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Lanthanum Inhibition of the Action of Oxytocin on the Water Permeability of the Frog Urinary Bladder: Effect on the Serosal and the Apical Membrane

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Summary. The effect of La^{3+} ion on the hydrosmotic response of the frog urinary bladder to oxytocin has been studied. When added to the serosal medium, in the concentration range 10^{-4} M to 10^{-3} M, La^{3+} inhibits the response to oxytocin, theophylline and cyclic-AMP, but not to hypertonicity. When added to the apical medium at a concentration greater than 5×10^{-3} M, La^{3+} exhibits an inhibitory effect only during respective stimulation, either by hormone or by hypertonicity, but has no significant effect on the developed response. The significance of this particular mode of action is discussed in terms of possible electrical potential variations of the apical membrane during stimulation.

It is well known that the action of the neurohypophysial hormones on the serosal side of the urinary bladder epithelium results in an increase in the water permeability of the apical membrane of the epithelial cells [5, 9, 12, 13, 15]. The mechanism of this action is not understood, although since the work of Handler and Orloff [8, 19–21] several of the intracellular biochemical steps have been elucidated.

While the mechanism by which the water permeability of the apical membrane itself is modified is unknown, hypotheses may be made concerning the possible structural transformation underlying the membrane permeability change.

It is currently accepted, on the basis of evidence obtained on model systems and on biological membranes [7, 23], that calcium plays an important role in maintaining the tight and ordered organization of biological membranes and therefore their low and selective permeability. It may be reasonably assumed that the apical membrane of epithelial cells has similar

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properties. Along this line of reasoning, the very large increase of water permeability induced by hormone may be the result of the displacement of calcium from its sites in the apical membrane. The ADH effect on cellular calcium movements [4, 26] brings some support to this idea.

If such a hypothesis is correct, it should be possible to inhibit this mechanism, replacing calcium in the apical membrane by a trivalent ion such as lanthanum. This ion is known to be able to take the place of calcium in biological membranes [14]. Being more strongly bound than calcium itself to the sites, it may not be displaced by the hormone.

In the present work, the effect of La^{3+} ions on the apical membrane of the urinary bladder epithelium, and its water permeability changes in response to hormonal stimulation was studied. It was found that La^{3+} effectively inhibits the osmotic water flux increase induced by the hormone. The very peculiar mode of action of La^{3+} throws some light on the mechanism involved in this permeability change.

Materials and Methods

Frogs *(Rana esculenta)* originating from Central Europe were purchased from Burgaud (85 St. Hilaire de Riez, France) and kept in running tap water at 20 to 22 °C. The bladders were removed from pithed frogs and mounted between two Lucite chambers of a two-channels device, so that an area of 1 cm^2 was exposed for each hemibladder. With this device, experiments and controls were conveniently run in parallel on the same bladder.

The serosal face was bathed with a Ringer's-Tris solution (NaCl 112 mM; KCl 5 mM; $CaCl_2$ 1 mM; Tris-HCl 20 mM, pH 7.8). The apical side was bathed either with the same solution or with the solution diluted 20 times, thus creating a large osmotic gradient across the epithelium. The net flux of water across the bladder was measured by the technique of Bourguet and Jard [3] in which the magnitude of the water bulk flow is recorded every minute. The chambers originally described were slightly modified for an easier access to both apical and serosal sides at any moment during the experiment.

The neurohypophysial hormone used was synthetic oxytocin (Syntocinon, Sandoz Pharmaceuticals, Basel, Switzerland).

Results

Permeability Study of Frog Urinary Bladder to Lanthanum Ions

It is known that Lanthanum ions do not cross the epithelial barrier of the frog skin or urinary bladder. Preliminary experiments were carried out on intact bladders mounted according to the method of Bentley [1] with ^{140}La, in the presence of concentrations of the nonradioactive species up

to 10 mM. These experiments show that La^{3+} does not significantly cross the membrane after a 15- to 18-hr incubation period, and that no detectable radioactivity enters the structure itself. (The radioactivity in the bathing medium in these experiments was 0.3 to 0.4 μC/ml.)

However, for permeability experiments, to determine the effect of La^{3+} on water fluxes, the bladder was mounted between two chambers as described previously. Although impermeant, La^{3+} ion might very well cross the structure in these conditions through artifactual leaks due to damages of the membrane during mounting.

As a matter of fact, when 10 mM La^{3+}, including 0.3 to 0.4 μC/ml of ^{140}La, was present in the medium bathing the apical side of the epithelium, small, variable, but significant radioactivity appears in the serosal medium. The concentration of La^{3+} reaches between 10^{-8} to 10^{-7} M in 20 min (as compared to a concentration of 10^{-2} M on the apical side). This amount never exceeds 10^{-6} M after 60-min incubation periods, and is independent of the permeability state of the epithelium, under varying doses of oxytocin in the serosal medium.

Lanthanum Effect on the Serosal Side of the Epithelium

Lanthanum, when present in the medium bathing the serosal side of the epithelium inhibits the hydrosmotic response of the bladder to a maximal dose of oxytocin (15 mU/ml). At a concentration of 10^{-4} M, La^{3+} reduces the response by 60% (Fig. 1*c*) and at a concentration of 10^{-3} M, the response to oxytocin is completely abolished (4 experiments) or at least reduced to 20% or less of the control (3 experiments). When added to the serosal medium after a complete development of the hydrosmotic response to oxytocin (Fig. 2), the effect of La^{3+} is quite comparable, although with slight quantitative differences: at 10^{-4} M, the response is depressed by only 40% (5 experiments), and this action is relatively slow, developing in approximatively 20 min. On the other hand, the response is rapidly and almost completely abolished, as in the previous experiments, by a concentration of 10^{-3} M.

In both types of experiments, below a concentration of 10^{-4} M, the complete removal of La^{3+} restored the ability of the epithelium to respond to oxytocin. After a treatment by 10^{-3} M La^{3+} and then removal, the response to oxytocin was always smaller (about 60% of the response before La^{3+}). In five experiments out of 10, epithelia were unable to respond any more to oxytocin, in spite of repeated washing and a long resting period.

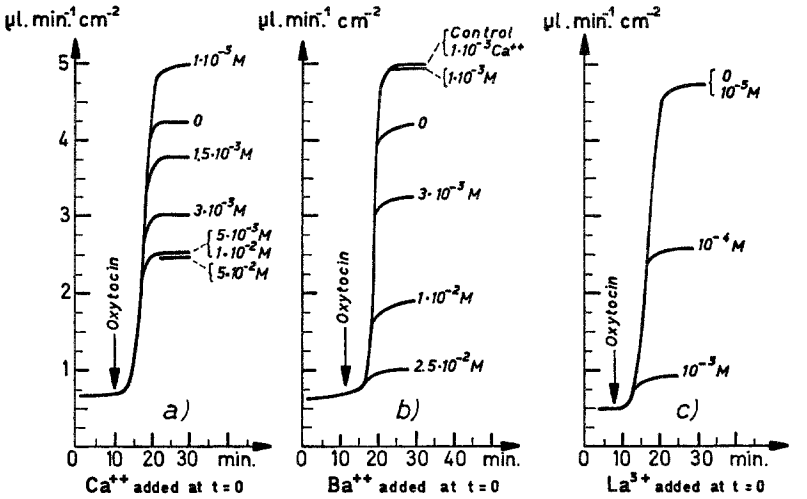

Fig. 1. Inhibitory effect of Ca^{2+}, Ba^{2+} and La^{3+} introduced in the serosal medium on the hydrosmotic response of urinary bladder to a maximal dose (15 mU/ml) of oxytocin. (*a* and *b*). Serosal medium: Ca^{2+}-free Ringer's solution; Ca^{2+} and Ba^{2+} added 10 min before oxytocin. (*c*) Serosal medium: normal Ringer's solution containing 1 mM Ca^{2+}. La^{3+} added 10 min before oxytocin

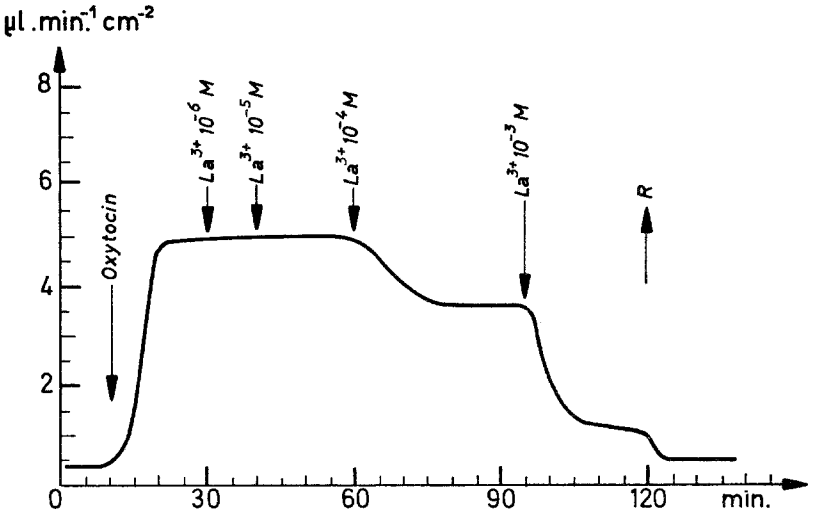

Fig. 2. Inhibitory effect of La^{3+} introduced in the serosal medium on a fully developed hydrosmotic response of urinary bladder to oxytocin. *Oxy* → introduction of, and *R* →, removal of oxytocin

Such an effect of a trivalent ion is not unexpected, since divalent ions, such as Ca^{2+}, normally present at a concentration of 1×10^{-3} M in the standard Ringer's solution, have inhibitory effects at higher concentrations.

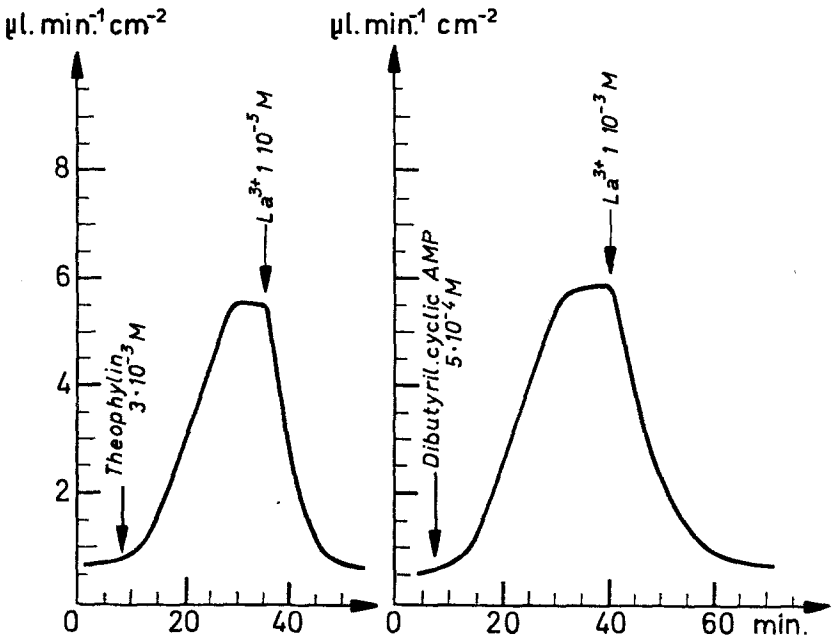

Fig. 3. Inhibitory effect of La^{3+} introduced in the serosal medium on a fully developed hydrosmotic response to theophylline or dibutyryl-cyclic-AMP

Fig. 1*a* and 1*b* show the inhibition produced by calcium and barium ions in the same conditions as La^{3+} in Fig. 1*c*. Calcium does not have a pronounced effect on the response of the bladder to oxytocin, a concentration of 5×10^{-3} M being necessary to reduce this response by 50%. A 10-fold increase of calcium concentration does not have any further significant effect. Barium ions are slightly more effective, depressing the response by about 50% at a concentration of 3×10^{-3} M, and inhibiting it completely at 2.5×10^{-2} M in spite of the hyperosmolarity due to its concentration in the serosal medium.

It is worthwhile noting that the response obtained when the serosal medium is completely free of divalent ion is significantly smaller than in the presence of 1×10^{-3} M calcium or barium. It appears that divalent ions at low concentration do not inhibit, but on the contrary increase the response to oxytocin.

The difference of about one order of magnitude between the divalent and trivalent ionic concentration effective in inhibiting the response to oxytocin might be readily understood if the action of these ions is to be ascribed to a general physico-chemical effect on the membrane. However,

this action seems to be more complex; Peterson and Edelman [22] have shown on the toad bladder that calcium ion inhibits the water flux increased by vasopressin, but not the increased Na^+ transport.

In the case of the frog bladder used in the present work, La^{3+} inhibits not only the response to oxytocin, but also the response to theophylline and to dibutyryl-cyclic-AMP as shown in Fig. 3. On the contrary, La^{3+} has practically no effect on the response to hypertonicity [2]. The increase of water flux induced by 220 mM of mannitol added to the serosal medium – which is of comparable magnitude to the flux induced by a maximal dose of oxytocin [24] – is not modified significantly by the presence of 10^{-3} M La^{3+}, which completely and irreversibly inhibits the response to the hormone.

Therefore, La^{3+} action appears not to be due to a general modification of the serosal membrane permeability; such a modification would affect equally the hydrosmotic response to every kind of stimulus, including hypertonicity. On the other hand, La^{3+} inhibition cannot be accounted for by a single action on the hormone receptor, since La^{3+} is able to reverse the hydrosmotic response induced by theophylline and dibutyryl-cyclic-AMP.

Lanthanum Effect on the Apical Side

As shown by the results obtained in the preceding section, the La^{3+} concentration which is necessary to obtain a significant effect on the serosal membrane (10^{-4} M), is at least two orders of magnitude greater than the maximum concentration which can be accumulated during the time course of an experiment by permeability through the structure, even when the La^{3+} concentration on the apical side is 10^{-2} M. Therefore, every effect observed when the ion is present on the apical medium would have to be attributed to its action on the apical membrane itself.

When present in the medium bathing the apical side of the epithelium, La^{3+} seems to have very little effect (Fig. 4*a*), as compared to control water flux measured without La^{3+}. The reduction of the response to a maximal dose of oxytocin introduced in the serosal medium by a La^{3+} concentration as high as 10^{-2} M (on the apical side) is not significant. In seven experiments, this reduction never exceeds 20%, and may be accounted for by the reduction of the osmotic pressure difference across the membrane, due to the addition of 40 milliosmols to the apical medium. The same reduction is obtained introducing 40 milliosmols of NaCl.

However, after 50 to 80 min, the hydrosmotic flux begins to fade slowly, in such a way that after 2 hr, a further reduction of about 20 to 25% is

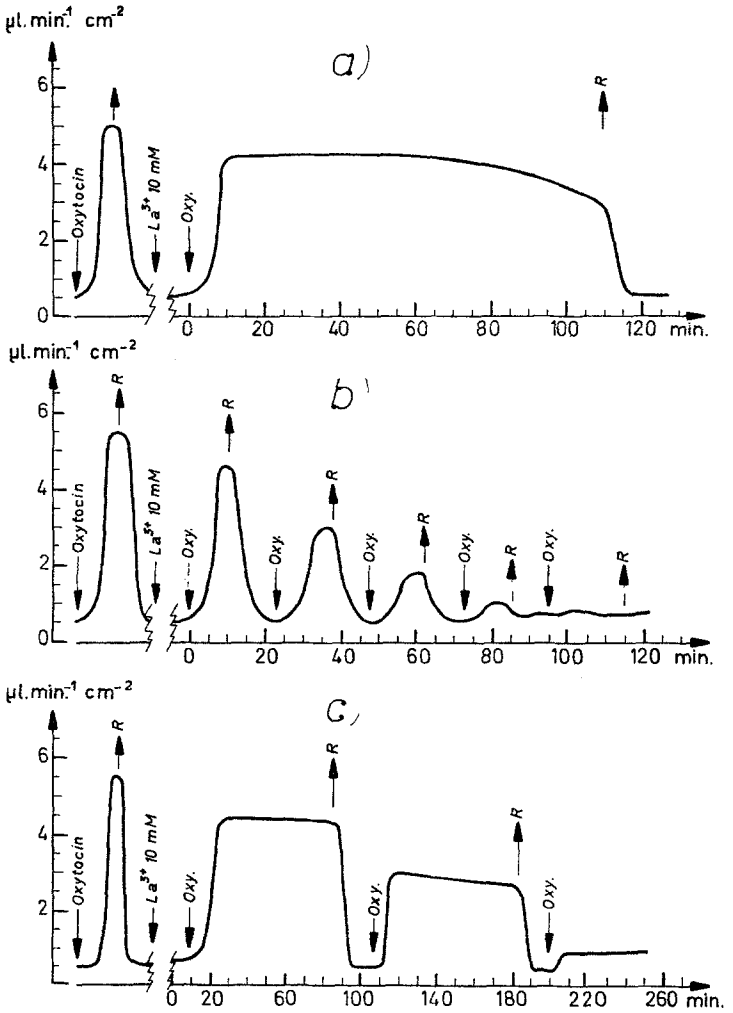

Fig. 4. Inhibitory effect of La^{3+} introduced in the apical medium on the hydrosmotic response of urinary bladder to a maximal dose of oxytocin. During the introduction of La^{3+} in the apical medium, no recording can be made, this period denoted by ⊣ ⊢, lasting 10 to 15 min. *Oxy* →, introduction of, and *R* →, removal of oxytocin in the serosal medium

observed. Such a slow drift of the water flux is not observed with the control during the same time.

A 60-min preincubation period of the apical side with La^{3+} does not result in an increased effect of the ion.

A quite different result is obtained (Fig. 4*b*) when oxytocin is not present continuously in the serosal medium, but only during short suc-

cessive oxytocin pulses separated by resting periods. After La^{3+} addition (10 mM) to the apical medium, oxytocin is added to the serosal side during 10 to 12 min, the necessary time for a full development of the hydrosmotic response, and then washed out. After 10 to 12 min during which the membrane returns to its resting permeability, oxytocin is added again in the same condition, washed out after 10 min and so on.

Under these conditions, the hydrosmotic response drops rapidly. After 3 or 4 pulses of oxytocin, the response was completely abolished (6 experiments). In four others, the response was reduced to less than 20% of the control response. On the other hand, the time course of the response remains identical to control.

Lowering the La^{3+} concentration in the apical medium results in a greater resistance to inhibition; with 5 mM La^{3+}, the inhibition was complete after four pulses in three experiments, but in four others, five to six pulses were necessary to obtain an 80 to 90% reduction only of the responses. Below 5 mM, the number of oxytocin pulses necessary to obtain a significant effect is much greater: with 3 mM, a reduction of less than 50% was obtained after 12 successive pulses, and 2 mM La^{3+} has no significant effect.

It must be noted that the response to oxytocin is not always completely abolished, even with a La^{3+} concentration of 10 mM. As just stated before, in four experiments out of 10 a small but significant response is obtained. In this latter case, more pulses do not result in a further decrease of the response, which remains at the same level in spite of repetitive stimulation.

It appears that La^{3+} can act on the apical side only when the membrane is in an instable state during the development of the response to hormone, or when returning to the normal impermeable state. This peculiar mode of action may be clearly seen in Fig. 4*c*: In this type of experiment, oxytocin was added successively three times on the same membrane for 60-min intervals and not for short pulses. In this case, while there is no significant decrease of the high water flux during each stimulation each new stimulation results in a smaller response.

The La^{3+} effect is completely independent of the water flux increase due to hormonal stimulation. In experiments where three successive pulses were applied to the preparation in the absence of any osmotic gradient across the membrane (by equilibration of Ringer's concentrations on both sides), the same inhibition of the response was obtained after the normal concentration gradient was restored.

At variance with what was observed on the serosal side of the membrane, La^{3+} on the apical side inhibits the membrane response to hypertonicity

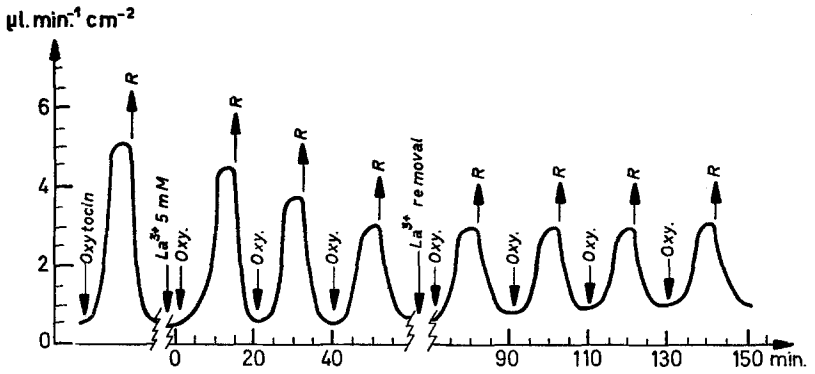

Fig. 5. Irreversibility of the La^{3+} inhibition of the hydrosmotic response of the urinary bladder. For explanation of symbols *see* Fig. 4

(220 mM mannitol added to serosal side) in exactly the same conditions of time and concentration as the response to hormone. This result indicates that La^{3+} affects directly the ability of the apical membrane to respond to stimulations.

Finally, the reversibility of the La^{3+} action on the apical membrane was tested. It appears that once the inhibition is obtained, removal of La^{3+} from the medium does not restore the ability of the membrane to respond to oxytocin. Furthermore, when submaximal concentrations of La^{3+} (5 mM) are applied, and washed out before complete action (that is after three oxytocin pulses), the membrane does not return to normal behavior. This is shown in Fig. 5 where the responses obtained after mild treatment with La^{3+} remain at exactly the same level reached during the last stimulation under the influence of La^{3+}.

The lanthanum effect on the apical side of the membrane appears to be completely irreversible. In an attempt to reverse the La^{3+} effect, EDTA was introduced on the apical side. EDTA complexes La^{3+} more effectively than divalent ions themselves [27]. It is known that by Ca^{2+} complexation, EDTA very rapidly destroys the epithelial structure by dissociating the cells [16]. Due to the slight hydrostatic pressure across the membrane (20 cm H_2O) which exist in our experimental set up, this action of EDTA manifests itself by an abrupt and extremely high increase in the water flux across the membrane.

The effect of EDTA (1.5 mM) in the medium bathing the apical side was studied comparatively on epithelia in contact with La^{3+} for 60 min or more either under continuous excitation by oxytocin or by successive oxytocin pulses. The results are shown in Fig. 6. Epithelia under continuous ex-

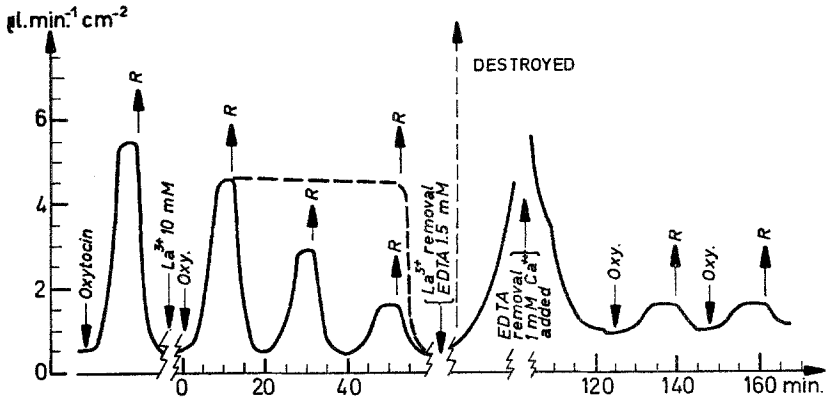

Fig. 6. Effect of EDTA on La^{3+}-treated epithelium (symbols: *see* Fig. 4). After complete inhibition by La^{3+}, epithelia are protected against dissociation by 1.5 mM EDTA. On the contrary (dotted line), uninhibited epithelia are not protected by La^{3+}, and destroyed within 3 to 4 min by EDTA. Note that EDTA does not reverse the La^{3+} inhibition

citation and therefore not inhibited are destroyed by EDTA within 2 to 3 min exactly as controls without La^{3+}. On the contrary, inhibited epithelia are resistant to EDTA. The water flux across them increases relatively slowly, in such a way that it is possible to stop the EDTA action by adding $CaCl_2$ to the apical medium, and to come back to a water flux comparable to resting flux before any treatment. However, the response to oxytocin is not restored.

Discussion

The inhibitory effect exhibited by La^{3+} ions introduced in the medium bathing the apical side of the urinary frog epithelium may be characterized as follows: The inhibition is obtained at relatively high La^{3+} concentration ($> 3 \times 10^{-3}$ M), and affects the response to hypertonicity as well as to oxytocin. The inhibition is irreversible, even after treatment by a chelating agent such as EDTA. However, the main characteristic is that La^{3+} has practically no effect on the membrane when it is either in the impermeable (resting state) or in the highly permeable state (under hormonal influence). La^{3+} ions seem able to exercise their effect only during the few minutes necessary for the membrane to switch from one state to the other.

At the beginning of this discussion, it must be pointed out that these characteristics – in many respects completely different from those of the La^{3+} action on the serosal side – indicate a specific action of the ion on the apical membrane. The concentration build-up of the ion during the

time course of an experiment from the apical into the serosal medium never reaches its threshold concentration of action on the serosal membrane. This demonstrates that La^{3+} present in the apical medium directly inhibits the change in permeability properties of the apical membrane of the epithelial cells. Thus, the La^{3+} effect may provide clues for the understanding of the mechanism by which this structure is able to increase its water permeability by more than an order of magnitude under hormonal influence.

Calcium is given a key role in maintaining the membrane structure and permeability. It is assumed that Ca^{2+} insures the ordered organization of the phospholipid bilayer and stabilizes proteolipid complexes.

Along this line, it is reasonable to assume that calcium plays such a role in the apical membrane, in the resting low permeability state. The highly permeable state, induced by hormone, may be due to a displacement of calcium from its sites, resulting in a membrane structural change.

The La^{3+} effect supports this assumption. La^{3+} is known to be able to displace calcium from its sites of fixation in biological membranes [11, 17, 18, 25, 26, 28]. Replacing calcium in the apical membrane, and being more strongly bound to the sites, La^{3+} will not be displaced under hormonal excitation and therefore will block irreversibly the membrane in the impermeable state.

Such a simple hypothesis does not account for the fact that La^{3+} has no effect on the resting membrane, or on the membrane in the permeable state, where the calcium sites are supposedly vacant and the membrane more permeable to hydrophilic solutes (including ions).

Another La^{3+} action characteristic has to be considered since a relatively high La^{3+} concentration is necessary to obtain the inhibitory effect, and that even at a concentration of 10^{-2} M, several oxytocin pulses (on the average of three) are necessary to reach a complete or nearly complete inhibition. This implies that during each membrane transformation, La^{3+} is able to replace only a fraction of the Ca^{2+} on their sites. The reason for this slow and progressive mode of action is to be found mainly in the membrane resistance to penetration by trivalent cations such as La^{3+}. In normal conditions, one may expect that due to its electrical charge, La^{3+} is completely prevented from penetrating the membrane structure. The fact that La^{3+} seems to have an immediate effect at lower concentration on the serosal side, indicates that in this case, the site of action is superficial.

It may be concluded that if La^{3+} acts only during the transition period between the impermeable and the permeable state it is because during this period, not only Ca^{2+} ions are more mobile, but mainly because the local

electric field is modified in such as a way that La^{3+} can have access into the membrane. It appears that the intracellular mechanism of the hormonal control on the membrane might provide a basis for such a modification. A consequence of hormonal action on the serosal membrane is the activation of a protein kinase present in the urinary bladder epithelial cell [10]. The target protein of this kinase is not known yet, but there is evidence that this kinase phosphorylates a membrane protein as suggested by Dousa and co-workers [6], in the case of the mammalian kidney collecting tubule; the phosphorylated protein may be finally responsible for the membrane permeability change.

Such a phosphorylated protein could eventually displace calcium from its sites and in this way promote a structural change. But the primary effect could be the accumulation of negative charges on the intracellular face of the apical membrane. As a result of this accumulation, the electric potential profile across the membrane will be modified in a way favorable to the penetration of external cations. Such an effect is only transient, since the excess of negative charges is very rapidly satisfied by counter-ions. It may be assumed that calcium ions, already present in the membrane matrix are the first to be displaced, thus promoting the structural change.

The exact localization of the structural change promoting the high permeability state is not known. It is not clear whether the water flux takes place across the apical membrane of the epithelial cells or across the tight junctions between the cells. The experiments carried out with EDTA point to the obvious La^{3+} action in stabilizing tight junctions and protecting them against the dissociating effect of chelating agents.

The two facts that La^{3+}-inhibited epithelia are not dissociated by EDTA and that this chelating agent is not able to reverse the La^{3+} inhibition of the hydrosmotic response may represent two unrelated phenomena. However, the hypothesis that the permeability changes may be located at the level of the intracellular tight junctions rather that at the level of the cellular membrane itself cannot be disregarded.

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