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Enhanced Sensitivity to Stimulation of Sodium Transport and Cyclic AMP by Antidiuretic Hormone after Ca^{2+} Depletion of Isolated Frog Skin Epithelium

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Summary. The role of Ca^{2+} in the stimulation by antidiuretic hormone (ADH) of active sodium transport across the isolated epithelium of frog skin was investigated. This has been done by bathing the blood side with Ca^{2+}-free solution containing 0.1 mM EGTA. This Ca^{2+} depletion halved the resistance but had no significant effect on the short-circuit current (SCC). The sensitivity of both cAMP- and SCC-stimulation to ADH was increased 40-fold by Ca^{2+} depletion. Sensitivity to stimulation by theophylline was only changed a little, while stimulation by exogenous cAMP was completely unaltered. The increase in sensitivity to ADH was dependent on the duration of preincubation in Ca^{2+}-free solution, which indicates that a slowly exchanging Ca^{2+} pool is involved in the determination of sensitivity to ADH. We suggest this pool is of cellular origin and the increased sensitivity is due to the decrease of a Ca^{2+} inhibition of the ADH-stimulated adenylate cyclase. But a direct effect of Ca^{2+} on binding of ADH to the receptor cannot be excluded. Our results are not compatible with the hypothesis that entry of extracellular Ca^{2+} is an obligatory step in the natriferic action of ADH, although it may be so in the hydroosmotic action of ADH. We also found the maximal response to ADH to be higher after Ca^{2+} depletion. This is in agreement with the hypothesis of intracellular Ca^{2+} as a modulator of the sodium permeability of the outward-facing membrane.

Key words active sodium transport · cyclic AMP · frog skin epithelium · antidiuretic hormone · cell Ca^{2+} · isoprenaline · theophylline · ADH receptor

Introduction

cAMP[1] has long been considered to be the second messenger for ADH, which stimulates active sodium transport and water permeability in amphibian bladder and skin preparations (Handler and Orloff, 1973). More recently Ca^{2+} has been included as a candidate, too (*see* Berridge, 1975), although the picture may be complicated by the involvement of Ca^{2+} in several steps in the chain of events leading from hormone binding to increased permeability. When added to the inside of the isolated frog skin the Ca^{2+} ionophore A23187 mimics ADH by stimulating the SCC (Nielsen, 1978; Balaban & Mandel, 1979). This effect of A23187 is dependent on the presence of Ca^{2+} in the IBS (Balaban and Mandel, 1979). Furthermore it has been found that verapamil, which blocks entry of Ca^{2+} into the cells, inhibits the stimulation by ADH of SCC (Bentley, 1974) and osmotic water flow (Humes, Simmons & Brenner, 1980). Thus Ca^{2+} might act as a second messenger for ADH alone or together with cAMP as speculated by us (Johnsen and Nielsen, 1981). In order to investigate this possibility we have chosen the approach to withdraw Ca^{2+} from the IBS and investigate the effects of this procedure on the stimulation of SCC by ADH, isop (a beta agonist), the phosphodiesterase inhibitor theo, and cAMP.

Materials and Methods

The experiments were performed on male and female frogs (*Rana esculenta*), which were kept at room temperature with free acess to water; they were fed twice a week with meal worms. The standard (control) incubation medium was a modified Ringer's solution (in mM: Na^+ 115, K^+ 2.5, Ca^{2+} 1, Mg^{2+} 1, Cl^- 118, CO_3^{2-} 2.5, PO_4^{3-} 1, glucose 5, pH = 7.8). In the Ca^{2+}-free solutions 1 mM $CaCl_2$ was omitted and 0.1 mM EGTA added.

The isolated epithelia were obtained after 3 hr of incubation of the skins in 0.3 mg collagenase/ml at room temperature.

SCC Measurements

The skin was divided into two symmetrical halves, and was treated with collagenase from the inside in a perspex chamber (7 cm^2). Thereafter the epithelia were cut along the edge (but not the underlying corium) and carefully lifted off. The epithelia

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[1] Abbreviations used in this paper: ADH, antidiuretic hormone; AVT, arginine vasotocin; cAMP, adenosine 3′, 5′ cyclic monophosphate; $H_{0.5}$, hormone concentration needed to elict half maximal response; IBS, inside bathing solution; isop, isoprenaline; SCC, short-circuit current, a measure of active sodium transport; theo, theophylline.

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were then mounted in perspex chambers exposing 1.5 cm^2 and with 2.5 ml bathing each of the two sides.

SCC was measured according to the technique of Ussing and Zerahn (1951), using an automatic voltage clamp, that compensated for the current-dependent potential drop between the potential-measuring electrodes. In order to monitor the resistance the epithelium was clamped at ± 5 mV (5 sec each) every second min. When the SCC had stabilized, the IBS of one of the epithelia halves was changed to Ca^{2+}-free medium. After 1 hr incubation in Ca^{2+}-free medium the experiments were carried out as described in Results.

Wet weights of 1.5 cm^2 epithelium varied from 7 to 20 mg; epithelial Ca^{2+} concentrations are around 2.5 nmol/mg (Zadunaisky and Lande, 1972; A.H. Johnsen, *unpublished results*). Allowing for up to 50 µl adhering control medium (a high estimate), less than a total of 100 nmol Ca^{2+} was present in the epithelium and in the inner chamber. Thus, the Ca^{2+}-concentration could not exeed 40 µM. The concentration of EGTA was 100 µM, leaving practically no free Ca^{2+} in the IBS. Influx of Ca^{2+} from the outer bathing solution was of no significance, since it has been reported to be in the range of 4 nmol/(1.5 cm^2 hr) (Zadunaisky & Lande, 1972).

cAMP Measurements

cAMP was measured as described by Johnsen and Nielsen (1978, 1980) with few modifications. In short the method was as follows:

The ventral skin was divided into four sections, each of which was divided into two symmetrical pieces. The skin pieces were transferred to test tubes (polyethylene, 100 × 15 mm) containing 5 ml collagenase solution. After the incubation each skin piece was placed on a glass plate and the intact epithelium scraped off by means of a microscope slide. The epithelium was transferred to fresh medium in a new test tube. After 1- to 2-hr equilibration the epithelium was transferred to another test tube containing standard or Ca^{2+}-free solution. Sixty min later AVT, isop or theo was added as desired.

After the incubation, cAMP was extracted by dropping the epithelium into 500 µl distilled water (100 °C), containing ^{3}H-cAMP as recovery marker, in a small test tube placed 2 min earlier in a boiling water bath. The tube was capped and 5 min later it was mixed on a vortex mixer and placed in an icebath. The tube was then centrifuged 10 min at 10,000 × *g*. The supernatant was placed on 1.4 g dry Al_2O_3 in a minicolumn (Evergreen Scientific) and eluted with 5 ml water. The eluate was lyophilized and disolved in buffer, and cAMP was measured by a binding protein assay (Geisler et al., 1977) in duplicates at one or two dilutions. The overall recoveries were typically 80%. The precipitate was dried at 70 °C and 50 mm Hg and the dry weight determined.

Materials

Collagenase (*C. histolyticum*) and N^6-2-0′-dibutyryl-cAMP was obtained from Boehringer. Synthetic AVT, the neurohypophysial hormone from amphibia, was obtained from Calbiochem. L-isoprenaline (Sigma) was disolved in 20 mM acetic acid and frozen in small aliquots. Immediately before use the stock solution was diluted in 1 mM acetic acid. Al_2O_3, 90 active neutral, was obtained from Merck, theophylline from Sigma and ^{3}H-cAMP from Amersham.

Results

Effect of Removal of Ca^{2+}

Substitution of the inside bathing solution (IBS) with a Ca^{2+}-free Ringer's solution containing

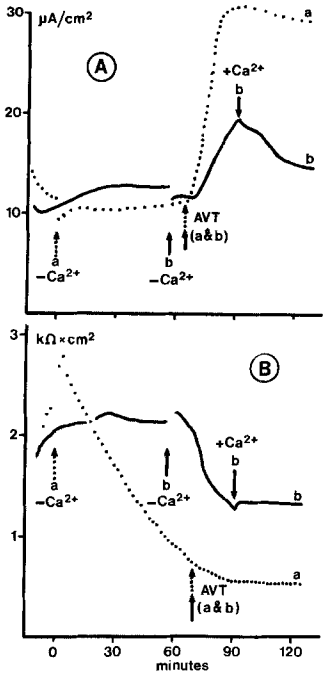

Fig. 1. Representative experiment showing effect of shift to Ca^{2+}-free IBS on SCC (*A*) and resistance (*B*) as well as time-dependence of the AVT-sensibilizing effect of this shift. The experiment was performed on paired halves of epithelium, *a* and *b*. *a* (dotted line) was preincubated 60 min in Ca^{2+}-free IBS and *b* (solid line) 7 min before both were stimulated by a submaximal dose of AVT (10^{-10} M, *cf.* Fig. 2). 25 min later 80 mM $CaCl_2$ was added to *b* to give a final concentration of 1.1 mM

0.1 mM EGTA had only minor effects on the SCC, while the resistance was drastically reduced (Fig. 1). After 60 min incubation with Ca^{2+}-free IBS, there was in most experiments a small decrease (around 10%) in the SCC compared with control halves. In all experiments the resistance decreased steadily after a lag-period (approximately 5 min) (Fig. 1 B). In mean the resistance fell to around half the starting value during this 60-min period, while the resistance of the control halves was nearly unchanged.

During 90-min incubation in Ca^{2+}-free medium the cAMP level of epithelia decreased with 0.35 ± 0.08 pmol/mg dry weight from 2.12 ± 0.14 in controls to 1.76 ± 0.18 in Ca^{2+}-free medium (mean $\pm$ SE, $n = 4$). This was also seen in the experiments shown in Figs. 2 and 3.

AVT

Sixty-min preincubation in Ca^{2+}-free medium increased the sensitivity of both SCC and cAMP level considerably (Fig. 2). The ratio of the mean of the $H_{0.5}$'s for stimulation of SCC was 38 (Fig. 2),

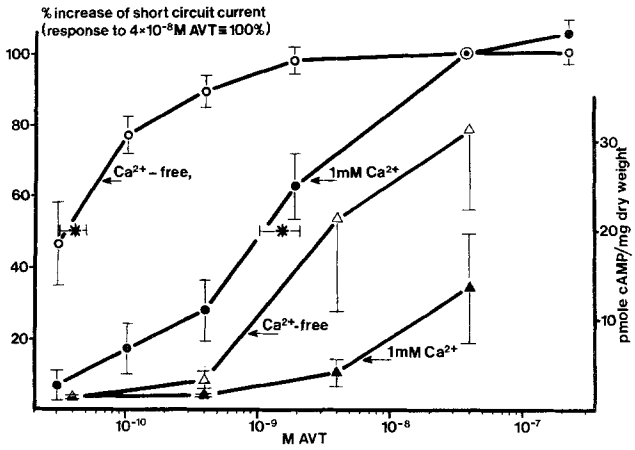

Fig. 2. Stimulation of SCC (circles) and total cAMP level (triangles) in response to AVT in control (1 mM Ca^{2+}, closed symbols) and Ca^{2+}-free (open symbols) Ringer's solution. SCC experiments were performed after 60-min preincubation as a cumulative response experiment: the epithelium was allowed to reach steady-state SCC before the next concentration of AVT was added. Responses (SCC after stimulation, SCC before addition of AVT) were calculated as percent of response to 4×10^{-8} M, which was defined as 100%. Asterisks show mean of $H_{0.5}$'s calculated from the individual experiments. cAMP measurements were performed on 8 two and two symmetrical pieces from each epithelium (*see* Methods section). Of each pair one was preincubated 60 min in Ca^{2+}-free and the other in control Ringer's solution and both thereafter incubated a further 30 min with one of 4 concentrations of AVT. Values are mean ±SE; $n=7$ for SCC measurements and $n=5$ for cAMP measurements

and a similar shift in sensitivity was found for the AVT stimulation of cAMP level. The shift in sensitivity of both parameters was subject to large variations, but a clear-cut drastic increase in sensitivity was found in each individual experiment. Five of the SCC experiments shown in Fig. 2 were performed on paired halves. Here the ratios of $H_{0.5}$ in Ca_2^{+}-free and control IBS ranged from 4 to 200 (Table 1). But it is noteworthy that this variation was mostly due to variation of $H_{0.5}$ of controls, while the $H_{0.5}$ obtained from the Ca^{2+}-depleted halves varied much less (Table 1).

In mean the control $H_{0.5}$ was 1.5×10^{-9} M. In two experiments performed on intact skins from the same batch of frogs in the same period with the same batch of AVT, $H_{0.5}$ was found to be 1.0 and 1.1×10^{-9} M. Thus the technique for isolating the epithelia did not alter the sensitivity to AVT.

In the 5 SCC experiments performed on paired halves the SCC rose 18.5 ± 4.0 μA/cm² (from 12.9 ± 4.1 to 31.3 ± 7.9) in the control halves compared with 26.7 ± 3.0 μA/cm² (from 12.1 ± 3.3 to 38.9 ± 4.7) in Ca^{2+}-depleted halves. The difference (8.3 ± 3.4 μA/cm²) is not significant ($0.05 < p <$ 0.1), but nevertheless suggestive. (Values are mean ±SE.)

Table 1. Comparison of $H_{0.5}$ for AVT stimulation of control ($+Ca^{2+}$) and Ca^{2+}-depleted ($-Ca^{2+}$) epithelia

Exp. no.	$H_{0.5}$ ($\times 10^{-11}$ M AVT)		
	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}/-Ca^{2+}$
1	10	2.3	4.3
2	173	5.2	32.3
3	265	2.7	98.1
4	380	1.8	211.1
5	34	5.7	4.9

Five individual dose-response experiments of AVT stimulation of SCC. The experiments are included in the curves of Fig. 2 and were performed as described in the legend to the Figure.

The increase in sensitivity to AVT was dependent on the duration of incubation in Ca^{2+}-free IBS. From Fig. 1 it is seen that after 60-min preincubation in Ca^{2+}-free IBS the response to a submaximal dose of AVT is much faster than after only 7-min preincubation, both lagtime and risetime being much shorter, and the magnitude of the response is considerably higher after the longer preincubation time. Furthermore, it is seen that readdition of Ca^{2+} to the skin half that was exposed for only 7 min to Ca^{2+}-free IBS slowly reversed the (after all) increased sensitivity. Thus to obtain full effect of changes of Ca^{2+} in the IBS rather long incubations (in the order of hours) are needed, which indicates that a slowly exchanging Ca^{2+} pool is involved in the determination of sensitivity to AVT.

Isoprenaline

To test the specificity of the enhanced sensitivity to hormonal stimulation after preincubation in Ca^{2+}-free IBS we also investigated the stimulation of SCC and cAMP level by the beta-adrenergic agonist, isoprenaline (isop).

After preincubation in Ca^{2+}-free IBS much lower (in mean approximately 30-fold) concentrations of isop were needed to stimulate the cAMP level than in control epithelial pieces (Fig. 3). This effect of Ca^{2+}-free incubation resembles that on AVT stimulation, but unlike AVT, preincubation in Ca^{2+}-free IBS caused only moderate increase in the SCC sensitivity to isop, in mean a 2.4-fold decrease of $H_{0.5}$ was found (Fig. 3).

Theophylline and Dibutyryl cAMP

The above-mentioned findings indicate that Ca^{2+} interacts with the chain of reactions leading from

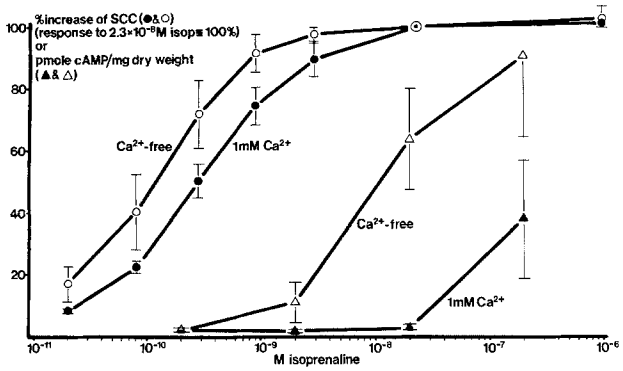

Fig. 3. Stimulation of SCC (circles) and total cAMP level (triangles) in response to isoprenaline in control (1 mM Ca^{2+}, closed symbols) and Ca^{2+}-free (open symbols) Ringer's solution. Details as in legend to Fig. 2. SCC response to 2.3×10^{-8} M defined as 100%. $n=4$ for SCC measurements and $n=6$ for cAMP measurements

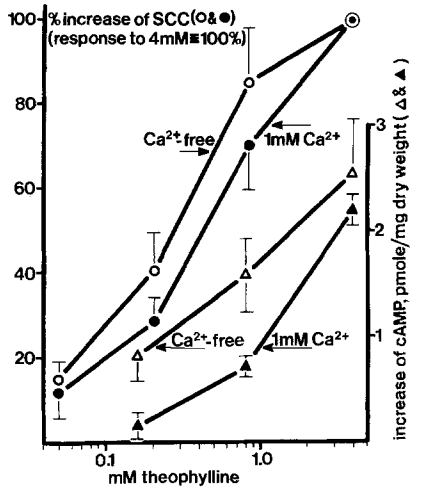

Fig. 4. Stimulation of SCC (circles) and cAMP level (triangles) by theophylline in control (1 mM Ca^{2+}, closed symbols) and Ca^{2+}-free (open symbols) Ringer's solution. Details as in legend to Fig. 2. SCC response to 4 mM defined as 100%. cAMP measurements are plotted as increase of cAMP above unstimulated control, since preincubation in Ca^{2+}-free solution lowered the cAMP level a little (*see text*). $n=4$ for both SCC measurements and cAMP measurements

AVT binding to increased sodium permeability—a chain where cAMP is supposed to be one step. Preincubation in Ca^{2+}-free medium induced a parallel shift in AVT sensitivity of both SCC and cAMP level. This indicates the sensitivity enhancement was not due to a post-cAMP interaction of Ca^{2+}. In other words, the correlation between cAMP level and SCC was not affected by Ca^{2+}. To verify this we increased cAMP level by theo which inhibits the break-down of cAMP and by N^6-2-0′-dibutyryl cAMP, an analog of cAMP which is more potent (probably due to more easy

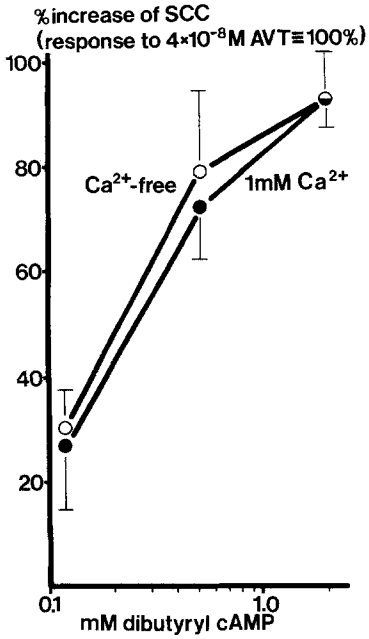

Fig. 5. Stimulation of SCC in response to dibutyryl-cAMP in control (1 mM Ca^{2+}, closed symbols) and Ca^{2+}-free (open symbols) Ringer's solution. Details as in legend to Fig. 2. Each experiment was terminated by the addition of 4×10^{-8} M AVT, the response to which was defined as 100%. $n=3$

penetration of the plasma membrane and greater resistance to break-down).

Preincubation in Ca^{2+}-free IBS induced an insignificant enhancement (1.5-fold) of the sensitivity to stimulation of SCC by theo (Fig. 4), but it was noteworthy that the response to theo proceeded faster in epithelia preincubated in Ca^{2+}-free IBS. For example, to reach half-full response to 0.85 mM theo control halves needed around 13 min, compared with around 7 min of the halves preincubated in Ca^{2+}-free IBS. The sensitivity to stimulation of cAMP level by theo was enhanced a little (approximately fourfold, Fig. 4). This was partly due to subtraction of a lower basal level, since preincubation in Ca^{2+}-free IBS reduced the unstimulated level by 0.35 ± 0.08 pmol cAMP/mg dry weight, as noted above.

Preincubation in Ca^{2+}-free IBS had no effect on the stimulation of SCC by dibutyryl cAMP (Fig. 5).

Discussion

Effect of Ca^{2+} Removal

The removal of Ca^{2+} (0.1 mM EGTA) from the IBS induced a marked fall in the resistance, but caused no significant change in SCC (Fig. 1). These findings resemble those of Curran, Zadunaisky and Gill (1961) on intact frog skin with 0.4–4 mM

EDTA in the IBS, but are somewhat in contrast to the report by Zadunaisky and Lande (1972) that Ca^{2+}-free IBS induced a rapid decay in both resistance *and* SCC of isolated frog skin epithelium even with no Ca^{2+} chelator present. There is no obvious reason why the sodium-transporting capacity was better preserved in our preparation. The only significant difference is that the epithelia of Zadunaisky and Lande (1972) were separated from the corium by means of pressure from the inside, while we dissected the epithelia off. However, the good preservation of sodium transport allowed us to study the role of Ca^{2+} in the IBS in the action of ADH.

Ca^{2+} depletion lowered the basal cAMP level a little, while the response to theo was increased. It is difficult to explain this in terms of change in only one of the enzymes, the synthezing adenylate cyclase or the degradating phosphodiesterase. But the observation can be explained if we assume that the activity of the unstimulated adenylate cyclase is lowered and the affinity of the phosphodiesterase to theo is increased by the Ca^{2+}-depleting procedure.

In the following discussion, cAMP is assumed to be a mediator of AVT stimulation of SCC.

Ca^{2+} depletion increased the sensitivity to stimulation of SCC by isop significantly (Fig. 3). But the degree of increase in sensitivity was much smaller than observed with AVT, although the stimulation of cAMP production was sensitized to the same degree as found with AVT (*cf.* Fig. 2 and 3). Thus the difference in the change of sensitivity after Ca^{2+} depletion is probably not due to different effects on the binding of isop and AVT to their respective receptors. This indicates that if isop stimulation of SCC is mediated by cAMP (Jard, 1974) the access of the AVT- and the isop-induced cAMP to the cAMP-regulated sites are different. But it has not been the aim of the present work to elucidate the explanation of this discrepancy.

Earlier experiments (Petersen & Edelman, 1964; Argy, Handler & Orloff, 1967) have shown that high (10 mM) Ca^{2+} inhibits the hydroosmotic response to submaximal ADH of toad bladders, but this inhibition was overcome by high ADH concentrations. The natriferic (SCC) response to ADH was not changed. In both papers these findings were interpreted in terms of two sets of receptors regulating each of the two responses. We might deal with two sides of the same phenomenon: inhibition by high Ca^{2+} concentration. If so the inhibition of the natriferic response must saturate at lower Ca^{2+} concentrations than that of the hydroosmotic response, i.e. the natriferic response is already inhibited at 1 mM Ca^{2+}. Furthermore, we must not forget that we compare two different tissues of two different species.

Post-AVT-binding Effect of Ca^{2+}?

Incubation in Ca^{2+}-free IBS resulted in a marked increase in sensitivity of cAMP level and sodium transport to AVT (Fig. 2). This may be 1) a direct effect caused by the decrease in extracellular Ca^{2+} concentration, or 2) an effect caused by a lowered cellular concentration following the wash-out. Effects exerted by extracellular Ca^{2+} are most likely to be on the binding of AVT to the receptor while intracellular Ca^{2+} is most likely to regulate the AVT-stimulated adenylate cyclase. Steps beyond cAMP production are not to be considered since Ca^{2+} depletion had hardly any effect on stimulation by theo (Fig. 4) or cAMP (Fig. 5). Ten mM Ca^{2+} in the medium reduced the increase in cAMP level in toad bladder following ADH (Omachi et al., 1974). Ca^{2+} inhibited the binding of ADH to purified plasma membranes of renal medulla (Campbell, Woodward & Borberg, 1972). One mM Ca^{2+} in the incubation medium halved the binding of a submaximal ADH dose compared with the binding in zero Ca^{2+}. An increased affinity of the receptor for AVT after Ca^{2+} depletion could explain our findings. But binding of ADH to membrane fractions of frog bladder was practically the same in Ca^{2+} concentrations from 0 to 1 mM (Roy et al., 1973). This finding on a, in many respects, similar tissue, strongly indicates that the increased sensitivity to AVT is due to a step later than binding of AVT to its receptor.

Further support to a post-AVT-binding explanation of the increased sensitivity comes from the observation that the full effect after shift to Ca^{2+}-free IBS was only slowly achieved (Fig. 1). This indicates that it is a slowly exchanging pool of Ca^{2+}, that determines the sensitivity to AVT. It seems more likely that such a slow pool is cellular *than* bound to the external membrane surface. In favor of this view is the finding of Zadunaisky and Lande (1972) that all the epithelial Ca^{2+} exchanges with a half-time around 20 min and total exchange after 1 h, which is the preincubation period we have used for Ca^{2+} depletion. In kidney cells Borle (1970) found two compartments with half-times of 1 and 30 min, respectively. He considered the fast compartment to be extracellular and the slow compartment to be the intracellular exchangeable Ca^{2+} pool.

That cellular Ca^{2+} regulates the stimulation of adenylate cyclase by ADH was strongly supported by the works of Campbell et al. (1972) on kidney preparations and by Bockaert, Roy and Jard (1972) on frog bladder. Both groups found that a certain (low) level of Ca^{2+} was necessary for ADH stimulation of adenylate cyclase. This effect of Ca^{2+} was unrelated to the binding of ADH to the preparations. Further support for the involvement of cellular Ca^{2+} in ADH stimulation of cAMP production comes from the recent work by Levine et al. (1981). In many processes where Ca^{2+} is involved the action is mediated by calmodulin. Levine et al. (1981) found that the cAMP response to ADH was almost doubled (although not significantly) after incubation of toad bladder segments with trifluoperazine, an inhibitor of calmodulin. These workers did not find any effect of trifluoperazine on SCC or the stimulation of SCC by ADH. But they used a maximal ADH dose, so if there was any enhanced response to ADH it might have been minor.

Thus the enhanced sensitivity to AVT after Ca^{2+} depletion may very well be due to the fact that the hormone-stimulated adenylate cyclase is somewhat inhibited by Ca^{2+}, maybe mediated by calmodulin. Washout of Ca^{2+} would then release the inhibition (if Ca^{2+} does not fall below the critical level) with an increased sensitivity to AVT as a result.

Ca^{2+} Entry in ADH Stimulation

Several different types of experiments indicate that entry of extracellular Ca^{2+} is part of the mechanism of stimulation by ADH. Pietras, Naujokaitis and Szego (1976) found that ADH increased the Ca^{2+} content of the granular cells of toad bladder. Nielsen (1978) and Balaban and Mandel (1979) found that the Ca^{2+} ionophore A23187 mimics ADH by increasing SCC when added to the inside. The supposed inhibitor of Ca^{2+} entry into cells, verapamil, inhibits ADH stimulation of SCC (Bentley, 1974).

That Ca^{2+}-free IBS instead of inhibiting the response to AVT increased the sensitivity to AVT of the isolated frog skin epithelium (Fig. 2) strongly disfavors the entry of Ca^{2+} as a step in the normal ADH stimulatory process. On the other hand, too much evidence involves Ca^{2+} as taking part in the action of ADH. Probably this Ca^{2+} stems from intracellular stores, and/or is connected to the hydroosmotic effect of ADH *(see below)*.

Na/Ca Exchange

Grinstein and Erlij (1978) proposed a Na/Ca exchange mechanism located at the inner surface of the frog skin epithelium as a regulator of the Na^+ permeability of the outer surface: increased cellular Na^+ would increase the inflow and thereby the concentration of Ca^{2+}, which then would shut down the Na^+ permeability of the outer surface. The evidence for this mechanism has been reviewed more extensively by Taylor and Windhager (1979). The mechanism would work as a down regulation after ADH stimulation and if the inflow of Ca^{2+} is hindered one would expect the response to ADH to increase. That the frog skin has a reserve of transport capacity after maximal ADH stimulation has been shown by the use of diphenylhydantoin (De Sousa & Grosso, 1973) and theo (Johnsen & Nielsen, *in preparation*). As mentioned above verapamil inhibited the response to ADH (Bentley, 1974), but this could have other explanations than a blockade of cellular Ca^{2+} uptake–blockade of mitochondrial uptake for instance. Another supposed Ca^{2+}-channel blocker is quinidine, which inhibited the basal sodium transport of toad bladder, but enhanced the absolute rise induced by ADH (Taylor, 1975).

The findings presented in this paper are by no means contrary to the above-mentioned hypothesis. However, the increased sensitivity to AVT reported in this paper is probably not beyond the cAMP production step, but as noted in the Results the absolute stimulation by AVT was approximately 40% higher after Ca^{2+} depletion than in controls, and this is in good agreement with the hypothesis of Ca^{2+} as an intracellular inhibitor of Na permeability. The Na/Ca exchange would also explain the increased Ca^{2+} content after ADH stimulation under standard conditions (Pietras et al., 1976).

Conclusion

Ca^{2+} depletion enhanced the sensitivity of the isolated frog skin epithelium to stimulation by AVT of the cAMP level and, probably as a consequence of this, SCC. We cannot distinguish between an effect of Ca^{2+} depletion on the binding of AVT to its receptor and on the stimulation of the adenylate cyclase, but we favor the latter possibility. Our results are not compatible with the hypothesis that the entry of extracellular Ca^{2+} is an obligatory step in the natriferic action of ADH. On the other hand, our results are in agreement with the hypo-

thesis of intracellular Ca^{2+} as a modulator of the sodium permeability of the outward-facing membrane.

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