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Effect of Amiloride on Chloride Transport Across Amphibian Epithelia

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Summary. Amiloride is found to inhibit chloride exchange diffusion in toad skin and passive chloride transport in frog skin. In both tissues, chloride transport is reactivated by substituting with KCl-Ringer's on the inside, so the effect of amiloride on chloride transport is secondary to its well-known inhibition of sodium transport. Removal of chloride from the outside bathing solution inhibits chloride outflux in both tissues. This is easy to explain in the case of the toad skin where chloride transport under short-circuit conditions occurs as exchange diffusion. In the frog skin this transeffect indicates that the chloride concentration at a location very near the outer surface is of significance for chloride permeability. The possibility is discussed that the chloride concentration in the outward facing membrane, or in compartments near to it, regulates chloride fluxes across frog skin.

In the short-circuited toad skin there is evidence that most of the chloride transport occurs *via* an exchange diffusion mechanism (Bruus, Kristensen, & Hviid Larsen, 1976; Hviid Larsen & Kristensen, 1978). This exchange diffusion can be inhibited by a variety of drugs: phloretin, acetazolamide, furosemide, and thiocyanate, all of which may be supposed to act by interfering directly with the chloride transporting mechanism (Kristensen & Hviid Larsen, 1978). None of the inhibitory effects on chloride transport could be explained as being secondary to the effect of the inhibitor on sodium transport (*loc. cit.*). As will be described in this paper, it appears, however, that amiloride also inhibits exchange diffusion of chloride across toad skin. It was therefore necessary to reinvestigate the possible interdependence of chloride and sodium transport. On the basis of this study it is suggested that the effect of amiloride on chloride transport may be explained by assuming that amiloride blocks sodium transport and thereby alters cellular electrolyte concentrations, especially chloride concentration which may be expected to decrease. This leads to the observed inhibition of chloride exchange diffusion.

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When frogs are kept in the cold room at about 4 °C they are not very permeable to choride, which seems to pass the skin passively *via* a paracellular route, and actively in very small quantities *via* a transcellular route (Kristensen, 1972, 1973). Frogs stored at higher temperatures have skins with much larger chloride permeabilities (Koefoed-Johnsen & Ussing, 1974). In such frog skins the behavior of chloride fluxes can be predicted by the flux ratio equation (Ussing, 1949), indicating passive chloride transport and not exchange diffusion. Also in this tissue (frog skin) it will be shown that amiloride inhibits chloride transport. As a working hypothesis it is suggested that chloride concentration at a location very near to or in the outward-facing membrane may be the factor controlling passive chloride transport through frog skin.

Materials and Methods

The toads (*Bufo bufo*) were treated as described in a previous paper (Bruus *et al.*, 1976).

The frogs (*Rana temporaria*) are kept in shallow tap water at about 4 °C until two weeks or more before the experiment. At this time they are transferred to a room with moist gravel on the floor, where they have free access to tap water, which is continuously renewed at a slow rate. The temperature is 20 °C, and 12-hr day and night periods alternate. Mealworms (*Tenebrio molitor*) are given *ad libitum* in a plastic jar from which the frogs can take them. Under these conditions the frogs will survive in good condition for a rather long time, our "oldest" frogs have been living there for nine months.

The chloride Ringer's solution (NaCl-R) is composed as follows (in mM): 113.5 Na^+, 1.9 K^+, 0.9 Ca^{++}, 114.8 Cl^-, 2.4 HCO_3^-, and pH=8.2 when aerated with atm air. In sulphate Ringer's (Na_2SO_4-R) and gluconate Ringer's, all chloride was substituted by either sulphate or gluconate. In potassium Ringer's all sodium was substituted by potassium (KCl-R).

All fluxes reported are unidirectional fluxes measured with ^{36}Cl obtained from the Danish Atomic Energy Commission (Risø, Denmark).

Results

Toad Skin

In short-circuited skins bathed with NaCl-Ringer's on both sides, it has been shown recently that chloride efflux is a good estimate of chloride exchange diffusion in toad skin, as in most cases chloride electrodiffusion is only a small fraction of the total efflux measured (Bruus *et al.*, 1976). The effect of amiloride on exchange diffusion was therefore studied by measuring the effect of the drug on isotopically determined chloride effluxes.

Table 1. Toad skin (*Bufo bufo*): The effect of amiloride (6×10^{-5} M, outside) on chloride efflux (measured with ^{36}Cl)[a]

Exp. No.	Efflux of chloride (nmoles $\times$ cm^{-2} $\times$ min^{-1})	
	Control	With amiloride
1	7.70	1.69
2	1.66	1.22
3	9.18	2.92
4	3.22	1.79

[a] NaCl-Ringer's on both sides. Each value is the average of three consecutive flux periods of 30 min. The skins were short circuited.

Table 2. Toad skin (*Bufo bufo*): The effect of removing sodium from the outside bathing solution on chloride efflux[a]

Exp. No.	Efflux of chloride (nmoles $\times$ cm^{-2} $\times$ min^{-1})	
	NaCl-Ringer's outside	KCl-Ringer's outside
1	11.54	2.35
2	2.69	0.88
3	10.58	3.36
4	2.99	1.00

[a] Sodium was removed by substituting with KCl-Ringer's, so chloride concentration was not changed. The fluxes were measured with ^{36}Cl under short circuit conditions.

Table 1 shows beyond any doubt that chloride efflux is markedly reduced by adding amiloride to the outside bathing solution to a concentration of 60 μM. The same result is obtained by removing Na^+ from the outside bathing solution (substituting with K^+) (Table 2). This already indicates that the effect of amiloride on chloride transport may be due to the effect of this drug on sodium transport. Fig. 1 shows the degree of inhibition of sodium and chloride transport as a function of amiloride concentration. The two response curves are not significantly different from each other, meaning that the effect of amiloride is due to the interference of the drug with one site (or with two sites having the same amiloride affinity). Similarly, Fig. 2 shows how chloride efflux varies with the sodium concentration in the outside bathing solution.

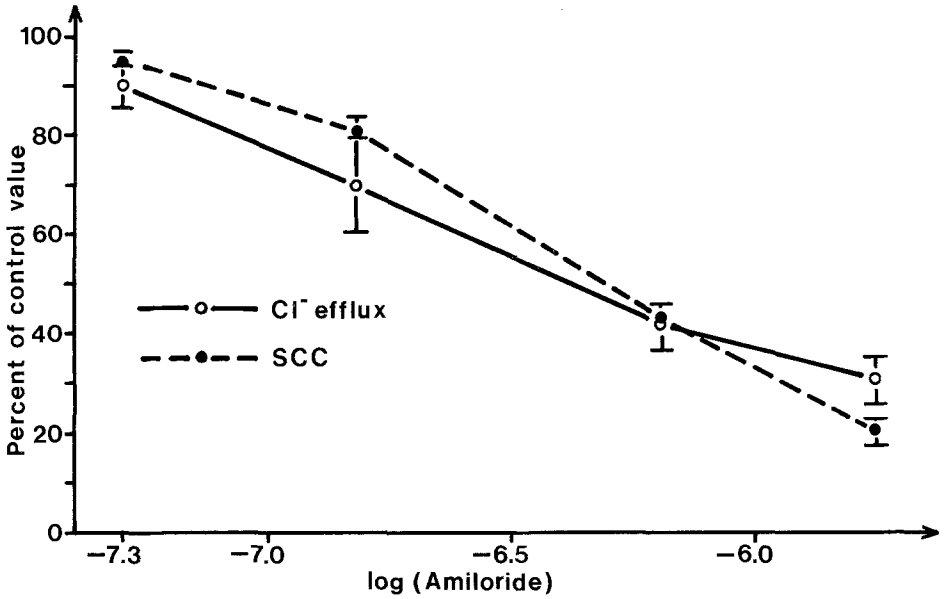

Fig. 1. *Toad skin.* The inhibition of chloride efflux ($\simeq$ exchange diffusion) and short-circuit current (=active sodium transport) by amiloride. Both magnitudes are given as % of their values before amiloride was added in the concentration steps indicated on the abcissa. (Average ± SEM for 6 toads)

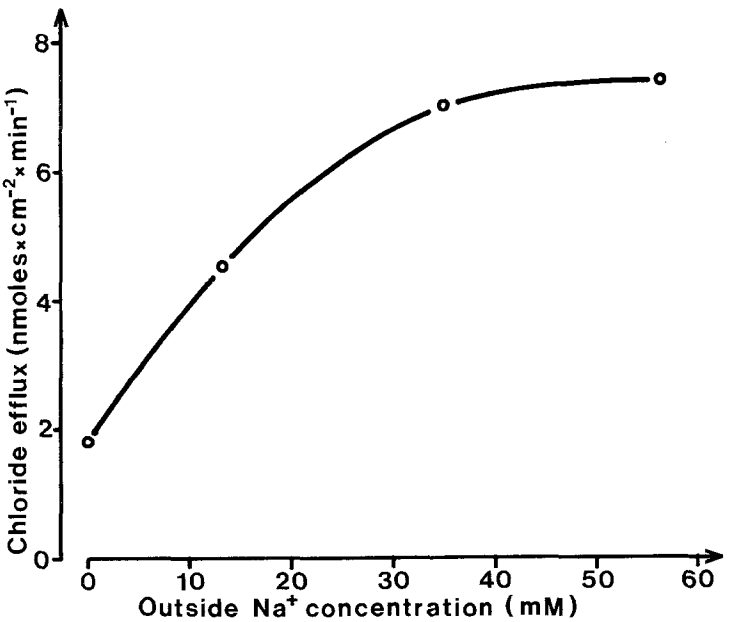

Fig. 2. *Toad skin.* The efflux of chloride at different outside sodium concentrations. (Average of 5 experiments)

Table 3. Toad skin (*B. bufo*): Activation of amiloride inhibited chloride efflux by raising the K^+ concentration on the inside[a]

Exp. No.	Period	Chloride efflux (nmoles $\times$ cm^{-2} $\times$ min^{-1})			
		Control	Amiloride	KCl	Sulphate outside
1	I	5.14	1.33	6.83	0.97
1	II	4.49	1.64	16.11	1.34
2	I	1.55	1.39	7.58	0.93
2	II	1.72	1.34	17.18	1.04
3	I	4.43	1.69	8.97	1.12
3	II	5.65	1.84	15.59	1.42
4	I	3.13	1.71	5.22	0.82
4	II	2.94	1.46	7.35	1.00

[a] The numbers given are 20-min fluxes, the first (*I*) starting 20 min after change of exptl. condition, the second (*II*) immediately after the first. Control: NaCl-Ringer's both sides. Amiloride: 10^{-4} M amiloride in outer bath. KCl: NaCl-Ringer's outside and a mixture of equal volumes of NaCl and KCl Ringer's inside. Sulphate outside: Inside as before, but Na_2SO_4-Ringer's in the outside compartment.

The function is very similar to that describing sodium influx across the mucosal barrier as a function of outside sodium concentration (*see*, e.g., Lindemann & Voûte, 1976).

Up to this point, the experiments are in accordance with the view that the changes following inhibition of sodium transport by amiloride lead to a reduction of chloride exchange diffusion. One simple way of explaining this would be that cellular chloride concentration is reduced either by adding amiloride or by removing sodium from the outside bathing solution. If this is the case, it should be possible to reverse the effect of amiloride by increasing cellular chloride concentration. This can be done by replacing the inside NaCl Ringer's with KCl Ringer's which is known to lead to a swelling of the skin epithelium (Ussing, Biber & Bricker, 1965). The effect of substituting on the inside with KCl Ringer's on amiloride-treated skins is shown in Table 3. It appears very clearly that this maneuver leads to a stimulation of chloride efflux to values significantly larger than those observed before amiloride was added. It is also observed that the stimulation of the efflux with KCl Ringer's requires some time, as the first flux period after substitution gives smaller fluxes than the following period. This is well in agreement with the time course for epithelial swelling under similar conditions (Ussing *et al.*, 1965). It is very important to note that when we now change

the outside solution to Na_2SO_4-Ringer's the chloride efflux is again reduced to values equal to those observed when the skin is bathed with sulphate on the outside and NaCl Ringer's on the inside (Bruus *et al.*, 1976). This means that even after activation by KCl on the inside, we are still dealing with a chloride transport following a route with the same characteristics as in the normal situation. So, substitution with KCl-Ringer's on the inside stimulates exchange diffusion of chloride, and does not open up new pathways for chloride transport across the skin.

Frog Skin

With respect to skins from frogs kept at temperatures higher than 10–14 °C, there is not much doubt that chloride transport is purely passive (Koefoed-Johnsen, Levi & Ussing, 1952). A few new experiments confirming this view are shown in Table 4. It is concluded that chloride transport through frog skin does not occur as exchange diffusion.

Nevertheless, it became clear that chloride fluxes in frog skin are also sensitive to amiloride. This is documented in Table 5, which clearly shows that amiloride inhibits both influx and efflux of chloride in this tissue. It is seen that amiloride has the largest effects when the chloride fluxes are large in the control period, whereas the effect may be absent in cases where the chloride fluxes are already small before amiloride addition.

When the inside solution under these conditions (amiloride outside) is changed to KCl-Ringer's, Table 6 shows that both influx and efflux

Table 4. Frog skin (*R. temporaria*): Flux ratio analysis of chloride fluxes[a]

Exp. No.	Clamping voltage (mV)	Flux ratio	
		Measured	Calculated
1	0	1.13	1.00
2	+60	11.54	10.40
3	−40	0.22	0.21
4	0	0.91	1.00
5	+60	8.20	10.40
6	0	0.86	1.00
7	+60	10.44	10.40

[a] Fluxes were determined on two symmetrical skin halves, influx in one, efflux in the other.

Table 5. Frog skin (*R. temporaria*): Effect of amiloride (10^{-5} M) on chloride influx and efflux measured with ^{36}Cl under short-circuit conditions

Exp. No.	Flux direction	Chloride fluxes (nmoles × cm^{-2} × min^{-1})	
		Control	Amiloride
1	in	6.52	3.05
2	in	9.34	5.16
3	in	10.91	2.27
4	in	7.75	2.04
5	in	2.62	1.44
6	in	4.37	3.57
7	in	22.00	5.57
8	in	3.38	3.77
9	out	11.80	3.94
10	out	12.36	2.88
11	out	8.62	3.31
12	out	12.53	2.25
13	out	20.59	5.78
14	out	3.11	1.04

Table 6. Frog skin (*R. temporaria*): Reactivation of chloride influx and efflux in amiloride-treated skins by substitution with KCl-Ringer's in the inside bath[a]

Exp. No.	Chloride fluxes (nmoles × cm^{-2} × min^{-1})					
	Control		Amiloride		KCl inside	
	Influx	Outflux	Influx	Outflux	Influx	Outflux
1	15.12	8.62	5.44	3.31	23.91	21.74
2	2.67	3.11	1.26	1.04	4.47	5.57
3	19.81	23.26	3.69	2.80	26.24	32.48
4	1.53	2.52	2.44	2.42	32.78	33.75

[a] Fluxes of chloride (^{36}Cl) were determined on short-circuited symmetrical halves.

of chloride are increased to levels above those observed under the control condition, i.e., the effect is equivalent to those occurring in the toad skin. It became, therefore, of interest to see how far this similarity reaches. As has been shown (Bruus *et al.*, 1976), removal of chloride in the outside bathing medium reduces chloride efflux in toad skin to very low values. It is evident from Table 7 that the same change performed on frog

Table 7. Frog skin (*R. temporaria*): The effect of removal of chloride in the outside bathing solution on chloride efflux (the *trans* effect)[a]

Exp. No.	Chloride efflux ($nmoles \times cm^{-2} \times min^{-1}$)	
	Control	Sulphate outside
1	12.13	1.70
2	9.76	0.56
3	6.39	2.24
4	24.72	1.79
5	15.94	1.31
6	2.48	1.25

[a] The outside NaCl-Ringer's was replaced by Na_2SO_4-Ringer's. Short-circuit conditions.

Table 8. Frog skin (*R. temporaria*): Reactivation with KCl-R of chloride efflux in skins bathed with Na_2SO_4-Ringer's on the outside[a]

Exp. No.	Chloride efflux ($nmoles \times cm^{-2} \times min^{-1}$)		
	Control (NaCl-R on both sides)	Na_2SO_4-R on the outside	KCl-R on inside Na_2SO_4-R on outside
1	6.39	2.24	26.05
2	24.72	1.79	36.63
3	15.94	1.31	31.12
4	2.48	1.25	8.74

[a] Short-circuit conditions.

skin also leads to a decrease of chloride efflux. In the case of frog skin, however, this trans-effect cannot be explained on the basis of exchange diffusion. This is most clearly shown by the experiments of Table 8, from which it is evident that the *trans*inhibition provoked by removing Cl^- from the outside solution can be counteracted by substituting with KCl-Ringer's on the inside.

The experiments reported so far indicate that the chloride concentration at some location in the epithelium is of significance for transepithelial chloride permeability. To get some further information in this direction, the effect of removal of chloride in the inside bathing solution was investigated. Table 9 shows that substitution with Na-gluconate-Ringer's on the inside most often results in a decrease in chloride influx, although

Table 9. Frog skin (*R. temporaria*): Chloride influx[a]

Exp. No.	Chloride influx ($nmoles \times cm^{-2} \times min^{-1}$)	
	Control	Gluconate inside
1	10.35	6.52
2	16.21	9.34
3	13.20	10.91
4	11.36	7.75
5	8.73	2.62
6	4.50	4.37
7	21.80	22.00
8	3.33	3.38

[a] The effect of removing chloride in the inside solution. Substitution is made with gluconate Ringer's. Short-circuit conditions.

Table 10. Frog skin (*R. temporaria*): Chloride influx[a]

Exp. No.	Chloride influx ($nmoles \times cm^{-2} \times min^{-1}$)			
	Control	Amiloride	K_2SO_4-R inside	KCl-R inside
1	27.14	4.98	18.10 32.58 39.11	31.88 39.90
2	23.04	2.09	18.81 33.15 37.58	23.52 19.25
3	15.01	4.39	9.52 20.30 23.53	30.27 19.19
4	21.45	4.27	24.15 43.63 44.00	56.58 43.40
5	22.78	4.41	– 34.72 –	–

[a] The effect of K_2SO_4-Ringer's on the inside. In the control and amiloride periods the numbers given are averages of two consecutive flux measurements of 20 min, the first of which starts 20 min after change of conditions. With K_2SO_4 Ringer's inside, the flux values are given for three periods individually, the first one starting immediately after changing to K_2SO_4. The same applies for the KCl Ringer's fluxes.

cases are found where the effect is small or absent. This means that concentration on the inside does not alone determine the chloride concentration at the location where it affects transepithelial chloride fluxes. This is even more evident from Table 10, where it can be seen that substitution with K_2SO_4-Ringer's for NaCl-Ringer's on the inside of the skin has a large stimulatory effect on chloride influx in amiloride-treated skins. The stimulation is of the same magnitude as that observed, when substitution is made with KCl-Ringer's on the inside. It is again observed that the chloride fluxes are not stimulated instantaneously when we change to K_2SO_4-Ringer's, but that attainment of a constant Cl^- flux is reached in about an hour.

Discussion

Superficially, the chloride transport systems of toad skin and frog skin have many properties in common. It is, however, important to note that the basic mechanism of chloride transport under short-circuit conditions are quite different: In frog skin chloride transfer occurs by passive diffusion, whereas the process in toad skin appears to have the characteristics of exchange diffusion. The results will, therefore, first be discussed separately for the two tissues.

Toad Skin

Earlier results obtained on this tissue have established that chloride transfer, under short-circuit conditions and under depolarization, mostly occurs by exchange diffusion (Bruus *et al.*, 1976; Hviid Larsen & Kristensen, 1978; Kristensen & Hviid Larsen, 1978). It was also found most likely that this mechanism is located at the outside facing membrane of the epithelium. Exchange diffusion of chloride will depend on the chloride concentration in the outer bathing solution and in the cells of the outermost living cell layer. If blocking of sodium transport with amiloride lowers cellular chloride concentration, this could be the explanation for the effect of this drug on chloride transport in toad skin.

Many attempts have been made to measure the potential difference between the transporting cells and either inside or outside solution of

anuran skins. Although many disagreements exist, it seems reasonable to expect that, under short-circuit conditions, the cells are negative with respect to their surroundings, the potential step across the two membranes being identical because of the short-circuit conditions. The potential steps may be formalized in many ways. In the open circuit skin, the total potential difference across the epithelium was described as the sum of two diffusion potentials (Koefoed-Johnsen & Ussing, 1958), one for sodium across the outward facing membrane, and one for potassium across the inward facing membrane. In the short circuited skin, it is less easy to formalize so strictly, because the formalism will depend on the detailed mechanism by which active sodium transport occurs across the inner membrane (electrogenic sodium pump?), and because under this condition the potential difference across the two membranes have to be of identical numerical value. The PD across the outward facing membrane could be visualized as the drop in voltage due to the sodium current through this membrane (*IR*-drop). That across the inward facing membrane has the direction of a potassium diffusion potential or an electrogenic sodium pump, but will be modified by the potassium current passing across it.

When amiloride is added, or when sodium is removed from the outside bathing solution, currents no longer pass through the two membranes, and the potential across the inward facing membrane will be the full Nernst potential for potassium. This means that the addition of amiloride makes the potential more negative in the cells than under normal symmetrical conditions. If it is assumed that chloride is in equilibrium between cells and inside, we will expect amiloride to induce a decrease in cellular chloride concentration. This again results in a reduction of chloride exchange diffusion.

The effect of substituting with KCl-Ringer's for the normal NaCl-Ringer's on the inside is likewise easy to explain. An increase of potassium concentration on the inside leads to a decrease in potassium diffusion potential, i.e., the cellular potential becomes less negative and eventually zero. This leads to a new chloride distribution with a cellular chloride concentration higher than under normal or amiloride conditions.

A very important observation is that, with KCl on the inside, removal of chloride in the outside solution still reduces chloride efflux to values similar to those reported to be due to an extracellular shunt pathway. This means that KCl added to the inside stimulates chloride transport through the normal pathway and does not form a new chloride pathway through the skin.

Frog Skin

Before discussing the effect of amiloride in this tissue, some of the general characteristics of chloride transport through it will be treated.

In skins from frogs stored in the cold (about 4 °C), it was easily shown that the permeability to passive chloride movements was independent of the chloride concentration in the outside bathing solution (Kristensen, 1972, 1973). It was suggested that passive chloride movements occurred *via* an extracellular pathway. When the frogs are stored for some days at room temperature prior to the experiments, the situation changes dramatically: The choride fluxes are now much larger, and they have been suggested to pass through a transcellular pathway (Koefoed-Johnsen & Ussing, 1974). The first flux ratio experiments performed on frog skin were made on skins from frogs kept at temperatures well above 4 °C, and they showed that chloride fluxes could be described by the flux ratio equation (Koefoed-Johnsen *et al.*, 1952). Also, the experiments of the present paper showed this to be the case. This is a strong argument in favor of the supposition that choride movements across the skin from warm adapted frogs occur by passive diffusion. It excludes the possibility that a simple exchange diffusion of chloride can be responsible for the transepithelial chloride fluxes observed.

It was therefore surprising to observe that removal of chloride in the outside bathing solution leads to a reduction of the efflux of chloride to levels near those we found to be due to electrodiffusion through the shunt pathway in toad skin. As chloride has been replaced by sulphate, the possibility exists that sulphate inhibits chloride transfer through an anion selective channel in the outward facing membrane. This is, however, ruled out by the fact that replacement of the inside Ringer's solution with KCl-Ringer's reactivates chloride efflux. That is, the reduction of chloride efflux cannot be a result of the presence of sulphate, but must be due to the absence of chloride.

It is interesting to compare the effect of removal of chloride on the two sides of the skin, because this may help us to localize the area of the skin, where removal of chloride is effective in inhibiting chloride fluxes. Eight experiments were performed, in which chloride in the inside bathing solution was replaced by gluconate. In five cases a clear reduction was seen in the influx, whereas no such change was observed in three. In the cases where a reduction was found, this was not nearly as large as the reduction observed in efflux, when the outside solution is changed from chloride to sulphate.

The substitution experiments thus indicate that chloride permeability is dependent on the chloride concentration at a location where chloride concentration is more sensitive to outside than to inside chloride concentration.

The fact that the substitution with KCl Ringer's on the inside affects chloride fluxes shows that this local regulatory chloride concentration is sensitive to changes in inside bath composition. An elevation of inside K^+ will depolarize the potential across the inward facing membrane. Because the skin is short circuited, depolarization of the potential across the inner membrane inevitably leads to a depolarization of the potential across the outward facing membrane. It may be suggested that the chloride concentration in a compartment located very near to the outside is the parameter controlling chloride fluxes through frog skin. This compartment could be the outward facing membrane itself or areas thereof.

When the frog skin is bathed on both sides with NaCl, some experiments (Nielsen, 1977) indicate that the potassium permeability of the inward facing membrane may be rate limiting for sodium transport. The potassium concentration in the epithelium will then be above the one predicted from the potential difference across the inward facing membrane. Indirect evidence in favor of this view is also found in the epithelial volume changes occurring as a result of changes in inside osmolarity (MacRobbic & Ussing, 1961), and direct evidence is found in microelectrode studies indicating that the potential difference across the inward facing membrane of toad bladder is much smaller than predicted from potassium activities measured with ion specific electrodes (Kimura *et al.*, 1977). Consequently, the potential across this membrane has to be in agreement with the chloride distribution. When we change to gluconate on the inside, we can no longer have chloride equilibrium, and the potential difference across the inward facing membrane switches to the higher PD determined by the potassium distribution. Because of the short circuit conditions, this results in a hyperpolarization of the outer membrane by exactly the same number of mV. This will result in a lower chloride concentration in the outward facing membrane, the final consequence being a decrease in chloride flux. It is reasonable to expect this effect to be rather variable, because it depends, among other things, on the extent to which the PD across the inner membrane is determined by chloride or potassium distribution.

As in the case of toad skin, it should also be considered if the cytoplasmic chloride concentration could be the direct regulatory factor. For example, chloride ions in the cells could regulate the opening of

anion channels by reacting with regulatory sites on the inside of the outward facing membrane.

It is clear that, with suitable permeabilities of the inward and outward facing membranes, the cellular chloride concentration must to some extent depend on the chloride concentrations in both bathing solutions. It is known e.g. (Ussing *et al.*, 1965), that substitution with KCl Ringer's on the inside induces a swelling of the epithelium. This swelling is faster when the outside is NaCl Ringer's than when it is sulphate Ringer's. It is, however, very difficult to understand that cellular chloride concentration should be as dependent on outside chloride concentration as indicated by the experiments where chloride is substituted with sulphate on the outside. And it is as difficult to see why a removal of inside solution chloride should not lead to a more pronounced reduction in cellular chloride concentration. We will therefore proceed with the discussion on the basis that it is not the cellular chloride concentration but the chloride concentration in the outward facing membrane that determines the chloride fluxes.

Let us first discuss what properties we have to postulate for the outward facing membrane in order to expect that chloride concentration within the membrane will change in parallel with the observed chloride fluxes, when changes in bathing solution are performed. For this purpose we may start with the simple homogenous membrane described by the constant field equation. In this case the concentration of an ion (C_j) at a point (x) in the membrane of thickness h may be described by the following equation (Sten-Knudsen, 1978):

$$C_j = \alpha_j \frac{C_j^{(c)} \cdot \exp\left(\frac{zFV}{RT}\right) - C_j^{(o)} - (C_j^{(c)} - C_j^{(o)}) \exp\left(\frac{zFVx}{RTh}\right)}{\exp\left(\frac{zFV}{RT}\right) - 1} \tag{1}$$

where $C_j^{(c)}$ and $C_j^{(o)}$ are the concentrations of the ions in the cells and in the outside bathing medium, respectively. α_j is the distribution coefficient and z, F, V, R and T have their usual meaning. The thickness of the membrane is h, and x denotes the vertical distance from the inside to a point in the membrane. In the following calculations we shall assume $\alpha_j = 1$ so that $C_j = C_j^{(c)}$ for $x = 0$ and $C_j = C_j^{(o)}$ for $x = h$. Fig. 3 shows some concentration profiles for chloride ions in a homogenous membrane with

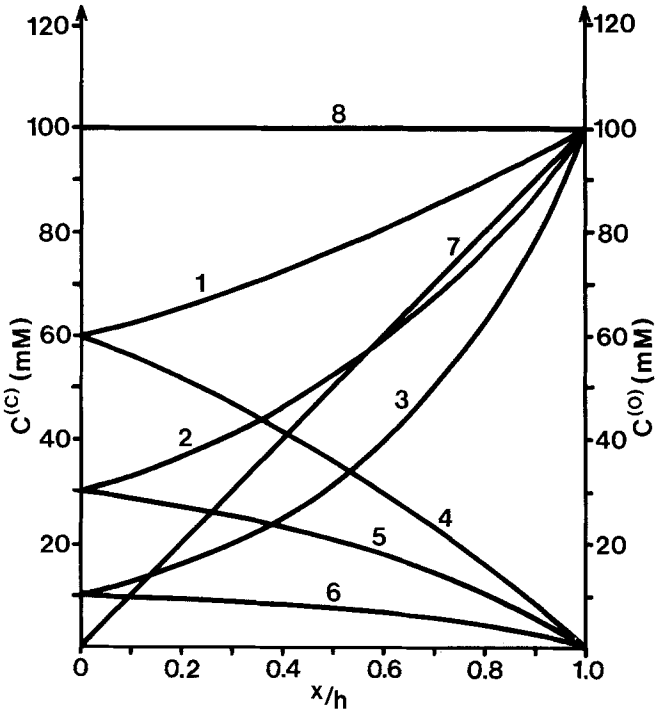

Fig. 3. Profiles of chloride concentration in a homogenous membrane with constant field properties, calculated from Eq. (1) and the assumptions given in the text. The intracellular potentials used in calculating the profiles were: (*1*): −20 mV; (*2*): −40 mV; (*3*): −60 mV; (*4*): −20 mV; (*5*): −40 mV; (*6*): −60 mV; (*7*): −2 mV; (*8*): −2 mV

constant electrical field. The conditions to which these cases apply are chosen so that they may have qualitative similarity to the conditions to which the outward facing membrane is submitted under the different experimental procedures. In the control period, the skin is bathed on both sides with NaCl-Ringer's, so $C_{Cl}^{(o)}$ is 100 mM (in fact, 115, but to simplify calculation we have used 100 mM). The intracellular potential is not known with certainty, but may be in the range from −20 to −60 mV. The intracellular chloride concentration also has to be estimated, and may be between 10 and 60 mM. The mentioned range of values are covered by curves 1, 2 and 3 of Fig. 3.

From this situation we now substitute on the outside with Na_2SO_4-Ringer's, i.e., $C_{Cl}^{(o)}=0$. If this is the only change, we now get profiles *4*, *5* and *6*. This shows that whatever control condition we start with, chloride concentration in most of the membrane will be reduced consider-

ably, most so, of course, in the regions nearest to the outer surface. Under these conditions we also measure a large drop in chloride efflux.

If we change the inside solution to chloride-free Ringer's, the situation is less well described. In the long run, of course, cellular chloride concentration in the whole epithelium will drop to very low values, but it is not easy to estimate with any degree of certainty what happens to the chloride concentration in the cytoplasm in contact with the outward facing membrane. It is, however, known that a change from chloride to sulphate on the inside leads to a slight reduction in cell volume. This means that the cellular potassium concentration is increased, and in the absence of chloride on the inside, the cells hyperpolarize.

A change from chloride to sulphate or gluconate on the inside may therefore be exemplified by a shift from profile *2* to profile *3* or even lower, again indicating a lowering of membrane chloride concentration. It is worth noting that the absolute reduction in this case depends on the control conditions: In a skin with a high cellular chloride concentration and a small cellular potential, the effect will be largest. These parameters may vary from skin to skin, and this variation could be the cause of the varying effect on chloride influx observed, when the inside is changed to gluconate from chloride.

The effect of amiloride on the cellular chloride concentration was already discussed in connection with toad skin. One further observation, which indicates that amiloride induces a hyperpolarization and a reduction of cellular chloride concentration, is that the outermost living cell layer shrinks after removal of sodium from the outer medium (Voûte & Hänni, 1973), this maneuver being equivalent to addition of amiloride. The cellular potential and chloride concentration may after amiloride addition therefore change qualitatively in the same direction, as happens when chloride is removed from the inner solution. The effects of amiloride on chloride fluxes are in agreement with this idea.

The activation of chloride fluxes occurring when KCl is substituted for NaCl on the inside is easily understandable on the basis of the present hypothesis: With KCl-Ringer's inside, the cells are depolarized to perhaps zero mV, resulting in an increase in cellular chloride concentration to that of the bathing solutions. The concentration of chloride in the outside bathing solution will be described by profile *8* of Fig. 3, showing that membrane chloride concentrations under these circumstances are the highest possible, given the chloride concentration in the Ringer's. Also, the chloride fluxes are under these circumstances larger than under control conditions.

The chloride concentration profile in the outer membrane of a skin with sulphate on the inside and amiloride added to the outside chloride Ringer's may be conceived to have a shape like curve *3*, but originating in $C_{Cl}^{(c)}=0$. When the inner membrane is depolarized with K^+ (as K_2SO_4) one would suppose the profile to shift to that given by curve *7*. Also the chloride influxes were found to be increased under these circumstances, and they even reached values comparable to those obtained with KCl-Ringer's inside, i.e., values higher than those obtained under control conditions. In the case of the simple hypothesis, this is only expected if the chloride concentration profiles in the control period can be described by curves like *3*. The K_2SO_4 activation may thus indicate that the chloride fluxes are not alone determined by the concentration of chloride in the membrane, but that also the influence of the potential gradient has to be considered. A voltage-dependent gating mechanism could be responsible for changes in permeability. This seems to be a possibility in toad skin under hyperpolarization (Hviid Larsen & Kristensen, 1978). But in frog skin such a mechanism alone cannot explain all results, e.g., the decrease in efflux observed upon removal of chloride in the outside solution. In this case one would, if anything, expect a depolarization of the mucosal membrane potential, and this would, with the potassium effects in mind, be expected to increase chloride permeability of this membrane.

The molecular mechanism that is responsible for the possible interdependence of membrane chloride concentration and chloride fluxes cannot be discussed on the basis of the present material. The fluxes may vary with chloride concentration as a physical consequence of the increase in membrane chloride conductance, or the chloride concentration may regulate chloride permeability by changing membrane structure. With respect to the effects of depolarization with K^+, it should be noted that they could be due to a specific action of this ion, although this did not seem to be the case in toad skin.

Finally, it is quite obvious that the working hypothesis discussed is a naive oversimplification. The outward facing membrane cannot be considered homogenous. But also in a nonhomogenous membrane concentration, profiles in "channels" or integral protein molecules will be determined by the potential difference and the compositions of the solutions in contact with the membrane. By using the outward facing membrane as an example, the roles of other anatomical structures located near to or in contact with this membrane are perhaps underestimated, but should not be forgotten.

Conclusions

One clear conclusion emerges from the present studies: Amiloride inhibits chloride transport in both toad skin and frog skin, and it does so, not by reacting with chloride transport systems, but because, as a consequence of sodium transport inhibition, the bioelectrical characteristics of the tissue are changed. It is therefore clear that the pathway of chloride transport in both tissues is located at epithelial cells which are also capable of transporting sodium. This means that chloride transport must pass through normal granulosa cells, or that a significant part of sodium transport occurs through the mitochondria-rich cells, which seem to be involved in chloride transport regulation (Voûte, 1977).

One clear difference between toad and frog skin under short circuit conditions is that, in the first case, chloride transport occurs as exchange diffusion and, in the second, as passive diffusion. There exists, however, the possibility that the molecular apparatus responsible for these two modes of transport may not be very different. It has been shown that chloride conductance of toad skin increases dramatically when the skin is hyperpolarized (Hviid Larsen & Kristensen, 1978), and there is pharmacological evidence (Kristensen & Hviid Larsen, 1978) that it is the exchange diffusion system which is transformed into a conductive system by hyperpolarization. The conductance activation could not be accounted for by simple "Goldman rectification", which means that a change in conductive chloride permeability is necessary to explain the results (Hviid Larsen & Kristensen, 1978). This change could be caused either directly by the change in potential difference across the membrane, or by a change of cellular or membrane chloride concentration. A more detailed comparison between the skins from the two animals may give us important clues to solve the question of chloride transport regulation.

The working hypothesis put forward also opens up new possibilities for understanding the functional links between the outward facing and the inward facing membrane. In the analysis of two-membrane models, it has to be taken into account that changes in the properties of one membrane as a simple physical consequence may lead to changes of the properties of the other membrane. Otherwise, experimental results may falsely be taken as evidence against the two-membrane hypothesis.

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References

Bruus, K., Kristensen, P., Hviid Larsen, E. 1976. Pathways for chloride and sodium transport across toad skin. *Acta Physiol. Scand.* **97**:31

Hviid Larsen, E., Kristensen, P. 1978. Properties of a conductive cellular chloride pathway in toad skin. *Acta Physiol. Scand.* **102**:1

Kimura, G., Urakabe, S., Yuasa, S., Miki, S., Takamitsu, Y., Orita, Y., Abe, H. 1977. Potassium activity and plasma membrane potentials in epithelial cells of toad bladder. *Am. J. Physiol.* **232**:*F*196

Koefoed-Johnsen, V., Levi, H., Ussing, H.H. 1952. The mode of passage of chloride ions through the isolated frog skin. *Acta Physiol. Scand.* **25**:150

Koefoed-Johnsen, V., Ussing, H.H. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298

Koefoed-Johnsen, V., Ussing, H.H. 1974. Transport pathways in frog skin and their modification by copper ions. *In*: Secretory Mechanisms of Exocrine Glands. N.A. Thorn and O.H. Petersen, editors. Munksgaard, Copenhagen

Kristensen, P. 1972. Chloride transport across isolated frog skin. *Acta Physiol. Scand.* **84**:338

Kristensen, P. 1973. Anion transport across frog skin. *In*: Transport Mechanisms in Epithelia. H.H. Ussing and N.A. Thorn, editors. p. 148. Munksgaard, Copenhagen, and Academic Press, New York

Kristensen, P., Hviid Larsen, E. 1978. Relation between chloride exchange diffusion and a conductive chloride pathway across the isolated skin of the toad (*Bufo bufo*). *Acta Physiol. Scand.* **102**:22

Lindemann, B., Voûte, C.L. 1976. Structure and function of the epidermis. *In*: Frog Neurobiology. R. Llinas and W. Precht, editors. Springer Verlag, Berlin, Heidelberg

MacRobbie, E.A.C., Ussing, H.H. 1961. Osmotic behaviour of epithelial cells of frog skin. *Acta Physiol. Scand.* **53**:348

Nielsen, R. 1977. The effect of the polyene antibiotic filipin and arginine vasotocin on the isolated frog skin. (This symposium)

Sten-Knudsen, O. 1978. Passive transport processes. *In*: Transport across Biological Membranes. H.H. Ussing, G. Giebisch, and D.C. Tosteson, editors. Springer Verlag (*in press*)

Ussing, H.H. 1949. The distinction by means of tracers between active transport and diffusion. *Acta Physiol. Scand.* **19**:43

Ussing, H.H., Biber, T.U.L., Bricker, N.S. 1965. Exposure of the isolated frog skin to high potassium concentrations at the internal surface. *J. Gen. Physiol.* **48**:425

Voûte, C.L. 1977. The MR cells as hormone receptors. (This symposium)

Voûte, C.L., Hänni, S. 1973. Relation between structure and function in frog skin. *In*: Transport mechanisms in epithelia. H.H. Ussing and N.A. Thorn, editors. Munksgaard, Copenhagen