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EFFECT OF OUABAIN ON ELECTRICAL CONDUCTANCE OF FROG SKIN

EVIDENCE AGAINST RECYCLING OF SODIUM

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Summary

Ouabain (10^{-4} M) added to the serosal solution of isolated frog skin not only stops the active transport of Na^+ but causes a dramatic increase in the electric conductance of the skin. The effect also appears when ouabain is added to amiloride-pretreated skins, ruling out the possibility of a cellular effect. The similarity between the effect of ouabain on normal and amiloride-pretreated skins indicates that no appreciable recycling of Na^+ across the basolateral membrane of frog skin takes place. The change of Na^+ efflux after ouabain is added to amiloride-pretreated skins parallels the change of electric conductance, indicating that besides blocking the Na^+ pump, ouabain affects the paracellular shunt pathway between the cells.

Introduction

Amphibian epithelia have been widely used in transport studies because of their functional simplicity. Na^+ is the only ion actively transported by most of these epithelia, and the correlation between sodium transport and metabolism enables direct study of the energetics of transport and of the effect of different drugs and hormones on the metabolic cost of transport.

An experimental difficulty in this type of study is the lack of an established method of differentiating between energy used for ion transport and energy used for other metabolic activities unrelated to transport. Once such a method is found, the energetics of transport under different conditions can be adequately assessed. A widely used approach to overcome this difficulty has been to block active Na^+ transport and to consider the remaining energy consumption as unrelated to transport. Active transport of Na^+, measured as short

circuit current, has been generally blocked by adding amiloride to the solution bathing the mucosal side of the epithelium. This method is only applicable, however, if no Na^+ ions can diffuse across the basolateral membrane of the cells from the serosal solution into the cells to be pumped back to the serosal solution by the (Na^+-K^+)-ATPase pump. These recycled Na^+ do not contribute to the net transepithelial Na^+ flux, but since energy is still used to pump them out, the 'non-transport related' energy consumption measured in the presence of such recycling is too high.

The model proposed by Koefoed-Johnsen and Ussing for Na^+ transport across tight epithelia [1] assumed that the basolateral membrane was totally impermeable to Na^+, no recycling taking place. Although the model remained acceptable in several respects, some corrections have been made in its formulation and the possibility of an important recycling of Na^+ has been investigated in several tissues with controversial results [2—7].

In toad bladder, a characteristically tight epithelium, it has been reported [2—4] that the contribution of the recycling of Na^+ (if such recycling exists) to the measurement of CO_2 production is within the experimental error of the measurement. Therefore, the CO_2 production measured after the short circuit current has been blocked by amiloride can be considered as unrelated to transport. The same thing has been claimed for the toad skin where recycling was estimated to be of the order of 5% of the active transport [5]. Measurement of permeability (P) of Na^+ and K^+ across the basolateral membrane of rabbit gallbladder gave a value of $P_{Na^+}/P_{K^+} = 0.044$ [6] also allowing one to consider the recycled Na^+ as insignificant.

A different situation was reported for the frog skin. Biber and Mullen [7] measured tracer effluxes across skins from *Rana pipiens* before and after treatment with ouabain and reported a very large increase of Na^+ efflux after the ouabain treatment. The fact that this increase was not paralleled by similar changes in the efflux of substances that are known to cross the skin by paracellular pathways such as sucrose, mannitol, and polyethylene glycol, was interpreted as ruling out any possibility of ouabain affecting paracellular pathways. Therefore the increase in Na^+ efflux was taken to be a measurement of recycled Na^+, since after blocking the pump with ouabain Na^+ supposedly cannot be pumped back anymore and leaks to the mucosal solution. If this explanation proves to be correct, the attempts to measure basal (non-transport related) energy consumption by using amiloride or Na^+-free mucosal solution in frog skin grossly overestimate it and underestimate the energy consumed by the pump. In this paper we try to clarify this question of recycling of Na^+ in frog skin.

Materials and Methods

Abdominal skins of frogs (*Rana pipiens*) were used. The frogs were double pithed, the skins carefully removed, divided in two symmetrical halves, washed and mounted without stretching between the two halves of modified Ussing chambers with an exposed area of 4.91 cm^2 and a volume of 20 ml. Calomel electrodes and silver-silver chloride electrodes were used for measuring potential difference and current respectively. The skins were short-circuited

during the entire experiment with a voltage clamp and the short circuit current was continuously recorded.

The total electrical conductance of the skins, k^t, was measured by clamping the skins to ±20 mV for 10 s every 5 min and recording the changes in current produced by the change in potential. k^t was calculated as $k^t = \Delta I/\Delta\psi$.

The protocol used in the first series of experiments was as follows: the two hemiskins were bathed in normal Ringer's solution, short-circuited, and allowed to stabilize for at least one hour. Following this $5 \cdot 10^{-5}$ M amiloride was added to the mucosal solution in order to block the transport of Na^+ and to measure the passive electrical conductance of the skins. Only those skins where amiloride decreased the short circuit current to less than 10% of the original value and where the passive conductance was less than 60% of the total conductance were considered, in order to minimize the possibility of edge damage. The two hemiskins were then washed with normal Ringer's solution and allowed to stabilize again. After that 10^{-4} M ouabain was added to the serosal solution of the experimental hemiskin and the values of short circuit current and total conductance monitored for up to 5 h.

The protocol for the second series of experiments was very similar to the previous one, the only difference being that amiloride was not washed out but ouabain was added to amiloride-pretreated hemiskins.

In several of the experiments of the second series, Na^+ efflux was measured simultaneously with the short circuit current and the total conductance. In these experiments, after the skins reached a steady state, 20 μCi of ^{22}Na were added to the serosal solution and samples were taken each half hour from both mucosal and serosal solution for counting. Four or five samples were taken with normal solution bathing the skins, amiloride was then added and three more samples taken, and finally ouabain was added and samples collected for at least 3 h.

The normal Ringer's solution used in all the experiments contained (mmol/l): NaCl, 110; $KHCO_3$, 2.5; $CaCl_2$, 1; glucose, 10.

Results

In 17 experiments, after the skins reached a steady state, a supramaximal dose of ouabain (10^{-4} M) was added to the serosal solution of one hemiskin, the other hemiskin being left untreated to serve as control, and the values of short circuit current and conductance were recorded for up to 5 h. It was found that while the short circuit current reached near zero values within one hour of ouabain application, the conductance did not fall in parallel but followed a time course independent of it (Fig. 1). During the first hour after ouabain application the conductance decreased slightly, never being less than 80% of its original value. (This should be contrasted with a decrease of 45% under the influence of amiloride observed in the same hemiskins.) After the first hour the total conductance increased dramatically, reaching a maximum 90 to 150 min after ouabain application, and then decreased slowly and levelled off at a value higher than the original value. Five hours after the addition of ouabain, the total conductance remained still higher that it was before the treatment with ouabain.

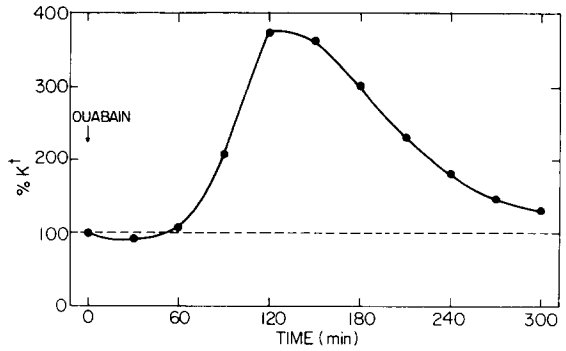

Fig. 1. Change of total conductance of frog skin produced by 10^{-4} M ouabain in the serosal solution, relative to the value in normal solution before ouabain. Standard errors have been omitted for simplicity. Out of 17 skins, two showed the maximum at 90 min, nine at 120 min, and six at 150 min. The passive conductance, measured with amiloride at the beginning of each experiment, averaged 55 ± 3% of the total value.

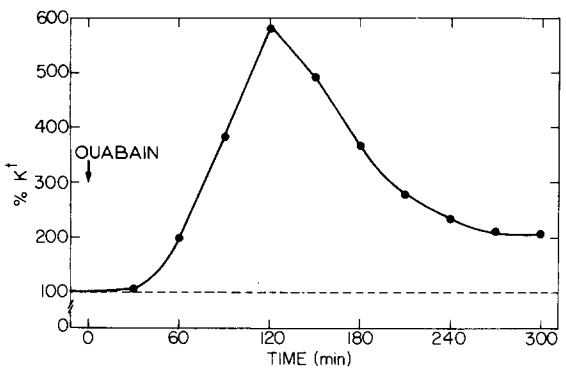

Fig. 2. Change of total conductance of amiloride-pretreated frog skin produced by 10^{-4} M ouabain in the serosal solution, relative to the value in amiloride-containing solution before ouabain. The values of the conductance after amiloride, considered here as a reference, averaged 53 ± 2% of the total conductance in normal solution. Standard errors have been omitted for simplicity. Out of 8 experiments, five showed the maximum at 120 min and three at 150 min.

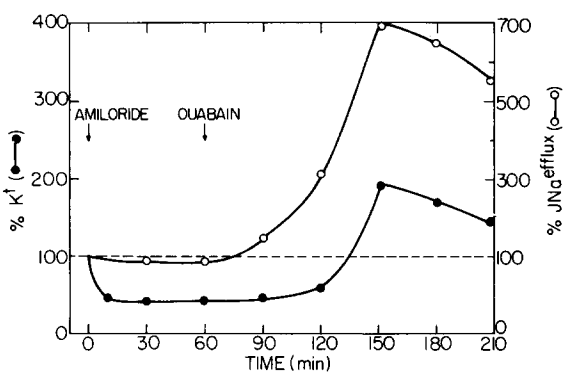

Fig. 3. Effect of amiloride and ouabain on total conductance (●———●) and Na^+ efflux (○———○) in frog skin. Changes are relative to the values in normal solution. The standard errors have been omitted for simplicity. The values of Na^+ efflux in normal conditions (our reference state) averaged 1.93 ± 0.7 nequiv./cm^2 per min. After amiloride, the conductance decreases to 42 ± 8% and the Na^+ efflux to 96 ± 11% (not significant). Out of five experiments, three showed the maximum after addition of ouabain at 120 min and two at 150 min.

Amiloride is known to block the movement of Na^+ across the mucosal membrane of the cells in both directions: from the mucosal solution into the cells [9] and from the cells out to the mucosal solution [10,11]. In 8 experiments, the experimental hemiskin was treated with amiloride ($5 \cdot 10^{-5}$ M final concentration) in the mucosal solution, and after a new steady state was reached, 10^{-4} M ouabain was added. As can be seen in Fig. 2 the changes in the conductance in amiloride-pretreated hemiskins were very similar to those of the non pretreated skins. One hour after adding ouabain, the conductance increased dramatically until reaching a maximum and then decreased slowly, being higher than the original value 5 h after ouabain application.

Fig. 3 shows the results of measurements of Na^+ efflux and the changes produced by ouabain on Na^+ efflux across amiloride-pretreated hemiskins. In these experiments the electrical conductance was measured at the same time as the Na^+ efflux. As can be seen, after treatment with amiloride the conductance decreased but no significant change occurred in Na^+ efflux. After adding ouabain, there was an increase in Na^+ efflux, and this increase and the subsequent decrease were closely parallel by the change in total conductance.

Discussion

Ouabain has been considered to be a specific blocker of the (Na^+-K^+)-ATPase that pumps Na^+ out of the cells, but the results shown here suggest that this is not the only effect. After addition of ouabain to frog skin, the active transport of Na^+, measured as short circuit current, disappears in about one hour, but the conductance of the skin does not behave as expected. During the first hour it decreased but it always remains higher than the passive conductance measured with amiloride, suggesting that during the blocking of the Na^+ pump an increase in passive conductance takes place. Not only that, after the short circuit current has been blocked, a dramatic increase of the conductance (now passive conductance) takes place, reaching a maximum and decreasing.

The first possible explanation of these results is to assume that in frog skin a substantial recycling of Na^+ occurs under normal conditions, and that after blocking of the pump by ouabain the Na^+ diffusing from the serosal solution into the cells cannot be pumped back again, and diffuses to the mucosal solution. This explanation, if true, would imply a very substantial recycling indeed, equivalent to or higher than the normal transepithelial transport.

That this is not the case is shown in Figs. 2 and 3. After treatment with amiloride, the time course of the passive conductance (because of ouabain) is similar to that observed in the absence of amiloride, the only difference being that no decrease of the conductance is observed during the first hour after ouabain. This would be expected if the initial decrease in conductance were produced by blocking an active transepithelial pathway that no longer exists after amiloride.

If the changes observed in Fig. 1 were due to a recycling of Na^+ unmasked by ouabain, amiloride should prevent the changes in conductance or at least reduce them very much. The fact that in the presence of amiloride the relative change of passive conductance is of the same order as in its absence rules out the possibility that the changes in conductance are due to recycling.

Fig. 3 shows that the effect of ouabain on the passive conductance is paralleled by changes in Na^+ efflux across a non-cellular pathway, since amiloride is known to block the movement of Na^+ in both directions across the mucosal membrane of the cells. This indicates that besides blocking the Na^+ pump ouabain affects the paracellular shunt pathway between the cells. It also shows that amiloride alone has no significant effect on Na^+ efflux, in agreement with previously reported results [9,10,12—14]. What is sure is that the changes of conductance and of Na^+ efflux produced by ouabain are not an indication of recycling of Na^+.

The model proposed by Koefoed-Johnsen and Ussing [1] for transport across tight epithelia assumed that the basolateral membrane of the cells was totally impermeable to Na^+, Na^+ being extruded from the cells by the action of a specific Na^+ pump, later identified as the (Na^+-K^+)-ATPase. In this model no recycling of Na^+ takes place and the energy consumption measured when the net active transport is blocked either by amiloride or Na^+-free mucosal solution is a reliable measurement of the basal, non-transport related, energy consumption. The model was subsequently revised [15] to include a diffusive shunt pathway between the cells and to include the possibility that all the Na^+ present in the cells may not be involved in active transepithelial transport, some diffusing from the serosal solution into the cells but not entering the transport pool. This compartmentalization of Na^+ has been shown to exist in toad bladder [16,17], where only 25% of the cellular Na^+ content is of mucosal origin and involved in transport. However, its existance has been denied in frog skin [11], where all the cellular Na^+ has been shown to be exchangeable with Na^+ of mucosal origin. This result contradicts previous reports claiming that compartmentalization also exists in frog skin [18,19]. The apparent difference in the properties of two tissues previously considered to have very similar characteristics makes it necessary to check each independently.

In toad bladder, it has been reported that recycling of Na^+, if it exists, must be negligible [2,3,4]. In order to reach this conclusion, the CO_2 production in the presence of amiloride or with Na^+-free mucosal solution was compared with the CO_2 production after further substitution for serosal Na^+, the rationale being that after amiloride no net transport of Na^+ takes place but serosal Na^+ may still be recycled and contribute to the measured CO_2 production. After replacing the serosal Na^+ the contribution of the recycled Na^+ should be observed. A similar conclusion about the non-existence of recycling in toad bladder was reached by analysing current-voltage relationships [20]. Cellular backflux of Na^+ was detected in toad bladder by imposing a concentration gradient of Na^+ and reversing the movement of Na^+ across the cell. The measured backflux was 0.5 $\mu A/cm^2$ when the gradient was imposed [21] and it is logical to think it would be less than this when the electrochemical gradient for Na^+ is zero.

In toad skin a small recycling of Na^+ was detected by efflux measurements [5], but it was so small (less than 5% of the normal short circuit current), that it was not detectable by standard oxygen consumption measurements. Such convincing results do not exist in frog skin. Recently, a slight decrease in oxygen consumption by amiloride-pretreated frog skins after addition of ouabain was reported [22]. The decrease was about 7% of the oxygen con-

sumption in the presence of amiloride, but because of the lack of a suitable control the idea of recycling was not raised explicitly. In any case this result is similar to the observation in toad skin and can be considered almost negligible.

Biber and Mullen [7] reported results on the changes of Na^+ efflux across the frog skin after treatment with ouabain, and claimed them to be a measurement of Na^+ recycling. They discarded the possibility of a paracellular effect because the efflux of nonelectrolytes (sucrose, mannitol, polyethylene glycol), known to cross the skin by a paracellular pathway, was not affected. Our results show that recycling is not the explanation and that it cannot be a cellular effect.

If the effect of ouabain on the electric conductance is intercellular and if, according to Biber and Mullen, the efflux of sucrose, mannitol, and polyethylene glycol is not affected by ouabain, there are two possible explanations. Either an opening occurs in the tight junction so small that mannitol cannot go through but smaller molecules can, or a change in the surface charge at the tight junction takes place affecting the movement of electrolytes only. It is known that after ouabain the Na^+ content of the epithelial cells increases and the K^+ content decreases both in frog skin [11,23] and in toad bladder [16, 17], but while the volume of toad bladder cells does not change a shrinkage of frog skin cells has been reported [23,24].

A tentative explanation of our results must take into account the possibility that progressive blocking of the pump activity by ouabain can change either the distribution of the surface charge or the dimensions of the tight junctions, facilitating the Na^+ efflux without altering the efflux of big nonelectrolytes. Changes in geometry, changes in KCl permeability [24] across the basolateral membrane, and/or changes in Na^+ permeability across the mucosal membrane (produced by changes in cellular ionic content) may explain the time course of the electrical conductance.

However, although the correct explanation of this behavior is not yet clear, three facts emerge from our results. First, the changes produced by ouabain on electrical conductance and on Na^+ efflux are not an indication of recycling of Na^+. Second, the similarity of the effect of ouabain on the conductance in the presence or the absence of amiloride suggests that recycling of Na^+, if exists, must be very small (a value as the suggested for toad skin [5] does not seem unreasonable). Third, because of the secondary effect of ouabain on the passive pathway, ouabain is not a good blocker in energetic studies since we require all the passive parameters to remain essentially unchanged while stopping the active transport. A corollary of this is that previous experimental studies based on the assumption that the only effect of ouabain is to block the Na^+ pump may require reconsideration.

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