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Roles of External and Cellular Cl^- Ions on the Activation of an Apical Electrodiffusional Cl^- Pathway in Toad Skin

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Summary. This study is concerned with the short-circuit current, I_{sc}, responses of the Cl^--transporting cells of toad skin submitted to sudden changes of the external Cl^- concentration, $[Cl]_o$. Sudden changes of $[Cl]_o$, carried out under apical membrane depolarization, allowed comparison of the roles of $[Cl]_o$ and $[Cl]_{cell}$ on the activation of the apical Cl^- pathways. Equilibration of short-circuited skins symmetrically in K-Ringer's solutions of different Cl^- concentrations permitted adjustment of $[Cl]_{cell}$ to different levels. For a given Cl^- concentration (in the range of 11.7 to 117 mM) on both sides of a depolarized apical membrane, this structure exhibits a high Cl^- permeability, $P_{(Cl)apical}$. On the other hand, for the same range of $[Cl]_{cell}$ but with $[Cl]_o = 0$, $P_{(Cl)apical}$ is reduced to negligible values. These observations indicate that when the apical membrane is depolarized $P_{(Cl)apical}$ is modulated by $[Cl]_o$; in the absence of external Cl^- ions, intracellular Cl^- is not sufficient to activate $P_{(Cl)apical}$. Computer simulation shows that the fast Cl^- currents induced across the apical membrane by sudden shifts of $[Cl]_o$ from a control equilibrium value strictly follow the laws of electrodiffusion. For each experimental group, the computer-generated I_{sc} *versus* ($[Cl]_{cell} - [Cl]_o$) curve which best fits the experimental data can only be obtained by a unique pair of $P_{(Cl)apical}$ and R_b (resistance of the basolateral membrane), thus allowing the calculation of these parameters. The electrodiffusional behavior of the net Cl^- flux across the apical membrane supports the channel nature of the apical Cl^- pathways in the Cl^--transporting cells. Cl^- ions contribute significantly to the overall conductance of the basolateral membrane even in the presence of a high K concentration in the internal solution.

Key Words toad skin · Cl channel · Cl transport · channel activation · mitochondria-rich cell

Introduction

Despite many previous studies dealing with the transepithelial movement of Cl^- ions in amphibian skins, several aspects of this subject remain unsettled (Lacaz-Vieira & Procopio, 1988*b*). One aspect not yet completely understood is the process which controls the permeability of the apical membrane of the Cl^--transporting cells to Cl^- ions, and halides in general. The present study focused on that process. The apical Cl^- pathways remain quiescent when the skin is short-circuited or depolarized, and are markedly activated in response to skin hyperpolarization above the spontaneous potential (Bruus, Kristensen & Larsen, 1976; Larsen & Kristensen, 1978; Larsen & Rasmussen, 1982; Harck & Larsen, 1986). Several studies suggest activation of the skin Cl^- conductance by external Cl^- ions (Kirschner, 1970; Mandel & Curran, 1972; Alvarado, Dietz & Mullen, 1975; Bruus et al., 1976; Rodriguez-Boulan et al., 1978; Kristensen, 1978; Petery, Rotunno & Cereijido, 1978; Biber, Walker & Mullen, 1980; Ehrenfeld & Garcia-Romeu, 1980; Kirschner, 1983; Katz & Larsen, 1984; Harck & Larsen, 1985).

It has also been proposed that activation of the skin Cl^- conductance is related to a voltage-dependent mechanism (Bruus et al., 1976; Larsen & Kristensen, 1978; Larsen & Rasmussen, 1982) located in the apical membrane of the mitochondria-rich cells (Voute & Meier, 1978; Katz & Larsen, 1984; Katz, Van Driessche & Scheffey, 1985; Foskett & Ussing, 1986; Katz & Scheffey, 1986; Spring & Ussing, 1986; Willumsen & Larsen, 1986; Larsen, Ussing & Spring, 1987). Accordingly, Cl^- permeation occurs via putative voltage-dependent apical membrane Cl^- channels, whose activation obeys a Hodgkin-Huxley kinetics of a single gating variable (Larsen & Rasmussen, 1982; Larsen & Rasmussen, 1983; Larsen & Rasmussen, 1985).

Previous studies (Harck & Larsen, 1986; Lacaz-Vieira & Procopio, 1988*b*) indicate that activation of the apical Cl^- pathways in response to depolarization of the apical membrane is strictly dependent on the presence of Cl^- ions in the external bathing solution, not occurring in Cl^--free external bathing media. Activation, on the other hand, can be induced in a previously depolarized apical membrane by step rising the Cl^- concentration of the external solution, the process following a sigmoidal time course (Lacaz-Vieira & Procopio, 1988*b*).

These observations indicate that the apical Cl^- pathways are not strictly voltage-gated entities, since Cl^- ions are essential for the activation process. The finding that partial activation of the apical Cl^- pathways results from increase of the external Cl^- concentration in the absence of apical membrane depolarization (Lacaz-Vieira & Procopio, 1988*b*) suggests that apical membrane voltage has an indirect role on the activation process affecting the Cl^- distribution across this structure.

The present study deals with the following aspects concerning the Cl^--transporting cells of the toad skin epithelium: (*i*) the relative importance of external and intracellular Cl^- ions as modulators of the apical Cl^- pathways; (*ii*) the electrodiffusional nature of the Cl^- flux across the apical membrane; and (*iii*) the contribution of Cl^- ions to the overall conductance of the basolateral membrane.

The main assumptions concerning this study can be briefly stated as: (*i*) The use of high $[K]_i$-Ringer's solution on the inner side markedly depolarizes the basolateral membrane and decreases its electrical resistance. Under this condition, transepithelial short circuit leads to apical membrane depolarization. (*ii*) Cl^- ions are assumed to be at Gibbs-Donnan equilibrium across the basolateral membrane. (*iii*) The ionic currents induced by fast changes of $[Cl]_o$ are assumed to go through the MR cells and to be driven by the Cl^- electromotive force at the apical membrane. (*iv*) The permeabilities of the apical membrane to K^+ and SO_4^{2-} are assumed to be negligible.

Materials and Methods

Abdominal skins of the toad *Bufo marinus ictericus* were used. The animals were double pithed prior to skin removal.

The experiments were performed according to a method previously described (Lacaz-Vieira, 1986; Lacaz-Vieira & Procopio, 1988*b*). A brief description of the method will thus be presented.

Circular skin fragments with the epithelial side facing upwards were mounted horizontally between two Lucite hemichambers exposing an area of 3.14 cm^2. Silicone grease and recessed rim compression minimized the effect of skin edge damage. The outer bathing solution was vigorously stirred at 3,000 rpm by a paddle placed 1.5 mm above the outer skin surface, in order to minimize unstirred layer effects and rapidly homogenize the outer bathing medium when solution changes were carried out. Solution changes in the external compartment were accomplished by a fast-flow procedure consisting of rapidly flushing, while aspirating, 100 ml of the new solution through the compartment, homogenization taking place with a half time of 1.3 sec.

A conventional voltage clamp (Department of Physiology, Yale University) with continuous feedback was used. Bipolar square voltage pulses of fixed magnitude and adequate frequency were applied at the summing point of the voltage-clamp operational amplifier by a monostable multivibrator circuit under the control of a ramp generator (Tektronix TM 503-RG 501) in order to monitor the skin electrical conductance. 3 M KCl agar-bridges and saturated calomel half-cells were used to access the electrical potential difference across the skin. The tip of the outer agar-bridge (approximately 250 μm in diameter) touched the outer skin surface tangentially, so that a negligible layer of solution was interposed between this sensing bridge and the outer skin surface. Equivalent precaution was taken regarding the inner voltage sensing bridge. 3 M KCl agar-bridges, adequately placed to give a uniform current density across the skin, were used for current passing. The clamping current was continuously recorded by a strip-chart recorder.

Computer simulation was carried out on a microcomputer (XT 2002, Microtec, Brazil) with programs written in Turbo-Pascal. Fitting computer-generated curves to the experimental data was done by trial and error, until achievement of a reasonable fitting, followed by a final step using the least-squares procedure.

In order to obtain the desired Cl^- concentration in the bathing solutions KCl and K_2SO_4-Ringer's solutions were mixed in adequate volume proportions. The compositions of the basic Ringer's solutions were (in mM): KCl-Ringer's KCl 115, $KHCO_3$ 2.5, and $CaCl_2$ 1.0, and K_2SO_4-Ringer's: K_2SO_4 57.5, $KHCO_3$ 2.5, and $CaSO_4$ 1.0, both with pH of 8.2 after aeration. No corrections were made to adjust the osmolarity of the bathing solutions due to Cl^- for SO_4^{2-} substitution. The results are presented as mean ± standard error of the mean.

Abbreviations

$E_{Cl(a)}$: Cl^- electromotive force of the apical membrane, equal to $RT/F \ln ([Cl]_{cell}/[Cl]_o)$.

$E_{Cl(b)}$: Cl^- electromotive force of the basolateral membrane, equal to $RT/F \ln ([Cl]_i/[Cl]_{cell})$.

E_{Cl}: Overall transepithelial Cl^- electromotive force, equal to $E_{Cl(a)} + E_{Cl(b)}$.

$fR_a = R_a/(R_a + R_b)$: Fractional apical membrane resistance.

G: Total skin electrical conductance. Calculated from the deflections of the clamping current induced by pulses of 300-msec duration and ±10 mV amplitude, as $G = \Delta I/\Delta V$, where ΔI and ΔV are the changes in the skin electrical potential difference and clamping current, respectively.

I_{sc}: Short-circuit current. The convention is such that a positive current corresponds to the transport of positive charge across the skin from the outer to the inner bathing solution.

$P_{(Cl)apical}$: Permeability of the apical membrane of the Cl^--transporting cells.

r: Donnan ratio.

R_a: Electrical resistance of the apical membrane of the Cl^--transporting cells.

R_b: Electrical resistance of the basolateral membrane of the Cl^--transporting cells.

$R_{Cl(b)}$: Electrical resistance of the basolateral membrane due to Cl^- ions.

$R_{K(b)}$: Electrical resistance of the basolateral membrane due to K^+ ions.

$V_a = (V_{cell} - V_o)$: Electrical potential difference across the apical membrane, cell potential referred to that of the outer solution.

$V_b = (V_i - V_{cell})$: Electrical potential difference across the basolateral membrane, inner potential referred to that of the cell compartment.

$[x]_j$: Concentration of species x in the compartment j ("o", "i", and "cell" represent outer, inner and cell compartments, respectively).

Results

The present work had the scope of analyzing the roles of external and cellular Cl^- concentrations on the activation of the apical pathways of the Cl^--transporting cells. We aimed at short-circuiting the apical membrane by the simultaneous use of high K concentration Ringer's solution and transepithelial short circuit (Fuchs, Larsen & Lindemann, 1977; Klemperer et al., 1986).

Both apical membrane depolarization (Larsen & Rasmussen, 1982) and presence of Cl^- ions in the external solution (Harck & Larsen, 1986; Lacaz-Vieira & Procopio, 1988*b*) are requisites for full activation of the apical Cl^- pathways. Changes of $[Cl]_{cell}$ and activation or inactivation of the apical Cl^- pathways are slow phenomena taking place in response to changes of $[Cl]_o$ (Lacaz-Vieira & Procopio, 1988*b*). Thus, in the present work, the possibility of rapidly imposing a Cl^- concentration gradient across a previously depolarized apical membrane allowed us to study inward and outward Cl^- currents and skin conductance before the occurrence of significant changes of $[Cl]_{cell}$ and activation or inactivation of $P_{(Cl)apical}$.

All experiments were carried out under transepithelial short circuit in skins previously equilibrated symmetrically in K-Ringer's of various Cl^- concentrations with the purpose of: (*i*) depolarizing the basolateral membrane and increasing its electrical conductance, and (*ii*) bringing $[Cl]_{cell}$ close to the Cl^- concentration of the internal solution, under the assumption of a passive Cl^- distribution across the basolateral membrane of the Cl^--transporting cells.

With KCl-Ringer's solution on the inner side, cell swelling occurs in the principal cells of the epithelium (MacRobbie & Ussing, 1961), the same possibly occurring with the MR cells. Cell swelling, however, does not seem to impair the preparation since after prolonged bilateral exposure (50 min or more) to KCl-Ringer's, the electrical conductance (4.35 ± 1.7 mS cm^{-2}, $n = 11$) is markedly reduced after skin equilibration in Cl^--free external solution (0.66 ± 0.39 mS cm^{-2}, $n = 5$). The complete reversibility of this effect, as shown in Fig. 3*A* and *B*, indicates functional integrity of the permeability barriers.

Responses to Fast Changes of the External Cl^- Concentration

Fast Current Responses

The experiments were carried out in four groups of skins equilibrated bilaterally in a control period with K-Ringer's having Cl^- concentrations equal to 11.7 mM (group A), 23.4 mM (group B), 58.5 mM (group C) and 117 mM (group D). Rapid imposition of an apical membrane Cl^- gradient was achieved in a test period by fast replacing (*see* Materials and Methods) the control external solution with a solution having a different Cl^- concentration. All test periods were preceded by a control period.

I_{sc} deflections caused by changes of $[Cl]_o$ were measured in reference to the steady-state level of I_{sc} of the control condition, i.e., under symmetric bathing solutions (for baseline currents different from zero, *see* Discussion).

Figures 1*A* and *B* shows for group B and D typical I_{sc} records for skins submitted to sudden shifts of $[Cl]_o$. The other groups yielded similar records. In all cases fast increments or decrements of $[Cl]_o$ relative to the control concentration values induced I_{sc} responses characterized by an initial fast current deflection (fast component) followed by a slow component whose time course is markedly influenced by the Cl^- concentration of the test solution. It is clear from Fig. 1*A* and *B* that the size of the fast component of the I_{sc} response increases with the Cl^- concentration difference imposed across the apical membrane.

Figure 2 depicts mean values of the fast component of I_{sc} (obtained according to the protocol of Fig. 1) as a function of the calculated (*see* Appendix) Cl^- concentration difference imposed across the apical membrane. It can be seen that in all groups the fast component of I_{sc} varied monotonical and nonlinearly with the transapical Cl^- concentration difference.

Slow Current Responses

The fast components of I_{sc}, as shown in Fig. 1*A* and *B*, are followed by slow components which evolve towards stationary conditions. For small decrements of $[Cl]_o$ (from the control level) the slow component is essentially a positive plateau, attained soon after the fast current deflection. For large decrements of $[Cl]_o$, on the other hand, the slow component is characterized by a slow decline of I_{sc} and G towards steady-state values. This behavior suggests a slow inactivation of the Cl^- pathways in response to reduction of $[Cl]_o$.

For increments of $[Cl]_o$ from the control level, the fast current component is followed by a negative current plateau when small concentration changes are imposed. For larger concentration changes a slow phase of activation ensues, characterized by a slow increase in magnitude of the negative I_{sc} and of G, suggesting a further activation of the Cl^- pathways.

Figure 3*A* and *B* shows typical I_{sc} records in

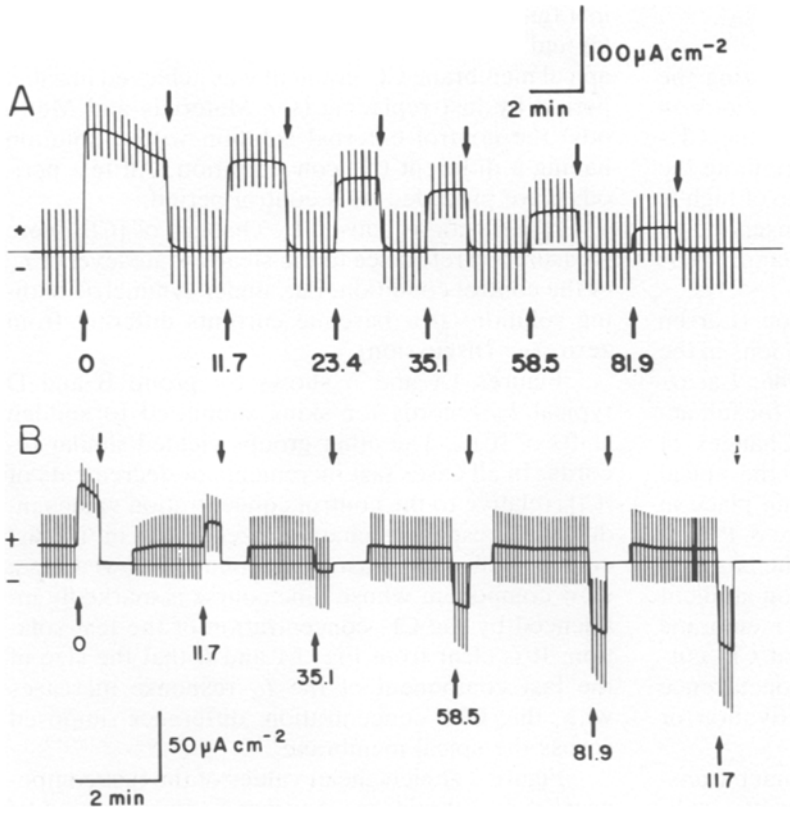

Fig. 1. Short-circuit current (I_{sc}) responses induced by sudden changes of the Cl^- concentration of the external solution, $[Cl]_o$ (SO_4^{2-} for Cl^- substitution) in two representative experiments. Prior to changes of $[Cl]_o$ the skins were equilibrated bilaterally in K-Ringer's solution of 117 mM Cl^- concentration (*A*) and of 23.4 mM (*B*) (control conditions). The lower arrows indicate the changes of the external solution and the new Cl^- concentration values (in mM), and the upper arrows the return to the control condition. The sign convention for currents is such that positive currents are inwardly directed. Vertical bars are deflections of the clamping current in response to offset pulses of ±10 mV in the clamping voltage, and are proportional to the skin electrical conductance, *G*. The horizontal line corresponds to $I_{sc} = 0$

response to fast and complete Cl^- removal from the external solution for two representative groups having different control Cl^- concentrations. Differently from the response to partial Cl^- substitution (as shown in Fig. 1), total Cl^- removal leads, after the fast current deflection, to a slow and complete reduction of I_{sc} accompanied by a marked and also slow reduction of *G*. The drastic fall of I_{sc} and *G* is highly suggestive of a slow and total inactivation of the apical Cl^- pathways due to the absence of external Cl^- ions. In contrast, partial reduction of $[Cl]_o$ (for example, from 117 to 35.1 mM, Fig. 3*A*, third run) leads I_{sc} to stabilize well above zero indicating a maintained residual activation of apical Cl^- pathways due to the presence of Cl^- ions in the outer solution.

After complete reduction of I_{sc} in response to total external Cl^- removal, rapid return of $[Cl]_o$ to the control value (Fig. 3*A* and *B*) leads to a slow and marked increase of skin conductance. This, however, is never accompanied by a concomitant negative current deflection. Absence of a negative I_{sc} deflection in response to return of $[Cl]_o$ to the control level, despite marked increase of *G*, suggests that Cl^- ions are in equilibrium across the apical membrane. This behavior strongly suggests that exposure of the outer skin surface to a Cl^--free solution does not lead to Cl^- depletion of the Cl^--transporting cells, and consequently $P_{(Cl)apical}$ virtually vanishes in the absence of external Cl^- ions.

Evidence for an Active Cl^- Transport Across the Basolateral Membrane

In Fig. 3*A*, second run, a positive transient I_{sc} response of small size obtains shortly after return of $[Cl]_o$ to 117 mM. This transient response might indicate that the Cl^- electrochemical potential in the Cl^--transporting cells rises above the equilibrium level following a long exposure of the outer skin surface to a Cl^--free medium. Due to this disequilibrium, subsequent addition of Cl^- ions to the external bathing solution, leading to activation of the apical Cl^- pathways, causes a transient discharge of cell Cl^- ions to the outer compartment, with $[Cl]_{cell}$ relaxing towards an equilibrium level. The above reasoning implies the assumption of an active Cl^- translocation at the basolateral membrane of the Cl^--transporting cells responsible for the uptake of Cl^- ions from the inner solution. The magnitude of this active Cl^- transport in the experiment of Fig.

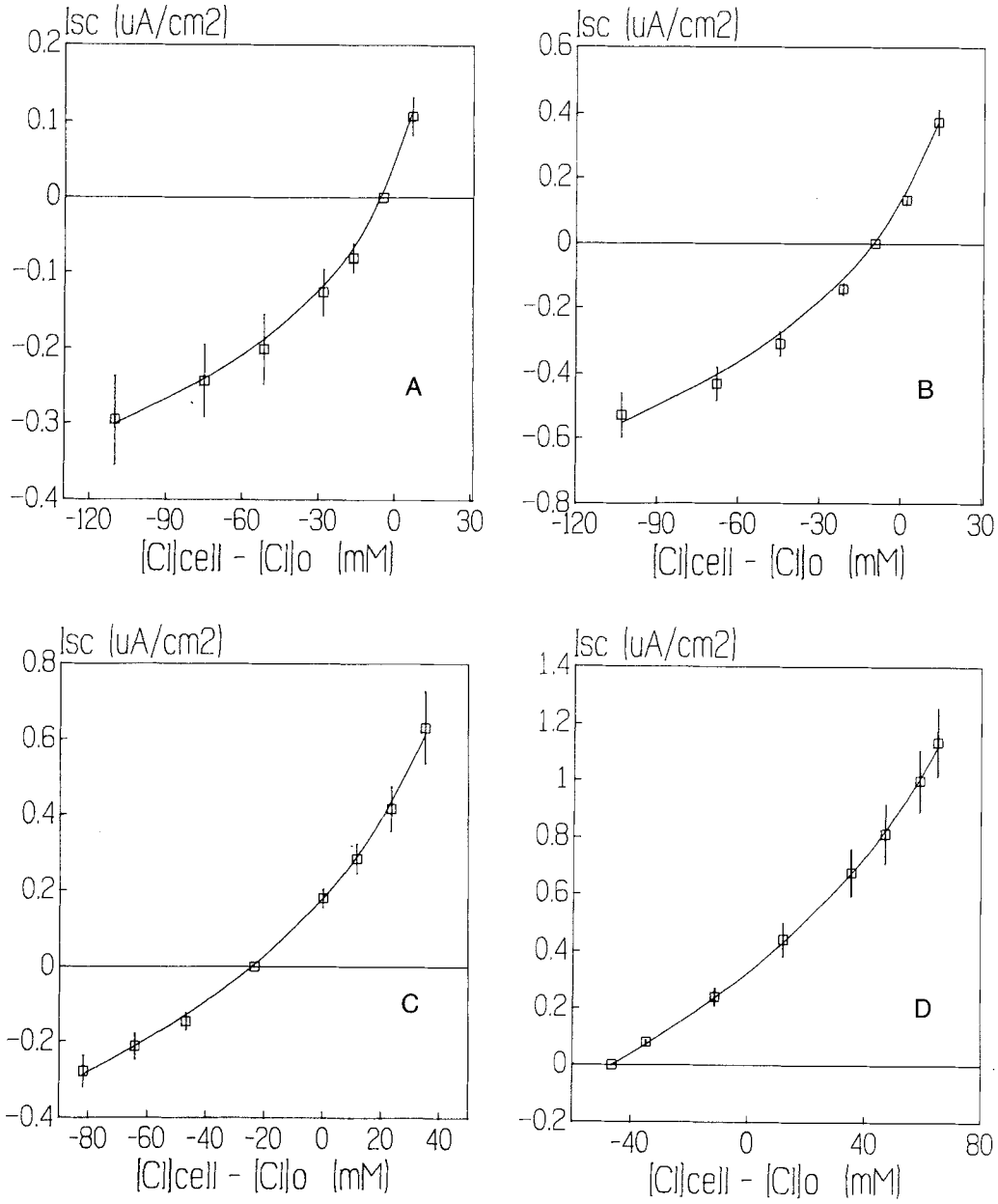

Fig. 2. Mean values of the fast component of the short-circuit current (I_{sc}) induced by sudden changes of the Cl^- concentration of the external solution (according to the experimental protocol of Fig. 1), as a function of the Cl^- concentration differences across the apical membrane. Four groups of skins initially equilibrated bilaterally in K-Ringer's solution of 11.7 mM Cl^- concentration (group A, $n = 7$), 23.4 mM (group B, $n = 6$), 58.5 mM (group C, $n = 6$), and 117 mM (group D, $n = 5$) were studied. Lines are best-fit theoretical curves obtained according to Eq. (A7) of the Appendix. $[Cl]_{cell}$ and $[Cl]_o$ are the cell and external Cl^- concentrations, respectively. Vertical bars are standard error or the mean

3*A* is of negligible importance to cause a significant I_{sc} in the control condition, though sufficient to rise the cell Cl^- electrochemical potential above that of the inner solution when the apical Cl^- pathways are shut up for a long period of time.

However, as exemplified in Fig. 3*B*, a positive I_{sc} (mean value of 11.7 ± 1.2 $\mu A\ cm^{-2}$, $n = 6$) has been consistently found in skins symmetrically equilibrated in K-Ringer's of 23.4 mM Cl^- concentration. Smaller currents were also observed in symmetrical conditions in skins equilibrated with Ringer's solution of 11.7 and 58.5 mM Cl^- concentration. The active current (Fig. 3*B*) virtually vanishes by complete removal of Cl^- ions from the external medium. This finding (*see* Discussion) suggests that the active current flows through a

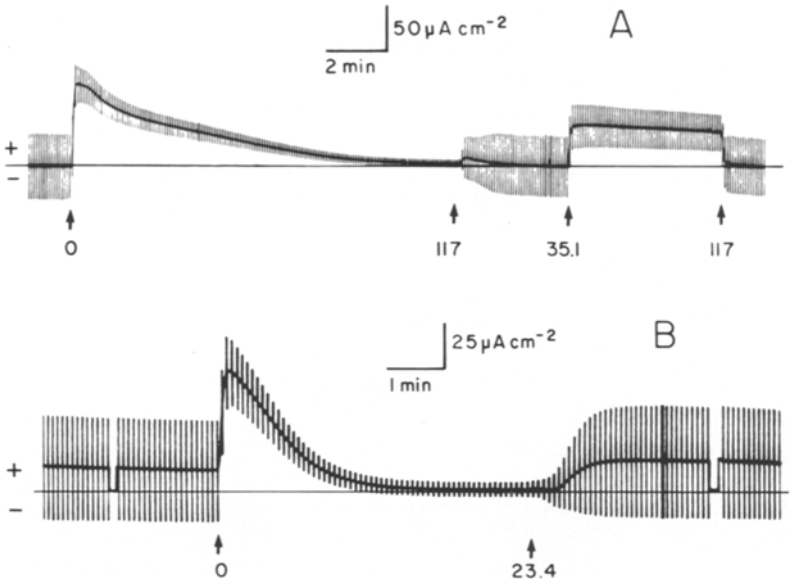

Fig. 3. Short-circuit current (I_{sc}) responses to sudden Cl^- concentration changes in the external bathing solution for two representative skins initially equilibrated bilaterally in K-Ringer's solution of 117 mM Cl^- concentration (*A*) and 23.4 mM (*B*). The arrows indicate the changes of the external solution and the new Cl^- concentration values (in mM). For conventions, *see* legend of Fig. 1

pathway modulated by $[Cl]_o$, most likely through the apical Cl^- channels of the Cl^--transporting cells.

Discussion

Activation of the apical Cl^- pathways in amphibian skins is a complex and still not completely understood subject (*see* Lacaz-Vieira & Procopio, 1988*a*, for a recent review). Apical membrane depolarization, achieved by skin hyperpolarization, markedly increases skin Cl^- permeability (Bruus et al., 1976; Larsen & Kristensen, 1978; Larsen & Rasmussen, 1982), being external Cl^- ions essential for activation to take place (Harck & Larsen, 1986; Lacaz-Vieira & Procopio, 1988*b*). On the other hand, partial activation of the Cl^- pathways obtains in response to addition of Cl^- ions to a Cl^--free external medium even in the absence of apical membrane depolarization (Lacaz-Vieira & Procopio, 1988*b*). The above evidence suggests that apical membrane voltage and external and/or intracellular Cl^- ions are implicated in the modulation of the Cl^- permeability of the apical membrane of the Cl^--transporting cells.

In previous studies of G_{Cl} activation induced by skin hyperpolarization both apical membrane voltage and intracellular Cl^- concentration probably did not remain constant during the activation process, as shown by the mathematical modeling of the Cl^- transport by the mitochondria-rich cells (Larsen & Rasmussen, 1983; Larsen & Rasmussen, 1985). Activation of G_{Cl} by a step elevation of $[Cl]_o$ under "fixed" apical membrane voltage was attempted in a previous work (Lacaz-Vieira & Procopio, 1988*b*) by the use of high K^+ concentration Ringer's solutions combined with transepithelial short circuit (Fuchs et al., 1977; Klemperer et al., 1986). Previous reports indicate, however, that a significant basolateral membrane potential and resistance persist under this condition (Benos, Hyde & Latorre, 1983; Delong & Civan, 1984).

In the present study we still aimed at short-circuiting the apical membrane by equilibrating skins on both sides with high K^+ concentration Ringer's solution under transepithelial short circuit. In this condition, with no current flowing, presumably complete depolarization of both apical and basolateral membranes obtains, bringing the cell Cl^- concentration to a Donnan equilibrium with the bathing solutions (*see* Appendix). On the other hand, complete short circuit of the apical membrane was certainly not achieved in the presence of transepithelial currents, due to the low $fR_a = R_a/(R_a + R_b)$ values, as shown in Table 1, since, from the model (*see* Appendix):

$$V_a = E_{Cl}(1 - R_a/(R_a + R_b)).$$

As shown in Results, fast and total removal of Cl^- ions from the outer solution causes two fundamentally distinct responses: (*i*) a fast inward current, due to an outward-directed Cl^- flux, which indicates that the apical Cl^- pathways are permeable in all control conditions, and (*ii*) a slow and progressive decline of I_{sc} towards near zero values accompanied by a substantial decrease of the total

Table 1.

Group	$[Cl]_{equil}$ (mM)	$P_{(Cl)apical}$ (cm $sec^{-1} \cdot 10^5$)	R_a (Ω cm^2)	R_b (Ω cm^2)	fR_a	n
A	11.7	1.72	1727	1360	0.56	7
B	23.4	3.10	480	520	0.48	6
C	58.5	2.40	248	450	0.35	6
D	117.0	2.82	106	280	0.27	5

Control skin parameters in the equilibrium condition (same solutions on both skin sides) obtained by fitting the experimental values of the short-circuit current, I_{sc}, *versus* Cl^- concentration difference across the apical membrane, through numerical solution of Eq. (A7). The experimental groups (A, B, C and D) refer to different control conditions obtained by equilibrating skins in K-Ringer's solution of different Cl^- concentrations, $[Cl]_{equil}$, on both sides. $P_{(Cl)apical}$ is the Cl^- permeability of the apical membrane. R_a and R_b are the electrical resistances of the apical and basolateral membranes, respectively. fR_a is the fractional apical membrane resistance, defined as $R_a/(R_a + R_b)$. n is the number of skins.

skin conductance, reflecting a slow inactivation of the apical Cl^- pathways. In contrast to total removal, partial reduction of the external Cl^- concentration to values down to 11.7 mM (lowest non-zero value tested) does not appreciably inactivate the apical Cl^- pathways. This is evidenced by the persistence of a stable positive I_{sc} compatible with a steady outward flow of Cl^- ions across the apical membrane. The above observations allow us to conclude that: (*i*) Activation of the apical Cl^- pathways requires, in addition to apical membrane depolarization, a minimum concentration of Cl^- ions at the outer surface of the apical membrane. According to the $P_{(Cl)apical}$ values shown in Table 1, this minimum value should be lower than the lowest nonzero value tested (11.7 mM). (*ii*) Independently of the $[Cl]_{cell}$ level, total removal of external Cl^- ions leads to a complete inactivation of the apical Cl^- pathways in agreement with the observation of Larsen et al. (1987). This shows that the presence of intracellular Cl^- ions, even in high concentration (117 mM maximum value tested) is not sufficient *per se* to activate the apical Cl^- pathways of a depolarized apical membrane. The fast component of I_{sc} (Fig. 1*A* and *B*) which result from sudden imposition of a Cl^- concentration difference across the apical membrane, most certainly reflects a Cl^- flux across this structure. Since activation and inactivation of the apical Cl^- pathways in response to fast changes of $[Cl]_o$ are slow phenomena (Lacaz-Vieira & Procopio, 1988*b*) the ensuing instantaneous currents reflect both the Cl^- driving force across the apical membrane and the apical Cl^- permeability in the equilibrium condition immediately preceding the Cl^- concentration change in the external solution. Had the apical membrane remained short-circuited during the sudden imposition of apical Cl^- gradients one would expect the fast I_{sc} responses to be a linear function of the Cl^- concentration difference across the apical membrane, according to Fick's law. This, however, is not observed experimentally, as shown in Fig. 2*A–D*. Deviations from linearity could in principle be due to: (*i*) electrical polarization of the apical membrane during current passage, indicating incomplete short-circuiting of this structure, or (*ii*) rapid changes of apical Cl^- permeability in response to sudden changes of $[Cl]_o$. This last view would imply a new phenomenon distinct from the known slow activation or inactivation by voltage (Larsen & Kristensen, 1978; Larsen & Rasmussen, 1982) or by external Cl^- ions (Lacaz-Vieira & Procopio, 1988*b*) of the apical Cl^- pathways. In the first case a significant fraction of the apical Cl^- electromotive force, $E_{Cl(a)}$, (*see* Appendix) would appear as a voltage across the apical membrane, leading to deviations from Fick's law. This voltage drop has, therefore, to be taken into account in the description of the Cl^- fluxes induced by sudden shifts of $[Cl]_o$.

Based on the above considerations we have analyzed the experimental data of Fig. 2 according to the formalism of electrodiffusion (Finkelstein & Mauro, 1963), as presented in the Appendix. The equivalent circuits of Fig. A1 depict a Cl^- transporting cell in a short-circuited skin suddenly exposed to a Cl^- gradient across its apical membrane. The relationship between I_{sc} and the Cl^- concentration difference across the apical membrane was obtained through numerical solution of Eq. (A7) with the variables defined by Eqs. (A1) to (A6), under assumption of a Donnan ratio equal to 1.66 (*see* Appendix). Adequate selection of $P_{(Cl)apical}$ and R_b values permitted a precise fitting of the experimental data by the theoretical curve as shown in Fig. 2 for the whole range of experimental values and for all four experimental groups. Each theoretical curve was found to be unique for a given pair of $P_{(Cl)apical}$ and R_b values, as discussed in the legend of Fig. 4. Therefore, a unique pair of $P_{(Cl)apical}$ and R_b values characterizes the skin for a given value of equilibrium Cl^- concentration. Table 1 shows $P_{(Cl)apical}$, R_a, R_b and fR_a values for the four experimental groups. Table 2 exemplifies for group D (skins equilibrated in K-Ringer's with 117 mM Cl^- concentration) a typical data output of the iteractive computer algorithm that solves Eq. (A7). It is clearly seen that V_a attains importantly large values when high currents are present. The following points are relevant in Table 1: (*i*) Contrary to what one would expect having a high K^+ concentration Ringer's solution bath-

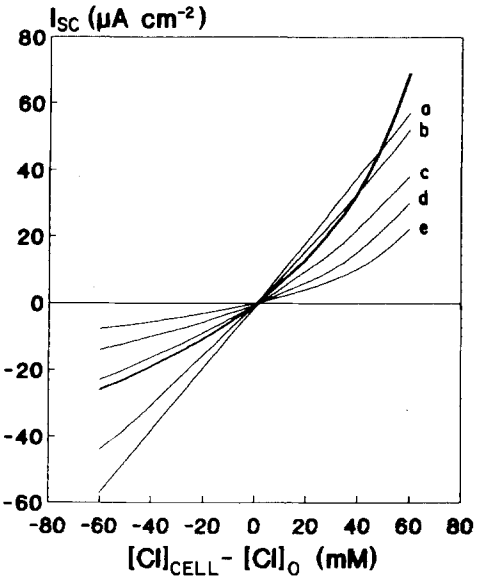

Fig. 4. Theoretical curves of short-circuit current, (I_{sc}), *versus* apical membrane Cl^- concentration difference, $[Cl]_{cell} - [Cl]_o$, obtained according to Eq. (A7) of the Appendix showing pictorially that a given curve can be obtained only by a unique pair of apical membrane Cl^- permeability, $P_{(Cl)apical}$, and resistance of the basolateral membrane, R_b. The thick curve corresponds to $P_{(Cl)apical} = 3 \times 10^{-5}$ cm/sec and $R_b = 600\ \Omega\ cm^2$. Thin curves were generated with a fixed $P_{(Cl)apical}$ equal to 1×10^{-5} cm/sec and R_b values of 10, 100, 500, 1,000 and 2,000 $\Omega\ cm^2$ (curves *a*, *b*, *c*, *d* and *e*, respectively)

ing the inner skin surface, the fractional apical membrane resistance, fR_a, is low for the Cl^--transporting cells indicating an extremely high apical Cl^- conductance in all four experimental groups. The reduction of fR_a with increasing equilibrium Cl^- concentration indicates that R_a is more sensitive than R_b to changes of the equilibrium Cl^- concentration. (*ii*) The calculated apical Cl^- permeability shows a maximum around 23.4 mM equilibrium Cl^- concentration conforming qualitatively with data of Harck and Larsen (1986) (obtained from Cl^- tracer studies) which indicate that the rate coefficients of Cl^- transfer exhibits a maximum around 60 mM $[Cl]_o$. (*iii*) Despite a high K^+ concentration in the inner solution, basolateral membrane conductance is still markedly affected by the Cl^- concentration of the inner Ringer's solution, R_b increasing with decrease of Cl^- concentration. This points to the importance of Cl^- ions to the overall conductance of the basolateral membrane, even when the contribution of K^+ is maximized by the use of high K^+ concentration in the inner bathing solution.

Figure 3*B* shows the existence of a steady-state positive I_{sc} in symmetrical (23.4 mM Cl^-) bathing solutions, implicating this current as being driven by an active ion-transport process. The possibility of an active inward transport of H^+ or K^+ ions is

Table 2.

$[Cl]_o$ (mM)	I_{sc} (μA cm^{-2})	fR_a	V_a (mV)
5.5	114.0	0.60	−31.5
11.7	100.0	0.53	−28.2
23.4	81.5	0.45	−23.0
35.1	67.7	0.40	−18.8
58.5	44.2	0.35	−11.9
81.9	24.0	0.31	−6.5
105.3	7.9	0.28	−2.1
117.0	0	0.27	0

Typical data output of the iteractive computer algorithm that solves Eq. (A7) relative to group D (skins equilibrated in K-Ringer's of 117 mM Cl^- concentration). $[Cl]_o$ is the Cl^- concentration of the external solution. I_{sc} is the instantaneous short-circuit current deflection induced by sudden change of $[Cl]_o$ from 117 mM to the corresponding values indicated in the first column. fR_a is the fractional apical membrane resistance, defined as $R_a/(R_a + R_b)$, and V_a is the voltage across the apical membrane, defined as $V_{cell} - V_o$.

unlikely since such active fluxes have been described in the opposite direction (Emilio & Menano, 1975; Procopio & Lacaz-Vieira, 1977; Nielsen, 1984). It is reasonable, therefore, to postulate that this positive current is due to a transepithelial active Cl^- transport in the outward direction. Strong evidence for such a view is that a maneuver which drastically reduces the apical Cl^- permeability (such as complete removal of external Cl^- ions) leads to complete abolishment of the active current and a pronounced decrease of skin conductance. Furthermore, return to a previous Cl^--containing external solution restores both I_{sc} and conductance to previous values. This strongly suggests that the active current exemplified in Fig. 3*B* flows indeed through an apical conductive pathway modulated by external Cl^- ions. An apparently paradoxical issue is that the active current, well characterized at 23.4 mM Cl^- concentration in the control Ringer's solution, practically does not exist at 117 mM. Evidence for an uphill Cl^- transport at 117 mM Cl^- concentration appears, however, in the transient positive current that obtains when $[Cl]_o$ is rapidly elevated to 117 mM after previous skin equilibration in a Cl^--free medium (Fig. 3*A*). Such transient current is compatible with a discharge of Cl^- ions which have been actively accumulated within the cells during the period when the apical Cl^- pathways were shut off by the absence of external Cl^- ions. An active outward directed transepithelial Cl^- movement modulated by the Cl^- concentration in the outer solution would be compatible with an uphill Cl^- entry in the cell across the basolateral membrane of the Cl^--transporting cells.

A primary active Cl^- transport has not been generally accepted; since Na^+ ions are absent in our bathing solutions, this excludes also the contribution of a Na/K/2Cl cotransport to the observed current. An apical neutral proton pump, possibly a H^+/K^+-ATPase similar to that described in the gastric mucosa (as reviewed by Sachs, 1977; Diamond & Machen, 1983; Sachs et al., 1984, 1988) and a Cl/HCO_3 exchanger in the basolateral membrane of the Cl^--transporting cells may be suggested as a possible mechanism. Further studies are warranted to characterize this system.

Evidences for active outward transport of Cl^- ions in amphibian skins have been obtained under adrenaline stimulation, being the mucous glands implicated (Koefoed-Johnsen, Ussing & Zerahn, 1952; Eskesen & Ussing, 1989). This possibility cannot be ruled out as an alternative explanation to our findings. However, this is unlikely since the active current is eliminated after Cl^- removal from the external side. If glands were playing a role in the genesis of the active current, the activation/deactivation kinetics should be much slower, since the apical surface of the gland cells are not readily accessible to changes in the outer medium composition. If glands were to be implicated, then one has to postulate the existence of a Cl^--activated Cl^--conductive pathway, located in the apical membrane of the gland cells. If a significant active Cl^- uptake does exist in the basolateral membrane of the MR cells then, with identical Cl^- concentration on both sides of the skin, the electrochemical Cl^- activity within the cells should be larger than in the external side in order to account for a passive Cl^- extrusion across the apical membrane. This would necessarily modify our assumption of a Cl^- equilibrium across the basolateral membrane. The maximum value for a deviation from that assumption can be estimated by taking the mean active Cl^- current of 11.7 $\mu A/cm^2$ and the mean $P_{(Cl)apical}$ for group C (23.4 mM Cl^-) which yields a cell Cl^- activity 4 mM above the equilibrium value, thus making unjustifiable any further correction beyond that for Donnan distribution.

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Appendix

Transepithelial Cl^- transport has been analyzed on the basis of the model depicted in Fig. A1 which assumes that the Cl^- movement occurs through a specialized cell type, distinct from the principal cells, which, according to several authors (Voute & Meier, 1978; Katz & Larsen, 1984; Katz et al., 1985; Foskett & Ussing, 1986; Katz & Scheffey, 1986; Spring & Ussing, 1986; Willumsen & Larsen, 1986; Larsen et al., 1987) might be the MR cells. The principal cells have been excluded from the model in view of the apparently virtual Cl^- impermeability of their apical membranes (Nagel, 1977; Ferreira & Ferreira, 1981; Giraldez & Ferreira, 1981; Nagel, Garcia-Diaz & Armstrong, 1981; Biber et al., 1985; Willumsen & Larsen, 1986).

The proposed equivalent circuit of the Cl^--transporting cells under transepithelial short circuit is shown in Fig. A1. In the present analysis the contribution of a paracellular current carried by Cl^- ions to the overall I_{sc} induced by a transepithelial Cl^- concentration difference was not taken into consideration. This is justified in view of our experimental results which show a negligible I_{sc} in skins having the apical Cl^- pathways in the inactive state (Lacaz-Vieira & Procopio, 1988*b*) and submitted to large transepithelial Cl^- concentration difference (Fig. 3*A* and *B*). Also considered negligible in our model were the SO_4^{2-} and K^+ permeabilities of the apical membrane of the Cl^--transporting cells. Accordingly, in the present working hypothesis, Cl^- is the only permeable species in the apical membrane. R_a and R_b denote, respectively, the electrical resistance of the apical and basolateral membranes. Numerical values of R_a, R_b, and of $P_{(Cl)apical}$ do not reflect actual cellular parameters of the Cl^--transporting

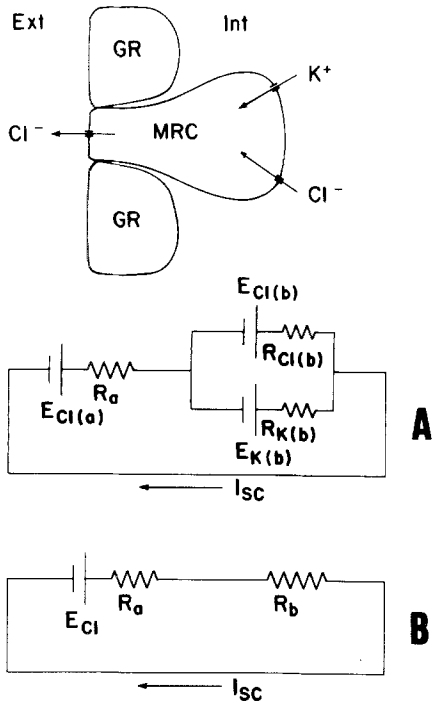

Fig. A1. Schematic diagram of a short-circuited skin depicting a mitochondria-rich cell (*MRC*) between two granular cells (*GR*). The diagram illustrates a maneuver of fast decrement of $[Cl]_o$ resulting in an outward-directed Cl^- flux across the apical membrane of the MR cell which is equivalent to the short-circuit current, I_{sc}. In the equivalent circuit *A*, R_a is the electrical resistance and $E_{Cl(a)}$, the Cl^- electromotive force, both referring to the apical membrane conductive Cl^- pathway. $R_{Cl(b)}$ and $R_{K(b)}$ are the resistances of the basolateral membrane to Cl^- and K^+ ions, respectively. $E_{Cl(b)}$ and $E_{K(b)}$ are the electromotive forces for Cl^- and K^+ ions across the basolateral membrane, being $E_{Cl(b)} = E_{K(b)} = (RT/F) \ln r$ (where r is the Donnan ratio). The circuit (*B*) is the equivalent Thevenin of circuit *A*, where $E_{Cl} = (RT/F) \ln ([Cl]_i/[Cl]_o)$

cells since they are calculated from the current density across the whole skin area, being, therefore, smeared all over the epithelial surface. If we assume, according to Larsen and Rasmussen (1985) the relative outer membrane area of the MR cells to be equal to 0.0076, then the real cellular parameters should be corrected by a factor of approximately 100. Thus, for example, the actual value of $P_{(Cl)apical}$ should be approximately 100 times higher than those presented in Table 1.

Due to basolateral membrane depolarization by high K concentration Ringer's solution on the inner side, equilibrium intracellular Cl^- and K^+ concentrations were assumed to obey a Gibbs-Donnan distribution (*see* last paragraph of Discussion for a possible error due to an active Cl^- mechanism). An estimated Donnan ratio (r) of 1.66 was calculated according to Schultz (1980) (Eq. (3.30) of that reference) from MR cell parameters used by Larsen and Rasmussen (1985), equivalent to a cellular concentration of 64 mM for nondiffusible anions with a mean valence of −2. An apical electromotive force, $E_{Cl(a)}$, results from the rapid imposition of an apical Cl^- electrochemical potential difference due to fast increments or decrements of $[Cl]_o$ from the equilibrium level. The apical, V_a, and basolateral, V_b, potential differences are defined, respectively, as: $V_a = V_{cell} - V_o$ and $V_b = V_i - V_{cell}$ being, at transepithelial short circuit,

$$V_a = -V_b. \tag{A1}$$

The short-circuit current, I_{sc}, which obtains in response to imposition of a Cl^- electrochemical potential difference across the apical membrane is considered to be entirely carried by Cl^- ions across this structure, and by an unknown proportion of K^+ and Cl^-, and possibly other ions in lesser extent, across the basolateral membrane. Under the above assumptions the following relations hold:

$$E_{Cl} = (RT/F) \ln \frac{[Cl]_i}{[Cl]_o} \tag{A2}$$

$$V_b = I_{sc} R_b. \tag{A3}$$

R_a is given by the electrodiffusion formalism (Finkelstein & Mauro, 1963), as:

$$R_a = \frac{(RT)^2(1 - X) \ln ([Cl]_{cell}/[Cl]_o X)}{P_{Cl} F^3 V_a([Cl]_{cell} - [Cl]_o X)} \tag{A4}$$

where:

$P_{Cl} = P_{(Cl)apical}$ (for short in the equation)

$$X = \exp(-FV_a/RT) \tag{A5}$$

$$[Cl]_{cell} = [Cl]_i/r. \tag{A6}$$

Finally, the short-circuit current can be expressed as:

$$I_{sc} = E_{Cl}/(R_a + R_b).$$