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Effect of the Polyene Antibiotic Filipin and the Calcium Ionophore A23187 on Sodium Transport in Isolated Frog Skin *(Rana temporaria)*

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Summary. Addition of filipin (50 μM) to the inside bathing solution of the frog skin resulted in a transient increase in the active sodium transport [measured as short-circuit current (SCC)]. The filipin-induced increase in the SCC required the presence of calcium. The calcium ionophore A23187 (4 μM) also induced a transient increase in the SCC. After the activation of the SCC by A23187, the SCC could not be activated by filipin. This indicates that the polyene antibiotic filipin acts as a calcium ionophore. Higher concentrations (40 μM) of A23187 resulted in a shrinking of the cells in the transporting cell layer. A23187 also increased the potassium-42 exchange in the isolated epithelium. It is suggested that calcium ionophores enhanced the intracellular calcium concentration; this increase in the calcium concentration resulted in an increase in the potassium permeability of the inward-facing membrane. The increase in the potassium permeability might explain the observed increase in the SCC.

In a previous paper (Nielsen, 1977) it has been shown that addition of the polyene antibiotic filipin to the outside of the isolated frog skin results in a nonspecific increase in the permeability of the outward-facing membrane and in the transepithelial permeabilities. Addition of the polyene antibiotic amphotericin B to the outside of the isolated frog skin and toad bladder had nearly the same effects as filipin, but the effects were somewhat smaller, the addition of amphotericin B to the inside of the toad bladder and frog skin had no effect (Lichenstein & Leaf, 1965; Nielsen, 1971). However, the addition of filipin to the inside resulted in an activation of the active sodium transport, whereas it had nearly no effect on the passive permeabilities (Nielsen, 1977).

In the work presented here the effect of filipin on the inside of the isolated frog skin has been investigated. It is shown that filipin (added to the inside) acts as a calcium ionophore. The calcium ionophore A23187 has the same effect as filipin. It is shown that A23187 increases the

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potassium permeability of the inward-facing membrane. It is suggested that this increase in the potassium permeability of the inward-facing membrane can explain the observed activation of the sodium transport induced by the ionophores.

Materials and Methods

The experiments were performed on female and male frogs (*Rana temporaria*). The frogs were kept partially immersed in tap water at about 4 °C. The skins were dissected from pithed animals and divided into two symmetrical halves. The skins were mounted in perspex chambers and bathed in stirred Cl^--Ringer's solution ($Na^+ = 115.0$, $K^+ = 2.5$, $Ca^{2+} = 1.0$, $HCO_3^- = 2.4$, and $Cl^- = 117.1$ mM, pH = 8.2) or SO_4^{2-}-Ringer's solutions, the SO_4^{2-}-Ringer's solutions had the same composition as the Cl^--Ringer's solution but Cl^- was replaced by 58.55 mM SO_4^{2-}. The epithelia were isolated as described by Johnsen and Nielsen (1978). The isolated epithelia was incubated in modified Ringer's solution ($Na^+ = 115$, $K^+ = 2.5$, $Ca^{2+} = 1.0$, $Mg^{2+} = 1$, $Cl^- - 116$, $CO_3^{2-} = 2.5$, $SO_4^{2-} = 1$, and $PO_4^{3-} = 1$ mM, pH = 7.8); glucose was added to a final concentration of 5 mM. Stock solutions of the ionophore A23178 were prepared by dissolving 2.5 mg A23187 in 125 µl acetone plus 900 µl 96% ethanol. The acetone/ethanol mixture was always added in the control experiment.

The short-circuit experiments were performed according to the method of Ussing and Zerahn (1951), using an automatic voltage clamp apparatus programmed to disconnect the short-circuit current every 5 min, thus allowing the potential to be measured for 60 sec.

Arginine-vasotocin(ADH) was obtained from Sandoz.

Measurement of Potassium Exchange

Isolated epithelia were preincubated for 1 hr in Ringer's solution with 10 µM amiloride. After preincubation the epithelium was transferred to a 50-ml flask containing 15 ml Ringer's solution with 10 µM amiloride, potassium-42, and inulin^{3}H. After 2, 5, 10, 15 and 20 min of incubation, the epithelium was removed from the flask, blotted on filter paper, and counted for 10 sec in a gamma counter; the epithelium was then transferred back to the flask, and the incubation was continued. After incubation the epithelium was blotted on filter paper and weighed. The epithelium was then extracted for at least 12 hr with 2 ml 0.1 M HNO_3. The amount of potassium in the extract was determined by flame photometry. The amount of inulin-^{3}H was determined by using a liquid scintillation counter. The total amount of ^{42}K in the epithelium was determined by using a sample from the Ringer's solution to which the ^{42}K was added, as standard.

Results

Addition of 50 µM filipin to the inside of the isolated frog skin resulted in a transient increase followed by a smaller activation of the short-circuit current (SCC) (Fig. 1). The increase in the transepithelial

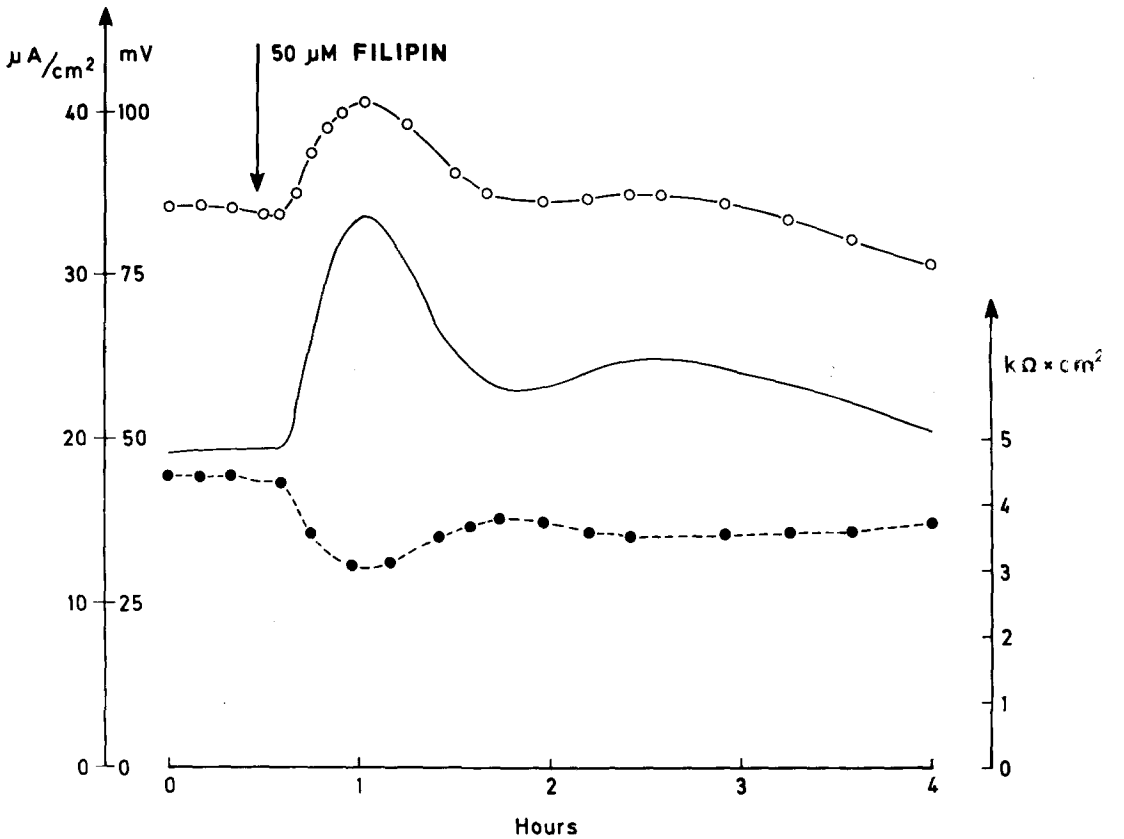

Fig. 1. At the arrow filipin was added to the inside to give a concentration of 50 μM. (——): Short-circuit current (μA/cm²); (o——o): potential (mV); (●——●): transepithelial resistance (kΩ × cm²)

potential (PD) was lower than the increase in the SCC; thus, there was a decrease in the transepithelial resistance. The increase in the SCC was caused by an activation of the active Na transport (Nielsen, 1977). The successive phases in the SCC (Fig. 1) did not correspond to changes in filipin concentrations in the incubation medium due to inactivation of the filipin since a similar pattern was observed when the inside bathing medium was replaced by fresh medium with filipin (data not given here). Furthermore, the removal of filipin from the inside medium after the SCC had reached the maximum had no qualitative effect on the filipin-induced changes in the SCC (Fig. 2). When the addition of filipin (50 μM) to inside bathing solution was repeated, the skin half where filipin already was present in the medium did not react, whereas the skin half (from which the filipin had been removed for some time) showed the usual filipin-induced changes in the SCC (Fig. 2). Thus, filipin released a

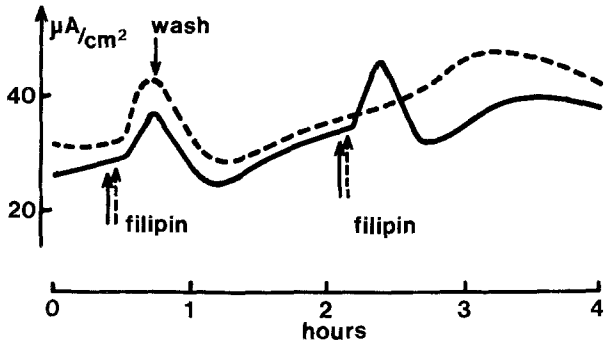

Fig. 2. Effect of filipin on the SCC. At the arrows filipin (50 μM) was added to both skin halves. At the arrow (*wash*) the medium bathing the skin half, represented by the full line, was changed to medium without filipin

"pool" which activated the SCC; when filipin was removed from the medium, the skin was able to regenerate this "pool".

Effect of Trypsin on the Filipin Response

Experiments were performed in which the inside of one of the skin halves was bathed with Ringer's solution containing trypsin. After 1 hr incubation the trypsin-containing solution was replaced by medium without trypsin. When the SCC was constant filipin was added to the inside, and when the SCC was constant again ADH was added. Table 1 shows that incubation with trypsin reduced the effect of filipin with 80% and the subsequent effect of ADH with 50%.

Thus, it was possible to remove the filipin receptor or effector with trypsin.

Table 1. Effect of trypsin on the filipin response

Trypsin (% increase in SCC)		Control (% increase in SCC)	
Filipin	ADH	Filipin	ADH
3.2±1.3	14.2±3.4	14.3±3.0	29.8±7.6

Values are the means±SE, $n=7$

One skin half was incubated with trypsin in the inside (0.04 mg/ml for 1 hr). After this incubation the inside was washed 3 times with new Ringer's solution. When the SCC was stable, filipin was added to the inside (50 μM); when the SCC had reached a new steady level, ADH was added to the inside (40 ng/ml). 3 of the skins were incubated in Cl^--Ringer's solution and 4 of the skins in SO_4^{2-}-Ringer's solution.

Effect of Antidiuretic Hormone

Table 2 shows a series of experiments where ADH was added to the inside of skin half *A*; after the SCC had reached a steady level filipin was added, and after that, diphenylhydantion (DPH). DPH was added to the outside. To skin half *B* filipin was added first and then ADH and DPH. Both filipin and ADH were added in a concentration which gave maximal stimulation of the SCC. Addition of DPH to the outside of the isolated frog skin activates the SCC, and the effect of DPH and maximal dose of antidiuretic hormone oxytocin are additive (de Sousa & Grosso, 1973). From the data in Table 2 it appears that the addition of ADH alone resulted in a 83% activation of the SCC, whereas the addition of filipin resulted in 31% activation. Furthermore, after the SCC had been stimulated by ADH the effect of filipin was abolished although it was possible to activate the SCC further by DPH. Since it is possible to abolish the effect of filipin by addition of ADH one can conclude that ADH and filipin stimulate the same cells. It is known that the intracellular level of cyclic AMP in toad bladder and in frog skin increases during the action of ADH (Handler *et al.*, 1965; Johnsen & Nielsen, 1978); however, the addition of filipin to the inside bathing solution had no effect on the intracellular level of cyclic AMP (Johnsen & Nielsen, 1978). Thus, the common step in the activation of the SCC by ADH and filipin is not cAMP.

Table 2. Effect of ADH on the filipin response

Expt No.	Skin half *A* (% increase in SCC)			Skin half *B* (% increase in SCC)		
	ADH	Filipin	DPH	Filipin	ADH	DPH
1	100	0	18	29	46	14
2	55	4	39	25	52	34
3	81	5	26	12	67	32
4	95	0	16	56	55	17
5	101	0	–	55	79	–
Means	86.4	1.8	24.8	35.4	59.8	24.3

The skins were incubated in Cl^--Ringer's solution. When the SCC was stable, ADH (40 ng/ml) was added to the inside of skin half *A*; when the SCC had reached a new steady level, filipin was added to the inside (50 μM); and when the skin again had reached a new steady level, diphenylhydantoin (DPH) (33 μg/ml) was added to the outside. Skin half *B* was treated in the same manner as skin half *A*, but first filipin was added, then ADH, and thereafter DPH.

Effect of Calcium and Calcium-Ionophore

Several studies on different tissues indicates there is an interaction between cyclic nucleotides and calcium in the control of cellular activity (*see* Berridge, 1975). Therefore, a series of experiments were carried out where one skin half was incubated in the presence and the other in the absence of calcium in the inside bathing solution. Filipin had no effect on the SCC when the skin was incubated in calcium-free solution (Fig. 3). Since the filipin-induced activation of SCC required Ca, it was investigated whether the calcium ionophore A23187 should have an effect on the SCC. Addition of 4 μM A23187 to the inside bathing solution resulted in an activation of the SCC and in a decrease in the transepithelial resistance (Fig. 4). A23187 induced either a stable or a transient activation of the SCC. In the experiment shown in Fig. 5 the addition of filipin to skin half *A* and A23187 to skin half *B* resulted in a transient activation of the SCC. After skin half *B* had reached a steady level, filipin was added and thereafter ADH. The addition of filipin after

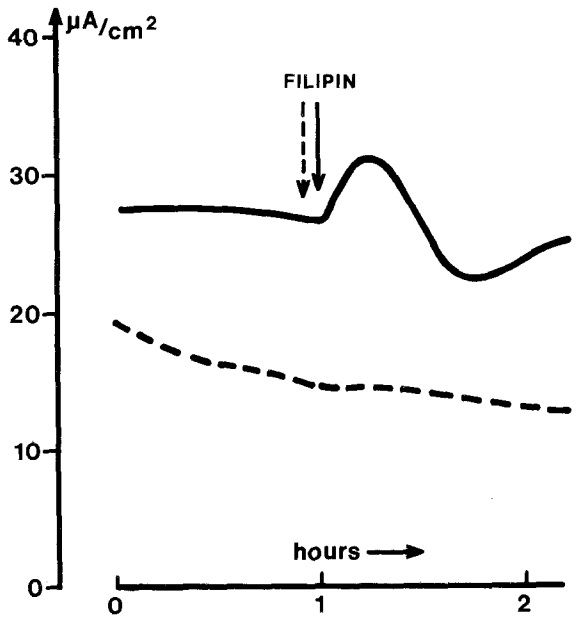

Fig. 3. (———): Skin half incubated in Cl^--Ringer's solution. (---------): Skin half incubated in Ca^{2+}-free Cl^--Ringer's solution with 0.1 mM EGTA. At the arrows filipin (50 μM) was added to the inside of both skin halves

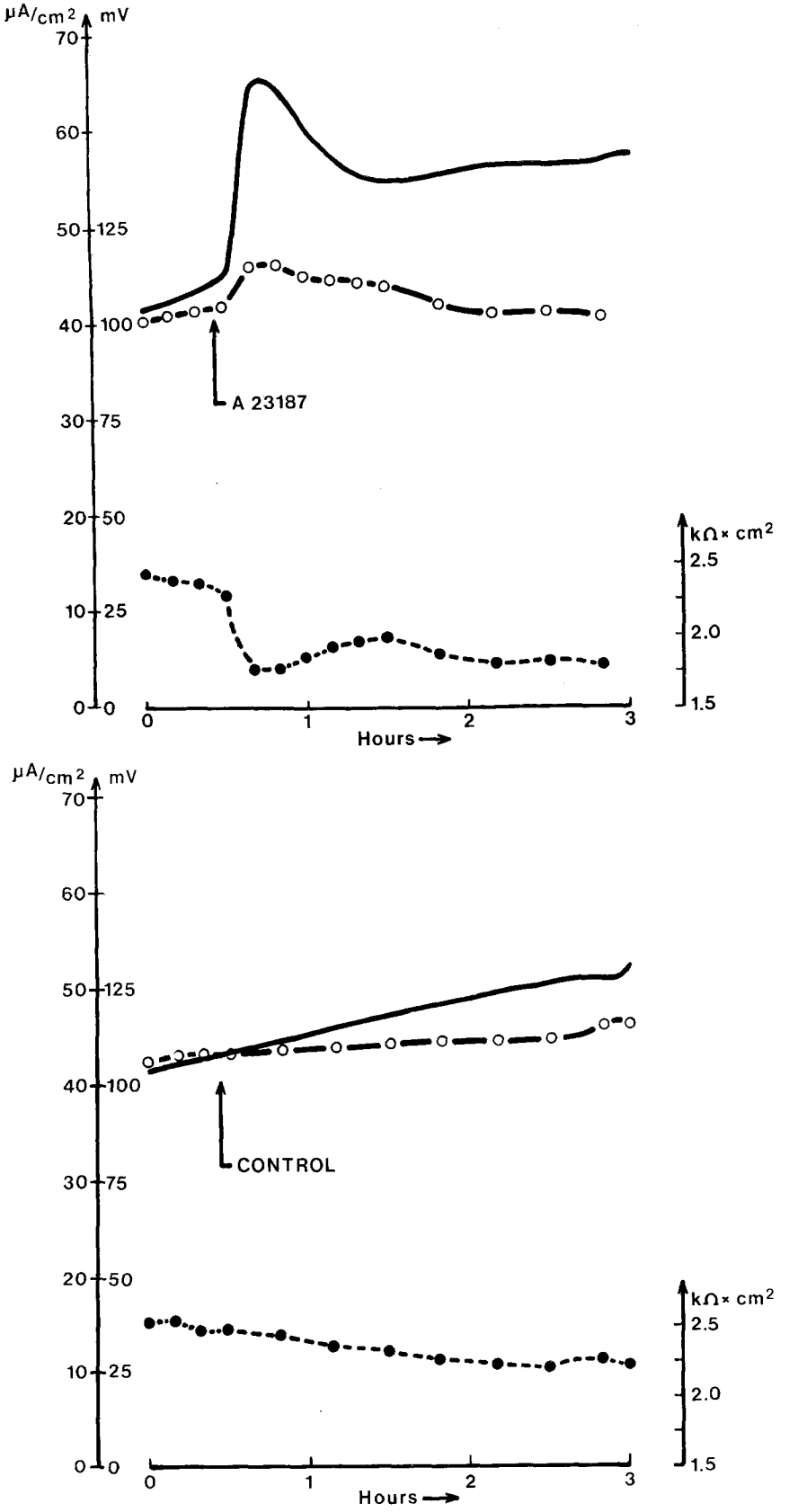

Fig. 4. Effect of 4 μM A23187 on the SCC and PD across the frog skin.
(———): SCC (μA/cm²); (○———○): mV; (●---------): kΩ/cm²

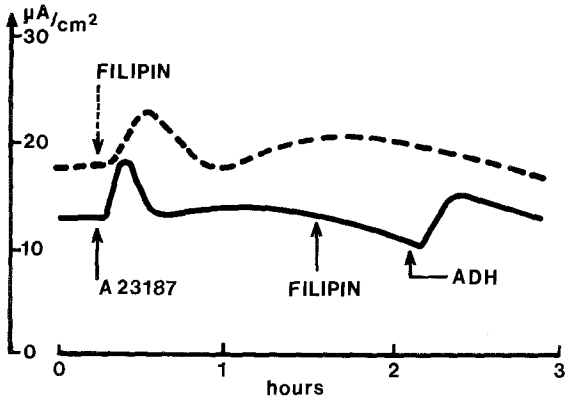

Fig. 5. Effect of filipin and A23187 on the SCC. Two skin halves were used for the experiment. Filipin (50 μM), A23187 (4 μM), and ADH (40 mg/ml) were added to the inside

A23187 had no effect on the SCC, whereas ADH enhanced the SCC (Fig. 5). In the experiments shown in Table 3 filipin was first added (to skin half *A*), and after the SCC had reached a steady level A23187 was added and then ADH. To skin half *B* (Table 3) A23187 was first added and then filipin and ADH. When A23187 was added to the skin filipin had no effect on the SCC (Table 3*B*); when filipin was added before A23187, filipin caused a reduction in the A23187 response (compare Table 3*A* and *B*). Since the filipin-induced activation of the SCC required the presence of Ca in the inside bathing solution, and the effect of filipin could be abolished by the calcium ionophore A23187, one can conclude that filipin when added to the inside of the frog skin acts as a calcium ionophore. Furthermore, an increase in the Ca permeability of the inward-facing membrane can result in an activation of the SCC.

Table 3. Effect of A23187 on the filipin response

Expt No.	Skin half *A* (% increase in SCC)			Skin half *B* (% increase in SCC)		
	Filipin	A23187	ADH	A23187	Filipin	ADH
1	29	28	–	42	0	32
2	41	13	48	46	8	39
3	37	47	21	56	0	5
4	48	10	33	17	0	23
Means	38.8	26.8		40.3	2	

When the SCC was stable, filipin (50 μM) was added to the inside of skin half *A*; when the SCC had reached a new steady level, A23187 (4 μM) was added to the inside; and when the skin again had reached a steady level, ADH (40 ng/ml) was added to the inside. Skin half *B* was treated in the same manner as skin half *A*, but A23187 was first added, then filipin, and thereafter ADH. The skins were incubated in Cl^--Ringer's solution.

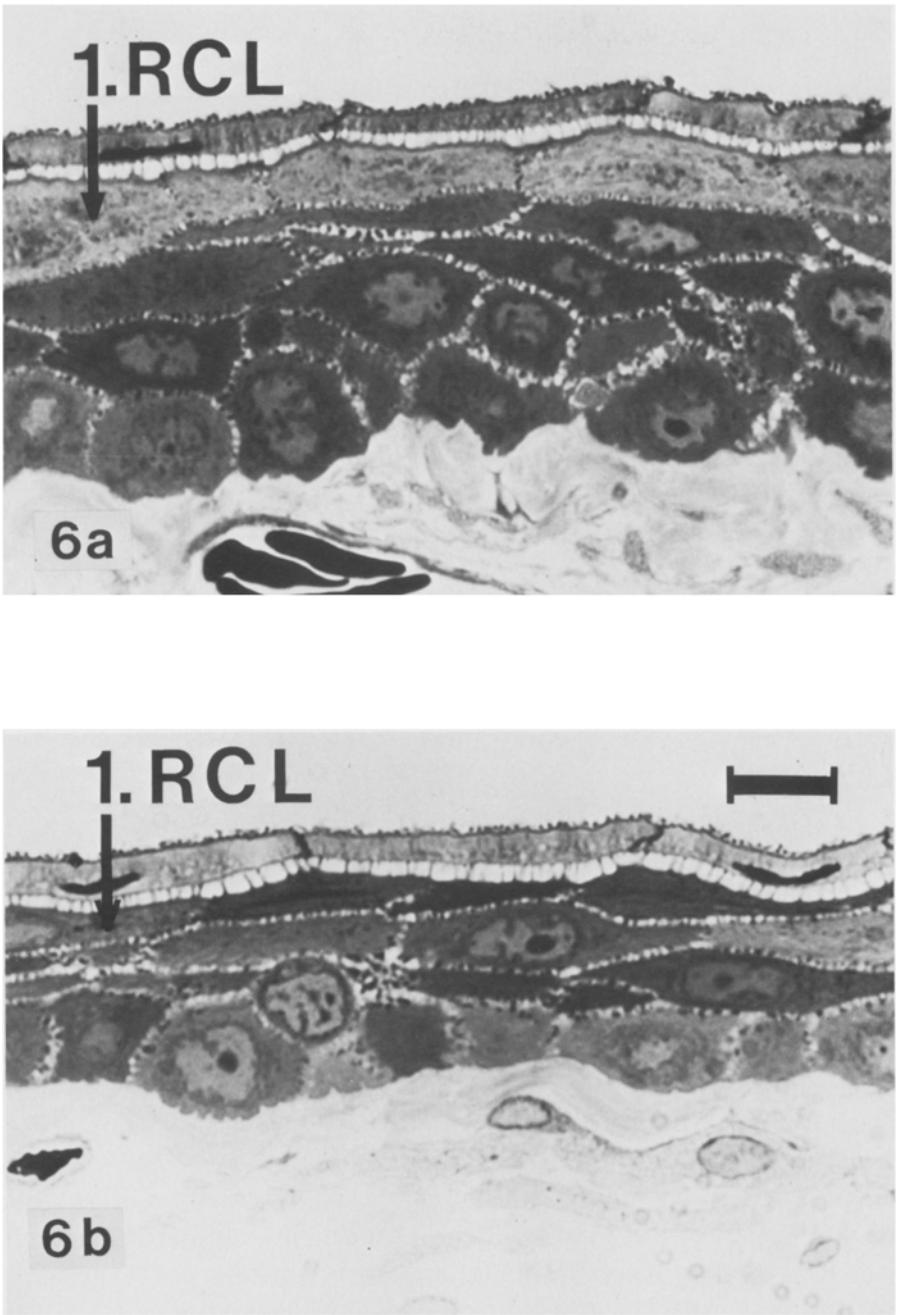

Fig. 6. Light micrographs of frog skin epithelium fixed with 1% OsO_4 during short circuiting of the frog skin. 1-μM sections stained with methylene blue. (*a*): control skin half. (*b*): incubated with 40 μM A23187. The bar on *b* corresponds to 10 μm. *1.RCL:* outermost layer of the *stratum granulosum*

Effect on Cell Volume

To investigate whether A23187 had an effect on the cell volume, experiments were performed where the skin was incubated with A23187 on the inside for 70–100 min. After incubation the skin was fixed by adding Ringer's solution with OsO_4 to both chamber halves. Addition of 4 μM A23187 had no visible effect on the cell volume. Addition of 40 μM A23187 to the frog skin resulted in a transient increase in the SCC, which in most cases was followed by a progressive decrease. When the skin halves were fixed, SCC had about the same density in the control skin half and the skin half to which the 40 μM A23187 was added. In the control skin half, the interspace system was open, and the cells in the outermost layer of the *stratum granulosum* (*1.RCL*) were swollen (Fig. 6*A*); it has been shown by Voûte and Ussing (1968) that a short-circuited epithelium had this appearance. In skin half to which the calcium ionophore A23187 was added, the interspace system was open but the *1.RCL* was shrunken. Such a shrinkage of the *1.RCL* could be due to A23187 increasing the K permeability in these cells. It has been shown by Reed (1976) that A23187 produces a rapid and extensive loss of potassium from rat erythrocytes during uptake of Ca.

Effect of A23187 on the Steady-State Exchange of Potassium-42

To investigate whether A23187 had an effect on the passive K flux of the inward-facing membrane, the steady-state exchange of ^{42}K was measured in the presence of 10 μM amiloride. Amiloride inhibits the active transepithelial sodium transport (Nielsen & Tomlinson, 1970; Salako & Smith, 1970), this inhibition is believed to be caused by an interaction of amiloride with the specific Na pathway (the sodium channels) in the outward facing membrane (Cuthbert & Shum, 1974). The K exchange rate increased in the presence of A23187 (Fig. 7). The curves in Fig. 7 can be graphically resolved into two first-order components. A part of the flux into the fast exchanging compartment results from K present in the extracellular space (the inulin space). The inulin space in these experiments contained 3.25% in the control and 3.97% in the presence of A23187 of the K present in the epithelium. Since the K present in the extracellular space exchanges at a high rate, one can treat this part as a blank value. If this is done, the experimental points of Fig. 7 agree well with curves representing the following empirical function:

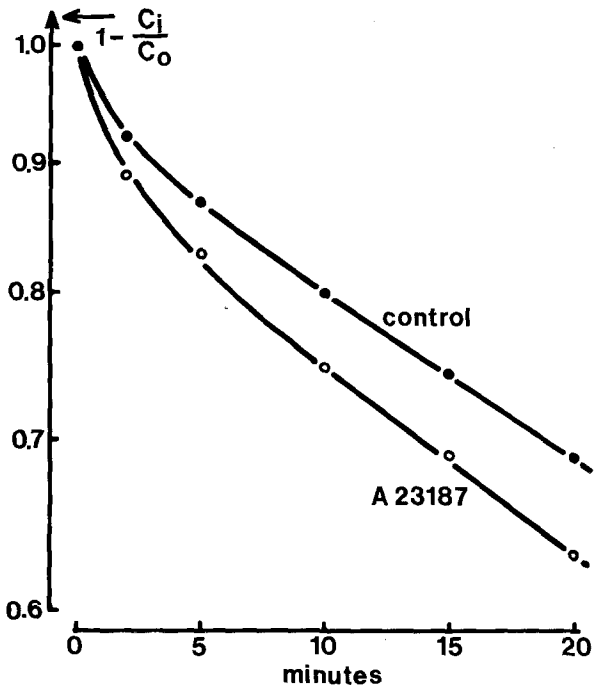

Fig. 7. Effect of A23187 (4 μM) on the apparent steady-state exchange of ^{42}K in the isolated epithelia in the presence of 10 μM amiloride. *Ordinate: Ci*, specific ^{42}K activity in the epithelium; *Co*, the specific ^{42}K activity in the medium. The epithelium was preincubated for 60 min with 10 μM amiloride at 21 °C. One half was thereafter incubated for 5 min in the presence of A23187, then transferred to the respective solutions labeled with ^{42}K. Values are the means of 4 experiments

The control:

$$1\text{-}Ci/Co = 0.03 + 0.06[\exp(-0.231)\,t] + 0.91[\exp(-0.0136)\,t] \quad (1)$$

with A23187

$$1\text{-}Ci/Co = 0.04 + 0.08[\exp(-0.288)\,t] + 0.88[\exp(\exp(0.0169)\,t]. \quad (2)$$

Where *Ci* is the specific ^{42}K activity in the epithelium and *Co* is the specific ^{42}K activity in the medium, *t* is time in min $0 < t \leqq 20$.

The potassium content in the control epithelium was 0.0412 μmol/mg wet wt, and A23187 K content was 0.0421 μmol/mg wet wt. From Eqs. 1 and 2 and the K content of the epithelium (excluding the K present in the inulin space), the K influx was calculated. The K influx in the control was 0.0649 μmol · (mg wet wt)$^{-1}$ · hr^{-1}, and in the presence of A23187 the influx was 0.0958 μmol · (mg wet wt)$^{-1}$ · hr^{-1}. The experiments were carried out under apparent steady-state conditions so the K influx is equal

to the K outflux; since the K outflux is passive, we can conclude that A23187 enhanced the passive K flux across the cell membrane. In the presence of ADH and 10 μM amiloride a small increase in the exchange rate was observed; the rate constant for the slow exchanging compartment was 0.0183 min^{-1} in the control and 0.0195 min^{-1} in the presence of ADH (40 ng/ml, mean of 5 expt).

In the isolated epithelium (frog skin) in the presence of amiloride Biber, Aceves and Mandel (1972) found a K influx of 0.289 $\mu mol \cdot hr^{-1} \cdot cm^{-2}$; in the experiments shown in Fig. 7, the mean wet wt was 4.5 $mg \cdot cm^{-2}$ which gave a K influx of 0.29 $\mu mol \cdot hr^{-1} \cdot cm^{-2}$. The K in the isolated epithelium was present in two compartments (Eq. (1), Fig. 7). The fast exchanging compartment contained 6% of the K and had a half time of 3 min; the slow exchanging compartment contained 91% of the K with a half time of 51 min. In the toad bladder Finn and Nellans (1972) found that 6% of the K was in a compartment with a half time of 2.5 min. In the absence of amiloride the following was found: in isolated frog skin, all K in one compartment with a half time of 35 min (Curran & Cereijido, 1965); in isolated epithelial cells, all K in one compartment, half time 49 min (Zylber, Rotunno, & Cereijido, 1975).

Discussion

From the data presented here it is seen that the filipin-induced activation of the SCC required the presence of calcium in the inside bathing solution (Fig. 3). Fig. 4 shows that the Ca ionophore A23187 had the same effect on the SCC. Furthermore, filipin had no effect if it was added after A23187. It is therefore concluded that the polyene antibiotic filipin acts as a calcium ionophore when it is added to the inside of the isolated frog skin. Van Zutphen, Demel, Norman and van Deenen (1971) have shown that filipin increases the Ca permeability of lipid bilayers.

Addition of the Ca ionophores to the isolated frog skin resulted in an activation of the active Na transport. The active Na transport across the amphibian skin is generally thought to occur in two steps: passive diffusion of Na across the outer border, followed by an active extrusion into the fluid bathing the inner border. For frog skin, Koefoed-Johnsen and Ussing (1958) suggested that the active mechanism is a sodium-potassium exchange pump. According to the two-membrane hypothesis (Koefoed-Johnsen & Ussing, 1958), frog skin can be treated as composed

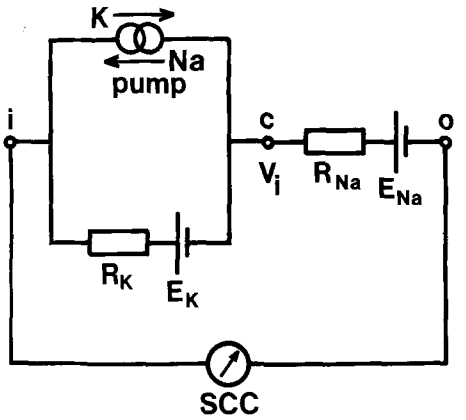

Fig. 8. Electrical analog of the two-membrane hypothesis

of an "outward-facing membrane", which is selectively permeable to sodium ions but impermeable to potassium ions, and permeable in a nonselective way to small anions like chloride. The "inward-facing membrane" is permeable to potassium and small anions, but impermeable to free sodium ions.

In Fig. 8 the two-membrane hypothesis is drawn as a simple electrical analog (all shunt resistances are excluded); according to this model the SCC is:

$$\mathrm{SCC} = (E_{\mathrm{Na}} + E_{\mathrm{K}})/(R_{\mathrm{Na}} + R_{\mathrm{K}}). \tag{3}$$

Where E_{Na} is the driving force for sodium across the outward facing membrane, R_{Na} is the resistance against the sodium movement. E_{K} is the driving force for potassium across the inward facing membrane, and R_{K} is the resistance against the potassium movement. According to this model, an increase in the SCC could be caused by: (i) a decrease in R_{Na} (increase in the Na permeability of the outward-facing membrane), (ii) a decrease in R_{K} (increase in the K permeability of the inward-facing membrane), (iii) an increase in the affinity of the Na/K exchange pump. An increase in the affinity of the Na/K pump would increase $(E_{\mathrm{Na}} + E_{\mathrm{K}})$. A polar cell with the same membrane properties as the membranes in the two-membrane hypothesis is used in the explanation of the results. It is assumed that the ionophores (filipin and A23187), by increasing the Ca concentration in the cell, increases the K permeability of the inward-facing membrane. The data in Fig. 7 indicates that A23187 increases the passive K flux across the cell membrane. In a short-circuited polar cell, assuming that chloride is passively distributed and in equilibrium, the

electrical potential inside the cell would be described by the following equation (Hodgkin, 1957):

$$PD_{cells} = -\frac{RT}{F} \ln \frac{[K_c] + \beta[Na_c]}{[K_0] + \beta[Na_0]} \tag{4}$$

$$\beta = P_{Na}/P_K \tag{5}$$

where P_{Na} and P_K are the permeabilities for sodium and potassium, K_c and Na_c the potassium and sodium concentration in the cell, and K_0 and Na_0 the potassium and sodium concentration in the Ringer's solution. If β in Eq. (4) is greater than zero, the Nernst diffusion potential for potassium (E_K) would be greater than PD_{Cell}. For isolated epithelial cells (from frog skin), Zylber *et al.* (1975) found $\beta = 0.34$. If $\beta = 0.34$ there would be a strong tendency for K to move out of the cell. An increase in the K permeability of the inward-facing membrane of the polar cell would make the cell more negative, the cell would hyperpolarize (PD_{cell} would move towards E_K). Since chloride was passively distributed across the cell membrane, chloride had to move out of the cell, and because of electroneutrality, cations had to follow; therefore, the cell would shrink, as observed in Fig. 6*b*. The hyperpolarization would continue until the cell had reached a new steady state. The hyperpolarization would result in an increased Na flow into the cell. Since the cell was polar, the increase in Na flow would occur across the outward-facing membrane; this might explain the transient increase in the SCC (Figs. 2 and 4). It has been shown by Gardos, Lassen and Pape (1976) that A23187 causes a large and sustained calcium, potassium dependent hyperpolarization of the *amphiuma* red cell membrane. The hypothesis given above would also explain the experiment shown in Fig. 2; when filipin was removed from the solution, the K permeability of the inward-facing membrane decreased and the cell increased its potassium concentration; when a new dose of filipin was added, the cell hyperpolarized again and a new transient increase in the SCC was observed.

It is generally assumed that ADH increases the SCC by increasing the Na permeability of the outward-facing membrane (*see* Andreoli & Schafer, 1976). The data in Table 2 shows that filipin did not activate the SCC after ADH; this might indicate that ADH also increased the K permeability of the inward-facing membrane. However, the ionophore might have effects on other parameters.

I wish to acknowledge the efficient help of Minna Rappold.

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