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PHYSIOLOGY

Involvement of Actin Filaments in the Effect of the Oxidized Glutathione and Drug Glutoxim on the Intracellular Ca^{2+} Concentration in Macrophages

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The redox control of signal transduction and gene expression is one of the fundamental mechanisms of cell physiology. The number of known redox-sensitive pathways of signal transduction constantly grows, and evidence available testifies to the fact that redox control of the state of a cell may be useful for treatment of AIDS and some cancers. For example, a pharmacological analogue of oxidized glutathione (GSSG), the pharmacological drug Glutoxim® (GSSG disodium salt with a nanoadditive of platinum (PHARMAVAM, Moscow)) is used in clinical practice as an immunomodulator and hemostimulator in the comprehensive therapy of bacterial and viral diseases, psoriasis, as well as in radio- and chemotherapies in oncology [1]. Nevertheless, the mechanisms that mediate the effects of GSSG and Glutoxim on cells remain unknown.

We were the first to find out earlier that GSSG and Glutoxim enhance the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) due to Ca^{2+} mobilization from the thapsigargin-sensitive Ca^{2+} stores and subsequent Ca^{2+} entry into the rat peritoneal macrophages [2, 3]. In addition, we have demonstrated the involvement of many proteins, such as tyrosine kinases [3, 4], tyrosine phosphatases [3, 4], phosphatidylinositol kinase [5], small G proteins of the ras family, phospholipase C, and protein kinase C [6], which are important enzymes of the phosphoinositide system of signal transduction, in the effect of GSSG and Glutoxim on $[Ca^{2+}]_i$ in macrophages.

The effect of phosphatidylinositol kinases, which are important for phosphoinositide metabolism, leads to dynamic reorganization of the actin filaments [7]. In addition, actin filaments are known to be characterized by a high redox sensitivity, and they readily undergo S-glutathionylation [8]. Another pharmacological GSSG analogue, the foreign product NOV-002 (GSSG in combination with cisplatin) has proved to be an inducer of S-glutathionylation of cellular proteins, mainly of actin. S-glutathionylation of actin alters the ratio between F- and G-actins, which leads to significant changes in the overall architecture of the cytoskeleton and in intracellular transport [9]. In this regard, it seems appropriate to study the possible involvement of actin filaments into the regulatory influence of GSSG and glutoxim on $[Ca^{2+}]$ in peritoneal rat macrophages.

The procedure of macrophage cultivation and an automated system for $[Ca^{2+}]_i$ measurement with the use of the fluorescent probe Fura-2AM was described earlier in detail [10]. The experiments were performed at a room temperature of 20–22°C on the second or third day of cell cultivation. The involvement of the actin cytoskeleton in the effect of GSSG and Glutoxim on $[Ca^{2+}]_i$ of peritoneal rat macrophages was studied using cytochalasin D and latrunkulin B [11], which are two structurally different agents capable of inducing depolymerization of microfilaments; calyculin A inducing condensation of submembrane actin filaments, was also used [12]. The results obtained with Glutoxim (100 μg/ml) can be seen in Figs. 1 and 2. Similar data were obtained with GSSG (100 μg/ml).

Rearrangement of actin filaments in response to Glutoxim was visualized using rhodamine–phalloidin. On the second day of macrophage cultivation, Glutoxim was added into the culture for 20 min (100 μg/ml). Control cell cultures were not treated with Glutoxim. To visualize the actin cytoskeleton, the cells were washed with a phosphate salt buffer solution (PBS) to remove the medium and, after fixation with 3.7% formalin for 15 min, they were again washed three times with PBS; afterwards, the cells were treated with 0.1% Triton X-100 for 10 min and then stained with rhodamine–phalloidin (Sigma, United States) at 37°C for 10 min. The preparations obtained were examined under an AxioObserver.Z1 microscope (Carl Zeiss, Germany) using the light with wave lengths of 550 and 605 nm to induce excitation and

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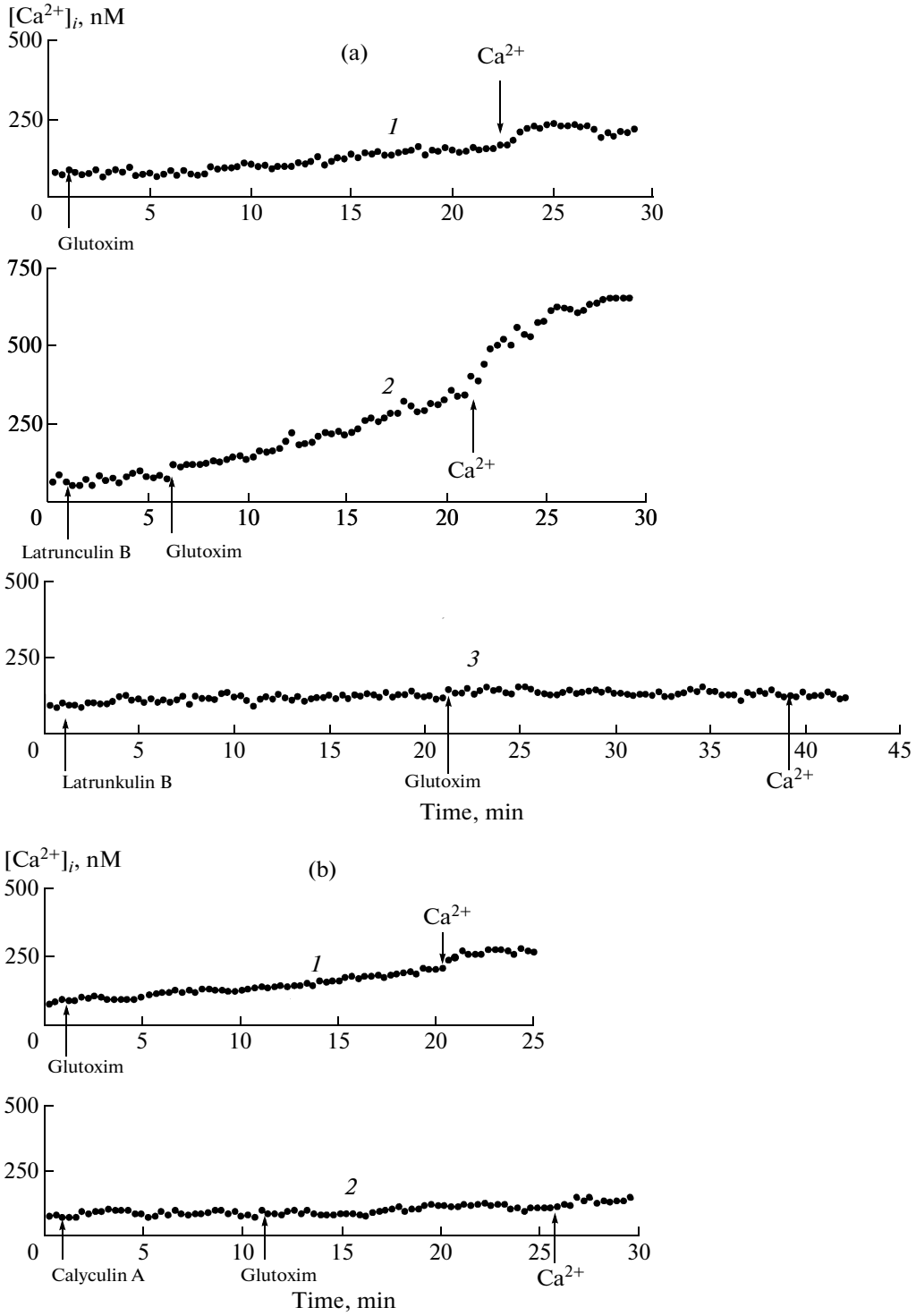

Fig. 1. The influence of (a) latrunculin B and (b) calyculin A on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in peritoneal macrophages treated with Glutoxim. Each recording is obtained for 40–50 cells and is typical of three- to seven experiments. Panels (a–b), see the text.

detect fluorescence, respectively. A 100× lens was used. The images were obtained with an ApoTome device. The image processing and plotting of the fluorescence distribution along the specified line were performed with the ImageJ software (http://rsb.info.nih.gov/ij).

The results demonstrate that macrophage incubation in the presence of Glutoxim (100 μg/ml) for 20 min leads to a significant increase in $[Ca^{2+}]_i$ due to Ca^{2+} mobilization from the intracellular Ca^{2+} stores. After addition of 2 mM Ca^{2+} into the external

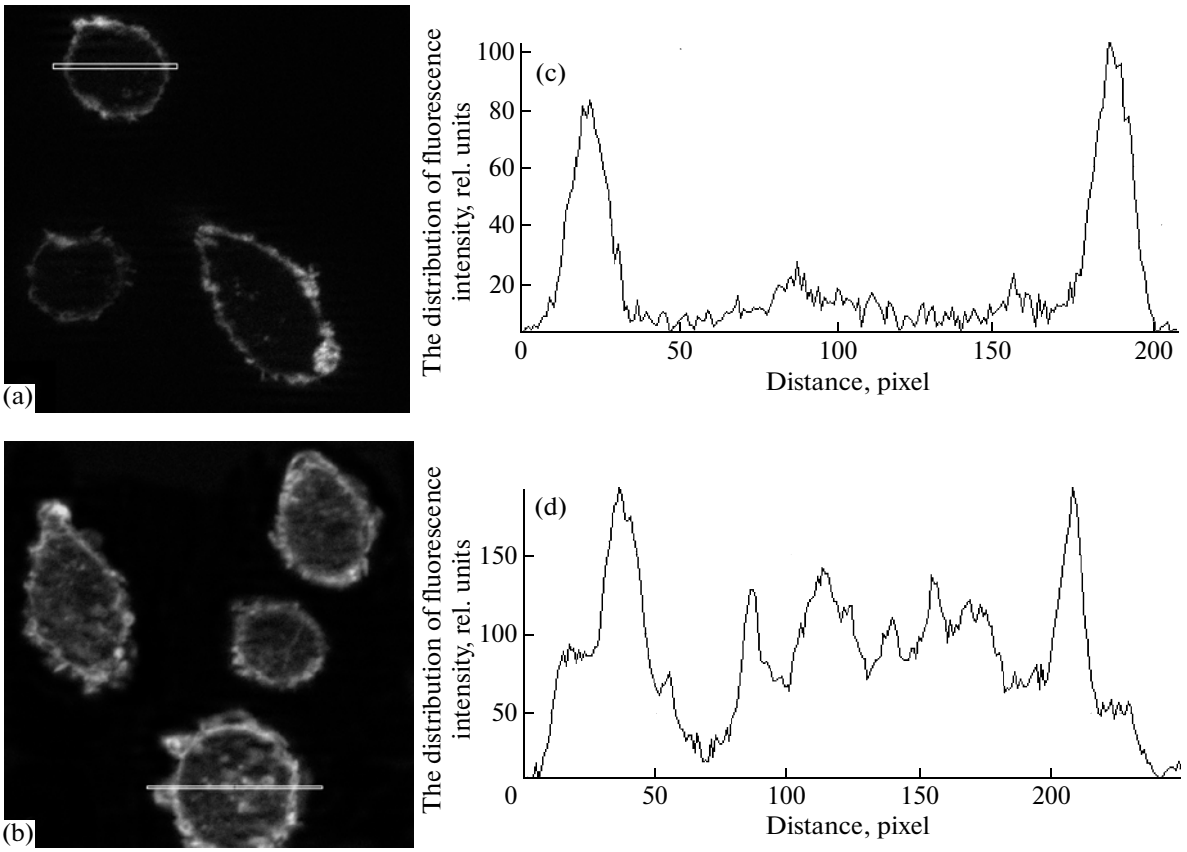

Fig. 2. Rearrangement of the actin cytoskeleton in the peritoneal macrophages treated with glutoxim. (a, b) Actin filaments in the control cells and in cells treated with Glutoxim (100 μg/ml) for 20 min, respectively. Rhodamine–phalloidin staining. Lens, 100×. (c, d) Distribution of fluorescence intensity along the specified line in the control cells and Glutoxim-treated cells, respectively.

medium, Ca^{2+} entered the cytosol, probably because of depletion of the Ca^{2+} stores (Figs. 1a, curve *1* and 1b, curve *1*). Preliminary cell incubation with 5 μM latrunculin B for 5 min before addition of 100 μg/ml Glutoxim led to enhancement of both phases of the Glutoxim-induced Ca^{2+} response (Fig. 1a, curve *2*). A longer preincubation of macrophages with latrunkulin B (for 20 min) resulted in an almost complete inhibition of the Glutoxim-induced increase in $[Ca^{2+}]_i$ and Ca^{2+} entry (Fig. 1a, curve *3*). Similar results were obtained with another agent capable of causing depolymerization of actin filaments (cytochalasin D at a concentration of 10 μg/ml). It has also been found that cell preincubation with 100 nM calyculin A for 10 min before addition of Glutoxim to the culture (100 μg/ml) led to an almost complete inhibition of both phases of the Ca^{2+} response to Glutoxim (Fig. 1b, curve *2*).

We assume that, during a short-term cell incubation with the agents depolymerizing microfilaments, only the cortical actin is disassembled, and, therefore transduction of the GSSG- or Glutoxim-induced signal from the plasmalemma to Ca^{2+} store remains undisturbed and, as a result, Ca^{2+} mobilization from it is still stimulated. In addition, rearrangement of submembrane actin facilitates the interaction between Ca^{2+} stores and store-dependent Ca^{2+} channels in the plasmalemma, which enhances the Ca^{2+} entrance from the external medium. Conversely, long-term cell treatment with latrunculin B or cytochalasin D led to disassembly of cytoplasmic actin filaments in addition to submembrane ones. Hence, the supportive role of the actin cytoskeleton in the interaction between Ca^{2+} stores and the plasmalemma becomes impossible, which results in inhibition of both phases of Ca^{2+} signals induced by GSSG or Glutoxim. The evidence obtained in this study is in agreement with the data on the effect of cytochalasin D and latrunculin A on the store-dependent Ca^{2+} entrance into platelets [12] and with our data on the effect of latrunculin B on the Ca^{2+} signals in peritoneal rat macrophages [13].

Rearrangement of actin cytoskeleton of macrophages in response to Glutoxim has been visualized by the morphological method, which confirms the involvement of actin cytoskeleton into the effect of GSSG and Glutoxim on $[Ca^{2+}]_i$. Figure 2 shows fluo-

rescent micrographs of actin stained with rhodamine–falloidin in (a) the control cells and (b) the cells treated with Glutoxim. It can be seen that, in the control cells, the elements of the actin cytoskeleton are localized mainly under the plasmalemma, and they form a clearly discernible cortical layer; the number of actin filaments in the cytosol is low (Fig. 2a).

In the macrophages treated with Glutoxim, rearrangement of actin filament is observed. Thus, the cortical layer becomes wider and "loose" and, in addition, the number of actin filaments in cytosol increases (Fig. 2b). This is confirmed by the plots that show the distribution of fluorescence intensity in the control cells and in the cells treated with Glutoxim (Figs. 2c and 2d, respectively).

Thus, the involvement of actin filaments in a signaling cascade triggered by GSSG or Glutoxim and resulting in an increase in $[Ca^{2+}]_i$ in macrophages has been demonstrated in this study.

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