J. Membrane Biol. 148, 1–11 (1995)

The Journal of
Membrane
Biology

pH- and Voltage-dependent Conductances in Toad Skin

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Received: 24 April 1995/Revised: 6 July 1995

Abstract. The present study focuses on two closely related topics on ion conductance in toad skins: (i) the interaction of apical protons with the apical voltage-dependent Cl^--activated channels of the mitochondria-rich cells, and (ii) the description and characterization of a novel subject, a voltage-dependent H^+-activated conductance.

The Cl^- conductance (G_{Cl}) is activated by tissue hyperpolarization (which leads to apical membrane depolarization) and the presence of Cl^- ions in the apical solution. Increasing apical proton concentration (from pH 8 to pH 4) impairs the process of activation of the Cl^- conductive pathway, slowing the kinetics of I_t activation and reducing the steady-stage values of G_t and I_t. This effect is markedly voltage-dependent since no effect is seen at $V_t = -100$ mv and is fully present at −50 mV. The voltage-dependence of the pH effect suggests that the critical protonation sites of the apical Cl^- channels are not freely exposed to the apical solution but dwell within the membrane electric field. An also coherent interpretation is that titration of apical proton binding sites affects the gating of the voltage-dependent Cl^- channels, shifting the conductance-*vs.*-voltage curve to more negative clamping potentials.

Tissue conductance in the absence of apical Cl^- ions can be importantly affected by the pH of the apical solution (pH_a), the effect being markedly dependent on the clamping potential. Generally speaking, the effect of rising apical proton concentration can be conspicuous at negative clamping potentials, while at positive potentials changes in tissue conductance were never observed. For a clamping potential of −100 mV, a turning point somewhere between $pH_a = 4$ and $pH_a = 3$ was observed. Apical acidification to pH 4 has no effect upon tissue conductance while apical acidification to pH 3 leads to a marked, slow and reversible increase of tissue conductance. A striking similitude exists between the voltage-dependent Cl^--gated conductance and the voltage-dependent proton-gated conductance regarding: (i) slow time courses of activation and deactivation, (ii) requirement for a negative clamping potential and the presence of a specific ion species in the apical solution for activation to take place, (iv) instantaneous ohmic behavior, and (v) steady-state rectification. However, so far the results do not permit one to conclude definitely that the voltage-dependent Cl^--gated conductance and the voltage-dependent proton-gated conductance share a common pathway.

Key words: Toad skin — pH — Ion conductance — Voltage dependence — Chloride conductance

Introduction

Epithelia have, among other functions, the ability to absorb or to secrete fluid. Cl^- channels play, among different functions, a key role in Cl^- absorbing or secreting epithelia, in cellular volume regulation, and in stabilization of membrane potential (Gogelein, 1989; Pusch & Jentsch, 1994). The chloride conductance (G_{Cl}) in amphibian skins has been associated with a voltage-dependent pathway (Bruus, Kristensen & Larsen, 1976; Larsen & Kristensen, 1978; Larsen, 1982; Larsen & Rasmussen, 1982, 1983, 1985; Lacaz-Vieira & Procopio, 1988*a,b;* Procopio & Lacaz-Vieira, 1990). Chloride channels presumed to be located in the apical membrane of the mitochondria-rich cells apparently are implicated in this process (Foskett & Ussing, 1986; Spring & Ussing, 1986; Larsen, Ussing & Spring, 1987; Larsen & Harvey, 1994). Activation of G_{Cl} results from depolarization of the apical membrane in response to whole tissue hyperpolarization and is critically dependent on the presence of Cl^- in the apical solution. In its absence,

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conductance activation does not take place even when the apical membrane is depolarized (Kristensen & Larsen, 1978; Harck & Larsen, 1986). In short-circuited tissues, G_{Cl} activation is induced by apical exposure to Cl^- or Br^-, whereas I^-, SCN^-, gluconate, and SO_4^{2-} are without effect. Once activated, the conductive pathway exhibit a poor anion selectivity indicating that it can be more properly referred to as an anion-permeable pathway than a specific Cl^- pathway (Larsen, 1991). So far, the available data indicate that regulation occurs at a Cl^- (or Br^-) ion-specific site remote from a poor anion-selective translocation site of the channel (Kristensen, 1982).

Besides being controlled by the potential difference and the apical Cl^- or Br^- concentration, the anion conductive pathway also seems to be modulated by a c-AMP-dependent process (Cuthbert & Painter, 1968; Mandel, 1975; Kristensen, 1983; Katz & Larsen, 1984; Katz & Van Driessche, 1987), c-AMP shifting the G_t-*vs.*-V curve along the V axis (Willumsen, Vestergaard & Larsen, 1992). The observation of a Lorentzian component in the power density spectrum of forskolin-treated frog skins that depends on the presence of Cl^- in the bathing solutions and are modified by Cl^- channel blockers also support the existence of c-AMP-activated Cl^- channels in frog skin (De Wolf, Van Driessche & Nagel, 1989).

Ion channels of plasma membrane of neurons (Hille, 1968; Tang, Dichter & Morad, 1990; Ueno, Nakaye & Akaike, 1992), muscles (Pietrobon, Prod'hom & Hess, 1989; Prod'hom, Pietrobon & Hess, 1989), epithelial cells (Kuwahara et al., 1989; Chang, Kushman & Dawson, 1991; Klaerke et al., 1993; Suzuki et al., 1994), limphocytes (Deutsch & Lee, 1989) organelles (Rousseau & Pinkos, 1990), and others (Wilmsen, Pugsley & Pattus, 1990; Todt & McGroarty, 1992; Todt, Rocque & McGroarty, 1992) are affected by the concentration of protons in the bathing solutions. These effects may result from changes of the kinetics of activation or inactivation, single-channel conduction, or ionic selectivity. Ion-conducting channels formed in lipid bilayers by diphtheria toxin are highly pH dependent, the channel's single-channel conductance and selectivity depending on proton concentration on either side of the membrane (Mindell et al., 1994*a,b*). The study of mutant channels unveiled an important aspect that a few charged residues, sometimes a single residue, is responsible for the pH effect (Mindell et al., 1994*a,b*). Cl^- channels in different structures are affected by the proton concentration in the bathing solutions. In colonic epithelial cells intracellular pH regulates G_{Cl} by modulating Ca^{2+} activation, external pH having no effect (Chang, Kushman & Dawson, 1991). Cl^--channels of the gastric parietal cell incorporated into lipid bilayers are active at low pH on the *trans* side, a low pH_{trans}, increasing channel open probability, but reduction of the pH on the *cis* side from 7.4 to 3 always resulted in loss of channel activity (Cuppoletti, Baker & Malinowska, 1993). A differential acidic pH sensitivity between normal and delta F508 CFTR Cl-channel activity in lipid bilayers has been reported and may be of significance to the understanding the cystic fibrosis defect since normal CFTR can function in the environment of acidic intracellular organelles, whereas the activity of mutant CFTR would be greatly reduced (Sherry, Cuppoletti & Malinowska, 1994).

The existence of a voltage sensor (charges or dipoles that move under the influence of the membrane electric field) is essential for the mechanism of voltage dependence of ion channels. Surface charges on ion channel proteins may affect their function in several ways (for review *see* (Green & Andersen, 1991; Latorre, Labarca & Naranjo, 1992; Jordan, 1993). They may influence conductance by changing the concentration of permeant counter-ions according to a Boltzmann distribution (MacInnes, 1961; Apell, Bamberg & Läuger, 1979; Hille, 1992), modulating gating kinetics (Behrens et al., 1989) or influencing transitions to subconductance states (Recio-Pinto et al., 1990). Thus, it can be expected that the function of ion channels might be modulated, among other variables, by the degree of protonation of the membrane surface charges which are highly affected by the concentration of hydrogen ions in the solutions bathing the membrane in which the channels are incorporated.

Materials and methods

Preparation

Abdominal skins of double-pithed toad *Bufo marinus* were used. A plastic ring of 20 mm diameter was glued to the apical surface of the skin with ethylcyanoacrylate adhesive (Super Bonder, Loctite Brasil Ltda). The fragment of tissue framed by the plastic ring was excised, immersed in Ringer solution and subsequently mounted in a modified Ussing's chamber (Castro, Sesso & Lacaz-Vieira, 1993), exposing an area of 0.5 cm^2. Hemichambers with a recessed rim filled with high viscosity silicone grease (Dow Corning High Vacuum Grease) prevented tissue edge damage (Lacaz-Vieira, 1986). Each chamber compartment was perfused with a continuous flow of solution (up to 25 ml/min) driven by gravity. Unstirred layers on the tissue surfaces were minimized by directing the incoming fluid towards the surfaces of the tissue. Each compartment was drained through a spillway open to the atmosphere, so that the pressure inside each compartment was kept constant at the atmospheric level. Rapid solution changes were obtained, without interrupting the voltage clamping, by switching the inlet tubings at their connections with the chamber.

Electrical Measurements

A conventional analogue voltage clamp (WPI DVC 1000) was used. Saturated calomel half-cells with 3 M KCl-agar bridges were used to measure the electrical potential difference across the skin. Current was passed through Ag-AgCl 3 M KCl electrodes and 3 M KCl-agar bridges, adequately placed to deliver a uniform current density across the skin.

The clamping current was continuously recorded by a strip-chart recorder. Clamping current and voltage were also digitized through an analog-to-digital converter (Digidata 1200 and Axotape 2.0, Axon Instruments) and recorded in a computer (Microtec 386 SX) for further processing. Current-voltage relationships were obtained by applying voltage steps, generated by a computer driven digital-to-analog converter connected to the external command port of the voltage clamp in order to impose clamping potentials of −200, 200, −180, 180, −160, 160, . . . −20, 20, and 0 mV, of 100 msec duration or less, on top of the previous holding potential.

Solutions

The inner bathing solution was NaCl Ringer in all experiments, with the following composition (in mM): NaCl 115, $KHCO_3$ 2.5, and $CaCl_2$ 1.0, with a pH of 8.2 after aeration. The apical bathing fluids were simple salt solutions, nonbuffered, without added Ca^{++} prepared with glass distilled water, having pH around 6.0 and free-Ca^{2+} concentration in the range of 1.5×10^{-7} and 2.0×10^{-7} M (Castro et al., 1993). Apical solution pHs were adjusted by adding HCl or H_2SO_4 (according to the main anion present in the apical solution) or KOH.

Statistics

The results are presented as mean ± SEM. Comparisons were carried out using Student's paired *t*-test. When more than two groups were compared, significance was determined by two-way analysis of variance followed by appropriate post-test comparison. The *P* values cited include Bonferroni's correction (Neter & Wasserman, 1974).

Abbreviations

G_t Total transmembrane electrical conductance, in mS/cm^2. Calculated from the deflections of the clamping current induced by shifts of the clamping potential of 300 msec duration, ±10 mV amplitude at 15-sec intervals, as $G_t = \Delta I_t/\Delta V_t$, where ΔV_t and ΔI_t are the changes in the electrical potential difference across the tissue and clamping current, respectively. I_t Clamping-current, in μA/cm^2. Positive (or inward) current corresponds to the transport of positive charges across the tissue, from the apical to the inner bathing solution. V_t Electrical potential difference across the tissue, in mV. The potential of the apical solution is referred to that of the inner solution. pH_a pH of the apical solution. $[Cl^-]_a$ Cl^- concentration in the apical solution.

Results

Effects of Apical pH and Clamping Potential on the Activation of the Cl^- Conductive Pathway

The present experiments aimed to evaluate the effects of pH_a on the activation of the voltage-dependent Cl^--gated G_{Cl}. The results show a marked interdependence of pH_a and clamping potential (V_t) on the activation of the Cl^- conductive pathway, as reflected by the steady-state levels of the clamping current (I_t), skin conductance (G_t), and the time course of I_t activation. The results compare the effects of two of pH_a values (8 and 4) at four different

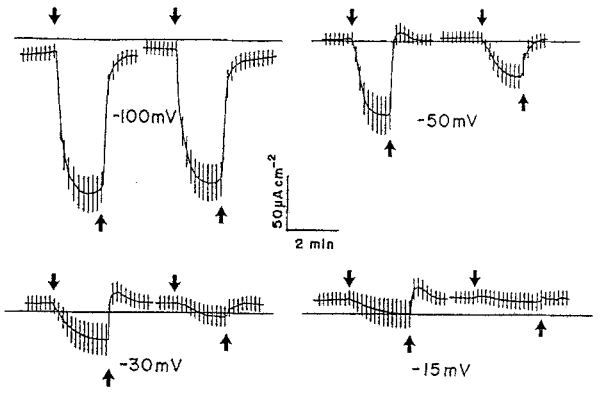

Fig. 1. I_t as a function of time for a representative experiment (of a group of 8 skins) performed in a single piece of tissue showing the effects of pH_a on the activation of the Cl^- conductive pathway. The skin was bathed on the inner side by NaCl Ringer. The four panels present similar sequences of two measurements each performed at the indicated clamping potential. The first measurement of each panel was carried out at $pH_a = 8$ and the second, at $pH_a = 4$. For each measurement, activation of I_t was induced by a sudden rise of apical Cl^- concentration, obtained by replacing the apical solution, initially K_2SO_4 57.5 mM, for a KCl 115 mM solution at the same pH (downward arrows indicates K_2SO_4 for KCl substitution). After being fully activated, the Cl^- pathway was deactivated by flushing the apical compartment with the initial bathing solution of K_2SO_4 (upward arrows indicates KCl for K_2SO_4 substitution). The vertical bars are deflections of I_t induced by V_t shifts of ±10 mV.

levels of V_t (−100, −50, −30 and −15 mV). A representative experiment of a group of 8 tissues is shown in Fig. 1. Tissue bathing solutions are described in the legend of Fig. 1. Activation of the Cl^--conductive pathway was induced by a sudden rise of $[Cl^-]_a$, while V_t was held at a preset level. It can be seen (Fig. 1) that the effect of a rise of $[Cl^-]_a$ on the activation of G_{Cl} is markedly affected by pH_a as well as V_t. Thus, for $V_t = -100$ mV the time course of I_t activation and the steady-state levels of I_t and G_t do not differ when experiments performed at $pH_a = 8$ and $pH_a = 4$ are compared. For higher V_t values (−50, −30 and −15 mV), in contrast, the level of activation (characterized by the steady-state values of I_t and G_t) is smaller and the time course of I_t activation is longer in experiments performed at $pH_a = 4$ as compared to experiments at $pH_a = 8$. These effects of apical solution acidification upon the activation of the Cl^--conductive pathway are entirely reversible.

Acidification of the apical solution not only affects the process of activation of the Cl^- conductive pathway, as shown above, but it also influences the steady-state level of activation previously attained at a more alkaline pH (normally $pH_a = 8$). This is shown for a representative experiment of a group of 6 skins in Fig. 2, where a skin clamped at −50 mV had the Cl^--conductive pathway activated by a sudden rise of $[Cl^-]_a$. With the Cl^--conductive pathway fully activated, acidification of the apical solution from pH 8 to pH 4 led to a conspicuous

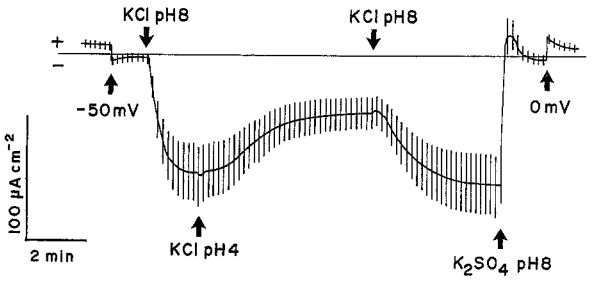

Fig. 2. I_t as a function of time for a representative experiment, of a group of 6 skins, showing the effect of pH_a on the steady-state level of activation of the Cl^- conductive pathway. The skin was bathed on the inner side by NaCl-Ringer, on the apical side by a solution of K_2SO_4 57.5 mM at pH 8, and short-circuited. Subsequently V_t was clamped to −50 mV and the Cl^- conductive pathway activated by replacing the apical solution by a KCl solution 115 mM at the same pH. When a steady state of activation was attained, the apical solution was replaced for approximately 6 min by a solution of KCl 115 mM at pH 4 and, subsequently, the apical solution was returned to pH 8. The vertical bars are deflections of I_t induced by V_t shifts of ±10 mV.

inactivation of the Cl^--conductive pathway that is characterized by a reduction of G_t and a shift of I_t to more positive levels. As shown in Fig. 2, this effect reverts completely when the apical solution is again returned to pH 8.

The results of experiments performed according to the protocol of Fig. 1 are summarized in Table 1 which shows mean steady-state values of I_t and G_t, as well as the half times of I_t activation for two pH_a values (8 and 4) and four levels of V_t (−100, −50, −30 and −15 mV). From the present results it can be concluded that activation of the Cl^- conductive pathway is significantly affected by pH_a and V_t.

To determine the effects of apical solution acidification (in the pH_a range of 8 to 4) on I_t and G_t in the absence of apical Cl^- ions, experiments were performed having a K_2SO_4 (57.5 mM) solution in the apical compartment and NaCl Ringer in the inner compartment. Table 2 shows that acidification of the apical solution from pH 8 to pH 4 leads to a small increase of I_t for all V_t values studied, except for $V_t = -100$ mV, while G_t is not significantly affected by apical acidification at any clamping potential.

Characterization of a Voltage-Dependent H^+-Gated Conductive Pathway

This study was carried out to analyze in more detail the effects of apical solution acidification upon I_t and G_t. The experiments were performed in the absence of apical Cl^- ions.

The results show the existence of a clear transition in the electrophysiological behavior of skins when pH_a is reduced to values below 4 and characterize the existence of a voltage-dependent proton-gated conductance which is not related to the presence of Cl^- ions in the apical solution.

The experiments were performed in skins bathed on the apical surface by a solution of K_2SO_4 (57.5 mM), on the inner surface by NaCl-Ringer and initially short-circuited. Acidification of the apical solution from pH 8 to pH 3 (Fig. 3, first run) and to pH 4 (Fig. 4, upper trace) were carried out, followed by a subsequent shift of V_t to −100 mV. In the short-circuited condition, apical solution acidification to pH 4 or pH 3 causes a minor positive deflection of I_t which rapidly reaches a steady-state level. In most cases, as shown in Fig. 3, the increase of I_t is only transient. G_t, on the other hand, is not affected by apical solution acidification at both pHs. A subsequent tissue hyperpolarization (V_t shift to −100 mV) has a completely distinct outcome according to the apical pH, indicating the existence of a turning point between $pH_a = 4$ and $pH_a = 3$. For $pH_a = 4$ (as well as for pH_as above 4, *not shown*), the skin responses to hyperpolarization ($V_t = -100$ mV) are ohmic (Fig. 4), being characterized by a negative deflection of I_t and constancy of G_t. Subsequent return to the short-circuited condition and to $pH_a = 8$ brings I_t to the initial control level. In contrast, at $pH_a = 3$ (Fig. 3), skin hyperpolarization to −100 mV causes an instantaneous ohmic response (similar to that seen at $pH_a = 4$, and characterized by a sharp negative deflection of I_t) that is followed by a slow and pronounced sigmoidal increase of I_t which attains a stationary condition with a half-time of 43 sec (n = 8 skins). This current response is accompanied by slow and marked increase of G_t. At $pH_a = 3$, a V_t shift from 0 mv to −100 mV causes G_t to increase from 0.94 ± 0.08 msec/cm^2 to 3.6 ± 0.22 msec/cm^2 ($P < 0.01$), and I_t to shift from 55.0 ± 3.7 mA/cm^2 to −236.0 ± 19.1 mA/cm^2 ($P < 0.01$). These changes of I_t and G_t which take place at $pH_a = 3$ in response to skin hyperpolarization may reflect a slow activation of a voltage-dependent H^+-gated conductive pathway. A slow inactivation occurs when the skin is returned to the short-circuited condition (Fig. 3), indicating that the system behaves in a totally reversible way. The sequence in which tissue is hyperpolarized and the apical solution acidified is irrelevant for the activation of the voltage-dependent proton-gated conductance. Thus, if a skin initially bathed by an apical solution of K_2SO_4 57.5 mM at $pH_a = 8$ is clamped to −100 mV, an ohmic response is observed. A subsequent acidification of the apical solution has a completely different outcome depending whether pH_a is shifted to a value above or below the turning point, which is somehow between $pH_a = 4$ and $pH_a = 3$. Apical acidification to pH 3 caused a slow sigmoidal increase of I_t accompanied by a slow increase of G_t towards steady-state values, as shown in Fig. 3 (second run). In contrast, apical acidification to pH 4 (Fig. 4, lower trace) or pHs above 4 (*not shown*) causes no significant effect upon I_t or G_t. Finally, return to pH_a

Table 1. Effects of pH_a and V_t upon the Cl^- conductive pathway

V_t (mV)	I_t (μA cm^{-2})		G_t (mS cm^{-2})		$t_{1/2}$ (s)	
	pH_a 8	pH_a 4	pH_a 8	pH_a 4	pH_a 8	pH_a 4
−100	−247.9 ± 37.2	−235.1 ± 35.3 ns	30.4 ± 3.4	31.1 ± 3.8 ns	18.2 ± 3.8	14.9 ± 1.9 ns
−50	−99.1 ± 23.6	−48.3 ± 12.3*	27.7 ± 5.0	18.6 ± 2.8*	19.3 ± 1.3	33.1 ± 1.2**
−30	−36.0 ± 13.4	−7.7 ± 4.6*	23.4 ± 4.6	15.5 ± 2.0*	21.5 ± 2.4	37.4 ± 2.7*
−15	−1.4 ± 3.1	7.8 ± 1.2*	11.2 ± 1.9	7.6 ± 0.8*	29.8 ± 5.6	38.8 ± 4.3*

Effects of pH_a and V_t on the steady-state levels of activation of the Cl^- conductive pathway, as indicated by the stationary values of I_t and T_p, and the time course of activation of the Cl^- conductive pathway, evaluated by the half-time of I_t activation ($t_{1/2}$). The experiments were carried out according to the protocol described in the legend of Fig. 1. *$P < 0.05$, **$P < 0.01$, n = 8.

Table 2. Effects of pH_a and V_t upon I_t and G_t in the absence of apical Cl^-

V_t (mV)	I_t (μA cm^{-2})		G_t (mS cm^{-2})	
	pH_a 8	pH_a 4	pH_a 8	pH_a 4
−100	−36.9 ± 2.6	−38.6 ± 5.9 ns	9.8 ± 0.9	11.9 ± 1.3 ns
−50	−2.6 ± 2.3	4.1 ± 1.9**	9.9 ± 1.5	9.5 ± 1.1 ns
−30	8.1 ± 2.7	14.7 ± 1.9**	9.6 ± 1.7	9.6 ± 1.2 ns
−15	9.5 ± 1.6	12.3 ± 1.2**	4.9 ± 0.6	4.9 ± 0.5 ns
0	27.4 ± 7.7	40.5 ± 5.6*	8.6 ± 1.0	9.2 ± 1.1 ns

Effects of pH_a and V_t on the steady-state levels of I_t and G_t in the absence of Cl^- ions in the apical solution. The skins were initially bathed on the inner side by NaCl-Ringer's and on the apical side by a solution of K_2SO_4 57.5 mM at pH 8 and short-circuited. V_t was then shifted to the desired potential and, subsequently, the apical solution had its pH reduced to 4. *$P < 0.05$, **$P < 0.01$, n = 8.

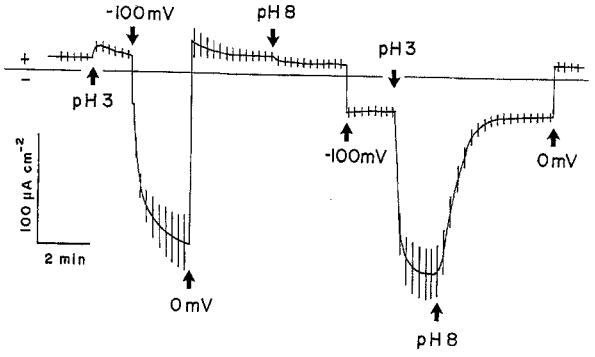

Fig. 3. I_t as a function of time for a representative experiment, of a group of 6 skins, showing the mutual effects pH_a (8 and 3) and tissue hyperpolarization to −100 mV on the activation of a voltage-dependent H^+-gated conductance. The skin was bathed on the inner side by NaCl-Ringer and on the apical side by a solution of K_2SO_4 57.5 mM. Initially, the apical solution had pH 8 and the preparation was short-circuited. Then pH_a and V_t were changed as indicated in the figure. The vertical bars are deflections of I_t induced by V_t shifts of ±10 mV.

= 8, despite keeping the tissue hyperpolarized, deactivates the conductance, G_t and I_t returning to their previous levels (Fig. 3).

The striking similarity between the activation of the Cl^--conductive pathway (Larsen & Rasmussen, 1982; Lacaz-Vieira & Procopio, 1988*a*; Larsen, 1991) and the activation of the voltage-dependent proton-gated pathway can be seen in Fig. 5, for a representative experiment, in which the voltage-dependent Cl^--gated conductance was activated followed by activation of the voltage-dependent proton-gated conductance.

Current-Voltage Curves of the Voltage-Dependent H^+-Gated Pathway

Steady-State Measurements

Skins bathed on the apical side by a K_2SO_4 solution (57.5 mM) and on the inner side by NaCl Ringer exhibit steady-state current-voltage curves that are highly dependent on the pH of the apical solution. In the pH_a range of 8 to 4, the steady-state current-voltage curves are linear, consistent with a preparation exhibiting a simple ohmic behavior. In contrast, experiments performed at $pH_a = 3$ show a highly nonlinear dependence, with current flowing more easily in the outward direction. Fig. 6*A* illustrates a representative experiment of a group of 8 skins in which the steady-state I_t-*vs.*-V_t-relationship is depicted for a single skin at two pH_a values. At $pH_a = 8$ the relationship is linear, while at $pH_a = 3$ the dependence is markedly nonlinear. This highly nonlinear behavior observed at $pH_a = 3$ is better illustrated for this group of skins in Fig. 6*B*, which shows mean G_t values increasing markedly in the negative range of clamping potentials, while at $pH_a = 8$ the $G_t \times V_t$ relationship is linear. These results show that the preparation exhibits a clear rectification when steady-state values are concerned. The time courses of I_t and G_t changes, induced by negative and positive clamping potentials, are shown for a representative experiment in Fig. 7.

"Instantaneous" I_t vs. V_t relationship

Determinations of the "instantaneous" I_t *vs.* V_t relationships (*see* Materials and Methods) were carried out at

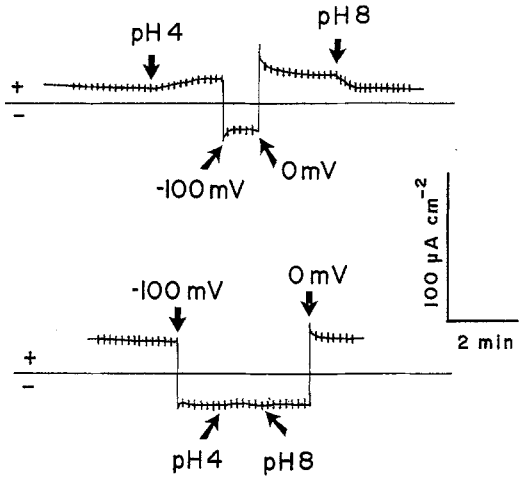

Fig. 4. I_t as a function of time for a representative experiment (of a group of 6 skins) showing the effects of pH_a (8 and 4) and skin hyperpolarization to −100 mV upon I_t and G_t. The skin was bathed on the inner side by NaCl-Ringer and on the apical side by a solution of K_2SO_4 57.5 mM. Initially, pH_a was set to 8 and the preparation short-circuited for both upper and lower traces. Then pH_a and V_t were changed as indicated in the figure. The vertical bars are deflections of I_t induced by V_t shifts of ±10 mV.

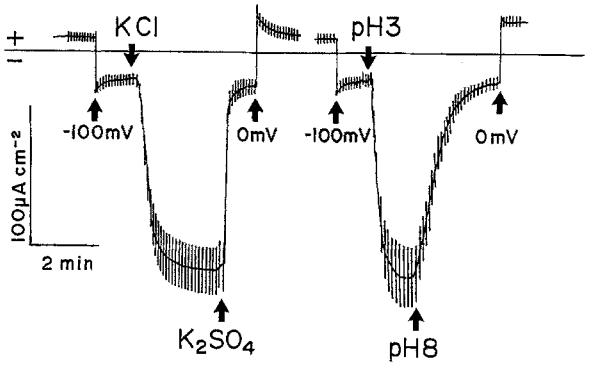

Fig. 5. A representative experiment performed in a single piece of skin in which the voltage-dependent Cl^--gated conductance and the voltage-dependent proton-gated conductance are subsequently activated. The skin was bathed on the inner side by NaCl-Ringer and on the apical side by a solution of K_2SO_4 (57.5 mM) with pH_a adjusted to 8. In the first run G_{Cl} was activated by shifting the clamping potential to −100 mV and replacing the apical solution by a KCl solution at pH 8. Conductance inactivation was obtained by returning to a K_2SO_4 solution on the apical side. In the second run the voltage-dependent proton-gated conductance was activated by shifting the clamping potential to −100 mV and replacing the apical solution by a K_2SO_4 solution at pH 3. Conductance inactivation was obtained by returning to a K_2SO_4 solution at pH 8 on the apical side. Between the first and second runs there was an interval of approximately 6 min. The vertical bars are deflections of I_t induced by V_t shifts of ±10 mV.

various pH_as, clamping potentials (holding potentials) and levels of activation of the voltage-dependent proton-gated conductive pathway. A representative experiment (Fig. 8*A*) of a group of 6 skins shows the time course of

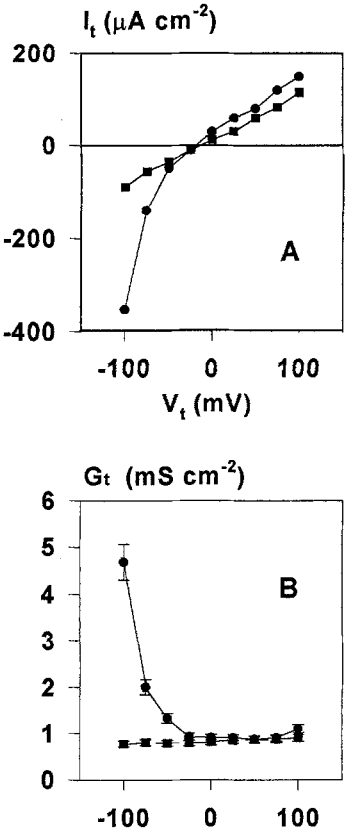

Fig. 6. (*A*) A representative experiment (of a group of 8 skins) showing steady-state current-voltage curves obtained at two pH_a values. Skins were bathed on the inner side by NaCl-Ringer and on the apical side by solutions of K_2SO_4 (57.5 mM) with pH_a adjusted to 8 (squares) or to 3 (dots). (*B*) Mean values of steady-state conductance-voltage curves for the same group of skins at the same conditions of panel A.

I_t and G_t activation and the instants data acquisition were carried out. Except for those measurements taken along the conductance deactivation period (*c* and *d* in Fig. 8*A*), the other determinations were performed when G_t and I_t had attained steady-state values. As the data acquisition intervals for the "instantaneous" I_t *vs.* V_t curves lasted 2 sec and the voltage pulses (100 msec each) were alternatively positive and negative, it can be assumed that the overall skin steady-state conditions (determined by the holding potential and pH_a) prevailing just prior to the sequence of voltage pulses also hold during the acquisition period. Fig. 8*B* shows for the same experiment illustrated in Fig. 8*A* that, for all conditions tested, current and voltage are linearly related. The behavior depicted by this representative skin is very similar to the other 5 skins studied in this group. From these results, it can be concluded that the voltage-dependent proton-gated conductive pathway does not display any instantaneous rectifying property.

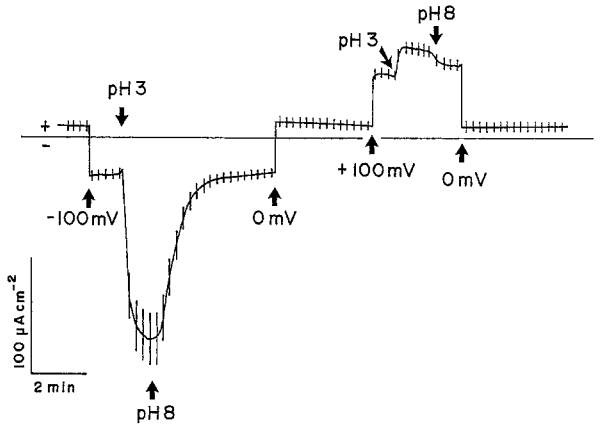

Fig. 7. I_t as a function of time for a representative experiment (of a group of 6 skins) showing the time course of activation of the voltage-dependent H^+-gated conductive pathway. The skin was initially short-circuited, bathed on the inner side by NaCl-Ringer and on the apical side by K_2SO_4 57.5 mM at pH 8. Subsequently, V_t and pH_a were changed as indicated in the figure. The vertical bars are deflections of I_t induced by V_t shifts of ±10 mV.

The Voltage-dependent H^+-gated Conductance and the Voltage-dependent Cl^--gated Conductance are Related to Two Functionally Distinct Pathways

A marked similarity is observed between the time courses of activation of the voltage-dependent Cl^--gated conductance and the voltage-dependent H^+-gated conductance (compare Fig. 3 and Fig. 5) and their steady-state and "instantaneous" current × voltage and conductance × voltage curves (compare Figs. 6, 7 and 8 with Fig. 3 in (Larsen, 1991)). To determine whether the conductive pathways activated in skins hyperpolarized to −100 mV by a rise of apical Cl^- or apical proton concentration are functionally related experiments were carried out in a group of skins initially short-circuited, bathed on the apical side by K_2SO_4 57.5 mM at $pH_a = 8$, and on the inner side by NaCl Ringer. Subsequently, V_t was clamped to −100 mV and a few minutes later the apical solution was replaced by KCl 115 mM at $pH_a = 8$, ensuing a conspicuous activation of G_{Cl}. When this conductance was fully activated, and a steady state had been reached, replacement of the apical solution by a K_2SO_4 solution at $pH_a = 3$ led to a sharp reduction of I_t amplitude, followed by a rapid increase toward a new highly conductive state (Fig. 9). The significance of this finding is presented in the Discussion.

Discussion

The present study focuses on two closely related topics on ion conductance in toad skins: (i) the interaction of apical protons with the voltage-dependent Cl^--activated conductance, and (ii) the description and characterization of a novel subject, a voltage-dependent H^+-activated conductance.

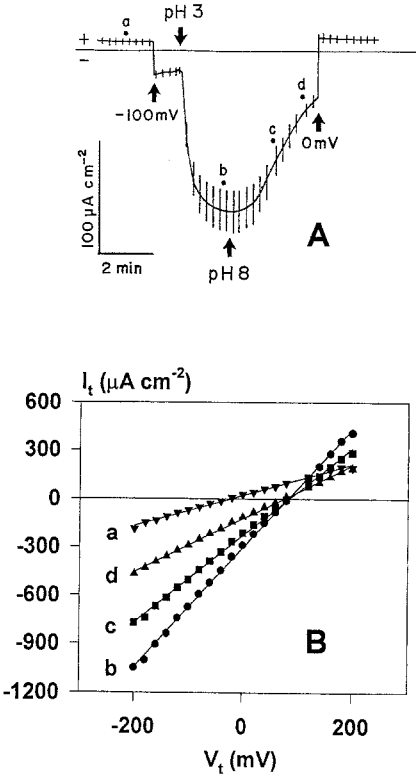

Fig. 8. (*A*) I_t as a function of time for a representative experiment showing activation of the voltage-dependent H^+-gated conductive pathway. The skin was initially short-circuited, bathed on the inner side by NaCl-Ringer and on the apical side by K_2SO_4 57.5 mM at pH 8. Subsequently, V_t was clamped to −100 mV and later the conductance activated by lowering pH_a to 3. Conductance deactivation was induced by returning pH_a to 8. The dots (*a, b, c* and *d*) mark the instants in which the instantaneous $I_t \times V_t$ relationships were measured. The vertical bars are deflections of I_t induced by V_t shifts of ±10 mV. (*B*) Instantaneous $I_t \times V_t$ relationships obtained at instants marked by dots (*a, b, c* and *d*) in *A*. The straight lines are regression lines fitted to the data points.

Effect of Apical Proton Concentration on the Activation of G_{Cl}

This project aimed to test the interaction of protons with the Cl^- channels, presumably located in the apical membrane of the MR cells of amphibian skins (Foskett & Ussing, 1986; Spring & Ussing, 1986; Larsen, Ussing & Spring, 1987; Larsen & Harvey, 1994) to evaluate the role of apical membrane fixed charges in the process of Cl^- channel activation.

The present study shows that increasing apical proton concentration (from pH 8 to pH 4) affects the process

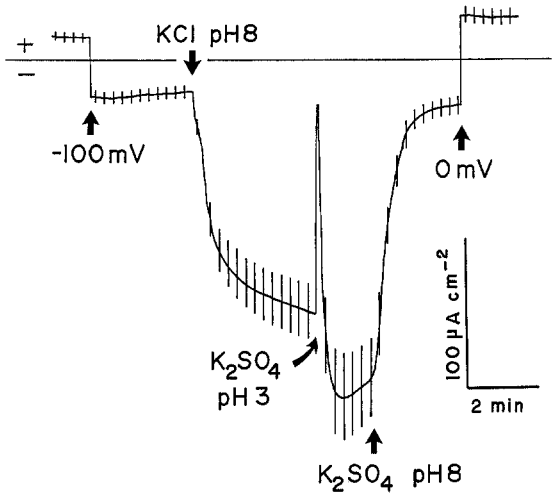

Fig. 9. I_t as a function of time for a representative experiment (of a group of 6 skins) showing that the conductance activated by hyperpolarized skins by apical Cl^- ions or by apical H^+ ions refer indeed to two functionally distinct pathways. The skin bathed on the inner side by NaCl-Ringer and on the apical side by K_2SO_4 57.5 mM at pH 8 was initially short-circuited. Subsequently, V_t was clamped to −100 mV and later the apical solution was replaced by KCl 115 mM at pH 8, ensuing a conspicuous activation of G_{Cl}. When this conductance was fully activated, replacement of the apical solution by K_2SO_4 at pH 3 leads to a sharp positive current deflection, compatible with the impermeability of the Cl^- pathways to SO_4^{2-}. Soon after, activation of the H^+-gated voltage-dependent conductive pathway takes place, characterized by a negative I_t deflection and increase of G_t.

of activation of the Cl^--conductive pathway since it slows down the kinetics of I_t activation and reduces the steady-state values of G_t and I_t (Figs. 1 and 2; Table 1). The voltage dependence of the pH effect might indicate that the critical protonation sites of the Cl^- channels are not freely exposed to the apical solution but are located within the membrane electric field. The concentration of protons at these binding sites are expected to conform to a Boltzmann distribution. This interpretation permits one to understand the absence of any effect when apical solution acidification is carried out at a clamping potential of −100 mV, while a conspicuous inhibition of G_{Cl} is observed when acidification is performed at −50 mV (Fig. 1 and Table 1). It is also conceivable that changes in the membrane electric field causes conformational changes in the Cl^- channel protein, exposing the proton binding sites, which otherwise were occluded. An also coherent interpretation would be that titration of apical proton binding sites affects the gating of the voltage-dependent Cl^- channels, shifting the conductance-*vs.*-voltage curve to more negative clamping potentials, in a way similar to what was observed for the Ca^{2+}-dependent K^+ channels in response to changes in the Ca^{2+} concentration in the *cis* side (Latorre, Vergara & Hidalgo, 1982). The absence of pH effect on tissue conductance at −100 mV would mean that at this potential the conductance-*vs.*-voltage curves, obtained at $pH_a = 4$ and $pH_a = 8$, are in their plateau regions, where the conductance is no longer affected by the potential difference across the membrane. In consonance with this interpretation is the fact that the apical Cl^- channels of the MR cells in toad skin epithelium are modulated by c-AMP, the effect also being a displacement of the conductance-*vs.*-voltage curve to more positive clamping potentials (Willumsen, Vestergaard & Larsen, 1992).

The effects of apical proton concentration and clamping potential upon G_{Cl} might result from the protonation of sites which modulate the kinetics of activation of the apical Cl^- channels. The reduction in the steady-state values of G_t and I_t in response to apical solution acidification cannot result from a decrease of the single-channel conductance due to a reduction of the effective Cl^- concentration at the channel entrance since protonation of outward-facing negative charges would have a converse effect, increasing the Cl^- concentration in that region. Therefore, this effect and the observed increase in the time course of I_t activation, are strong indications that the protonation of membrane fixed charges affects the Cl^- channel itself. Additional evidence that the Cl^- channels are affected is that apical acidification from pH 8 to pH 4 in the absence of apical Cl^- ions does not effect tissue conductance for all tested clamping potentials (Table 2).

Voltage-dependent Proton-gated Conductance

In addition to the effects of apical protons on the activation of the Cl^- conductive pathway, the present study describes and characterizes the existence in the toad skin of a voltage-dependent proton-gated conductance. This conductance was characterized in the absence of apical Cl^- ions to rule out any contribution of Cl^- to the overall tissue conductance, since G_{Cl} is also activated by tissue hyperpolarization (Larsen & Rasmussen, 1982; Lacaz-Vieira & Procopio, 1988*b*).

Apical solution acidification carried out in Cl^--free apical solution revealed a new and interesting aspect of the skin electrophysiological behavior, i.e., that tissue conductance in the absence of apical Cl^- ions can be importantly affected by the pH of the apical solution, the effect being markedly dependent on the clamping potential. Generally speaking, the effect of rising apical proton concentration can be conspicuous at negative clamping potentials, while at positive potentials changes in tissue conductance were never observed even for apical pHs as low as pH 3. In the negative range of clamping potentials, a turning point pH can be clearly seen. Thus, for a clamping potential of −100 mV, the turning point is between pH 4 and pH 3, since apical acidification to pH 4 has no effect upon tissue conductance while apical acidification to pH 3 leads to a marked, slow and revers-

ible increase of tissue conductance (Fig. 3). Both a low apical pH and a negative clamping potential are needed to induce conductance activation. Meeting only one of these requisites is not enough to activate the conductive pathway (Fig. 3). This feature enables us to characterize the conductance as a voltage-dependent proton-gated conductance. The location of this conductance is so far uncertain. Nevertheless, due to the striking similitude between the voltage-dependent Cl^--gated conductance (*see* Larsen, 1991) for review) and the voltage-dependent proton gated conductance (in (i) time course of activation, (ii) requirement for a negative clamping potential and the presence of a specific ion species in the apical solution for activation to occur, (iii) instantaneous ohmic behavior, and (iv) steady-state rectification), a tempting assumption would be to associate the voltage-dependent proton-gated conductance to the apical Cl^- channels of the mitochondria-rich cells, and consider sulfate as a charge carrier. It is known that the Cl^- channel of the MR cells when activated is poorly selective, behaving more properly as an anion channel (Harck & Larsen, 1986). Supporting the assumption that SO_4^{2-} is a charge carrier is the fact that sulfate, apparently as a monovalent species (KSO_4^- or HSO_4^-), is permeable across the apical Cl^- channels, the ratio of the anion rate coefficients, k_{Cl}:k_{SO4} being 1:0.035 (Larsen & Simonsen, 1988). In our present case, however, due to the low apical pH, the specificity of the modifier site and/or the translocation site could have been altered, the channels no longer needing apical Cl^- for activation and the SO_4^{2-} permeability increasing. Interaction of protons with ion channels may drastically alter their behavior. Thus, in chick dorsal root ganglion cells, a proton-induced transformation of calcium channel, resulting in alterations of the gating and permeability properties, has been described (Konnerth, Lux & Morad, 1987; Davies, Lux & Morad, 1988). Also, in hypothalamic neurons of the rat, a proton-gated current increased as extracellular pH decreased, the maximum response occurring near pH 4. The properties of this proton-operated channel were similar to those of the voltage-gated Na^+ channel rather than the Ca^{2+} channel (Ueno, Nakaye & Akaike, 1992) suggesting a modification of the Na^+ channels. Porin, the channel-forming protein of the outer membrane of Gram-negative bacteria, shows a pH-induced change of channel size, switching occurring over a very narrow range of pH (Todt, Rocque & McGroarty, 1992). Ion-conducting channels formed in lipid bilayers by diphtheria toxin (Mindell et al., 1994*a*) and colicin N (Wilmsen, Pugsley & Pattus, 1990) are highly dependent on pH. These studies show that minor pH-induced changes in the molecule may lead to drastic changes of function. A direct influence of pH on the conformation of channel proteins is a possibility to explain our results. It is conceivable that, in our case, ion channels of the apical membrane could have been altered by the combined action of low apical pH and membrane depolarization, leading to an increase of tissue conductance. Yet, it is known that ions are able to interact both with protein channels as well as with the lipid component of the membrane (Cai & Jordan, 1990). The pH dependence of tissue conductance could, in principle, result from a partial titration of the negative charges of membrane lipids (phosphates or carboxyl groups), which would alter the lipid packing of the apical membrane where the channels are incorporated. It is known that H^+ is able to cause dramatic changes in lipid ordering when its concentration in solution reaches a threshold value (Ohki & Duax, 1986; Van Dijck et al., 1978). Recently, it has been shown that pH changes may also affect molecular packing of pure phosphatidylcholine bilayers (Massari et al., 1991).

A paracellular contribution to the voltage-dependent proton-gated conductance cannot be discarded. In skins of frog (*Rana esculenta* and *Rana temporaria*) (Fischbarg & Whittembury, 1978) and toad (*Bufo marinus*) (Gonzales et al., 1978) a critical apical pH value, lower than 2.5, was observed. Below this critical pH, the permeability of the paracellular pathway for sucrose increases and the total electrical resistance decreases reversibly. Our present results show that the critical apical pH value, or turning point, is highly dependent on the clamping potential. The voltage-dependence of the pH effect cannot be explained in terms of a rise of H^+ ion concentration within the tight junctions leading to an increase in their permeability, since the polarity of the applied potential is opposite to that necessary for an effect to occur. This observation weakens the arguments in favor of a paracellular route for the voltage-dependent proton-gated conductance.

The results presented so far show a remarkable likeness between the voltage-dependent Cl^--activated anion conductance (*see* Larsen, 1991 for review) and the voltage-dependent proton-activated conductance described in the present study (Fig. 5). The most important characteristics of these two conductances are that both: (i) rely on the presence of a certain type of ion in the apical solution (Cl^- or H^+, respectively); (ii) are activated by tissue hyperpolarization; (iii) present a slow time course of activation and deactivation; (iv) the steady-state I_t-*vs.*-V_t relationship shows a marked rectification, with G_t increasing at the negative potential range, and (v) the instantaneous I_t-*vs.*-V_t relationship is linear for all conditions studied. These similarities suggest that both conductances could indeed be related to a common pathway. The result shown in Fig. 9 is apparently conflicting with this interpretation, suggesting two independent systems, since the transient reduction of I_t, when the apical solution of KCl at pH 8 is replaced by a solution of K_2SO_4 at pH 3 might indicate that G_{Cl} deactivates while the voltage-dependent proton-gated conductance is activated. However, a more plausible interpretation, in con-

sonance with the rest of the data, is that the lowering of apical pH could have altered the selectivity of the anion channel so that sulfate becomes a permeant ion. In this case, the transient reduction of I_t (Fig. 9) would be the result of the time taken for the new steady state to be reached.

In summary, the present study shows that increasing apical proton concentration impairs G_{Cl} activation in toad skin, this being a clearly voltage-dependent effect. In addition, we describe and characterize the existence of a voltage-dependent apical proton-gated conductance that might be closely related the voltage-dependent apical Cl^--gated conductance well characterized in amphibian skins (for review *see* (Larsen, 1991; Lacaz-Vieira & Procopio, 1988*a*). These two conductances share very similar characteristics, as slow time courses of activation, highly nonlinear steady-state current-voltage curves and linear instantaneous current-voltage curves.

I greatly appreciate helpful discussions with Antonio Carlos Cassola and Erik Hviid Larsen. This project was supported by grants 94/0300-1 from Fundação de Amparo à Pesquisa do Estado de São Paulo, and 521869/94-3 NV from Conselho Nacional de Desenvolvimento Cientifico e Tecnológico, Brazil.

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