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Hydrosmotic Salt Effect in Toad Skin: Urea Permeability and Glutaraldehyde Fixation of Water Channels

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Summary. The "hydrosmotic salt effect" (HSE), the reversible dependence of skin osmotic water permeability upon the ionic concentration of the outer bathing solution, is known to induce the appearance of sucrose-impermeable pathways in the apical membrane of the outermost epithelial cell layer. Diffusional ^{14}C-urea permeability, measured in the $J_v = 0$ condition to prevent solvent drag effects, indicates that the newly formed pathways induced by HSE are narrower than the size of the urea molecule, being therefore highly selective for water molecules. After mild glutaraldehyde (2% solution) fixation of the apical membrane structures, the water channels induced by the HSE are no longer affected by the ionic strength of the outer solution. This indicates that the channel-forming membrane protein can be fixed in different configurations with the water channels in the open or closed states.

Key Words toad skin · hydrosmotic salt effect · water permeability · glutaraldehyde fixation · urea permeability

Introduction

The reversible dependence of osmotic water permeability (L_{PD}) of amphibian skin upon the ionic concentration of the outer bathing solution has been termed "hydrosmotic salt effect" (HSE) [5]. It was demonstrated that pathways for water movement appear in the apical boundary of the skin when the ionic strength of the outer solution is increased. These new pathways are not permeable to sucrose [5].

The HSE shows exponential onset and offset time courses [5] which are clearly distinct from the sigmoidal time evolution associated with the increase or decrease in osmotic permeability of amphibian epithelia [5–7, 10, 11] induced by addition or removal of antidiuretic hormone (ADH). These differences support the hypothesis that the increase in water permeability in the course of the HSE is not due to incorporation of new water permeability units, or channels, into the apical border of the most superficial epithelial cells, as accepted for the ADH effect [8, 9, 18], but seems to be due to modification of existing structures leading to the opening of water pathways [5]. In artificial collagen membranes, the permeability to solutes and water is strongly influenced by the ionic strength of the bathing solution, and this effect is mediated by membrane phase transition [4].

For the HSE, it is conceivable that integral membrane proteins in the apical membrane of the most superficial cell layer change their configuration as a function of the external solution ionic strength, leading to the observed changes in water permeability. As an alternative to explain the HSE, it is also conceivable that increases in the ionic strength of the outer solution would result in a progressive "melting" of the structured water bound to the vicinity of existing water channels within the apical membrane causing an increase in their water permeability.

To distinguish between these two possibilities, we attempted to stabilize the membrane protein structures [20] by mild treatment with glutaraldehyde [12–15, 19] and the results indicate that this stabilization locks the water channels in open or closed states. This fact strongly supports the notion that the HSE is associated with changes in membrane protein conformation mediated by the ionic strength of the outer solution.

Materials and Methods

The experiments were carried out in abdominal skins of the toad *Bufo marinus ictericus* bathed on the inner side by NaCl-Ringer's solution and on the outer side by distilled water or KCl solutions. The osmotic flow was measured by the gravimetric

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Table. Diffusional ^{14}C-urea permeability (P_D^{urea}) in toad skins for different external bathing solutions[a]

Skin	Outer bathing solution ($P_D^{urea} \times 10^4$ cm min^{-1})		
	Sucrose (220 mOsM)	KCl (60 mOsM) plus sucrose (160 mOsM)	KCl (180 mOsM) plus sucrose (40 mOsM)
1	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
2	1.3 ± 0.1	1.5 ± 0.1	1.7 ± 0.1
3	1.1 ± 0.0	1.2 ± 0.0	1.3 ± 0.0
4	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
5	1.0 ± 0.0	1.1 ± 0.0	1.2 ± 0.0
6	1.9 ± 0.2	2.1 ± 0.0	2.2 ± 0.0

[a] P_D^{urea} was measured in efflux experiments in the $J_v = 0$ condition. The inner bathing solution was NaCl-Ringer's solution. The values in the Table are the mean of ten consecutive determinations, each lasting three minutes.

method, using a rotating bell-shaped chamber of 18 cm^2 of area [5]. A piece of skin was tightly fixed to the chamber border with several turns of string, and the lateral area of the chamber covered with a Teflon® ribbon to isolate the edge-damaged area and prevent undue amount of solution to be retained in this region during weighing. The osmotic flow J_v was calculated by the slope of the regression line of chamber weight measured every 5 min as a function of time, assuming the relative density of the transported solution to be equal to 1. L_{PD} was calculated as

$$\left(\frac{J_v}{\Delta\pi}\right)_{\Delta P=0}.$$

The mild glutaraldehyde (E. Merck, Darmstadt) fixation [12–14] of the apical membrane structures was performed with 2% glutaraldehyde in water or KCl solution without buffer, according to the experimental protocol, for a period of 2 min followed by thoroughly rinsing the outer membrane surface with distilled H_2O or KCl solution to remove the fixative.

The permeability to ^{14}C-urea was determined in the absence of an osmotic gradient across the skin to prevent solvent drag of urea [3]. The isotopic flux experiments were performed in a specially designed chamber with an area of 3.14 cm^2 in which the outer bathing solution was vigorously stirred by means of a stainless steel propeller with the blades rotating at 3000 rpm, 1.5 mm above the outer skin surface. The isotope, 30 μCi of ^{14}C-urea (New England Nuclear), was added to the inner compartment, the solution being stirred by a magnetic stirrer. After an equilibration period of 30 min, the external solution was removed by suction every 3 min and replaced by fresh solution of the same composition. An aliquot of each sample was counted in a liquid scintillation counter (Beckman LS-8000) in Bray's solution. Special precautions were taken to prevent the effect of skin edge damage on the labeled urea efflux by using hemichambers with silicone gaskets [22].

NaCl-Ringer's solution had the following composition (in mM): NaCl 115.0; $KHCO_3$ 2.5 and $CaCl_2$ 1.0, with pH 8.2 after aeration and osmolality of 220 mOsM. The osmolalities of the outer bathing solutions—KCl, sucrose, or KCl plus sucrose—were calculated from their molar concentration according to tables (Handbook of Chemistry and Physics, CRC Press, 59th edition, 1959, p. D-265). The results are presented as mean ± standard error. n is the number of experiments.

Results

Hydrosmotic Salt Effect and Urea Permeability

These experiments were carried out to evaluate the specificity to urea, a small hydrophilic molecule, of the pathways induced in the apical membrane by the rise of the ionic strength of the outer solution.

Three successive periods of ten determinations each were performed with the following external solutions (in mOsM): sucrose 220, KCl 60 plus sucrose 160, and KCl 180 plus sucrose 40. As shown in the Table, no significant increase in urea permeability was observed.

Glutaraldehyde Fixation of Skins with Low or High Osmotic Permeability

Two experimental groups of skins were submitted to glutaraldehyde fixation. In group A the fixation was performed with the skins under low osmotic permeability condition due to the sole presence of water in the outer compartment. In group B, the fixation was performed in skins with high osmotic permeability induced by an outer bathing solution of high ionic strength (KCl 170 mOsM). For both groups, three successive experimental periods of approximately 30 min each were carried out in each skin according to the following protocol, where the composition of the outer bathing solution is indicated:

	Group A	**Group B**
1st period	H_2O	KCl 170 mOsM
Fixation	H_2O + glutaraldehyde (2%)	KCl 170 mOsM + glutaraldehyde (2%)
2nd Period	H_2O	KCl 170 mOsM
3rd Period	KCl 170 mOsM	H_2O

The osmotic permeabilities of the skin for the two groups in the three periods are presented in the Figure. The effect of the fixative was to lock the skin permeability at a higher or lower value according to the condition of the prefixation period (1st period), rendering the skin insensitive to further changes in the ionic strength of the outer bathing solution.

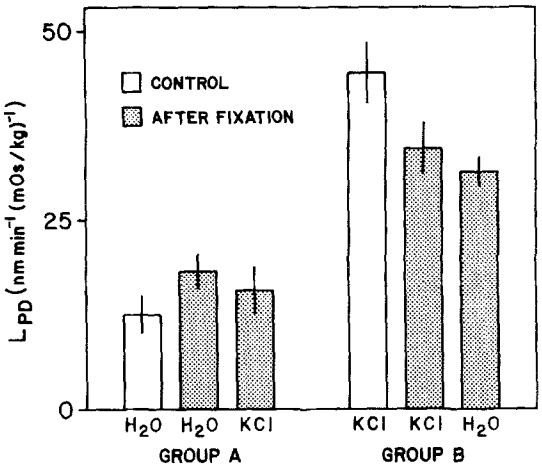

Fig. Glutaraldehyde fixation of skins with low (group A) or high (group B) osmotic permeability (L_{PD}). In A, L_{PD} was first measured with distilled water as the outer solution (control condition). Fixation was then performed for 2 min with 2% glutaraldehyde in water, and L_{PD} was again determined with distilled water and with 170 mOsM KCl. In B, L_{PD} was measured with 170 mOsM KCl in the outer compartment (control condition). Fixation was then performed for 2 min with 2% glutaraldehyde in 170 mOsM KCl, and L_{PD} was again measured with 170 mOsM KCl and with distilled water. NaCl-Ringer's solution bathed the inner skin surface in both groups. $n = 6$ skins for each group

Discussion

The hydrosmotic salt effect cannot be explained by a paracellular permeability increase due to tight junction opening since no effect of this condition upon diffusional sucrose permeability was observed [5]. In amphibian skin, different procedures known to increase paracellular permeability, such as the presence of hypertonic [16], or acid [5, 17] solutions in the outer compartment, Li^+ for Na^+ substitution in the outer solution [2], or the effect of vanadate [1], are accompanied by an increase of the skin sucrose permeability. The lack of sucrose permeability increase during the HSE supports the notion that the new pathways are located in the apical membrane of the outermost cell layer, and are sufficiently small to restrict the movement of this probing solute [5].

In order to better characterize the size of the newly formed pathways which appear in the course of the HSE, urea, a hydrophilic solute, was used as a probe molecule of small dimension (molecular radius of 2.3 Å) [21].

In the urea experiments, the solutions used to bathe the outer skin surface contained KCl in sufficient concentrations to induce large increases in skin water permeability as can be deduced from the Figure (*compare* bars 1 and 4) and from a previous publication [5]. However, the results shown in the Table indicate that urea permeability is not affected in the course of the HSE. This result as well as the absence of changes in sucrose permeability [5] strongly support the view that the pathways induced in the apical border of the skin in the course of the HSE are highly specific to water molecules.

The second aspect of this study was an attempt to distinguish between the two possibilities raised to explain the HSE: 1) that membrane proteins in the apical membrane of the outermost superficial cell layer undergo structural changes increasing the size or the density of apical membrane water channels; or 2) that the increase in the outer bathing solution ionic strength would lead to a progressive "melting" of the structured water bound to the entrance of the water channels of the apical membrane, here assumed to be of fixed size.

Glutaraldehyde should not affect water permeability in the second hypothesis mentioned above. On the other hand, due to its cross-linking properties, glutaraldehyde would stabilize the putative protein structures which form the walls of the water channels, locking them in closed or open states, according to the prefixation condition.

The water channels which can be reversibly modulated by the ionic strength of the outer solution in fresh skins [5] are no longer affected by the ionic strength after glutaraldehyde treatment (Figure), indicating that the channel-forming membrane protein can be fixed in different configurations with the water channels in the open or closed states.

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