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The Effect of Glutoxim on Na^+ Transport in Frog Skin: The Role of Cytoskeleton

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Abstract—Using the voltage-clamp technique, the possible implication of cytoskeleton in the effect of glutoxim, a pharmacological analog of oxidized glutathione (GSSG), on Na^+ transport in the skin of frog *Rana temporaria* was investigated. It was shown for the first time that skin preincubation with nocodazole, a microtubular disrupter; cytochalasin D, actin filament disrupter; or protein phosphatase PP1/PP2A inhibitor calyculin A significantly decreased the stimulatory effect of glutoxim on Na^+ transport. The results suggest the involvement of microtubules and microfilaments in the regulatory effect of glutoxim on Na^+ transport in frog skin and that reorganization of actin filaments or microtubules leads to inhibition of the stimulatory effect of glutoxim on Na^+ transport in frog skin epithelia.

Keywords: glutoxim, transepithelial Na^+ transport, cytoskeleton.

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Amphibian skin and other isolated epithelium systems are classic models for studying the mechanisms of ion transport across biological membranes. Na^+ transport in osmoregulated epithelia is a complex multicomponent system that supports electrolytic and water homeostasis. Various protein components of this system are targets of oxidative stress.

Many researchers have focused on the functioning of reduction–oxidation (redox) system in cells, as well as the impact of oxidizers and reducers on various cellular processes under normal and pathological conditions. The gathered experimental data show that the regulation of the cellular redox condition is important for treatment of various diseases, including AIDS and cancer (Sen et al., 1998). The influence of oxidizing and reducing agents has been demonstrated for a number of epithelial tissues. It is known that key Na^+ transporting proteins, such as amiloride-sensitive epithelial Na^+ channels (ENaC), Na^+/K^+-ATPases, and Na^+/H^+ exchangers are targets of oxidizing and reducing agents (Boldyrev and Bulygina, 1997; Firsov et al., 1999). However, the molecular mechanisms of oxidizers and reducers impact on various components of Na^+ transepithelial transport are poorly studied.

Novel binuclear catalytic agents containing nanoadditions of d-metals currently are widely used in clinical practice. Glutoxim is a synthetic biologically active compound, disodium salt of oxidized glutathione (GSSG) with platinum nanoaddition. Glutoxim is widely applied as an immunomodulator and hemostimulator for treatment of bacterial and viral infections (Zhukov et al., 2004), psoriasis (Korsunskaya et al., 2003), and cancer irradiation and chemotherapy (Filatova et al., 2004). Another GSSG analogue, NOV-002 (GSSG in combination with cisplastin in a ratio of 1000 : 1), is a GSSG imitator and induces receptor-mediated activation of proteins involved in hematopoiesis (Townsend et al., 2008).

We found that Na^+ transport in frog skin is modulated by various oxidizing agents, such as cystamine, cystine, GSSG, and glutoxim® (FARMA-VAM, Russia), its synthetic analog (Krutetskaya et al., 2008). It was demonstrated for the first time that GSSG and glutoxim applied to the basolateral surface of frog skin mimicked the action of insulin and stimulated transepithelial Na^+ transport. However, the mechanisms underlying GSSG and glutoxim stimulation of Na^+ transport remain obscure.

It is known that insulin stimulates transepithelial Na^+ transport. The process is initiated by the hormone binding with the receptor having tyrosine kinase activity and localized in the basolateral membrane of epithelial cells (Cox and Singer, 1977). We revealed that insulin effect on Na^+ transport in the frog skin was dependent on tyrosine kinase and tyrosine phosphatase activity and involved phosphatidylinositol kinases and protein kinase C (Melnitskaya et al., 2006a). Cytoskeleton plays an important role in insulin signaling. Actin cytoskeleton mediates morphological, metabolic, and nuclear manifestations of insulin

Abbreviations: ENaC—amiloride-sensitive epithelial Na^+ channels, GSH—reduced glutathione, GSSG—oxidized glutathione, PP1—serine/threonine protein phosphatase PP1, PP2A—serine/threonine protein phosphatase PP2A.

action (Tsakiridis et al., 1994). It is known that cytoskeleton proteins, such as actin (Dalle-Donne et al., 2003) or tubulin (Burchill et al., 1978; Wang et al., 2001), are highly redox sensitive and are easily glutathionylated. Thereby, it seems reasonable to examine whether microfilaments and microtubules are implicated in glutoxim regulation of Na^+ transport in frog *Rana temporaria* skin. Nocodazole (microtubule depolymerizer), cytochalasin D (microfilament depolymerizer), and calyculin A, a protein phosphatase PP1/PP2A inhibitor, were used in the experiments. Calyculin A induces rearrangements of actin (Yano et al., 1995; Rosado and Sage, 2000) and microtubule (Yano et al., 1995) cytoskeleton in various cells.

MATERIALS AND METHODS

Experiments were performed on frog *Rana temporaria* males during the period from November to March. The skin from frog abdomen was cut and placed in a Ussing chamber (World Precision Instruments, Inc., Germany) with a 12-mm inner orifice. The chamber was filled with Ringer's solution for cold-blooded animals: 110 mM NaCl, 2.5 mM KCl, 3.0 mM $CaCl_2$, 5 mM Tris-HCl, pH 7.4. Experiments were carried out at room temperature (22–23°C).

Frog skin electrical parameters were measured with automatic device for voltage-clamp and registration of current-voltage relations (Krutetskaya et al., 2003). To measure current-voltage relations, the ramp 20 mV/s voltage was applied to the skin. In the intervals between the measurements of current-voltage relations, the skin transepithelial potential (V_T) was sustained at 0 mV (short-circuit regime) or at open-circuit potential V_{OC} ($V_{OC} = V_T$ at transepithelial current $I_T = 0$). Short-circuit current I_{SC} ($I_{SC} = I_T$ at $V_T = 0$), V_{OC}, and transepithelial conductance g_T were determined from current-voltage relations.

Na^+ transport was evaluated as amiloride-sensitive I_{SC}. For this reason, at the end of each experiment, 20 μM amiloride, an ENaC blocker, was added to the solution washing the apical skin. It is known that amiloride within the concentration 20–100 μM selectively blocks ENaC (Bentley, 1968). The information gained from the experiments was treated with specific software.

The reagents were purchased from Sigma Co. (United States). Cytochalasin D (2 mg/mL), calyculin A (25 μM), and nocodazole (5 mM) stock solutions were prepared in dimethyl sulfoxide. Amiloride (10 mM) and glutoxim (50 mg/mL) stock solutions were prepared in water. The agents were applied to the apical or basolateral skin surfaces. Agents producing microfilament or microtubule reorganization were added 30–40 min before glutoxim application.

Results were statistically treated with Student's *t*-test. The data are presented as $x \pm s_x$. The figure illustrates the results of typical experiments.

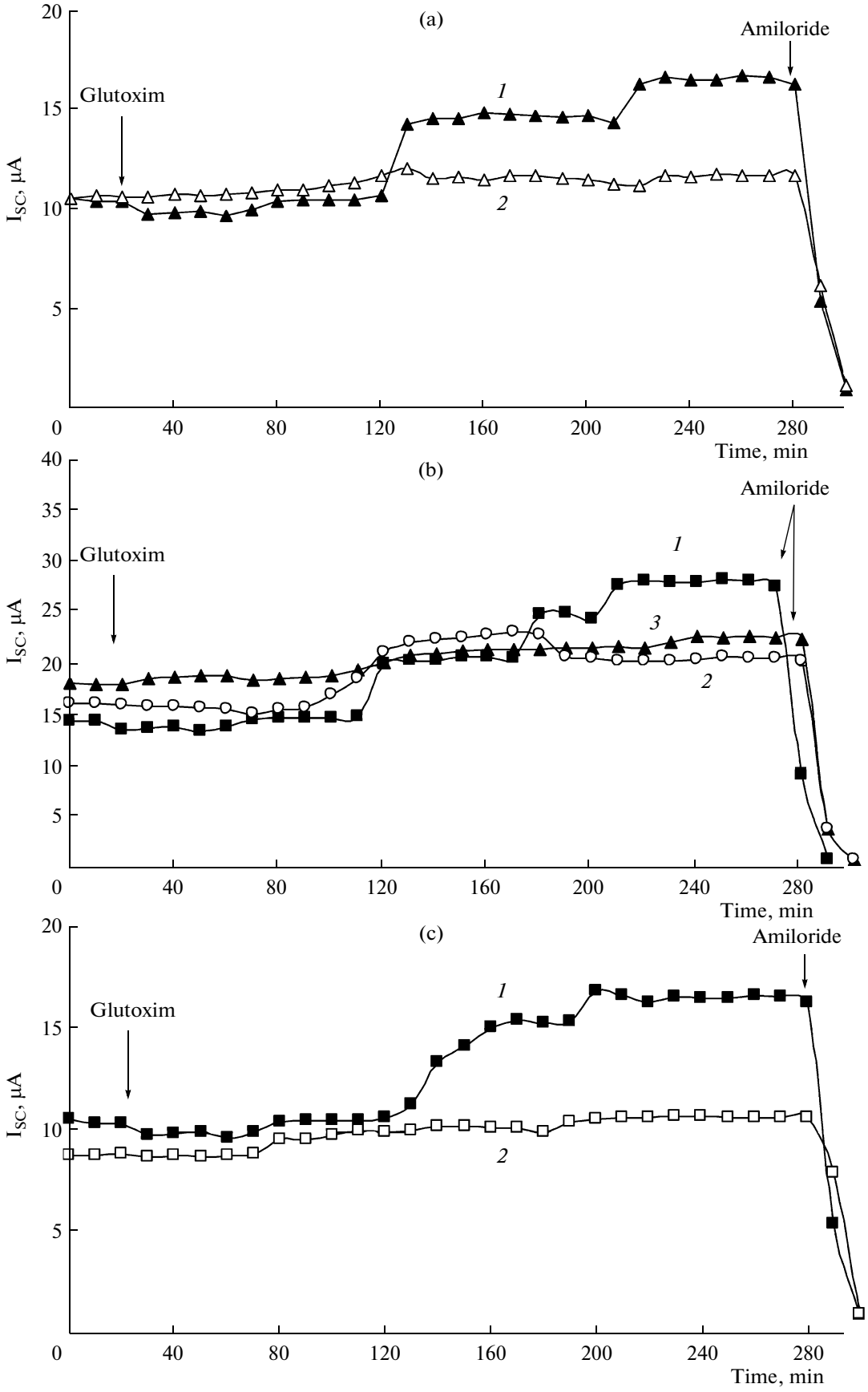
RESULTS AND DISCUSSION

Glutoxim action on Na^+ transport in frog skin. The mean values of frog skin electrical characteristics in control (from ten experiments) are: $I_{SC} = 14.58 \pm 0.91$ μA, $V_{OC} = -38.01 \pm 2.74$ mV, and $g_T = 0.38 \pm 0.01$ mS. One hundred micrograms per milliliter glutoxim applied to the basolateral surface of the intact frog skin like insulin stimulated Na^+ transport. After glutoxim application, I_{SC} increased on average by $31.24 \pm 8.32\%$, whereas V_{OC} increased by $38.04 \pm 5.15\%$ (data from ten experiments). The g_T value was not altered. Based on these, as well as previous, data (Melnitskaya et al., 2006a; Krutetskaya et al., 2008), it is possible to suggest that glutoxim interacts with extracellular domains of insulin receptor in the basolateral membrane of epithelial cells and induces its transactivation, resulting in increased Na^+ transport in frog skin. The data we obtained coincide with the literature data. Thus, GSSG and glutoxim induced transactivation of the epidermal growth factor receptor and activation of its tyrosine kinase activity in human epidermal carcinoma A431 cells (Burova et al., 2005; Vasilenko et al., 2006).

The effect of microtubule disrupter nocodazole. Nocodazole almost totally attenuated the stimulatory action of glutoxim on Na^+ transport in the frog skin (figure a). Skin apical surface treatment with 25 μM nocodazole for 30 min before application of 100 μg/mL glutoxim to the frog skin basolateral surface increased I_{SC} by $9.01 \pm 1.02\%$ and V_{OC} by $10.12 \pm 1.21\%$. The g_T value was not changed.

Na^+ transport in frog skin was found to be dependent on the functional and structural organization of actin and tubulin cytoskeleton (Krutetskaya et al., 2006; Melnitskaya et al., 2006a). It was demonstrated that amitotic drugs (colchicine, colcemid, vinblastin) inhibited I_{SC} in the skin of *R. temporaria* frog. It is known that colchicine, colcemid, and vinblastin bind with the same site of the tubulin dimer and increase its nucleotide triphosphatase activity. The binding of the drugs in a complex with tubulin dimer to the fast-growing microtubule ends diminishes the rate of monomer joining to the end. Nocodazole is more specific than colchicine, and its binding is more reversible (Fulton, 1987).

The microtubule structure reorganization with amitotic agents reduced hormone-produced (aldosterone, vasopressin) stimulation of Na^+ transport (Verrey et al., 1995). Microtubules probably play an important role in exo- and endocytosis by regulation of ENaC density in the apical membrane. Microtubule disruption attenuates the insulin effect on cellular processes (Eyster et al., 2006). However, there is evidence that tubulin cysteine residues are targets of oxidants. It has been shown that GSSG in physiologically higher concentrations inhibited microtubule assembly (Burchill et al., 1978). Our results support the idea that there is implication of the tubulin cytoskeleton in glutoxim regulation of Na^+ transport in frog skin.


(a)
Glutoxim
Amiloride
1
2
I_{SC}, μA
Time, min
(b)
Glutoxim
Amiloride
1
2
3
I_{SC}, μA
Time, min
(c)
Glutoxim
Amiloride
1
2
I_{SC}, μA
Time, min

Dependence of glutoxim-induced changes in the short circuit current I_{SC} on the cytoskeleton integrity and PP1 and PP2A serine/threonine protein phosphatase activity. Curve *1* (a–c) I_{SC} after intact skin basolateral surface exposure to 100 μg/mL glutoxim; curve *2*—I_{SC} after preliminary treatment of the skin apical surface with 25 μM nocodazole (a), 5 μg/mL cytochalasin D (b) or 25 nM calyculin A (c); curve *3*—20 μg/mL cytochalasin D. At the end of each experiment, 20 μM amiloride, a ENaC blocker, were added to the solution washing out the skin apical surface.

The effect of microfilament disrupter cytochalasin D. Preliminary treatment with 5 or 20 μg/mL cytochlasin D dramatically reduced glutoxim stimulation of Na^+ transport in frog skin (figure b). The mean values of electrical characteristics of frog skin exposed to glutoxim on the skin basolateral surface pretreated with cytochalasin D for 30 min on the apical surface were the following (from ten experiments): I_{SC} was increased by 25.25 ± 2.12 and 12.12 ± 1.14%; V_{OC} increased by 28.31 ± 3.31 and 16.14 ± 4.08%, respectively, for 5 or 20 μg/mL cytochalasin D. The g_T value was not altered. These results show that the cytochalasin D effect is dose-dependent. Thus, inhibition of glutoxim-stimulated Na^+ transport is higher with 20 μg/mL than with a dose of 5 μg/mL of cytochalasin D. Moreover, cytochalasin D in low concentrations modifies the kinetics of glutoxim-affected Na^+ transport: a slight increase in the initial phase and dramatic inhibition of the second phase of glutoxim stimulation effect is observed (figure b, curve *2*). Preliminary treatment of frog skin with a higher dose (20 μg/mL) of cytochalasin D inhibited both phases of the glutoxim stimulation effect on the Na^+ transport in frog skin (figure (b), curve *3*).

We reported that cytochalasin D, cytochalasin B and dihydrocytochalasin B produced dose-dependent inhibition of Na^+ transport in *Rana temporaria* skin (Krutetskaya et al., 2006; Melnitskaya et al., 2006b). Here, we have shown that structural rearrangements of the actin cytoskeleton modulated glutoxim effect on Na^+ transport in frog skin.

It is known that actin cytoskeleton is involved in the regulation of Na^+ transepithelial transport and the activity of various Na^+ transport proteins. ENaC plays a key role in Na^+ transport across epithelial systems. There is increasing information suggesting that cytoskeleton plays an important role in the regulation of this type of channel activity. It was shown that ENaC is colocalized with actin filaments and actin-binding proteins (ankyrin and spectrin) (Cantiello et al., 1991; Smith et al., 1991). Furthermore, it was revealed that ENaC directly interacted with SH_3 domain of α-spectrin due to the proline-rich region in the C-terminal of the ENaC α-subunit (Rotin et al., 1994). According to the literature data, ENaC activity is regulated rather by short actin filaments than by monomer G- or fiblillar F-actin. The influence of microfilaments on ENaC activity may be also mediated by the channel lipid microenvironments (Cantiello et al., 1991; Prat et al., 1992) or associated with actin's function as an important component of signal cascades (Cantiello et al., 1993; Janmey, 1998). Moreover, the impact of actin cytoskeleton architecture on ENaC activity significantly depends on the experimental object. Thus, in A6 cells, pharmacological agents able to disrupt F-actin, e.g., cytochalasins, stimulate Na^+ transport (Cantiello et al., 1991; Prat et al., 1992; Rehn et al., 1998). On the other hand, in amphibian skin and bladder, microfilament disruption with cytochalasins inhibits Na^+ transport and reduces the transepithelial potential (Els and Chou, 1993; Chou and Els, 1995).

The effect of calyculin A, a protein phosphatase inhibitor. The figure (c) illustrates the kinetics of I_{SC} modification with consecutive addition to frog skin calyculin A, glutoxim and amiloride, a ENaC blocker. It is seen that treatment of the skin apical surface with 25 nM calyculin A for 30 min before addition of 100 μg/mL glutoxim to the solution washing out the skin basolateral surface almost totally eliminates the drug's stimulating effect on I_{SC} via frog skin. The average I_{SC} and V_{OC} increase after the calyculin A and glutoxim combined exposure was (from ten experiments) 10.14 ± 2.08 and 14.44 ± 3.67%, respectively. The g_T value was not altered. It follows from these results that calyculin A, an inhibitor of PP1- and PP2A-type protein phosphatases, modulates the glutoxim effect on Na^+ transport in frog skin.

It is known that protein phosphatases regulate the activity of a large number of structural and regulatory proteins modifying the level of their phosphorylation/dephosphorylation. Our previous results and literature data show that the ENaC activity and Na^+ transport in epithelial cells are regulated by various serine/threonine and tyrosine kinases, as well as phosphatidylinositol kinases (Garty and Palmer, 1997; Becchetti et al., 2002; Krutetskaya et al., 2003; Melnitskaya et al., 2006b). The results of this paper agree with the literature data. Thus, it was reported that okadaic acid and calyculin A, two structurally different inhibitors of PP1- and PP2A-type protein phosphatases modulated Na^+ transport in cultured A6 cells derived from distal segments of frog *Xenopus laevis* kidney. It was found that okadaic acid and calyculin A changed the kinetic characteristics and density of expressed ENaC but almost did not alter the phosphorylation level of the channel subunit (Benos and Stanton, 1999). These results, as well as some other reports, show that PP1- and PP2A-type protein phosphatase inhibitors influence the membrane Na^+ permeability of epithelial cells not by direct modulation of ENaC activity, but via a number of other proteins. Literature data show that cytoskeleton proteins are possible messengers in this process. It is known that various inhib-

itors of PP1/PP2A-type protein phosphatases, such as okadaic acid, calyculin A and microcistine, induce dynamic structural rearrangements of actin and tubulin cytoskeleton. Thus, in platelets, neutrophils, and other cells, these agents induce reorganization of microtubules into short microtubule bundles (Yano et al., 1995). Caliculin A and okadaic acid affect the actin cytoskeleton architecture. They facilitate phosphorylation of actin-binding ERM proteins, such as ezrin, radixin, or moezin, regulating the association of these proteins with actin filament proteins and plasma membrane (Rosado and Sage, 2000).

We demonstrated that calyculin A, like nocodazole, microtubule disruptor, almost totally attenuated gluoxim stimulation of Na^+ transport in frog skin. Hence, it is possible to suggest that calyculin A action's on the glutoxim effect is realized through cytoskeleton elements.

Various Na^+-transporting proteins have many cysteine residues that are targets for intra- and extracellular oxidizing and reducing agents. In reabsorbing epithelia, ENaC play a critical role in Na^+ transport. Extracellular domains of ENaC α-, β-, and γ-subunits have highly conservative fragments containing cysteine residues that play an important role in maintaining of the channel tertiary structure and ENaC translocation to the plasmalemma (Benos and Stanton, 1999; Firsov et al., 1999). Transmembrane, as well as N- and C-terminal domains of ENaC subunits, have cysteine residues available from the cytosol side for SH-reactive compounds (Kellenberger et al., 2005). Numerous cysteine residues located in various ENaC segments determine its redox sensitivity and are the targets for intra- and extracellular oxidizing and reducing agents. Addition of 20 μM amiloride, a ENaC blocker, into the solution washing the apical surface of frog skin at the end of each experiment almost completely inhibited I_{SC} (figure). This implies that glutoxim affects Na^+ transport predominately by modulation of ENaC activity.

Thus, our results show that any changes in the actin and tubulin cytoskeleton structure reduce glutoxim-induced Na^+ transport stimulation in the frog skin. These as well as our previous data (Melnitskaya et al., 2006a, 2006b, 2009, 2010; Krutetskaya et al., 2008) allow it to be supposed that glutoxim may interact with cysteine-rich domains of the insulin receptor in the basolateral membrane of epithelial cells, induce its transactivation, and trigger the signal cascade including tyrosine kinases, phosphatidylinositol kinases, protein kinase C, and cytoskeleton elements that results in stimulation of Na^+ transport in frog skin.

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