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Intracellular calcium regulates basolateral potassium channels in a chloride-secreting epithelium

(patch clamp/quin-2/tracheal epithelium/epinephrine)

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ABSTRACT **The two individual cell membranes of epithelia are functionally coupled, so that changes in apical membrane conductance are paralleled by changes in basolateral K^+ conductance. However, the signal that regulates basolateral K^+ conductance, thereby coupling the two membranes, is unknown. We tested the hypothesis that the cellular calcium concentration, $[Ca^{2+}]_c$, may regulate basolateral K^+ conductance in canine tracheal epithelium, a Cl^--secreting epithelium that shows marked membrane coupling. Three findings support the hypothesis. First, the intracellular Ca^{2+} antagonist 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) attenuated the secretory response. Second, the secretagogue epinephrine increased $[Ca^{2+}]_c$, as measured with quin-2. Third, we found a K^+ channel that was activated by Ca^{2+} on the cytosolic side of the membrane. Thus, cytosolic Ca^{2+} regulates the basolateral K^+ conductance and may be the signal responsible for functional coupling of the two cell membranes.**

Vectorial ion transport by epithelia is made possible by the segregation of the individual ion transport processes (channels, carriers, and pumps) to either the apical or basolateral membrane. However, ion transport processes at opposite sides of the cell do not function independently: there is a functional linkage between the membranes, so that changes in the rate of ion transport at one membrane are coupled to changes in the rate of ion transport at the opposite membrane (1). This coupling, or coordination of ion transport at the two cell membranes, has been most widely appreciated in Na^+-absorbing epithelia (2, 3). In such epithelia, the apical membrane contains an entry step (channel or carrier) that allows Na^+ to enter the cell passively, down a favorable electrochemical gradient. The basolateral membrane contains the Na^+ pump (Na^+,K^+-ATPase) that actively extrudes Na^+ and a K^+ conductance that recycles K^+. When the Na^+ conductance of the apical membrane is altered, parallel changes in basolateral K^+ conductance follow (4–6).

Similar interactions between the conductance of the two cell membranes occur in Cl^--secreting epithelia. In Cl^--secreting epithelia the apical membrane is Cl^- conductive and the basolateral membrane is K^+ conductive. The mechanism of active Cl^- secretion involves neutral cotransport of Cl^- and Na^+ into the cell across the basolateral membrane; uphill Cl^- entry is thus energized by Na^+ movement down its electrochemical gradient. Chloride then exits passively, down its electrochemical gradient across the Cl^--conductive apical membrane. Sodium that enters the cell with Cl^- is extruded via the basolateral Na^+,K^+-pump, while K^+ that enters in exchange for Na^+ is recycled back across the K^+-conductive basolateral membrane. When apical Cl^- conductance increases or decreases, the magnitude of the basolateral K^+ conductance follows in parallel (7–9).

The physiologic significance of coupling basolateral K^+ conductance to changes in apical conductance is twofold. First, it prevents a large, sustained depolarization of the cell, which would otherwise diminish the electrical driving forces required for passive ion movement at the apical membrane. Second, it prevents large changes in cell volume, which would otherwise occur when the rate of transport changes. Thus, the transport-related regulation of basolateral K^+ conductance is critical not only to the ion transport functions of the cell but also to cell viability.

Despite their importance, the factors that regulate the basolateral K^+ conductance so that it parallels apical membrane conductance are unknown. We have previously shown that the K^+ conductance is neither directly coupled to Na^+ pump activity nor regulated simply by membrane voltage (10). The present study tested the hypothesis that the basolateral K^+ conductance is regulated by the cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_c$. This hypothesis would be consistent with reports of Ca^{2+}-activated K^+ conductances in a variety of excitable cells (11), in exocrine glands (12), and in the apical membrane of cortical collecting tubule cells (13). However, there is no direct evidence to support this hypothesis in tracheal epithelium; the only indirect evidence is the observation that the Ca^{2+} ionophore A-23187 stimulates Cl^- secretion (14).

MATERIALS AND METHODS

Intact canine tracheal epithelia were obtained and mounted in Ussing chambers, as previously described (9). For studies in Ussing chambers the bathing solution contained the following, in mM: 118.9 NaCl, 20.4 $NaHCO_3$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 glucose (pH 7.4 at 37°C when bubbled with 95% O_2/5% CO_2). Indomethacin (1 μM) and amiloride (100 μM) were present in the mucosal bathing solution throughout to minimize the baseline rate of Cl^- and Na^+ transport (7, 15, 16); thus changes induced by secretagogue are due to changes in the rate of Cl^- secretion.

Primary cultures of canine tracheal epithelium were prepared and grown as previously described (17, 18). In culture, these cells form epithelial sheets that retain most of the morphological characteristics and the hormonally responsive ion transport properties of the intact trachea. The culture medium consisted of a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient F-12 medium with 5% fetal calf serum, nonessential amino acids (KC Biological, Lenexa, KS), and insulin at 5 μg/ml. For studies designed to measure $[Ca^{2+}]_c$, primary cultures of tracheal epithelium were grown in T-75 tissue culture flasks (Becton Dickinson, Labware, Oxnard, CA) and used 4–10 days after plating. For patch clamp studies cells were grown in 35-mm Petri dishes

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Abbreviation: TMB-8, 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride.

at a seeding density of 2×10^4 cells cm^{-2} for 1–3 days before use. All culture surfaces were coated with human placental collagen (17, 18).

To measure $[Ca^{2+}]_c$ we used the fluorescent Ca^{2+} indicator quin-2 (19). To load quin-2 into the cells, the culture medium was removed and replaced with a HCO_3^-/Ringer's solution that contained 50 μM quin-2 acetoxymethyl ester (Behring Diagnostic, San Diego, CA) for 30 min. The cells were then rinsed and removed from the culture vessel by incubation in phosphate-buffered saline containing 0.25% trypsin for 20 min. After washing and spinning, the cells were incubated in the regular culture media for 1 hr before use. The cells were then suspended at 2.5×10^6 cells ml^{-1} in Hepes-buffered Ringer's solution and placed in a quartz cuvette that contained a motor-driven rotary mixing unit. Fluorescence was measured with a Perkin–Elmer fluorescence spectrophotometer: 340-nm excitation wavelength, 5.5-μm slit width, and 480-nm emission wavelength, 22-μm slit width. The presence of intracellular quin-2 was confirmed by finding the characteristic peak excitation wavelength (340 nm) of the free acid form. Determinations of $[Ca^{2+}]_c$ were made by using techniques similar to those described by Tsien *et al.* (20). The fluorescence of quin-2-loaded cells was measured during a baseline period and then the cell membranes were disrupted with digitonin (10 μM), allowing a saturating concentration of Ca^{2+} (1 mM) to yield maximal quin-2 fluorescence. Minimum quin-2 fluorescence was obtained by quenching with Mn^{2+} (0.5 mM) or chelating Ca^{2+} with EGTA (2 mM).

To examine single K^+ channel currents, we used the patch-clamp technique (21). Our technique for constructing pipettes and seal formation is similar to that described by Hamill *et al.* (21). We used a Dagan model 8900 patch clamp amplifier (Minneapolis, MN). Currents were filtered at 500–700 Hz by an eight-pole Bessel filter (to eliminate high-frequency noise that distorted the tracings) and recorded on a strip chart recorder (model 220, Gould, Cleveland, OH). The 90% rise time for a 2-cm deflection on the strip chart recorder was 3.5 msec. Results were analyzed by hand. Typical seal resistance was 10–60 GΩ. During seal formation and when recordings were being made in the cell-attached mode, bath solution contained the following, in mM: 140 Na^+, 5 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, and 144.8 Cl^-. All bath and pipette solutions contained 10 mM Hepes, buffered to pH 7.4. Experiments were performed at room temperature (21–23°C).

RESULTS

Effect of TMB-8. Our first experiments were designed to determine if a purported intracellular Ca^{2+} antagonist would diminish the Cl^--secretory response of trachea. 8-(Diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) has been reported to stabilize intracellular Ca^{2+} and/or inhibit Ca^{2+}-dependent processes in a variety of nonepithelial cells (22–24). Six pairs of epithelia were studied: TMB-8 (100 μM) was added to both bathing solutions for 20 min in one tissue, and the second tissue served as a control. Subsequent addition of epinephrine (10 nM) to the submucosal solution increased the short-circuit current by 44 ± 8 $\mu A \cdot cm^{-2}$ (mean ± SEM) in control tissues but only 23 ± 6 $\mu A \cdot cm^{-2}$ in the TMB-8-treated tissues ($P < 0.05$ by paired *t* test). TMB-8 also attenuated the epinephrine-induced increase in transepithelial conductance: conductance increased 1.1 ± 0.1 $mS \cdot cm^{-2}$ in control tissues but only 0.6 ± 0.1 $mS \cdot cm^{-2}$ in TMB-8-treated tissues. These results are consistent with the idea that Ca^{2+} plays a regulatory role in the secretory process. However, the evidence must be considered indirect because this agent may have more than one effect.

Measurement of $[Ca^{2+}]_c$. If Ca^{2+} is a physiologically important regulator of the K^+ conductance, then the intracellular Ca^{2+} concentration $[Ca^{2+}]_c$ should increase after addition of secretagogue. By measuring the fluorescence of quin-2-loaded cells, we found that baseline $[Ca^{2+}]_c$ was 108 ± 14 nM (range 65–164 nM, $n = 7$). Addition of epinephrine increased $[Ca^{2+}]_c$, as shown by the tracing from a representative experiment in Fig. 1. The mean $[Ca^{2+}]_c$ increased to a peak of 126 ± 15 nM ($P < 0.05$, paired *t* test) at 58 ± 17 sec. The changes in $[Ca^{2+}]_c$ were small and at least partly transient. Therefore, it is difficult to be sure of the absolute magnitude of changes in $[Ca^{2+}]_c$. These difficulties may result from the use of a cell suspension, because isolated suspended cells may not function identically to cells in an epithelial sheet. Moreover, the properties of quin-2, which has a continual decay in fluorescence and which may buffer changes in $[Ca^{2+}]_c$ (19), might tend to minimize the observed changes. Despite these limitations, the important point is that epinephrine increased $[Ca^{2+}]_c$.

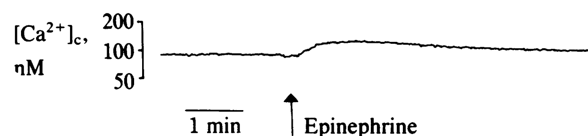

FIG. 1. Effect of 1 μM epinephrine on $[Ca^{2+}]_c$.

K^+ Channel. The increase in $[Ca^{2+}]_c$ induced by epinephrine and the attenuation of the epinephrine secretory response by TMB-8 are consistent with the hypothesis that $[Ca^{2+}]_c$ regulates the basolateral K^+ conductance. To directly test this hypothesis, we examined single K^+ channel currents in primary cultures of tracheal epithelium. When recording in the cell-attached conformation with a 135 mM KCl pipette solution, we observed single-channel currents that reversed (Fig. 2) near the expected (18) resting potential of the cell (41 to 60 mV, $n = 9$). These channels were observed in 30–50% of patches. Fig. 2 also shows the single-channel current–voltage relation when the bath and pipette solutions were 130 mM KCl. The current–voltage relation rectified (conductance decreased at outward currents) and had a conductance of 19.4 ± 0.5 pS ($n = 12$, mean ± SEM) at 0 mV with symmetrical solutions. The channel was highly selective for K^+ over both anions and Na^+, as determined from the shift in reversal potential. As shown in Fig. 2, decreasing external K^+ from 135 mM to 45 mM, by substitution of Na^+, shifted the reversal potential to the value expected (−27.9 mV) for a purely K^+-selective membrane within the accuracy

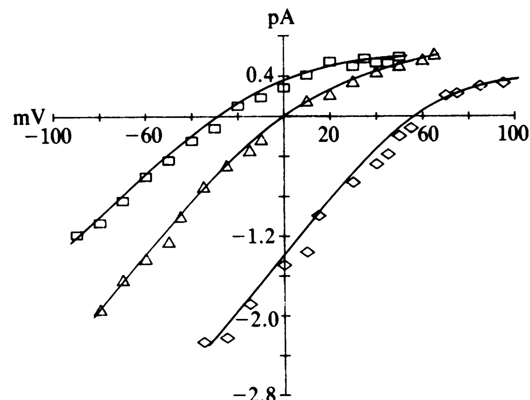

FIG. 2. Single-channel current-voltage (*I–V*) relations. ◇, *I–V* relation of the channel obtained from a cell-attached patch (pipette solution: 135 mM KCl/1 mM $CaCl_2$). △, *I–V* relation of the same patch in the excised, inside-out configuration and bathed in symmetrical K^+ solutions (bath: 135 mM KCl/1 μM $CaCl_2$). □, *I–V* relation of an excised inside-out patch bathed in asymmetric K^+ solutions (pipette: 45 mM KCl/90 mM NaCl/1 mM $CaCl_2$; bath: 135 mM KCl/1 μM $CaCl_2$). The reversal potential expected for this K^+ gradient is −28 mV. Lines were drawn by eye.

limits of our measurements. In addition, when internal K^+ was replaced by Na^+ or Cs^+, the channel would not carry outward current. Finally, substitution of SO_4^- or gluconate for external Cl^- did not alter the conductive properties.

To determine if epinephrine increases K^+ channel activity, we added epinephrine (1 μM) to the bathing solution while recording from a cell-attached patch. Fig. 3 shows that K^+ channel activity was minimal but increased dramatically after addition of epinephrine ($n = 6$). After addition of epinephrine, K^+ channel activity often increased transiently and then decreased, but it remained at a level greater than observed before addition of epinephrine. The observation that the change in K^+ channel activity was partly transient is consistent with the finding of transient changes in $[Ca^{2+}]_c$, and the observation that stimulation of secretion in cultured epithelial monolayers has a transient component (17). Although in the cell-attached configuration the absolute voltage across the membrane patch is not precisely defined, we did not observe any substantial effect of voltage on channel activity in either cell-attached or excised modes. Although the frequency response of our recording system and method of analysis would prevent us from observing subtle changes in channel kinetics, it is clear that there was not a major voltage-activation of the channel in the physiologic range. Thus, the epinephrine-induced increase in channel activity cannot be explained by a change in membrane voltage. These findings indicate that the K^+ channel is regulated by an intracellular second messenger. They also suggest that this channel is the one involved in the response to secretagogues.

This K^+ channel was found in patches from isolated cells and from cells at the edge of a cluster of confluent cells and in freshly dissociated cells. It was not observed in the apical membrane of cells that had grown to confluency. This observation suggests that the K^+ channel is localized to the basolateral membrane in confluent cells (and therefore is not accessible to the pipette) but, in the absence of a continuous tight junctional complex, it may be located on any part of the cell surface. In support of this conclusion, the tight junction is the point of segregation of proteins that are confined to either the apical or the basolateral cell membrane (25, 26). While these arguments are not definitive, when taken together with the observations that apical membrane of intact and cultured tracheal epithelium has minimal, if any, K^+ conductance (27, 28), and the similarities between the properties of this channel and those predicted from studies in the intact epithelium (see below), they suggest that we are dealing with the channel responsible for the basolateral K^+ permeability.

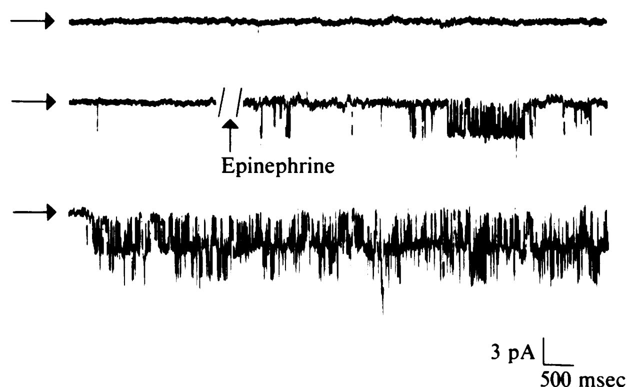

FIG. 3. Effect of epinephrine on K^+ channel activity in a cell-attached patch. Tracing is a continuous record except for a 14-sec gap when epinephrine (1 μM) was added. The holding potential was -100 mV (membrane hyperpolarized). Pipette solution was, in mM, 182 K gluconate, 10 $CaCl_2$, and 10 $MgCl_2$. Downward deflections represent inward current—i.e., cation movement from the external side of the membrane to the cytoplasmic side. The arrows indicate the current level when all channels are closed. After the addition of epinephrine it is apparent that there are two (and possibly three) channels in the patch.

FIG. 4. Effect of A-23187 on K^+ channel activity in a cell-attached patch. Tracings are from a single representative patch. Tracings were taken under control conditions, after addition of A-23187 (5 μM) (tracing begins 32 sec after A-23187 was added), and after the bathing solution was changed to a Ca^{2+}-free one (tracing begins 36 sec after the change). By 42 sec after the change to a Ca^{2+}-free solution, channel activity had stopped and no openings were observed during the subsequent 88 sec of observation. See also legend of Fig. 3.

Ca^{2+} Activation of the K^+ Channel. To directly examine the effect of $[Ca^{2+}]_c$ on channel activity, we did two types of experiments. First, we examined the effects of Ca^{2+} on K^+ channel activity in cell-attached patches. Fig. 4 shows a representative recording taken from a cell-attached patch. Under control conditions the channel openings were rare (none were seen in this tracing). Within seconds of addition of A-23187 to the bath solution (which contains 1.2 mM Ca^{2+}) channel openings were frequent, and it is apparent that the patch contains at least two channels. The bathing solution was then changed to a Ca^{2+}-free solution (5 mM EGTA and nominal Ca^{2+}). After the change in solution, the frequency of channel opening continually decreased, and by 42 sec after the change, no further openings were observed. Similar observations were made in three patches.

To examine the effect of Ca^{2+} on the channel in the absence of the cell, we used inside-out patches of membrane and varied the Ca^{2+} concentration bathing the cytosolic surface of the membrane between 10^{-8} and 10^{-3} M. As shown in Fig. 5, the frequency of channel opening and duration of the open state increased when $[Ca^{2+}]$ increased. The increased channel activity occurred most prominently between 10^{-7} and 10^{-6} M Ca^{2+}, in the same range as the $[Ca^{2+}]_c$ measured with quin-2. We did not observe channel opening at $[Ca^{2+}]$ of 10^{-8}

FIG. 5. Effect of $[Ca^{2+}]_c$ on K^+ channel activity. Tracings were obtained from a single representative inside-out patch held at -100 mV (membrane hyperpolarized). Bathing and pipette solutions were symmetric 135 mM KCl, and $[Ca^{2+}]$ in the pipette solution was 1 mM. Ca^{2+} concentrations in the bath (the cytoplasmic surface of the membrane) are as listed. $[Ca^{2+}]$ of 1 μM or less were achieved by buffering 1 mM Ca^{2+} with EGTA [assuming a dissociation constant of 10^{-7} M (29)]. At 10^{-3} M Ca^{2+} and occasionally at 10^{-6} M Ca^{2+}, there is evidence of two channels in the patch.

M or less, suggesting that the channel opening requires internal Ca^{2+}. The effects of Ca^{2+} were completely reversible and did not alter the channel conductance. Similar observations were made on seven patches.

DISCUSSION

The results suggest that $[Ca^{2+}]_c$ is the intracellular second messenger that regulates the K^+ channel in tracheal epithelium. Hille (30) has recently listed four criteria that indicate a channel is regulated by a second messenger. They are as follows: (*i*) There is a response to stimuli applied only to parts of the cell away from the channel. Our finding that a hormone, epinephrine, or the Ca^{2+} ionophore A-23187, applied while recording from a cell-attached patch, increases the probability of K^+ channel opening, fulfills this criteria. (*ii*) There is production of internal second messenger by the stimulus. We found that epinephrine increases $[Ca^{2+}]_c$; A-23187 does the same. (*iii*) The response is mimicked by applied second messenger. An increase in $[Ca^{2+}]_c$ on the cytoplasmic surface of inside-out patches activated this K^+ channel. (*iv*) There is a loss of response when messenger accumulation is prevented. Our results in regard to this criteria are not as direct; however, two findings suggest that this is the case. First, TMB-8, which is purported to interfere with intracellular Ca^{2+} handling, attenuated the response of secretagogue. Second, when channel activity had been increased by A-23187, removal of Ca^{2+} from the bath diminished and then abolished channel opening. Thus, these findings indicate that cytosolic Ca^{2+} may be the physiologic second messenger that regulates basolateral K^+ conductance and suggest that cytosolic Ca^{2+} may be the signal that links the basolateral conductance to changes in apical membrane conductance (1–3). However, it is also possible that other intracellular second messengers may regulate the probability of channel opening alone or in a synergistic manner with $[Ca^{2+}]_c$.

The properties of this K channel are consistent with the properties expected from previous studies in intact and cultured tracheal epithelium. In intact or cultured epithelia, secretagogues, including epinephrine, increase the K^+ conductance of the basolateral membrane (7–9, 18). The increase in K^+ conductance is readily explained by our data. We have also recently found that the basolateral K^+ permeability is not regulated by membrane voltage or the activity of the Na^+,K^+-pump (10). The voltage insensitivity of this K^+ channel, as well as its functioning in excised patches (i.e., in the absence of pump activity), are thus consistent with previous results.

The properties of the K^+ channel we have described are different from those of most other K^+ channels (for reviews, see refs. 11, 30, 31). Petersen and Maruyama (31) have described a Ca^{2+}-activated K^+ channel in the basolateral membrane of exocrine gland cells. Hunter *et al.* (13) have also described Ca^{2+}-activated K^+ channel in the apical membrane of epithelial cells from cortical collecting tubule. However, those were large channels (approximately 200 pS) that were also voltage-activated. Thus, they belong in a class or family of large Ca^{2+}-activated K^+ channels, called maxi-K channels (11). The channel we describe is much smaller and has a nonlinear current–voltage (*I–V*) relation, even with symmetrical K^+ solutions. From this standpoint, it somewhat resembles the inwardly rectifying K^+ channel in cardiac ventricular cells (32). The inward rectifier has a single channel conductance of 27 pS, similar to that which we report; however, current rectification by the inward rectifier is even more pronounced for outward currents. Moreover, in contrast to the K^+ channel we describe, the inward rectifier is voltage sensitive (33). Thus, the conductive properties of this K channel, as well as the finding that it is Ca^{2+}-activated but voltage insensitive, suggest that it is different from the properties of previously described channels.

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