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An inwardly rectifying chloride channel in ragweed-sensitized canine tracheal epithelial cells

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Abstract. The single channel inside-out patch clamp technique was used to characterize ion channels in the apical membranes of ragweed-sensitized and control canine tracheal epithelial cells maintained in primary culture. Patches were obtained from single isolated cells or from cells at the edges of confluent sheets. A new type of chloride channel was seen in sensitized cells but not in control cells. The channel showed inward rectification in symmetric chloride solutions with conductance varying from 95 pS to 52 pS over the range of −60 mV to 60 mV membrane potential. Channel gating was voltage dependent with maximal opening at about −30 mV. Kinetic analysis showed that distributions of closed and open times could both be well fitted by the sums of three exponential components. Rate constants for transitions between the states of a linear kinetic model were calculated, with only one rate being significantly voltage dependent. The possible significance of this channel is discussed.

Key words: Airway epithelium – Asthma – Apical membrane – Ion channel kinetics

Introduction

Four groups of chloride channels have so far been characterized in mammalian airway epithelia, with conductances of approximately 10, 20, 45 and 350 pS (Frizzell et al. 1986; Duszyk et al. 1989, 1990; Shoemaker et al. 1986). The 45 pS channel shows outward rectification in symmetric chloride solutions, whereas the other channels have linear current voltage relationships. Two types of cation channels have also been identified in the apical membranes of human airway epithelial cells with conductances of approximate 25 pS and 8 pS (Man et al. 1989), while potassium channels of 300 pS have been seen in the basolateral membranes (Kunzelmann et al. 1989).

We have obtained small numbers of samples of tracheal tissues from mature ragweed-sensitized dogs with chronic sustained non-allergic airway hyperresponsiveness, a canine model of asthma (Becker et al. 1989). Airway tissues were also obtained from control littermates. The single channel patch-clamp technique was used to characterize chloride channels in excised patches from apical membranes of epithelial cells. The aim of this study was to investigate the effects of ragweed-sensitization on the numbers or types of chloride channels present in the apical membrane.

The four types of chloride channels described above were all found in control cells and in ragweed-sensitized cells. However, ragweed-sensitized apical membranes also contained a type of chloride channel which has not been described before. This paper describes the conductance and kinetic properties of the novel channel, and discusses its possible function.

Materials and methods

Epithelial tissues were obtained from resected canine tracheas. Tissues were treated with 0.1% protease and 0.1% DNase in calcium-free minimum essential medium (MEM) at 4 °C for 16 to 24 h. The enzymes were neutralized by 10% fetal bovine serum (FBS) for 30 min, and then the cells were detached from the epithelial strips by gentle mechanical agitation. Free cells were filtered through a Nitex nylon mesh (60 μm), centrifuged at 150 g for 10 min, pelleted and then resuspended in 10% FBS in MEM. Trypan blue exclusion was used to test viability, which was normally greater than 90%. The cells were washed once more and then plated at low density on Falcon Primaria plates at 37 °C in a culture medium containing MEM-F12 supplemented with insulin (1 μg/ml), transferrin (7.5 μg/ml), hydrocortisone (100 n*M*), cholera toxin (10 ng/ml), T_3 (0.1 n*M*), epidermal growth factor (2.5 ng/ml), endothelial cell growth substance (10 ng/ml), and antibiotics (gentamycin 50 μg/ml, streptomycin 50 μg/ml) (Wu et al. 1985). Experiments were performed on isolated cells, or those at the edges of confluent sheets. The temperature was kept at 37 ± 1 °C during all experi-

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ments by means of a thermostatically controlled stage. Experiments were performed with cells not more than 6-day old, usually between 1 and 4 days after plating.

Apical membranes were uppermost in the cells, as confirmed by the presence of beating cilia. Patches were obtained from the upper surfaces of the cells, so that recordings were assumed to originate from apical membranes. However, the inclusion of other cell surfaces cannot be completely excluded.

Single channel currents were recorded from excised patches of membrane in the inside-out configuration using a List EPC-7 amplifier (Hamill et al. 1981). Pipets were fabricated from thick walled microfilament borosilicate glass using a two stage Narishige pipet puller, coated with Sylgard (Dow Corning), and fire polished with a Narishige microforge. Pipet resistances were 16–20 MΩ when filled with 140 m*M* NaCl solution and gave seal resistances of about 30 GΩ. Pipet offset potentials were measured and corrected before forming a seal. All potentials are reported relative to zero in the extracellular solution, and positive currents are outwards throughout.

Channel current signals from the amplifier were fed into a digital VCR recorder adaptor (Medical System, PCM-1) and stored on video tape. A digital computer sampled the data at 10 kHz with a 12-bit analog-to-digital convertor. Filtering was carried out after sampling by a Gaussian digital filter.

The procedures used for data analysis were based largely on those described by Colquhoun and Sigworth (1983). The half-amplitude criterion was used as a threshold to distinguish between open and closed states. Event durations were corrected for filter rise-time by a polynomial approximation (Colquhoun and Sigworth 1983). Distributions of open and closed times were created and the probability of the channel being in the open state was calculated. Only openings longer than the filter dead-time were used to compute the mean channel current amplitude. Histograms of open and closed intervals were created using the square root of the number of events versus the log binwidth of event durations (Sigworth and Sine 1987). Distributions were corrected for sampling promotion error by the probabilistic redistribution method (Korn and Horn 1988). The distributions were fitted with probability density functions of the form:

$$f(t) = \sum_{j=1}^{k} (a_j/\tau_j) \exp(-t/\tau_j), \quad (1)$$

where k is the number of exponential components and a_j and τ_j are the fitted amplitude and time constant parameters. The number of fitted parameters for a given value of k is $2k-1$. The data were fitted using the maximum likelihood method (Colquhoun and Sigworth 1983) assuming that each event had a duration equal to the mid point of its bin. The number of significant components k, was determined from a likelihood ratio test (Horn 1987). This test assumes that twice the log likelihood ratio has a Chi-squared distribution with 2 deg of freedom when comparing exponential models with k and $k+1$ components.

Results

From the tissues available to us, 100 successful patches were obtained from control cells (4 animals) and 117 successful patches from ragweed-sensitized cells (5 animals). The four types of chloride channels which have been described before in airway epithelia (10, 20, 45 and 350 pS) were all identified in both the control and ragweed-sensitized cells which we examined. In addition, a new inwardly rectifying chloride channel was seen in 8 patches from the ragweed-sensitized cells. Four of these patches also contained a 20 pS channel, a 350 pS channel or both. No patches were seen with two or more inwardly rectifying channels together. A characteristic feature of this channel was a rapid run-down of activity, usually leading to silence in less than 3 min. However, in 2 cases the channel was active for more than 30 min, allowing complete kinetic analysis.

Figure 1 shows typical recordings of this channel at different voltages. The ionic selectivity of the channel was determined by replacing sodium ions in the bath by choline ions, which are impermeant to cation channels (Duszyk et al. 1989). There was no change in channel current after replacement of sodium ions by choline, indicating that the channel is permeant to chloride ions under physiological conditions.

Figure 2 shows the current-voltage relationship for the inwardly rectifying chloride channel. All of the data for this figure were obtained from a single patch but from several different recordings over a period of approximately 30 min. The total number of openings used to compute the mean values varied from 2 459 at 20 mV to 10 598 at −40 mV. The inward rectification of the channel is evident in this data, with the slope varying from 95 pS at −60 mV to 52 pS at 60 mV. Of the other seven inwardly rectifying channels, five had current-voltage relationships which lay within the standard deviations of Fig. 2. However, the remaining two had inwardly rectifying conductances of about 60% of these values.

The open probability of the inwardly rectifying channel varied with the steady membrane potential applied to the patch. The probability of the channel being open as a

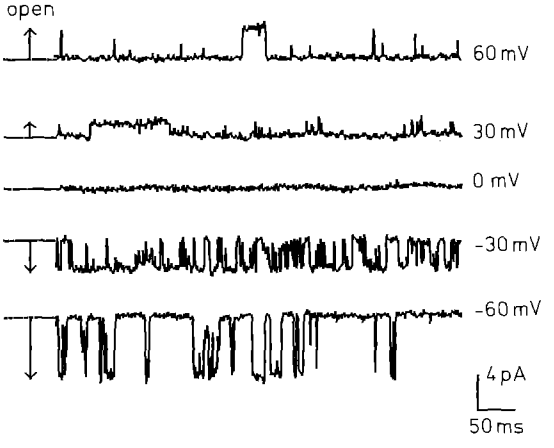

Fig. 1. Examples of single channel recordings of the inwardly rectifying chloride channel in symmetric solutions containing 146 m*M* chloride at different membrane potentials

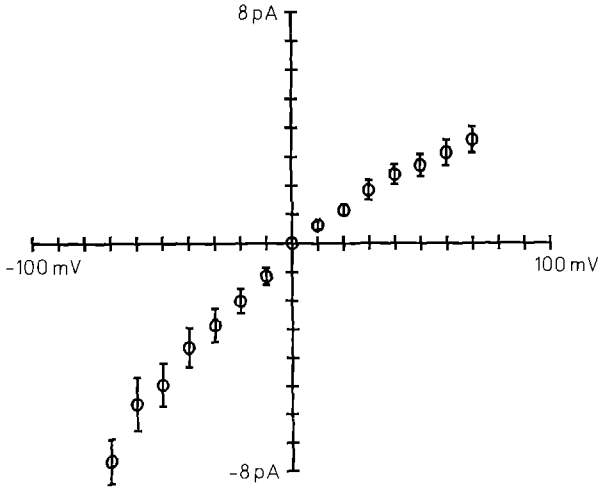

Fig. 2. Current-voltage relationships for an inwardly rectifying chloride channel. The pipet contained 140 m*M* choline chloride and the bath contained 140 m*M* NaCl. The remaining components in the pipet and the bath were: 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$ and 5 m*M* Hepes (pH 7.2). The data points show means and standard deviations from two to five different recordings on the same patch

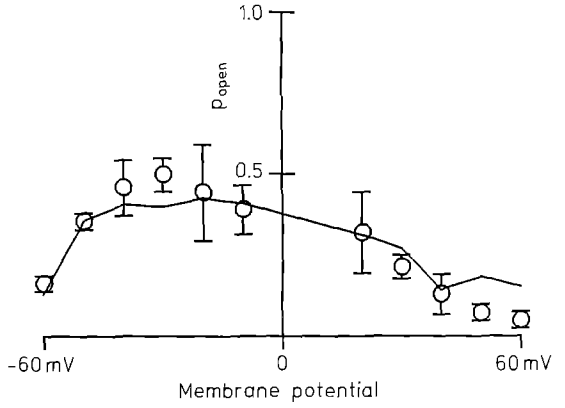

Fig. 3. The probability of the inwardly rectifying channel being open as a function of membrane potential. The data points show means and standard deviations from the same recordings as in Fig. 2. The solid line shows the predicted open probability of the linear kinetic model of (2) with three open and three closed states using the rate constants from Figs. 5 and 6

function of transmembrane potential is shown in Fig. 3. The data present mean values and standard deviations from the same recordings used in Fig. 2. The solid line shows the predicted open probability calculated from a kinetic model of the channel to be described below. Over the range of tested membrane potentials (±60 mV), the probability of the channel being open was highest (approximately 0.46) at −30 to −40 mV and decreased at other membrane potentials. The short durations of most of the other recordings prevented us from obtaining complete open probability records. However, the incomplete data from these channels were in good agreement with Fig. 3. All channels showed strongly increased activity at negative holding potentials.

Kinetic analysis of channel activity was performed on 38 separate recordings from 1 patch. The number of transitions between open and closed states for each separate recording was between 334 and 5 728 events. An example of kinetic analysis is shown in Fig. 4 with distributions of open and closed states being fitted by (1). For each recording, probability density histograms were constructed for both open and closed durations using the Sigworth and Sine (1987) technique. Single, double and triple exponential functions (1) were fitted to the experimental data using the maximum likelihood technique. Discrimination between possible models was performed by the log likelihood ratio test (Horn 1987) using a chi-squared distribution. The null hypothesis was that adding an additional exponential component did not increase the likelihood. Double exponentials were favored over single exponentials for both open and closed durations in 100% of the recordings. For open durations, triple exponentials were favored over double exponentials in 30 of the 38 recordings at the level of $\alpha = 0.05$. For closed durations, triple exponentials were favored in 36 cases. Although it is possible that four exponentials might have provided even better fits in some cases, it did not seem appropriate to risk over-parameterizing the limited amount of data available.

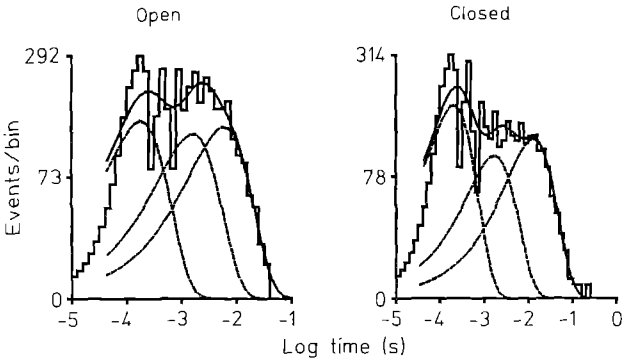

Fig. 4. An example of distributions of open and closed durations of an inwardly rectifying chloride channel, plotted on square root versus log time axes. The data were obtained from a channel held at −50 mV. The total number of events was 5728. The solid lines represent fits from (1) with sums of three exponentials. The dashed lines show the separate components of the fits, with their peaks corresponding to individual time constants along the abscissa. Durations with times shorter than the fitted lines were not reliable because of the bandwidth of the recordings

The above analysis indicates that channel has at least three open states and three closed states. From all possible models with this number of states we attempted to analyze the behavior of the channel in terms of the following linear sequential model for simplicity:

$$C_1 \underset{k_2}{\overset{k_1}{\rightleftarrows}} C_2 \underset{k_4}{\overset{k_3}{\rightleftarrows}} C_3 \underset{k_6}{\overset{k_5}{\rightleftarrows}} O_1 \underset{k_8}{\overset{k_7}{\rightleftarrows}} O_2 \underset{k_{10}}{\overset{k_9}{\rightleftarrows}} O_3 \qquad (2)$$

where C_1, C_2, C_3 and O_1, O_2, O_3 are closed and open states, respectively. The general relationship between the time constants and amplitudes calculated from the open and closed time distributions and the rate constants of the model is given by Colquhoun and Hawkes (1977). The analytical solution for this particular case is given in the Appendix.

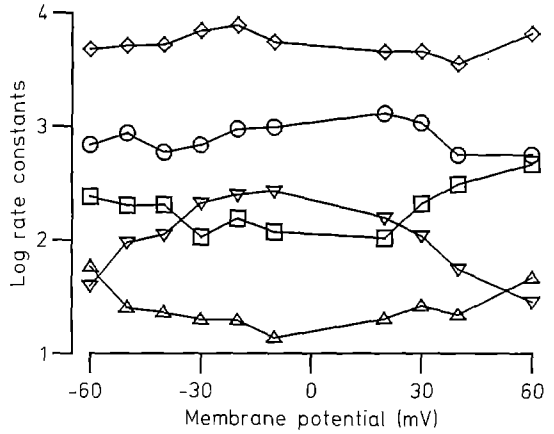

Fig. 5. Calculated rate constants for the closed states of (2) versus membrane potential. Logarithms to the base 10 of the actual rate constants in units of s^{-1} are shown. (k_1 *circles*, k_2 *squares*, k_3 *diamonds*, k_4 *upright triangles*, k_5 *inverted triangles*)

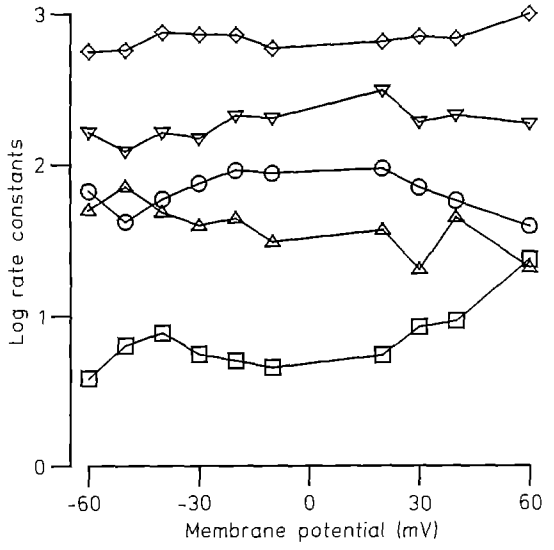

Fig. 6. Calculated rate constants for the open states of (2) versus membrane potential. Logarithms to the base 10 of the actual rate constants in units of s^{-1} are shown. (k_6 *circles*, k_7 *squares*, k_8 *diamonds*, k_9 *upright triangles*, k_{10} *inverted triangles*)

The calculated rate constants as functions of membrane potential are shown in Figs. 5 and 6. Of all the measured rate constants, k_4, k_5, k_6 and k_7 were most clearly voltage dependent. k_5 was maximum at about -20 mV, k_4 was minimum at about -10 mV, k_6 was maximum at about 0 mV, and k_7 increased monotonically with membrane potential. However, the major voltage dependence of the channel seemed to involve k_5, the rate of leaving the dominant closed state, C_3. This rate peaked in the region of maximum open probability.

The equilibrium probabilities of each state, C_1–O_3 were also calculated from the measured rate constants at each membrane potential (see Appendix). The results of these calculations indicated that the channel spends more than 90% of its time in the two connected states $C_3 \leftrightarrows O_1$. The sum of equilibrium probabilities of the three open states represents the total probability of seeing an open channel. These values were calculated and are superimposed on the experimental open probability data in Fig. 3. They show good agreement over most of the experimental membrane potential range.

Discussion

The small amount of tissue available for this study made it impossible to determine the relative contributions of the various chloride channel types in these cells, or to perform detailed studies of ionic selectivity. Eight clear recordings of inwardly rectifying chloride channels were obtained from 117 patches on cells from ragweed-sensitized dogs. Such channels were not seen in 100 patches from control littermates, have not been reported in any previous studies of canine airway epithelia, and were not found in an extensive survey of human airway epithelia (Duskzy et al. 1989). An inwardly rectifying chloride channel has been found previously in rabbit bladder epithelium (Hanrahan et al. 1985). However, the channel described here has significantly larger conductance, about 93 pS at -50 mV compared with 64 pS, and it also has a different dependence of open probability on membrane potential. This data, while not conclusive, suggests that the channel is unique to ragweed-sensitized tissues. If this channel is unique to cells from the ragweed-sensitized airway, it would be interesting to know if it is only produced in these cells or whether it is merely inactive in normal cells. We hope to conduct further studies to investigate these possibilities in the future, but ragweed-sensitized tissues are difficult to obtain.

The distributions of open and closed durations of the channel were usually best approximated by a sum of three components, suggesting that the channel has a minimum of three open and three closed states (Colquhoun and Sigworth 1983). The simplest model of channel gating with this number of states is the linear scheme of (2), and analysis in terms of this scheme was able to accurately predict the experimental open probability (Fig. 3). The model suggests that the channel spends most of its time in transitions between the closed state C_3 and the open state O_1 with the rate of leaving C_3 being most affected by the steady membrane potential.

The physiological role of this channel could be important. It is most active at negative voltages, and the rectification gives a maximum chloride conductance at negative voltages. Significant numbers of such channels would therefore generate a large chloride ion permeability at the normal membrane potentials found in epithelial cells. Chloride permeability in turn, is thought to be crucial in the control of mucus properties and mucociliary clearance. The rectification and voltage sensitivity of the channel contrast strongly with the properties of the other known chloride channels of airway epithelia (Frizzell 1987; Gögelein 1988), which are either voltage insensitive or increase their net conductance with depolarization.

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Appendix

Fitting the open or closed distributions of single channel recordings with the sum of k exponential functions, as shown in (1) leads to the measurement of $2k$-1 experimental parameters. These parameters can then be used to calculate rate constants for models of channel behavior in which k discrete open or closed states are connected by simple chemical reactions. A general solution to the problem of calculating the rate constants of the kinetic model from the amplitudes and time constants of the exponentials has been given by Colquhoun and Hawkes (1977, 1981). For models with three or less closed or open states, a solution is always available but may require numerical techniques for the solution of multiple simultaneous nonlinear equations. For the model used in this paper (2) it is possible to derive a complete solution for the rate constants k_1-k_{10}.

For a sequence of three closed states, the relationship between the calculated parameters from the distribution and the rate constants of the model are given by (Colquhoun and Hawkes 1981):

$$Q_1=K_1+K_2+K_3+K_4+K_5$$

$$Q_2=K_5\cdot(K_1+K_2+K_3)+K_1\cdot(K_3+K_4)\cdot K_2\cdot K_4$$

$$Q_3=K_1\cdot K_3\cdot K_5$$

$$Q_4=K_5\cdot(L_6^2-L_6\cdot(K_{10}+K_2+K_3)+K_1\cdot K_3)$$

$$Q_5=K_5\cdot(L_7^2-L_7\cdot(K_1+K_2+K_3)+K_1\cdot K_3)$$

$$Q_6=K_5\cdot(L_8^2-L_8\cdot(K_1+K_2+K_3)+K_1\cdot K_3),$$

where:

$$Q_1=L_6+L_7+L_8,\quad Q_2=L_6\cdot L_7+L_6\cdot L_8+L_7\cdot L_8,$$

$$Q_3=L_6\cdot L_7\cdot L_8,\quad Q_4=A_6/E_6,\quad Q_5=A_7/E_7,$$

$$Q_6=A_8/E_8,\quad E_6=1/((L_7-L_6)\cdot(L_8-L_6)),$$

$$E_7=1/((L_6-L_7)\cdot L_8-L_7))\quad\text{and}$$

$$E_8=1/((L_6-L_8)\cdot(L_7-L_8)).$$

Here L_6, L_7 and L_8 are the time constants and A_6, A_7 and A_8 are the corresponding amplitudes of the fitted exponentials. Of the last three equations only two are independent since $A_6+A_7+A_8=1$. The solution of the above system is:

$$K_5=Q_3/P_2$$

$$K_4=Q_1-L_7-P_1\cdot P_2-K_5$$

$$K_1=P_2\cdot K_4/((K_4+K_5)\cdot(L_7+P_1\cdot P_2)+P_2-Q_2)$$

$$K_3=P_2/K_1$$

$$K_2=L_7+P_1\cdot P_2-K_1-K_3,$$

where: $P_1=(1-Q_3/Q_5)/L_7$ and $P_2=L_6\cdot(L_6-L_7)/(Q_4/Q_3+L_6\cdot P_1-1)$. Similar solutions can be derived for open states. The probability of the channel being in a given state can be calculated by assuming that the system is in equilibrium, leading to:

$$[C_1]=K_2\cdot K_3\cdot K_6\cdot K_8\cdot K_{10}/D$$

$$[C_2]=K_1\cdot K_4\cdot K_6\cdot K_8\cdot K_{10}/D$$

$$[C_3]=K_1\cdot K_3\cdot K_6\cdot K_8\cdot K_{10}/D$$

$$[O_1]=K_1\cdot K_3\cdot K_5\cdot K_8\cdot K_{10}/D$$

$$[O_2]=K_1\cdot K_3\cdot K_5\cdot K_7\cdot K_{10}/D$$

$$[O_3]=K_1\cdot K_3\cdot K_5\cdot K_7\cdot K_9/D,$$

where:

$$D=K_2\cdot K_4\cdot K_6\cdot K_8\cdot K_{10}+K_1\cdot K_4\cdot K_6\cdot K_8\cdot K_{10}$$
$$+K_1\cdot K_3\cdot K_6\cdot K_8\cdot K_{10}+K_1\cdot K_3\cdot K_5\cdot K_8\cdot K_{10}$$
$$+K_1\cdot K_3\cdot K_5\cdot K_7\cdot K_{10}+K_1\cdot K_3\cdot K_5\cdot K_7\cdot K_9.$$

The total probability of the channel being open is $[O]=[O_1]+[O_2]+[O_3]$.

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