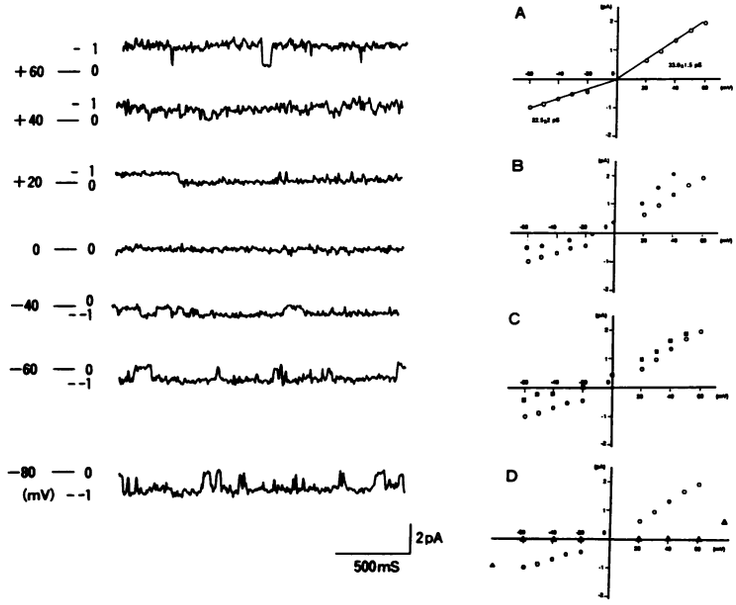

Figure 2. I-V relation of single Cl^- channel by inside-out mode. The pipette and bath contained the external and internal NaCl solutions, respectively, as described in Methods. The Cl^- channel of the apical membrane was activated by depolarization stimuli (−60 mV) in the cell-attached mode. A typical recording of current to negative value of applied voltage (membrane potential) is shown after excision (*left*). (*A*) Current-voltage relation of six determinations are plotted (○). (*B*) Current-voltage relation obtained after changing the bath solution to 40 mM NaCl (•). (*C*) Current-voltage relation obtained when the bath solution consisted of 40 mM NaCl and 90 mM Na-gluconate (■). (*D*) Current-voltage relation obtained after addition of DIDS (10^{-4} M) to the bath (*right*).

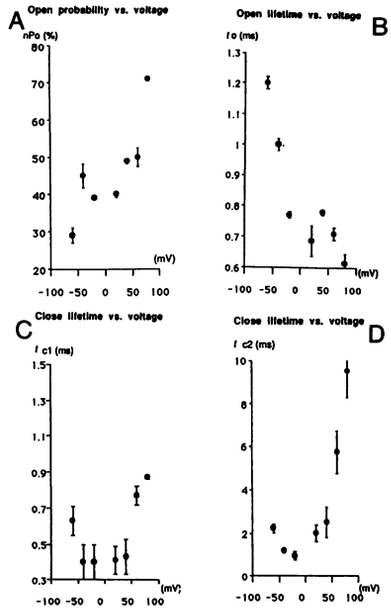

Figure 3. Kinetics of Cl^- channel. The pipette and bath contained the external and internal CsCl solution, respectively (see Methods). The Cl^- channel of the apical membrane was activated by depolarization stimuli (−60 mV) in the cell-attached mode. The kinetics of the Cl^- channel was calculated from 10-s recordings by the computer and plotted to negative value of applied voltage (membrane potential). We compared the results with one and two exponential fits for close lifetime, and selected the better one. (*A*) Open probability of the channel. (*B*) Open lifetime of the channel. (*C*) Fast component of the close lifetime of the channel. (*D*) Slow component of the close lifetime.

the close lifetime was not affected by voltage, while its slow component was a function of voltage. In contrast to the open lifetime, the more positive the membrane potential, the longer the close lifetime.

Next, we made an inside-out membrane patch from cells without activated Cl^- channels with membrane potential of −60 to +60 mV. To determine whether the Cl^- channel is regulated by PKA, PKC, or Ca^{2+}, we maintained excised patches at −60 mV to avoid voltage activation and then added PKC or PKA plus their cofactors, ATP (1 mM) or ATP and DiC8 (10 μg/ml) with 500 nM Ca^{2+} to the bath, respectively. After activation of the channel, we maintained the voltage at +50 mV to observe the currents clearly. Fig. 4 shows the results of the influence of the protein kinases. nPo was calculated at maximal channel activation. A catalytic subunit of PKA (membrane phosphorylation activity of 420 pmol/min per ml) significantly activated the channels for a few minutes, and then decayed to the basal level. 10–20 s elapsed between the addition of the catalytic unit and the observed stimulation. PKC was dissolved in the internal solution, possessing membrane phosphorylation of 3 pmol/ml per min. PKC activated the channels for at least 3 min without decay. There was an ~ 10–20-s delay until stimulation. Subsequent addition of alkali phosphatase (from bovine kidney) to enhance dephosphorylation inhibited the evoked channel. This suggests that the activation by PKC is a result of membrane phosphorylation and not depolarization. PKC also activated the channels in the presence of cAMP-dependent PKA inhibitor (400 U, inhibits 400 pmol phosphorylation, $n = 3$). Thus PKA and PKC may independently activate the Cl^- channel. Unlike the protein kinases, Ca^{2+} ion (500 nM, 1,000 nM) did not activate the channel but did increase noise ($n = 3$, data not shown). We also examined the influence of these reagents and depolarization on the open-close lifetime of the Cl^- channel (Table I). Compared to voltage activation, PKA slightly altered the slow component of the close lifetime, whereas PKC altered it remarkably and further modified the fast close lifetime. The open lifetime of the voltage-activated channel was identical to those of the kinase-activated channels.

Based on the above data, cultured renal proximal tubule cells have an apical Cl^- selective channel, which appeared to be activated by PKA and PKC-induced membrane phosphorylation but not by Ca^{2+}. However, it is not clear that these pathways are used in vivo in response to PTH stimulation. Therefore, we used the cell-attached patch clamp to address the question.

Cell-attached patch clamp. Fig. 5 shows the activation of the Cl^- channel by PTH (10^{-8} M) while cell attached. The external solution supplemented with 3 mM α-ketoglutarate and 1 mM NaH_2PO_4 to maintain the internal ATP store was used for the bath and pipette solutions. The basal activity of the channel at a membrane potential of −60 mV was used as a

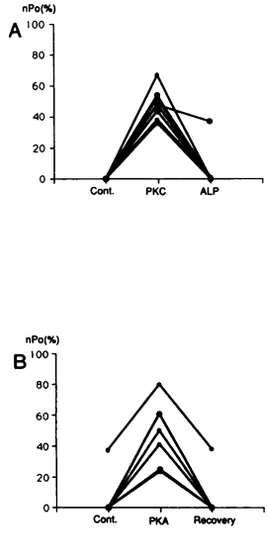

Figure 4. Effects of PKA and PKC on the open probability of Cl^- channel. The pipette and bath contained the external and internal CsCl solution, respectively (see Methods). (*A*) Effect of PKC on Cl^- channel. We maintained excised inside-out patches at −60 mV and added purified PKC (with an activity of membrane phosphorylation of 3 pmol/ml per min) and DiC8 (10 μg/ml) to the bath solution with 1 mM ATP and 500 nM Ca^{2+}. After activation of the channel, the currents were observed at 50 mV for 1 min and then alkaline phosphatase (1 U/ml) was added. Open probabilities are calculated during an appropriate 10 s at 50 mV. (*B*) We maintained excised inside-out patches at −60 mV and added PKA catalytic subunit (with an activity of membrane phosphorylation of 420 pmol/ml per min) to the bath solution with 1 mM ATP and 10 nM Ca^{2+}. After activation of the channel, the currents were observed at 50 mV for 2 min. Spontaneous disappearance of the channel is marked as recovery.

Table I. Effects of the Various Activation Procedures of the Cl Channel on Its Lifetime

	n	to	tc1	tc2
		ms	*ms*	*ms*
Inside-out patch				
Voltage	6	0.7±0.02	0.57±0.2	9.19±0.7
PKA	6	0.61±0.036	0.88±0.027	7.8±1.0
PKC	7	0.69±0.0022	0.55±0.028*	1.3±0.9*
Cell-attached patch				
cAMP	5	0.7±0.03	1.02±0.02	9.24±0.8
PTH	8	0.7±0.008	0.94±0.05	5.6±0.7
PMA	7	0.67±0.01	0.77±0.11*	1.56±0.46*

to, open lifetime; tc1; fast component of close lifetime; tc2, slow component of close lifetime. * $P < 0.01$.

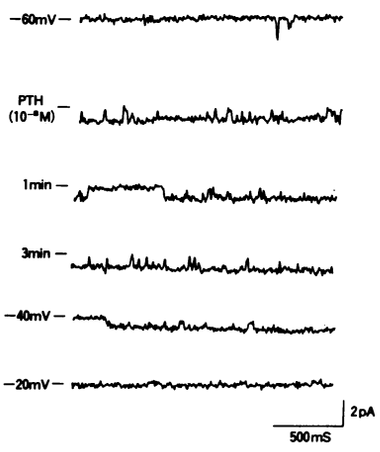

Figure 5. Representative traces of the Cl^- channel by the cell-attached mode. The bath contained the external NaCl solution plus 1 mM $NaHPO_4$ and α-ketoglutarate. The pipette contained the external CsCl solution (see Methods). We maintained the cell-attached patch at −60 mV and then added PTH (10^{-8} M). After activation of the Cl^- channel for 3 min, the applied voltage was changed to 20 mV through 40 mV.

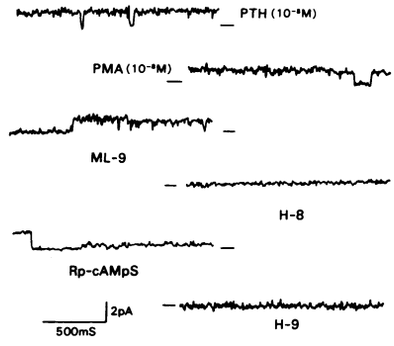

Figure 7. Effects of blockers on the Cl^- channel. The procedures for the recordings are the same as for Figs. 5 and 6. PTH and PMA activated the channel at the holding potential of −50 mV. After activation of the Cl^- channel by PTH (10^{-8} M), we added either ML-9 (10 μM), H-8 (10 μM), Rp-cAMP (10 μM), or H-9 (10 μM) to the bath. Representative traces for PTH and PMA-activation and for effects of the blockers on the PTH-activated channel are shown with a zero current mark (—).

control. The activation continued for at least 3 min. Next we changed the potential to −40 and −20 mV and found the reversal potential at around −20 mV. This reversal potential is comparable to the Cl^- gradient, when assuming an intracellular Cl^- concentration of ~ 20 mM (20). Outward rectification was the same as that observed in excised membranes. The dose-responses of the activation by PTH, dibutyryl-cAMP, and PMA are shown in Fig. 6. After an initial period of 30 s to verify the absence of spontaneous activation, we added the reagents to the bath. PTH and PMA at concentrations of 10^{-9} M could activate the channel significantly, while > 10^{-7} M of dibutyryl cAMP (n = 5) was required for the activation. DiC8 at the same concentration as PMA also activated the channel in dose-dependent way (n = 3).

Holding the potential at −50 mV, we measured the influence of these reagents on the open-close lifetimes. Table I shows the influences of PTH, PMA, and dibutyryl cAMP in cell-attached patches on the Cl^- channel lifetimes. The findings were similar to those obtained by the inside-out patch clamp; both PMA and PTH significantly altered the slow component of the close lifetime.

We performed blockade experiments next to explore the contribution of PKA and PKC to the PTH mechanism of action. Doses of blocking reagents used here were those suggested as effective by previous reports (19, 21, 22). After activation of the Cl^- channel by PTH (10^{-8} M), the various reagents described below were added 1 min later and recordings made. Representative traces at a potential of +50 mV are shown in Fig. 7. PTH and phorbol ester (PMA) similarly activated the Cl^- channel. Myosin light chain kinase inhibitor, ML-9 (10 μM), did not affect the PTH stimulation of the Cl^- channel. A blocker of PKA, Rp-cAMPS (10 μM), incompletely and transiently (for 30 s) inhibited the PTH activation of the Cl^- channel. On the other hand, H-8 (10 μM), a blocker of both protein kinase A and C, and H-9 (10 μM), a specific blocker of PKC, almost completely blocked the PTH-activation of the Cl^- channel. Furthermore, the inhibition was sustained. The specificity of these blockers also was tested. The PMA-activated Cl^- channel was blocked by the subsequent addition of H-9, but not affected by Rp-cAMPS. On the other hand, the cAMP-activated Cl^- channel was inhibited by the addition of Rp-cAMPS but not by H-9. The effects of the blockers on nPo are summarized in Table II. No activation of the Cl^- channel was seen in controls. Once PTH activation of the Cl^- channel was observed, the various blockers were added, the inhibitory effects of the blockers (usually observed within 1 min) on nPo were then calculated. From our results, it appears that PTH at a concentration of 10^{-8} M activates the Cl^- channel through protein kinase A and C pathways.

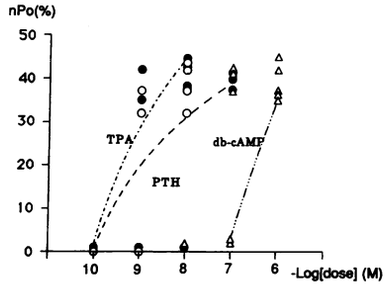

Figure 6. Dose response of the open probability of Cl^- channel to the reagents. The procedures for the recordings are the same as for Fig. 5. After activation of the Cl^- channel by the three reagents for 3 min, the applied voltage was changed to −50 mV to provide distinct traces for analysis. Open probabilities are calculated 10 s at the beginning of −50 mV.

Table II. Blockade Experiments of PTH-activated Cl^- Channel by Several Reagents

nPo	Control	PTH	Blockade	n
ML-9	0	38.5±5	39.0±6	3
RpcAMPS	0	42.6±3.5	12.0±10*	4
H-8	0	39.5±6	0‡	4
H-9	0	40.0±4	0‡	4
	Control	RpcAMPS	H-9	n
Dibutyryl-cAMP	63.6±4.2	16.0±3.5‡	51.2±5.0	4
PMA	46.3±5.2	51.3±5.6	18.0±4.6‡	4

* $P < 0.05$; ‡ $P < 0.01$.

Discussion

Location of Cl^- channel. The proximal tubule of the kidney is the nephron site where the major portion, between 2/3 and 3/4 of the filtered sodium salt is reabsorbed. The mechanism of fluid transport across the PCT has been investigated in detail in vivo by micropuncture or in vitro by microperfusion methods for over a decade. Many transporters as well as ion permeable channels that contribute to NaCl transport are present in this segment. Cl^- permeability also has been investigated in a number of the studies. It was initially thought that a gradient of $[Cl^-]_{lumen}/[Cl^-]_{peritubular} > 1.0$ and $[Na^+]_{lumen}/[Na^+]_{peritubular} = 1$ yielded a lumen positive potential that was the driving force for Cl^- to exit to the lumen and Na^+ to be passively reabsorbed in the cortical proximal tubules (23). Edelman et al. (24) have detected the presence of this Cl^- excretion by a Cl^- selective electrode in vivo in *Necturus* convolutions. Warnock and Yee (25) found a voltage-dependent Cl^- flux in rabbit cortical brush border membrane vesicles, which has been supported recently by microperfusion methods (26). Thus Cl^- can be secreted to the lumen by a driving force for Cl^- and recycled through paracellular pathways in Cl^- permeable segments.

Patch clamp analysis is useful for the study of ion channels. The PCT can be studied by this method but the brush border is an obstacle for giga-seal. Therefore, the basolateral membrane of the PCT is the only side capable of being patch clamped. Available data in cell-attached patch studies indicate that there is only a nonspecific Cl^- permeable cation channel in the basolateral cut-end of rabbit proximal tubules (2). Thus a Cl^--specific channel may not exist in the basolateral membrane. Since the apical luminal membrane was not tested, the presence of a Cl^--specific channel was not ruled out.

To explore the presence of a Cl^- channel by the patch clamp method in this study, the PCT has been cultured to produce cells with less abundant brush border surfaces so that we could make a giga-seal of the apical membrane. In fact, most of the Cl^--specific channels have been described in cultured cells, e.g., A6 renal epithelia (4) and in distal tubular cell lines (3, 27). In cultured proximal tubular cells, the Cl^- conductance of whole cells (28) and a single Cl^- channel (29) also has been demonstrated recently. Although the cultured cells may have some advantages for the patch clamp studies, fully differentiated transport polarity requires that cells should be grown on permeable supports (30). Therefore, this study, though suggesting the presence of a Cl^- channel in the apical membrane, may not conclusively prove its existence in intact PCT. In addition to the patch clamp, protein reconstitution techniques with artificial membranes have been developed for the study of channels. Landry et al. (31) have provided evidence from reconstituted bovine renal cortical vesicles that there are three size of Cl^- channels possessing conductances of 400±50 pS, 240 pS, and 26 pS. While the number and size of the Cl^- channel in the rabbit renal cortex have not been investigated with this technique, a channel size of 26 pS is compatible with our findings. However, since the brush border membrane is hardly conductive to the creation of artificial membranes, the reconstituted channels may be located in endosomal vesicles (32).

Regulation and role of Cl^- channel. We first tested the presence of Cl^- conductance in whole cells. Since they appeared less conductive to Cl^-, the Cl^- conductance of the PCT is probably very low until activated by an agonist. The resting and evoked current-voltage relationships were similar in the PCT and in rat peritoneal mast cells (33). In these small cells, the 50-pS channel provides a whole cell conductance of ~ 500 pS, indicating roughly 10 channels per cell. We examined the effects of PTH and forskolin on whole cell currents. They showed activated conductance of ~ 250 pS at −30 mV. By similar calculation with a single channel conductance, ~ 10 channels were activated in a single cell.

This Cl^- channel revealed several characteristics that are shown in fluid-secreting epithelia. Outward-rectification of the single Cl^- channel was observed in our study. This outward rectification in cell-attached patch clamping was not modified by membrane excision, which is also observed in human nasal epithelial cells (7). In contrast, outward rectification is altered by excision in the basolateral membrane of the fluid-reabsorbing thick ascending limb of the mouse kidney (3). Furthermore, the relationship of open-time probability to voltage and the open-close lifetimes were similar to the previous findings in fluid-secreting epithelia (8).

A number of studies have suggested that the Cl^- channel in fluid-secreting as well as reabsorbing epithelia is stimulated by cAMP (34). Addition of dibutyryl cAMP stimulates the Cl^- channel, thereby making it easier to detect (3). More directly, the PKA catalytic subunit in the presence of Mg-ATP keeps Cl^- channel open by membrane phosphorylation in the absence of Ca^{2+}. The findings with whole cell and inside-out patch clamping with internal Ca^{2+}-free media in this study clearly indicate that PKA activated the channel independently of Ca^{2+}. In contrast to the previous findings by others (12, 13), the contribution of PKA to the activation of Cl^-, the channel was transient with the internal addition of this reagent. This was also observed by the transient blockade with Rp-cAMPS in cell-attached experiments. Addition of cAMP or its analogue to the cells in the whole cell and cell-attached experiments, however, provided continuous activation. One explanation for the transient activation may be the spontaneous dephosphorylation of the channel by a membrane-bound enzyme. We added PKA followed by okadaic acid (3 nM, $n = 4$) to prevent the dephosphorylation and thereby keeping the channel open for at least 2 min or longer, although okadaic acid itself did not affect the channel activation. Thus transient events may be at least partly due to spontaneous dephosphorylation of PKA.

Regulation of the Cl^- channel by PKC also has been demonstrated. Regulation of the Cl^- channel in airway epithelia has been investigated in details (12, 13). The independent contribution of PKC to channel activation should be carefully addressed, however, since Ca^{2+} is often a cofactor in the activation of the kinase, and PTH increases the intracellular free calcium with the activation of PKC. Our purified PKC from rabbit renal cortex phosphorylated lysine-rich histone only in the presence of 500 nM Ca^{2+}. Thus we tested whether the purified PKC activated the Cl^- channel of the excised membrane in the presence of Ca^{2+}. The stimulatory effect of PKC was reversed by alkaline phosphatase, indicating a true contribution of membrane phosphorylation to the activation mechanism (Fig. 4). Since the PTH-evoked channel was completely blunted by a blocker of PKC, PTH activated the Cl^- channel at least partly via the PKC pathway. In contrast, Ca^{2+} itself did not activate the Cl^- channel in the inside-out membrane, and the PTH-evoked Cl^- channel was not altered by the Ca^{2+}-dependent myosin light chain kinase inhibitor. Thus it appears

that the activation of the Cl^- channel was unlikely to depend on a rise in intracellular Ca^{2+} alone. In our preliminary data, however, DiC8 (10^{-8} M) inhibited rather than stimulated whole cellular Cl^- conductance in the absence of Ca^{2+} in the intracellular solution, and this effect was reversed by subsequent addition of H-9 ($n = 6$). Therefore, PKC may have opposite dual actions on the channel, by means of coexistent Ca^{2+} concentrations. This bipolar action by PKC on the Cl^- channel has been also reported in human and canine airway epithelial (12). There are several types of the C kinase known (35). In fact, a subclass of PKC might act in nominally free Ca^{2+} solution while other types of PKC require Ca^{2+} for their function (36). It is not known whether the former type of PKC exists in the kidney.

PTH activated the apical Cl^- channel via activation of these protein kinases. Modification of the Cl^- channel by hormones is reported in airway epithelial cells, in which a β-adrenergic agonist increases the magnitude of apical Cl^- conductance via PKA and PKC pathways and leads to enhanced Cl^- secretion. 1-34 PTH, like β-adrenergic hormones, induces second messengers, PKA, PKC, and a rise in intracellular Ca^{2+} of this renal segment (11), and may enhance Cl^- secretion. Although the inhibitory action of PTH on fluid reabsorption may be primarily due to the suppression of Cl^--bearing transporters, Cl^- secretion by this channel also may be involved in this inhibitory mechanism. Recent data in opossum kidney cell lines indicate that the induction of second messengers by PTH is achieved at $< 10^{-9}$ M, and that the physiologic concentration of PTH is $\sim 10^{-11}$ M. PTH concentrations of 10^{-9} M induce PKC and a rise in Ca^{2+}, while a higher dose is required for PKA activation (37). Our data suggest that PTH at 10^{-9} M could activate the channel. Thus PKC may play a cardinal role and PKA a secondary role in Cl^- secretion by PTH.

In addition to activation by this membrane phosphorylation, Schwiebert et al. (27) have suggested that the Cl^- channel is activated by the Giα protein in renal distal tubules. Since recent data have suggested that the Giα protein exists on the luminal membrane of proximal tubules, this novel mechanism of regulation and its physiologic significance in Cl^- channel activation should be examined in the future.

The physiologic function of the present Cl^- channel still remains obscure. When the Cl^- channel is activated, the membrane potential will depolarize to -20 mV, and then Cl^- will outflux. This modification of the membrane potential may then influence a cation flow. For instance, agonist-stimulated Cl^- flow has been considered to be the driving force of delayed Ca^{2+} influx into mast cells (38). PTH increases intracellular Ca^{2+}, which then activates the maxi-K^+ channel and results in K^+ outflux. It is possible that the Cl^- channel will open to neutralize the cation flow. The Cl^- channel also may contribute to cell volume regulation. When cells are exposed to a hypotonic solution, they swell and then recover their size toward normal. The recovery, termed regulatory volume decrease, accompanies K^+ and Cl^- efflux. The cells we have used have a potent volume regulatory mechanism (39), and their maxi-K^+ channel is activated by a hypotonic attack (16). The Cl^- channel may be thus concomitantly activated to enhance an outflux of this anion and thereby play a role in regulatory volume decrease.

In conclusion, we explored the chloride conductance of cultured rabbit renal proximal convoluted cells and found an apical Cl^- channel. This Cl^- channel was outward rectified, activated by depolarization, protein kinase A, and protein kinase C, but not by Ca^{2+}. 1-34 PTH activated the channel by the PKA and PKC pathways. This channel may excrete the Cl^- ion into the lumen under physiologic conditions. The channel may also play a role in cell volume regulation or in the modification of the flow of other ions.

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