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# Involvement of Tyrosine and Phosphatidylinositol Kinases in Oxidized Glutathione and Glutoxim Regulation of $Na^+$ Transport in Frog Skin

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**Abstract**—The role of tyrosine and phosphatidylinositol kinases in oxidized glutathione (GSSG) and its pharmacological analogue, glutoxim, regulation of $Na^+$ transport in *Rana temporaria* frog skin was investigated by the voltage-clamp technique. It was shown for the first time that the preincubation of the skin with tyrosine kinase inhibitor genistein or with two structurally distinct phosphatidylinositol kinase inhibitors, wortmannin and LY294002,significantly decreased the stimulatory effect of GSSG or glutoxim on $Na^+$ transport. The data suggest that GSSG and glutoxim can transactivate insulin receptor in the basolateral membrane of epithelial cells and trigger the signaling cascade, which involves tyrosine and phosphatidylinositol kinases, which stimulates $Na^+$ transport in frog skin.

*Key words*: $Na^+$ transport, oxidized glutathione, glutoxim, tyrosine kinases, phosphatidylinositol kinases.

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*Abbreviations used*: ENaC, amiloride-sensitive epithelial $Na^+$-channels; GSH, reduced glutathione; GSSG, oxidized glutathione; PI-3-kinases, phosphatidylinositol-3 kinases; PI-4-kinases, phosphatidylinositol-4-kinases

Amphibian skin and other isolated epithelial systems are useful objects to study mechanisms of the transepithelial ion transport. Amphibian skin and the urinary bladder are comparable to the distal regions of kidney canals in electrolyte transport and their reactions to some hormones (Natochin, 1982). It makes possible to extrapolate data on these objects to clarify the mechanisms of ion and water transport in kidney cells. $Na^+$ transport in osmoregulated epithelia is a complex multicomponent system which creates and maintains electrolytic and water homeostasis. Various protein components in this system may be targets for the oxidative stress.

Nowadays the functioning of cellular oxidation–reduction (redox) systems and the influence of oxidizing and reducing agents on different cellular processes in normal and pathological conditions are of particular interest. The current data suggest that modulation of the redox state may prove to be an important strategy for the treatment of AIDS and some forms of cancer (Sen, 1998). It was demonstrated that oxidizing and reducing agents mediated $N^+$ transport in epithelial cells. In epithelial cells of frog kidneys (cell line A6), hydrogen peroxide stimulated $Na^+$ transport (Markadieu et al., 2005), whereas the active oxygen species decreased $Na^+$ transport in epithelial human carcinoma cells (Wang et al., 2000).

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 for oxidants and reducing agents (Boldyrev and Bulygina, 1997; Firsov et al., 1999). However, molecular mechanisms of transepithelial $Na^+$ transport regulation by oxidizing and reducing agents are poorly understood.

ENaC play a crucial role in $Na^+$ transport within the reabsorbing epithelia. The extracellular domains of ENaC channels include $\alpha$, $\beta$ and $\gamma$ subunits enriched with highly conserved cysteine residues, which are important for maintaining the tertiary structure of the channel and the ENaC transfer to the plasmalemma (Benos and Stanton, 1999; Firsov et al., 1999). The transmembrane, as well as N- and C-terminal domains of ENaC subunits, have cysteine residues available for intracellular SH-reactive compounds (Kellenberger et al., 2005). Because of numerous cysteine residues in various segments of ENaC, the channel is redox sensitive and, therefore, serves as a target for both intra- and extracellular oxidizing and reducing agents. Extracellular and cytoplasmic domains of insulin receptor $\alpha$ and $\beta$ subunits in basolateral membrane of epithelial cells also have numerous cysteine residues which redox modification modulates receptor autophosphorylation followed by tyrosine phosphory-

lation of protein substrates (Wilden and, Pessin, 1987; Ullrich and Schlessinger, 1990; Garant et al., 1999). Glutathione (γ-glutamyl cysteinyl glycine) is presented in reduced (GSH) and oxidized (GSSG) forms (Sies, 1999); it is a universal tripeptide found in the majority of plants, microorganisms, and all mammalian tissues. A number of cellular proteins (receptors, channels, enzymes, transcription factors, oncogenes) are subjected to S-glutathionylation (Ghezzi, 2005; Biswas et al., 2006). Glutathionylation regulates the activity of key signaling proteins, including protein kinase C (Ward et al., 2002), protein kinase A (Brennan et al., 2006), receptor and cytoplasmic tyrosine kinases and tyrosine phosphatases (Staal et al., 1994; Rao et al., 2000), Ras -proteins (Mallis et al., 2001), actin cytoskeleton elements (Wang et al., 2001). GSH functions in cells as a reducing agent and antioxidant (Hayes and McLellan, 1999), whereas GSSG could have a receptor-mediated effect on cellular processes (Burova et al., 2005; Vasilenko et al., 2006).

Pharmaceutical agent glutoxim, which is a GSSG disodium salt with the addition of nano platinum, has found clinical application as an immunomodulator and a hemostimulator in complex therapy in cases of bacterial and viral diseases (Zhukov et al., 2004), psoriasis (Korsynskaya et al., 2003), and the radio- and chemotherapy of oncological diseases (Filatova et al., 2004). Another GSSG analogue, NOV-002 (GSSG in combination with cysplatin in 1000 : 1 ratio), has receptor-modulated action on cells and induces the activation of proteins involved in hematopoiesis (Townsend et al., 2008).

Previously, we showed that the $Na^+$ transport in frog skin is regulated by various oxidants, such as cystamine, cystine, GSSG, and its synthetic analogue glutoxim (FARMA-VAM, Moscow, Russia) (Krutetskaya et al., 2008). It was demonstrated for the first time that GSSG and glutoxim applied to the basolateral surface of frog skin imitated the insulin action and stimulated transepithelial $Na^+$ transport. However, the mechanisms of GSSG and glutoxim regulation of $Na^+$ transport remain obscure.

It is known that the effect of insulin on $Na^+$ transport is initiated by hormone binding with the receptor with intrinsic tyrosine kinase activity localized in the basolateral membrane of epithelial cells (Cox and Singer, 1977). We found that the effect of insulin on $Na^+$ transport depended on tyrosine kinase and tyrosine phosphatase activities and involved phosphatidylinositol kinases (PI-kinases) and protein kinase C (Melnitskaya et al., 2006a). Moreover, it was reported that GSSG and glutoxim induced transactivation of the epidermal growth factor receptor and activation of its intrinsic tyrosine kinase activity in epidermoid cacinoma cells A431 (Burova et al., 2005; Vasilenko et al., 2006).

The purpose of the present study was to determine the role of tyrosine kinases and PI-kinases in GSSG and glutoxim regulation of $Na^+$ transport in frog *Rana temporaria* skin. Genistein, an inhibitor of protein tyrosine kinases (Akiyama, Ogawara, 1991), and two structurally distinct phosphatidylinositol-3-kinase (PI-3 kinases) and phosphatidylinositol-4-kinase (PI-4 kinases) inhibitors, wortmannin and LY294002 (Vlahos et al., 1994; Pacold et al., 2000), were used in the study.

## MATERIALS AND METHODS

Experiments were performed on frog *R. temporaria* males between November and March. Abdominal skin was removed and fastened to the hollow polyethylene tube with an inner diameter of 0.8 mm. The tube with skin was placed into a modified Ussing chamber with apical skin surface being turned into an external solution. The chamber was filled with Ringer's solution for cold-blooded animals as follows: (mM), 100 mM NaCl, 2.5 mM KCl, 3 mM $CaCl_2$, 5 mM Tris-HCl, pH 7.4. Experiments were performed at room temperature (22–23°C).

Electric parameters of frog skin were measured with automated device for voltage-clamp and registration of volt-ampere relations (I–V relations) (Krutetskaya et al., 2003). To measure I–V relations, the transepithelial potential, VT, was changed periodically to a series of nonzero values. In intervals between measurements of I–V relations, the skin transepithelial potential ($V_T$) was kept at 0 mV (short-circuit regime) or at open-circuit potential ($V_{oc}$) ($V_{oc} = V_T$ at transepithelial current $I_T = 0$). Skin electrical parameters were determined from I–V relations: short-curcuit current $I_{sc}$ ($I_{SC} = I_T$ at $V_T = 0$), $V_{oc}$ and transepithelial conductance $g_T$.

$Na^+$ transport was measured as amiloride-sensitive $I_{sc}$. To ensure that $Na^+$ transport was the source of $I_{SC}$, the ENaC blocker, amiloride (20 μM) was added to the apical bath in the end of all experiments. It is known that 20–100 μM amiloride selectively blocks ENaC (Bentley, 1968). Information was computerized and treated with proper software. The reagents used in experiments were from Sigma (United States). Genistein (100 mM), wortmannin (1 mM) and LY294002 (50 mM) stock solutions were prepared in DMSO. Amiloride (10 mM), GSSG (50 mg/ml) and glutoxim (50 mg/ml) stock solutions were prepared in water. Pharmacological agents were added to apical or basolateral skin surfaces. Tyrosine kinase (genistein) and phosphatidylinositol kinase (wortmannin and LY294002) inhibitors were applied 30–40 min before oxidizing agents (glutoxim or GSSG) were added to the solution.

Statistical analysis was performed using Student's t-test. The data are presented as $x \pm s_x$. The figures show the results of typical experiments.

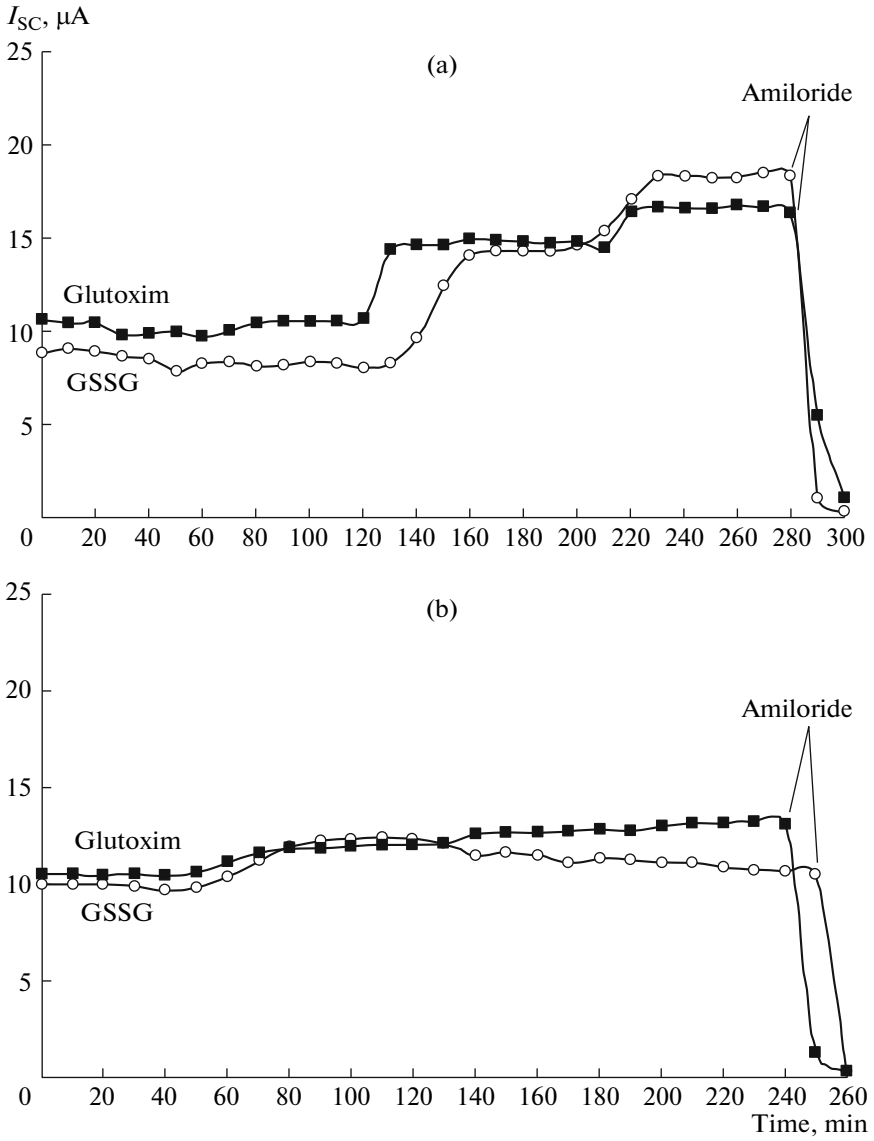


**Fig. 1.** Kinetics of changes in the short circuit current $I_{sc}$ after treatment of the frog skin basolateral surface with 100 μg/ml glutoxim or 100 μg/ml GSSG following by application to skin apical surface 20 μM amiloride, a blocker of epithelial $Na^+$-channels: (a) $I_{sc}$ after glutoxim or GSSG addition to intact frog skin; (b) $I_{sc}$ after preincubation of apical skin surface with 100 μM genistein, an inhibitor of tyrosine kinases, for 30 min.

## RESULTS AND DISCUSSION

**$Na^+$ transport in frog skin exposed to GSSG and glutoxim.** In a series of ten experiments, the control values of electrical characteristics of frog skin were as follows: $I_{sc} = 14.58 \pm 0.91$ μA; $V_{oc} = -38.01 \pm 2.74$ mV; $g_T = 0.36 \pm 0.01$ mS. It was shown that the basolateral application of GSSG or glutoxim (100 μg/ml) to intact frog skin stimulated $Na^+$ transport similar to insulin. On average (the results of ten experiments), $I_{sc}$ increased by 40.37 ± 11.24% and 30.31 ± 1.04% and $V_{oc}$ by 48.05 ± 10.34% and 29.64 ± 1.13% for GSSG and glutoxim, respectively (Fig. 1a). $g_T$ did not change.

**GSSG and glutoxim effect in the presence of tyrosine kinase inhibitor genistein.** Insulin regulated transepithelial $Na^+$ transport is mediated by the activation of the receptor with intrinsic tyrosine kinase activity in the basolateral membrane of epithelial cells (Rodriguez-Commes et al., 1994). Specific tyrosine kinase inhibitors (geninstein, tyrphostin-23, lavendustin A) significantly reduce the insulin stimulation of $Na^+$ transport in various epithelial cells (Hagiwara et al., 1992; Matsumoto et al., 1993; Rodriguez-Commes et al., 1994; Krutetskaya and Lebedev, 1998; Melnitskaya et al., 1998).

To elucidate the possible role of tyrosine kinases in the effect of GSSG or glutoxim on $Na^+$ transport in

frog skin a specific tyrosine kinase inhibitor, isoflavonoid genistein, was used (Akiyama and Ogawara, 1991). It is known that genistein competes with ATP for tyrosine kinase binding. Genistein inhibits a wide range of receptor and cytoplasmic tyrosine kinases, probably due to their highly conservative ATP-binding domain (Akiyama and Ogawara, 1991).

It was demonstrated that genistein significantly reduced $Na^+$ transport stimulated by GSSG or glutoxim (Fig. 1b). Thus, after the preincubation of frog apical skin surface with 100 μM genistein for 30 min before oxidants application $I_{sc}$ increased by 10.05 ± 2.11% and 16.81 ± 3.48%, and $V_{oc}$ by 11.05 ± 1.25% and 18.25 ± 4.32% for GSSG and glutoxim (100 μg/ml), respectively. $g_T$ did not change. These data show that GSSG and glutoxim effect on $Na^+$ transport in frog skin is presumably mediated by tyrosine kinase activation.

Two types of tyrosine kinases are distinguished, i.e., receptor and cytoplasmic (Hunter, 1996). Activated receptor tyrosine kinases transduce the information by protein phosphorylation and protein–protein interactions with molecules having SH2- and SH3-domains (Schlessinger and Ullrich, 1992). Cytoplasmic kinases are found in both the cytoplasm and cell nucleus; they are engaged in intracellular signaling. Thus, tyrosine kinases of Src, Jak, and Fak families are directly involved in the process of transmembrane signal transduction and serve as catalytic subunits of membrane receptors without intrinsic tyrosine kinase activity (Hunter, 1996).

Receptor and cytoplasmic tyrosine kinases and tyrosine phosphatases are engaged in the regulation of transepithelial $Na^+$ transport and ENaC activity (Tilly et al., 1993; Davis et al., 2001). In various reabsorbing epithelia tyrosine kinases mediate the regulation of ENaC activity by insulin (Hagiwara et al., 1992; Matsumoto et al., 1993; Rodriguez-Commes et al., 1994) and growth factors (insulin-like and epidermal) (Davis et al., 2001; Tong and Stockand, 2005). In amphibian kidney cells (A6 cells), tyrosine kinases are also involved in the increase of $Na^+$ transport stimulated by the decrease in osmotic pressure in the solution surrounding the cellular apical surface (Niisato et al., 2000). The results of many experiments demonstrated that, in A6 cells, tyrosine kinases are involved mostly in the regulation of ENaC insertion in membranes (Matsumoto et al., 1993; Niisato et al., 2000). However, in cell cultures of distal segments of mammalian nephrons receptor tyrosine kinase activation by epidermal growth factor decreases $Na^+$ transport due to the reduction of ENaC open probability (Tong and Stockand, 2005). Taking into account that the genistein inhibit a broad spectrum of tyrosine kinases, it is possible to suggest that both the receptor and cytoplasmic tyrosine kinases may be involved in $Na^+$ transport regulated by GSSG and glutoxim.

Studies on the GSSG effect on cells at concentration close to or higher than the concentration determined outside the cells showed that GSSG could have a receptor-mediated effect on cellular processes (Filomeni et al., 2002, 2005; Burova et al., 2005). Thus, it was shown that, in human epidermoid carcinoma A431 cells, GSSG and glutoxim caused the transactivation of epidermal growth-factor receptor and the activation of its intrinsic tyrosine kinase activity (Burova et al., 2005; Vasilenko et al., 2006). It can be supposed that, in frog skin, GSSG and glutoxim could also induce the transactivation of receptor tyrosine kinases, e.g. insulin receptor localized in basolatheral membranes of epithelial cells. Extracellular domains of insulin receptor enriched in cysteine are the possible targets for GSSG and glutoxim applied from the basolateral surface.

Tyrosine kinases and tyrosine phosphatases are the most redox-sensitive enzymes. Oxidative stress and decrease in GSH/GSSG ratio change tyrosine kinase and tyrosine phosphatase activity (Rao et al., 2000; Forman and Torres, 2002), which results in an increase phosphorylation of proteins at tyrosine residues. Moreover, tyrosine phosphatases themselves can be targets for covalent modifications with GSSG and glutoxim. The oxidation of conservative cysteine residues in the catalytic domain and cysteine residues in functionally important SH2 domains of tyrosine phosphatases leads to inhibition of tyrosine phoshatases (Fischer et al., 1991; Walton and Dixon, 1993; Barford et al., 1994; Filomeni et al., 2002). The inhibition of tyrosine phosphatases may activate tyrosine kinases and enhance the protein tyrosine phosphorylation.

On the other hand, it was reported that the activation of receptor tyrosine kinases in various cells activated phagocytosis (Wang et al., 2000). Therefore, it can be supposed that the transactivation of receptor tyrosine kinases by GSSG and glutoxim can stimulate phagocytosis and the penetration of GSSG and glutoxim molecules in epithelial cells, which, in turn, can modify activity of various redox-sensitive molecules.

**Effects of GSSG and glutoxim in presence of PI-kinase inhibitors.** It is known that the autophosphorylation of the insulin receptor releases the binding sites for proteins with SH2- and SH-3 domains and facilitates the phosphorylation of tyrosine residues of various endogenous substrates (Cadena and Gill, 1992; Saltiel, 1996). A key endogenous substrate for the insulin receptor tyrosine kinase is the insulin receptor substrate (IRS). Phosphorylated IRS binds to proteins with SH2-domains, such as PI-kinases, tyrosine phosphatases, and phospholipase Cγ (Cadena and Gill, 1992). Its known that PI-kinases play an important role in the insulin regulation of $Na^+$ transport in various epithelial systems (Markadieu et al., 2004), particularly in frog skin (Melnitskaya et al., 2006a).

Previously, we showed that the effect of PI-kinases inhibitors on $Na^+$ transport in frog skin depended on the concentration of the agent and the application site (apical or basolateral skin surface) (Krutetskaya et al., 2006). To elucidate the possible involvement of PI in

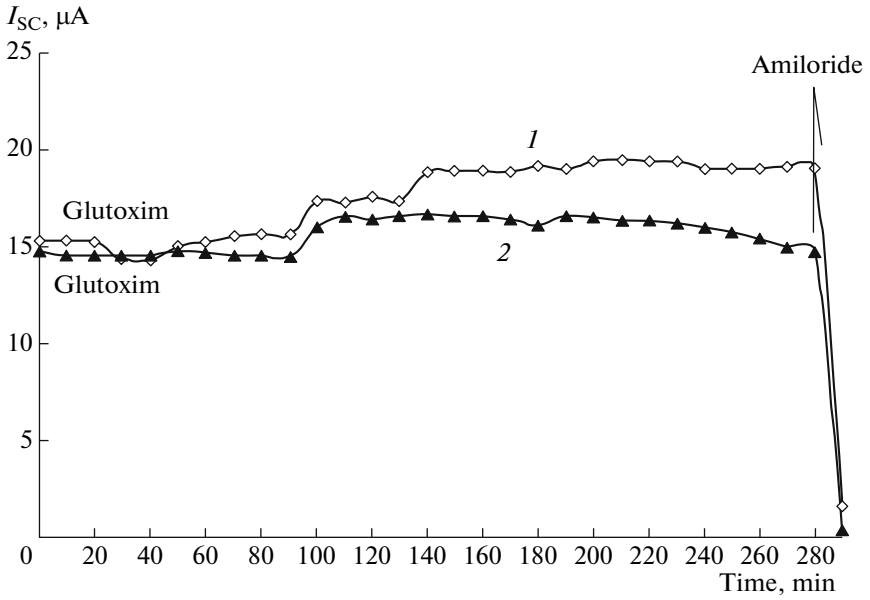


**Fig. 2.** Kinetics of changes in short-circuit current $I_{sc}$ in frog skin after application of 100 μg/ml glutoxim to basolateral skin surface preliminary treated with phosphatidylinositol kinase inhibitors followed by addition to apical skin surface of 20 μM amiloride, a blocker of epithelial $Na^+$-channels: (*1*) $I_{sc}$ after preincubation of apical skin surface with LY294002 (200 nM, 30 min); (*2*) $I_{sc}$ after preliminary incubation of basolateral skin surface with wortmannin (1 μM, 30 min).

GSSG and glutoxim regulation of $Na^+$ transport in frog skin, we performed series of experiments for each oxidant in which wortmannin (500 nM and 1 μM) or LY294002 (100 and 200 nM) were preliminary added to the solution surrounding apical or basolateral surface of the frog skin; afterwards, GSSG or glutoxim (100 μg/ml) were applied from the basolateral surface of the frog skin.

It was found that the preliminary incubation of frog skin with wortmannin or LY294002 for 30–40 min significantly reduced the GSSG- or glutoxim-mediated stimulation of $Na^+$ transport. Figure 2 demonstrates the effect of preincubation with 200 nM LY294002 applied to apical skin surface and 1 μM wortmannin applied to the basolateral skin surface on $I_{sc}$ in frog skin exposed to glutoxim. It can be seen that both agents considerably reduce glutoxim-mediated stimulation of $Na^+$ transport.

The mean values (10 experiments) of electrical characteristics of frog skin exposed to wortmannin (in various concentrations) applied to apical surface were as follows: $I_{sc}$ increased by 8.45 ± 1.29% and 3.36 ± 0.24% and $V_{OC}$ increased by 9.34 ± 2.08% and 4.01 ± 1.23% for 500 nM and 1 μM wortmannin, respectively. Preincubation of apical surface of frog skin with LY294002 before glutoxim application increased $I_{sc}$ by 13.84 ± 3.48% and 11.42 ± 4.04%; $V_{OC}$ was augmented by 15.01 ± 3.43% and 12.34 ± 4.32% for 100 and 200 nm LY294002, respectively.

In all experiments $g_T$ value was unaltered. Similar results were obtained when 100 μg/ml GSSG was applied to the frog skin pretreated with the PI-kinase 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 concentration of PI-kinase inhibitors considerably (as compared to higher concentrations) the stimulatory effect of glutoxim or GSSG on $Na^+$ transport. It is known that wortmannin and LY294002 are highly efficient PI-kinase inhibitors. Low concentrations of these agents irreversibly inhibit all known types of PI-kinases, whereas at higher (submicromolar) concentrations wortmannin and LY294002 also inhibit PI-kinases (Vlahos et al., 1994). 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 that specifically inhibit PI-3-kinases suggests that either PI-4-kinases are involved more actively in this process than PI-3-kinases or that the activation of PI-4-kinase is an earlier stage in the realization of the stimulatory effect of GSSG and glutoxim on $Na^+$ transport in *Rana temporaria* frog skin. It is possible that PI-4-kinases can attenuate the inhibition of PI-3-kinase by phosphorylating phosphatidylinositol 3-phosphate remaining in cells.

It is known that various major $Na^+$-transporting proteins contain numerous cysteine residues that are targets for intra- and extracellular oxidizing and reducing agents (Boldyrev and Bulygina, 1997; Benos

and Stanton, 1999; Firsov et al., 1999; Kellenberger et al., 2005). However, after the addition of ENaC blocker amiloride (20 μM) to the solution surrounding the apical surface of frog skin at the end of each experiment, $I_{sc}$ was completely blocked (Figs. 1, 2). This indicates that the effect of GSSG and glutoxim on $Na^+$ transport is associated primarily with the modulation of ENaC activity.

Thus, we showed for the first time that tyrosine kinases and PI-kinases are involved in the effects of GSSG and glutoxim on $Na^+$ transport in the skin of the *Rana temporaria* frog. Based on the results of this work and our previous studies (Krutetskaya et al., 2008; Melnitskaya et al., 2008), it can be assumed that GSSG and glutoxim may interact with the cysteine-rich domains of the insulin receptor in 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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