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Characterization of K^+ Channels in the Basolateral Membrane of Rat Tracheal Epithelia

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Abstract. To study K^+ channels in the basolateral membrane of chloride-secreting epithelia, rat tracheal epithelial monolayers were cultured on permeable filters and mounted into an Ussing chamber system. The mucosal membrane was permeabilized with nystatin (180 μg/ml) in the symmetrical high K^+ (145 mM) Ringer solution. During measurement of the macroscopic K^+ conductance properties of the basolateral membrane under a transepithelial voltage clamp, we detected at least two types of K^+ currents: one is an inwardly rectifying K^+ current and the other is a slowly activating outwardly rectifying K^+ current. The inwardly rectifying K^+ current is inhibited by Ba^{2+}. The slowly activating K^+ current was potentiated by cAMP and inhibited by clofilium, phorbol 12-myristae 13-acetate (PMA) and lowering temperature. This is consistent with the biophysical characteristics of I_{SK} channel. RT-PCR analysis revealed the presence of I_{SK} cDNA in the rat trachea epithelia. Although 0.1 mM Ba^{2+} only had minimal affect on short-circuit current (I_{sc}) induced by cAMP in intact epithelia, 0.1 mM clofilium strongly inhibited it. These results indicate that I_{SK} might be important for maintaining cAMP-induced chloride secretion in the rat trachea epithelia.

Key words: Trachea — K^+ channels — Basolateral membrane — cAMP

Introduction

Transepithelial Cl^- secretion which occurs in the airway epithelium is dependent on two types of ion channels, Cl^- channels at the apical membrane and K^+ channels at the basolateral membrane (Welsh, 1987). Cl^- enters the cells across the basolateral membrane via an electrically neutral Na-K-2Cl cotransporter. Cl^- then exits across the apical membrane through Cl^- channels. Na^+ entering the cell is recycled by the basolateral Na-K-ATPase. Recycling of K^+ through basolateral K^+ channels serves two important functions (Smith & Frizzell, 1984; Welsh, 1987). First, it prevents accumulation of K^+ that could cause ceil swelling. Second, K^+ efflux hyperpolarizes the cell, thereby maintaining the driving force for Cl^- exit across the apical membrane. Thus an increase or decrease in the apical Cl^- conductance is followed closely by an increase or decrease in the basolateral K^+ conductance (Smith & Frizzell, 1984; Shorofsky, Field & Fozzard, 1983). This coordination of the limiting membrane conductances is critical to the secretory functions of the cell. A similar relationship between transport rates and basolateral K^+ conductance is evident in Na^+-absorbing epithelia (Davis & Finn, 1982; Diamond, 1982).

Although recent studies have suggested the presence of two types of K^+ channels including inwardly rectifying K^+ channels in tracheal epithelia (Butt, Clapp & Frizzell, 1990; McCann & Welsh, 1990), their molecular identities are still unknown. Furthermore, information about which K^+ channels are activated during Cl^- secretion is sparse.

In this study, we cultured tracheal epithelial cells on permeable supports and used nystatin to permeabilize the apical membranes of rat tracheal epithelial cells. This procedure functionally isolates the basolateral membrane conductance. During measurement of the macroscopic K^+ current of the basolateral membrane under a transepithelial voltage clamp, we detected at least two types of K^+ current. One is an inwardly rectifying K^+ current and the other is outwardly rectifying K^+ current that is acti-

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vated slowly. The latter was important for maintaining cAMP-induced Cl^- secretion.

Materials and Methods

Materials

LHC-8 medium was purchased from Biofluids (Rockville, MD), 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), $BaCl_2$, glybenclamide, tetraethylammonium chloride (TEA), CsCl, nystatin, antibiotics and fetal bovine serum from SIGMA (St. Louis, MO), clofilium from RBI and BAPTA/AM from Molecular Probes (Eugene, OR). Glybenclamide, BAPTA/AM and nystatin were dissolved in DMSO. The final concentration of DMSO in Ringer solution was <0.2% which does not have any effect on I_{sc}.

Primary Cell Culture

The isolation and culture of rat tracheal epithelia was done as described previously (Schwiebert et al., 1995). Briefly, male Wistar rats were anesthetized. Freshly excised tracheas were incubated at 4°C for 18–24 hours in a CaMg-free, serum-free modified Eagle's minimum essential medium (MEM) containing 0.1% protease XIV (Sigma), and 50,000 U/ml penicillin and 50,000 μg/ml streptomycin (Gibco-BRL solution of both antibiotics). The epithelial cells were removed from the airways by scraping the epithelial surface of the filleted tracheas with a sterile cell scraper, and the cells were recovered by centrifugation. The cells were then washed 3× with fresh MEM medium (*see above*) containing 10% fetal bovine serum (Gibco-BRL) to neutralize the protease and 1× with LHC-8 medium (Biofluids) containing 5% fetal bovine serum (Gibco-BRL) and 50,000 U/ml penicillin and 50,000 μg/ml streptomycin. After the final wash, the cells were resuspended in LHC-8 medium, and final cell suspension (about 10^6 cells) was added to each permeable filter support (12 mm SNAPWELL, Costar, Cambridge, MA). The cells were incubated in an atmosphere of 5% CO_2-95% air at 37°C in the LHC-8 medium containing 5% fetal bovine serum (Gibco-BRL) and 50,000 U/ml penicillin and 50,000 μg/ml streptomycin. Cells were cultured for 6–10 days before insertion into a modified, circulating Ussing chamber system constructed to accept SNAPWELL filters (World Precision Instruments, Sarasota, FL). Characteristically, transepithelial resistance, measured by EVOM epithelial ohmmeter or by Ohm's law after steady-steady voltage and current were recorded after recording was begun for a particular monolayer, was between 1,000 and 2,000 Ω/cm^2.

Electrophysiology

Transepithelial electrophysiologic measurements were performed in a modified Ussing chamber constructed to accept SNAPWELL filter (World Precision Instrument, Sarasota, FL). I_{sc} was measured with a DVC-1000 voltage/current clamp (World Precision Instrument) with a voltage clamp mode. The filters which are above 1800 Ohm/cm^2 in transepithelial resistance measured by EVOM Epithelial Ohmmeter (WPI, Sarasota, FL) were used for all experiments. The bath solution in intact monolayers was a nominally bicarbonate-free Ringer's solution that was composed of (in mM): 140 NaCl, 2.3 K_2HPO_4; 0.4 KH_2PO_4; 1.2 $CaCl_2$; 1.2 $MgCl_2$; 10 HEPES; and 10 glucose (pH 7.4). Potassium current flowing through the basolateral membrane was measured in a high-K^+ gluconate solution which bathed both the apical and serosal chambers. High-K^+ gluconate solution was made by substitution K^+ gluconate for NaCl in the normal Ringer solution. Nystatin (180 μg/ml) was added to the luminal side to permeabilize the apical membrane in the symmetrical high-K^+ solution. Routinely, 10–20 min was necessary to permeabilize fully the mucosal membrane to impose symmetrical or asymmetrical ion gradients on the basolateral membrane. To prepare a voltage-current curve of channels, currents were elicited by 0.8 sec or 8 sec test potentials from −100 to +100 mV in 20 mV increments in the symmetrical or asymmetrical high K^+ gluconate solution.

Cloning of I_{SK} Channel from Rat Trachea

Total RNA was extracted from tracheal mucosa as previously described (Jung et al., 1994) and the cDNA was synthesized using oligo dT primers. Based on the nucleotide sequence for the rat I_{SK} channel (Takumi, Ohkubo & Nakanishi, 1988), a pair of oligonucleotide primers were synthesized. Sense primer was 5′aggatggccctgtccaattcca-3′ and antisense primer was 5′-gttcatgacagtggcttcagtt-3′. PCR was performed for 35 cycles (94°C, 1 min; 52°C, 1 min; 72°C, 1 min) in 100 μl of a reaction mixture containing 20 pmole of each primer. PCR products were cloned into TA cloning vector (Invitrogen,) by standard techniques. The nucleotide sequence of the inserted region of the plasmid was determined by the dideoxy chain-termination method (Sanger, Nicklen & Coulson, 1977) with a modified T7 DNA polymerase (Sequenase).

Data Analysis

Data are presented as mean ± SE. When it was necessary, statistical comparison between different groups were made using unpaired-Student's *t* test. $P < 0.05$ was considered significantly different from the control.

Results

The basal transepithelial potential difference (PD) of the intact tracheal epithelial monolayer was 24.23 ± 1.79 mV (serosal side ground) and the basal short-circuit current (I_{sc}) was 9.55 ± 0.68 μA ($n = 105$) in the symmetrical normal Ringer solution. To record basolateral K^+ current with eliminating other ion currents, both mucosal and serosal bath solution were filled with symmetrical high K^+ gluconate solution (K^+ 145 mM). The mucosal membrane was treated with nystatin (180 μg/ml) for 10–20 min and then currents were recorded by a voltage clamp. Figure 1*A* shows the representative trace of K^+ current through the basolateral membrane of rat tracheal monolayer at 37°C and 28°C. Holding potential was 0 mV. Traces elicited by steps to −100, −80, −60, −40, +40, +60 and +100 mV are shown. At 37°C inward rectifying K^+ current was observed, and slowly activating outward current was unexpectedly observed at +80 and +100 mV of depolarization pulses. The current recorded at 28°C showed inactivation of inwardly rectifying K^+ current shortly after channel activation within 500 msec. The current was recorded within 1 min after

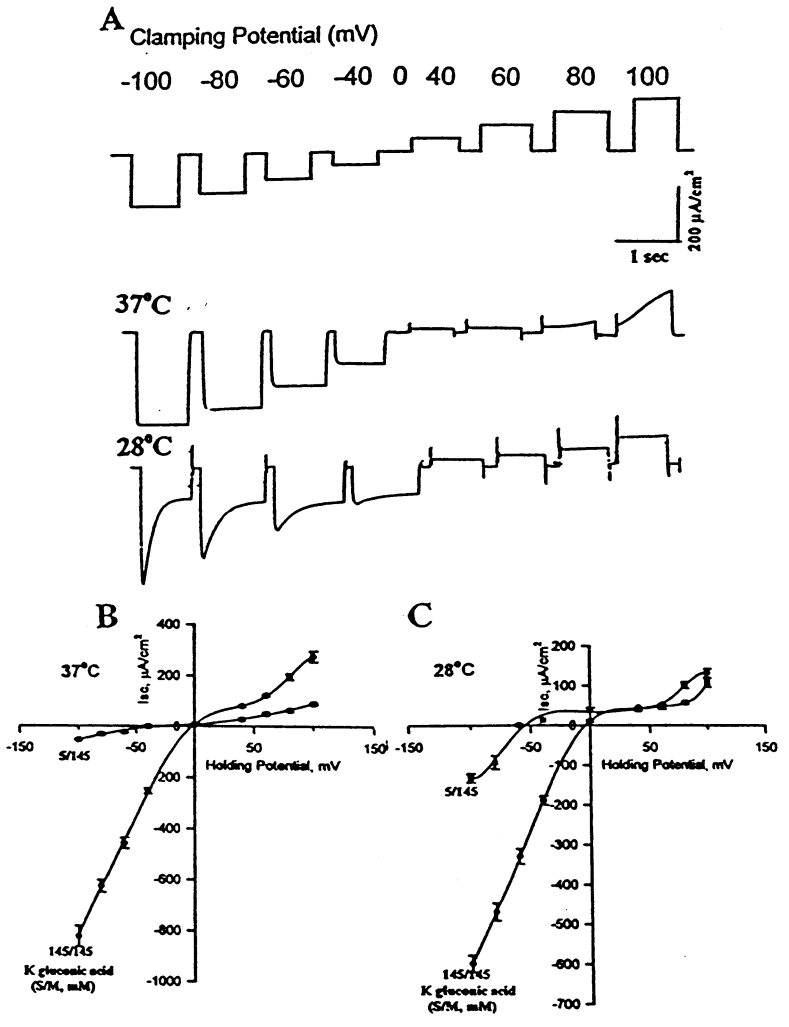

Fig. 1. Inwardly rectified basolateral K^+ current in rat tracheal epithelia after permeabilization of mucosal membrane. (*A*) Representative currents at different clamping voltages applied after nystatin (180 μg/ml) permeabilization of the mucosal membrane at 37°C and 28°C. Currents were elicited by 0.8-sec clamping pulses from a holding voltage of 0 mV; pulses were from −100 to 100 mV. Bilateral K^+ gluconate (145 mM) solution was used. (*B* and *C*) Corresponding *I–V* plots of inwardly rectifying K^+ current at 37C and 28°C. Current was recorded according to the different K^+ concentrations in the serosal bath. K^+ gluconate concentration of the serosal bath was reduced by substituting mannitol for K^+ gluconate while keeping 145 mM K^+ in the mucosal bath. Instantaneous currents recorded at 28°C were plotted for (*C*). All values are mean ± SEM ($n = 12$)

changing 37°C solution to 28°C solution. This inactivated current could not be restored even after readministration of the 37°C solution. Fig. 1 *B* and *C* show the *I–V* relationship of K^+ currents according to the different K^+ concentrations in the serosal bath at 37°C and 28°C, respectively. K^+ gluconate concentration of the serosal bath was reduced by substituting mannitol for potassium gluconate. We noticed that the slowly activating outward current at +80 and +100 mV clamping potential was not observed at 28°C, which suggests slowly activating outward current may reflect different channel recording from inwardly rectifying current. Therefore, further characterization of the slowly activating outward K^+ current was done. At first we tested whether Ba^{2+} inhibits the inward K^+ current. Ba^{2+} (0.1 mM) was added to a serosal bath solution while +100, 0 and −100 mV voltage clamping were being applied alternately. As shown in Fig. 2, 0.1 mM Ba^{2+} strongly inhibited the inwardly rectifying current without affecting the outward current. Therefore, biophysical characterization of slowly activating outward K^+ current was done in the

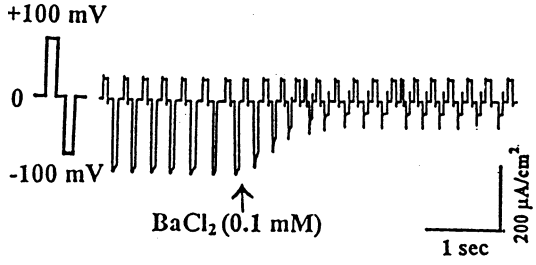

Fig. 2. Inhibition of inwardly rectifying K^+ current by serosal Ba^{2+} in rat tracheal epithelia after permeabilization of mucosal membrane. Currents were recorded in the presence of Ba^{2+} (0.1 mM) at the serosal bath solution after nystatin (180 μg/ml) permeabilization of the mucosal membrane at 37°C, while +100, 0 and −100 mV pulses were being applied alternatively. Bilateral high K^+ (145 mM) solution was used.

presence of 0.1 mM Ba^{2+}. Figure 3 shows the representative trace of K^+ current through the basolateral membrane at 37°C in the presence of serosal 0.1 mM Ba^{2+}. Currents were recorded by voltage clamp in the symmetrical high K^+ solution after mucosal permeabilization

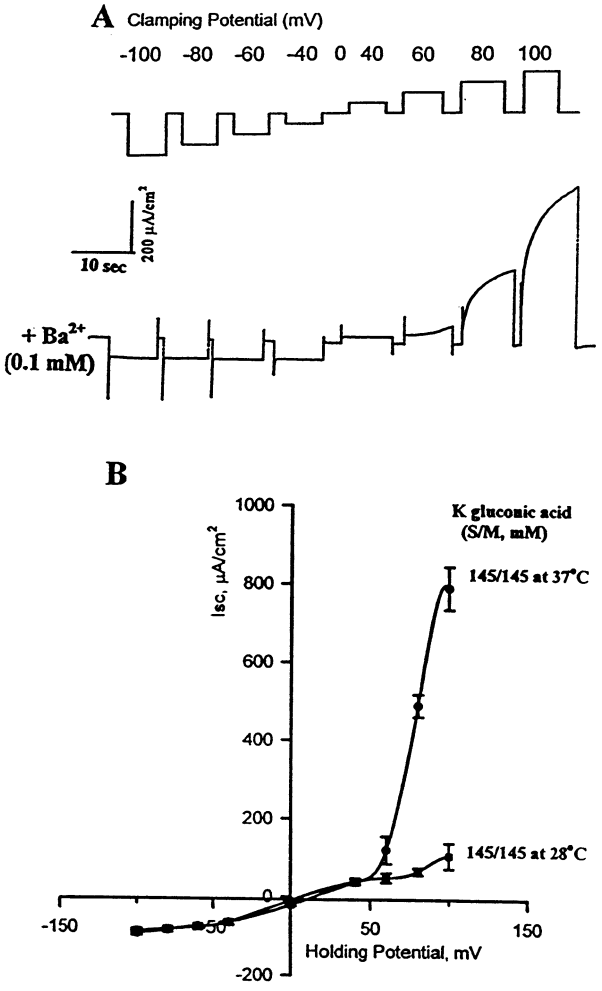

Fig. 3. Outwardly rectified basolateral K^+ current in rat tracheal epithelia after permeabilization of mucosal membrane. (*A*) Representative traces of outwardly rectified basolateral K^+ current. In the presence of seroal Ba^{2+} (0.1 mM), 8 sec different clamping pulses from a holding voltage of 0 mV were applied after nystain (180 μg/ml) permeabilization of the mucosal membrane at 37°C. Bilateral K^+ gluconate (145 mM) solution was used. Note different time scale from that of Fig. 1*A*. (*B*) *I–V* plots of outwardly rectified basolateral K^+ current at 37°C and 28°C. All values are mean ± SEM ($n = 12$)

with nystatin. Holding potential was 0 mV. Traces elicited by steps to −100, −80, −60, −40, +40, +60 and +100 mV are shown. All the experimental conditions of Fig. 3 are the same as those in Fig. 1 *A* except the serosal Ba^{2+} and the slow chart speed. At this condition the slowly activating current was evident and current-voltage relationship showed a strong outward rectification. To test if the current is proton conducting, the current was measured in the solution in which K^+ gluconate was replaced by Na^+ gluconate. In the Na^+ gluconate solution the outward K^+ current was significantly decreased. The permeability ratio of K^+/Na^+ was 0.21 ± 0.006 ($n = 3$). The outward K^+ current was strongly inhibited by lowering temperature to 28°C, whereas the amplitude of instantaneous inwardly rectifying K^+ current was slightly affected (Fig. 1*C* and 3*B*). The characteristic feature of slowly activating and strong outwardly rectifying current indicates that this current may be from mink (I_{SK}) channel which was first cloned from kidney epithelial cell (Takumi, Ohkubo & Nakanishi, 1988).

To examine this possibility, we tested the effect of clofilium, the most potent inhibitor of I_{SK} current (Folander et al., 1990; Attali et al., 1992). The application of high voltage clamping is necessary to record I_{SK} current in symmetrical high K^+ gluconate solution (Fig. 3). We could not observe the inhibitory effect of clofilium under these experimental conditions. To record I_{SK} current at the range of the lower test potential, 140 mM sodium gluconate/5 mM K^+ gluconate-solution was used as the serosal bathing solution while maintaining high K^+ gluconate solution in the apical bathing solution. As shown in Fig. 4 *A*, pretreatment of clofilium (0.1 mM) for 10 min strongly inhibited this outward current without affecting reversal potential. The disappearance of the blocking effect of the clofilium under symmetrical high K^+ gluconate solution suggests the possibility that clofilium and K^+ compete for the same site in the pore. Further experiments are needed to address the question. However, nifedipine (0.1 mM), diltiazem (0.1 mM), quinidine (0.1 mM), Ba^{2+} (0.1 mM), TEA (0.1 mM), glybenclamide (0.1 mM), Ni^+ (0.1 mM), Cs^+ (0.1 mM) and DPC (0.1 mM) little affected the slowly activating outward K^+ current. BAPTA/AM (50 μM) and ionomycin (1 μM) did not affect the current either (*data not shown*). As it has been reported that I_{SK} is potentiated by PKA (Blumenthal & Kaczmarek, 1992) and is inhibited by PKC (Busch et al., 1992), we measured the effect of cAMP and PMA on the K^+ conductance. As shown in Fig. 4 *B*, the pretreatment of 0.2 mM 8-Br-cAMP for 20 min potentiated K^+ conductance during depolarization without affecting reversal potential, whereas PMA (1 μM, 20 min) strongly inhibited this K^+ conductance.

To confirm the presence of I_{SK} in rat tracheal epithelia, we did RT-PCR of tracheal RNA. Recently, I_{SK} cloned from heart and uterus has the exactly same sequence with that of kidney I_{SK} cDNA (Folander et al., 1990). This suggested that I_{SK} in a variety of tissues has the identical sequence. Therefore, we used primers which were designed from the sequences of kidney I_{SK} cDNA to I_{SK} cDNA of tracheal epithelia. From RT-PCR the expected size of PCR products was amplified and DNA sequencing revealed that the sequences of the I_{SK} cDNA in the trachea is identical with the known I_{SK} cDNA (Fig. 5).

To evaluate the physiological role of I_{SK}, the effects of Ba^{2+}, PMA and clofilium on I_{sc} induced by cAMP were observed in the intact monolayer filled with the symmetrical Normal Ringer solutions in the both serosal

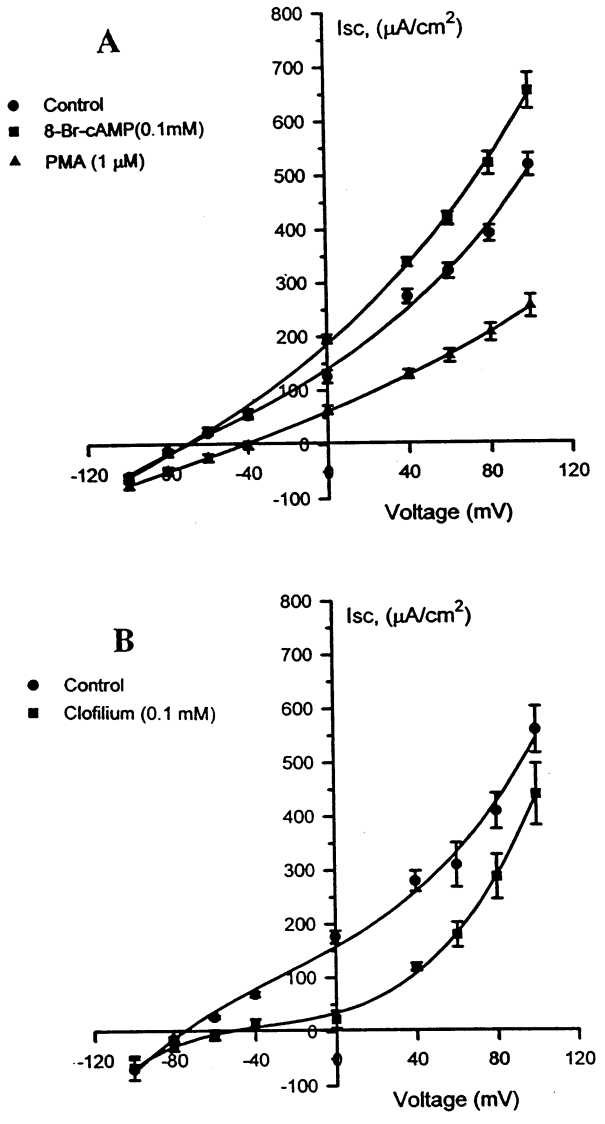

Fig. 4. (*A*) and (*B*). Effect of clofilium, 8-Br-cAMP and PMA on outwardly rectified K^+ currents in rat tracheal epithelia after permeabilization of mucosal membrane. Currents were recorded at different clamping pulses applied after nystatin (180 μg/ml) permeabilization of the mucosal membrane at 37°C in the presence or absence of each drug. Asymmetrical K^+ gluconate (5/145 mM:Serosal/Mucosal) solution was used. All values are mean ± SEM ($n = 4$)

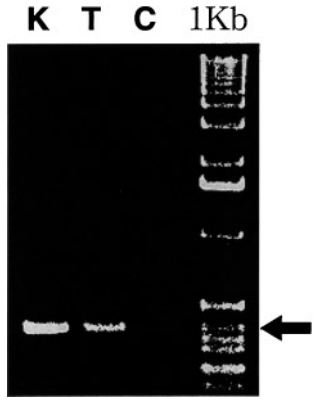

Fig. 5. RT-PCR amplification of I_{SK} cDNA from rat trachea. One μg of total RNA which was isolated from rat tracheal monolayers (T) or kidney (K) were reverse transcribed using oligodT primers and amplified using I_{SK} primers. PCR products were analyzed by electrophoresis using 1.5% agarose gel. As the negative control for the PCR reactions (*C*) water was added instead of tissue RNAs for reverse transcription.

and apical bath (Fig. 6). Although 0.1 mM Ba^{2+} minimally inhibited 8-Br-cAMP induced I_{sc}, the pretreatment of 1 μM PMA and 0.1 mM clofilium inhibited it by more than 80%. This suggests significant participation of I_{SK} during cAMP-mediated Cl^- secretion in the intact tracheal epithelia.

Discussion

In this study, we demonstrated that at least two types of K^+ currents are present in the basolateral membrane of rat tracheal epithelia. Both K^+ currents could be recorded separately in the permeabilized monolayer with different biophysical characteristics. One is an inwardly rectifying K^+ current. The presence of Ca^{2+}-sensitive inward rectifying K^+ current has been reported for dog trachea (McCann et al., 1990) and T84 cells (Tabcharani et al., 1994). Yet each had different electrophysiological properties from the classical Ca^{2+}-activated K^+ channel. In our experiments the inwardly rectifying K^+ current is highly sensitive to Ba^{2+}, which is a typical characteristic of the IRK family (Fig. 2).

The unexpected slowly activating outward K^+ current that we observed in the rat tracheal epithelia was the primary focus of this study. The slow activation by depolarization, the inhibition by clofilium, PMA and low temperature (Busch & Lang, 1993), and the activation by cAMP are consistent with the biophysical characteristics of I_{SK}. The presence of I_{SK} in tracheal epithelia is confirmed by the RT-PCR. cDNA which is responsible for I_{SK} was first cloned in 1988 from renal tissue and subsequent localization study revealed that it is also present in the heart, uterus, submandibular gland and pancreas (Takumi et al., 1988; Folander et al., 1990). However, the physiological role of the channel is still unresolved. In this study, we observed that the inhibition of I_{SK} current by clofilium strongly affected cAMP-stimulated chloride secretion indicating that I_{SK} current is important for maintaining Cl^- secretion during cAMP stimulation, perhaps by maintaining the resting potential. A similar roles of I_{SK} current has been suggested for the proximal tubule of the kidney (Gruenberg & Clague, 1992), colon (Attali et al., 1995) and heart (Varnum et al., 1993), although depolarization in those tissues resulted from different mechanisms. Na^+ absorption has been reported to be important for the tracheal epithelial function.

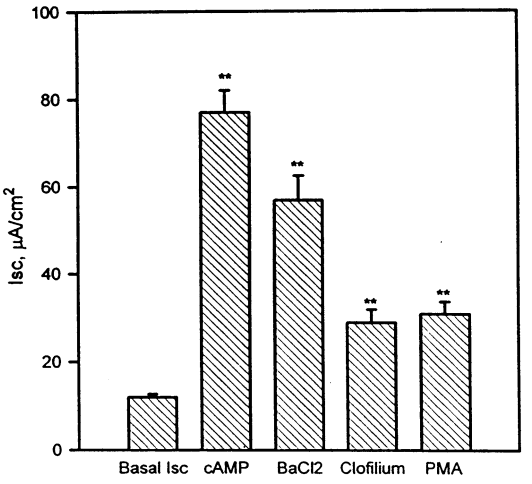

Fig. 6. Effect of $BaCl_2$ (0.1 mM), clofilium (0.1 mM) and PMA (1 μM) on 8-Br-cAMP-induced I_{sc} in intact tracheal monolayers. I_{sc} was measured in bilateral NaCl (140 mM) Ringer solutions. The reagents were pretreated to the serosal bath solution for 10 min before adding 0.2 mM 8-Br-cAMP. All values are mean ± SEM ($n = 4$).

Amiloride sensitive-Na^+ current contributed in the basal I_{sc} in this system, but there was no significant increase of amiloride-sensitive Na^+ current in the presence of cAMP in this system. We did not do further experiments on the relation of cAMP activated-Na^+ current and I_{SK}. However, it can be expected that I_{SK} may be stimulated if Na^+ absorption is stimulated in rat tracheal epithelia.

Whether or not mink protein (the name of protein which is responsible for I_{SK} current) itself is a K^+ channel is still controversial. Although reports using site-directed mutagenesis seem to indicate that the mink is a K^+ channel (Takumi et al., 1991; Goldstein & Miller, 1991), Attali et al. (1993) demonstrated that *Xenopus* oocyte injected with high concentrations of cRNA of mink can induce a ''phopholemman-like'' conductance current in addition to the I_{SK} current. Furthermore, CHIF (channel-inducing factor), which was cloned from rat colon, induces I_{SK}-like current in oocytes, although it has a completely different amino acid sequence from that of I_{SK} (Attali et al., 1995). One possibility is that CHIF and I_{SK} are regulatory proteins that activate the same endogenous oocyte K^+ channel.

Several investigators reported that K^+ current activated during Cl^- secretion by cAMP was also inhibited by Ba^{2+} (Butt et al., 1990; McCann & Welsh, 1990). However, in this experiment we observed an I_{SK} current in the presence of Ba^{2+}. The discrepancy of these results can be explained by differences in the concentration of Ba^{2+} used. We used only 0.1 mM Ba^{2+}, whereas others used more than 2 mM. 0.1 mM Ba^{2+} completely inhibited inwardly rectifying K^+ current in this experiment. However, we cannot exclude the possibility of the presence of another K^+ channel which is regulated by changes in intracellular Ca^{2+} concentration, because clofilium could not completely inhibit I_{sc} by cAMP and 0.1 mM Ba^{2+} partially inhibited it (Fig. 5). In this study, an I_{SK} current was not affected by agents that manipulate Ca^{2+}-signaling pathway. Although an increase in $[Ca]_i$ has been reported to increase I_{SK} current in oocytes at 22°C (Busch et al., 1992), a subsequent study showed that I_{SK} current is temperature-sensitive and at higher temperatures the role of $[Ca]_i$ is negligible (Busch & Lang, 1993).

In summary, we identified two K^+ currents in the basolateral membrane of rat trachea based on electrophysiological characteristics. The slow activation of the outwardly rectifying current resembled I_{SK}, and the presence of I_{SK} cDNA was confirmed by RT-PCR. This current has an important role for maintaining Cl^- secretion by cAMP.

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