INTRACELLULAR CALCIUM AS A MODULATOR

OF TRANSEPITHELIAL PERMEABILITY

TO WATER IN FROG URINARY BLADDER

MARCOS A. HARDY. From the Division of Nephrology, Department of Medicine, University of Miami School of Medicine, Miami, Florida 33152

ABSTRACT

The divalent cation ionophore A 23187 was used to evaluate the action of intracellular calcium on net transepithelial water movement across the isolated frog urinary bladder. Incubation with the ionophore increases the net basal water flux in a dose-dependent fashion but independent of the extracellular calcium concentration. Bladders pretreated with A 23187 and exposed thereafter to an increase in calcium concentration exhibit a water permeability that under certain conditions can be comparable to that achieved with antidiuretic hormone (ADH). Lowering the serosal calcium at the peak of the hydrosmotic responses to both ADH and A 23187 inhibited the maintenance of the net water flux. The action of a supramaximal dose of ADH is blunted in bladders pretreated with A 23187, while the hydrosmotic effects of a submaximal dose are enhanced when the ionophore is added together with the hormone.

The results show that an increase in transepithelial water movement can be triggered by calcium and that serosal calcium is needed to sustain the response. This hydrosmotic response may be dependent upon the rate at which intracellular calcium concentrations change and on the absolute concentration attained.

It is suggested that calcium is involved in the action of ADH on water permeability and may act as a modulator of the hydrosmotic response.

KEY WORDS   frog urinary bladder · antidiuretic hormone · water permeability · divalent cation ionophore A 23187 · calcium · excitation-response coupling

In almost every system in which a cellular response to a stimulus is followed by a rise in adenosine 3′,5′-monophosphate (cyclic AMP), a concomitant change in the metabolism of cell calcium has been observed. Calcium ions are involved in the excitation-response sequence of different cells, and their role as second messengers and/or coupling factors (particularly in interrelationship with cyclic nucleotides) has been proposed as a generalized mode of intracellular communication by which a wide variety of cells are activated by specific extracellular messengers (14).

In the urinary bladder of amphibia, the addition of antidiuretic hormone (ADH) to the serosal (or blood) side elicits an increase in the permeability to sodium (natriferic effect), water (hydrosmotic effect) and other small solutes on the apical (or luminal) membrane. This excitation-permeability coupling has been described as dependent on the activation by the hormone of a membrane-bound adenylate cyclase, the resulting increase in the intracellular concentration of cyclic AMP being the mediator that triggers a poorly defined chain of events leading to the final permeability changes (7, 11).

In regard to the possible involvement of calcium in this system, it has been reported that associated with the action of ADH there is a transient increase in the washout of ^{45}Ca of preloaded toad

bladders (3, 16) as well as an oscillatory phenomenon of calcium uptake and release by homogenates of epithelial cells of the bladder (16). Also, the natriferic effect of ADH can be inhibited by incubation of the toad bladder with the divalent cation ionophore A 23187 (21). But the possibility of a role of calcium on the hydrosmotic response can only be derived from indirect evidences. The hydrosmotic response can be modified by agents and treatments (such as ouabain, metabolic inhibitors, lithium, quinidine, lanthanum, and changes in calcium concentrations of the medium) (2, 5, 7–9, 18, 22) which have been shown in other cells to modify either the intracellular ionic calcium or the calcium permeability of the membranes.

These and other results lead to the hypothesis that the compartmentalization of intracellular calcium may be important in the regulation of the hydrosmotic response (5, 6) and that changes in intracellular ionic calcium could trigger and inhibit the effects of ADH on water transport (8, 9).

The possibility that intracellular calcium plays a role in net transepithelial movement of water across the urinary bladder of amphibia was examined using the divalent cation ionophore A 23187. This antibiotic facilitates the translocation of divalent cations across cell membranes, produces a diffuse rise in cytoplasmic ionic calcium (14), and doubles the calcium uptake by isolated epithelial cells of the urinary bladder of the toad (21).

MATERIALS AND METHODS

Urinary bladders were excised from doubly pithed frogs (*Rana grylio*, Everglades Frogs, Ochopee, Fla.). One hemibladder was used as control and the other as experimental preparation. Net water fluxes along a fixed osmotic gradient were measured by gravimetry with the hemibladders mounted as sacs (2). The millimolar composition of the serosal anuran Ringer's solution was: Na, 110; K, 5; Ca, 2; Cl, 124; glucose, 10; and Tris, 5; pH 8.0–8.2. The solution bathing the mucosal side of the bladder was a 1:10 dilution of the serosal Ringer's; thus it always contained 0.2 mM calcium. When calcium concentration was changed in the serosal medium, the osmolality was kept constant by adjusting the NaCl concentration. The surface area of the preparation was calculated by weight, assuming that each hemibladder represented a perfect sphere, and the net water fluxes were expressed in microliters per square centimeter of bladder per hour ($\mu l \cdot cm^{-2} \cdot h^{-1}$). The results reported in the text are the mean ± SEM of six experiments; each experimental point in the figures is the mean of six experiments. The serosal concentrations of A 23187 were attained by adding an adequate amount of a 10^{-3}

M solution of the ionophore prepared in a 1:1 dimethyl sulfoxide (DMSO)-ethanol solvent. Equivalent amounts of the solvent were added to control bladders. The hydrosmotic response in frog bladders is usually best obtained with oxytocin rather than with vasopressin (15). In these particular frog bladders, no difference was found between the action of oxytocin (Sigma Chemical Co., St. Louis, Mo.) and that of vasopressin (Pitressin, Parke Davis & Co., Detroit, Mich.). With 20 mU/ml of either agent, a hydrosmotic response was obtained with a maximal effect between 15 and 25 min. The peaks were 166.76 ± 7.53 $\mu l \cdot cm^{-2} \cdot h^{-1}$ with vasopressin and 172.55 ± 13.30 $\mu l \cdot cm^{-2} \cdot h^{-1}$ with oxytocin. The results reported here are those obtained with vasopressin.

RESULTS

The net water flux of the unstimulated frog bladder was 2.09 ± 1.60 $\mu l \cdot cm^{-2} \cdot h^{-1}$. The addition of A 23187 to the serosal medium elicited an increase in net water flux (Fig. 1 and 2b). This response was dependent on the concentration of the ionophore; thus, with 1 × 10^{-6} M and 2 × 10^{-6} M the net water fluxes were 23.07 ± 3.78 and 31.68 ± 4.06 $\mu l \cdot cm^{-2} \cdot h^{-1}$, respectively, after 30 min. With 5 × 10^{-6} M and 1 × 10^{-5} M, the results were 49.21 ± 4.84 and 68.41 ± 8.80 $\mu l \cdot cm^{-2} \cdot h^{-1}$, respectively, also after 30 min.

These results, obtained with 2.0 mM calcium in the serosal medium, were not significantly different if A 23187 was added to a medium containing 0.2 or 20.0 mM calcium. The hydrosmotic response was greatly enhanced if, 20 or 30 min after adding the ionophore, the serosal calcium concentration was raised 5- to 10-fold. When 2 × 10^{-6} M of A 23187 was added to a serosal medium containing 0.2 mM calcium and then the calcium concentration was increased to 2.0 mM, a response of 85.78 ± 12.61 $\mu l \cdot cm^{-2} \cdot h^{-1}$ was obtained. The same concentration of the ionophore added to a 2.0 mM calcium medium which was afterwards increased to 10.0 mM yielded a response of 82.07 ± 10.52 $\mu l \cdot cm^{-2} \cdot h^{-1}$.

When 2 × 10^{-6} M A 23187 was added to a 2.0 mM calcium medium which was then abruptly increased to 20.0 mM calcium, a 145.96 ± 19.73 $\mu l \cdot cm^{-2} \cdot h^{-1}$ response was observed. This stimulation of the net water flux obtained by increasing calcium in the presence of the ionophore was similar to the 151.02 ± 12.89 $\mu l \cdot cm^{-2} \cdot h^{-1}$ water flux obtained in the paired hemibladders with 20 mU/ml ADH (Fig. 1a).

When this last experiment was repeated and, at the peak of the responses to ADH and to the ionophore, the calcium concentration on the se-
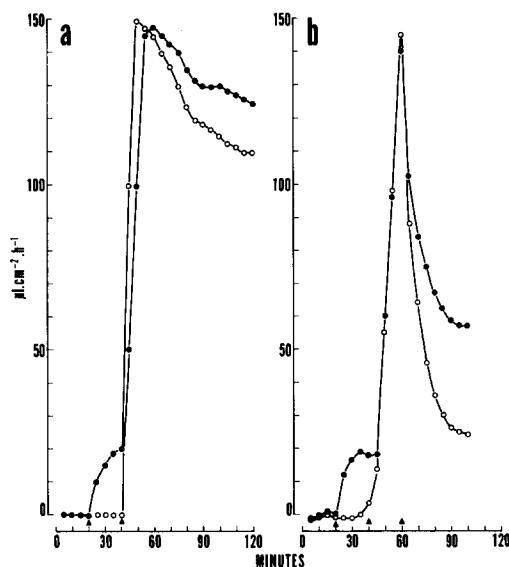
FIGURE 1 Control hemibladders (O—O); experimental hemibladders (●—●). (a) First arrow: 2 × 10⁻⁶ M A 23187 added to experimental and diluent to control hemibladders. Second arrow: 20 mU/ml ADH added to control hemibladders and serosal calcium concentration raised from 2.0 to 20 mM in experimental hemibladders. (b) First and Second arrows: same as in Fig. 1a. Third arrow: serosal calcium concentration reduced to 0.2 mM in both control and experimental hemibladders. ADH and A 23187 are not removed.

rosal side was lowered to 0.2 mM, an inhibition of the hydrosmotic effects was obtained (Fig. 1b), in spite of the continuous presence of the hormone or the ionophore.

Fig. 2a depicts the interaction between a submaximal dose of ADH and A 23187. An increase in net water flux to 9.86 ± 3.68 μl·cm⁻²·h⁻¹ was obtained with 2 mU/ml of ADH. The response to the same dose of ADH was enhanced up to 58.33 ± 5.07 μl·cm⁻²·h⁻¹ when added together with 1 × 10⁻⁶ M A 23187.

In contrast, Fig. 2b shows that hemibladders preincubated for 60 min with the same concentration of the calcium ionophore exhibited an inhibition of the hydrosmotic response to a maximal dose of ADH (a peak of 73.06 ± 10.61 μl·cm⁻²·h⁻¹ in the ionophore-pretreated experimental hemibladders as opposed to the 147.70 ± 6.32 μl·cm⁻²·h⁻¹ peak of the control).

The possibility that high serosal calcium could reverse the inhibition produced by preincubation with A 2318 was tested. Control and experimental hemibladders were preincubated for 60 min with

1 × 10⁻⁶ M A 23187 in 2.0 mM serosal calcium. After that period, 20 mU/ml ADH were added to both hemibladders, but in the experimental hemibladders at the same time that ADH was added serosal calcium was raised to 20.0 mM. In this experiment, the hydrosmotic response had a peak of 86.18 ± 10.06 μl·cm⁻²·h⁻¹ in the control and 49.28 ± 9.84 μl·cm⁻²·h⁻¹ in the experimental. High serosal calcium blunted even more the already A 23187-inhibited response. In another set of experiments, it was observed that, in the absence of A 23187, if serosal calcium is increased to 20.0 mM at the same time that ADH is added, the response is also blunted (161.73 ± 10.36 μl·cm⁻²·h⁻¹ in ADH-treated control hemibladders and 119.74 ± 6.90 μl·cm⁻²·h⁻¹ in ADH + calcium 20.0 mM-treated experimental hemibladders).

DISCUSSION

The results obtained seem paradoxical. The intracellular calcium concentrations attained by adding different concentrations of A 23187 (a) produced hydrosmotic responses comparable to those that can be evoked by submaximal doses of ADH; (b)
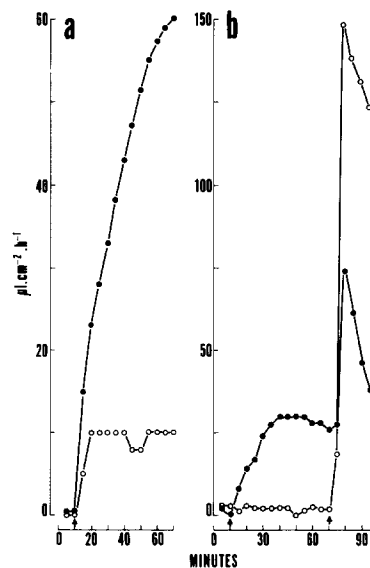

FIGURE 2 Control hemibladders (O—O); experimental hemibladders (●—●). (a) At arrow, 2 mU/ml ADH plus diluent added to control and 2 mU/ml plus 1 × 10⁻⁶ M A 23187 to experimental hemibladders. (b) First arrow: 1 × 10⁻⁶ M A 23187 added to experimental and diluent to control hemibladders. Second arrow: 20 mU/ml ADH added to both control and experimental hemibladders.

blunted by 50% the hydrosmotic response elicited by a maximal dose of ADH; and (c) had an additive effect on the action of a submaximal dose of ADH. To obtain with calcium an effect similar to that obtained with a maximal dose of the hormone, extracellular calcium must be abruptly increased after the membrane has been rendered permeable with the ionophore.

A possible explanation is that whilst the hydrosmotic response might be mediated by the absolute level of free intracellular calcium attained, it may also be critically dependent upon the rate at which calcium increases, as has been suggested for the K⁺-induced contractions in smooth muscle (20). Accordingly, A 23187 would rapidly increase intracellular calcium if calcium in the bath is increased after the addition of the ionophore, whereas a much slower rise in cytoplasmic calcium would occur when the ionophore alone is added to a bladder at any given level of extracellular calcium.

This hypothesis might be also applicable to the result obtained in some other tissues in which A 23187 was used to simulate the effect of hormones. In the pancreas, parotid glands (17) and neurohypophyses (19) of the rat, a much faster rate of secretion is obtained if extracellular calcium is raised after preincubation with A 23187 than if the ionophore is added to a medium already containing calcium (see also discussion on page 79, reference 17).

A feature of many calcium-activated processes is an inhibition of the response as intracellular ionic calcium concentration is elevated (14). If the action of ADH on water permeability involves changes in intracellular calcium, the inhibition of the response to ADH in bladders that were pre-treated with A 23187 could be explained by supposing that, in bladder cells in which the calcium concentration has been previously raised, the hormone can induce little further changes in cytosolic calcium; thus its action on water permeability would be inhibited. This could also account for the inhibition of the serotonin-evoked secretion in the fly salivary gland by pretreatment with A 23187 (13), and the inhibition of ADH action by treatments that increase intracellular calcium, such as the ones mentioned in the introductory paragraph. Thus, the hydrosmotic response evoked by ADH (and maybe the cellular responses to hormones in different tissues) could be dependent on the previous intracellular ionic calcium concentration.

It is possible that if the hydrosmotic action of ADH on the amphibian urinary bladder is produced through sudden changes in concentration and/or redistribution of compartmentalized intracellular ionic calcium, this in turn could lead to a high calcium concentration in the cell that acts as a negative feedback, inhibiting the hydrosmotic response to ADH. If this is the case, it may represent a mechanism for self-limiting the hormonal effect on water permeability, thus modulating the response.

It has been pointed out that if a cell response similar to the physiological activation is obtained through A 23187, the result is taken as evidence that, in the normal cell response, Ca²⁺ plays a role as a coupling factor, though the nature of the calcium pool involved in the normal effect cannot be inferred (14). The sudden inhibition of the developed hydrosmotic responses to ADH or A 23187 produced by a reduction of the calcium concentration on the serosal side shows that extracellular calcium is needed to sustain the response. It has been shown in epithelial cell "ghosts" of frog bladder that the absence of calcium does not modify the binding of ADH to receptor sites, but reduces the activation of adenyl cyclase by the hormone (11). There is no evidence in the intact tissue of an altered activity of adenyl cyclase or a drop in cellular cyclic AMP (cAMP) if serosal calcium is lowered during the fully developed hydrosmotic response to ADH. In the case of the ionophore-induced hydrosmotic response (such as the one in Fig. 1a) the cell content of cAMP is lowered from a basal value of 16.0 ± 1.3 fmol/mg protein to 10.4 ± 0.4 fmol/mg protein ($N = 5, P < 0.01$) at the peak of the response (D. C. Lehotay and M. A. Hardy, unpublished observation). This means that, through A 23187, Ca²⁺ can trigger a cAMP-independent increase in water permeability. Thus, at least in the case of the ionophore + calcium-induced hydrosmotic response, it is the calcium uptake which seems to be responsible for the effect, and not the activation of adenyl cyclase.

An exocytotic process is triggered by ADH at the apical membrane of the cells. Masur et al. have suggested that in this respect the behavior of the urinary bladder resembles the behavior of a gland, and that this process may be related to the permeability changes elicited by the hormone (12). Exocytosis is a common feature of neurotransmitter release and gland secretion, which is described as a calcium-mediated phenomenon (1, 4). Suggestively, the increased ⁴⁵Ca efflux observed in bladders under the action of ADH (16)

occurs only at the apical membrane (3).

As ADH also induces contraction in some types of smooth muscle cells, Bentley hypothesized that the action of ADH on transepithelial water movement and on muscle contraction may involve similar cellular events (2). In all types of muscle cells, calcium is the coupling factor in the excitation-contraction sequence. So calcium could be the common factor in the excitation-response coupling to ADH, in both muscle and epithelia. Calcium could also be the mediator of the hydrosmotic effect triggered by serosal hypertonicity (10).

REFERENCES

1. BAKER, P. F. 1974. Excitation-Secretion Coupling. *In* Recent Advances in Physiology. R. J. Linden, editor. Churchill Livingstone, Edinburgh and London. (No. 9) Chap. 2. 51–86 pp.
2. BENTLEY, P. J. 1966. The Physiology of the Urinary Bladder of Amphibia. *Biol. Rev.* **41**:275–316.
3. CUTHBERT, A. W., and P. Y. D. WONG. 1974. Calcium release in relation to permeability changes in toad bladder epithelium following antidiuretic hormone. *J. Physiol. (Lond.).* **241**:407–422.
4. DOUGLAS, W. W. 1974. Exocytosis and the Exocytosis-Vesiculation Sequence, *In* Secretory Mechanisms of Exocrine Glands. Alfred Benzon Symposium. VII. N. A. Thorn and O. H. Peterson, editors. Munskgaard A/S, Copenhagen. 116–129 pp.
5. DOUSA, T. P., and H. VLATIN. 1976. Cellular actions of vasopressin in the mammalian kidney. *Kidney Int.* **10**:46–63.
6. FERGUSON, D. R. 1971. Cellular mode of action of Neurohypophysial Hormones. *In* Neurohypophysial Hormones. *Ciba Found. Study Group.* **39**:109–115.
7. HANDLER, J. S., and J. ORLOFF. 1973. The mechanism of action of Antidiuretic Hormone. *In* Handbook of Physiology. Sect. 8: Renal Physiology. J. Orloff and R. W. Berliner, editors. American Physiological Society, Washington, D. C. Chap. 24. 791–844 pp.
8. HARDY, M. A. 1977. Effect of Ca^{2+} on the hydrosmotic action of vasopressin. *Physiologist.* **20**:41.
9. HARDY, M. A., and J. FUNES. 1977. Toad urinary bladder: action of metabolic inhibitors on unidirectional water flux. *Bioph. J.* **17**:89a. (Abstr.)
10. HARDY, M. A., and J. J. BOURGOIGNIE. 1977. Effects of Quinine and A 23187 on the hydrosmotic response to serosal hypertonicity. Tenth Annual Meeting of the American Society of Nephrology. Washington, D. C.
11. JARD, S., and J. BOCKAERT. 1975. Stimulus-response coupling in neurohypophysial peptide target cells. *Physiol. Rev.* **55**:489–536.
12. MASUR, S., E. HOLTZMAN, and R. WALTER. 1972. Hormone-stimulated exocytosis in the toad urinary bladder. *J. Cell Biol.* **52**:211–219.
13. PRINCE, W. T., H. RASMUSSEN, and M. J. BERRIDGE. 1973. The role of calcium in fly salivary gland secretion analyzed with the Ionophore A 23187. *Biochim. Biophys. Acta.* **329**:98–102.
14. RASMUSSEN, H., and D. B. P. GOODMAN. 1977. Relationships between calcium and cyclic nucleotides in cell activation. *Physiol. Rev.* **57**:421–509.
15. SCHWARTZ, I. L., and L. M. LIVINGSTON. 1964. Cellular and Molecular Aspects of the Antidiuretic Action of Vasopressins and Related Peptides. *In* Vitamins and Hormones. R. S. Harris, I. G. Wool, and J. A. Loraine, editors. Academic Press, Inc., New York. Vol. 22. 261–358 pp.
16. SCHWARTZ, I. L., and R. WALTER. 1969. Neurohypophysial Hormone – Calcium interrelationships in the toad bladder. *In* Protein and Polypeptide Hormones. M. Margoulies, editor. Excerpta Medica Foundation, Amsterdam. 264–269 pp.
17. SELINGER, Z., S. EIMERL, N. SAVION, and M. SCHRAMM. 1974. A Ca^{++} Ionophore (A 23187) simulating hormone and neurotransmitter action in the rat parotid and pancreas glands. *In* Secretory Mechanisms of Exocrine Glands. Alfred Benzon Symposium. VII. N. A. Thorn and O. H. Peterson, editors. Munskgaard A/S, Copenhagen. 68–78 pp.
18. SINGER, I., and E. FRANKO. 1973. Lithium-induced ADH resistance in toad urinary bladders. *Kidney Int.* **3**:151–159.
19. THORN, N. A. 1974. Role of calcium in secretory processes. *In* Secretory Mechanisms of Exocrine Glands. Alfred Benzon Symposium. VII. N. A. Thorn and O. H. Peterson, editors. Munskgaard A/S, Copenhagen. 305–326 pp.
20. VAN BREEMEN, C. 1977. Calcium requirement for activation of intact aortic smooth muscle. *J. Physiol. (Lond.).* **272**:317–329.
21. WIESMANN, W., S. SINHA, and S. KLAHR. 1977. Effects of ionophore A 23187 on base-line and vasopressin-stimulated sodium transport in the toad bladder. *J. Clin. Invest.* **59**:418–425.
22. WIETZERBIN, J., Y. LANGE, and C. M. GARY-BOBO. 1974. Lanthanum inhibition of the action of oxytocin on the water permeability of the frog urinary bladder: effect on the serosal and the apical membrane. *J. Membr. Biol.* **17**:27–40.