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Neurohypophyseal hormones and sodium transport

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Neurohypophyseal hormones are known to affect the transfer characteristics of some amphibian epithelia to salt and water. The general view of sodium transport across toad bladder is that sodium enters the cells passively down a concentration gradient from the mucosal solution, and is pumped into the serosal solution by an unsaturated ion pump. Neurohypophyseal hormones are believed to stimulate transport by increasing the permeability of the mucosal surface to sodium ions.

Measurements of the amount of sodium in the transport pool by tissue pool labelling has shown that the electrochemical gradient may not always be favourable for sodium entry across the mucosal border. Furthermore, other experiments have demonstrated that most of the labelled sodium pool, presumably intracellular, is beyond the active transport step. These difficulties have led to suggestions of alternative models for the active transport of sodium and for the actions of neurohypophyseal peptides.

In this paper the effects of amiloride on short circuit current and sodium fluxes in isolated toad bladder is described, and an alternative view of the transport process is suggested.

1. Introduction

Neurohypophyseal hormones increase sodium transport across a number of amphibian and reptilian epithelia. The isolated frog skin (*Rana temporaria*) and isolated toad bladder (*Bufo marinus*) are the most extensively studied of these. Models proposed for epithelial sodium transport in frog skin (Koefoed-Johnsen & Ussing 1958) and toad bladder (Leaf 1964) envisage that sodium ions enter through the outer or mucosal surface of the tissue, into the cells and are removed across the serosal border of the epithelium by an ion pump. Thus sodium transport involves a two-step process, passive diffusion down an electrochemical gradient through the outer membranes, and active transport across the serosal membranes. This simple model has been modified for frog skin to take account of transport in more than one epithelial layer (Ussing & Windhager 1964).

Sodium transport in toad bladder increases in a curvilinear fashion with an increase in sodium in the mucosal bathing fluid. The system appears to obey simple saturation kinetics with a K_m value of 20 mmol/l sodium. Neurohypophyseal hormones increase the sodium permeability of the mucosal surface of the bladder and, since the apparent K_m for sodium remains at 20 mmol/l, it is concluded that they either increase the number of permeability sites in the mucosal surface or alternatively increase the turnover rate at each site (Frazier, Dempsey & Leaf 1962).

2. The sodium transport pool

If the entry of sodium into transporting epithelia is a passive process it is vital to demonstrate that there is a favourable electrochemical gradient for sodium entry. Various types of measurement have been used to estimate the sodium transport pool in epithelia, i.e. the amount of sodium that has entered the tissue and is awaiting transport. The most common method is tissue pool labelling in which the tissue is exposed on the mucosal surface to Ringer solutions containing radiosodium. The amount of sodium in the pool awaiting transport is estimated

from the amount of radiosodium in the tissue, which is assumed to have the same specific activity as the mucosal bathing solution.

Alternatively the sodium pool can be determined from a plot of the accumulated inward sodium flux versus time. After the flux has become constant the sodium pool may be estimated from the difference between this curve and the straight line of the same slope but passing through the origin (Andersen & Zerahn 1963). In yet another method the tissue is exposed to radiosodium on the outer surface until equilibrium is reached. The radiosodium is then removed from the outer solution and its disappearance from the tissue into the serosal bathing solution is measured against time. The rate of disappearance of the radiosodium from the tissue has been found to follow an exponential time course in both bladder (Essig & Leaf 1966) and frog skin (Andersen & Zerahn 1963). By these various methods it has been shown that the sodium pool is increased by neurohypophyseal hormones, a finding which is consistent with the view that hormones increase the mucosal sodium permeability.

3. Difficulties with existing models

The exponential disappearance of the labelled sodium pool shown for frog skin and toad bladder was believed to represent the active transport of sodium from a pool into which it had entered passively. However metabolic inhibitors, which cause an immediate reduction of short circuit current, failed to influence the rate of loss of sodium from the tissue (Essig & Leaf 1966; Zerahn 1969). Apparently, all the sodium in the tissue had already passed the transport stage, and it was not possible to detect any sodium awaiting transport. Although it is difficult to find the concentration of sodium in the pool, since the volume of the pool is unknown, estimates have been made of sodium concentrations in the tissue and have proved embarrassingly large. For example Zerahn (1969) found the sodium concentration in the epithelial layers of frog skin was 18 mmol/l when the mucosal solution contained no sodium. Rotunno, Pouchon & Cereijido (1966) obtained a value of 97 mmol/l in frog skin with 1 to 10 mmol/l Na in the bathing solution. In toad bladder a concentration of 38 mmol/l was found intracellularly when the mucosal bathing solution contained 24 mmol/l (Crabbé & De Weer 1965). When the mucosal surface was bathed in normal Ringers (115 mmol/l) the intracellular sodium was 75 mmol/l (Leaf 1965). This value is again large considering the magnitude of the potentials which can be recorded across the mucosal surface. It is true that much of the intracellular sodium may be bound, however Janaček, Morel & Bourguet (1966) found an intracellular sodium concentration of about 30 mmol/l using cation selective microelectrodes. Since transporting epithelia are able to transport sodium from mucosal bathing solutions with as little as 1 mmol/l sodium it may be that sodium may move up both an electrical and chemical gradient into the cell.

4. Amiloride, short circuit current and sodium fluxes

The potassium sparing diuretic amiloride (*N*-amidino-3,5-diamino-6-chloropyrazine carboxamide) causes a rapid and reversible inhibition of short circuit current (s.c.c.). The concentration causing a 50% reduction in s.c.c. is about 10^{-7} mol/l. The evidence indicates that the primary mode of action of the material is to prevent sodium entry across the mucosal surface and with steady-state conditions the net sodium flux equals the s.c.c. (Ehrlich & Crabbé 1968; Crabbé & Ehrlich 1968; Nagel & Dörge 1970; Salako & Smith 1970*a*, *b*).

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An attempt has been made to look for pools or compartments in toad bladders from and into which transport may be taking place by measuring the time course of the s.c.c. transient in response to amiloride. The s.c.c. was measured automatically and a special cell was devised so that amiloride could be added rapidly to the mucosal bathing solution. In addition, the serosal surface was rapidly perfused with Ringer solution so that mucosal to serosal fluxes of ^{22}Na could be measured or vasopressin could be applied to the serosal surface.

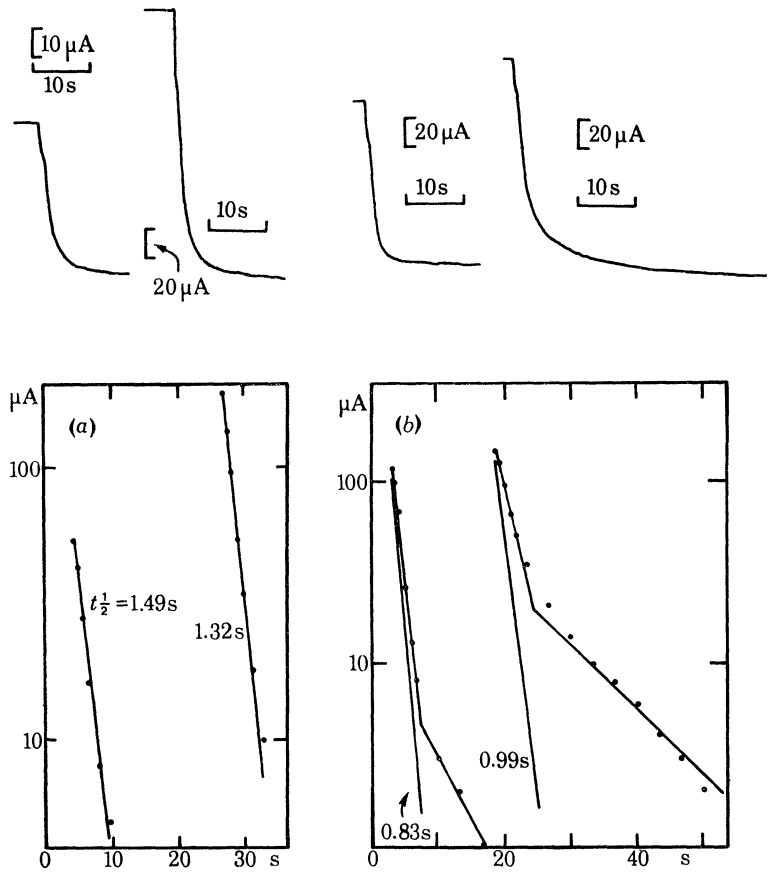

FIGURE 1. (*a*) S.c.c. transient from bladder before and after exposure to ADH (50 mu/ml). The final concentration of amiloride was 2×10^{-4} mol/l. Logarithmic plots of s.c.c. against time are shown below the transients. (*b*) S.c.c. transients from bladder as in (*a*). Note in this experiment the logarithmic plots had a double exponential form.

The time course of the s.c.c. transient when 2×10^{-4} mol/l amiloride was rapidly mixed with the mucosal fluid was either a single or double exponential (figure 1*a*, *b*), which may represent the transport of sodium out of one or two compartments with first-order kinetics. A series of experiments were carried out on frog skins with different concentrations of amiloride. No difference was found in the time course of the fast component with concentrations amiloride of between 10^{-3} and 10^{-5} mol/l. In experiments where no second component was observed it cannot be assumed it was absent, but rather that it was not apparent at s.c.c. greater than a few μA. From the time courses of the transients 'amiloride pools' or the amount of sodium which can be transported after adding amiloride was calculated.

The pool (P, in μmol) is given by

$$P = \frac{\text{s.c.c. } (t = 0)\ 60t_{\frac{1}{2}}}{0.693F},$$

where s.c.c. ($t = 0$) is the short circuit current at the time of adding amiloride, $t_{\frac{1}{2}}$ is the half-time of the fast component in minutes and F is the Faraday constant. Nine experiments were carried out in which the s.c.c. transient was recorded before and after arginine vasopressin (50 mu/ml). Table 1 gives mean value for the half-time of the fast component in these experiments, together with the corresponding pool size and the s.c.c. It can be seen that the pool size is significantly

TABLE 1. TABLE SHOWING MEAN VALUES FOR THE S.C.C., HALF-TIME OF THE S.C.C. TRANSIENT AND THE 'AMILORIDE POOL' IN NINE BLADDERS BEFORE AND AFTER ADDITION OF ADH (50 mu/ml).

The final concentration of amiloride in these experiments was 2×10^{-4} mol/l. The mean wet mass of the bladders was 52 mg (dry mass 8 mg).

	control	ADH (50 mu/ml)	
s.c.c.	51 μA	107 μA	
$t_{\frac{1}{2}}$	1.8 s	1.4 s	n.s.
pool	0.0013 μequiv.	0.0021 μequiv.	$P < 0.05$

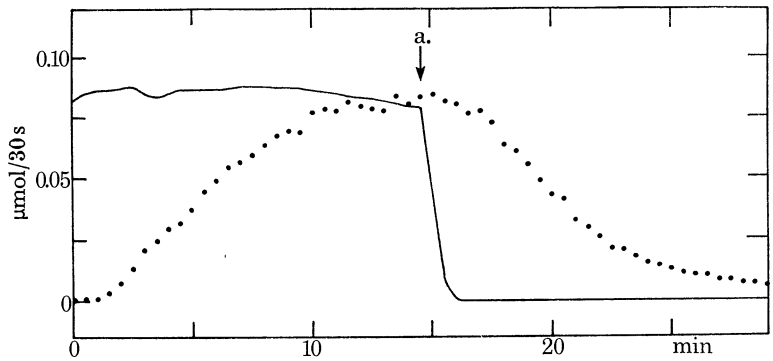

FIGURE 2. Sodium transport measured as s.c.c. (——) and as mucosal to serosal flux of ^{22}Na (···). The isotope was added at $t = 0$. Amiloride (2×10^{-4} mol/l) was added at $t = 15$ min. The serosal solution was choline chloride Ringer, and contained 50 mu/ml vasopressin.

increased by vasopressin, but that the half-times are not significantly different. The pool sizes measured in this way are considerably smaller than those obtained by tissue pool labelling. For example, Crabbé & De Weer (1969) gave a value of 0.12 μmol/10 mg dry mass in sixty determinations, while Frazier *et al.* (1962) quoted a value of 9.3 μmol/g tissue water in ten determinations.

In an attempt to understand what is happening during the transient response to amiloride mucosal to serosal fluxes of ^{22}Na were measured. The principle of the experiment can be readily understood from figure 2. A small amount (40 μCi) of ^{22}Na was added to the mucosal bathing solution when the s.c.c. was steady and samples were collected every 30 s until the flux and s.c.c. were equivalent. Amiloride (2×10^{-4} mol/l) was then added and the collection of samples continued.

Three estimates of the transport pool can be made from this experiment as outlined previously and as shown in figure 3. The cumulative influx curve gave a value in this experiment of

0.8 μmol, and the curve relating to the disappearance of labelled sodium from the tissue after amiloride gave a value of 0.83 μmol. The s.c.c. transient gave a value of only 0.034 μmol, one twenty-fifth of value given by the other methods. It can also be seen from figure 2 that the time course for the increase in ^{22}Na appearing in the serosal solution is the same as that for the decrease in ^{22}Na appearing in the serosal solution after mucosal entry was blocked by amiloride.

One immediate question is the location of the large sodium pool present in the tissue after the s.c.c. has fallen to zero. It can be shown that part, at least, of the pool is intracellular by the use of ouabain. If experiments similar to that described above are carried out but ouabain is added for some time before amiloride, then the disappearance of sodium from the tissue is very much delayed (figure 4).

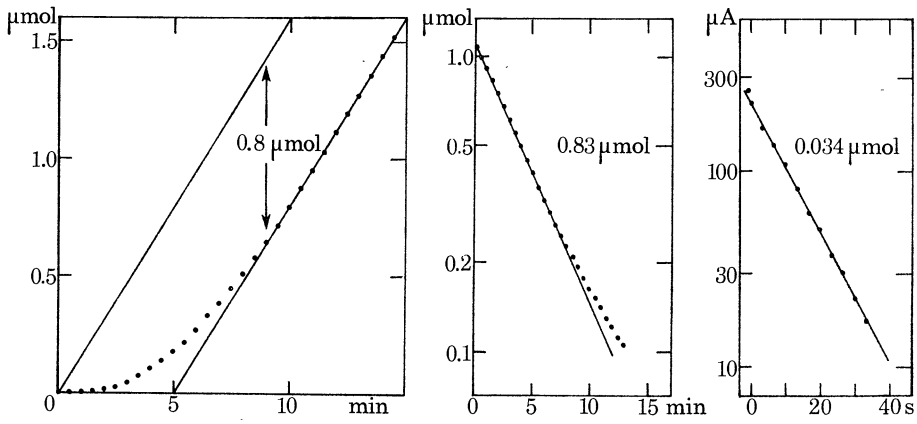

FIGURE 3. Determination of various sodium pools from the data shown in figure 2. (*a*) Estimation of sodium pool from the accumulated inward flux. (*b*) Estimation of sodium pool from sodium efflux from the tissue after amiloride. (*c*) Estimation of sodium pool from the s.c.c. transient after amiloride.

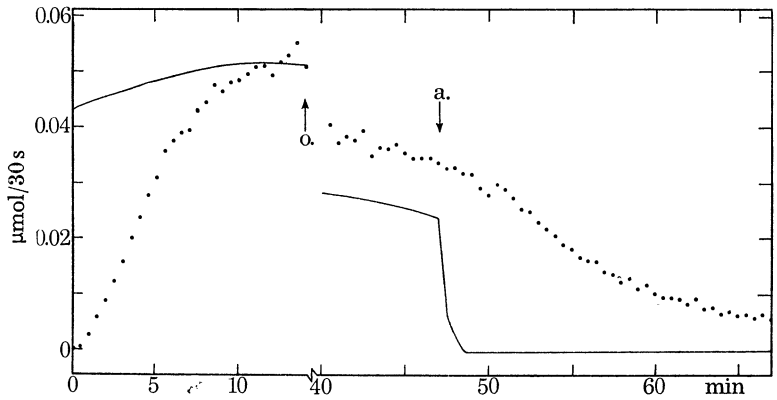

FIGURE 4. As figure 2, except ouabain (10^{-4} mol/l) was added to the serosal fluid at $t = 15$ min (shown by first arrow). Amiloride (2×10^{-4} mol/l) was added (at the second arrow) to the mucosal fluid approximately 30 min after ouabain. Note, s.c.c. and sodium flux for the period 15–40 min are not shown.

5. CONCLUSIONS

The transport pool as determined by tissue pool labelling has been found to behave in a predictable way to hormones and other agents which either increase or decrease mucosal entry or serosal exit from the tissue (Crabbé & De Weer 1969). For example, Civan and Frazier (1968) produced particularly clear evidence that neurohypophyseal hormones increase the sodium

conductance of the mucosal surface of toad bladders, and there have been many demonst ration of increased tissue pool labelling following hormone treatment. Yet the tissue pools in skins and bladders have been found to behave as transported pools (Essig & Leaf 1966; Zerahn 1969).

From the response of the s.c.c. in bladders to amiloride it has been possible to detect one, and in some bladders two, other pool(s) with half-times in the order of seconds, rather than minutes as is found with labelled pools. The half-times of the s.c.c. transients to amiloride was not changed by treating bladders with vasopressin, while the size of the amiloride pool was increased.

One interpretation of the present results is as follows. There may be two sodium pools in series, a small pool located at or near the mucosal surface and a large pool towards the serosa, into which sodium enters from the small pool and in which it is diluted or delayed before it appears in the serosal fluid. The suggestion that the small pool represents sodium awaiting transport rather than diffusion is strengthened by the failure of amiloride at different concentrations to influence the half-times for this pool. In this model sodium would be transported actively from each pool but would not generate a transepithelial in passing into the serosal fluid. The possibility must be considered that the small pool is located at the cell border.

Note added following the meeting (14 *December* 1970)

These experiments have shown that after amiloride the s.c.c. does not reflect the passage of sodium across the serosal border of the epithelium, yet it is clearly from an ouabain sensitive pool. Similarly the s.c.c. does not reflect the appearance of sodium in the serosal bathing fluid after DNP (Essig & Leaf 1966), an agent having little effect on epithelial resistance.

It has been suggested that separation of charge across a barrier located at the mucosal border might not generate a transepithelial potential due to the short circuiting effect of ions passing in shunt paths, particularly *after* the mucosal conductance had been decreased by amiloride.

If this were so then the current measured by the clamp would be less than the charge transferred, while the time course of charge transfer would be accurate; i.e. it would be inappropriate to use the s.c.c. ($t = 0$) for calculation of pool size. From the data given in figures 3 and 4 it can be seen that $t_{\frac{1}{2}}$ is 22.9 times faster for the small pool compared to the large. Since at steady state the flux through both series pools would be the same then the large pool should be 0.79 µmol, in good agreement with that found from sodium efflux. Similar agreement was found in four other experiments. Alternatively the conductance of various shunt paths may be changing during the s.c.c. transient so that the time course of the transient is inappropriate. However the $t_{\frac{1}{2}}$ of the fast component varied only slightly and non-systematically while the amiloride concentration was changed from 10^{-3} to 10^{-5} mol/l. It is reasonable to conclude that charge separation occurring near the mucosal border is monitored across the epithelium after amiloride.

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