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PHYSIOLOGY

Involvement of Phosphatidylinositol Kinases in the Effect of Oxidized Glutathione and Drug Glutoxim on Na^+ Transport in Frog Skin

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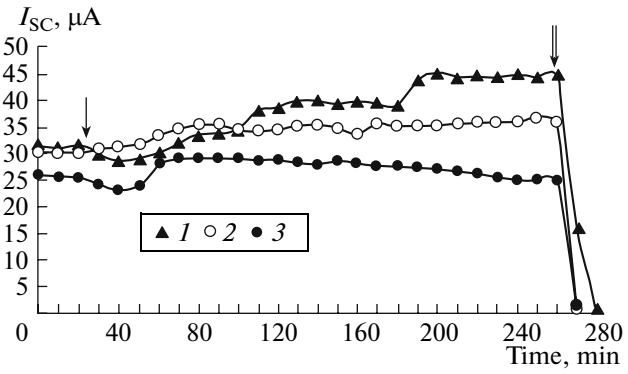
Amphibian skin and other isolated epithelial systems are classic model objects for studying the mechanisms of ion transport through biological membranes. The transport of Na^+ ions in osmosis-regulating epithelia is a complex multicomponent system whose functioning ensures the creation and maintenance of electrolyte and water homeostasis. Various protein components of this system may be targets for oxidative stress. Earlier, we showed that Na^+ in frog skin is modulated by different oxidizing agents [1]. It was discovered that oxidized glutathione (GSSG) and its pharmacological analogue glutoxim® (FARMAVAM, Moscow), applied onto the basolateral surface of frog skin, mimic the effect of insulin and stimulate the transepithelial Na^+ transport. In addition, we were the first to show, with the use of the tyrosine kinase inhibitor genistein, that tyrosine kinases are involved in the stimulatory effect of GSSG and glutoxim on Na^+ transport in frog skin [2].

It is known that the effect of insulin on Na^+ transport is induced by the hormone binding to the receptor possessing own tyrosine kinase activity, which is located in the basolateral membrane of epithelial cells [3]. Earlier, we showed that the effect of insulin on Na^+ transport in frog skin depends on the activity of tyrosine kinases and tyrosine phosphatases and is regulated by phosphatidylinositol kinases (PI kinases) and protein kinase C [3]. In view of this, it was reasonable to study a possible role of PI kinases in the regulation of Na^+ transport in the frog (*Rana temporaria*) skin by GSSG and glutoxim using two structurally different inhibitors of phosphatidylinositol 3-kinases (PI 3-kinses) and phosphatidylinositol 4-kinases (PI 4-kinases) wortmannin and LY 294002 [4].

The current–voltage characteristics (CVCs) of frog skin were recorded with an automatic voltage recording device [5]. CVCs were used to determine the electric parameters of the skin: the short-circuit current I_{SC} ($I_{SC} = I_T$ at $V_T = 0$, where I_T is the transepithelial current), the open-circuit voltage V_{OC} ($V_{OC} = V_T$ at $I_T = 0$, where V_T is the transepithelial voltage), and the transepithelial conductance g_T. The transport of Na^+ ions was estimated as amyloride-sensitive I_{SC} [5]. The figures show the results of representative experiments with the use of glutoxim (100 μg/ml). Similar data were obtained for GSSG (100 μg/ml).

We found that the addition of 100 μg/ml GSSG or 100 μg/ml glutoxim to the basolateral surface of intact frog skin stimuated Na^+ transport. On average (as assessed by the results of ten experiments), the I_{SC} value increased by 40.37 ± 11.24 ($p < 0.05$) and $20.31 \pm 1.04\%$ ($p < 0.01$), whereas the V_{OC} value increased by 48.05 ± 10.34 ($p < 0.05$) and $19.64 \pm 1.13\%$ ($p < 0.01$) for GSSG and glutoxim, respectively. The g_T did not change. Earlier, we showed that the effect of PI kinase inhibitors on Na^+ transport in frog skin depends on the



Kinetics of changes in the short-circuit current (I_{SC}) after the addition of 100 μg/ml glutoxim to the basolateral surface of intact and phosphatidylinositol kinase inhibitor-treated frog skin and subsequent application of the epithelial Na^+-channel (ENaC) blocker amyloride. (*1*) Changes in I_{SC} after the addition of glutoxim to intact skin; (*2*) changes in I_{SC} after the addition of glutoxim to the skin pretreated for 30 min with 200 nM LY 294002 at the apical surface; and (*3*) changes in I_{SC} after the addition of glutoxim to the skin pretreated for 30 min with 1 μM wortmannin at the basolateral surface. The single arrow indicates the addition of glutoxim to the solution; the double arrow, the addition of 20 μM amyloride from the apical surface.

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concentration of agents and the application site (apical or basolateral skin surface) [6]. With allowance for this fact, we performed four series of experiments for each oxidizing agent. In the first and third series of experiments, the apical surface of frog skin was preincubated for 30 min with wortmannin (500 nM and 1 μM for the first series) or LY 294002 (100 nM and 200 nM for the third series). In the second and fourth series of experiments, wortmannin and LY 294002 were added at the same concentrations form the basolateral skin surface. Thereafter, in each series of experiments, 100 μg/ml GSSG or 100 μg/ml of glutoxim was added from the basolateral surface of frog skin.

We discovered that preincubation of frog skin with wortmannin or LY 294002 significantly decreased the stimulatory effect of glutoxim on Na^+ transport (figure). For example, in the first series of experiments, after the addition of glutoxim to the frog skin pretreated with glutoxim at different concentrations, I_{SC} increased by 8.45 ± 1.29 ($p < 0.01$) and $3.36 \pm 0.24\%$ ($p < 0.01$), and V_{OC} increased by 9.34 ± 2.08 ($p < 0.01$) and $4.01 \pm 1.23\%$ ($p < 0.01$) for 500 nM and 1 μM wortmannin, respectively. In the third series of experiments, I_{SC} increased by 13.84 ± 3.48 ($p < 0.01$) and $11.42 \pm 4.04\%$ ($p < 0.01$), and V_{OC} increased by 15.01 ± 3.43 ($p < 0.01$) and $12.34 \pm 4.32\%$ ($p < 0.01$) for 100 and 200 nM LY 294002, respectively. In the second series of experiments, I_{SC} increased by 10.45 ± 2.48 ($p < 0.01$) and $6.33 \pm 2.24\%$ ($p < 0.01$), and V_{OC} increased by 11.31 ± 3.33 ($p < 0.01$) and $7.12 \pm 2.28\%$ ($p < 0.01$) for 500 nM and 1 μM wortmannin, respectively. In the fourth series of experiments, I_{SC} increased by 22.73 ± 9.48 ($p < 0.01$) and $19.39 \pm 8.01\%$ ($p < 0.01$), and V_{OC} increased by 26.51 ± 9.14 ($p < 0.01$) and $21.34 \pm 9.21\%$ ($p < 0.01$) for 100 and 200 nM LY 294002, respectively. In all experiments, the g_T value did not change. Similar results were obtained when 100 μg/ml GSSG was applied onto the frog skin pretreated with the PI inhibitors.

Our results indicate that, in all experimental variants, PI kinase inhibitors concentration-dependently modulated the effect of GSSG and glutoxim on Na^+ transport in frog skin. For example, preincubation of frog skin with low concentrations of PI kinase inhibitors considerably suppressed (as compared to higher concentrations) the stimulatory effect of glutoxim or GSSG on Na^+ transport. It is known that wortmannin and LY 294002 are highly efficient PI kinase inhibitors. Low concentrations of these agents irreversible inhibit all known types of Pi 3-kinases, whereas at higher (submicromolar) concentrations wortmannin and LY 294002 also inhibit PI 4-kinases [7]. Thus, the results of our experiments indicate that PI kinases are involved in the regulation of Na^+ transport in frog skin by GSSG and glutoxim. However, the fact that both inhibitors are less efficient at low concentrations, at which they specifically inhibit PI 3-kinases, suggests that PI 4-kinases are involved more actively in this process or that the activation of PI 4-kinases is an earlier stage in the realization of the stimulatory effect of GSSG and glutoxim on Na^+ transport in the frog (*R. temporaria*) skin. Possibly, PI 4-kinases may attenuate the inhibition of PI 3-kinases by phosphorylating phosphatidylinositol 3-phosphate remaining in the cell.

It is known that various major Na^+-transporting proteins, such as epithelial amyloride-sensitive Na^+-channels (ENaC) or Na^+/K^+-ATPases, contain numerous cysteine residues, which are the target for intracellular and extracellular oxidizing and reducing agents [8, 9]. However, after the addition of the ENaC blocker amyloride (20 μM) to the solution surrounding the apical surface of frog skin at the end of each experiment, I_{SC} was completely blocked (figure), indicating that the effect of GSSG and glutoxim on Na^+ transport is associated primarily with modulation of ENaC activity.

Thus, we were the first to demonstrate the involvement of PI kinases in the stimulation of Na^+ transport in the frog (*R. temporaria*) skin by GSSG and glutoxim. On the basis of the results of this work and our earlier studies [1, 2], it can be assumed that GSSG and glutoxim may interact with the cysteine-rich domains of the insulin receptor in the basolateral membrane of epithelial cells, induce its transactivation, and trigger the signaling cascade including tyrosine kinases and PI kinases. This results in ENaC stimulation and enhancement of Na^+ transport in frog skin.

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