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The effects of anions on sodium transport

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1. Substitution of chloride by isethionate reduces the short circuit current (SCC) and increases the potential of isolated frog skin. In sodium isethionate Ringer antidiuretic hormone and choline chloride increase the SCC, whereas theophylline is ineffective.

2. Frog skins treated on the outside with copper ions always show an increased potential when bathed in normal Ringer solution. The SCC may be moderately increased or decreased.

3. Theophylline increases skin thickness and cell volume in non-short-circuited skins.

4. The ways in which the theophylline-induced increase in chloride permeability affects sodium transport is discussed, together with the requirements for a permeant anion in both short- and open-circuited skins.

Both antidiuretic hormone (ADH) and theophylline stimulate active sodium transport across the isolated frog skin. In two recent papers (Cuthbert & Painter, 1968a, b) evidence was presented suggesting that theophylline increases active transport of sodium ions across various epithelia by increasing chloride permeability, rather than by inhibiting phosphodiesterase. The latter view is held by those who propose an intrecellular role for cyclic-3′,5′-AMP as a mediator of sodium permeability (Orloff & Handler, 1967). The cyclic nucleotide is degraded to 5′-AMP by phosphodiesterase (Butcher & Sutherland, 1962) and presumably intracellular levels of the nucleotide rise when phosphodiesterase enzyme activity is abolished with methyl xanthines.

In this paper further experiments are presented which prove that chloride permeability is a factor in sodium transport across frog skin and three possible mechanisms for the anion effect on sodium transport are proposed.

Methods

Measurements of short circuit current and skin potential

Measurement of short circuit current and potential in pieces of frog (*Rana temporaria*) abdominal skin (4·5 cm^2) were made by the standard procedure of Ussing & Zerahn (1951). Short circuit current was measured continuously and skin potential intermittently by temporarily switching off the short-circuiting current.

Determination of tissue volume

Tissue volume and thickness were estimated from measurements of tissue weight and area. For these purposes pieces of frog abdominal skin (10–12 cm^2) were tied to glass frames and suspended from a torsion balance so that the skins were immersed in Ringer solution bubbled with air. Every 3 min the vessel containing the Ringer solution was lowered and the skin allowed to drain for 1 min before the weight was recorded. Ten weighings were made at 3 min intervals, after which theophylline was added to the Ringer solution. Ten further weighings, taking 30 min, were then made and in some experiments weighing was continued for a further 60 min. Standard errors for weight/cm^2 of skin before and after theophylline were calculated and the differences in the means tested for significance. Skin weights were converted to thicknesses assuming a skin density of 1.

Demonstration of changes in cell volume

Changes in cell volume of pieces of frog skin were demonstrated by the Principle of Archimedes. Pieces of frog skin (8–12 cm^2) were tied to glass frames and suspended in Ringer solution containing 5% w/v sucrose from the arm of an electronic microbalance (Elford, Farrant & Piper, 1966) by a nylon filament. The microbalance was sensitive to 1–2 μg. Water entering or leaving the cells caused a change in both real weight and upthrust on the tissue, because the Ringer solution containing the impermeant sucrose had a density greater than one. Thus the microbalance recorded a decrease in apparent weight when the cells swelled and an increase in apparent weight when cell shrinkage occurred. Changes in extracellular space had no effect on the apparent weight.

When the skins were first suspended in sucrose-Ringer solution there was a loss of apparent weight due to cell shrinkage. Approximately 1 hr later shrinkage had stopped, or was occurring at a very slow rate. Theophylline was added to the bathing solution either immediately after the tissues were suspended, or after 1 hr. Thus the effects of theophylline on cell volume were determined in tissues in which cells were already shrunken or were in the process of shrinking. It is not possible to determine the effects of theophylline on cell volume in normal cells by this method. Obviously the suspending solution must have a specific gravity greater than that of the intracellular fluid. Also the difference in specific gravity must be achieved using low molecular weight substances, such as sucrose, which can penetrate the extracellular spaces. Therefore the osmotic effect on the tissue using this method is unavoidable.

Addition of drugs to the bathing solution presented considerable problems. Of the several methods tried, the most successful used a motor driven ram so arranged as to drive two syringes in opposition. As theophylline (0·8 or 1·6 ml. 5×10^{-2}M solution) was driven from one syringe into the bathing solution an equal volume of bathing solution was drawn into the other syringe. This method avoided wetting a previously dry part of the nylon thread, which otherwise caused a considerable artefact.

The drug had to move through 5 cm of bathing fluid before reaching the tissue, so that the time course of the response was slow, and it is unlikely that a uniform distribution of drug was achieved during the experiments. The drug spread to the tissue by diffusion and by convection currents generated when the drug solution

was pumped into the system. It is claimed that the results with the microbalance demonstrate changes in cell volume rather than the extent or time course of these changes.

Determination of inulin space in frog skin

Pieces of frog abdominal skin (about 10 cm^2) were dissected and placed either in Ringer solution or Ringer solution with 10^{-3}M theophylline and bubbled with air for 60 min. After this, individual skins were blotted, weighed on a torsion balance, and placed for exactly 30 min in solutions identical with that from which they were removed except in that they contained 0·5% inulin. After removal from the inulin solutions the skins were blotted and placed in 100 times their own weight of distilled water for 2 hr. The inulin leached out of the skins was estimated colorimetrically by the method of Bacon & Bell (1948). The inulin space was estimated from a standard curve prepared using known inulin concentrations. Theophylline did not interfere with the colorimetric estimation of inulin.

Solutions

The Ringer solution used contained NaCl, 111 mM; KCl, 2 mM; $CaCl_2$, 1 mM; NaH_2PO_4, 0·08 mM; $NaHCO_3$, 2·4 mM; and glucose 11·1 mM, and was made from Analar grade reagents. When sodium isethionate Ringer was used sodium chloride was replaced by sodium isethionate, potassium chloride by potassium sulphate and calcium chloride by calcium sulphate (2 mM). Since isethionic acid (hydroxyethylsulphonic acid) is monobasic no correction for tonicity was necessary. Theophylline hydrate (BDH) and Pitressin (Parke Davis) were used. The latter was purified by vacuum distillation over a mixture of calcium chloride and wax shavings to remove chlorbutol.

Results

Effects of removal and addition of chloride

In the first experiments skins were mounted in Ringer solution and the short circuit current and skin potential (outside negative) were monitored for a control period. After this the bathing solution on both sides of the skin was changed to sodium isethionate Ringer, after which short-circuiting was continued. The results are illustrated in Fig. 1. Sodium isethionate caused the short circuit current to fall abruptly to a new steady value. At the same time the skin potential showed a sharp increase. Addition of theophylline (10^{-3}M) had no effect on either skin potential or short circuit current in skins bathed in sodium isethionate Ringer. whereas it invariably caused an increase in short circuit current and fall in potential in skins bathed in normal Ringer solution (Cuthbert & Painter, 1968a, b). In the continued presence of sodium isethionate Ringer and theophylline, antidiuretic hormone (ADH, 300 m-u/ml.) had the usual effect on short circuit current and potential.

Addition of a small quantity of chloride (10 mM) together with an impermeant cation in the form of choline chloride caused a considerable increase in the short circuit current of frog skins mounted in sodium isethionate Ringer. In these conditions the skin potential was also affected, showing a rise (Fig. 2). This potential

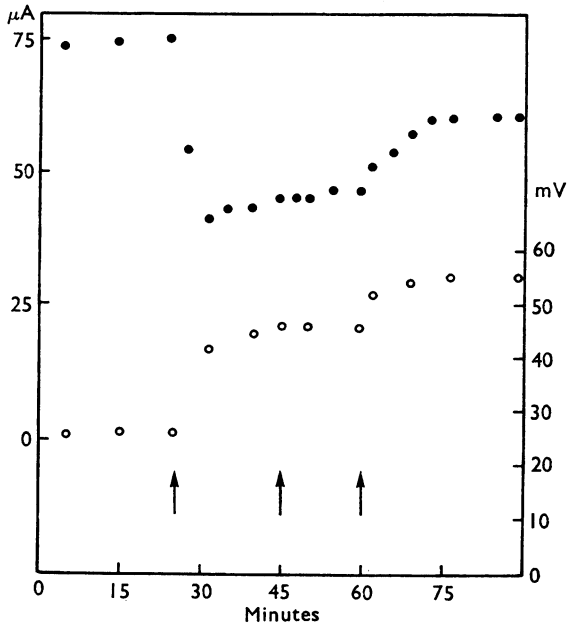

FIG. 1. Short circuit current (●) and skin potential (○) of 4·5 cm² of frog skin. At the first arrow the bathing solution was changed from Ringer to sodium isethionate Ringer solution. In this latter solution theophylline (10^{-3}M) and ADH (300 m-u./ml.) were added to the inside bathing solution at the second and third arrows respectively.

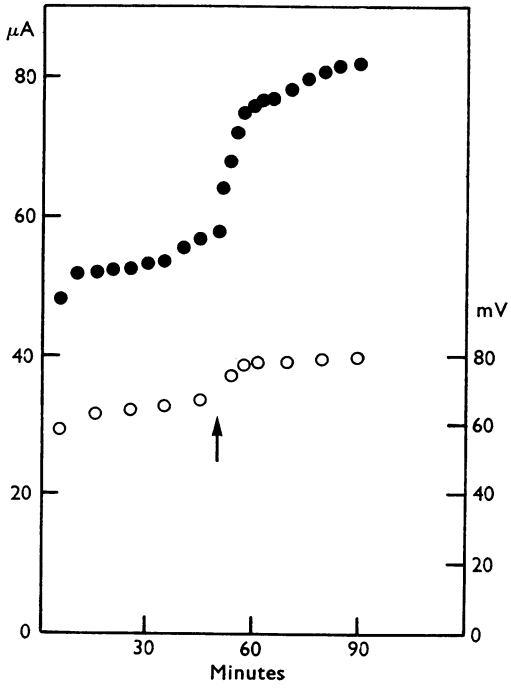

FIG. 2. Short circuit current (●) and potential (○) of 4·5 cm² frog skin bathed in sodium isethionate Ringer. At the arrow 10 mM choline chloride was added to the outside bathing solution.

change may have been due to a reduction in intracellular sodium consequent on an increase in short circuit current after adding choline chloride.

Effects of reducing chloride permeability

Koefoed-Johnsen & Ussing (1958) measured the permeability of frog skin to chloride ions using ^{36}Cl. They found that the chloride permeability was reduced when copper ions (10^{-5}M) were applied to the outside surface, and, in consequence, the skin potential was increased by a reduction of the shunting effect of chloride ions. The values quoted by these authors indicate a wide variation in the chloride permeability of different skins.

In these experiments copper ions produced small, but we think significant, changes in short circuit current as well as increasing the skin potential. Examination of Table 1 shows that in all five experiments with copper ions the skin potential increased, while the short circuit current was decreased in three experiments and increased in the two others. Higher concentrations of copper ions (10^{-3}M) caused a rapid reduction of both the skin potential and short circuit current when applied to the outside of the skin.

TABLE 1. *Effect of copper ions on short circuit current (SCC, μA/4·5 cm²) and skin potential (mV) of frog skin*

Expt.	$CuSO_4$ (M)	Initial SCC	% decrease SCC	Initial potential (mV)	% increase potential
466	10^{-5}	102·5	4·9	88	5·7
468	10^{-5}	97·5	5·1	40	37·5
473	10^{-5}	128·0	−3·2	93	51·1
470	10^{-4}	190·0	12·6	71	16·9
471	10^{-4}	120·0	−16·7	65	52·5

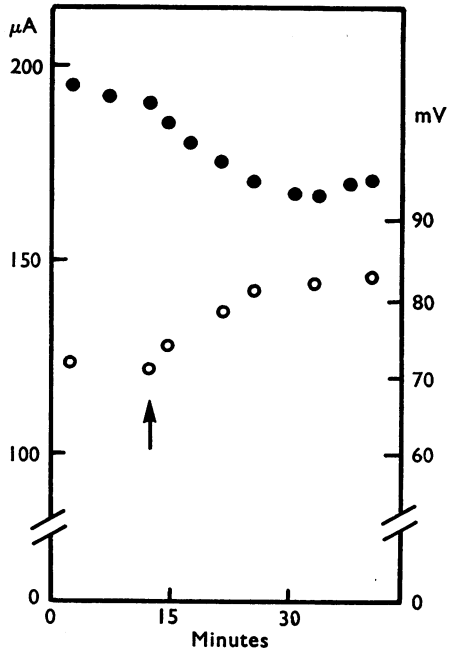

FIG. 3. Short circuit current (●) and skin potential (○) of 4·5 cm² of frog skin. At the arrow copper sulphate (10^{-4}M) was added to the outside bathing solution.

The time course of the copper ion effect is shown in Fig. 3. The effect was immediate and both the short circuit current and potential approached new values with a similar time course.

Effects of theophylline on tissue volume and inulin space

The results of three experiments in which the effect of theophylline on tissue volume was investigated are shown in Fig. 4. The weight/cm^2 before and after theophylline has been calculated from the total tissue weight and the skin area. If the swelling of the tissue occurs mainly by an increase in thickness, a likely assumption, then the weight/cm^2 can be converted directly to skin thickness, assuming a tissue density of 1. In all three experiments illustrated there was a highly significant ($P<0{\cdot}0001$) increase in skin weight after treatment with theophylline (10^{-2} to 10^{-4}M). The extent of the swelling was similar with all three concentrations of theophylline used. In other experiments skins were exposed to similar concentrations of theophylline for 90 min. Results similar to those already cited were obtained, and the theophylline effect was maintained over this time. This is important because skins were exposed for 90 min to theophylline during determinations of inulin space.

The increases in skin thickness with theophylline shown in this work (8 to 11 μ) are of the same order as those seen by Ussing (1965) using short-circuited skins subjected to osmotic gradients. Ussing measured skin thickness by a direct microscopic method.

It was necessary to show whether the increase in cell weight was due to an increase in extracellular space or to an increase in cell volume. The inulin space of six pieces of normal skin and six pieces exposed to theophylline (10^{-3}M) for 90 min was determined. The inulin space of normal skin was found to be $24{\cdot}8 \pm 2{\cdot}4\%$ ($\pm$S.E.). In skins treated with 10^{-3}M theophylline the inulin space was $31{\cdot}5 \pm 1{\cdot}8\%$ ($\pm$S.E.). The difference between the two mean values was statistically significant ($P<0{\cdot}05$). It can be shown that if all the weight increase illustrated in Fig. 4 were due to an increase in the size of the extracellular space then, at the most, a 2·5% increase in inulin space would be expected. The observed 6·7% increase in inulin space can therefore more than account for the increase in skin weight. Probably inulin can gain access to parts of the tissue after treatment with theophylline not available before addition of the drug. In view of these results a more direct measure of cell swelling was investigated.

Effects of theophylline on cell volume

In the section on methods the authors were at pains to point out that the apparent weight method has been used to demonstrate cell swelling rather than to rigorously quantify this parameter.

The apparent weight of skins together with their frames suspended in sucrose-Ringer (specific gravity 1·022) were determined as described in methods. The output from the microbalance was fed directly to a pen-recorder. Figure 5 illustrates two experiments in which theophylline was added to skins suspended from the microbalance. In (a) the skin had been in the sucrose-Ringer solution for 1 hr and cell shrinkage (gain in apparent weight) was slow. Addition of theophylline to give final concentrations of 1 and 3×10^{-4}M caused, after a delay for mixing, a reduction

in apparent weight (cell swelling). In (b) the record was started at the time the skin was placed in sucrose-Ringer solution. The rate of gain of apparent weight was greater than in (a). Addition of theophylline to give final concentrations of 1, 3 and 5×10^{-4}M caused either a reduction in apparent weight or reduced the rate of apparent weight increase. Control experiments in which small volumes of Ringer were added caused only mechanical artefacts.

From measurements of skin area, the loss in apparent weight and the density of sucrose-Ringer, an estimate of the increase in skin thickness caused by theophylline can be made. The values are for curve (a) 5·7 μ and for curve (b) 3·0 μ. These values may be compared with the thickness increases estimated from weight measurements (8–11 μ).

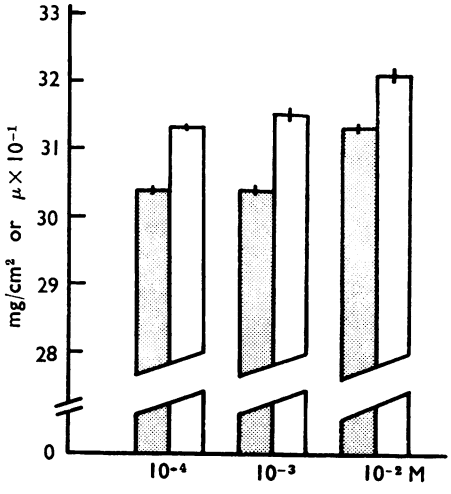

FIG. 4. Changes in skin weight (mg/cm²) and skin thickness ($\mu \times 10^{-1}$) caused by theophylline (10^{-4}, 10^{-3} and 10^{-2}M). The hatched columns represent mean values for ten weighings before theophylline was added to the bathing fluid. The open columns represent mean values for ten weighings after addition of theophylline. The vertical bars at the top of each column are standard errors. The difference of the means for each experiment was highly significant ($P>0.0001$).

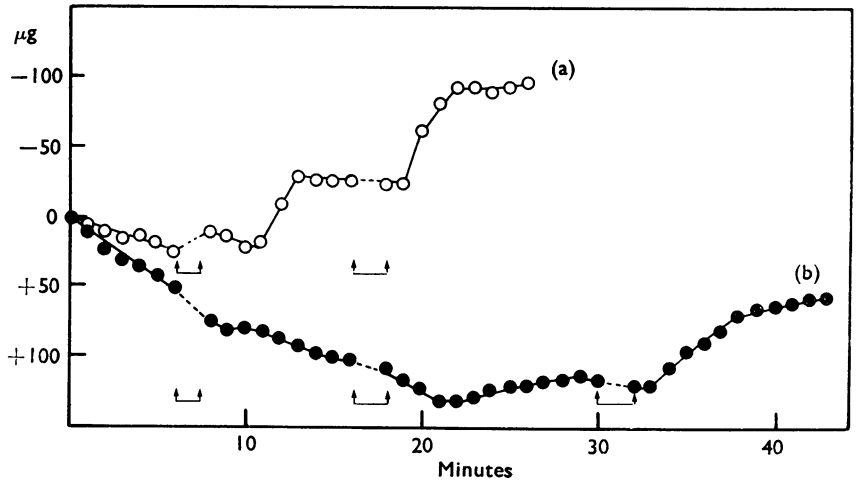

FIG. 5. Changes in apparent weight of pieces of frog skin suspended in sucrose-Ringer solution. In (a) (8 cm² skin), theophylline was added at the arrows to give final concentrations of 1 and 3×10^{-4}M. In (b) (9·7 cm² skin), theophylline was added at the arrows to give final concentrations of 1, 3 and 5×10^{-4}M. Estimated increases in skin thickness were 5·7 μ (a) and 3·0 μ (b).

Discussion

In a previous paper (Cuthbert & Painter, 1968b) it was shown that theophylline increased the chloride permeability of the outer facing membranes of frog skin. This conclusion was based on the ability of theophylline to cause a potential fall in choline chloride Ringer, but not in sodium isethionate Ringer. This effect alone was considered sufficient to explain the increase in sodium transport under open circuit conditions by removal of anion drag. But theophylline increases sodium transport in frog skin and other epithelia under short circuit conditions (for example Baba, Smith & Townshend, 1967). We consider that this effect too can be explained by an increase in chloride permeability.

In these experiments substitution of isethionate for chloride caused a reduction in the short circuit current, while the potential increased because of a reduction in the shunting effect of chloride. This indicates that permeant anions are partly responsible for the amount of sodium transported by the skin. Recently, Ferreira (1968) came to a similar conclusion when he substituted sulphate and gluconate for chloride, provided in the case of the former that tonicity was maintained by addition of glucose. Theophylline was found to be without effect in sodium isethionate Ringer, whereas ADH remained effective. This result shows that phosphodiesterase inhibition is unlikely to be the mechanism by which theophylline increases sodium transport in short-circuited epithelia.

Three mechanisms by which an increase in sodium transport may result from increased chloride permeability in short-circuited epithelia are envisaged. The first considers the outer membrane as a mosaic of sodium and chloride permselective sites in which the potential is positive inside with respect to outside in the open-circuited condition. Sodium entering the cell from the outside is pumped away by an ion pump on the inner facing membranes. The leak currents of Na^+ and Cl' across the outer membrane in open circuit steady state conditions are given by

$$I_{Na} = g\mathrm{Na} \cdot (E_1 - E_{Na})$$
$$I_{Cl} = g\mathrm{Cl} \cdot (E_1 - E_{Cl})$$

where g refers to conductances and E_{Na} and E_{Cl} the equilibrium potentials for Na^+ and Cl' respectively. E_1 is the potential of the outer facing membranes. Under short-circuited conditions the potential across the whole skin, and across the outer facing membranes is zero, or nearly so (Frazier, 1961 ; Janacek, Morel & Bourguet, 1968). Under these conditions the leak currents reduce to

$$I_{Na} = g\mathrm{Na} \cdot (-E_{Na})$$
$$I_{Cl} = g\mathrm{Cl} \cdot (-E_{Cl})$$

If chloride conductance increases to $g'\mathrm{Cl}$ then I_{Cl} increases to

$$I'_{Cl} = g'\mathrm{Cl} \cdot (-E_{Cl})$$

carrying with it a negative charge to the inside of the membrane and thus increasing the electrical gradient for sodium ions. The increased sodium ion flow so induced enables the membrane potential to remain at zero. In other words the chloride inrush carries with it sodium ions by cation drag. Providing the sodium pump is not saturated extra sodium ions are then removed across the inner facing membranes.

Do chloride ions *per se* have other effects on sodium transport ? In the skin of *Leptodactylus ocellatus* it was found that sodium transport at a constant outside sodium concentration depended on the chloride concentration of the outer bathing

solution (Fischbarg, Zadunaisky & de Fisch, 1967). The effect was not directly on the outer surface of the skin, for in washout experiments there was no change in either chloride or sodium permeability. The authors concluded that chloride had a direct effect on the pumping mechanisms. In these experiments it has been shown that reduced chloride permeability resulting from treatment with copper ions can increase or decrease the short circuit current. By the previous argument only a decrease in short circuit current should result. However, if chloride has an intracellular effect on the pumping mechanism it may be that it has an optimal cencentration for this effect. Changes in chloride permeability could then either make the intracellular chloride concentration nearer to or further from this optimum. The skin potential should always increase due to reduction of the short circuit across the skin (Table 1).

Finally, the third way in which chloride permeability might affect sodium transport is by an effect on cell size. As a consequence of increased chloride permeability sodium chloride concentration within the cell increases. Since the inner facing membranes are highly water-permeable water flows into the cell down an osmotic gradient. Ussing (1965) showed that the short circuit current depended on skin thickness, shrinkage causing a reduction in short circuit current and swelling causing the converse. These findings might be interpreted in terms of the increased availability of pumping sites in turgid cells with unfolded membranes. This work has shown that an increase in chloride permeability, due to theophylline, does in fact increase cell size in open-circuited skins. The apparent extracellular space is also increased by theophylline, but it must be concluded that the swelling of the cells induced by theophylline alters the extent to which inulin can enter the tissue spaces. It is significant that the swelling induced by theophylline when measured by two independent methods and by osmotic gradients is of the same magnitude.

We conclude that an increase in chloride permeability in sodium transporting epithelia increases sodium transport partly by inducing an increased sodium influx by cation drag and partly by an effect on cell size. We consider that phosphodiesterase inhibition and endogenous 3′5′-AMP accumulation may be unimportant for the effect of methyl xanthines on sodium transport in this tissue.

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