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EVIDENCE THAT pH-TITRATABLE GROUPS CONTROL THE ACTIVITY OF A LARGE EPITHELIAL CHLORIDE CHANNEL

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Summary. The effects of pH changes were examined on the properties of a large, voltage dependent, epithelial Cl^- channel from bovine tracheal cells. Alkaline solutions in the range pH=7.4-9.2 had no detectable effects on channel conductance or gating. However, acid solutions significantly reduced channel open probability, raising the voltage required to open the channel. Analysis of channel activity in the acidic pH range suggested that at least one charged group on the channel with an apparent pK=6.09, is responsible for its voltage dependence. Neutralization of this charge does not eliminate the voltage dependence, but changes the energy difference between the closed and open states. The absence of any change in channel conductance over this wide pH range suggests that the protonation site is far removed from the channel permeation pathway.

Apical membrane Cl^- channels play important roles in the regulation of salt and water transport in a variety of salt secreting epithelia. Several groups of different ion channels have been identified in the apical membranes of epithelial cells, including a large conductance (340-450 pS) anion channel (1,2). Although the physiological role of these channels is not clear, several reports indicate that they are regulated by endogenous GTP-binding proteins, and may be involved in transepithelial fluid secretion in the lung (1).

In the present study we examined the behavior of large conductance Cl^- channels during intracellular pH changes. Our results indicate that pH-modifiable electrostatic interactions modulate the kinetics, but not the conductance of the large epithelial Cl^- channel.

MATERIALS AND METHODS

Apical membrane proteins were prepared from bovine tracheae according to established methods (3). Apical membrane vesicles were separated from the basolateral fraction by a magnesium precipitation method. Measurements of the alkaline phosphatase activity in the apical fraction showed 31.2±5.9 (n=6) times enrichment, compared to the homogenate. Proteins were solubilized with CHAPS, and used to prepare giant liposomes by the hydration-dehydration method (4,5).

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Standard patch-clamp recording techniques were used (6). Pipettes were made from borosilicate microfilament glass, and tips were coated with Sylgard (Dow Corning) to reduce capacitance, and fire-polished. Pipette resistance was about 12 MΩ. Currents were detected with a List EPC-7 amplifier and pipette offset potentials were measured and corrected before forming a seal. Exchange solutions were provided by a multiple pathway flow system with a very small dead space to minimize exchange times. All potentials were reported relative to zero in the extracellular solution, and positive currents were outwards throughout. Pipette solution contained (in mM) 140 choline Cl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH=7.4. Standard bath solution contained 140 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH=7.4. The pH of the bath solution was monitored continuously using a solid state micro pH electrode (Whatman).

The procedures used for computer data analysis were based largely on those described by Colquhoun & Sigworth (7). 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. Distributions of current amplitudes were created and the histograms were fitted to the sum of one to five Gaussian distributions, each Gaussian reflecting a conductance or closed level. The probability of the channel being open was calculated as a percentage of a total time spent in any conductance state compared to total recording time.

RESULTS

Figure 1A shows single channel recordings from a large conductance epithelial Cl^- channel at different bath pH values. The mean conductance for this type of channel was 396±27 pS (n=19), and was unaffected by pH changes in the bath solution (Fig. 1B). The channel was significantly

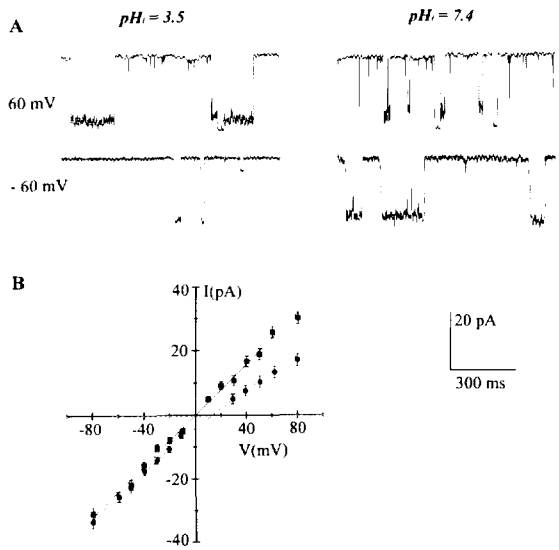

Fig 1. A) Single-channel recordings of a large conductance Cl^- channel at ±60 mV. Dotted lines indicate closed conditions in all cases. Bath pH is shown above the tracings. B) Current-voltage relationships under symmetrical pipette/bath 140 mM NaCl (Squares) and asymmetrical 70/140 mM NaCl (circles) conditions. The data are presented as the means±SD of 12 recordings in symmetrical and 4 in asymmetrical solutions (pH=7.4). The solid line shows a linear regression fit giving a channel conductance of 397.4±16.1 pS. The dashed line represents the best fit of the GHK equation under nonsymmetrical NaCl conditions and gives a channel permeability ratio of P_{Na}/P_{Cl}=0.11±0.04.

more selective for anions than cations. When 140 mM NaCl in the bath solution was replaced by 70 mM NaCl, fitting the Goldman-Hodgkin-Katz (GHK) current equation (8) to the current-voltage relationship gave a permeability ratio P_{Na}/P_{Cl} =0.11±0.03 (n=4). Similar P_{Na}/P_{Cl} permeability ratios were obtained at pH 6.5, 7.4 and 9.2. At each pH studied, the channel was more active at depolarizing than at hyperpolarizing voltages. The voltage-dependence of a channel gating f(V), is described by the Boltzmann function (9):

$$f(V) = \frac{1}{1+\exp(-N_z F(V-V_o)/RT)} \tag{1}$$

where N_z is a number of charges which traverse the membrane during the transition between the open and closed states, V is the voltage, V_o is the voltage at which channel free energy between open and closed states is zero, F, R, and T are the Faraday constant, the gas constant and the temperature, respectively.

The effects of low pH on channel behavior suggested that the primary effect of protons was on channel gating, reducing channel open probability (Fig. 1). This effect could be caused by stabilization of channel closed states, or by destabilization of channel open states. Let a channel protein has N_h acidic groups X, that can bind protons according to the reaction:

$$X + N_h H^+ \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} XH^+_{N_h} \tag{2}$$

Then, the fraction of X in the unprotonated form is given by:

$$\frac{X}{X + XH^+_{N_h}} = \left(1+10^{(pK-N_h pH)}\right)^{-1} \tag{3}$$

where $pK = -log(k_{-1}/k_1)$.

If we assume that P_o is proportional to the number of groups in the unprotonated form, then:

$$P_o = f(V)\left(1+10^{(pK-N_h pH)}\right)^{-1} \tag{4}$$

where $f(V)$ is the function given by equation (1).

In the general case, if a pH-independent component of P_o, called P_{in} exists, then P_o is given by:

$$P_o / P_{max} = P_{in} / P_{max} + f(V)\left(1 - P_{in} / P_{max}\right)\left(1+10^{(pK-N_h pH)}\right)^{-1} \tag{5}$$

where P_{max} is the maximal value of P_o.

The values of P_{max} and P_{in} were estimated to be 0.93, and 0.04, respectively. Figure 2 presents the best fits of Eq. 5 to the experimental data. The fitted parameters, V_o, N_z, pK, and N_h, were determined for different pH values. N_z was not significantly affected by pH variations, and the best fit was obtained for N_z = 0.78±0.15. This indicates that titration does not affect channel voltage gate. V_o changed from -48.36 mV at pH=9.2, to -2.72 mV at 3.5. This change suggests that, in the absence of an electric field, the free energy difference between channel open and closed states

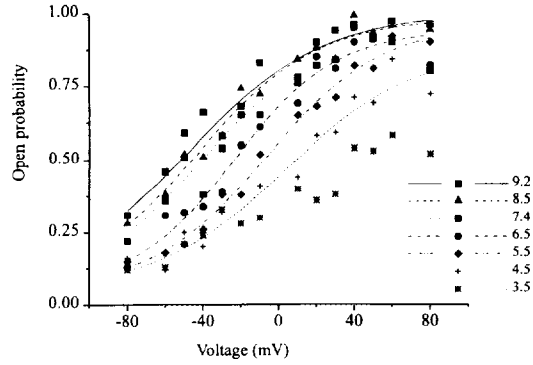

Fig 2. Single channel open probability versus voltage at different bath pH values. The data represent mean values from 6(3.5), 6(4.5), 5(5.5), 8(6.5), 11(7.4), 3(8.5) and 8(9.2) different experiments. Standard deviations varied between 0.08 and 0.29 and were omitted for clarity. The lines through the data were obtained from equation (5).

decreased with an increase in bath pH. N_h was not significantly affected by pH or voltages changes, and was equal to 1.27 ± 0.34. This value is not significantly different from one, indicating that there is probably a single proton binding site on the channel protein. However, since N_h gives a minimum estimate of binding sites, we cannot exclude the possibility that other sites with lower affinities are also involved. The mean value of pK was 6.09±0.42, and did not represent any significant pH-dependence at the 95% confidence level. This value of pK indicates that histidine or some acidic amino acids could probably be involved in the pH modulation of channel activity.

DISCUSSION

Our results show that the large epithelial Cl^- channel has at least one titratable site located on the intracellular site of the membrane that can be modified by changes in solution pH. The fact that channel conductance is pH insensitive, indicates that the protonation site is probably not close to the channel permeation pathway. If the titratable groups were located within the channel pore, then one would expect that the protonation-deprotonation reaction would lead to a flickering current signal in single channel recordings (8). Alternatively, if the protonation reaction were too fast to be resolved in patch-clamp experiments, one would expect a decrease in channel current, as was observed in L-type Ca^{++} channels (9). If the protonation site were located near the channel entrance, then changes in electrostatic interactions caused by pH would affect the distribution of ions near the channel pore, and therefore its measured conductance. However, no significant change in channel conductance was observed (Fig. 1). These results also indicate that both the conduction pathway and the channel entrance are electrostatically isolated from the charged head groups on the membrane lipids, because these would also be titrated by the pH changes.

Since all of these experiments were performed with ion channel proteins incorporated into liposomes, it is possible that the channel sometimes adopted the opposite orientation to

normal. However, there is strong experimental evidence to suggest that detergent-solubilized proteins incorporate into lipid bilayers in a unidirectional orientation determined solely by protein features (10). Other reports showed that channels in excised patches from liposomes were activated by exposure to NaF, similarly to native cells (11), and that variations in pH were only effective on the same side of the bilayer as the addition of channel proteins (12). Additionally, studies of protein orientation after reconstitution into liposomes indicated that between 75% and 95% of all proteins preserved their original orientations in the cell membrane (13). Therefore, we can probably assume that channel orientations after reconstitution into liposomes were similar to their original orientations in the native cells.

The parameter N_z can be interpreted as a measure of the minimum number of charged groups on the protein which detect the applied electric field and allow the protein to respond to the field. A decrease in this parameter indicates neutralization of some of these charges, and a reduction in the channel's ability to respond to the electric field. The fact that N_z did not change appreciably with pH suggests that pH does not affect the voltage dependence of the gating reaction.

Most of the changes in channel activity were measured while the pH was decreasing from 7.4 to 3.5. Therefore, the possible identities of the gating charges associated with the channel protein can be limited to the side chains of amino acids such as histidine, aspartic acid, glutamic acid, and the terminal carboxyl groups. The intrinsic pK values of these groups within proteins have been reported to be 5.2-6.4, 3.0-4.7, 4.3-4.5, and 3.5-4.3, respectively (14). Our evaluation of the number of groups involved suggests that at least one titratable amino acid residue, probably histidine, is involved in channel gating.

Large-conductance anion-selective channels have been described in a variety of cell types but their physiological function is unknown. It is possible that these channels play a transient role in internal ion regulation in cells, or in intracellular pH regulation by being permeable to HCO^-_3 ions. However, their relatively great abundance in a large number of cell types, and regulation by physiologically important enzymes such as G-proteins, suggests that they are important in cell physiology. These channels will normally experience a significant variation in the pH of their environment, so that knowledge of their pH sensitivity is crucial to understanding their normal function.

REFERENCES

1. Kemp, P.J., MacGregor, G.G., and Olver, R.E. (1993) Am. J. Physiol. 265, L323-L329.
2. Xu, B. and Lu, L. (1994) J. Membr. Biol. 142, 65-75.
3. Langridge-Smith, J.E., Field M., and Dubinsky W.P. (1983) Biochim. Biophys. Acta 731, 318-328.
4. Keller, B.U., Hedrich, R., Vaz W.L.C., and Criado M. (1988) Pflügers Arch. 411, 94-100.

5. Duszyk, M., Liu, D., French, A.S., and Man, S.F.P. (1993) Eur. Biophys. J. 22, 5-11.

6. Hamill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. (1981) Pflügers Arch. 391, 85-100.

7. Colquhoun, D. and Sigworth, F.J. (1985) In Single-channel recording (B. Sakmann and E. Neher, Eds.), pp. 191-236. Plenum Press Publ. Co. New York.

8. Hille, B. (1992) Ionic channels of excitable membranes, Sinauer Associates Inc. Sunderland, Mass.

9. Prod'hom, B., Pietrobon, D., and Hess, P. (1987) Nature 329, 243-246.

10. Brunen, M. and Engelhardt, H. (1993) Eur. J. Biochem. 212, 129-135.

11. Duszyk, M., Liu, D., Kamosinska, B., French, A.S. and Man, S.F.P. (1995) J. Physiol. (in press).

12. Hanke, W. and Miller, C. (1983) J. Gen. Physiol. 82, 25-45.

13. Gennis, R.B. (1989) Biomembranes, molecular structure and function, Springer, New York.

14. Creighton, T.E. (1993) Proteins. Structure and molecular properties, W.H. Freeman & Co. New York.