The Effect of Ca and Antidiuretic Hormone on Na Transport across Frog Skin

I. *Examination of interrelationships between Ca and hormone*

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ABSTRACT Ca added to the solution bathing the outside of isolated frog skin causes a decrease in net Na transport across the skin while antidiuretic hormone (ADH) causes an increase. Possible interrelations between the effects of these agents have been examined. The decrease in Na transport caused by Ca was the same before and after treatment of the skin with ADH and the increase in transport caused by ADH was unaffected by the presence of Ca. The relationship between Ca concentration and degree of inhibition of Na transport was not appreciably altered by ADH. These results indicate that Ca and ADH do not compete but act independently at two different sites and these sites appear to be located on the same barrier to Na movement in the skin. Further, Ca causes a decrease in Cl influx across the short-circuited skin but ADH has no effect on Cl movement, again suggesting that the actions of these agents are independent.

Ca and antidiuretic hormone (ADH) are known to alter permeability properties of biological membranes, and some recent evidence has suggested that the effects of these two agents may be interrelated. Bentley (1) and Peterson and Edelman (2) have found that the increase in water permeability of the toad bladder caused by ADH could be partly reversed by addition of Ca to the bathing solutions. Whittembury, Sugino, and Solomon (3) observed that the permeability of *Necturus* kidney slices to glycerol was increased by ADH and that this increase could be completely reversed by raising the Ca concentration from 1 to 10 mM. These observations suggested that there might be a competition between Ca and ADH in their effects on membrane properties and such a possibility must be taken into account in any attempt to explain

the effects of these agents. Both Ca (4) and ADH (5, 6) are known to alter the rate of active Na transport across the isolated frog skin, but the mechanisms involved are unclear. In an attempt to gain further insight into the mode of action of these agents, experiments have been carried out to examine possible interrelationships between them in their effects on the Na transport system of frog skin.

METHODS

The skin of *Rana pipiens* was mounted between lucite chambers as a flat sheet having an area of 7.1 cm^2. Many of the experiments required the use of paired skins obtained from the same frog by splitting the skin longitudinally along back and abdomen to yield two pieces as nearly symmetric as possible. The chambers employed were equipped with two sets of electrodes and were similar in design to those described by Ussing and Zerahn (6). The electrical potential difference across the skin was measured with a pair of calomel electrodes and a Keithley model 610 A electrometer. Current was passed through the skin from an external source *via* Ag-AgCl electrodes and was measured with a Weston microammeter. After mounting in the chamber, the skins were allowed to equilibrate with the bathing solutions for at least 1 hour and were then short-circuited (6). Throughout the experiments, the short-circuit current has been taken as a measure of net Na transport across the skin. Previous investigations have indicated that this current is equal to the net Na flux when the skin is treated with ADH (6) or Ca (4) as well as under control conditions (6).

The skins were initially bathed on both sides with a Ringer's solution containing 92 mM NaCl, 23 mM choline chloride, 2.4 mM $NaHCO_3$, and 2 mM KCl, but no added Ca. The effect of Ca on short-circuit current was tested by increasing the Ca concentration in the outside bathing solution only. The Ca content of this solution was altered, without changing the Na or Cl concentrations, by replacing part or all of the choline chloride by $CaCl_2$. The usual Ca concentration employed was 11.3 mM, but in one series of experiments, the effects of several different Ca concentrations were tested on the same skin using the method previously described by Curran and Gill (4). In all experiments, the Ca-free control solution bathing the outside of the skin contained 0.5 mM ethylene diaminetetraacetate (di-Na salt) as previously reported (4). Commercial ADH (Pitressin, Parke, Davis and Co.) was added to the inside bathing solution, usually at a concentration of 0.25 U/ml.

In some experiments, unidirectional influx of Cl across the skin was measured using Cl^{36}. Tracer was added to the outside solution and samples were withdrawn periodically from the inside solution. The samples were dried on aluminum planchets and counted on a windowless flow counter. Control flux was measured for two 30 minute periods, either Ca or ADH was then added as described above, and flux was measured for two 30 minute periods.

RESULTS AND DISCUSSION

The initial series of experiments tested the effect of a single Ca concentration (11.3 mM) on Na transport in the presence and in the absence of ADH. Since

the effect of Ca added to the outside solution was completely reversible, Ca could be tested before and after ADH treatment of the same skin, thereby eliminating the necessity for comparing different pieces of skin. The results of one experiment are shown in Fig. 1. Addition of Ca caused a decrease in short-circuit current which was reversed when the outside solution was replaced with Ca-free Ringer's. ADH caused an appreciable increase in current and subsequent treatment with Ca again caused a decrease of approximately the same magnitude as that observed before treatment with ADH. The

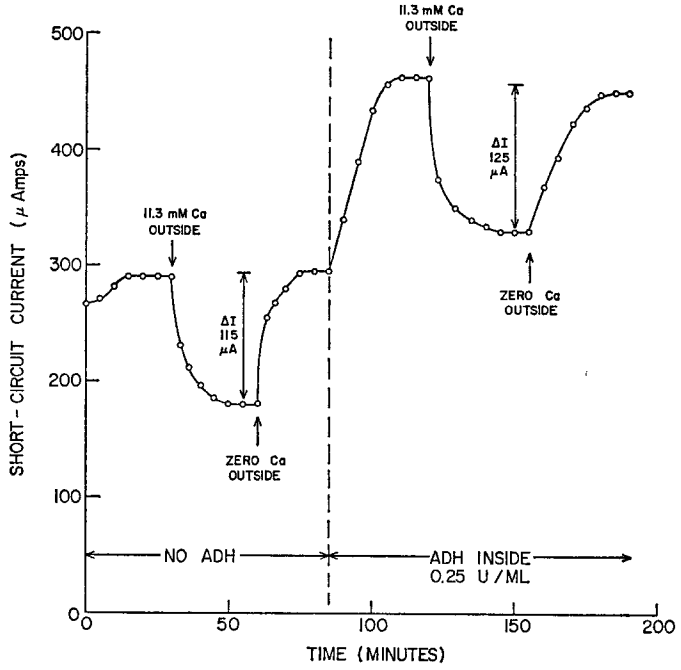

FIGURE 1. Effect of Ca on net Na transport across frog skin before and after treatment with ADH. Ca was added to the outside solution only and ADH was added to the inside solution.

results of 8 such experiments are summarized in Table I in which the short-circuit current has been expressed as net Na flux in terms of μeq/hr.cm^2. Treatment with ADH caused a 50 per cent increase in Na transport, but the absolute decrease in transport caused by Ca was not significantly altered by ADH. Thus, the effect of Ca added to the outside solution appears to be independent of the presence of ADH.

In a second series, the converse experiment was performed to test the effect of ADH in the presence and absence of Ca. Since the effect of ADH was less easily reversed than that of Ca, paired skins from the same frog were used. After mounting and equilibration, one skin was treated with 11.3 mM Ca in

the outside solution and the other skin was kept in Ca-free Ringer's solution. ADH was then added simultaneously to the inside solution of both skins and the resulting increases in short-circuit current were observed. The results of 7 experiments are summarized in Table II. ADH caused approximately the same increase in current in both Ca-treated and control skins, indicating that the action of ADH on Na transport is independent of the presence of Ca in the outside solution.

The results of these experiments appear to suggest an important difference in the effect of ADH on toad bladder and frog skin. Experiments on toad bladder (7) have indicated that Ca must be present in the bathing solutions in order to obtain any increase in short-circuit current upon addition of ADH,

TABLE I

EFFECT OF CA ON FROG SKIN TREATED WITH ADH

Experiment No.	Decrease in net Na flux caused by Ca (11.3 mM)*		
	No ADH	ADH (0.2 U/ml)	Δ‡
		μeq/hr. cm²	
1	0.28	0.24	−0.04
2	0.36	0.41	0.05
3	0.45	0.55	0.10
4	0.83	0.85	0.02
5	0.39	0.37	−0.02
6	0.29	0.33	0.04
7	0.63	0.70	0.07
8	0.63	0.66	0.03
Mean ±SE	0.48±0.08	0.51±0.08	0.03±0.02

* Mean values for net Na flux under the different conditions were, in μeq/hr. cm², control 1.28, Ca 0.80, ADH 1.90, ADH + Ca 1.39.

‡ Δ = decrease (ADH) − decrease (no ADH).

while in the present experiments on frog skin, appreciable effects of ADH have been observed in the absence of Ca. However, this discrepancy may simply be a reflection of the difference in function of the bladder and the skin in Ca-free solutions. In the bladder, removal of Ca from the bathing solutions causes a decrease in short-circuit current (7). On the other hand, the frog skin appears to retain sufficient Ca for normal function when bathed with nominally Ca-free solutions (4), although a decrease in Na transport does occur if a chelating agent is added to the inside solution under these conditions (8). Thus, we cannot conclude that the effect of ADH is completely independent of Ca in the skin, since the skin appears to retain sufficient Ca for normal function in the absence of this ion in the bathing solutions.

In fact, some experiments carried out in the present series have suggested

that there may be a Ca requirement for ADH action in the skin. The results presented in Table II were obtained during the winter at a time when ADH was found to give a sustained increase in Na transport. A similar series was attempted in late spring, and the effect of ADH was found to be quite transient. Addition of the hormone caused the usual increase in current, but current fell rapidly again, usually to a level below that before addition of the hormone. In 5 experiments, some differences were observed between control and Ca-treated skins. The effect of ADH was definitely more sustained in Ca-treated skins and current did not fall below the control level. There is, at present, no adequate explanation for this observation, but it may reflect a relative deficiency of Ca in some critical region of the skin. The results of these experiments have not been included in Table II because the transient nature of the ADH effect made quantitative comparison of control and Ca-treated skins impossible.

TABLE II

EFFECT OF ADH ON FROG SKIN TREATED WITH Ca

Experiment No.	Increase in net Na flux caused by ADH (0.2 U/ml)*		
	No Ca	Ca (11.3 mM)	Δ‡
		μeq/hr. cm²	
1	0.16	0.26	0.10
2	0.52	0.56	0.04
3	0.52	0.42	−0.10
4	0.14	0.13	−0.01
5	0.60	0.65	0.05
6	0.23	0.18	−0.05
7	0.20	0.23	0.03
Mean ±SE	0.34±0.08	0.35±0.08	0.01±0.03

* Mean values for net Na flux under the different conditions were, in μeq/hr. cm², control 0.59, ADH 0.96, Ca 0.41, Ca + ADH 0.77.

‡ Δ = increase (Ca) − increase (no Ca).

Under the more optimal conditions of a sustained ADH effect, Ca and ADH appear to act independently in altering Na transport across the skin. However, these experiments cannot be considered an adequate test of possible interrelationships, particularly since they are based on only one concentration of each agent. In fact, the results presented in parts of Fig. 1 and Tables I and II taken by themselves, might suggest the existence of competition between Ca and ADH. Thus, addition of Ca to the solution bathing the outside of a skin treated with ADH causes a decrease in Na transport, an apparent reversal of the ADH effect. Conversely, addition of ADH to a skin treated with Ca causes an increase in transport apparently reversing the original Ca

inhibition. However, the observation that the magnitude of the change in Na transport caused by one agent is not altered by the presence of the other does not seem to be consistent with a competition.

In order to test for competition more directly, a series of experiments were carried out in which various Ca concentrations were studied in the presence and absence of ADH. In each experiment the effects of 5 different Ca concentrations were tested on paired skins from the same frog. One piece of skin served as a control while the other was treated with ADH throughout the experiment. The results can be most conveniently expressed in terms of the

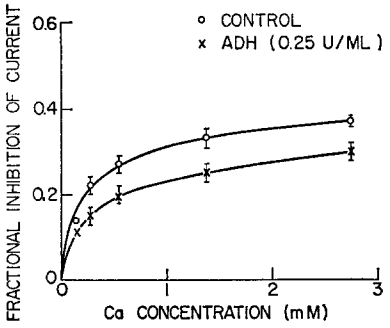

FIGURE 2. Fractional inhibition (α) of Na transport as a function of Ca concentration in the outside solution. The points represent the average values obtained in 8 experiments using a pair of skins from the same frog for each experiment, one serving as control and one treated with ADH. The bars indicate ± one standard error of the mean.

fractional decrease or inhibition of current caused by Ca. Fractional inhibition (α) is defined by the relationship

$$\alpha = 1 - \frac{I}{I_o}$$

in which I is current in the presence of Ca and I_o is the control current observed in Ca-free Ringer's solution. The averaged results of 8 experiments are shown in Fig. 2 in which α is plotted against Ca concentration in the outside solution. The effect of Ca approached a maximum with increasing concentration in both control skins and in those treated with ADH. As previously discussed (4) this indicates that the total Na transport can be divided into two parts, one sensitive to Ca and one insensitive to Ca. To compare properly the effect of Ca in the two cases, only the Ca-sensitive portion of the current should be considered since the maximum possible inhibition is different for the different conditions. The results may then be summarized in terms of the fractional inhibition of the Ca-sensitive portion of the current (α') which is defined by the relationship

$$\alpha' = 1 - \frac{I - I_\infty}{I_o - I_\infty} = \frac{\alpha}{\alpha_m}$$

in which I_∞ is the Ca-insensitive part of the current and α_m is the maximum value of α at high Ca concentrations. As discussed by Curran and Gill (4) the value of α_m may be obtained from the intercept of a plot of the reciprocal of α against the reciprocal of the Ca concentration and the values of α' at each Ca concentration may then be calculated from the above relationship. The values of α' thus obtained are shown in Fig. 3 as functions of the Ca concentration. There is no significant difference between the points for control skins and those treated with ADH, indicating that the two agents do not compete. If there were any significant competition between Ca and ADH for the same site of action, the points for ADH-treated skins would be shifted toward the right relative to the points for control skins.

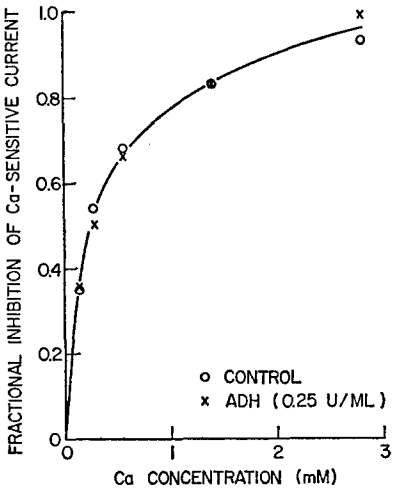

FIGURE 3. Fractional inhibition of Ca-sensitive part of Na transport (α') for control and ADH-treated skins as a function of Ca concentration in the outside solution. The points were calculated from the data in Fig. 2 using the method described in the text.

The absence of competition was confirmed in 4 experiments which were carried out to examine the influence of different ADH concentrations. Paired skins were again used; both were treated with a much lower ADH concentration (0.005 U/ml) than in previous experiments and one skin was then treated with 11.3 mM Ca. Finally, additional ADH (0.1 U/ml) was added to both skins. The averaged results of these experiments are presented in Fig. 4. The initial ADH treatment produced nearly maximal effect on Na transport and transport decreased upon addition of Ca. A considerable increase in ADH concentration failed to produce a significant reversal of the Ca inhibition again indicating that there is no competition between these agents.

The results presented in Figs. 3 and 4 show that Ca added to the outside solution and ADH added to the inside solution must affect Na transport at different sites since there is no competition between them. The results presented in Tables I and II also indicate that the action of each agent is entirely independent of the presence of the other. This indicates that Ca does not affect the ADH-stimulated part of the Na transport and, conversely, that

ADH does not alter that portion of the transport which is sensitive to Ca. For example, if Ca were altering the ADH-stimulated part of the transport, it should have a greater effect on ADH-treated skins than on control skins, but the data in Table I indicate that the effect is the same in both cases. This independent nature of the two effects offers additional information concerning the arrangement of sites of action on the over-all path of Na transport through the skin. These sites could be arranged in parallel or in series; a parallel arrangement would indicate that both sites were on the same barrier to Na movement while a series arrangement would imply different barriers. The observation that Ca and ADH act independently can only be explained by a parallel arrangement of their sites of action. If the sites were arranged in

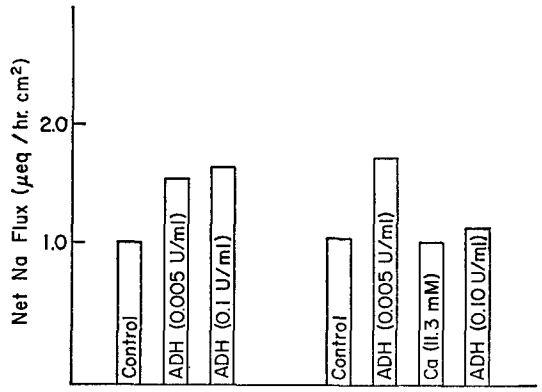

FIGURE 4. The effect of increasing ADH concentration on Na transport in skins previously treated with both Ca and a low dose of ADH. The results are the average of 4 experiments. The height of each bar represents the steady state Na transport under the condition indicated within the bar. Paired skins from the same frog were used in each experiment. One skin was treated only with 2 concentrations of ADH while the other was treated with Ca between the two ADH treatments and the Ca remained present during the rest of the experiment.

series, the ADH-stimulated portion of Na transport would necessarily be sensitive to treatment of the system with Ca, and the agents would not appear to act independently even if there were no direct competition between them. This conclusion suggests that the sites of action of Ca and ADH on Na transport must both be located on the same barrier to Na movement but, as discussed above, there must be two separate sets of sites.

These conclusions apply only to Na transport and not necessarily to the effects of Ca and ADH on the movement of other substances across the skin. Peterson and Edelman (2), in their studies of the effects of Ca and ADH on water permeability of the toad bladder, have obtained results indicating a competition between these agents. The difference between these results and the present ones could be ascribed to differences between the frog skin and

toad bladder or to the fact that Peterson and Edelman studied the effect of Ca added to the serosal (or inside) solution rather than to the outside as in the present experiments. On the other hand, an equally possible explanation is suggested by the observations of Bourguet and Maetz (9) who have obtained evidence indicating that the effect of ADH on water movement across frog skin and toad bladder may involve a different mechanism than its effect on Na transport. The observation of a competition between Ca and ADH in effects on water movement across toad bladder but not on Na transport across frog skin may be a reflection of different mechanisms of action in the two cases.

TABLE III

EFFECT OF Ca AND ADH ON Cl INFLUX ACROSS SHORT-CIRCUITED FROG SKIN

Experiment No.	Cl influx			
	Control	Ca (11.3 mM)*	Control	ADH (0.20 U/ml)*
		μeq/hr. cm²		
1	0.13	0.09	0.11	0.10
2	0.10	0.05	0.06	0.07
3	0.08	0.05	0.11	0.07
4	0.10	0.05	0.09	0.07
5			0.04	0.04
6			0.04	0.06
7			0.08	0.07
8			0.16	0.20
9			0.09	0.09
Mean ±SE	0.10±0.01	0.06±0.01	0.09±0.01	0.09±0.02

* In these experiments Ca decreased mean net Na flux from 1.51 to 1.03 μeq/hr. cm² and ADH increased mean net Na flux from 1.51 to 2.22 μeq/hr. cm².

Previously reported experiments suggested, however, at least one other case in which the effects of Ca and ADH on frog skin might be independent. Ussing (10) had reported that ADH had no consistent effect on Cl flux across the short-circuited skin, but Curran and Gill (4) found that Ca decreased Cl flux. A series of experiments were carried out in an attempt to confirm these results for *Rana pipiens*. Cl influx across the short-circuited skin was measured under control conditions and following treatment with either Ca or ADH. The results are summarized in Table III. Ca caused a decrease in Cl influx of approximately the same magnitude as the effect on Na transport as previously reported (4). ADH, on the other hand, caused no change in Cl flux even though there was an appreciable increase in Na transport. The effects of Ca and ADH on Cl flux are, therefore, markedly different, again suggesting differences in their action on permeability properties. However, similar experiments must be carried out on water movement before any conclusion

can be drawn concerning the relationship between the effects of Ca and ADH in this case. The present experiments show only that the two agents do not compete in their effects on Na transport and that they have entirely different effects on Cl diffusion across the skin.

As discussed above, the effects of ADH and Ca on Na transport appear to be located on the same barrier to Na movement even though they are added to the solutions bathing opposite sides of the skin. There appear to be at least two such barriers in the skin (11–13), and Koefoed-Johnsen and Ussing (13) have suggested that these are the outward and inward facing membranes of the transporting cells. The present experiments cannot distinguish between effects on these two barriers. However, previous evidence suggests that both Ca and ADH may affect a barrier located at the outer side of the skin. ADH has been shown to alter the permeability of the outward facing membrane of the frog skin to water (14) and the permeability of the analogous membrane in the toad bladder to water, urea, and Na (15, 16). Less direct evidence has led to the suggestion that Ca alters the permeability of the outer membrane of the frog skin to Na, Cl, and water (4, 17). On the basis of these considerations, a working hypothesis of the mechanism by which Ca and ADH could affect Na transport can be suggested. This hypothesis represents a combination of previous suggestions made by Curran and Gill (4), Skou and Zerahn (18), and Leaf (15) and is based on the model of the frog skin proposed by Koefoed-Johnsen and Ussing (13).

We shall assume that both Ca and ADH alter the Na permeability of the outer membrane of the transporting cells which is considered to present only a passive barrier to Na movement. Further, the active transport system itself, assumed to be located at the inward facing membrane, will be considered far from saturated and able to transport whatever Na is presented to it by passage through the outer membrane. On the basis of this model, the decrease in Na transport caused by Ca could be explained by a decrease in Na permeability of the outer membrane which would reduce the rate of Na entry into the transporting cells. The active transport system itself will not be altered by Ca and its continued function would then reduce the steady state Na concentration in the cell and this, in turn, would lead to a decrease in the rate of active Na transport. Conversely, the effect of ADH could be explained in terms of an increase in Na permeability of the outer barrier followed by an increase in Na entry and in Na concentration in the cells. This would result in an increase in net active Na transport. This hypothesis suggests that the Na permeability of the outer membrane of the skin plays an important role in controlling the rate of net active Na transport across the skin. A similar suggestion has been made by Frazier, Dempsey, and Leaf (16) regarding Na transport across the toad bladder.

The specific mechanisms by which Ca and ADH might alter permeability

of the outer membrane are not clear, nor is the relationship between the two sites of action which appear to be located on this barrier. The results of measurements on Cl fluxes suggest that the changes brought about by ADH occur at a part of the barrier which is not involved in Cl movement. Both sets of sites may be involved in water movement since Ca decreases net water flow across the skin under an osmotic pressure gradient (17) while ADH increases it (19). At present, there is no evidence indicating whether or not these two parts of the barrier behave differently with respect to Na. The suggestion has been put forward that ADH causes changes in permeability by increasing the size of pores in the membrane (19, 20). It is possible that Ca could decrease pore size by binding and steric hindrance, and some indication of such an effect has been obtained in studies on kidney slices (3). If this view is correct for frog skin, the pores affected by ADH would appear to be negatively charged since they seem to be impermeable to Cl, while those affected by Ca seem to be permeable to both Na and Cl.

In conclusion, the present experiments have shown that Ca and ADH do not compete in their effects on Na transport across the frog skin. The sites at which Ca and ADH act appear to be located on parallel paths of Na movement and are apparently on the outward facing permeability barrier of the skin. The effects of both agents could be explained in terms of changes in the Na permeability of this outer barrier with no direct effect on the active Na transporting system itself. The experiments described in the following paper were designed to investigate these suggestions concerning the mechanisms of Ca and ADH action in more detail.

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