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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Epoxygenase Inhibitors Attenuate the Stimulatory Effect of Glutoxim on Na^+ Transport in Frog Skin

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Abstract—Using voltage-clamp technique, the involvement of epoxygenases in immunomodulatory drug glutoxim regulation of Na^+ transport in frog skin was investigated. We have shown for the first time that preincubation of the frog skin with epoxygenase inhibitors econazole or proadifen almost completely inhibits the stimulatory effect of glutoxim on Na^+ transport. The data suggest the involvement of the enzymes and/or products of epoxygenase oxidation pathway of arachidonic acid metabolism in glutoxim effect on Na^+ transport in frog skin epithelium.

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The skin of amphibians is a classic model object for studying the mechanisms of ion transport through biological membranes. Na^+ transport in osmoregulatory epithelia is a complex multicomponent system whose functioning ensures the creation and maintenance of electrolytic and water homeostasis. Protein components of this system may be a target for oxidative stress [1, 2]. Previously [3], we found that Na^+ transport in the epithelial cells of the frog skin is modulated by oxidizing and reducing agents. In the cited paper, we for the first time showed that oxidized glutathione (GSSG) and drug Glutoxim® (G, disodium salt of GSSG with a nano additive of a *d*-metal, PHARMA-VAM, Russia), when applied to the basolateral surface of the frog skin, mimic the effect of insulin and stimulate the transepithelial transport of Na^+.

In reabsorbing epithelia, arachidonic acid (AA) and its derivatives (eicosanoids) are involved in the regulation of ion and water transport [4]. There are three major pathways of AA metabolism: cyclooxygenase-, lipoxygenase-, and epoxygenase-dependent (cytochrome P-450-dependent) [5]. We previously showed that inhibitors of cyclooxygenases [6] and lipoxygenases [7] attenuate the stimulatory effect of G on Na^+ transport in the frog skin. However, it is known that epoxygenases contain numerous conserved cysteine residues and may be a target for oxidizing and reducing agents [8]. In view of above, it was reasonable to study the role of epoxygenases in the regulation of Na^+ transport in the frog skin epithelium by G, which was the subject of this communication.

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In the experiments, we used effective epoxygenase blockers—antifungal agents of imidazole nature econazole and proadifen (SKF525A). It is known that, at the micromolar concentration, econazole and proadifen block the cytochrome P-450-dependent oxygenases [9].

Experiments were performed on male frogs *Rana temporaria* in the period from November to March. Abdominal frog skin was cut and placed in an Ussing chamber (World Precision Instruments, Inc., Germany) with an inner opening diameter of 12 mm. The experiments were performed at room temperature (22–23°C). The current–voltage characteristics (I–V relations) of the frog skin were recorded using an automated voltage-clamp device [3]. On the basis of I–V relations, the electrical parameters of the skin were determined: the short-circuit current I_{SC} ($I_{SC} = I_T$ at $V_T = 0$, where I_T is the transepithelial current), the open-circuit potential V_{OC} ($V_{OC} = V_T$ at $I_T = 0$, where V_T is the transepithelial potential), and the transepithelial conductance g_T. The transport of Na^+ ions was assessed by the value of the magnitude of amiloride-sensitive I_{SC}. The reagents used in the experiments were from Sigma-Aldrich (United States). Proadifen and econazole were added 30–40 min before the addition of G to the solution. Statistical analysis was performed using Student's *t* test.

The values of the electrical characteristics of the frog skin in the control were as follows (hereinafter, data are represented as $M \pm m$, n (number of tests) = 10): $I_{SC} = 29.79 \pm 4.36$ μA, $V_{OC} = -64.13 \pm 10.08$ mV, and $g_T = 0.49 \pm 0.09$ mS. We established that G (100 μg/mL) applied to the basolateral surface of the frog skin, similarly to insulin, stimulates Na^+ transport (Fig. 1, curve *1*). After the application of G, I_{SC} increased by

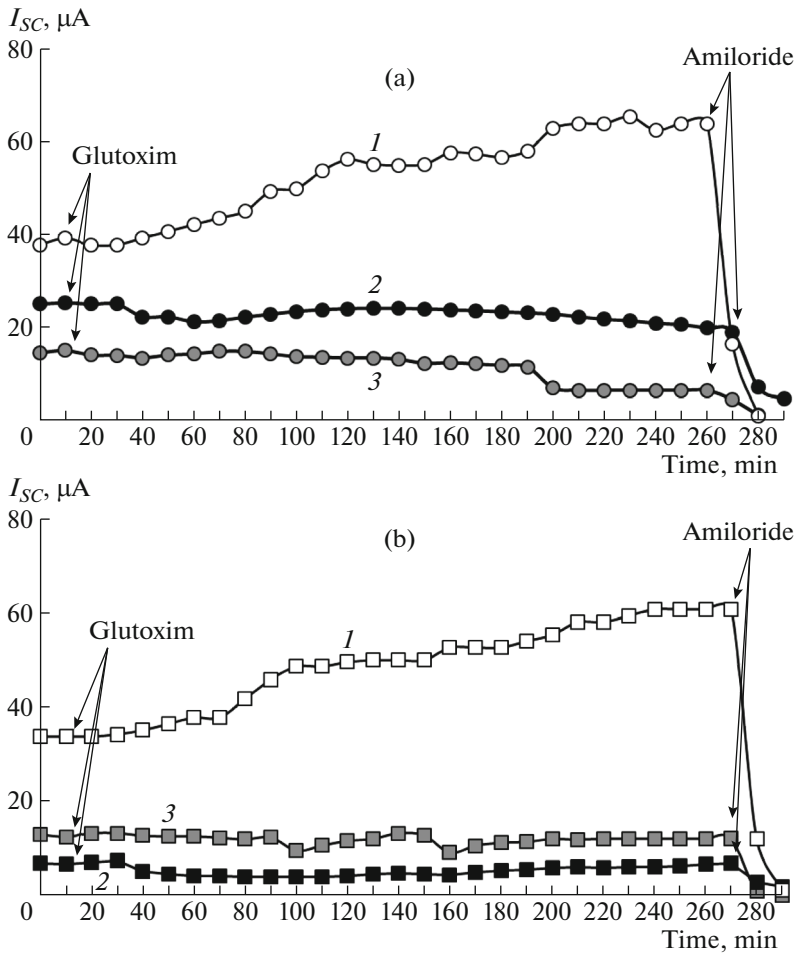

Fig. 1. Kinetics of changes in the short-circuit current I_{SC} through the frog skin in response to glutoxim (G) and epoxygenase blockers applied from (a) apical or (b) basolateral skin surface. Designations: (*1*) I_{SC} after the addition of 100 μg/mL G to the basolateral surface of intact skin; (*2*) I_{SC} after the addition of G to the frog skin pretreated with 100 μM proadifen for 30 min; (*3*) I_{SC} after the addition of G to the frog skin pretreated with 50 μM econazole for 30 min. At the end of each experiment, the ENaC blocker amiloride (20 μM) was added to the solution bathing the apical skin surface. The figure shows the results of typical experiments.

$38.42 \pm 7.45\%$, V_{OC} increased by $43.24 \pm 7.08\%$, and g_T did not change.

We found that the pretreatment of the apical or basolateral surface of the frog skin with the epoxygenase blockers econazole (50 μM) or proadifen (100 μM) for 30 min before the addition of 100 μg/mL G to the basolateral surface of the skin almost completely inhibited the stimulatory effect of G on Na^+ transport (Table 1, Fig. 1). Thus, in this study, we for the first time showed using the frog skin epithelium that two structurally different epoxygenase inhibitors suppress the effect of G on Na^+ transport, which indicates the involvement of the enzymes and/or the products of the epoxygenase pathway of AA oxidation in the effect of G on Na^+ transport in the frog skin epithelium.

Our results are consistent with the published data. It is known that the products of the epoxygenase pathway of AA oxidation (primarily epoxyeicosatrienoic acids) are involved in the regulation of transport functions of osmoregulatory epithelia and pathogenesis of hypertension [10–12]. Recently, data on the involvement of epoxygenases and their products in the insulin receptor signaling cascades were published [13, 14]. For example, it was found that overexpression and increased activity of epoxygenases CYP2J2 and CYP2J3 leads to potentiation of signaling from the insulin receptor and insulin receptor substrate IRS-1 in cells of different types (liver, muscle, heart, and kidney cells) [13, 14]. Our previous results [3] and published data [15] suggest that the regulatory effect of disulfide agents (including GSSG and G) on Na^+ transport is due to their ability to interact with the cysteine-rich domains of the insulin receptor in the basolateral membrane of epithelial cells, which causes the transactivation of the receptor and triggers the signaling cascades that stimulate the membrane Na^+ transport in the frog skin. In this regard, it can be assumed

Table 1. Effect of glutoxim (G) on the electrical characteristics of frog skin

Blocker, concentration	Electrical characteristics	Changes in the electrical characteristics after application of G on frog skin pretreated with epoxygenase blockers from the apical surface, %	Changes in the electrical characteristics after the application of G on frog skin pretreated with epoxygenase blockers from the basolateral surface, %
Proadifen, 100 μM	I_{SC}	↓ 10.56 ± 2.65	↓ 26.79 ± 9.01
	V_{OC}	↓ 9.55 ± 1.34	↓ 38.61 ± 12.67
	g_T	↓ 8.62 ± 1.07	↑ 34.95 ± 10.91
Econazole, 50 μM	I_{SC}	↓ 25.05 ± 8.32	↓ 8.09 ± 1.01
	V_{OC}	↓ 31.23 ± 11.45	↓ 22.05 ± 7.54
	g_T	↑ 6.28 ± 0.12	↓ 21.43 ± 8.02

The arrows indicate the increase (↑) or decrease (↓) in the electrical characteristics of the skin after the application of G as compared to the control. Data are represented as $M \pm m$, $n = 10$.

that the suppression of the stimulatory effect of G on the Na^+ transport in the frog skin by epoxygenase inhibitors may also be due to the suppression and/or attenuation of the signal transmission from the insulin receptor.

It is known that many Na^+-transporting proteins contain numerous cysteine residues, which are targets for intra- and extracellular oxidants and reducers [1, 2]. In our experiments, the addition of 20 μM amiloride, a blocker of amiloride-sensitive epithelial Na^+-channels (ENaC), to the solution bathing the apical skin surface caused a complete inhibition of Na^+ transport (Fig. 1). This fact suggests that the effect of G on Na^+ transport is mainly due to the modulation of ENaC activity.

Thus, the results obtained in this study and earlier [6, 7] suggest that the signaling cascade that is triggered in the frog skin by G and results in the stimulation of Na^+ transport involves all three pathways of AA oxidation—cyclooxygenase-, lipoxygenase-, and epoxygenase-dependent. Moreover, the obtained data on the inhibition of the stimulatory effect of G on Na^+ transport by econazole and proadifen suggest that a combined use in clinical practice of the immunomodulator G and antifungal agents that inhibit epoxygenase is undesirable, because it may attenuate the therapeutic effect of G.

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