THE N-SHAPED CURRENT-POTENTIAL CHARACTERISTIC IN FROG SKIN

III. IONIC DEPENDENCE

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ABSTRACT A ramp voltage clamp measurement described previously is used to detect alterations in the frog skin current-potential (I-V) characteristic following removal or replacement of various ions in the solutions bathing the skin. The ionic requirements for the maintenance of a negative-slope I-V property are the following: Ca^{++}, Na^{+}, and Cl^{-} must be in the outside solution; K^{+} and Cl^{-} must be in the inside solution. Removal of any one of these ions from its respective solution results in the decay and eventual disappearance of the negative slope.

The similarity between the I-V characteristic following Ca^{++} removal with EDTA from the outside solution and the I-V relation in a refractory skin suggests that the loss (refractory state) and recovery of the negative slope is a consequence of unbinding and subsequent rebinding of Ca^{++} to membrane sites. The role of the univalent ions is not clear—presumably some or all of these ions constitute the current through the skin; however, some of these ions may also be involved in maintaining a membrane condition necessary for the existence of a negative slope I-V relation. Further, excitation does not appear to be a direct consequence of the Na^{+} pump.

INTRODUCTION

The development of a quasi-steady state negative resistance during voltage clamping of frog skin has been reported (Fishman and Macey, 1968 *b*). Further, a direct and rapid (5 msec) method of displaying the N-shaped current-potential (I-V) characteristic in real time with a ramp voltage-clamp system has been shown (Fishman and Macey, 1968 *c*). We now use this technique to describe the changes which occur in the I-V characteristics of frog skin following removal or replacement of the various ions in the solutions which bathe the skin. The implications of these results for the nature of the excitable process (spike generation under current clamp) are also discussed.

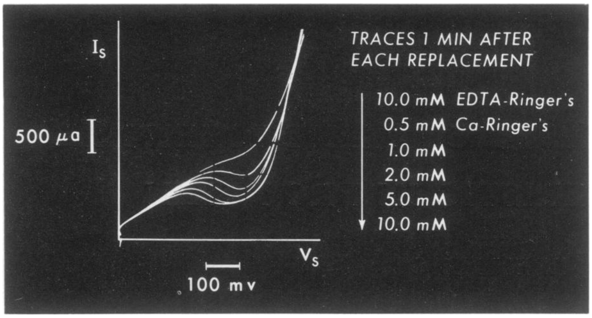

FIGURE 1 The effect of $[Ca^{++}]_0$ on the I-V characteristic of frog skin. The $CaCl_2$ in the outside Ringer's solution was replaced with 10 mM EDTA or its concentration varied as indicated.

METHODS

A 1 cm^2 disc of abdominal frog skin (*Rana pipiens*) was isolated and exposed to 5 ml of Ringer's solution on each side by placing it between two symmetrical chamber halves made of Lucite. The Ringer's solution was composed as follows: 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 8 mM Tris, 2 g/liter glucose, pH 8.0, Λ = 12.5 mmho/cm. The other experimental solutions used were made by modifying or adding to the Ringer's solution as indicated in the text and figure legends. All experiments were done at room temperature.

Fast solution changes (each chamber-half volume could be replaced within 2 sec) were accomplished by allowing an influx of new solution under pressure into a chamber half while simultaneously withdrawing solution by opening the chamber-half connection to a vacuum line.

The ramp voltage-clamp system described previously (Fishman and Macey, 1968 *b, c*) was used to produce I-V plots on the cathode-ray tube of a Tektronix 564 storage oscilloscope (Tektronix, Inc., Beaverton, Ore.). However, in these experiments a saw-tooth ramp pulse of 100 v/sec slope (500 mv peak and 5 msec duration) was used exclusively as the command to the voltage-clamp system.

RESULTS

Variation and Removal of Outside Ca^{++}

The effect of outside calcium concentration, $[Ca^{++}]_0$, on the I-V relation is presented in Fig. 1. Each curve was obtained after the skin had recovered from prior stimuli and 1 min after the change in $[Ca^{++}]_0$ had been made. There is no negative slope with 10 mM EDTA (ethylenediaminetetraacetate) outside. A negative slope occurs with 0.5 mM Ca^{++} in the outside Ringer's solution and increases with increasing $[Ca^{++}]$ until the calcium concentration reaches 10 mM where it begins to fall. There is an optimum $[Ca^{++}]_0$ which gives a maximum negative slope in each skin, but it varies from skin to skin. The minimum $[Ca^{++}]_0$ required to produce a negative slope depends upon the outside solution ionic strength (as did spike generation [Fishman and Macey, 1968 *a*]) and is also skin dependent.

The coordinates of the valley of each I-V curve in Fig. 1 shift toward lower

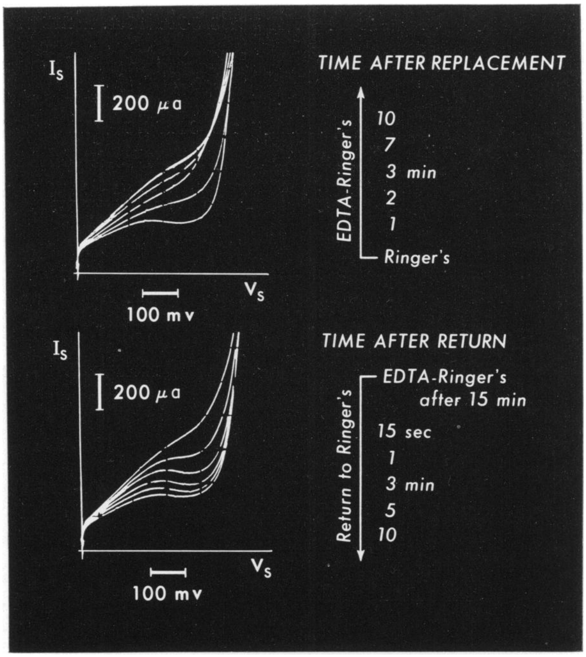

FIGURE 2 (top) Kinetic behavior of the I-V characteristic following replacement of outside Ringer's solution with a Ca^{++} free Ringer's solution containing 10 mM EDTA. Ringer's solution maintained inside. (bottom) After 15 min with EDTA-Ringer's solution outside, Ringer's solution (with Ca^{++}) is returned to the outside; the original N-shaped I-V curve also returns.

values with increasing $[Ca^{++}]_0$. A similar result was obtained on the squid axon membrane by Ehrenstein and Gilbert (1966) and on frog node of Ranvier by Müller-Mohnssen and Balk (1965). Also notice that the low resistance (reciprocal slope) state at high skin potentials is relatively invariant with $[Ca^{++}]_0$ or other ionic concentrations as will be seen in subsequent data.

The kinetic alteration in the I-V curve after the outside Ringer's solution was replaced with EDTA-Ringer's solution ($\Lambda = 13.6$ mmho/cm) is seen in Fig. 2 (top). Following replacement (in 20 skins), the negative slope I-V relation collapses rapidly (usually within 5 sec) and approaches a linear positive resistance. The effect of calcium removal from the outside skin surface on the I-V characteristic is thus similar to moving the skin potential into the low resistance state as described previously (Fishman and Macey, 1968 *c*). When the EDTA-Ringer's solution was replaced with Ringer's solution (with calcium), the negative slope returned quickly (15 sec) and eventually reverted to the original I-V curve prior to EDTA treatment; the Ca^{++} effect is fully reversible.

The I-V data and spike generation data (Fishman and Macey, 1968 *a*), for removal of outside calcium with EDTA, correlate very well. Both the negative slope and spike generation disappear rapidly (2–5 sec) and reversibly upon replacement of outside Ringer's solution with EDTA-Ringer's solution. Further correspondence between ramp clamp and current clamp data is illustrated in Fig. 3. This skin was originally inexcitable (Fig. 3 *A*) and showed no negative slope (Fig. 3 *C*) in the I-V plane. Replacement of the outside Ringer's solution with low ionic strength sucrose solution led to the appearance of both the current clamp spike (Fig. 3 *B*) and the negative slope (Fig. 3 *C*). The excellent correlation between these two

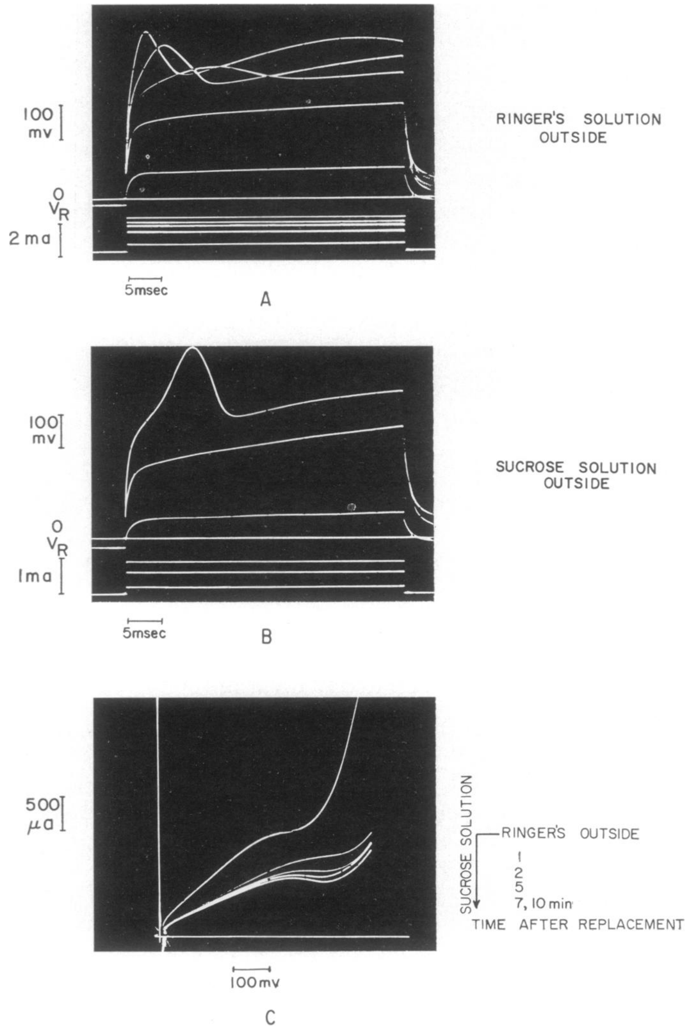

FIGURE 3 Comparison of current-clamp and voltage-clamp data for Ringer's solution outside followed by sucrose solution (10 mM NaCl, 200 mM sucrose, 10 mM Tris, pH 8.0, Λ = 1.6 mmho/cm) outside. (*A*) Ringer's solution both sides; (*B*) Sucrose solution outside; (*C*) The I-V characteristic with Ringer's solution on both sides followed by kinetic behavior of this characteristic after replacing outside Ringer's with sucrose solution.

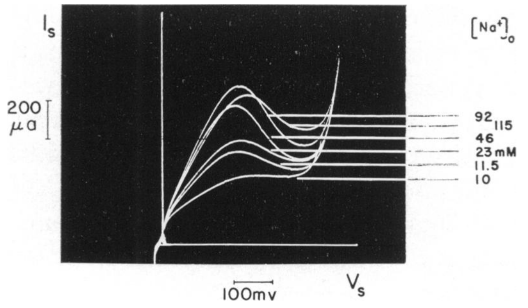

FIGURE 4 The effect of $[Na^+]_0$ on the I-V characteristic. Ringer's and K^+ Ringer's (KCl substituted for NaCl) were combined to give the various $[Na^+]_0$. Ringer's solution maintained on the inside. Traces 1 min after each replacement.

different types of data demonstrates that the ramp clamp presentation of the I-V relation is very useful in characterizing excitability in frog skin.

Observation of the effectiveness of other divalent ions (5 experiments with each ion) in Group II*a* of the periodic table to substitute for Ca^{++} showed that Sr^{++} was almost an exact substitute for Ca^{++}. Mg^{++} and Ba^{++} gave negative slope I-V curves but of reduced slope when compared to the same concentration of Ca^{++}. The major effect of Mg^{++} and Ba^{++} was to increase the time for the skin to recover the negative slope after entering the low resistance region of the I-V curve; they also prolong the spike refractory period as expected from the I-V data. The order of effectiveness of these ions in producing a negative slope and spikes in frog skin is $Ca^{++} > Sr^{++} > Mg^{++} > Ba^{++}$.

Variation and Replacement of Outside Na^+

In studying the effect of sodium concentration, $[Na^+]_0$, on the I-V characteristic, both choline$^+$ and K^+ were used as impermeable substitute ions for Na^+ with the same results. Fig. 4 shows there is no negative slope and a high subthreshold resistance with 10 mM $[Na^+]_0$. This agrees with Finkelstein's report (1964) that the spike changed to an overshoot response and the subthreshold resistance increased with Na^+ removal. However, these effects required about 3 min in contrast with Finkelstein's results which occurred within 10 sec. With increasing $[Na^+]_0$ the negative slope increases and the subthreshold resistance decreases until a $[Na^+]_0$ of 92 mM. A further increase in $[Na^+]_0$ causes a negative slope decrease and a subthreshold resistance increase (four out of seven skins). The I-V changes for $[Na^+]_0$ beyond 80 mM were not always as large as in Fig. 4. The optimum $[Na^+]_0$ for a maximum negative slope ranged from 70–90 mM.

Replacement of Outside Cl^-

The $SO_4^=$ and acetate$^-$ ions were used to replace all Cl^- in the Ringer's solution (10 skins). The typical kinetic behavior of the I-V characteristic following the replacement of outside Ringer's solution with $SO_4^=$-Ringer's solution is shown in Fig. 5. There is a large increase in subthreshold skin resistance and a loss of the negative slope 3 min after the replacement. Return of Ringer's solution to the outside restores the I-V curve to its original state in about 3 min (in half the skins a slight negative slope persists for 15–20 min after the replacement of Cl^-). Replacement of Cl^- by acetate$^-$ gave the same results. The effect of total Cl^- replacement on the I-V relation is thus similar to the effect produced by replacing Na^+ with K^+ or choline$^+$.

Since this result was unexpected, current clamp and step voltage-clamp measurements were also undertaken. A comparison of the three types of measurements on the same skin (before and after Cl^- replacement by $SO_4^=$) show the same information (Fig. 6). The spike changes into an overshoot response and the subthreshold resistance increases; the current response crossings in the step voltage-clamp data become uncrossed and the current values indicate a higher skin resistance; the ramp-clamp data also shows the loss of the negative slope and the increase in skin resistance.

Finkelstein (1964) reported that replacement of Cl^- by $SO_4^=$ did not have any effect upon spike generation. However, he did not completely remove the Cl^-

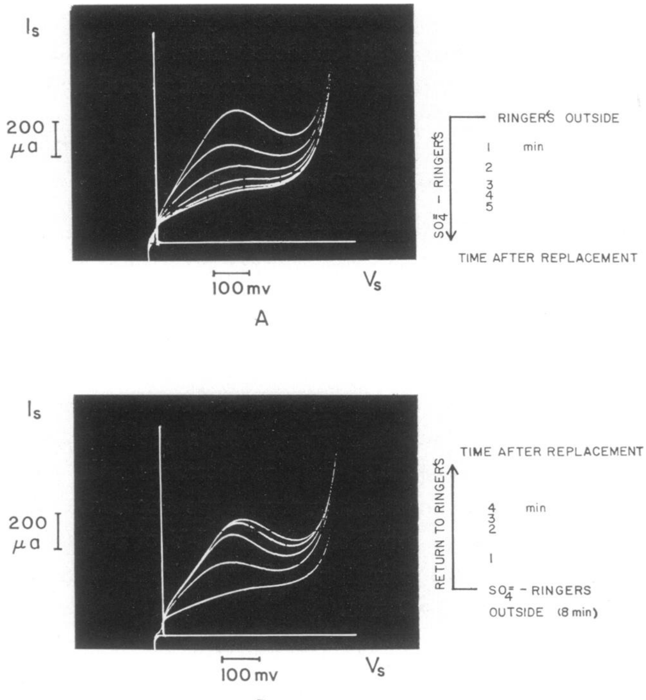

FIGURE 5 (*A*) The kinetic behavior of the I-V characteristic following replacement of outside Ringer's solution with $SO_4^=$ Ringer's solution (57.5 mM Na_2SO_4, 2.5 mM K_2SO_4, 2 mM $CaSO_4$, 8 mM Tris, 27g/liter sucrose, Λ = 9.7 mmho/cm). Ringer's solution maintained inside. (*B*) Return of Ringer's solution into the outside after 8 min in $SO_4^=$ Ringer's solution.

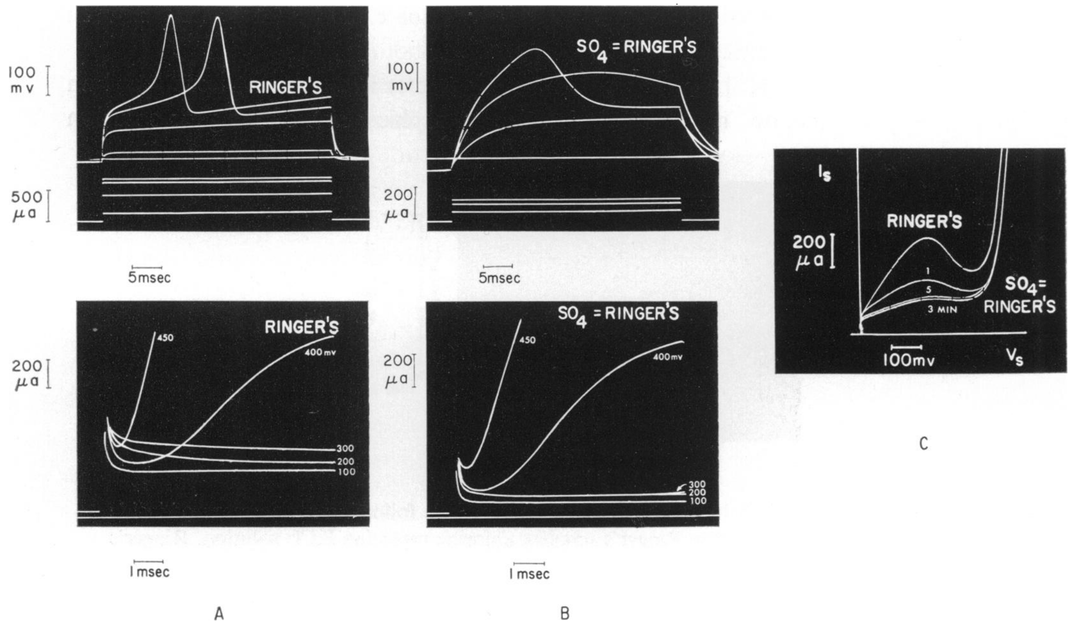

FIGURE 6 Comparison of current-clamp, step voltage-clamp, and ramp voltage-clamp data with Ringer's solution outside and after replacement with $SO_4^=$ Ringer's solution (no Cl^-). Ringer's solution maintained on the inside.

(2.5 mM $CaCl_2$ was in his $SO_4^=$-solution) and he did not state how long after the replacement spikes could be elicited. A loss of excitability occurred in these experiments when Cl^- was totally replaced by $SO_4^=$. It appears, then, that both external Na^+ and Cl^- are necessary for excitability; the replacement of either one results in the disappearance of the negative slope I-V relation.

Inside Ion Replacement

No changes were observed in the negative slope portion of the I-V curve for variations in $[Na^+]_i$. The significant alterations in excitability occurred for elimination of $[K^+]_i$ or $[Cl^-]_i$. Results with Ringer's solution on the outside and the inside K^+ replaced by choline$^+$ (four skins) are shown in Fig. 7. The effect is similar to that produced by replacement of Na^+ or Cl^- in the outside solution with impermeable substitutes. However, substantially longer time (1 hr) is required to see the K^+ effect. The short-circuit current also decayed to zero in this period.

The replacement of inside Cl^- by $SO_4^=$ (two skins) was found to have the same effect on the I-V characteristic as replacement on the outside. The only significant difference was in the decay time of the negative slope after the replacement. Replacement of $[Cl^-]_i$ by $SO_4^=$ required 20 min whereas the same effect after a similar outside replacement required only 3 min. These results probably reflect the fact that the excitable membrane is located on the outside skin surface (Lindemann and Thorns, 1967; Fishman and Macey, 1968 *a*).

An increase in the $[K^+]_i$ well above normal (60 mM) in three skins produced an interesting phenomenon. In the first minute after replacement of Ringer's solution

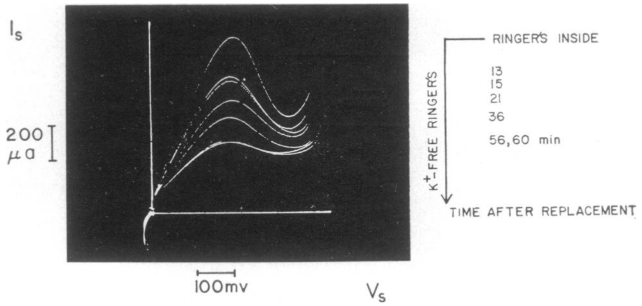

FIGURE 7 The kinetic behavior of the I-V characteristic following replacement of inside Ringer's solution with K^+ free Ringer's (choline chloride replacing KCl) solution. Ringer's solution maintained on the outside.

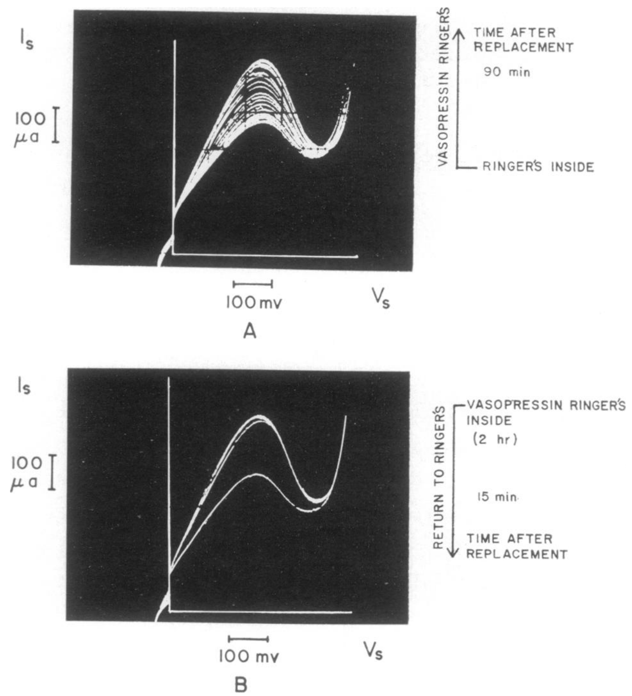

FIGURE 8 (*A*) The kinetic behavior of the I-V characteristic following replacement of inside Ringer's solution with vasopressin-Ringer's (20 pressor units pitressin/liter). Ringer's solution maintained on the outside. (*B*) After several wash outs of the inside vasopressin-Ringer's solution.

by high K+-Ringer's solution (60 mM NaCl, 60 mM KCl, 2 mM $CaCl_2$, 8 mM Tris pH 8.0), the skin subthreshold resistance decreased and the negative slope disappeared. Within 3 min the skin subthreshold resistance increased to approximately its original value and after 5 min, the negative slope reappeared and began to increase, eventually (25 min) reaching a larger slope than the skin originally had in Ringer's solution. During the negative slope rise the subthreshold resistance decreased while both the current and threshold voltage points (coordinates of the peak in the N-shaped curve) increased.

Drugs and Transport Inhibitors

Replacement of inside Ringer's solution by vasopressin-Ringer's (20 pressor units of pitressin/liter of Ringer's solution) (Fig. 8 *A*) in four skins produced a very large and continuous increase in the negative slope for about 1.5 hrs. There was also a decrease in the subthreshold resistance and an increase in the short-circuit current

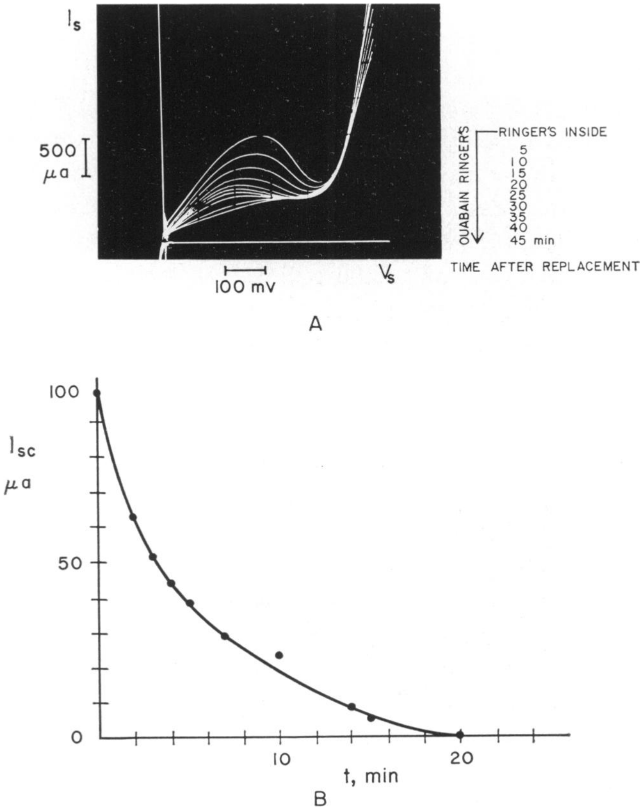

FIGURE 9 (*A*) Replacement of inside Ringer's solution with ouabain-Ringer's (1 mM ouabain/liter) solution. Ringer's solution maintained outside. (*B*) The decay of short-circuit current, I_{sc}, after the replacement.

(I_{sc}). Several wash outs of the inside skin surface with Ringer's solution returned the I-V relation to its original state in 15 min (Fig. 8 *B*).

Inhibitors

The introduction of KCN (1 mM/liter of Ringer's solution) to the inside solution of four skins caused a disappearance of the negative slope as the short-circuit current approached zero. This is in agreement with Finkelstein's report (1964), however, in one skin the negative slope disappeared before I_{sc} reached zero while in the other experiments it disappeared after I_{sc} reached zero.

Introduction of ouabain (1 mM/liter of Ringer's solution) to the inside solution depressed I_{sc} to zero within 25 min (Fig. 9 *B*), whereas the negative slope (Fig. 9 *A*) remained for 35 min in five out of six skins. The action of physostygmine (eserine) (10 mM in the inside Ringer's solution) in two skins was similar to ouabain, i.e., the negative slope slowly decayed and disappeared as the subthreshold resistance increased. However there was no change in I_{sc}.

DISCUSSION

The ionic requirements for the maintenance of a negative-slope I-V characteristic in frog skin are the following: Ca^{++}, Na^{+}, Cl^{-} must be in the outside solution; K^{+} and Cl^{-} must be in the inside solution. Removal of any one of these ions from its respective solution results in the decay and eventual disappearance of the negative slope. Furthermore, the different effects upon the I-V relation produced by the removal of Ca^{++} in comparison to the removal or replacement of the univalent ions suggest different roles for these ions.

Role of Ca^{++} in Producing the Frog Skin I-V Curve

The importance of Ca^{++} in excitation of frog skin by current clamp has been emphasized in earlier publications (Finkelstein, 1964; Fishman and Macey, 1968 *a*). In general Ca^{++} removal affects the I-V relation by lowering the subthreshold resistance and causing the disappearance of the negative slope. Further a low resistance state appears at substantially lower membrane potentials (Fig. 2) than in the presence of Ca^{++}. This low resistance state resembles a breakdown phenomenon (Fishman and Macey, 1968 *c*) because it occurs at a critical potential and is relatively insensitive to the ionic composition of the bathing solutions.

The above description of the I-V relation during Ca^{++} removal also applies to I-V plots obtained during the absolutely refractory phase in a skin bathed in Ringer's (containing Ca^{++}) solution. In a normal Ringer's environment the membrane persists in this condition only briefly (during the refractory period), but apparently when Ca^{++} is removed (unbound from the membrane?) by EDTA, the membrane passes into this state and remains there indefinitely. Reintroduction of Ca^{++} shows that this effect is fully reversible. These facts suggest that the normal process of excitation and recovery in response to the passage and subsequent removal of current involves the unbinding and rebinding of Ca^{++} to membrane sites.

Univalent Ions

According to the Koefoed-Johnsen Ussing hypothesis or its later modifications (Farquhar and Palade, 1966), the outer cell membrane is permeable to Na^+ and Cl^- while the inner cell membrane is permeable to K^+ and Cl^-. These permeabilities correspond to the specific requirements of each surface for negative slope characteristics and spike production, and it would be reasonable to assume that some or all of these ions are the principal current carriers during excitation.

The evidence suggests considerable interaction between the individual Na^+, K^+, and Cl^- currents. This follows because removal of any one of these ions is sufficient to completely abolish the negative slope and excitation. Removal of a specific ion would be expected to alter the electrochemical potential profiles of other ions through single membranes and possibly through the entire skin. The existence of these interactions would preclude the possibility of decomposing the total current into individual Na^+, K^+, and Cl^- currents by simple replacement experiments.

Introduction of the high K^+ Ringer's solution on the inside probably results in a large initial water flow across the K^+ permeable inner skin border, and the initial disappearance of the negative slope may be related to this flow. The short-circuit current did not change significantly with high K^+ Ringer's solution inside; the pump is probably not affected. The high $[K^+]_i$, however, could have reduced the leak of K^+ across the inner border and produced a build up of intracellular $[K^+]$. Thus the enhanced negative slope may have been a consequence of an increase in intracellular $[K^+]$.

A similar response (enhanced negative slope) was produced by application of vasopressin. This could simply reflect the interruption of a larger Na^+ and Cl^- current across the inner surface membranes. Alternatively, since vasopressin causes an increase in short-circuit current we would expect a greater exchange of K^+ for Na^+ which could raise intracellular $[K^+]$. Again we assume conditions which may produce an increased intracellular $[K^+]$ with an enhanced negative slope. An important role for intracellular $[K^+]$ is also suggested by the large time required for changes in K^+ concentrations in the bathing medium to exert their effects.

Finally, the relationship between active ion transport and excitation must be considered. Although there is a parallel decay of the pump and the negative-slope characteristic following the introduction of inhibitors, a study of the time course of these decays shows that in some skins the pump stops before the disappearance of a negative slope whereas in other skins the reverse is the case. Further, Cl^- is required for a negative slope whereas it is not required for active ion transport. Thus we conclude, contrary to Finkelstein (1964), that there is no direct relationship between pump and excitability in frog skin. There may, however, be an indirect relationship such as in nerve where the pump maintains long term ionic concentration gradients (e.g. high intracellular K^+) across the membrane.

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REFERENCES

EHRENSTEIN, G., and D. L. GILBERT. 1966. *Biophys. J.* **6**:553.
FARQUHAR, M. G., and G. E. PALADE. 1966. *J. Cell Biol.* **30**:359.
FINKELSTEIN, A. 1964. *J. Gen. Physiol.* **47**:545.
FISHMAN, H. M., and R. I. MACEY. 1968 *a*. *Biochim. Biophys. Acta.* **150**:482.
FISHMAN, H. M., and R. I. MACEY. 1968 *b*. *Biophys. J.* **9**:127.
FISHMAN, H. M., and R. I. MACEY. 1968 *c*. *Biophys. J.* **9**:140.
LINDEMANN, B., and U. THORNS. 1967. *Science.* **158**:1473.
MÜLLER-MOHNSSEN, H., and O. BALK. 1965. *Nature.* **207**:1255.