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Exchange Diffusion, Electrodiffusion and Rectification in the Chloride Transport Pathway of Frog Skin

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Summary. Measurements of chloride flux ratios across frog skin at different clamping voltages showed that chloride transport at clamping voltages from 0 mV to and beyond the spontaneous potential is probably electrodiffusion. At reversed potentials a significant fraction of chloride transport could be described formally as exchange diffusion. Chloride conductance was found to be highly voltage dependent, being largest at hyperpolarizing clamping voltages. The transition from the less conducting state to the more conducting one was studied by recording the time course of the current after a step change in clamping voltage from 0 mV to hyperpolarizing voltages. The shape of the curve is sigmoidal, and the relative rate of change of current increases with increasing hyperpolarization. It is proposed that the change in conductance is governed by the same mechanism as in the toad skin, namely a change in chloride permeability due to voltage gating of chloride channels. The time course of transepithelial conductance after addition of amiloride to the outside solution indicates that a fraction of the decrease in conductance is due to closure of chloride channels caused by the change in intracellular potential due to the inhibition of the sodium channels.

Key words frog skin · chloride · flux ratio · exchange diffusion · rectification · gating

Introduction

In the past few years the most interesting information about the mechanism of chloride transport through the isolated amphibian skin originates from experiments made mainly on five species, namely the toad *Bufo bufo* and the frogs *Rana temporaria*, *R. esculenta*, *R. pipiens*, and *Leptodactylus ocellatus*. In all these species it was shown that chloride efflux is stimulated by chloride in the outside bathing solution (short-circuit conditions). In the toad skin this phenomenon has been attributed to exchange diffusion (Bruus, Kristensen & Larsen, 1976; Kristensen, 1978). This conclusion is in accordance with a more recently published flux ratio analysis showing that the flux ratio between 0 and 140 mV ($\psi_o - \psi_i$) is equal to one (Larsen, Rasmussen & Willumsen, 1981). Information in the literature indicates that chloride transport in *Rana temporaria* is electrodiffusional (Ussing, 1949; Koefoed-Johnsen, Levi & Ussing, 1952; Kristensen, 1978), and this led to the conclusion that chloride in the outer solution stimulates the opening of conducting chloride channels in the outward-facing membrane, probably by reacting with a regulatory site that determines the degree of opening of the channels. A similar working hypothesis has been put forward to explain that the efflux of chloride is stimulated by external chloride in skins of *Leptodactylus ocellatus* (Ques-von Petery, Rotunno & Cereijido, 1978). The trans-effect observed in *R. pipiens* (Biber, Walker & Mullen, 1980) was believed mainly to be due to an augmented exchange diffusion.

The experimental basis on which it has been concluded that chloride transport in *R. temporaria* and *R. esculenta* predominantly is electrodiffusional is open to some criticism, because the flux ratio experiments referred to above, in all but one case, were done in the range of spontaneous transepithelial potential differences.

A critical reinvestigation is therefore desirable, mainly with the aim of getting a quantitative measurement of any exchange diffusion that may play a role in chloride transfer. For this purpose *R. temporaria* or *R. esculenta* are more suited than *L. ocellatus*, because the intensity of their inward active transport is orders of magnitudes smaller than that of *L. ocellatus*, and because it is small compared to the large passive movements (exchange and/or electrodiffusion) of chloride.

It was observed simultaneously both in *Rana* (Kristensen, 1978) and in *Leptodactylus* (Ques-von Petery et al., 1978) that chloride efflux is dependent on the sodium concentration in the outside solution. In both cases this was suggested to be due to the depolarizing effect of the sodium diffusion potential across the outward-facing membrane of

the cells in question. As a matter of fact, the effect of sodium on transepithelial chloride permeability is predicted by the two-membrane hypothesis, as shown by computer model analysis (Kristensen, 1982) even when the individual chloride permeabilities of the two serially arranged membranes were assumed constant.

Work on the toad skin (Bruus et al., 1976; Larsen & Kristensen, 1978; Larsen et al., 1981) has, however, shown that chloride permeability of one of the two membranes (probably the outward-facing one) is highly voltage dependent. This has not yet been reported for frog skin, but would have important consequences for our understanding of the effect of sodium on chloride transport, because such a mechanism may lead to an even more pronounced effect of sodium than predicted from the simple two-membrane hypothesis with constant chloride permeabilities.

The aim of the present study therefore was to reinvestigate chloride transfer in frog skin (*Rana*) in search of the presence of exchange diffusion, and to investigate the voltage dependence of the chloride transport pathway.

It turned out that in many cases exchange diffusion may play a role at reversed transepithelial voltages, while its presence is hardly detectable at normal polarity. Chloride conductance is usually highly voltage dependent, or if not, it can be made so by adding theophylline to the bathing solutions. The effect of sodium on chloride transport involves, in some cases at least, changes in chloride permeability. These in turn are due to changes in transmembrane potentials caused by addition to or removal of sodium from the outside bathing solution on top of the conductance changes predictable by the constant field theory applied on a two-membrane system.

Materials and Methods

The frogs used in this study were in most cases *R. esculenta* that were kept at room temperature (about 20° C) and fed with mealworms. A few experiments were carried out on skins from *R. temporaria*, which were transferred from the cold (4° C) to room temperature at least one week before they were used for experiments. The two species have been found to behave identically towards all experimental maneuvers, but *R. esculenta* are easier to keep at room temperature, because they are more willing to eat.

The skins (abdominal) were mounted in conventional, conical Lucite® chambers (Koefoed-Johnsen, Ussing & Zerahn, 1952).

The bathing solutions were ordinary frog Ringer's composed as follows (mM); 113.5 Na^+, 1.9 K^+, 0.9 Ca^{++}, 114.8 Cl^-, 2.4 HCO_3^-, at pH 8.2 when aerated with atm air. Variations in chloride concentration were achieved by substituting chloride with the equivalent amount of sulfate or gluconate.

Simultaneous bidirectional fluxes of chloride were measured with ^{36}Cl (Risø, Denmark) on two laterally symmetrical halves of the skin from one animal, one being used for influx, the other for efflux measurement.

The clamping experiments were performed with a conventional automatic voltage clamper, which allows the clamping voltage to be changed from 0 mV to any desired clamping voltage in a small fraction of a second. Square pulses in both directions of preselected voltage, duration, and distance can be superimposed on the clamping voltage; the resulting current responses are used for estimation of tissue conductance, especially under short-circuit conditions, where no other fast measurent of this parameter is possible. Usually the square pulse cycle of 20 mV was repeated every 15 to 30 sec, depending on the recording speed.

The measurements of tissue conductance obtained in this way are (though often used) not at all well defined, because they include loss or gain of ions from the cellular compartment. In this paper the method is therefore only used to indicate changes in tissue conductance.

In work with isolated skins it has been customary to define the transepithelial potential difference with respect to the outside. This is opposite to the practice used for intestine, kidney tubules, etc., and has the disadvantage of giving negative slopes of the current-voltage relations. In the present paper the potential difference is therefore given with respect to the blood side or inside solution. The spontaneous potential difference thereby becomes negative.

Results

As already mentioned in Materials and Methods the experiments described in this paper have been performed exclusively on skins from frogs stored at room temperature. This has to be kept in mind, when comparisons are made with earlier experiments reported in the literature, because frogs stored in the cold are not only quantitatively but also qualitatively different from warm-adapted frogs.

The Trans-Effect

Some experiments describing the effect of outside chloride concentration on chloride efflux have been presented earlier (Kristensen, 1982), but it is considered reasonable to present all the experiments performed in order to have a more complete description of chloride transport characteristics in the present paper.

The transepithelial permeability for chloride, calculated from the isotopically measured efflux, depends in nearly all cases linearly on outside chloride concentration. The two species investigated (*R. temporaria* and *R. esculenta*) behave identically (Fig. 1).

An exchange diffusion characterized by a large half-saturation constant would exhibit the trans-effect observed. Thus a large part of the unidirectional fluxes could be due to such a mechanism.

At the same time it is indisputable that the largest fraction of the electrical shunt in frog skin is due to chloride (Koefoed-Johnsen, Levi & Ussing, 1952). An additional argument for a conductive chloride transport comes from the observation that Cu^{++} in small concentrations (10^{-5} M) in the outside solution inhibits chloride fluxes and stimulates an increase in transepithelial potential (Koefoed-Johnson & Ussing, 1958). These conclusions were, however, drawn from results obtained under open-circuit conditions. Experiments shown below indicate that the fraction of transport that can be explained by assuming exchange diffusion may vary with transepithelial voltage difference. Therefore it was found necessary to perform experiments where the change in chloride conductance could be compared with the change in electrically measured conductance of the tissue. Table 1 shows a series of experiments where the effects of Cu^{++} on chloride efflux and on bioelectric parameters have been measured. The skins were kept short-circuited with identical concentrations on both sides, which means that chloride conductance can be calculated from the equation derived by Ussing and Zerahn (1951):

$$g_i = z^2 F^2 J_i / RT \qquad (1)$$

where g_i is the partial conductance of the ith ion, J_i is the unidirectional flux of that ion, while z, F, R and T have their usual meaning. It appears that inhibition of chloride transport is accompanied by a decrease in total transepithelial conductance and an increase in transepithelial potential difference, while the short-circuit current is only slightly affected. The decrease in total tissue conductance is linearly related to the decrease in chloride conductance ($\Delta g_{CL} = -0.052 + 0.715\ G_T$; $r = 0.959$), but it obviously only accounts for 71.5% of the decrease in total conductance. The nature of this extra conductance has not yet been subjected to experimental investigation.

Flux-Ratio Analysis

These experiments were performed on two halves of the skin from each animal, one being used for efflux measurement, the other for influx measurement. With this procedure it is clear that erroneous results are sometimes obtained because of differences in chloride permeability between the two halves. It is not possible to make any corrections for this because this would require knowledge about the dependence of the unidirectional fluxes on transepithelial potential difference. The results of the analysis are presented in Fig. 2*A–D*, and they show that the experimentally observed flux ratios in many cases agree well with those predicted by the flux ratio equation (Ussing, 1949), especially in the voltage range from 0 to −90 mV. In the reversed potential range some flux ratios are larger than expected for free diffusion. It is worth noting that in some of these cases the slope of the line relating the logarithm of the flux ratio to the trans-

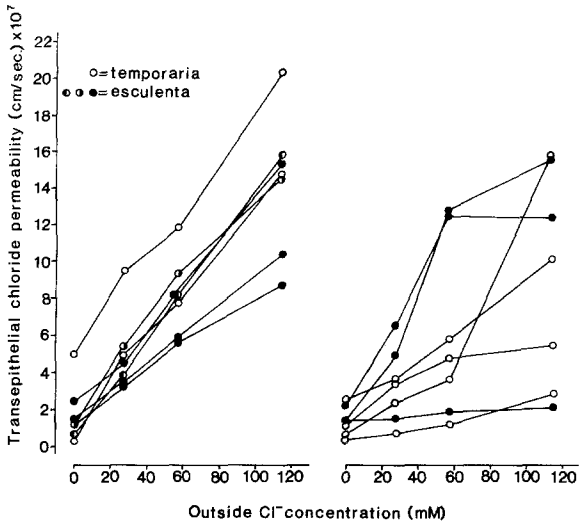

Fig. 1. Variation of transepithelial chloride permeability with outside chloride concentration under short-circuit conditions. The inside bathing solution was NaCl-Ringer's, while the chloride concentration in the outer chamber was varied by substitution with sulfate. For clarity the experiments have been divided into two groups. The permeability was calculated from the isotopically measured effluxes. The lines connect points representing results from one animal

Table 1. Effect of the addition of Cu^{++} to the outside bathing solution on chloride conductance and total tissue conductance of frog skin[a]

Exp. no.	Before Cu^{++}		After Cu^{++}		Δg_{Cl} mS/cm²	ΔG_{tot} mS/cm²
	PD (mV)	SCC (μA/cm²)	PD (mV)	SCC (μA/cm²)		
1	−10.5	25.7	−31.7	21.4	−0.94	−1.25
2	−23.0	16.4	−97.0	18.6	−0.36	−0.58
3	−31.0	32.1	−93.0	30.0	−0.44	−0.72
4	−21.0	25.7	−58.7	29.3	−0.29	−0.72
5	−93.0	15.7	−102.0	21.4	−0.01	+0.04
6	−22.8	21.4	−42.0	29.3	−0.15	−0.23
7	−38.0	23.6	−86.5	30.7	−0.19	−0.30

[a] Short-circuit conditions. Cu^{++} added as sulfate to a final concentration of 10^{-5} M. The electrical conductance of the tissue was calculated from the slope of the current-voltage relation obtained at clamping voltages +30, +10, 0, −10 and −30 mV. The chloride conductance was calculated with Eq. (1) from the isotopically measured efflux of chloride. G_{tot} = electrically determined skin conductance. g_{Cl} = chloride conductance.

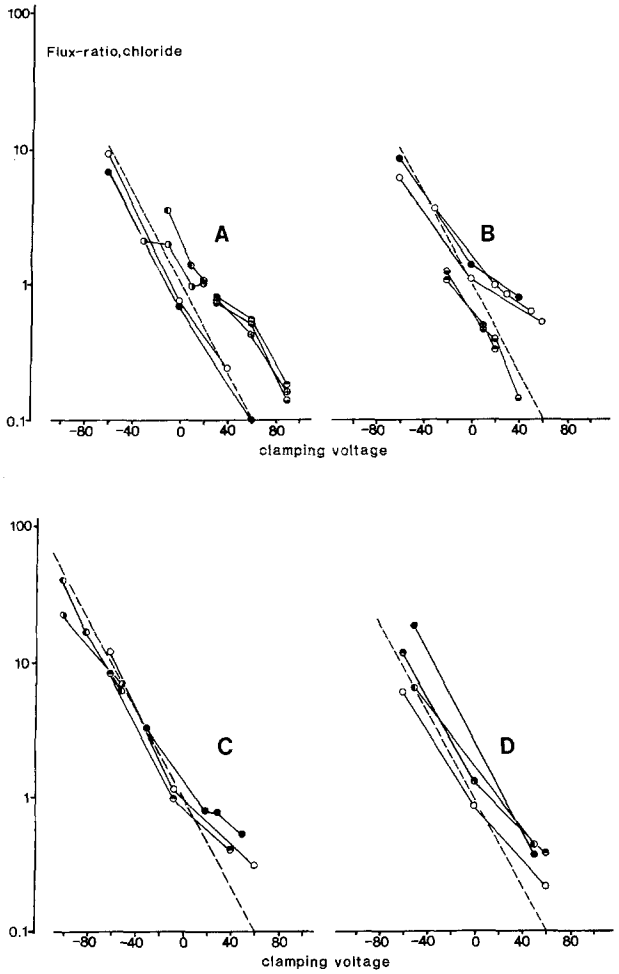

Fig. 2. The Variation of flux ratio of chloride with transepithelial clamping voltage (*R. esculenta*). The skins were bathed on both sides with NaCl-Ringer's. Chloride influx and efflux were measured with ^{36}Cl on two symmetrical skin halves from the same animal. Points representing measurements at different voltages on a skin from one animal are connected by lines. For the sake of clarity, the experiments are shown in four groups. The broken lines indicate the expected variation of flux ratio with voltage for a passively moving ion according to the flux ratio equation (Ussing, 1949)

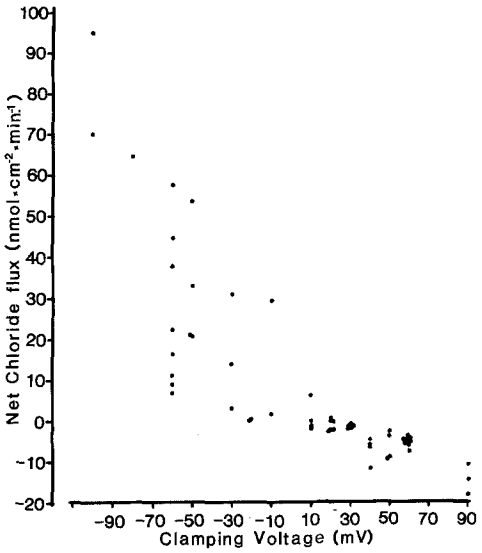

Fig. 3. Chloride net flux in skins of *Rana esculenta* as a function of transepithelial clamping voltage. The net fluxes were obtained as the difference between the unidirectional fluxes measured in the experiments of Fig. 2

epithelial clamping voltage has a break near 0 mV, indicating that we have passed from a situation at hyperpolarization where exchange diffusion is not detectable to a situation where it may account for a considerable fraction of the chloride fluxes (reversed potential range). It must however be noted that in a number of skins the observed flux ratios do not differ much from the predicted ones even at reversed clamping voltages.

The experimental material collected in the flux ratio experiments allows us to look at the dependence of net chloride flux on clamping potential. This relationship is depicted in Fig. 3, and although large scatter is observed it shows that chloride conductance can attain much larger values at hyperpolarizing voltages than at reversed voltages. This property is described in more detail in the following section.

Voltage-Dependent Chloride Conductance

The transition from a less conducting state to a more conducting state can only be followed electrically because the rate of change of conductance is too fast to be followed by isotope methods. In order to approach measurements of chloride currents alone, the transitions were studied in skins where sodium transport was blocked with amiloride (10^{-4} M). Figure 4 shows that in such skins the net flux of chloride accounts for the clamping current. All studies of the time dependence of chloride conductance have been performed by recording the time course of the clamping current after a step change in clamping voltage from zero to negative values (normal or hyperpolarizing range). In the legend to Fig. 5 the experimental procedure is described. A description of several current-time curves is given below, but one point deserves to be commented upon. As described in Fig. 5, the vertical bars are current changes due to 2-sec voltage pulses of 20 mV and allow estimates of instantaneous tissue conductance. When, after steady state is achieved, the clamping voltage is stepped back to 0 mV, the time course of the deactivation of chloride conductance can be followed. The kinetics of this inactivation will not be described in

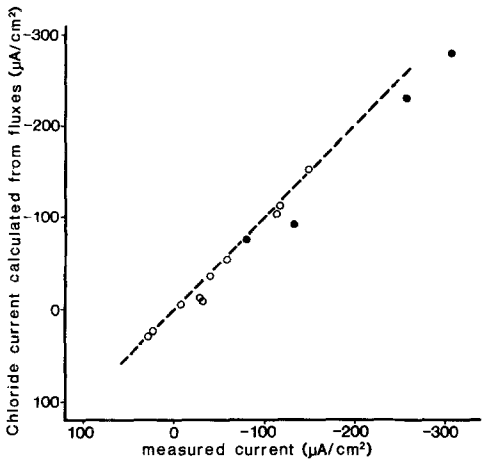

Fig. 4. Equality between chloride net current, calculated from the unidirectional fluxes, and actually recorded clamping currents in the absence of sodium transport. The open circles represent experiments where sodium transport was blocked with amiloride. The filled circles are from experiments where the outside was bathed with a Ringer's solution in which all sodium had been replaced by potassium. The broken line indicates the 1:1 relation

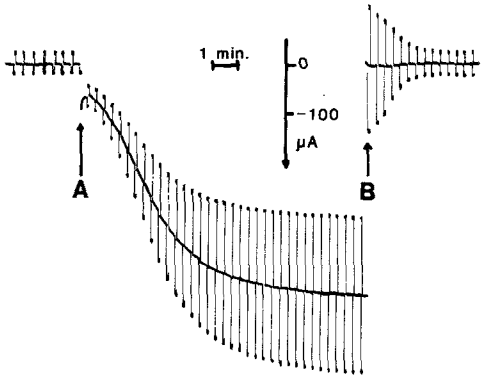

Fig. 5. Typical recording of current change after a step change in clamping voltage. NaCl-Ringer's on both sides and amiloride (10^{-4} M) on the outside. Starting at the left side of the recording, the clamping voltage is initially zero, and the same holds for the clamping current. At the arrow labeled *A*, the clamping voltage is changed in a stepwise manner to −60 mV (in this example). It is kept there until a steady-state current is obtained. At *B* the clamping voltage is again changed back to zero. The vertical lines are current responses to square-wave voltage deflections of ±20 mV which are superimposed on the clamping voltage. They allow calculation of an "instantaneous" tissue conductance and are of special interest at a clamping voltage of 0 mV, because they allow a control to be made of the reversion of the conductance activation before the next hyperpolarizing clamping step is applied. The time constant for the current response to the square pulses is about 0.5 sec, so the current is read at the end of the 2-sec voltage pulses

detail, but is observed to last some minutes. The duration of this inactivation will, however, be of significance later, when the role of sodium for chloride permeability is discussed.

Figure 6 shows the results of several attempts to record the time course after a step change in clamping voltage. It is observed that the skins respond very individually with respect to the shape of the curve and with respect to the magnitude of the conductance activation. After a step change in voltage the current increases immediately to a new value because of the increase in driving force. Thereafter it increases slowly with time, often with an initial delay, until it reaches a new steady level in the course of several minutes. In many cases, instead of the initial delay, a transient decrease is seen. Examination of the current-time curves indicates that the relative rate of change of current (and therefore also conductance) increases with increasing hyperpolarization. One way to quantitize this is to measure the time spent in reaching half-maximum (steady-state) current ($T_{1/2}$). In those cases where a transient decrease in negative current occurs immediately after the clamping voltage is stepped to negative values, $T_{1/2}$ was measured from the time where the current was at its minimum. The current at this point is much more well defined than the current immediately after a voltage change. Figure 7 shows that the half-time for the conductance activation increases with increasing clamping voltage.

In those cases where current-time relations have been obtained at different voltages on the same skin, we can draw curves relating the steady-state current with the clamping potential. A series of such I/V relations are shown in Fig. 8. Usually the clamping experiments were carried out in the order of increasing hyperpolarization. The reversibility of the conductance changes was checked after each clamping by observing the conductance decrease after return to the holding potential (0 mV). The time courses as well as the steady-state currents will be reasonably reproducible for about 3 to 4 hr, but in some skins prolonged hyperpolarization at large voltages results in larger $T_{1/2}$ values and smaller currents in subsequent trials. It should be remembered that they almost exclusively reveal the properties of the chloride pathway, because amiloride is present in the outside solution. The variation from one animal to the other is often large, but most of the curves exhibit very significant rectification, the conductance being many times larger at negative than at positive clamping voltages. In some cases, however, chloride conduc-

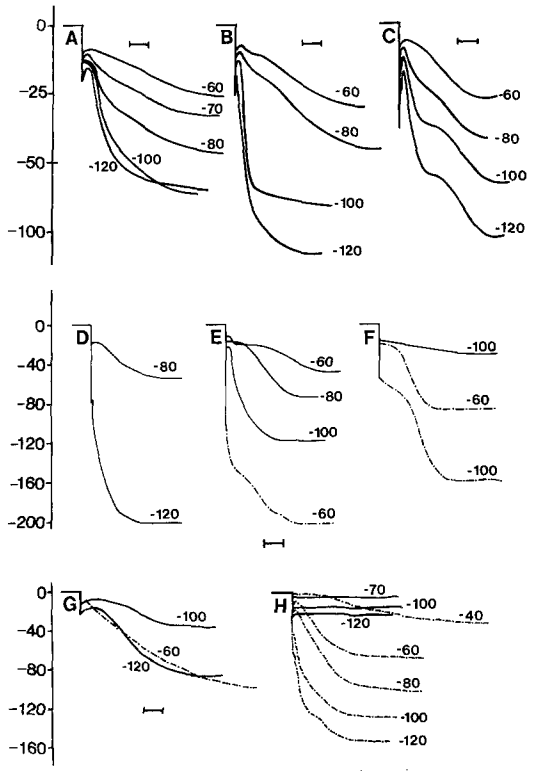

Fig. 6. Time courses of clamping current after step changes in clamping voltages, obtained as described in Fig. 5. The ordinates give the clamping currents in $\mu A/cm^2$, and the small horizontal bars give the length of one min. Each letter corresponds to the skin from one animal (*R. esculenta*). The broken lines were obtained after theophylline (2.4 mM) had been added to both bathing solutions. The solid lines were obtained on untreated skins. NaCl-Ringer's on both sides and amiloride (10^{-4} M) on the outside

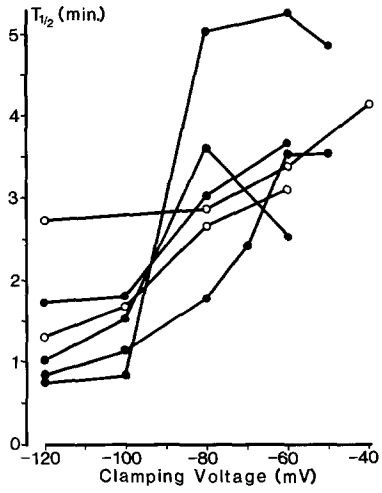

Fig. 7. Half-time for current activation plotted against clamping voltage. Filled circles: no treatment. Open circles: with theophylline (2.4 mM) added to the bathing solutions

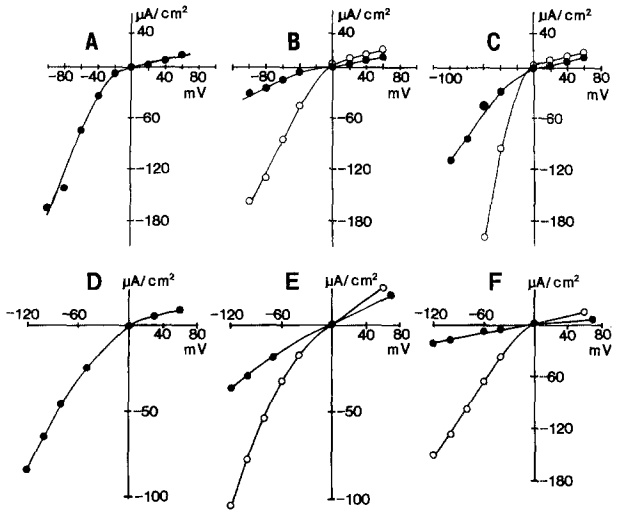

Fig. 8. Some examples of steady-state current-voltage relations obtained on skins from *R. esculenta* bathed with NaCl-Ringer's on both sides and with amiloride added to the outside (10^{-4} M). In all measurements the current has been measured as a function of time (as in Fig. 5) to ascertain that steady state was achieved. By comparison with Fig. 6 the time required is from 5 to 12 min. Filled circles: untreated skins. Open circles: theophylline added to the bathing solutions (2.4 mM) of the same skin

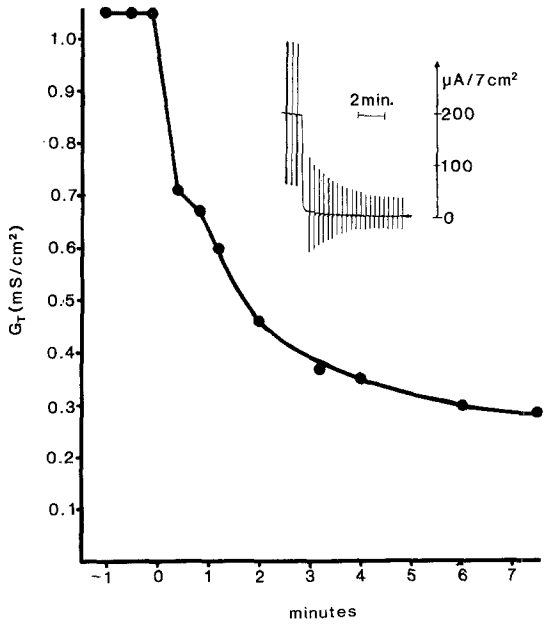

Fig. 9. Time-dependence of instantaneous transepithelial conductance after the addition of amiloride (10^{-4} M) at time = 0 min to a skin bathed with NaCl-Ringer's on both sides and short circuited. The insert is the actual experimental recording, where the height of the vertical lines are proportional to the conductance. The points of the lower curve have been calculated by dividing the height of the current responses with the pulse height of the square-wave voltage signal (in this case 20 mV)

tance is low and hardly stimulated by hyperpolarization. In such cases, addition of theophylline (2.4 mM) transforms the skin so as to become rectifying. This indicates that theophylline activates the "native" chloride pathway.

Changes in sodium concentration in the outside bathing solution is believed to affect the voltage drop across the outward-facing membrane. It is therefore possible that the effect of sodium on chloride transport is not only due to the depolarization of the intracellular potential per se, but that the effect is amplified through the action of voltage on chloride permeability. That this is the case is shown in Fig. 9. When amiloride is added, a sudden drop in tissue conductance is observed, followed by a much slower decrease over several minutes. This slow decrease is similar to the decrease observed when the clamping voltage is changed from large negative values to zero (Fig. 5). The slow phase of conductance decrease after amiloride addition may therefore be due to inactivation of chloride conductance caused by the hyperpolarizing effect of amiloride. The slow phase of conductance change cannot be due to changes in sodium conductance, because any remaining sodium conductance would result in the presence of a positive short-circuit current, and this is virtually zero one minute after amiloride addition.

Discussion

The results presented show beyond any doubt that the chloride conductance of frog skin is voltage dependent. This is most clearly disclosed by the relationships between clamping current and clamping voltage shown in Fig. 8. As the experiments have been carried out in the presence of amiloride in the outside bathing solution, the clamping current is practically equal to the chloride current (Fig. 4). The possibility that the rectification is a result of amiloride treatment can be excluded by the finding that the relationship between isotopically determined chloride net flux and potential is strongly nonlinear also in the absence of amiloride (Fig. 3). Both Fig. 8 and Fig. 3 show, however, that the degree of rectification of the chloride pathway varies considerably from skin to skin. The observation that theophylline can induce rectification in skins which were characterized by an almost linear I/V curve led us to the conclusion that chloride conductance is potentially voltage dependent in all skins provided that the chloride transport system is in an activated state. The nonlinearity of the theophylline-stimulated conductance is in itself in accordance with the assumption that theophylline stimulates a cellular transport pathway, as it is unlikely that a paracellular pathway can exhibit rectification, when the concentration of the permeant species is the same on the two sides. In *R. temporaria* the permeability of the paracellular route is not potential dependent (Kristensen, 1972). Probably the theophylline-stimulated pathway is identical to the native rectifying pathway present in most skins.

Most of the chloride transport in *R. esculenta* is probably localized to a transcellular pathway. The effects of amiloride, benzimidazolylguanidine and removal of outside sodium on chloride transport mentioned below are in accordance with this assumption. Also the specific action of outside chloride on chloride efflux (Fig. 1 and Kristensen, 1982) supports this view, and indicates that only about 10 percent of the chloride transport may occur through paracellular pathways or via pathways formed by edge damage.

The large variation observed with respect to rectification of the chloride transport pathway indicates that this pathway is controlled by several factors. If we assume that the cells through which the transport is taking place have an outside membrane that contains the normal type of sodium channels, chloride transport is expected (Kristensen, 1982) to be dependent on the number of open sodium channels, because the transepithelial chloride permeability increases, when the intracellular potential is depolarized, even if the chloride permeabilities of the two serially arranged membranes do not change. That such a mechanism could be involved is indicated by much experimental evidence: Amiloride inhibits chloride transport (Candia, 1978; Kristensen, 1978), reduction in outside sodium concentration reduces chloride permeability (Macey & Meyers, 1963; Kristensen, 1978) and the sodium channel activator benzimidazolylguanidine (BIG) stimulates chloride transport (Kristensen, 1981). The activation of the chloride transport system with theophylline indicates that this pathway is also under metabolic regulation, probably via the cAMP system. This conclusion is based on the fact that theophylline stimulates chloride transport in the presence of amiloride, which means that this stimulation cannot be secondary to the effect of theophylline on sodium permeability. In skins not treated with amiloride, theophylline may stimulate chloride transport directly via its action on the cAMP level and indirectly via the effect of the drug on sodium permeability and thereby on the intracellular potential. It has been reported that the chloride conductance in

toad skin is sensitive to reduced oxygen tensions (Larsen & Kristensen, 1977). Preliminary observations (*unpublished*) indicate that this is also the case in frog skin. The link between metabolism and regulation of passive transport pathways remains to be resolved. Experiments by Voûte and Meier (1978) indicate that the natural stimuli that may be involved in chloride permeability regulation are the catecholamine hormones.

The presence of rectification in an epithelium can be explained in two different ways (Larsen & Kristensen, 1978; Larsen et al., 1981; Larsen, 1982). One is that the chloride permeability of the two serially arranged membranes (the outward- and inward-facing, respectively) remain constant, but that the intracellular chloride concentration increases due to hyperpolarization. Model calculations on the basis of the two-membrane hypothesis have shown that increases in cellular ionic composition alone can lead to an increase in chloride conductance. The other possibility is the less trivial one that the change in potential difference across one of the membranes resulting from the transepithelial hyperpolarization induces an increase in chloride permeability of that membrane and thereby of the whole tissue.

The multitude of shapes of current versus time curves obtained after a step change in clamping voltage in the frog skin makes it hardly possible to perform a strict mathematical analysis aimed at showing the presence of voltage-gated chloride channels. But in the formulation of gating mechanisms presented by Hodgkin and Huxley (1952) it appears that the rate of conductance activation increases with increasing hyperpolarization. We have used the time spent in reaching half steady-state current ($T_{1/2}$) to quantify the rate of conductance increase and have found that it decreases with increasing hyperpolarization (Fig. 7), qualitatively in agreement with the classical gating theory. A computer model analysis (Larsen et al., 1981) shows that for the trivial case (Goldman-type rectification with constant permeabilities) the relationship between $T_{1/2}$ and V_{clamp} is inverse of the relationship obtained here. It is also noteworthy that the initial delay or even decrease observed after clamping is not predicted by analysis of the two-membrane hypothesis with constant permeabilities (Larsen et al., 1981). On the basis of the qualitative arguments presented above it seems fair to conclude that the rectification of the chloride transport pathway in frog skin is mainly due to changes in permeability of one of the serially arranged membranes of the cells responsible for chloride transport. The results presented do not allow us to decide whether the gating process takes place in the outside- or inside-facing membrane. As it has been proposed that chloride passes the skin via the mitochondria-rich cells in warm-adapted frogs (Voûte & Meier, 1978; Kristensen, 1981) it would not be fruitful to discuss the localization on the basis of the overall electrophysiological properties of the tissue, because knowledge about the voltage divider ratio of these cells is not available. If, however, the trans-effect (Fig. 1) is due to interaction of chloride ions with the molecular entity responsible for the voltage-dependent conductance, it is reasonable to propose that it is localized to the outward-facing membrane (for further discussion *see* Larsen, 1982)

The flux ratio analysis shows that electrodiffusion accounts for chloride transport at negative clamping voltages. At positive clamping voltages many experimentally observed flux ratios are larger than predicted by the flux ratio equation. This means that a fraction of the chloride fluxes in this region may be attributed to exchange diffusion. If it is assumed that transport occurs by both electrodiffusion and by exchange diffusion, it is possible to make an estimate of the magnitude of the exchange component. The observed flux ratio R^{obs} is related to the electrodiffusional fluxes J^{el}_{in} and J^{el}_{out} and to the exchange flux J^{ex} (equal in the two directions) by Eq. (2):

$$R^{obs} = (J^{el}_{in} + J^{ex})/(J^{el}_{out} + J^{ex}) = J^{obs}_{in}/J^{obs}_{out} \quad (2)$$

where J^{obs}_{in} and J^{obs}_{out} are the observed fluxes. The theoretical flux ratio R^t is by definition equal to the ratio between the electrodiffusional fluxes. So the following relation holds:

$$R^t = (J^{obs}_{in} - J^{ex})/(J^{obs}_{out} - J^{ex}) \quad (3)$$

which allows calculation of the exchange flux:

$$J^{ex} = (R^t \times J^{obs}_{out} - J^{obs}_{in})/(R^t - 1). \quad (4)$$

The calculation cannot of course be made at a clamping voltage of 0 mV because R here will always be one. In Table 2 are shown calculations of the apparent exchange flux in experiments where the flux ratios at negative voltages were in accordance with expectations, while deviations were observed at positive voltages. The chloride transport accounted for by exchange diffusion amounts to a considerable fraction of the unidirectional fluxes at positive clamping voltages.

A deviation from flux ratio can also be caused by an active transport in the inward direction. In *R. temporaria* this transport may attain rates of 1 nmole/cm min, but is in most cases only a frac-

Table 2. Calculation of exchange diffusion component of chloride fluxes in frog skin

Exp. no.	Clamping voltage mV	Influx	Efflux	Observed ratio	Expected ratio	Exchange flux ($nmol \times cm^{-2} \times min^{-1}$)
		($nmol \times cm^{-2} \times min^{-1}$)				
1	+40	5.21	6.58	0.79	0.205	4.86
	−60	25.26	3.03	8.34	10.75	0.56
2	+30	5.20	6.27	0.83	0.305	4.73
	−30	19.74	5.66	3.48	3.28	−0.52
3	+30	5.49	7.25	0.76	0.305	4.72
	−30	44.97	13.86	3.24	3.28	0.22
4	+50	7.45	16.60	0.449	0.138	2.72
	−50	24.55	3.95	6.22	7.25	0.654
5	+60	3.43	9.30	0.37	0.093	2.78
	−60	49.3	4.82	10.23	10.75	−0.12
6	+60	4.76	9.23	0.51	0.093	4.26
	−60	10.77	1.78	6.05	10.75	0.78
7	+60	4.76	9.23	0.51	0.093	4.26
	−60	63.00	5.26	12.00	10.75	−1.17

tion of that (Kristensen, 1972). This is also the case in *R. esculenta (unpublished)*. If it is assumed that the influx is composed of active transport and electrodiffusion, the rates of active transport necessary to explain the flux ratios obtained would be about 4 nmoles/cm min. Such an active transport was not present in the skins used for this analysis as evidenced by the flux ratio at 0 mV, which was very near to one. In the experiments reported here, the active transport must have been too small to be detected by the method used. It is unlikely, therefore, that the deviations from expected flux ratio at positive voltages are due to active transport. Provided that the deviation from flux ratio predictions can be explained by exchange diffusion, the calculations indicate that exchange diffusion is voltage dependent, being at its maximum when the conductance of the chloride pathway is at its minimum. It is clear that for an exchange diffusion the flux ratio is always one, but the unidirectional fluxes may very well change with voltage across an epithelium, because intracellular chloride concentration will do so. So, the "reciprocal" relationship between the intensity of electrodiffusion and exchange diffusion does not allow the conclusion that the exchange mechanism bears any relationship to the molecular structures responsible for the conductive transfer of chloride ions. Such a relationship has been proposed in the case of the toad skin, based mainly on the effects of various inhibitors on the two forms of chloride transport (Kristensen & Larsen, 1978). A similar comparison has not been performed on frog skin, because it has been difficult to find drugs suited for the purpose and because of the variability of the exchange diffusion contribution. In a number of cases the flux ratio equation actually predicts the ratios obtained at positive voltages, indicating an absence of exchange diffusion in these cases.

While the situation seems to be clear with respect to the role of exchange diffusion at clamping voltages different from zero, the question about the presence of exchange diffusion under short-circuit conditions is much more difficult to deal with. In this case, with identical solutions on the two sides, the flux ratio will be the same whether transfer is due to one mechanism or the other. In Table 1 it was attempted to compare the change in partial chloride conductance and the total tissue conductance caused by the addition of Cu^{++} to the outside solution. As already shown by Koefoed-Johnson and Ussing (1958), Cu^{++} inhibits chloride fluxes in frog skin and at the same time induces an increase in the spontaneous transepithelial potential difference. If Cu^{++} inhibits conductive chloride transport only, the change in chloride conductance is expected to be equal to the change in total tissue conductance. If Cu^{++} inhibits both electrodiffusion and exchange diffusion one would expect the calculated change in chloride conductance to be larger than the observed change in total conductance. It is found that the decrease in chloride conductance is smaller than the decrease in total conductance. The simplest explanation for this is that Cu^{++} inhibits other ionic pathways which contribute to tissue conductance. The ions present are chloride, sodium, potassium, and bicarbonate, but the sodium pathway is not inhibited by Cu^{++}, and the concentrations of potassium and bicarbonate are small. It is therefore very unlikely that other pathways should play a larger role for tissue conductance

than indicated by the difference between the decrease in chloride and tissue conductance. It is suggested that most of the chloride fluxes under short-circuit conditions contribute to tissue conductance. The linear relationship between chloride and tissue conductance supports this view. This means that the trans-effect described cannot be due to stimulation of exchange diffusion alone, but must be explained as a stimulation of a conductive pathway as well. Comparison of the transport of different anions with their trans-effect on chloride efflux (Kristensen, 1982) supports this view. A similar conclusion was put forward to explain the trans-effect observed in skins of *Leptodactylus ocellatus* (Ques-von Petery et al., 1978).

The experiments performed on *Rana pipiens* (Biber et al., 1980) have led to conclusions that differ from those presented here. They found that about 75% of the chloride transport at short-circuit conditions was due to exchange diffusion. This is very nearly the same as I find in skins clamped at reversed potentials. The difference between the two species may be due to a difference in voltage sensitivity of the exchange mechanism, so that this is also in function under short-circuit conditions in *R. pipiens*. A flux ratio analysis, like the one performed here on *R. esculenta*, may solve this question.

As already indicated above, the voltage dependence of the chloride permeability has relevance for our understanding of the effect of sodium on chloride permeability. Candia (1978) and Kristensen (1978) suggested that the effect of amiloride on chloride permeability may be due to the hyperpolarization of the intracellular potential. A computer calculation on the basis of the two-membrane hypothesis confirmed this view. If, however, the change in voltage drop across the membrane responsible for the rectifying properties of the chloride pathway activates ("gates") chloride permeability, the effect will be amplified. The time course of conductance change observed when amiloride is added (Fig. 9) supports the idea that permeability changes do take place. The direct action of amiloride is inhibition of the sodium channels and thereby abolishing the sodium diffusion potential across the outward-facing membrane. This leads to the fast initial drop in conductance. This drop is composed of two contributions: (1) the direct drop in sodium conductance due to closure of the sodium channels, and (2) the concomitant decrease in chloride conductance as predicted by the constant field approach applied to two membranes in series. The subsequent slow inactivation occurs over a time period like the one needed for inactivation of chloride conductance after the clamping voltage is stepped from a hyperpolarizing value to zero (Fig. 5). On this basis it seems fair to conclude that changes in outside sodium concentration or addition of amiloride affects the permeability of a membrane for chloride. Biber et al. (1980) mention that chloride efflux is not affected by removal of sodium from the outside solution. This may be due to a displacement of the current-voltage relation of the chloride transport pathway towards the open-potential range. Though chloride permeability theoretically will be dependent on the presence of sodium, even when the permeability of the outward-facing membrane is constant, the effect of sodium will be larger and easier to observe when sodium (via its effect on the intracellular potential) affects membrane permeability. The simultaneous displacement of the current-voltage relation and the exchange-voltage relation is not unlikely if the two types of transport involve the same complex of molecular entities as has been suggested for toad skin (Kristensen & Larsen, 1978).

The experiments presented in this paper have clearly shown that chloride conductance in frog skin is highly voltage dependent, probably because chloride permeability changes with voltage. This phenomenon is important for our understanding of epithelial function especially with respect to different effectors (be they drugs, hormones, or ions) on transport and conductance, and for understanding the dynamics of the interrelationship between sodium and chloride transfer. The presence and significance of transport pathways with similar properties in other epithelia besides toad and frog skin remains to be evaluated.

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