*J. Pharm. Pharmac.*, 1971, **23**, 262–269 *Received October 7, 1970*

# Modifications of the responses to antidiuretic hormone by hydrolytic enzymes

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Proteolytic enzymes (trypsin and α-chymotrypsin) and lipolytic enzymes (phospholipases A & C) have been used to treat both surfaces of the skins of frogs and toads. The effects of the enzymes on sodium transport (frog skin) and hydraulic flow (toad skin) have been measured, together with the effects on the responses to ADH. With the experimental conditions used, proteolytic enzymes reduced resting sodium transport but had no major effect on the responsiveness to ADH. Conversely these enzymes had no effect on resting hydraulic flow but reduced or abolished the responses to ADH. Phospholipases applied to the inner surfaces of skins affected neither water nor sodium movement but abolished the responses to ADH. Phospholipases applied to the outside enhanced the effect of ADH on water flow but had no effect on the increased sodium movement caused by ADH. The results are discussed in terms of the possible removal of calcium binding sites by phospholipases.

Proteolytic and lipolytic enzymes have been used to treat the skins of frogs (*Rana temporaria*) and toads (*Bufo marinus*) to see how these agents affect the active transport of sodium and hydraulic flow down an osmotic gradient. In addition the responses of epithelia to the antidiuretic hormone, arginine vasopressin (ADH) have been tested following enzymic treatment. It was hoped to gain some insight into the nature of the permeability changes which result from hormone stimulation.

## METHODS

Sodium transport was measured by the short circuit current (SCC) technique of Ussing & Zerahn (1951) using a standard procedure. The skin area was 4·5 or 7 $cm^2$. Experiments were performed in pairs with one skin serving as control. In some instances test and control skins were taken from the same animal. After exposure of the test skin to enzyme the effect of the hormone was tested. ADH and enzyme were added simultaneously to the appropriate side of the control skin. Amphibian skins are relatively insensitive to the mammalian hormone, arginine vasopressin, compared to the natural hormone, arginine vasotocin. Hence ADH was added only once to each tissue in high concentration so that a maximal response was obtained.

Water flow was measured volumetrically by observing the movement of a meniscus along a 1 ml graduated pipette. The pipette was sealed into an open-ended chamber which was closed off by the tissue such that the outer surface of the skin was within the chamber. The inner skin surface was bathed in aerated Ringer solution in a beaker. The solution bathing the outer skin surface was a ten times dilution of Ringer solution, thus the skin was subjected to a pressure gradient of 200 mOsm. The skin area was 6·2 $cm^2$.

The Ringer solution had the following composition (mM) NaCl, 111; KCl, 1·9; $CaCl_2$, 1·08; $NaH_2PO_4$, 0·083; $NaHCO_3$, 2·4 and glucose, 11·1. This solution had a

pH of 7·6 when bubbled with air. In the experiments on water flow $Na_2HPO_4$ was substituted for $NaH_2PO_4$ to give a pH of 8·0. The effects of ADH on water permeability are maximal at this pH.

The following enzymes were used: α-chymotrypsin (EC 3.4.4.5, salt free, 11 500 ATEE u/mg, Seravac Labs Ltd.), trypsin (EC 3.4.4.4, salt free, 10 000 BAEE u/mg, Seravac Labs Ltd.), phospholipase C (EC 3.1.4.3, α-toxin of *Cl. welchii*, phosphatidylcholine choline phosphohydrolase, Mann Research Labs Inc.) and phospholipase A (EC 3.1.1.4) *Naja naja* venom, phosphatide acyl-hydrolase, Koch-Light Labs Ltd.).

ADH was Pitressin (Parke Davis & Co.).

## RESULTS

### *Proteolytic enzymes and sodium transport*

Trypsin, EC 3.4.4.4 (0·1 mg/ml) and α-chymotrypsin, EC 3.4.4.5 (1 mg/ml) produced a progressive fall in short-circuit current (SCC) and transepithelial potential (PD) when applied to the inner surface of frog skin. Addition of ADH after this always produced an increase in SCC which was related to the ability of the skin to transfer sodium. The percentage increase in SCC has been calculated relative to the SCC which would have existed, at the time of the peak response, if ADH had not been added (Fig. 1). Table 1 gives the percentage increases in SCC in six paired experiments in which the initial SCC had fallen by 25 to 85% of its initial value after treatment with α-chymotrypsin.

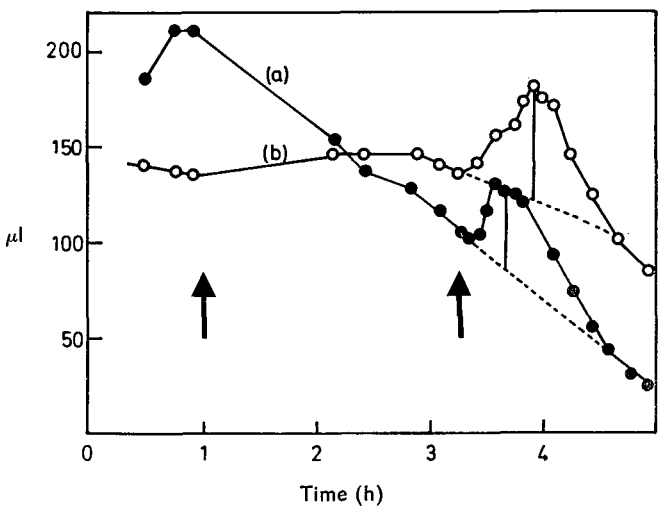


FIG. 1. SCC (μA) of two pieces of frog skin (4·5 cm²). Skin (a) was bathed on the inner surface with α-chymotrypsin (1 mg/ml) (enzyme added at first arrow). Responses to ADH (0·6 u/ml) was tested in both skins 2 h after addition of the enzyme (second arrow). The increase in SCC caused by hormone is indicated by the vertical lines on the figure at the times of the peak responses.

There was no significant difference between the responses of test and control skins. Similar results were obtained when trypsin (0·1 mg/ml) was used to treat the inner surface of skins (Table 1).

Table 1. *The effect of α-chymotrypsin* (1 mg/ml) *and trypsin* (0·1 mg/ml) *applied to the inner surface of frog skin on the response to ADH.*

| % increase (SCC) to ADH (0·6 u/ml) after α-chymotrypsin (1 mg/ml) | % increase (SCC) to ADH (0·6 u/ml) in control | % reduction in SCC caused by α-chymotrypsin | Duration of exposure to α-chymotrypsin (min) |
|---|---|---|---|
| 45 | 44 | 79 | 150 |
| 39 | — | 50 | 120 |
| 40·5 | 89 | 0 | 120 |
| 16 | 112 | 85 | 180 |
| 48 | 45 | 50 | 220 |
| 30 | 50 | 75 | 210 |
| 120 | 84 | 25 | 45 |
| 48·4 ± 12·6 (s.e.) | 70·7 ± 11·6 (s.e.) | $0·3 > P > 0·2$ | |
| % increase (SCC) to ADH (0·6 u/ml) after trypsin (0·1 mg/ml) | % increase (SCC) to ADH (0·6 u/ml) in control | % reduction in SCC caused by trypsin | Duration of exposure to trypsin (min) |
| *47 | 33 | 43 | 75 |
| *36 | 48 | 55 | 150 |
| 34 | 18 | 57 | 120 |
| 39 (mean) | 33 (mean) | | |

*Test and control skin was taken from the same animal.

Proteolytic enzymes also caused a progressive fall in SCC and PD when applied to the outer surface of frog skin. Table 2 shows the values for the percentage increases in SCC with ADH in five treated and five control skins.

The average response was greater in treated skins than in controls, however this difference was not significant due to the wide variation in values. When the SCC becomes very low, for example the last experiment in Table 2, other factors apart

Table 2. *The effects of α-chymotrypsin* (1 mg/ml for 2h) *applied to the outer surface of frog skin on the response to ADH* (0·6 u/ml)h.

| % increase (SCC) to ADH (0·6 u/ml) after α-chymotrypsin (1 mg/ml) | % increase (SCC) to ADH (0·6 u/ml) in control | % reduction in SCC caused by α-chymotrypsin |
|---|---|---|
| 120 | 78 | 50 |
| 154 | 43 | 56 |
| 29 | 12 | 60 |
| 18 | 0 | 20 |
| 0 | 100 | 90 |
| 64·2 ± 30·5 (s.e.) | 46·6 ± 19·0 (s.e.) | $0·6 > P > 0·5$ |

from the ability of ADH to affect its receptor may be responsible for the diminished response. Fig. 2A,B illustrates this point. It can be seen that when the SCC had been reduced to 90% of its original value ADH had no effect on SCC but did cause an increase in conductance (ratio of SCC to PD) greater than that in the control skin.

*Lipolytic enzymes and sodium transport*

Experiments were performed with phospholipase A (naja venom) EC 3.1.1.4 and phospholipase C EC 3.1.4.3 with a protocol similar to that with proteolytic enzymes. In these experiments aeration of the Ringer solution was stopped on the side to which the enzyme was added since bubbling inactivates these enzymes (Macfarlane &

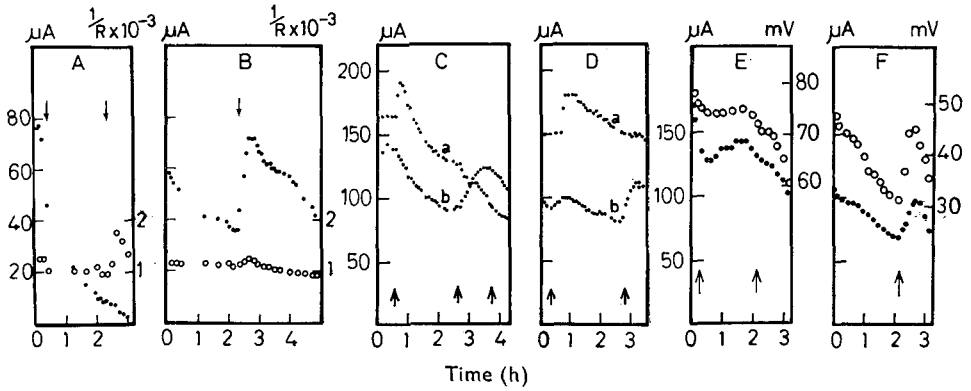


FIG. 2. A and B. SCC ($\mu$A) and conductance (ratio SCC/PD) of two pieces of frog skin (4·5 cm$^2$). Skin (A) was bathed on the outer surface with $\alpha$-chymotrypsin (1 mg/ml) (enzyme added at first arrow). Responses to ADH (0·6 u/ml) were tested in both skins (at second arrow).

C and D. SCC ($\mu$A/4·5 cm$^2$) in two paired experiments in which naja venom (50 $\mu$g/ml) was applied to the inside of test skins (C) at the first arrow. In (C) the responses to ADH (0·1 u/ml and 0·6 u/ml) are shown at the second and third arrows. In (D) the response to ADH (0·1 u/ml) is shown at the second arrow. In (C) both skins were taken from the same animal. Note the stimulation produced by the enzyme.

E and F. SCC ($\mu$A closed circles) and PD (mV open circles) of two pieces of frog skin. In (E) the skin was exposed on the inner surface to phospholipase C (50 $\mu$g/ml) (first arrow). After 2 h ADH (0·3 u/ml) was added to inside solution bathing the test and control skins (F). Note the increase in SCC caused by the enzyme.

Left hand ordinates on B, D and F are the same as those on A, C and E respectively.

Knight, 1941). Unlike the proleolytic enzymes the lipolytic enzymes, in a concentration of 50 $\mu$g/ml, had little effect on SCC and PD when applied in the inner bathing solution apart from an initial stimulant action, after which the SCC fell at a rate comparable to the control. The fall in SCC was most probably due to the reduced oxygen tension since it fell in the control preparations not exposed to enzyme. Aeration was always stopped in control and test skins at the same time.

In spite of the high SCC's prevailing in test skins exposed to enzyme the response to ADH was abolished. This is shown in Fig. 2 C, D and Table 3. The inhibition of the response to ADH by phospholipase A was highly significant.

In six experiments with phospholipase C (50 $\mu$g/ml over 0–120 min) the development of the ADH (0·3 u/ml) inhibition was studied. Complete inhibition of the

Table 3. *The effects of phospholipase A* (50 $\mu$g/ml) *applied to the inner surface of frog skin on the response to ADH* (0·3 u/ml).

| % increase (SCC) to ADH (0·3 u/ml) after phospholipase A (50 $\mu$g/ml) | % increase (SCC) to ADH (0·3 u/ml) in control | Duration of exposure to phospholipase A (min) |
|---|---|---|
| 39 | 72 | 120 |
| 6 | 38 | 135 |
| *0 | 71 | 120 |
| *7 | 55 | 120 |
| 19 | 79 | 120 |
| 0 | 65 | 60 |
| 0 | 17 | 120 |
| 0 | 100 | 120 |
| 0 | 0 | 120 |
| 7·9 ± 4·4 (s.c.) | 55·2 ± 10·6 (s.c.) | 0·001 > *P* |

* Test and control skins came from the same animal.

response was achieved with a 2 h exposure (% increase in SCC to ADH: 57, 48, 13, 26, 0, 0, to 0, 30, 60, 90, 120, 120 min exposure respectively). As with phospholipase A the response to ADH was abolished after treatment with phospholipase C at a time when the SCC remained high (Fig. 2E,F). Phospholipase C (100 $\mu$g/ml for 2 h) was used to treat the outer surface of frog skins in five paired experiments. There was no effect on the response to ADH in this series. The average % increase in SCC in the control preparations was 32·7% and in the enzyme treated preparations was 33%. In three further unpaired experiments the outer surfaces of skins from the same batch of frogs were exposed to phospholipase C 500 $\mu$g/ml for 2 h. The average percentage increase in SCC in these experiments was 24·8%. Over the period of exposure the SCC of those skins exposed on the outer surface to phospholipase C was maintained. Thus no effects of this enzyme on either resting or stimulated sodium transport were demonstrated when it was applied to the outer skin surface.

*Water movement, enzymes and ADH*

In initial experiments the hydraulic flow in frog skin was found to be small (5 $\mu$l/cm$^2$h$^{-1}$) and the increase caused by ADH was small and irregular (mean increase of 1 $\mu$l/cm$^2$h$^{-1}$ in 17 experiments). In consequence we used the skins of toads (*Bufo marinus*) which have higher resting and stimulated rates of water flow. The size of the animals was such that test and control skins were always taken from the same animal. When the effects of enzymes were to be tested on the outer surface of the skins the skins were first exposed to enzyme in the apparatus used for SCC measurements for 1 h. After this the skins were mounted in the apparatus for water flow measurement. This procedure was necessary since ADH is added to the serosal skin surface which must, of consequence, be accessible during flow measurement. In all experiments the Ringer solution bathing the outer skin surface was diluted ten times. Table 4 gives the results of 16 paired experiments in which test and control skins were taken from the same animal.

Table 4. *The effects of enzymes* (*α-chymotrypsin* 1 mg/ml *and phospholipase C* 50 $\mu$g/ml) *on hydraulic flow in toad skin.* Numbers of experiments indicated in parentheses. ADH concentration was 0·1 $\mu$/ml.

| Enzyme treatment | Test skin | Control skin | |
|---|---|---|---|
| α-Chymotrypsin inside .. | 46·3 ± 5·1 (4) ($\mu$l/cm$^2$h$^{-1}$) | 56·9 ± 14·5 (4) ($\mu$l/cm$^2$h$^{-1}$) | $0·6 > P > 0·5$ |
| α-Chymotrypsin inside + ADH .. .. .. | 34·9 ± 12·2% (4) | 186·9 ± 40·5% (4) | T/C = 0·19 $0·01 > P > 0·005$ |
| α-Chymotrypsin outside .. | 62·0 ± 20·1 (4) ($\mu$l/cm$^2$h$^{-1}$) | 46·6 ± 5·5 (4) ($\mu$l/cm$^2$h$^{-1}$) | $0·5 > P > 0·4$ |
| α-Chymotrypsin outside + ADH .. .. .. | 35·8 ± 5·3% (4) | 93·3 ± 11·2% (4) | T/C = 0·38 $0·02 > P > 0·01$ |
| Phospholipase C inside .. | 34·4 ± 2·8 (4) ($\mu$l/cm$^2$h$^{-1}$) | 40·3 ± 6·9 (4) ($\mu$l/cm$^2$h$^{-1}$) | $0·5 > P > 0·4$ |
| Phospholipase C inside + ADH .. .. .. | 41·4 ± 19·8% (4) | 190·8 ± 102% (4) | T/C 0·22 $0·2 > P > 0·1$ |
| Phospholipase C outside .. | 54·4 ± 7·5 (4) ($\mu$l/cm$^2$h$^{-1}$) | 46·1 ± 7·4 (4) ($\mu$l/cm$^2$h$^{-1}$) | $0·6 > P > 0·5$ |
| Phospholipase C outside + ADH .. .. .. | 124·4 ± 17·8% (4) | 68·2 ± 19·3% (4) | T/C 1·82 $P > 0·001$ |

The following features can be found in Table 4. Exposure of either surface of toad skin to either phospholipase C (50 $\mu$g/ml) or $\alpha$-chymotrypsin (1 mg/ml) for 1 h had no statistically significant effect on hydraulic flow in the absence of ADH. The wide variation in flow rates (even although two pieces of skin are from the same animal) probably masks a slight increase in flow rate caused by $\alpha$-chymotrypsin applied to the inner skin surface. Further evidence for this is that when $\alpha$-chymotrypsin was applied to the inner surface, after a control period, there was a definite increase in flow (Fig. 3A). In all the experiments the flow rates were linear for a

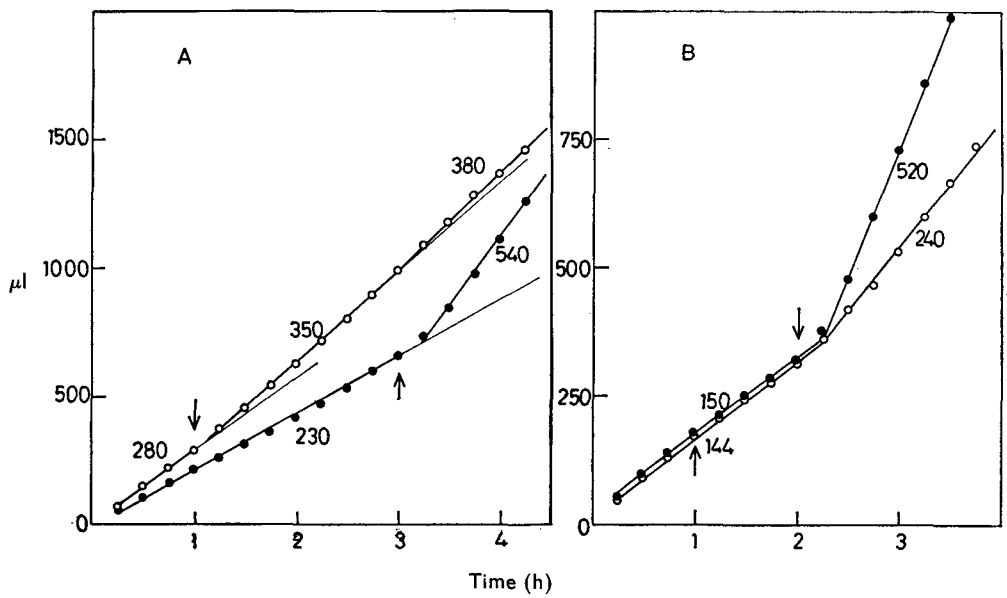


FIG. 3. Hydraulic flow across toad skin. A. $\alpha$-Chymotrypsin (1 mg/ml) or, B, phospholipase C (50 $\mu$g/ml) was added to the inner bathing solution of the test skin (open circles) at the first arrow. ADH (0·1 $\mu$/ml) added to both skins at the second arrow. The numbers on the curves indicate flow rates in $\mu$l/h.

particular condition. Fig. 3A,B are typical of all the experiments. Both types of enzyme caused a significant and similar inhibition of the ADH response when the enzymes were applied to the inside. Obviously this is a function of both concentration of enzyme and of duration of exposure. For example, phospholipase C, 50 $\mu$g/ml completely abolished the ADH response when the duration of exposure was increased to 90 min. Chymotrypsin significantly inhibited the response to ADH when the enzyme was applied to the outer surface of the skin. A striking effect was seen when phospholipase C was used to treat the outer face, when the response to ADH was almost doubled.

## DISCUSSION

It was not expected that very specific lesions would result when enzymes were applied to tissues, however, in the absence of specific pharmacological antagonists efforts to discover the location, nature and diversity of receptors are made difficult. Fortunately these results show that enzymes can have differential effects on sodium transport and hydraulic flow in epithelia from which some conclusions can be made.

Phospholipases, in the concentrations used, had no inhibitory effect on either SCC

or hydraulic flow when applied to the inner surface of skins, while the responses to ADH were inhibited or abolished. The degree of inhibition was similar whether SCC or water flow was measured (when using the same enzyme concentration for the same duration) although it must be remembered that two different tissues were used. The current view of the action of ADH is that it stimulates adenylcyclase bound on the inner facing membranes to generate cyclic 3′5′-AMP which then diffuses across the cell to affect the permeability of the outer facing membranes (Orloff & Handler, 1967). The increase in SCC and water flow caused by theophylline is taken as evidence for the continual endogenous production of cyclic AMP. Normally this is converted to cyclic 5′-AMP by phosphodiesterase, an enzyme inhibited by theophylline. The inhibition of the ADH response by phospholipases may mean that adenyl cyclase is inactivated or that some ADH-receptor grouping associated with the cyclase is affected. If the former then endogenous production of cyclic AMP is not necessary for resting transport and flow. If the latter then the receptor grouping may not be obligatory for the functioning of the cyclase.

The idea of multiple specific receptor groups associated with adenyl cyclase has found much favour to explain how a variety of different hormones can work through a common system (Sutherland, Robinson & Butcher, 1968). There are, however, alternative views. Rasmussen (1970) has discussed the possibility that multiple and perhaps sequential second messengers may be involved in hormone action, for instance calcium ions. There is considerable evidence that calcium can bind to acidic groupings in phospholipids (for references see Cuthbert, 1967) and lipolysis might remove calcium on binding sites. In addition, Bentley (1959) has suggested that the ADH receptor may have an absolute requirement for calcium. Although others (Hays, Singer & Malamed, 1965) have attributed the fall in SCC following calcium removal as due to disaggregation of the epithelial layer it seems unlikely that this effect would inhibit hydraulic flow caused by ADH, as was found by Bentley. There is much confusion about whether adenyl cyclase is a calcium requiring enzyme (for references see Breckenridge, 1970) and it has been reported that ADH can mobilize bound calcium in toad bladder epithelial cells (Schwartz & Walker, 1969). The possibility remains that the prime effect of ADH may be to mobilize membrane bound calcium and the inhibition of ADH by phospholipases may be relevant to this action.

It is worth remembering that phospholipase enzymes themselves cause an initial stimulation of SCC, which might result from calcium liberation due to removal of binding sites.

Proteolytic enzymes appear to affect the active transport mechanisms rather than the ADH receptor when applied to the inner surface. The ability of ADH to stimulate SCC was retained in proportion to the ability of the skin to transport sodium. Marchesi & Palade (1967) found that trypsin treatment of red cells inactivated ($Na^+$–$K^+$) activated ATPase. Proteolytic enzymes applied to the inner surface severely inhibited the stimulation of water flow caused by ADH while the enzymes themselves caused an increase in flow. The contrasting effects of proteolytic enzymes on the SCC and flow responses to ADH may indicate separate "water" and "sodium" receptors. Others have concluded that two types of adenyl cyclase are needed to explain the functioning of these epithelia (Petersen & Edelman, 1964).

It is clear from a variety of evidence (for example, MacRobbie & Ussing, 1961; Frazier & Hammer, 1963) that the permeability changes to sodium and water follow-

ing addition of ADH occur at the outer facing membranes. The mechanism by which the permeability is changed is not clear but may result from the formation of one or more messengers within the cell, which after traversing the cell from the inner facing membranes affect the permeability of the rate limiting outer membranes. When the outer surfaces were treated with proteolytic enzymes there was no significant difference in the resting flow rate, however, the response to ADH was significantly impaired. In contrast treatment of the outer surface with proteolytic enzymes did not inhibit, and may even have enhanced, the SCC response to ADH (Table 2, Fig. 2A,B). These results would indicate that the different mechanisms are involved in the permeability increase at the outer faces following ADH and supports the idea that sodium ions and water move through separate channels (Lichtenstein & Leaf, 1965). It is not possible on the evidence to decide whether the different permeability changes are a consequence of two second messengers or a single messenger acting on different structures.

As well as the ADH receptor having an absolute requirement for calcium an excess of this ion in the outer bathing solution is known to depress the SCC and flow responses to ADH (Curran & Gill, 1961; Gill & Nedergaard, 1961). This effect of excess calcium is thought to be due to a stabilizing effect of calcium on the outer facing membranes. The enhancement of the flow response to ADH following treatment of the outer surface with phospholipase C may be connected with mobilization of membrane bound calcium by the enzyme, however the SCC responses to ADH were not enhanced by this treatment.

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