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Identification and Regulation of Whole-Cell Cl^- and Ca^{2+}-Activated K^+ Currents in Cultured Medullary Thick Ascending Limb Cells

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Abstract. The whole-cell patch-clamp technique has been used to study membrane currents in cultured rabbit medullary thick ascending limb (MTAL) epithelial cells. A Ca^{2+}-activated K^+ current was characterized by its voltage-dependent and Ca^{2+}-dependent properties. When the extracellular K^+ ion concentration was increased from 2 to 140 mM, the rereversal potential (E_k) was shifted from -85 to 0 mV with a slope of 46 mV per *e*-fold change. The Ca^{2+}-activated K^+ current is blocked by charybdotoxin (CTX) in a manner similar to the apical membrane Ca^{2+}-activated K^+ channel studied with the single channel patch-clamp technique. The results suggest that the Ca^{2+}-activated K^+ current is the predominant, large conductance and Ca^{2+}-dependent K^+ pathway in the cultured MTAL cell apical membrane. The biophysical properties and physiological regulation of a Cl^- current were also investigated. This current was activated by stimulation of intracellular cAMP using forskolin and isobutyl-1-methylxanthine (IBMX). The current-voltage (*I-V*) relationship of the Cl^- current showed an outward-rectifying pattern in symmetrical Cl^- solution. The Cl^- selectivity of the whole-cell current was confirmed by tail current analysis in different Cl^- concentration bath solutions. Several Cl^- channel blockers were found to be effective in blocking the outward-rectifying Cl^- current in MTAL cells. The cAMP-dependent Cl^- transport in MTAL cells was further confirmed by measuring changes in the intensity of Cl^- sensitive dye using fluorescence microscopy. These results suggest that the Cl^- channel in the apical or basolateral membrane of MTAL cells may be regulated by cAMP-dependent protein-kinase-induced phosphorylation.

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Key words: MTAL epithelial cells — Ca^{2+}-activated K^+ current — Cl^- current — SPQ fluorescence — Patch clamp

Introduction

One of the important roles the mammalian kidney plays is to maintain body salt and water balance. The separation of salt and water absorption in the nephron is dependent upon the thick ascending limb of Henle's loop. The ion transport in Henle's loop is unique. The transport properties of this portion of Henle's loop are critically important to dilute the urine and generate the medullary osmotic gradient that is an essential energy source for the formation of countercurrent multiplication. In vitro microperfusion and single channel patch-clamp studies have suggested that a number of transport mechanisms are involved in the MTAL epithelial cells. In the apical plasma membrane of the medullary thick ascending limb (MTAL), water permeability is relatively low compared to other parts of the kidney, and ions such as K^+, Na^+ and Cl^- are reabsorbed from the apical plasma membrane into epithelial cells via an electroneutral and furosemide-sensitive Na^+ $2Cl^-K^+$ cotransporter (Hebert, Culpepper & Andreoli, 1981*a*, 1986*a*,*b*; Greger et al., 1984). In the basolateral membrane, Na^+ is actively pumped out of the cell by the Na^+-K^+-ATPase which provides the electrochemical driving force for Cl^- ions across the basolateral membrane. The Na^+-K^+-ATPase can be inhibited by addition of ouabain to, or removal of K^+ ions from, the extracellular solution (Burg & Green 1973; Hebert et al., 1981*a*; Rocha & Kokko, 1973).

A Ca^{2+}-activated K^+ channel also is present in the apical membrane and plays a role in K^+ recycling. Recently, it has been proposed that the apical membrane K^+ conductance may be involved in cell volume regulation under low osmotic conditions because evidence has shown that the Ca^{2+}-activated K^+ channel can be activated by stretching of the membrane at cell-attached patches (Taniguchi & Guggino, 1989). Single channel properties of the Ca^{2+}-activated K^+ channel in cultured MTAL epithelial cells have been well studied (Guggino et al., 1987; Cornejo, Guggino & Guggino, 1989). This channel is highly selective to K^+, very sensitive to charybdotoxin (CTX) and Ba^{2+}, and has a large single channel conductance of 150 pS in 140 mM symmetrical KCl solutions. The gating mechanism of the Ca^{2+}-activated K^+ channel has also been elucidated using the single channel patch clamp.

A Cl^- conductance in MTAL cells has been reported from in vitro microperfusion experiments (Hebert, 1981*b*; Greger et al., 1983). The Cl^- conductance was enhanced by a decrease in the bath pH (Yoshitomi et al., 1987), by addition of Ba^+ in the perfusion solution (Yoshitomi et al., 1987), and by application of ADH in the basolateral membrane (Wirz, 1957; Levitin et al., 1962; Oberleithner et al., 1983). Using cell-attached and cell-free patch clamp, the single Cl^- channel has been found in the basolateral membrane of the rat TAL segment (Greger, Bleich & Schlatter, 1990). These results indicate that Cl^- channels in MTAL epithelial cells play a role in: (i) hormonal regulation of ion transports; (ii) maintenance of resting membrane potential during decreased K^+ conductance; (iii) maintenance of intracellular pH when extracellular pH is changed; and (iv) cell volume regulation. We know that Cl^- channels serve important functions in the MTAL of Henle's loop; however, no direct evidence about the Cl^- channel has been gained by patching at the whole-cell level.

The present studies were undertaken to investigate the characteristics and regulations of the Ca^{2+}-activated K^+ channel and Cl^- channel using the whole-cell patch clamp. Our results indicate that whole-cell currents in MTAL cells are activated by either depolarization membrane potential and increasing intracellular Ca^{2+} concentration or by phosphorylation induced by an increase in intracellular cAMP. The biophysical properties and regulation of the whole-cell Ca^{2+}-activated K^+ current and Cl^- current found in MTAL cells are consistent with previous studies about these channels from single channel recording and in vitro microperfusion of renal tubules.

Materials and Methods

Culture of Kidney Epithelial Cells

The medullary thick ascending limb (MTAL) cells were obtained from a MTAL cell line originally derived from GBR-MAL1 (clone A3) of rabbit kidney (Green et al., 1985). These cells can grow for more than 80 passages under culture conditions, with a doubling time of 1.4 days. Culture conditions and procedures are described in the following sections.

Culture Medium

Cells were grown in Coon's medium supplemented with (in 200 ml): 1.5% L-15 (20 ml), 5% transferrin (0.2 ml), 0.018% hydrocortisone (0.2 ml), 0.0017% selenium (0.2 ml), 0.01% spermine (0.2 ml), 0.0000034% T3, 0.5% pituitary extract (0.2 ml), 29% glutamine (2 ml), heat-inactivated rabbit serum and calf serum (1 ml and 1 ml), and pen-strep 100 μg/ml.

Culture Matrix

Cells were cultured on coated culture flasks or dishes. Flasks or dishes were coated with a thin layer of 0.1% collagen (Vitrogen 100) and then dried overnight under UV light.

Subculture

Cells were detached from culture dishes by treatment with 0.05% trypsin (Sigma) for 15 min at 37°C; the enzyme reaction was then stopped by addition of medium containing 5% heat-inactivated calf serum. Suspended cells were seeded to coated dishes or flasks at a final density of 5×10^4 per ml, or were frozen in medium containing 10% DMSO at a final density of 5×10^5 per ml. The cell freezing process was controlled at a rate of 1°C/1 min to −80°C. Frozen cells were stored in liquid nitrogen.

Fluorescence Microscopy Measurements

For Cl^- transport experiments, cultures of MTAL cells grown on coverslips were washed with Phosphate Buffered saline (PBS) and loaded with 5 mM SPQ (Molecular Probes) by incubating cells at room temperature (23°C) for 6 min in hypotonic loading solution. The hypotonic loading solution contained a 50 : 50 mixture of distilled water and "solution 1" consisting of (in mM): 110 NaCl, 5 KCl, 2 Mg-gluconate, 2 Ca-gluconate, 20 Na-gluconate and 5 HEPES pH 7.4. Experiments were performed in a continuously perfused chamber at a speed of 10 ml per min. Perfusion solutions were switched between solution 1 (with 112 mM Cl^-) and "solution 2" (Cl^- free) containing (in mM): 105 Na-gluconate, 5 K-gluconate, 2 Mg-gluconate, 2 Ca-gluconate, 50 mannitol and 5 HEPES pH 7.4. SPQ fluorescence in the cell was finally quenched by a bath solution in the presence of valinomycin and KSCN. Changes in SPQ fluorescence were measured using a Nikon inverted epifluorescence microscope (Diaphot, Japan). Fluorescence of single cell images was collected with an image intensifier (VideoScope International, LTD) coupled to a Newvicon video camera (Dage MTI) and processed by the Image-1/FL Quantitative Fluorescence System (Universal Imaging Corpora-

tion). Fluorescence was excited at a wavelength of 435 nm. The filter cube (Nikon, DM400) contained a 400 nm dichroic mirror. Fluorescence emission was measured using a 510 barrier filter. Sixteen frames were averaged for each acquisition cycle before the acquired image was processed. Fluorescence intensity was calculated according to a pseudocolor scale.

Patch-Clamp Studies at Whole-Cell Configuration

Whole-Cell Patch Clamp

The MTAL cells were detached from culture dishes by treatment with 0.05% trypsin or 0.05% collagenase for 15 min at 37°C, and then resuspended in 10 ml Ringer solution (*see* Solutions). Patch pipettes were fabricated from Micro-hematocrit capillary tubes (1.12 mm ID, 1.5 mm OD, VWR Scientific) using a Narishige puller (Model PP-83). The patch electrode was fire-polished on a microforge using a heating filament over which a gentle stream of nitrogen air was blown. Patch pipettes were checked using the "bubble number." Typically, our bubble number was about 5 ml for whole-cell patches after fire-polishing. The bubble numbers correspond to pipette resistances of between 2–4 MΩ for whole-cell patches when the pipette was filled with 150 mM KCl saline. Whole-cell patch-clamp experiments were performed in a continuously perfused chamber. After the addition of 2–3 drops of suspended cells into the chamber, only single, isolated cells were studied, using an EPC7 patch-clamp amplifier (List, Electronics) grounded with a Ag/AgCl/Cl agar bridge. Typically, the resistance of our "tight seal" was between 50 to 100 GΩ. The resistance was measured by putting a 10 Hz square wave into the patch clamp and recording the resulting current pulses. A tight-sealed whole cell model was confirmed by measurement of the input resistance 5 to 10 min after the membrane was disrupted; this resistance should be between 5 to 10 GΩ. We found that a waiting period (approximately 10 min after the whole-cell model was obtained) is quite important to allow the pipette solution to uniformly diffuse into the intracellular compartment. Cell membrane potentials (V_m) were measured at zero membrane current (I_m = 0) under the current clamp model.

Cancellation of Whole-Cell Capacitance Transient

To make a good whole-cell recording, the transient capacitance current was cancelled by using the C-SLOW and G-SERISES controls. Adjustment of the C-SLOW control narrows the transient current, and then a further adjustment of the G-SERISES control was satisfactory to cancel the transient capacitance current. In our whole-cell recording experiments on MTAL cells, the mean value for whole cell capacitance was 27 pF, and for compensation of series resistance, the T_M was 300 μS.

Solutions

Whole-cell patch-clamp experiments were performed in different solutions dependent upon recording of the Ca^{2+}-activated K^+ current or the Cl^- current. For the Ca^{2+}-activated K^+ current, the pipette solution (the intracellular solution) contained (in mM): 140 KCl, 2 Mg-ATP, 10 EGTA and 10 HEPES pH 7.2. The Ca^{2+} concentration was calculated by using a Ca^{2+}-selective electrode. The final Ca^{2+} concentration was titrated from 10 nM to 1 μM by addition of $CaCl_2$. Bath solution was composed of (in mM): 140 NaCl, 2 KCl, 1 $CaCl_2$ and 5 HEPES pH 7.4. In ion selectivity experiments, K^+ concentration was altered by isotonically replacing Na^+. For the Cl^- current, experiments were performed in symmetrical Cl^- solutions. The pipette solution was composed of the following (in mM): 140 CsCl, 2 Mg-ATP, 10 EGTA, 10 HEPES, titrated by CsOH to pH 7.2. Bath solution was composed of (in mM): 70 $BaCl_2$, 50 NMDG (*N*-Methyl-D-Glucamine), 5 HEPES pH 7.4, or 85 NaCl, 60 TEA-Cl, 1 CaCl, 10 HEPES, adjusted by NaOH to pH 7.4 (NaCl is replaced by NaOH for low Cl^- concentration bath, and cations are substituted by Ba^{2+} or Cs^+ for selectivity experiments). Ringer solution contained (in mM): 140 NaCl, 2.3 K_2HPO_4, 0.4 KH_2PO_4, 1.3 $CaCl_2$, 1.2 $MgCl_2$-$6H_2O$, 5 glucose, and 10 HEPES (pH 7.4). Forskolin (Sigma), IBMX (Sigma), DPC (Fluka) and 9-AC (Fluka) in stock solution were freshly added into the bath solution shortly before the performance of each experiment.

Abbreviations

DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DPC, diphenylamine-2-carboxylic acid; 9-AC, anthracene-9-carboxylic acid; CTX, charybdotoxin; IBMX, isobutyl-1-methylxanthine; SPQ, 6-methoxy-1-(3-sulfonatopropyl) quinolinium.

Results

Ca^{2+}-Activated K^+ Current in MTAL Cells

The whole-cell current was activated by depolarization of the membrane potential from a holding potential of −100 to +100 mV in 20 mV increments. Data were recorded in 2 mM extracellular K^+ bath solution wth 140 mM K^+ in the pipette. The intracellular Ca^{2+} concentration (equivalent to the Ca^{2+} concentration in the patch pipette) varied from 7 nM to 1 μM (Fig. 1*A* and *B*). This whole-cell current demonstrates both voltage- and intracellular Ca^{2+}-dependent activation, followed by a very slow inactivation process. After extracellular application of 50 nM CTX, the amplitude of the current was inhibited by more than 50% (n = 4, Fig. 1*C*).

The selectivity of the current to K^+ ions was examined by tail current analysis. Extracellular K^+ concentrations were varied from 2 to 140 mM by isotonic substitution of extracellular Na^+ concentrations. Reversal potential of the current was changed from −85 to 0 mV when the extracellular K^+ concentration was increased from 2 to 140 mM (Fig. 2*A*). Reversal potentials measured in different concentrations of extracellular K^+ were plotted as a function of extracellular K^+ concentration (Fig. 2*B*). The calculated slope for each 10-fold change of extracellular K^+ concentration was 46 mV (n = 4), which is characteristic for a K^+-selective channel.

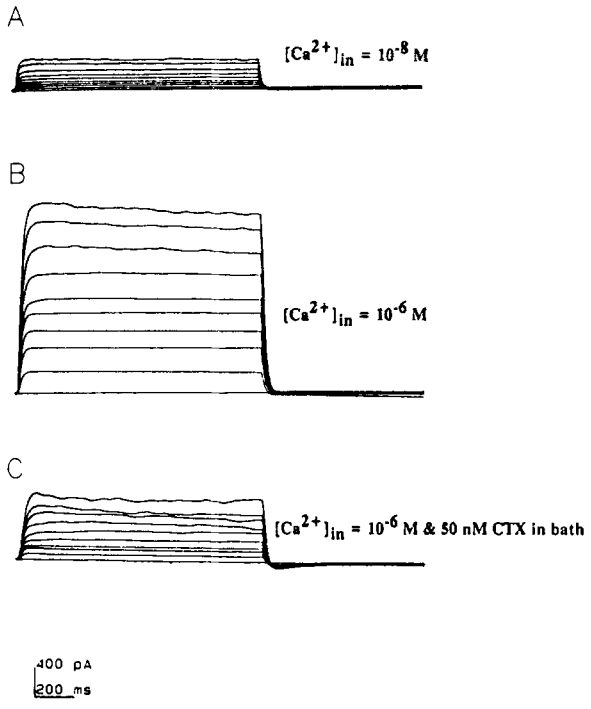

Fig. 1. Identification of Ca^{2+}-activated K^+ channels at the whole-cell level in the membrane of rabbit kidney MTAL epithelial cells. A pulse protocol to depolarize the membrane potential from a holding potential of −100 to +100 mV at 20 mV increments, was utilized to activate the whole-cell current. The pipette (internal) solutions contained 140 mM KCl and varying concentrations of Ca^{2+} as follows: (*A*) 10 nM Ca^{2+} in the pipette for cell #1; (*B*) 1 μM Ca^{2+} in the pipette for cell #2; (*C*) 1 μM Ca^{2+} in the pipette (cell #2) with perfusion of 50 nM CTX in the bath.

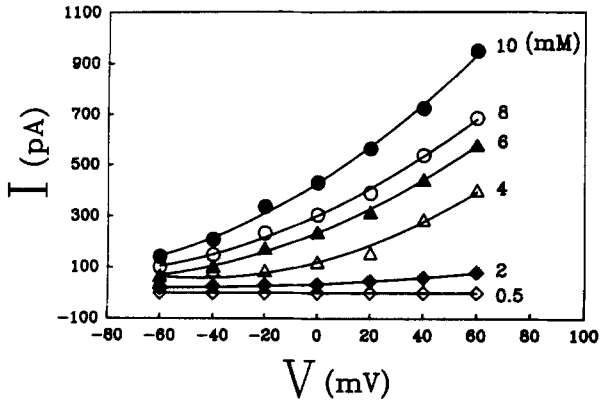

Fig. 3. Effect of the external Ca^{2+} concentration gradients on the Ca^{2+}-activated current in the presence of 1 μM Ca^{2+} ionophore. The experimental conditions are 140 mM KCl and 7 nM Ca^{2+} in the pipette solution, 2 mM KCl and Ca^{2+} concentration varied from 0.5 to 10 mM in the bath solution. Extracellular Ca^{2+} concentrations are indicated at the right end of each curve.

Dependence of the K^+ current upon Ca^{2+} was observed in four independent experiments involving alterations in extracellular Ca^{2+} concentration in the presence of the Ca^{2+} ionophore A23187 (1 μM). Figure 3 shows the relationship between the current and membrane potential at different extracellular Ca^+ concentrations in the presence of the Ca^{2+} ionophore. The outward K^+ current was activated by depolarizing membrane potential and was greatly enhanced when the extracellular Ca^{2+} concentration was increased from 0.5 to 10 mM. These data are consistent with previous studies that suggest the ac-

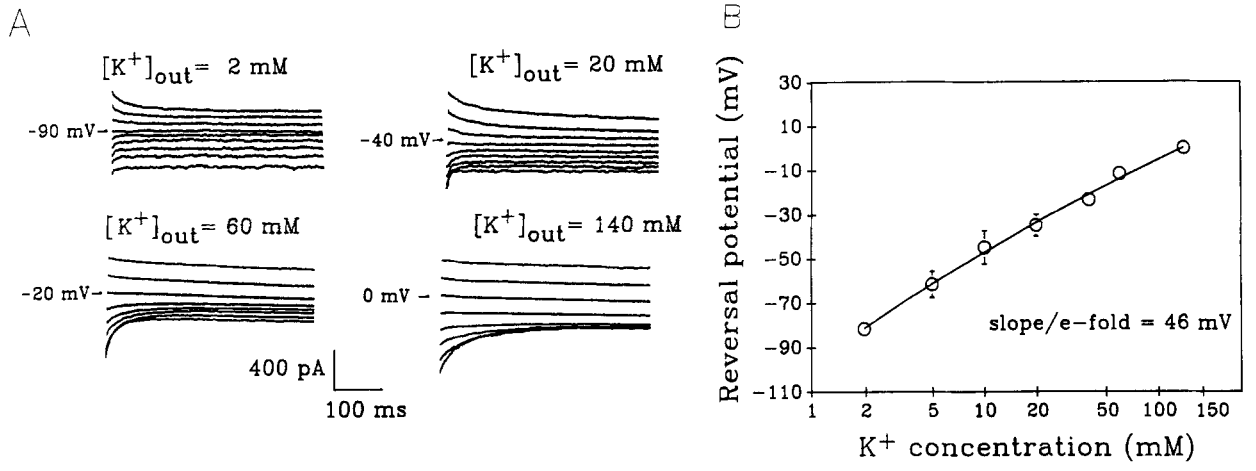

Fig. 2. K^+ selectivity of the Ca^{2+}-activated current in MTAL cells as determined by the tail current analysis using a pulse protocol to depolarize the membrane potential from a holding potential of −100 to +60 mV for 200 msec and then to repolarize the membrane potential to negative potentials. The pipette solution contained 140 mM KCl and 1 μM Ca^{2+}. (*A*) The K^+ concentration in the extracellular solution was changed from 2 to 140 mM. Reversal potentials in response to changes of the K^+ concentration are indicated at the left side of each recording. (*B*) Reversal potential obtained from the tail current analysis plotted as a function of the K^+ concentration in the bath. Data were collected from four independent experiments and plotted as means with SE bars. The slope of the regression line is 46 mV.

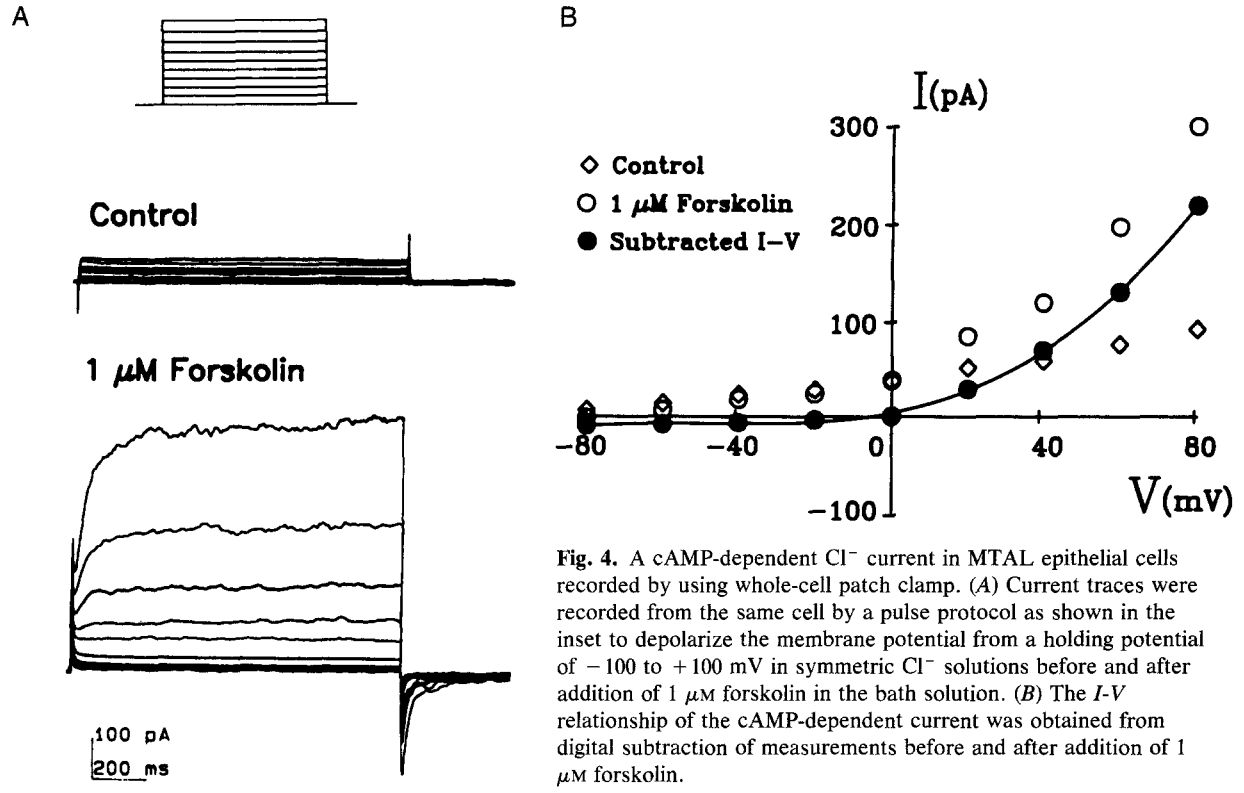

Fig. 4. A cAMP-dependent Cl^- current in MTAL epithelial cells recorded by using whole-cell patch clamp. (*A*) Current traces were recorded from the same cell by a pulse protocol as shown in the inset to depolarize the membrane potential from a holding potential of −100 to +100 mV in symmetric Cl^- solutions before and after addition of 1 μM forskolin in the bath solution. (*B*) The *I-V* relationship of the cAMP-dependent current was obtained from digital subtraction of measurements before and after addition of 1 μM forskolin.

tivation of the K^+ channel in MTAL cells is Ca^{2+} and voltage dependent.

Cl^- Current in MTAL Cells

A cAMP-dependent Cl^- current in MTAL cells was observed by using the whole-cell patch clamp. Activation of the Cl^- channels in the MTAL cell membrane was dependent upon the stimulation of intracellular cAMP by forskolin and/or IBMX. A pulse protocol was used to depolarize the membrane potential from a holding potential of −100 to +100 mV in 20 mV increments. Under the whole-cell clamp condition, the Cl^- channel activity was not readily observed when applying depolarization pulses from a holding potential of −100 mV. But, after addition of 1–10 μM forskolin to the continuously perfused bath solution, a large membrane current was activated using the same pulse protocol (Fig. 4*A*). A digital subtraction method was also utilized to analyze data recorded before and after applications of forskolin, which allowed us to eliminate the linear leakage current (Fig. 4*A*). For further investigation, a combination of forskolin and IBMX was used as an alternative stimulation to activate the Cl^- channel in some experiments. Current-voltage (*I-V*) curves before and after forskolin stimulation are plotted in Fig. 4*B*. The net current was obtained by subtracting the current before forskolin from the current obtained after forskolin stimulation. The net current showed an outwardly rectified relationship with a reversal potential of 0 mV in the symmetrical 140 mM Cl^- bath solution. In some patches, the Cl^- current was directly activated after application of several large depolarization pulses. The average current amplitudes observed from independent experiments are 180.6 ± 38 pA at a membrane potential of +40 mV (n = 20) and 419.4 ± 86 pA at +60 mV (n = 20).

Ion selectivity of the cAMP-stimulated current to Cl^- ions was confirmed by performing experiments with various concentrations of Cl^- in the bath solution (Fig. 5). In the symmetrical Cl^- solution, the reversal potential observed from the *I-V* curve was 0 mV and shifted to −20 and +20 mV when altering extracellular Cl^- concentration to 300 and 60 mM, respectively. Tail current recording was used to further analyze the Cl^- ion selectivity. Tail currents of the cAMP-dependent Cl^- current were recorded at 140 and 60 mM extracellular Cl^- concentrations to determine the selectivity of the current (n = 8). The reversal potential of the tail current was also shifted approximately 20 mV toward positive membrane potential when the extracellular Cl^- concentration was switched from 140 to 60 mM. These

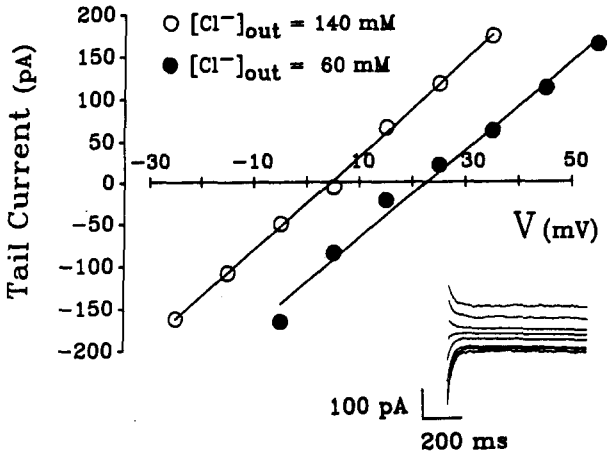

Fig. 5. Effect of extracellular Cl^- concentrations on the reversal potential of the cAMP-dependent Cl^- whole-cell current measured by the relaxation potential recording. The membrane potential was depolarized from a holding potential of −100 to +60 mV and then dropped to −50 mV in 10 mV decrements. Current-voltage relationships of the tail current are obtained in bath solutions with 140 mM Cl^- (open circles) and 60 mM Cl^- (filled circles). For low extracellular Cl^- experiments, bath Cl^- was replaced by methanesulfonic acid and the current traces are illustrated in the inset with a reversal potential between 20 to 30 mV.

experiments showed that Cl^--selective anion channels are responsible for the current.

The current was inhibited by epithelial Cl^- channel blockers, such as DIDS, 9-AC and DPC. In a continuous recording in which on-off pulses from a holding potential of −100 to +60 mV were applied, the Cl^- conductance was increased after introducing forskolin into the bath solution. Figure 6*A* shows that 200 μM DIDS blocked more than 50% of the outward rectifier current. The blockade of DIDS is not reversible (*data not shown*). The peak conductance of the current was reached within 10 min and then inhibited by addition of 200 μM 9-AC in the bath (Fig. 6*B*). The effect of the other Cl^- channel blocker, DPC, on the current was also examined. The dose-response curve for DPC blocking is plotted in Fig. 6*C*. Data were collected from four or five independent experiments and fitted with the single site binding saturation equation with a half-inhibition concentration (IC_{50}) of 200 μM at a membrane potential of +60 mV.

The membrane permeability to Cl^- ions in single cells was also studied by measuring the fluorescence intensity of intracellular SPQ. MTAL cells were loaded with SPQ in hypotonic solution and then transferred to a continuously perfused chamber. Figure 7 shows the time courses of intracellular SPQ fluorescence upon extracellular Cl^- removal from the perfused bath solution in the absence of (control) and in the presence of 20 μM forskolin and 100 μM IBMX. The intensity ratio (F/F_0) provides a quantitative indication for changes of intracellular Cl^- activity. F/F_0 is the fraction of the total SPQ fluorescence signal (F_0) remaining after quenching by Cl^- ions. The F/F_0 value slowly increased and reached a steady-state following removal of extracellular Cl^- ions in control cells (open circles in Fig. 7). Addition of forskolin and IBMX in the perfusion chamber induced a very fast increase of SPQ fluorescence intensity within 2 min after removal of Cl^- from the bath. The increase in F/F_0 after stimulation with forskolin and IBMX suggests that there is a cAMP-dependent Cl^- efflux in the MTAL cell membrane.

Discussion

Ca^{2+}-Activated K^+ Current in MTAL Cells

A Ca^{2+}-activated K^+ current in MTAL cells has been identified and studied with respect to biophysics and regulation. Previous single channel studies have demonstrated that a large conductance K^+ channel in the apical membrane of MTAL cells can be activated by an increase in the intracellular Ca^{2+} (Guggino et al., 1987; Cornejo et al., 1989) or by membrane stretch in the presence of the extracellular Ca^{2+} (Taniguchi & Guggino, 1989). Our data suggest that the activity of the Ca^{2+}-activated K^+ channel at the whole-cell level stays higher when the membrane potential is depolarized to positive potentials. On the other hand, the K^+ channel spends more time in the open state when the Ca^{2+} concentration in the bath solution is increased in the presence of Ca^{2+} ionophore A23187 (Fig. 3). This study demonstrates as clearly as those single channel studies that the activation of the Ca^{2+}-activated K^+ current is dependent upon both membrane potential and intracellular Ca^{2+} concentration (Barrett et al., 1982). Tail current experiments demonstrate that reversal potentials are shifted 46 mV per *e*-fold change in the extracellular K^+ concentration, as would be expected for a K^+ equivalent potential. This provides evidence that the whole-cell current was carried by K^+ ions. In addition, the Ca^{2+}-activated K^+ current is inhibited by CTX, a specific channel blocker for the large conductance Ca^{2+}-activated K^+ channel (Miller et al., 1985). Our data are consistent with previous single channel studies in this cell lineage, and with the heterologous expressed K^+ current in *Xenopus* oocytes after injection of size-fractionated mRNA from MTAL cells (Lu, Montrose & Guggino, 1990).

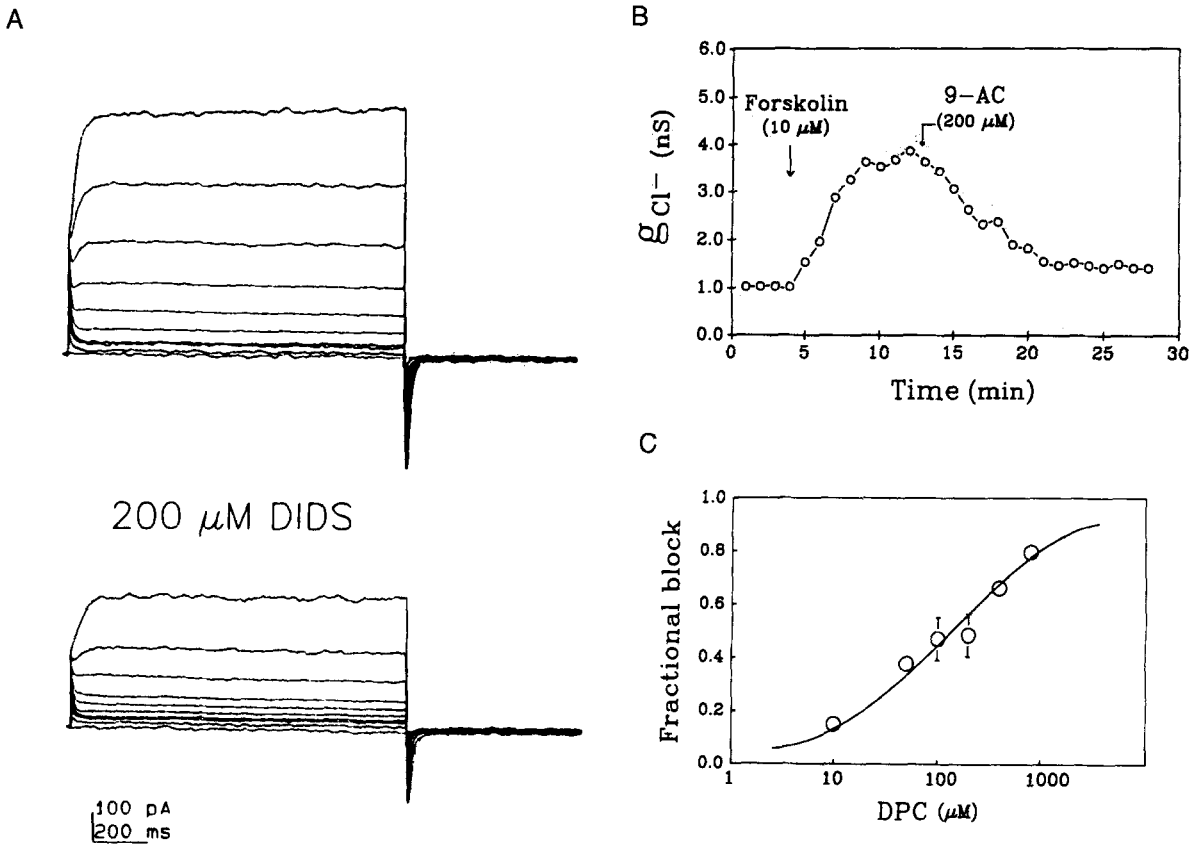

Fig. 6. Effect of Cl^- channel blockers on the cAMP-dependent Cl^- current. (*A*) Cl^- currents were recorded by depolarizing the membrane potential from +100 to −100 mV in 20 mV increments and inhibited by perfusion of 200 μM DIDS in the bath solution. (*B*) A continuous recording of cAMP-dependent current was inhibited by 200 μM 9-AC. The membrane potential was depolarized from −100 to +40 mV at 4 min intervals. (*C*) Dose-response curve of the Cl^- current blocked by DPC in the bath solution. Data represent mean ± SEM from at least four cells at each point. Some SEM bars fall within the dots. The fractional block of the current was calculated by $1 - I/I_0$, where I and I_0 represent currents measured in the presence of the blocker and in the absence of the blocker, respectively.

Cl^- Current in MTAL Cells

A Cl^-current was observed in MTAL cells when cAMP in the cell was stimulated by forskolin and IBMX. The stimulated Cl^- current has a very slow inactivation time course. In the symmetrical Cl^- concentration between intracellular and extracellular compartments, the *I-V* relationship showed an outwardly rectifying pattern. This is similar to the *I-V* relationships of cAMP-activated Cl^- currents in airway epithelial cells (McCann, Li & Welsh, 1989) and Ca^{2+}-activated Cl^- current in intestinal T84 epithelial cells (Cliff & Frizzell, 1990). As mentioned, a Cl^- conductance has been observed in the mammalian kidney medullary thick ascending limb of Henle's loop (TALH) from in vitro microperfusion experiments. The Cl^- conductance was enhanced by phosphorylation of the cAMP-dependent protein kinase (PKA) and was not inhibited by furosemide, an inhibitory reagent for Na^+-$2Cl^-$-K^+ cotransport (Schlatter & Greger, 1985). Epithelial Cl^- channel blockers such as 9-AC block the Cl^- conductance from both apical and basolateral membranes, suggesting that the Cl^- conductance may be carried by Cl^- channels in either the apical or basolateral membrane (Schlatter & Greger, 1985). Our data demonstrated that the cAMP-activated Cl^- current in MTAL cells is also sensitive to various epithelial Cl^- channel blockers, such as 9-AC, DPC or DIDS.

Previous experiments have suggested that the NaCl reabsorption and water dilution in medullary TALH are regulated by hormones. Our data demonstrate that the Cl^- current in MTAL cells is activated by forskolin and IBMX. Both forskolin and IBMX increase the intracellular cAMP by different mechanisms. Forskolin activates the adenylate cyclase to stimulate the intracellular cAMP and IMBX inhibits the phosphodiesterase to prevent the degradation

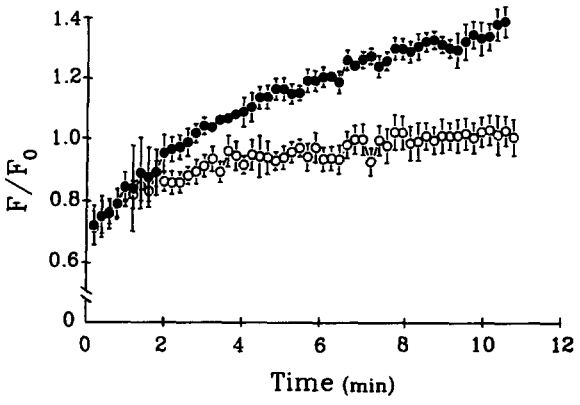

Fig. 7. Change in fluorescence of SPQ-loaded MTAL cells following stimulation of intracellular cAMP. The relative intensity of fluorescence in SPQ-loaded MTAL cells was measured by perfusion of the Cl^- free solution at zero min for the control (open circles) and for cells stimulated with 20 μM forskolin and 100 μM IBMX (filled circles). Data are presented as means ± SE for 26 control and 32 cAMP-stimulated MTAL cells. Values for cAMP-stimulated MTAL cells were significantly greater than values for control MTAL cells ($P < 0.05$).

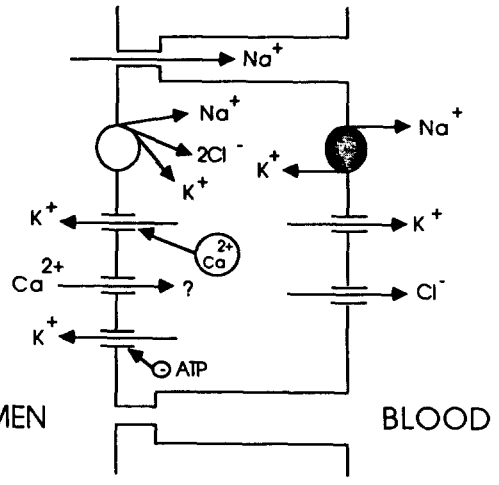

Fig. 8. Model for ion transport in MTAL epithelial cells. Basically, there are electroneutral cotransporters, ATP-sensitive K^+ channels and Ca^{2+}-activated K^+ channels in the apical membrane and Na^+-K^+-ATPase, K^+ channels and Cl^- channels in the basolateral membrane. Na^+ ion is also transported through those tight junction channels.

of the intracellular cAMP. The positive effects of forskolin and of IBMX indicate that the second messenger systems for the interaction of hormone-regulated receptors in cultured MTAL cells are still intact. It has been known that antidiuretic hormone (ADH) not only stimulates water reabsorption in the collecting duct in the kidney (Grantham & Burg, 1966), but it also appears to enhance the reabsorption of NaCl by an increased intracellular cAMP level in the kidney medullary TALH (Eberhard & Greger, 1985). PGE_2 has also been found to modulate the rate of NaCl reabsorption in medullary TALH (Fine & Trizna, 1977; Stokes, 1979). It has been proposed that the mechanism of PGE_2 modulation involves the interaction of PGE_2 with ADH in medullary TALH (Culpepper & Andreoli, 1983).

Finally, mechanisms involved in ion transport in MTAL cells are summarized in Fig. 8. Basically, there are the electroneutral and furosemide-sensitive $Na^+2Cl^-K^+$ cotransporter (Hebert et al., 1981*a*, 1986*a,b*; Greger et al., 1984), a Ca^{2+}-activated K^+ channel (Guggino et al., 1987; Cornejo et al., 1989), a K^+ channel regulated by intracellular ATP concentration (Bleich, Schlatter & Greger, 1990; Wang, Schwab & Giebisch, 1990; Wang et al., 1990; Wang, Sackin & Giebisch, 1992), and possibly a dihydropuridine-sensitive Ca^{2+} channel in the apical membrane (Montrose-Rafizadeh & Guggino, 1991). In the basolateral membrane, Na^+ is actively pumped out of the cell by the Na^+-K^+-ATPase (Burg & Green, 1973; Rocha & Kokko, 1973; Hebert et al., 1981*a*). A recent study has suggested that the Na^+-K^+-ATPase is parallel to the activity of ATP-sensitive K^+ channels by the coupling modulator-ATP in renal tubule cells (Tsuchiya et al., 1992). A K^+ channel is also present in the basolateral membrane of MTAL cells (Wang et al., 1992). A previous single channel study has suggested that the Cl^- channel is located in the basolateral membrane of the rat TAL segment (Greger, Bleich & Schlatter, 1990). Our whole-cell patch-clamp data suggest that a cAMP-regulated Cl^- conductive channel is present in cultured MTAL epithelial cells; this channel may play an important role in facilitating the exit of reabsorbed Cl^- ions from MTAL cells.

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