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ROLES OF Na^+ AND Ca^{2+} IN THE INHIBITION BY LOW pH OF THE HYDROSMOTIC RESPONSE TO SEROSAL HYPERTONICITY IN TOAD BLADDER

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Summary

The hydrosmotic responses to serosal hyperosmolar solutions were studied in isolated urinary bladders of toads (*Bufo marinus*) at different pH values to see whether the increase in water flux obtained with serosal hypertonicity in urinary bladders of amphibia is due to non-specific mechanisms or whether it is related to specific ionic regulation.

The increase in net water flux elicited by serosal hypertonicity (Ringer + 250 mM mannitol $\simeq$ 430 mosM) at pH 8.2 is inhibited by lowering the pH to 6.6. The same inhibition is obtained if pH is lowered when the response is fully developed. The inhibition of the development is reversible upon return to pH 8.2, provided serosal Na^+ and Ca^{2+} are present. The reversibility is incomplete in Ca^{2+}-free solutions and is completely abolished in Na^+-free solutions.

It is concluded that the hydrosmotic response to serosal hypertonicity in the amphibian bladder is not a passive phenomenon. It is suggested that during the osmotic challenge there is an increase in a H^+-sensitive Na^+ permeability at the basolateral membrane which may be related to the intracellular events that lead to the permeability changes. Serosal Ca^{2+} could be important in the maintenance of the water permeability in the developed response to hypertonicity.

A hypothesis is presented concerning the possibility of similar mechanisms in the modulation of the hydrosmotic responses to antidiuretic hormone and to serosal hypertonicity.

Introduction

Serosal hypertonicity inhibits the transepithelial Na^+ transport and increases the transepithelial movement of water in urinary bladders of amphibia [1,2].

It has been suggested that its hydrosmotic effect may be transduced by reactions that are common with those that produce the hydrosmotic response to antidiuretic hormone but that are distal to the generation of cyclic AMP [3—8]. In this context, Kregenow et al. [9] suggested that 'the response to hypertonicity could prove useful in illuminating the more distal events in a cyclic AMP-mediated, hormonally-induced transport process'. However, the events that follow a hypertonic challenge and their relationship to permeability changes are unknown.

In order to characterize the responses to serosal hypertonicity in the amphibian urinary bladder, experiments were performed to test whether in the toad urinary bladder, the increased water permeability produced by serosal hypertonicity may be dependent on the pH of the medium in which the response is elicited.

Methods

The experiments were carried out in bladders isolated from toads from the Dominican Republic (*Bufo marinus*, National Reagents, Bridgeport, CT).

The bilobed bladders were cut in half and mounted as sacs (mucosa inwards), filled with 2.5 ml diluted Ringer (see below) and were used one half as control and the other as experimental. Net water fluxes under the imposed osmotic gradient were monitored gravimetrically [1]. Weight changes were recorded at 15-min intervals and converted into water fluxes by assuming that each hemibladder represented a perfect sphere. Results are expressed in μl water lost per surface unit of hemibladder per h. ($\mu l \cdot cm^{-2} \cdot h^{-1}$).

The composition of the serosal Ringer solution was 110 mM Na^+, 5 mM K^+, 1.1 mM Ca^{2+}, 123 mM Cl^-, 5 mM Tris, 10 mM glucose (pH 8.2). The mucosal Ringer solution was a 1 : 10 dilution of the serosal Ringer solution.

The serosal Ca^{2+}-free Ringer solution had the same composition except that $CaCl_2$ was omitted. The absence of Ca^{2+} can produce tissue disrupture, thus in the Ca^{2+}-free experiments $CaCl_2$ was added to the mucosal Ringer to a final concentration of 0.5 mM, which is about 25 times the minimal concentration needed to maintain tissue integrity [10—12].

The composition of the serosal (Na^+-free)-Tris Ringer solution was: 155 mM Tris, 5 mM K^+, 1.1 mM Ca^{2+}, 108 mM Cl^-, 10 mM glucose (pH 8.2). The mucosal Ringer solution was a 1 : 10 dilution of the serosal Na^+ Ringer solution, thus containing 11 mM NaCl. Tris instead of choline was chosen to substitute isoosmotically for Na^+ since choline increases the production of CO_2 by the toad urinary bladder, whereas Tris does not and it has been suggested that choline itself may be used as a metabolic substrate and/or activate the metabolism of the bladder [13].

Serosal Ringer solutions had an osmolality of 250—255 mosM/kg H_2O; mucosal Ringer solutions's osmolality was 30—35 mosM/kg H_2O. Thus, the mucosa-to-serosa osmotic gradient in basal (unstimulated) conditions was about 220 mosM.

Hypertonic Ringer solutions to which 250 mM mannitol was added had an osmolality of 420—430 mosM/kg H_2O; thus, when hypertonicity was created, the osmotic gradient across the hemibladders was about 400 mosM.

During the experiments the serosal solutions were changed every 15 min. The pH changes were adjusted with HCl. Results are presented as the means ±S.E. of nine experiments, unless otherwise noted. The challenge with serosal hypertonicity was always done at t = 15 min.

Results

The basal water flux of the bladders in serosal Ringer solution was 2.78 ± 0.02 $\mu l \cdot cm^{-2} \cdot h^{-1}$ (n = 22).

Lowering the serosal pH from 8.2 to 5.0 had no effect on basal net water fluxes. Below pH 5.0, transient increases in the transepithelial water flux were observed. This increase was maximal at pH 3.5, presenting a peak of 17.03 ± 0.18 $\mu l \cdot cm^{-2} \cdot h^{-1}$ 20 min after lowering the pH and then spontaneously returning to the previous basal value.

If the bladders were maintained at pH 3.5 for up to 90 min, returned to pH 8.2 for 15 min and then challenged with antidiuretic hormone, they displayed a normal hydrosmotic response, showing that the integrity of the bladder was maintained even at such a low pH.

When serosal hypertonicity was created, the water flux increased in a 2-step fashion. This phenomenon was more clearly seen in the response of a single bladder (Fig. 1). First, there was a sudden small increase in water movement of about 10—15 $\mu l \cdot cm^{-2} \cdot h^{-1}$; followed by a secondary increase in water flux that shows a sigmoidal shape and which plateaued at 50—70 $\mu l \cdot cm^{-2} \cdot h^{-1}$ 60 min after the serosal Ringer solution became rendered hyperosmotic.

The first rise in water flux can be attributed to the sudden increase in

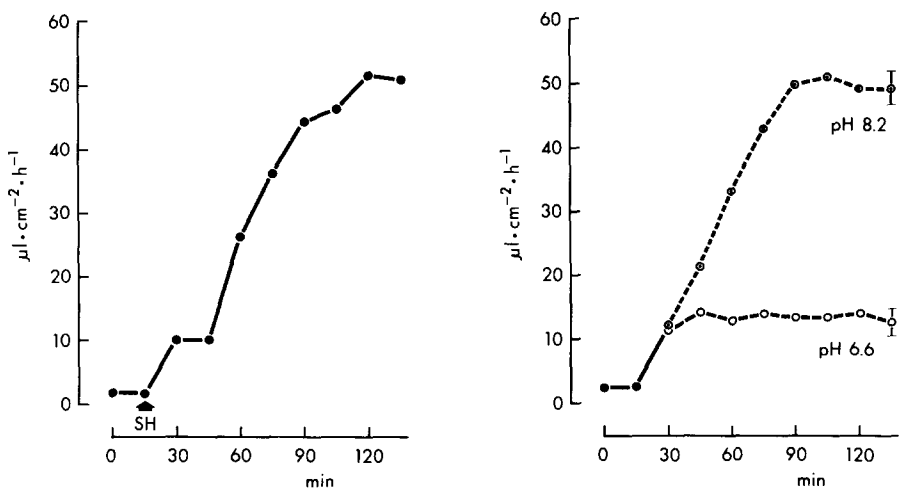

Fig. 1. Hydrosmotic response of a single urinary bladder. Mannitol (250 mM) added at t = 15 min to the serosal Ringer solution. SH, serosal hypertonicity.

Fig. 2. Inhibition by low pH of the development of the hydrosmotic response to serosal hypertonicity. After two periods of monitoring the basal water flux (●) (n = 18) half of the group of hemibladders were challenged with hypertonic serosal Ringer solution (at t = 15 min); the controls at pH 8.2 (⊙) and the experimentals at pH 6.6 (○).

osmotic gradient, while the second sigmoid-shaped enhancement is the development of the response per se (cf. ref. 14). Due to the variability of the first increase, it is not noticeable when the mean of several responses are plotted, but it can be observed when the development is inhibited.

The development of the response elicited by serosal hypertonicity at pH 8.2 is suppressed if, at the same time that mannitol is added, the pH of the serosal Ringer solution is lowered to 6.6 (Fig. 2). The water flux that remained above basal values was probably due to the increased osmotic gradient, whereas the secondary increase (i.e. the development of the response) is completely inhibited.

When pH was lowered from 8.2 to 6.6 after the response to hypertonicity had developed (60 min after addition of the hypertonic Ringer solution), the water flux was inhibited. The plateau achieved by the control was 49.62 ± 2.51 $\mu l \cdot cm^{-2} \cdot h^{-1}$ while the plateau of the experimental hemibladders fell to 18.03 ± 1.25 $\mu l \cdot cm^{-2} \cdot h^{-1}$, 60 min after lowering the pH (Fig. 3).

The reversibility of the inhibition produced by low pH was tested by challenging all the hemibladders with serosal hypertonicity at pH 6.6 during 60 min. After this period, half of the hemibladders were transferred to hypertonic Ringer solution at pH 8.2. Upon reduction of the serosal H^+ concentration, the hydrosmotic response developed immediately, reaching the same plateau values achieved by the response elicited at pH 8.2 (Fig. 4).

What could be the mechanism by which low serosal pH inhibits the

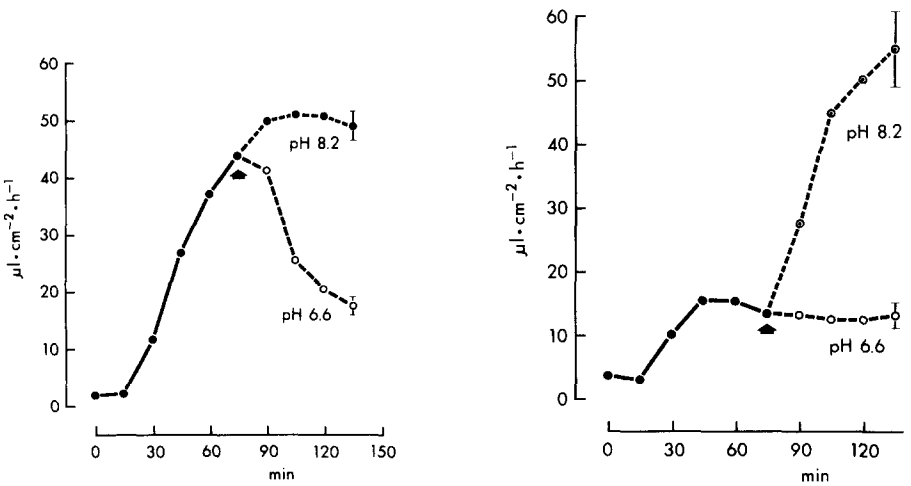

Fig. 3. Inhibition by low pH of the developed hydrosmotic response to serosal hypertonicity. At $t = 15$ min the hemibladders ($n = 18$) were challenged with hypertonic solution at pH 8.2 (●). When the response had developed half of the group was transferred to hypertonic solution at pH 6.6 (○) and the other half remained at pH 8.2 (◎).

Fig. 4. Reversibility of the inhibition by low pH of the development of the hydrosmotic response to serosal hypertonicity. All the hemibladders ($n = 18$) were placed at $t = 15$ min in serosal Ringer solution at pH 6.6 (●). After 60 min (arrow), half of the group was transferred to serosal hypertonic Ringer solution at pH 8.2 (◎), while the other half remained at pH 6.6 (○).

hydrosmotic response to serosal hypertonicity? Low pH affects the ionic permeabilities of excitable and non-excitable membranes, thus the inhibition of the development of the hydrosmotic response to hypertonicity by low pH could be due to H^+ causing a decrease in the cationic permeability of the basolateral membrane.

Low pH has the following affects: (1) it decreases the rate constant of Na^+ influx into erythrocytes [15]; (2) in axons, it lowers the Na^+ conductance and shifts the voltage dependence of Na^+ activation [16]; (3) it blocks the Na^+ permeability of dark-adapted rod outer segments of frog retina [17]; (4) it increases the effective resistance of skeletal muscle [18]; (5) it blocks the Ca^{2+} influx into nerve terminals [19] and smooth muscle [20]. Thus, the roles of serosal Na^+ and Ca^{2+} were tested on the reversibility of the inhibition of the hydrosmotic response to hypertonicity by low pH.

The hyperosmotic challenge was done at pH 6.6 and the water flux monitored during 60 min. After this period, during which the development of the response was suppressed, all hemibladders were transferred to hypertonic Ringer solutions at pH 8.2; the controls to full strength hypertonic Ringer solutions and the experimentals to hypertonic Ringer solution from which $CaCl_2$ was omitted or NaCl isoomotically substituted by Tris.

After transferring the bladders from hypertonic solution (pH 6.6) to a Ca^{2+}-free hypertonic solution (pH 8.2), the response started to develop as in the

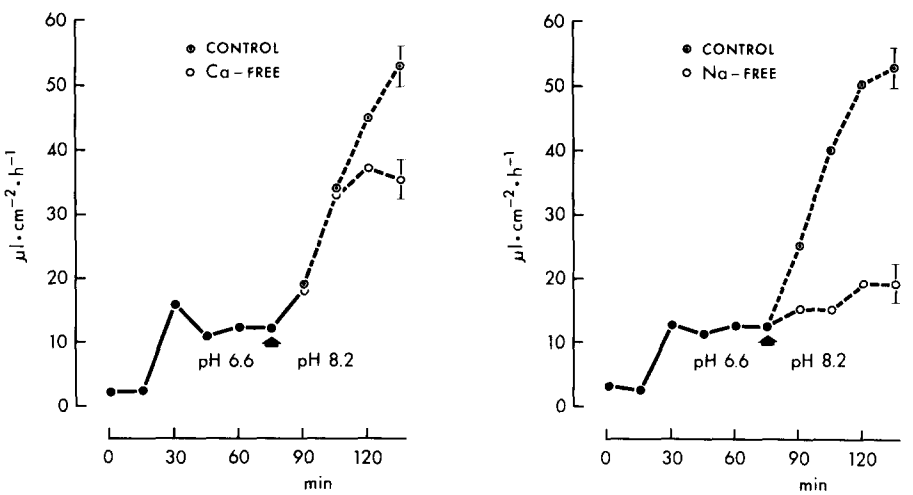

Fig. 5. Effect of withdrawing $CaCl_2$ from the serosal Ringer solution on the reversibility of the inhibition by low pH of the development of the hydrosmotic response to serosal hypertonicity. At t = 15 min, the hemibladders (n = 18) were placed in the serosal hypertonic solution at pH 6.6 (●); after 60 min (arrow) all were transferred to serosal hypertonic Ringer solution at pH 8.2, half with (⊛) and half without (○) serosal Ca^{2+}.

Fig. 6. Effect of isoosmotic substitution of serosal NaCl by Tris on the reversibility of the inhibition by low pH of the development of the hydrosmotic response to serosal hypertonicity. At t = 15 min, the hemibladders (n = 18) were placed in the serosal hypertonic solution at pH 6.6 (●); after 60 min, (arrow) all were transferred to serosal hypertonic Ringer solution at pH 8.2, half with (⊛) and half without (○) serosal Na^+.

control, but the plateau achieved a lower value (35.12 ± 3.02 $\mu l \cdot cm^{-2} \cdot h^{-1}$) than the control ($52.20 \pm 3.20$ $\mu l \cdot cm^{-2} \cdot h^{-1}$) (Fig. 5).

The absence of Na^+ had a dramatic effect (Fig. 6), completely inhibiting the development of the reversal, the water flux remaining at the level of that created by the large osmotic gradient (19.32 ± 3.25 $\mu l \cdot cm^{-2} \cdot h^{-1}$).

Discussion

The results clearly demonstrate that the hydrosmotic response to hypertonicity is not just the result of non-specific mechanisms related to cell shrinkage or mechanically induced alterations of the membranes and other structures, since the same mechanical forces are still operating at low pH but are unable to induce a permeability change to water.

Hypertonicity exerts in duck erythrocytes a norepinephrine-like response that has been shown to be totally independent of cyclic AMP [9]. In frog bladder, indirect analysis of the relationship between levels of intracellular cyclic AMP and the response to hypertonicity suggests that cyclic AMP has a facilitating effect but is not a necessary condition [4,5]. This suggestion stems from the fact that inhibitors of adenyl cyclase in amphibian bladder, like norepinephrine and prostaglandin E_1, can inhibit the development of the response if added previous to the hypertonic challenge but has no effect if added when the response is already developed. Thus, lowering the cyclic AMP content of the cell can affect the development, but the developed response seems to be independent of cyclic AMP.

In the present experiments, an increase in the serosal H^+ concentration blunted the development of the hydrosmotic response to serosal hypertonicity and the same effect was obtained by the low serosal pH on the developed response. Thus, it is likely that the effects of H^+ on the osmotically-induced water movements in the urinary bladder are mediated independently of their action on adenyl cyclase.

The inhibition of the hydrosmotic response to serosal hypertonicity by low pH is reversible, but a full reversibility is observed only when both serosal Na^+ and Ca^{2+} are present. This suggests that, in spite of the presence of Na^+ and Ca^{2+} in the serosal Ringer solution, an increase in the serosal H^+ concentration interferes in some fashion with the availability of Na^+ and Ca^{2+} to the basolateral membrane. Thus, serosal Na^+ and Ca^{2+} would be needed to obtain a full serosal hypertonicity-induced hydrosmotic response. This hypothesis has been confirmed by preliminary experiments. The development of the response to serosal hypertonicity at pH 8.2 was completely inhibited when serosal Na^+ is isosmotically substituted by Tris. The absence of serosal Ca^{2+} did not modify the development, but the response plateaued at a lower level than the control.

From these preliminary observations and the results here reported, it is concluded that serosal Na^+ is an essential requirement for the development of the hydrosmotic response to serosal hypertonicity, and serosal Ca^{2+} is needed in the maintenance of water flow in the developed response.

For the time being, the mechanism by which an increased serosal H^+ concentration blunts the response can only be a matter of speculation.

Isolated cells of toad bladder possess a net negative charge, displaying an

amphoteric behavior; their electrophoretic mobility is reduced by divalent cations and H^+ compete with Ca^{2+} for binding to anionic groups at the cell surface [11]. The displacement of Ca^{2+} by H^+ at the basolateral membrane could play a role in the inhibition of the developed response to hypertonicity.

On the other hand, the entry of Na^+ into the cells could be a requisite in order for serosal hypertonicity to produce a net transepithelial water flow. The basolateral membrane of the epithelial cells of the urinary bladder may have Na^+ channels, the conductance of which might be increased by hypertonicity. If these Na^+ channels have negative fixed charges, like those described in other systems [16,17,21], the protonization would screen the charges. Consequently, their permeability to Na^+ would be reduced by low pH. The outcome would be the same as when serosal Na^+ is absent: the development of the response to hypertonicity is inhibited.

The hydrosmotic response of the amphibian bladder to antidiuretic hormone also has specific ionic requirements. (1) Can be inhibited by low serosal pH [1,2], and our laboratory has recently presented data to suggest that an increased serosal H^+ concentration may inhibit the response by acting on both pre- and post-cyclic AMP steps [22]. (2) A Ca^{2+}-free serosal solution does not seem to affect the development of the antidiuretic hormone-induced net water flux, but reduces the magnitude of the peak response and inhibits the plateau of the osmotic water flow [23—25]. (3) Na^+-free serosal solutions inhibit the development of the response [1,2], and it was suggested that Na^+ plays a role in a post-cyclic AMP step [26].

The striking similarities of the ionic dependencies of the hydrosomotic responses to antidiuretic hormone and serosal hypertonicity support the hypothesis that both stimuli share common cellular mechanisms that produce an increase in transepithelial water permeability [3—8].

A significant entry of Na^+ from the serosal side into the epithelial cells has been a matter a controversy [27]. Electron microprobe analysis of toad urinary bladder epithelium [28] and frog skin [29] revealed that the cellular Na^+ is almost exclusively exchangeable from the mucosal side, but after antidiuretic hormone a significant fraction of the total cellular Na^+ seems to originate from a serosal influx (Rick, R., Dörge, A. and Roloff, C., personal communication).

Although a relationship between changes in the concentration of intracellular Na^+ and the effects of antidiuretic hormone has, in principle, been ruled out [30], the question is far from clear.

An entry of Na^+ across the basolateral membrane into the cells after the hormonal or the hypertonic challenge might play a role in triggering the excitation-permeability coupling.

Antidiuretic hormone can produce changes in the compartmentalization of cell Ca^{2+} in rat hepatocytes [31], mitochondria of rat kidney [32], homogenates of toad bladder cells [33] and toad bladder epithelium [33,34].

I have suggested that at least part of the system might work with the type of interactive organization postulated by Rasmussen and Goodman [35] for a variety of cellular responses in which cyclic nucleotides are involved. That is, Ca^{2+} can trigger, modulate and interact at different levels with the reactions that result in a permeability change at the apical membrane [25].

An increase in the concentration of cytosolic Na^+ can release bound Ca^{2+}

from intracellular pools [36—38]. In the urinary bladder of amphibia, an increase in the concentration of cellular Na^+ after the hormonal or the hypertonic challenge could lead to an increase in intracellular ionic Ca^{2+} concentrations which in turn could critically participate in the mechanisms of modulation of the permeability change to water.

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