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CELL BIOPHYSICS

Microtubular Disrupter Nocodazole and Vesicular Transport Inhibitor Brefeldin A Attenuate the Glutoxim Effect on Na^+ Transport in Frog Skin

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Abstract—Using the voltage-clamp technique, a possible role of microtubules and vesicular transport in the effect of pharmacological analogue of oxidized glutathione, drug glutoxim, on Na^+ transport in the frog *Rana temporaria* skin was investigated. It was shown for the first time that the disrupter of microtubules nocodazole or inhibitor of vesicular transport brefeldin A similarly modulate (completely inhibit) the stimulatory effect of glutoxim on Na^+ transport. The data suggest the involvement of reorganization of microtubules and vesicular transport in the regulatory effect of glutoxim on Na^+ transport.

Keywords: Na^+ transport, glutoxim, nocodazole, brefeldin A

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INTRODUCTION

The skin of amphibians and other isolated epithelial systems are classical model objects for investigating the mechanisms of ion transport across biological membranes. Transport of Na^+ in osmoregulating epithelia presents as a complicated, multicomponent system, the work of which ensure creation and maintenance of electrolyte and water homeostasis. Various protein components of this system, such as amiloride-sensitive Na^+ channels (ENaC), Na^+/K^+-ATPases or Na^+/H^+ exchangers may be a target for oxidative stress [1]. Redox regulation of Na^+ transport has been shown for a number of epithelial tissues, yet the molecular mechanisms of the influence of oxidants and reducers on various components of the system of transepithelial Na^+ transport are practically unstudied.

In recent time new disulfide-containing agents with nanoadditions of *d*-metals have been widely used in clinical practice. Thus a drug Glutoxim® ("FARMA VAM", Moscow) – disodium salt of oxidized glutathione (GSSG) with nanoaddition of platinum – has found broad application as immunomodulator and hemostimulator in complex therapy of bacterial and viral infections, psoriasis, in radiation and chemotherapy of oncological diseases [2].

Earlier we have shown that Na^+ transport in the skin of frog *Rana temporaria* is modulated by various disulfide-containing oxidizing agents, such as cystamine, cystine, GSSG and glutoxim. For the first time it was demonstrated that GSSG and glutoxim applied to the basolateral surface of frog skin mimic the action of insulin and stimulate transepithelial Na^+ transport [3]. With the use of pharmacological agents influencing the structural elements and components of signalling systems in the cell, we have shown that glutoxim regulation of Na^+ transport in frog skin involves tyrosine kinases, phosphatidylinositol kinases [4, 5], protein kinase C [6], serine/threonine protein phosphatases PP1/PP2, elements of actin and tubulin cytoskeleton [7, 8], and also products and/or enzymes of the cyclooxygenase pathway of arachidonic acid oxidation [9].

It is known that processes of exo- and endocytosis play an important role in modulation of the activity of ENaC and other Na^+- transporting proteins. It has been established that processes of removal/insertion and delivery of ENaC subunits to the membrane involve various structural and signalling elements, such as microtubules and microfilaments [10], regulators of protein synthesis [11] and components of vesicular [12]. In this connection, it appeared interesting to investigate the possible participation of vesicular transport transport and microtubules in regulation by glutoxim of Na^+ transport in frog skin. In experiments we used a specific inhibitor of vesicular transport brefeldin A and microtubule disrupter nocodazole.

EXPERIMENTAL PROCEDURE

Experiments were conducted on frog *R. temporaria* males in the period from November to March. Skin from the frog abdomen was cut off and placed into an Ussing chamber (World Precision Instruments, Inc., Germany) with the inner orifice diameter of 12 mm.

The chamber was filled with cold-blood Ringer solution containing 110 mM NaCl, 2.5 mM KCl, 3 mM $CaCl_2$, 5 mM Tris-HCl, pH 7.4. Experiments were conducted at room temperature (22–23°C).

For measurement of electrical parameters of frog skin, the automated device for voltage-clamp and recording the current-voltage characteristics was used [3]. For measurement of current-voltage characteristics, linearly changing voltage (ramp) was applied to the skin at a rate of 20 mV/s. In the intervals between measurements of current-voltage characteristics, transepithelial potential (V_T) of the skin was maintained at 0 mV (short-circuit mode) or at open-circuit potential V_{oc} ($V_{oc} = V_T$ at transepithelial current $I_T = 0$). From current-voltage characteristics the electrical parameters of skin were determined: short-circuit current I_{sc} ($I_{sc} = I_T$ at $V_T = 0$), V_{oc} and transepithelial conductance g_T.

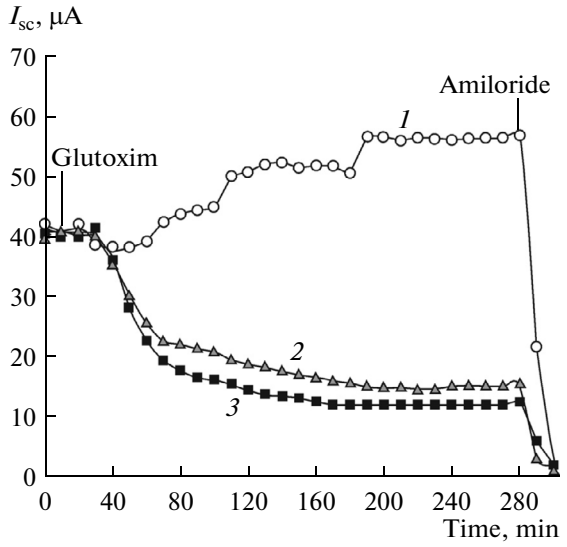
Transport of Na^+ was estimated as amiloride-sensitive I_{sc}. In this connection at the end of every experiment into the solution bathing the apical surface of the skin we added ENaC blocker amiloride (20 μM).

Reagents from Sigma (USA) were used. A stock solution of nocodazole (5 mM) was prepared in dimethyl sulfoxide. A stock solution of brefeldin A (50 mM) was prepared in alcohol. Stock solutions of amiloride (10 mM) and glutoxim (50 mg/mL) were prepared in water. Drugs were added to the apical or the basolateral surface of the skin. Nocodazole or brefeldin A were added 30–60 min before introducing glutoxim into solution.

Statistical analysis was conducted with the use of Student's *t*-test. Data are presented as $x \pm s_x$. The figure presents the results of typical experiments.

RESULTS AND DISCUSSION

The values of electrical characteristics of frog skin in control on average (by the data of 10 experiments) are: $I_{sc} = 39.16 \pm 3.45$ μA, $V_{oc} = -110.65 \pm 12.41$ mV, $g_T = 0.35 \pm 0.02$ mS. It is shown that glutoxim (100 μg/mL) applied to the basolateral surface of intact skin, like insulin, stimulates Na^+ transport. On average (by the results of 10 experiments) after glutoxim application the I_{sc} increases by 31.24 ± 8.32%; V_{oc} – by 38.04 ± 5.15%; the g_T does not change. On the basis of results obtained in the present work and earlier [3–9], it may be supposed that glutoxim can interact with cysteine-rich extracellular domains of the insulin receptor in the basolateral membrane of epithelial cells, cause its transactivation and trigger a signalling cascade leading to an increase of Na^+ transport in frog skin. The results obtained agree well with literature data. Thus in human epidermal carcinoma A431 cells, GSSG and glutoxim cause transactivation of the receptors of epidermal growth factor and activation of its intrinsic tyrosine kinase activity [13, 14].



Changes in short-circuit current I_{sc} across frog skin in response to glutoxim: *1* – I_{sc} after adding 100 μg/mL glutoxim to the basolateral surface of intact skin; *2* – I_{sc} after adding glutoxim to skin pretreated (for 30 min) with 50 μM nocodazole from the apical surface; *3* – I_{sc} after adding glutoxim to skin pretreated (for 60 min) with 50 μM brefeldin A from the apical surface; at the end of every experiment, 20 μM ENaC blocker amiloride was added into the solution bathing the apical surface of the skin.

It is shown that nocodazole and brefeldin A completely prevent the stimulating action of glutoxim on Na^+ transport in frog skin (figure). Thus on average, by the data of 10 experiments, after pretreatment of the apical surface of the skin with 50 μM nocodazole for 30 min before adding 100 μg/mL glutoxim to the basolateral surface of the skin the I_{sc} decreases by 35.46 ± 7.09%, V_{oc} – by 29.15 ± 6.34%, while g_T – by 18.03 ± 6.38%. In a similar way, in the case of preincubation of the apical surface of the skin with 50 μM brefeldin A for 60 min before adding 100 μg/mL glutoxim, I_{sc} decreases by 41.62 ± 9.35%, V_{oc} – by 35.45 ± 8.34%, while g_T – by 20.81 ± 6.34%.

Earlier we have shown that GSSG or glutoxim applied to the basolateral surface of frog skin mimic the effect of insulin and stimulate Na^+ transport, evoking a biphasic increase of I_{sc} [3]. The figure presents the kinetics of I_{sc} change upon adding 100 μg/mL glutoxim to the basolateral surface of intact skin (curve *1*), and also after adding glutoxim to skin pretreated from the apical surface with 50 μM nocodazole (curve *2*) or 50 μM brefeldin A (curve *3*). It is seen that nocodazole or brefeldin A completely suppress both phases of the stimulating action of glutoxim on I_{sc} in frog skin.

It is known that various Na^+-transporting proteins contain numerous cysteine residues, which are targets for intra- and extracellular oxidizing and reducing agents. In reabsorbing epithelia a key role in Na^+

transport is played by ENaC. In the extracellular domains of α-, β- and γ-subunits of ENaC there are highly conserved fragments containing cysteine residues, which play an important role in maintaining the tertiary structure of the channel and translocation of ENaC to the plasmalemma [1, 15]. Transmembrane and also N- and C-terminal domains of ENaC subunits contain cysteine residues accessible to the action of SH-reactive compounds from the cytosol side [16]. Numerous cysteine residues localized in various segments of ENaC determine its redox-sensitivity and are a target for intra- and extracellular oxidizing and reducing agents. Introduction at the end of every experiment into the solution bathing the apical surface of the skin of ENaC blocker amiloride (20 μM) caused complete suppression of I_{sc} (figure), which suggests that the influence of glutoxim on Na^+ transport is associated prevalently with modulation of ENaC activity.

The data obtained are evidence that nocodazole and brefeldin A in a similar way modulate the effect of glutoxim on Na^+ transport. Thus both agents essentially reduce the electrical characteristics of frog skin and completely suppress the stimulating action of glutoxim on Na^+ transport in frog skin.

The results obtained are consistent with literature data. It is known that microtubule disrupter nocodazole and vesicular transport inhibitor brefeldin A are effective inhibitors of the processes of intracellular transport. It is shown that disruption of microtubules by antimitotic agents reduces the stimulating effect on Na^+ transepithelial transport of various hormones (aldosterone, vasopressin) [17], in particular, fully prevents many cellular effects of insulin [18].

It has been established that microtubules play an important role in processes of exo- and endocytosis, taking part in modulation of the activity of many Na^+-transporting proteins, including regulation of ENaC density in the apical membrane. At the same time there are data that functionally important SH-groups of tubulin are a target for the action of oxidants. It is shown that at concentrations above physiological, GSSG inhibits microtubule assembly in vitro [19].

On the other hand, the metabolite of fungi *Eupenicillium brefeldianum* brefeldin A is a reversible inhibitor of translocation of proteins from the endoplasmic reticulum to the Golgi complex, and also induces reversible structural rearrangements in the Golgi complex [20]. The effect of brefeldin A is associated with its ability to inactivate small G-proteins of the Arf family [21], which play a key role in regulation of vesicular transport in cells and are an important regulatory component of several pathways of intracellular transport. Arf proteins play an important role in modulation of the movement of membranes and membrane organelles, rearrangements of the cytoskeleton, and also processes of endocytosis [22].

Thus, the data obtained by us suggest an important role of processes of vesicular transport and rearrangements of the tubulin cytoskeleton in glutoxim modulation of Na^+ transport in frog skin. This, in its turn, allows suggesting that glutoxim regulation of Na^+ transport in frog skin involves processes of removal/insertion or delivery of ENaC subunits, requiring participation of microtubules and movement of membranes or membrane organelles. The results obtained also allow to regard small G-proteins of the Arf family as important signalling elements in glutoxim regulation of Na^+-transporting proteins.

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