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Single chloride channel currents from canine tracheal epithelial cells

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Patch-clamp techniques were used to characterize the properties of anion-selective channels in canine tracheal epithelial cells that had been maintained in primary culture. Gigaohm seals (10–30 GΩ) were obtained in single isolated cells or cells at the edge of a confluent sheet, and channels were studied in the cell attached or the inside-out, excised patch configuration. Pretreatment with isotonic KCl caused the cells to round-up and allowed us to have better success in obtaining good seals. Based on conductance, anion-cation selectivity and voltage-dependent kinetic properties, four anion channel types could be detected in symmetrical solutions of 0.15 M NaCl: (i) a 30–50 pS Cl^- channel of high selectivity, active at negative potentials and inactivated by large positive potentials; (ii) an approx. 20 pS Cl^- channel of high selectivity, active at positive potentials and inactivated at negative potentials; (iii) an approx. 250 pS channel of moderate selectivity ($P_{Cl}/P_{Na} = 4$) that was not voltage-dependent, and (iv) an approx. 10 pS Cl^- channel with characteristics similar to (iii) above, but remaining somewhat active at large negative voltages. All excised patches were exposed to relatively high calcium concentrations on the intracellular side. Channel activity was increased in tracheal cells treated with 1 mM cAMP, suggesting that at least one of these channels plays a role in the increase of the apical membrane Cl^- conductance that is mediated by cAMP and elicited by agonists of active Cl^- secretion.

Introduction

The cellular conductance pathways that mediate transepithelial current flow during electrogenic Cl^- secretion have been characterized in canine tracheal epithelium using microelectrode techniques [1–3]. Agents that stimulate electrogenic Cl^- secretion by increasing intracellular cAMP levels (e.g., epinephrine, prostaglandins) elicit a 10-fold increase in apical membrane Cl^- conductance (G_a^{Cl}), which permits net Cl^- exit from cell to mucosal solution. The increase in G_a^{Cl} is the first detectable response to secretogogues and is followed by a secondary increase in basolateral membrane conductance to K^+ (G_b^K), which closely parallels the increase in short-circuit current associated with Cl^- secretion. During steady-state secretion, transepithelial current flow (positive in the mucosa-to-serosa direction) is comprised of Cl^- exit across the apical membrane and K^+ exit across the basolateral membrane. Accordingly, it is anticipated that Cl^- and K^+ channels gated by cAMP (and/or other cellular mediators) will be detected in the apical and basolateral membranes respectively, of tracheal cells and other Cl^--secreting epithelia.

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Three additional anion selective channels might be anticipated in electrophysiological records from tracheal epithelial cells. The first channel type is that associated with the volume regulatory decrease (RVD) seen following exposure of certain lymphoid and epithelial cells to hypotonic solutions [4,5]. This channel selects against anions as large as sulfate or gluconate [6], inactivates over a period of 30 min [7] and is inhibited by permeant calmodulin antagonists and sulfhydryl reagents [5] and also by oligomycin, dipyrimadole, H_2DIDS (4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonate) and NIP-taurine (*N*-(2-nitro-4-isothiocyanophenyl)taurine) [8]. This channel is particularly likely because the cells of this study had been volume expanded by pretreatment with isotonic KCl. The second is the calcium-dependent anion selective channel [9], since 2 mM calcium was present in the bath solution in certain of the experiments. Finally, the resting anion permeability such as seen in red blood cells [5] might be apparent.

In the present study, we evaluated the feasibility of applying patch-clamp techniques to canine tracheal epithelial cells in primary culture. We focus on the characteristics of Cl^- channels studied using excised patch techniques on tracheal epithelial cells and assess the channel types that may be involved in cAMP-stimulated Cl^- transport across the apical membrane.

Methods

Canine tracheal cells were isolated using the methods described by Widdicombe and co-workers [10,11]. In brief, tracheal segments were preincubated in a Ca^{2+}- and Mg^{2+}-free Hepes-buffered Ringer solution containing 1 mM dithiothreitol for 15–20 min. Mucosal strips were obtained by blunt dissection, minced and digested during three successive periods in fresh solutions containing 1g% bovine serum albumin, 20 mg% collagenase type I, 10 mg% DNAase type I, 2% pen-strep, 1% gentamycin and 1 mM dithiothreitol. Cells were collected at incubation times of 90, 180 and 240 min. The vascular perfusion technique of Widdicombe et al. [11] was omitted from our protocol, so that the 90 min fraction contained significant numbers of red blood cells. Since this fraction also exhibited the poorest epithelial cell viability (about 50%), it was usually discarded and the 180 and 240 min fractions (showing greater than 90% viability) were pooled and cultured. The cells were preplated in plastic dishes to remove fibroblasts and were subsequently plated at a concentration of $2.5 \cdot 10^4$ cells/ml on coverslips coated with human placental collagen contained in 35 mm dishes. Cells were incubated in a 1 : 1 mixture of Dulbecco's modified minimum essential medium and Hamms F12, fortified with 5% fetal bovine serum and antibiotics, as above. They were incubated at 37°C in a 5% CO_2 atmosphere until use and refed three times per week.

Cells were prepared in Birmingham and used there or transported from Alabama to Mississippi at ambient temperatures. All patches were obtained within 10 days of plating, before the epithelial cells had grown to confluency.

Experiments were performed as described by Dwyer and Farley [12]. Briefly, the coverslip bearing the cells was placed in a chamber maintained at 11°C. The cells were volume expanded by exposure to isotonic KCl for 1–2 h; gigaohm patches were more easily formed after this pretreatment when standard Kimax No. 51 borosilicate glass capillaries were used [13]. The individual cell to be studied was visualized using phase contrast or Hoffmann modulation optics, and the cell attached gigaohm seal was formed as described by Hamill et al. [14]. Inside-out patches were obtained by abruptly lifting the pipette from the cell. Single channel currents were amplified by a List EPC-5 voltage clamp (Greenvale, NY) and recorded on FM tape. Analyses were performed manually using a digital oscilloscope (Model 3091, Nicolet Instrument Corporation, Madison, WI) and the records were displayed on a Hewlett-Packard *X-Y* plotter (Model 7475A).

All experimental solutions were buffered to pH 7.4 with 5 mM Hepes. Solution composition (in mM) bathing excised membrane patches is given as follows: pipette concentration//bath concentration. Transmembrane voltages are expressed with the pipette as reference, i.e., the sign is that of the membrane interior. A similar convention applies to statements regarding membrane sidedness, i.e., "inside" solution refers to that bathing the cytoplasmic membrane surface. Sufficient data

were derived from a total of 21 patches to characterize the channel type or types that were active. An additional five patches were tested for sensitivity to epinephrine.

Results

The studies of Coleman et al. [10] have demonstrated clearly that epithelial cells isolated from dog trachea and cultured on a collagen substrate form confluent sheets of cells and retain the capacity for cAMP-stimulated Cl^- secretion characteristic of the intact epithelium. In general, the cultured cells rarely display cilia, but do form apical microvilli and cell junctions and assume a roughly cuboidal shape in the monolayer (cell height is approx. 8 μm). In general, we studied cells before they reached confluence and obtained patches on either single isolated cells or cells growing at the edge of a confluent sheet. With the cells in this configuration, conductance pathways are likely to be distributed at random across the plasma membrane, so that the area of membrane studied cannot be designated as apical or basolateral. Using this approach, gigohm seals were formed with applied suction. The seal resistances were uniformly highest (10–30 GΩ) when the cells were caused to round up by pretreatment with isotonic KCl [13]. In agreement with the findings of Nelson et al. [15], our success at forming seals was further improved after treatment with cAMP. Therefore, our initial studies were carried out using cells that had been pretreated for 1–2 h with buffered, isotonic KCl solutions containing 1 mM cAMP to maximize the likelihood of obtaining high resistance, channel-bearing patches. At the beginning of the experiment, this solution was replaced by one containing 151 mM NaCl and 5 mM Hepes. Using this approach, a number of different channel types were regularly observed, including two channels that have been described elsewhere: the Ca^{2+}-activated K^+ channel (approx. 20 pS) and the 400 pS nonspecific cation channel [16]. However, in this study we concentrated on the characteristics of observable anion channels.

The single-channel events of Fig. 1 were recorded in the cell attached mode, and illustrate the activities of three types of anion channels. Comments regarding their frequency of appearance and anion-cation selectivity were derived from other, more detailed excised-patch studies where these channels were also encountered. In fact, many patches exhibited more than one type of anion-selective channel. Examples for this paper were chosen from among those patches in which only one channel type appeared. The larger channels could be identified by their characterisitc conductance and voltage-dependent kinetics. The smallest channel was unambiguously apparent in several patches where it was the sole active channel type; in other patches where larger channels were active, we often could not be certain whether or not this smaller channel was also present.

Panel A of Fig. 1 shows the most common channel; it was seen in 16/21 patches. This approx. 40 pS Cl^- channel activated at negative membrane potentials and inactivated at large positive potentials. The selectivity and conductance of this channel will be discussed in more detail below. A second channel type (panel B) activated at positive potentials and inactivated at negative voltages, characteristics opposite to those of the channels illustrated in panel A. This channel was seen in 5/21 patches and ionic substitution experiments revealed that this channel was highly anion-selective (data not shown). However, because its conductance was only about 20 pS, more detailed analyses were often precluded by the activities of other channels in the same patch. A third channel type (panel C) was smaller than the others (approx. 10 pS) and was encountered in 7/21 patches. This channel was open a greater percent of time at positive than at negative membrane potentials. Ostensibly, this channel was similar to that shown in panel B, but differed from it in showing activity at large negative voltages (e.g., -160 mV, panel C). The channel illustrated in panel B inactivated completely under these conditions.

We also detected a large anion-selective channel in excised membrane patches (3/21) which was open at all potentials. Fig. 2A shows a sample record in which channel conductance was approx. 270 pS at positive voltages and at negative voltages was approx. 200 pS. This channel exhibited rapid open-closed transitions (flickering) particularly at negative voltages. This channel was spon-

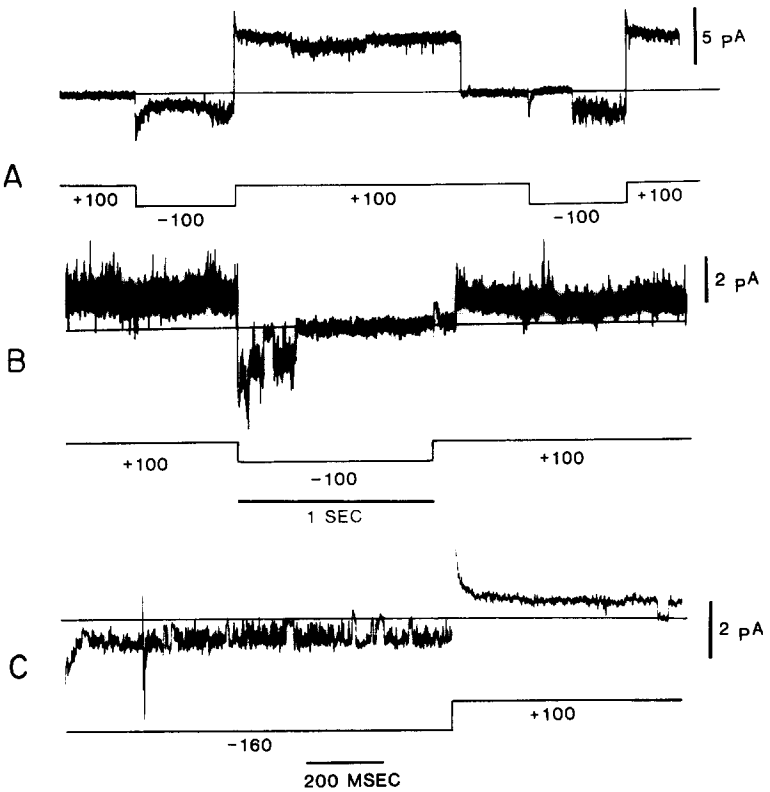
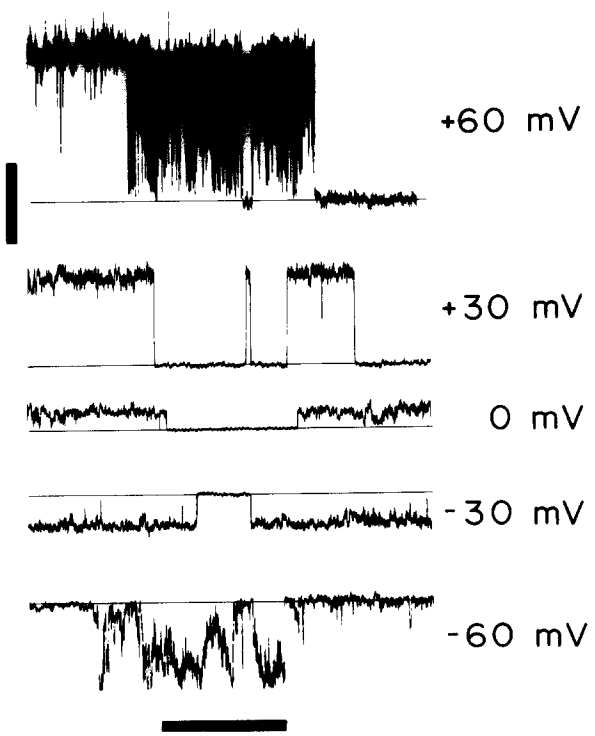


Fig. 1. Single channel records from tracheal epithelial cells. These records were obtained from cell attached patches, each on a different cell. The channel types differed in voltage sensitivity, kinetics of opening and selectivity, as discussed in the text. Both the bath and the pipette solution contained 151 mM NaCl and 5 mM Hepes; the pipette also contained 2 mM $CaCl_2$; bath temperature was 11°C. Cells were pretreated with 140 mM KCl and 1 mM cAMP before the experiment. The time-scale is 400 ms for panels A and B and 200 ms for panel C. Baselines are drawn through the closed state current; capacity transients are seen in the current records at the voltage transitions. Channel openings are upward deflections during the positive holding potentials and downward deflections during the negative holding potentials.



taneously active for many minutes after the patch was excised, but lapsed into silence following a sudden onset of the flickering illustrated in the tracing at +60 mV. At zero potential, current was outward when the external solution contained twice the concentration of NaCl as the internal solution; the reversal potential was approx. −10 mV. If the channel were perfectly selective for anions or cations, the reversal potential would have been −17 or +17 mV, respectively. Thus, by the Goldman-Hodgkin-Katz voltage equation [17], the channel was able to select for Cl^- over Na^+ in a ratio of 4 : 1.

The anion-selective channel most commonly

Fig. 2. Records from a large, moderately anion-selective channel. This channel was observed in an inside-out patch bathed by 151//76 NaCl. Calcium (2 mM) was present in both the pipette and bath solutions. Cells were pretreated in 140 mM KCl plus 1 mM cAMP for 1 h. Calibration bars are 10 pA and 1 s; baselines are drawn through the closed state current.

encountered in this study is that of Fig. 1A and is illustrated in greater detail by the records and data of Fig. 3. This channel was markedly voltage-sensitive; channels turned on at voltages more negative than +60 mV and became inactivated at potentials of +80 to +120 mV. The record in Fig. 3 (upper left quadrant) begins with a period of noticeable channel activity during which the membrane patch was held at −100 mV. Switching to +100 mV caused the two channels to turn off. The lower right quadrant shows this record (same patch) at a later time. After channel activity had ceased at +100 mV, returning to −100 mV activated one channel (within this time frame) after a brief delay. At intermediate voltages (Fig. 4C) the channels opened and closed repetitively.

The selectivity of this channel was determined by ionic substitution experiments. The current-voltage curves in Fig. 3 were obtained from a single patch, first with 76 and then with 301 mM NaCl in the bath. The current record in the upper left panel shows channel activity at −100 mV; two distinct steps off were apparent a short time after the membrane potential was switched to +100 mV. The converse is shown in lower right

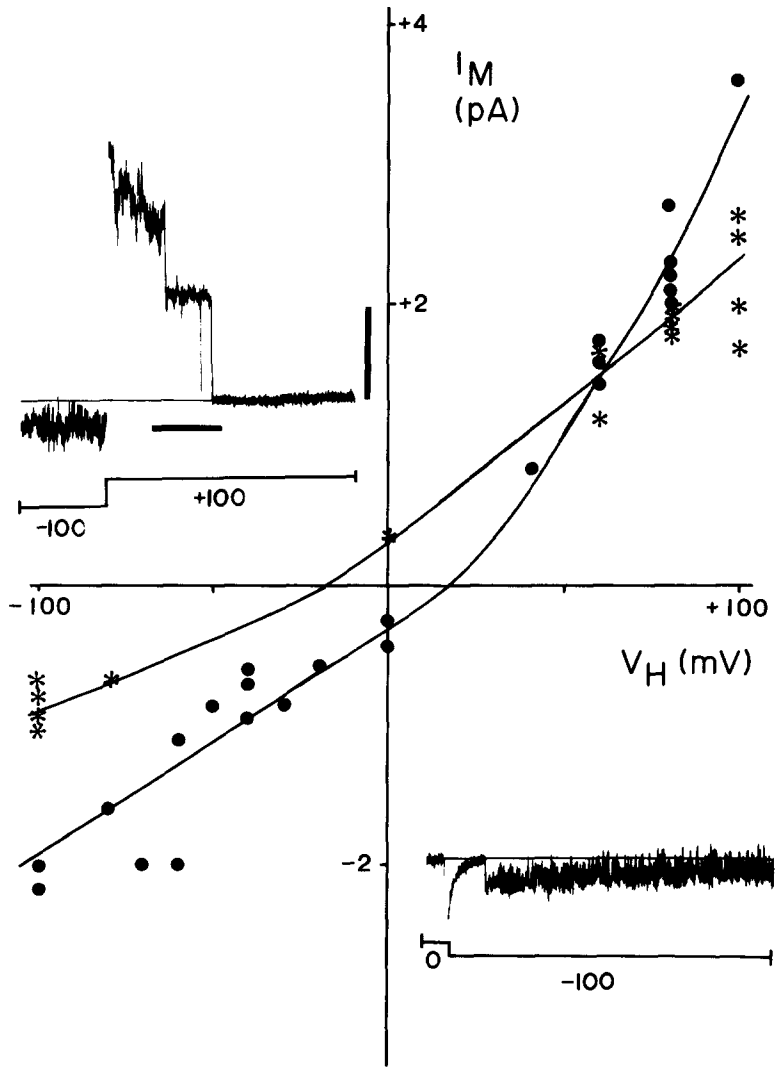


Fig. 3. A highly selective anion channel that is inactivated by positive potentials. This record was obtained with an inside-out patch from a cell pretreated with 140 mM KCl plus 1 mM cAMP. No calcium was present in the bath solution. Calibration bars are 2 pA and 250 ms; baselines are drawn through the closed state current. The line was drawn by eye.

panel, where activation was seen shortly after switching to −100 mV. The open channel current-voltage curves obtained at NaCl concentration of 151/176 (filled circles) and 151/301 (empty circles) NaCl show that 2 : 1 and 1 : 2 NaCl gradients yield reversal potentials of approx. −17 mV and +17 mV, respectively, as predicted for an anion-selective channel. This 4-fold increase in bath NaCl concentration shifted the reversal potential in the positive direction by 34 mV, the value expected for a purely anion-selective channel. Outward currents were larger than inward currents even when the inside Cl^- concentration was higher than that outside; that is, the slope conductance was greater at positive than at negative voltages. Increasing the internal Cl^- concentration 4-fold increased both the inward and outward currents by approx. 2-fold.

The frequency with which this channel was encountered depended on the prior history of treatment with cAMP or epinephrine. In several experiments cells were pretreated with 140 mM KCl, but were not exposed to cAMP or epinephrine. This channel was seen in only 7/26 patches in cells that had not been exposed to either agonist. The channel was seen in 13/36 patches formed after a 1 h exposure to 1 mM cAMP and in 3/5 patches formed after a similar treatment with 1 μm epinephrine.

Fig. 4 illustrates the voltage dependence of the single channel closing kinetics for the channel type shown in Figs. 1A and 3. The records in Fig. 4 were generated by summing records of the currents that followed voltage-steps to +100, +80 or +60 mV from voltages of −100, −80 or −60 mV, respectively. The channels had opened during the time when the patch was at the negative holding potential. After a step to +100 mV the number of open channels fell rapidly to zero; a total of 28 closures were apparent in individual records. At +80 mV the decay in channel activity occurred more slowly and only \15 closures were seen after the step to +80 mV, with the final closure occurring several seconds after the end of the record shown here. At +60 mV there was seldom a total loss of channel activity, even after several minutes. Thus, more channels were opened on average by the pulse to −100 than to −80 mV. The voltage dependence with the pulses from −60 mV to +60 mV is less certain, since the channels never completely closed at +60 mV.

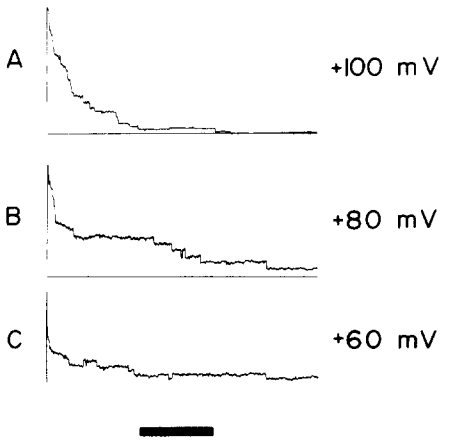


Fig. 4. Channel kinetics – positive off. The average of four current records obtained after a voltage step to +100, +80 or +60 mV shown in panels A, B and C, respectively. The channels were of the type illustrated in Figs. 1A and 3. These records are all from the same patch, which had at least seven active channels. The scale for the vertical axis is arbitrary as a result of the averaging algorithm. A baseline is provided in panels A and B; no baseline is possible for panel C, since there was never a period where all the channels were closed simultaneously.

All these channels returned to their open state within a few hundred milliseconds after the voltage was stepped to −100 mV. However, the longer the channel remained closed at positive potentials, the slower it was to open at −100 mV. Very prolonged periods of closure led to a complete cessation of further channel activity.

Discussion

Canine tracheal cells in primary culture provide a useful preparation for characterization of the cation and anion channels involved in electrogenic Cl^- secretion and volume regulation. This report identifies four types of anion-selective channel detected in these cells. The channel having the greatest conductance (approx. 250 pS) was not highly anion-selective. In records where discrete openings could be defined, the approx. 250 pS channel showed a moderate anion selectivity ($P_{Cl}/P_{Na} = 4$). Two highly selective anion channels of low conductance (approx. 10 and approx.

20 pS) were active at positive membrane potentials, but less active in the physiological voltage range and inactive at large negative voltages. This voltage-dependent kinetic behavior suggests two possibilities. First, because the activity is less in the physiological range, the channel may not be involved in cAMP-dependent Cl^- secretion. Second, the low activity under the conditions of this study may reflect a non-optimum stimulation of the channel, perhaps due to a lacking metabolic factor or a deficient regulatory unit.

A fourth channel whose conductance was 30–50 pS was open at all but the most positive potentials. Channels that had opened at negative potentials closed within seconds following a step to +60 mV or greater; the more positive the step, the more rapidly the channels closed. Up to seven of these channels have been observed in a single patch. When these channels were present, it was apparent that the same number of channels could be activated at negative voltages as were subsequently inactivated at extreme positive potentials, particularly when there were only 1–3 channels in the patch. Thus, the steps on and the steps off reflect the activity of the same channels. Under conditions of volume expansion, the frequency with which this channel was observed increased from 25% to 80% following treatment with cAMP or epinephrine.

Single channel records obtained from excitable cell membranes reveal anion-selective channels with a variety of conductance and selectivity properties. Some of these show enhanced activity at positive potentials [18,19], similar to the small conductance channels illustrated in records 1B and 1C. In contrast, *Aplysia* neurons exhibit a resting Cl^- conductance that is activated by hyperpolarization [20]; this voltage dependence is similar to that of the anion channel most frequently observed in our studies. Using an outside-out patch technique, Chesnoy-Marchais and Evans [21] found a small conductance (10–15 pS) anion channel with this voltage sensitivity, but it was present infrequently.

There are several preliminary reports of anion channel activity in epithelia, although most of these are from absorptive tissues. Nelson et al. [15] reported on the properties of an approx. 360 pS (0.1 M NaCl) channel from cultured A6 epithelia whose selectivity for Cl^- over Na^+ was about 9. Its kinetics resemble those of the high conductance, moderately selective anion channels shown in Fig. 2, but the channel detected in A6 cells was relatively inactive in excised patches at voltages exceeding ±20 mV. Hanrahan et al. [22] detected an anion channel in cultured mammalian bladder cells prior to confluency whose voltage dependence resembles that of the channel most frequently encountered in tracheal cells (Fig. 1A, 3, 4). In excised patches, this channel became less active at depolarizing voltages greater than +20 mV, and was inactivated completely with larger positive voltage steps. However, the conductance of this channel was approx. 375 pS (0.15 M KCl), much larger than the tracheal cell channels that exhibit similar voltage sensitivity. Gogelein and Greger [23] observed a 10–15 pS anion-selective channel in the plasma membrane of proximal renal tubule cells that was inactivated by depolarizing voltages. The properties of these channels resemble those illustrated in Figs. 1B and 1C as well as those detected in excitable membranes.

Most relevant to this study is that of Greger et al. [24], performed using shark rectal gland tubules, an epithelium where active Cl^- secretion is activated by cAMP. Cl^- channels were recorded from patches of apical membrane during stimulation by cAMP, forskolin and adenosine. Their conductance was estimated at 20–50 pS (0.3 M NaCl), values similar to the predominant anion channel in tracheal cells. However, their voltage sensitivity has been studied over a limited range thus far (±50 mV), so that one cannot discern whether inactivation by large depolarizing voltages is a characteristic of channels from rectal gland as well as tracheal epithelium.

Acknowledgements

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References

1 Smith, P.L. and Frizzell, R.A. (1984) J. Membrane Biol. 77, 187–199
2 Welsh, M.J. (1983) Am. J. Physiol. 244, C377–384
3 Welsh, M.J., Smith, P.L. and Frizzell, R.A. (1983) J. Membrane Biol. 71, 209–218
4 Foskett, J.K. and Spring, K.R. (1985) Am. J. Physiol. 248, C27–C36
5 Sarkadi, B., Cheung, R., Mack, E., Grinstein, S., Gelfand, E.W. and Rothstein, A. (1985) Am. J. Physiol. 248, C480–C487
6 Grinstein, S., Clarke, C.A., Dupre, A. and Rothstein, A.J. (1982) Gen. Physiol. 80, 801–813
7 Sarkadi, B., Mack, E. and Rothstein, A. (1984) J. Gen. Physiol. 83, 513–527
8 Sarkadi, B., Mack, E. and Rothstein, A. (1984) J. Gen. Physiol. 83, 497–512
9 Cuthbert, A.W. (1985) Pflügers Arch. 405, S127–S130
10 Coleman, D.L., Tuet, I.K. and Widdicombe, J.H. (1984) Am. J. Physiol. C355–C359
11 Widdicombe, J.H., Basbaum, C.B. and Highland, E. (1981) Am. J. Physiol. 241, C184–C192
12 Dwyer, T.M. and Farley, J.M. (1984) Biophys. J. 45, 529–539
13 Standen, N.B., Stanfield, P.R., Ward, T.A. and Wilson, S.W. (1984) Proc. R. Soc. Lond. B. 221, 455–464
14 Hamill, O., Marty, A., Neher, E., Sakmann, B. and Sigworth, F. (1981) Pflügers Arch. 391, 85–100
15 Nelson, D.J., Tang, J.M. and Palmer, L.G. (1984) J. Membrane Biol. 80, 81–89
16 Latorre, R. and Miller, C. (1983) J. Membrane Biol. 71, 11–30
17 Hodgkin, A. and Katz, B. (1949) J. Physiol. (Lond.) 108, 37–77
18 Blatz, A.L. and Magleby, K.L. (1983) Biophys. J. 43, 237–241
19 Blatz, A.L. and Magleby, K.L. (1985) Biophys. J. 47, 119–123
20 Chesnoy-Marchais, D. (1983) J. Physiol. (Lond.) 342, 277–308
21 Chesnoy-Marchais, D. and Evans, M.G. (1984) J. Physiol. (Lond.) 357, 64P
22 Hanrahan, J.W., Alles, W.P. and Lewis, S.A. (1984) Biophys. J. 45, 300a
23 Gogelein, H. and Greger, R. (1985) Pflügers Arch. 403 (Supplement), R10
24 Greger, R., Schlatter, E. and Gogelein, H. (1985) Pflügers Arch. 403, 446–448