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DOES INTRACELLULAR SODIUM REGULATE SODIUM TRANSPORT ACROSS THE MUCOSAL SURFACE OF FROG SKIN?

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Summary

A method has been devised to functionally remove the serosal membrane of frog skin. Skins treated in this way have no spontaneous potential. However, if sodium gradients are placed across the tissues diffusion potentials and hence short-circuit currents of either sign, depending on the direction of the gradient, could be recorded. These short-circuit currents were completely inhibited by amiloride only from the mucosal face. However, the concentration of amiloride causing 50% inhibition of the short-circuit current (K_m) in treated skins was $2.3 \cdot 10^{-3}$ M, when a sodium gradient was applied from serosa to mucosa, whereas both in untreated skins without a sodium gradient and in treated skins with a mucosal to serosal sodium gradient, the K_m of amiloride was $2 \cdot 10^{-7}$—$4 \cdot 10^{-7}$ M. The mechanism by which amiloride is able to inhibit the short-circuit currents of either sign is discussed.

Amiloride, a K^+-sparing diuretic, has proved to be a suitable probe for investigating the Na^+-translocating mechanisms in tight Na^+-transporting epithelia [1—5]. Recently, it has been reported that raising intracellular Na^+ concentration in frog skin reduces the availability of the sites which can be labelled with [^{14}C]amiloride [6]. Furthermore, frog skin shows adaptive changes in transporting capacity [7—9]. It would be informative to have a preparation in which the Na^+ concentration on either side of the mucosal membrane could be controlled precisely. The experiments reported here give an account of attempts to remove functionally the serosal membrane while leaving the mucosal membrane intact. Subsequently by adjusting

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the Na^+ gradients across the treated skins it is possible to examine the effects of amiloride on the diffusive short-circuit current.

Abdominal skins of the leopard frog (*Rana pipiens*) were used throughout; all the experiments were performed between July and September. Tissues were mounted vertically in an Ussing-type chamber and bathed on both sides with normal Ringer's solution of the following composition 111 mM NaCl/5 mM Tris-HCl buffer (pH 7.6)/1 mM KCl/1 mM $CaCl_2$/11.1 mM glucose. Na^+-free and 1.1 mM Na^+-Ringer's solution were also used, and these solutions were made simply by omitting the required amount of NaCl from the above without any correction for the change in tonicity. All the solutions were bubbled with air and had a pH of 7.6. The skins were voltage clamped at zero potential by an automatic voltage clamp (Schema Versatae S/V 360c). In all instances the skin area was 8.55 cm^2. The short-circuit current was monitored throughout the experiment and the tissues were allowed to equilibrate with the bathing solutions for at least 1 h until the current was steady. Cumulative dose-response curves to amiloride were recorded under various conditions (Fig. 1A) and the results expressed as percentage inhibition of short-circuit current (Fig. 1B). Each curve required about 10 min to complete. The purpose of these experiments was to develop a preparation in which the serosal membranes were removed functionally while the mucosal surface of the transporting epithelium remained intact. If this could be achieved, then it might be possible to examine the diffusive short-circuit current across the mucosal face in the presence of Na^+ gradients of either direction.

Using six different skins bathed on both sides with normal Ringer's solution, the mean short-circuit current was 14.9 ± 2.5 $\mu A/cm^2$ and the concentration of amiloride applied in the mucosal solution which was required to reduce the current by 50% (K_m) was $2.1(\pm 0.1) \cdot 10^{-7}$ M. Amiloride was effective only when added to the mucosal face in keeping with the proposed mode of action of this drug [10].

The following technique was devised in an attempt to functionally remove the serosal membrane. Phospholipase (50 μg/m) was added to the serosal bathing solution and incubated with the skin for 1 h. Following this, the enzyme was washed away and the serosal fluid replaced with Na^+-free Ringer's solution for an additional hour. The combined enzyme treatment and hypotonic shock produced skins with no transepithelial potential when bathed on both sides with normal Ringer's solution.

However, when such skins were subject to Na^+ gradients, potentials appeared across the tissues and short-circuit currents could be recorded. Experiments were performed in which the solution bathing one side of the skin was normal Ringer's solution contained 111 mM Na^+ while the other solution contained no Na^+. The gradient was applied at different times in both directions and sufficient time allowed for the current to equilibrate before the blocking drug was added.

When the mucosal solution contained 111 mM Na^+ while the serosal bathing solution was Na^+-free, there was a diffusive short-circuit current which was sensitive to amiloride. The K_m for amiloride under these conditions was $4.3 \cdot 10^{-7}$ M (mean of two observations) which is not very different

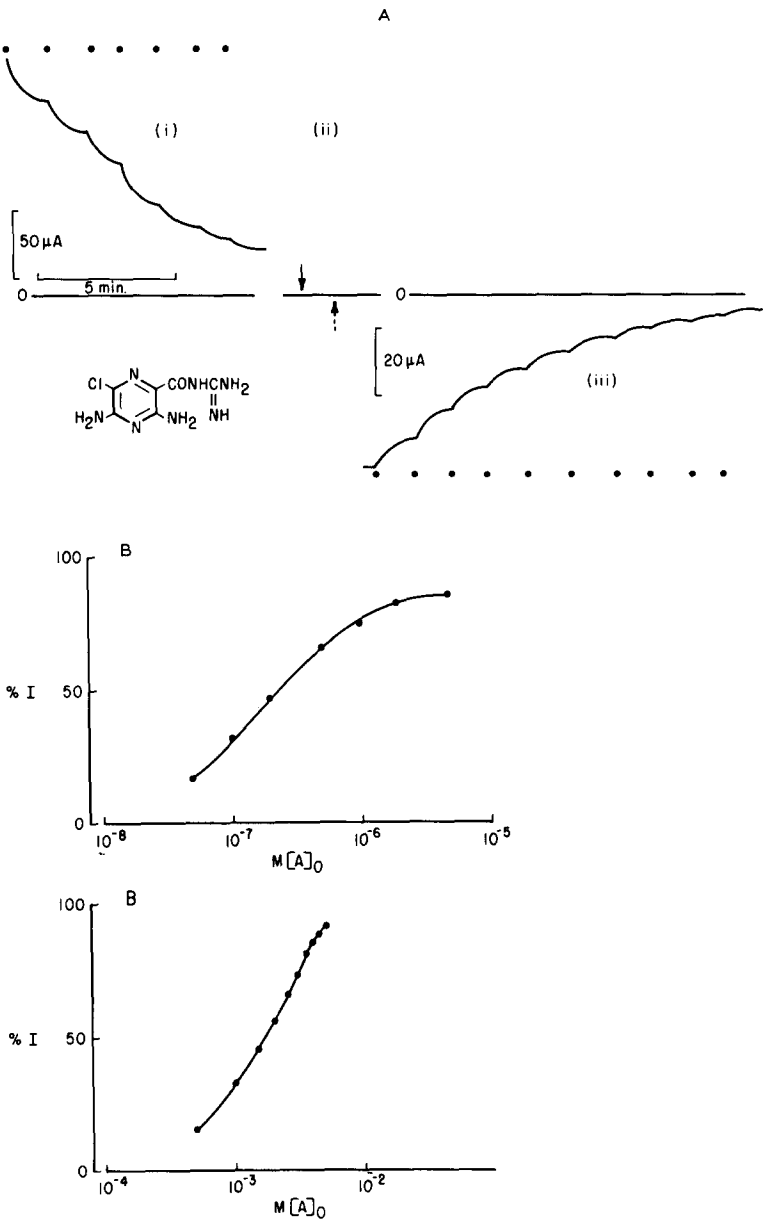

Fig. 1. A. (i) Effect on short-circuit current of cumulative doses of amiloride under usual conditions, i.e., when both surfaces were bathed with normal Ringer's solution (pH 7.6). The cumulative concentrations of amiloride from left to right were 50, 99, 197, 483, 957, 1896 and 4636 nM. The horizontal line represents the zero current level. The chemical structure of amiloride is shown below the horizontal line. (ii) After the drug was washed away and the current recovered to its original level, a sodium gradient from the serosal (111 mM Na^+) to mucosal (no Na^+) side was applied. Under these conditions the short-circuit current was close to zero and addition of 10^{-3} M amiloride either to the mucosal (↓) or serosal bathing solution (↑) produced no effect on the short-circuit current. (iii) Effect of cumulative doses of amiloride on short-circuit current in the same skin after the combined enzyme and hypotonic shock treatment and with a sodium gradient from the serosal (111 nM Na^+) to mucosal (no Na^+) side. The cumulative concentrations of amiloride from left to right were 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mM. Note that the current obtained in (iii) was in the opposite direction to that obtained in (i). B. Logarithmic concentration-response curves of percentage inhibition (%I) vs. mucosal concentration of amiloride ($M[A]_0$) using the data in Fig. 1A (i) and (iii).

from that ($4.4 \cdot 10^{-7}$ M, mean of two observations) obtained under control conditions from the same preparations but before enzyme treatment. When the Na^+ gradient was applied in the reverse direction, i.e., serosal to mucosal, in treated skins a negative short-circuit current was obtained. This current had a mean value of 5.8 ± 0.7 $\mu A/cm^2$ (6 experiments). This is smaller than the current obtained in treated skins when the gradient is in the mucosal to serosal direction (17.7 $\mu A/cm^2$, mean of two observations) or in untreated skins with no gradient (14.9 $\mu A/cm^2$, mean of six observations). The negative current was not inhibited by amiloride applied to the serosal face even at a concentration as high as 10^{-3} M. Furthermore, amiloride applied to the mucosal side under these conditions caused 100% inhibition of this negative short-circuit current, suggesting that the treatment to remove the serosal membrane does not open a non-specific leak pathway for sodium movement. Although the negative current was still sensitive to amiloride when the drug was added to the mucosal face, there was a significant decrease in sensitivity, the K_m being $2.3(\pm 0.2) \cdot 10^{-3}$ M (6 experiments).

Fig. 2. shows three concentration-response curves for amiloride in a single skin. The curves for the normal condition, i.e., no Na^+ gradient and for a mucosal to serosal gradient can be superimposed, whereas when the gradient is in the serosal to mucosal direction the curve is moved to the right.

These experiments have shown that treatment of frog skin on the serosal side with phospholipase C and hypotonic shock produces a preparation with no spontaneous short-circuit current but one which shows amiloride-sensitive currents in the presence of Na^+ gradients. We have assumed these diffusive currents are caused by Na^+ but have not confirmed this by flux measurements. The identity of the K_m for amiloride and inhibition characteristics when the Na^+ gradient is in the mucosal to serosal direction to that which obtains under normal conditions suggests the integrity of the mucosal membrane is intact. When the gradient is from serosal to mucosal,

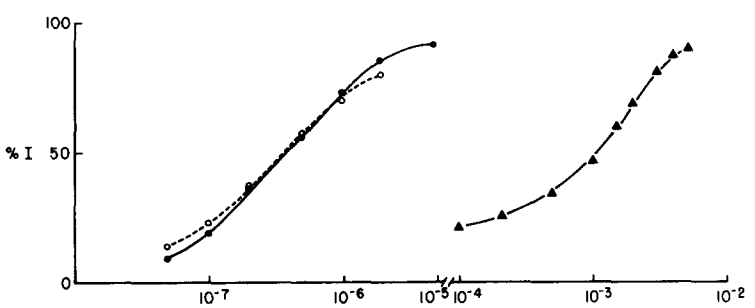

Fig. 2. Inhibition of short-circuit current by amiloride in a single skin. Closed circles represent values obtained under usual conditions, i.e., both surfaces were bathed with normal Ringer's solution. Under these conditions the basal current was +15.9 $\mu A/cm^2$ and the K_m for amiloride obtained from the curve was $3.8 \circ 10^{-7}$ M. Open circles represent values obtained after the combined enzyme and hypotonic shock treatment with a Na^+ gradient from the mucosal to serosal side; the basal current and the K_m for amiloride were +15.7 $\mu A/cm^2$ and $3.5 \cdot 10^{-7}$ M, respectively. Closed triangles represent values obtained from the treated skin but with a Na^+ gradient from the serosal to mucosal side; the basal current and the K_m for amiloride in these instances were −3.04 $\mu A/cm^2$ and $1.1 \cdot 10^{-3}$ M, respectively.

the failure of amiloride to affect the current when added to the serosal bathing solution implies that the recognition site for the drug cannot transfer to the internal face of the mucosal membrane. However, the change in K_m under these experimental conditions suggests that application of high Na^+ concentrations to the inside of the mucosal face alters the binding characteristics of the amiloride binding site. This is consistent with the finding that the amount of [^{14}C] amiloride binding which can be detected at the mucosal face is reduced by manipulations which raise the Na^+ concentration inside the cell [11] and which also correlates with a change in mucosal permeability. It thus appears that the properties of the amiloride binding sites are dependent upon the direction of the Na^+ gradient across the mucosal membrane. From biophysical studies, Helman and Fisher [12] found it necessary to include in the equivalent circuit of the mucosal membrane two diodes conducting in opposite directions. The relative resistive values of these components depended on conditions. It is possible that they represent a biophysical correlate of the modification in amiloride sensitivity seen in this study, which is improved by altering sodium gradients. Since this work was completed Dawson and Al-Awqati [13] have reported for toad urinary bladder, that small negative or 'reversed' currents could be obtained with a serosal to mucosal sodium gradient without modification of the serosal membrane. The negative current was sensitive to amiloride but quantitative relationships were not investigated.

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