ISSN 1990-519X, Cell and Tissue Biology, 2012, Vol. 6, No. 3, pp. 240–247. © Pleiades Publishing, Ltd., 2012.
Original Russian Text © L.S. Kurilova, Z.I. Krutetskaya, O.E. Lebedev, N.I. Krutetskaya, V.G. Antonov, 2012, published in Tsitologiya, 2012, Vol. 54, No. 2, pp. 135–142.

The Involvement of Actin Cytoskeleton in Glutoxim and Molixan Effect on Intracellular Ca^{2+}-Concentration in Macrophages

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Received August 8, 2011

Abstract—Glutoxim and molixan belong to new generation of disulfide-containing drugs with immunomodulatory, hepatoprotective and hemopoetic effect on cells. Using Fura-2AM microfluorimetry, two structurally distinct actin filament disrupters latrunculin B and cytochalasin D, and calyculin A, which causes actin filaments condensation under plasmalemma, we have shown the involvement of actin cytoskeleton in the intracellular Ca^{2+}-concentration increase induced by glutoxim or molixan in rat peritoneal macrophages. Morphological data obtained with the use of rhodamine-phalloidine demonstrated that glutoxim and molixan cause the actin filaments reorganization in rat peritoneal macrophages.

Keywords: peritoneal macrophages, glutoxim, molixan, intracellular Ca^{2+}-concentration, actin filaments.

DOI: 10.1134/S1990519X12030066

A number of disulfide-containing drugs affecting redox-state and having prominent physiological effect have clinical value. Thus, a synthetic analogue of oxidized glutathione (GSSG) pharmacological drug glutoxim (GSSG disodium salt with platinum nanoaddition, PHARMA-VAM, Moscow, Russia), is applied as an immunomodulator and hemostimulator in therapy of bacterial and viral diseases (Zhukov et al., 2004), psoriasis (Korsunskaya et al., 2003; Chermoshentsev et al., 2003), radio- and chemotherapy of oncological disorders (Filatova et al., 2004). Another disulfide-containing drug molixan (complex of glutoxim with inosine nucleoside) has the similar application. However, the cellular and molecular mechanisms underlying these drugs action are poorly understood.

Earlier we found that GSSG and glutoxim increase intracellular Ca^{2+}-concentration, $[Ca^{2+}]_i$, inducing Ca^{2+}-mobilization from thapsigargin-sensitive Ca^{2+}-stores and subsequent Ca^{2+}-entry into the rat peritoneal macrophages (Kruretskaya et al., 2007a; Kurilova et al., 2008). Later we demonstrated that the same effect on $[Ca^{2+}]_i$ is observed in macrophages treated with molixan (Krutetskaya et al., 2010). In addition, it was found that tyrosine kinases, tyrosine phosphatases (Krutetskaya et al., 2007b, Kurilova et al., 2008), phosphatidylinositol kinases (Krutetskaya et al., 2008), small G-proteins from Ras family as well as phospholipase C and protein kinase C, the key molecules of phosphoinositol signaling pathway (Krutetskaya et al., 2009) are the critical components of the signaling cascade, triggered by GSSG and glutoxim and leading to $[Ca^{2+}]_i$ increase in macrophages.

It is known that phosphatidylinositol kinases which play an important role in phosphoinositide metabolism induce the reorganization of actin filaments (Foster et al., 2003). Actin filaments are highly redox-sensitive and easily glutathionylated (Dalle-Donne et al., 2003). NOV-002, another GSSG analogue (GSSG in combination with cisplatin), induces S-glutathionylation of cell proteins, mostly actin. NOV-002 effect on actin filaments is time- and dose- dependent. Actin S-glutathionylation that alters the F-and G-actin ratio induces drastic changes in the total cytoskeleton architecture and intracellular transport (Townsend, 2007; Townsend et al., 2008). It can be supposed that actin cytoskeleton components are also targets for glutoxim and molixan.

The purpose of the present study was to elucidate the possible involvement of actin filaments in glutoxim and molixan regulation of $[Ca^{2+}]_i$ in rat peritoneal macrophages. Actin cytoskeleton involvement in the signaling cascade triggered by glutoxim or molixan in macrophages supposes its reorganization and redistribution. Therefore, the other purpose of this study was to examine actin cytoskeleton reorganization in macrophages exposed to glutoxim or molixan.

MATERIALS AND METHODS

Cells. Experiments have been performed on cultivated resident peritoneal macrophages from Wistar rats. These cells were isolated from peritoneal cavity of 180–250 g rats as described earlier (Conrad, 1981; Randriamampita and Trautmann, 1987). Freshly isolated cells have spherical shape and diameter of 10–

20 μm. Cell suspension was placed in culture dishes with 10 × 10 mm quartz glasses and cultivated for 1–3 days in 199 medium (pH 7.2) with 20% bovine serum, 3% glutamine, penicillin (100 units/mL), streptomycin (100 μg/mL) at 37°C. Testing α-naphthyl esterase activity revealed that, at least, 96% cells in monolayer were macrophages. Experiments were performed at 22–24°C 1–2 days after cell plating. Quartz glasses with cells were placed in experimental chamber filled with the physiological solution: 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES-NaOH, pH 7.3–7.4. Calcium-free medium contained 1 mM EGTA and 0 mM $CaCl_2$. Details of macrophage cultivation procedure were described previously (Krutetskaya et al., 1997b).

Reagents. Glutoxim and molixan were provided by PHARMA-VAM Company (Russia). Glutoxim (50 mg/mL) and molixan (50 mg/mL) stock solutions were prepared in water. Cytochalasin D (2 mg/mL), latrunculin B (5 mM) and calyculin A (50 μM) stock solutions were prepared in dimethyl sulfoxide. All reagents were purchased from Sigma-Aldrich Company (United States).

$[Ca^{2+}]_i$ was measured with Fura-2AM fluorescent probe (Sigma-Aldrich, United States). Macrophages were incubated for 45 min in saline containing 2 μM Fura-2AM at the room temperature. Glasses with stained cells were washed with physiological solution and transferred to the experimental chamber fixed on the table of Lumam-KF luminescent microscope (LOMO, Russia). Fura-2AM fluorescence was excited at 337 nm with LGI-503 nitrogen laser. The laser was located one side of the microscope at an angle of 30° to the experimental chamber, that allowed to concentrate the laser beam directly to the object. The fluorescence intensity was registered with SFN-10 spectrophotometer at 510 nm. The signal from FEU-79 was enhanced with the specially constructed amplifier and recorded with the IBM PC digital computer using the original software. Cells were visualized with objective 10× and aperture 0.4. At this magnification the registered area has 40–50 cells. To avoid photobleaching, the measurements were performed every 20 s, irradiating the object for 2.5 s. $[Ca^{2+}]_i$ values were calculated according to the Grynkiewicz's equation (Grynkiewicz et al., 1985). Figs. 1–4 present the results of typical experiments.

Visualization of actin cytoskeleton in macrophages. On the second day of macrophage cultivation the cells were exposed to 100 μg/mL glutoxim or molixan for 20 min. Control cells were incubated in the medium without drugs. To visualize actin cytoskeleton, the cells were washed with phosphate saline buffer (PBS), fixed with 3.7% formalin for 15 min, washed three times with PBS, treated with 0.1% Triton X-100 for 10 min and then stained with rhodamine-phalloidin (Sigma-Aldrich, United States) for 10 min at 37°C. The preparations obtained were examined using AxioObserver.Z1 inverted microscope (Carl Ziess, Germany) under objective 100×, exciting and registering fluorescence at 550 and 605 nm, respectively. Images were obtained with ApoTome device. Images were analyzed and fluorescence intensity along the specified line was plotted using ImageJ software (http://rsb.info.nih.gov/ij).

RESULTS AND DISCUSSION

Effect of actin filament depolymerizing agents cytochalasin D and latrunculin B. Actin filament involvement in glutoxim and molixan action on $[Ca^{2+}]_i$ in rat peritoneal macrophages was examined using cytochalasin D and latrunculin B, two structurally different agents inducing microfilament depolymerization (Spector et al., 1983).

In control experiments it was shown that macrophage incubation with 100 μg/mL glutoxim for 20 min in calcium-free medium resulted in progressive $[Ca^{2+}]_i$ increase due to Ca^{2+}-mobilization from intracellular Ca^{2+}-stores. Addition of 2 mM Ca^{2+} to the external medium induced Ca^{2+} entry into cytosol, probably mediated by Ca^{2+}-store depletion (Fig. 1a; Fig. 2a; Fig. 3a). Cell incubation with 10 μg/mL cytochalasin D for 5 min before 100 μg/mL glutoxim addition enhanced glutoxim induced $[Ca^{2+}]_i$ increase due to Ca^{2+}-mobilization from the store and subsequent Ca^{2+}-entry (Fig. 1b). Longer (20 min) preincubation of macrophages with cytochalasin D almost completely inhibited $[Ca^{2+}]_i$ increase and Ca^{2+}-entry induced by glutoxim (Fig. 1c). Similar results were obtained with 5 μM latrunculin B, another agent inducing actin filament depolymerization (Fig. 2).

The enhancement of glutoxim induced $[Ca^{2+}]_i$ increase by short-term incubation with cytochalasin D or latrunculin B is in agreement with the data we reported previously on the effect of phenylarsine oxide (PAO), a SH-oxidizing reagent, on $[Ca^{2+}]_i$ in macrophages (Krutetskaya et al., 1997a). It was found that short-term (6 min) incubation of peritoneal macrophages with agents disrupting actin filament structure (20 μg/mL cytochalasin B or 40 μM phalloidin) induced progressing increase of $[Ca^{2+}]_i$ in cells treated with PAO. The addition of cytochalasin B or phalloidin during already developed PAO-induced Ca^{2+}-response leads (with 1–2 min delay) to further essential increase in $[Ca^{2+}]_i$. The data suggested that reorganization in actin filament structure stimulated PAO-induced Ca^{2+}-response (Krutetskaya et al., 1997a).

Thus, we have shown that the short-term preincubation of macrophages with actin depolymerizers leads to the enhancement of $[Ca^{2+}]_i$ increase induced by glutoxim. It can be suggested that short-term incubation of cells with microfilament depolymerizing agents causes disassembly of only cortical actin. It facilitates glutoxim-induced signal transduction from plasmalemma to Ca^{2+}-store and thereby enhances

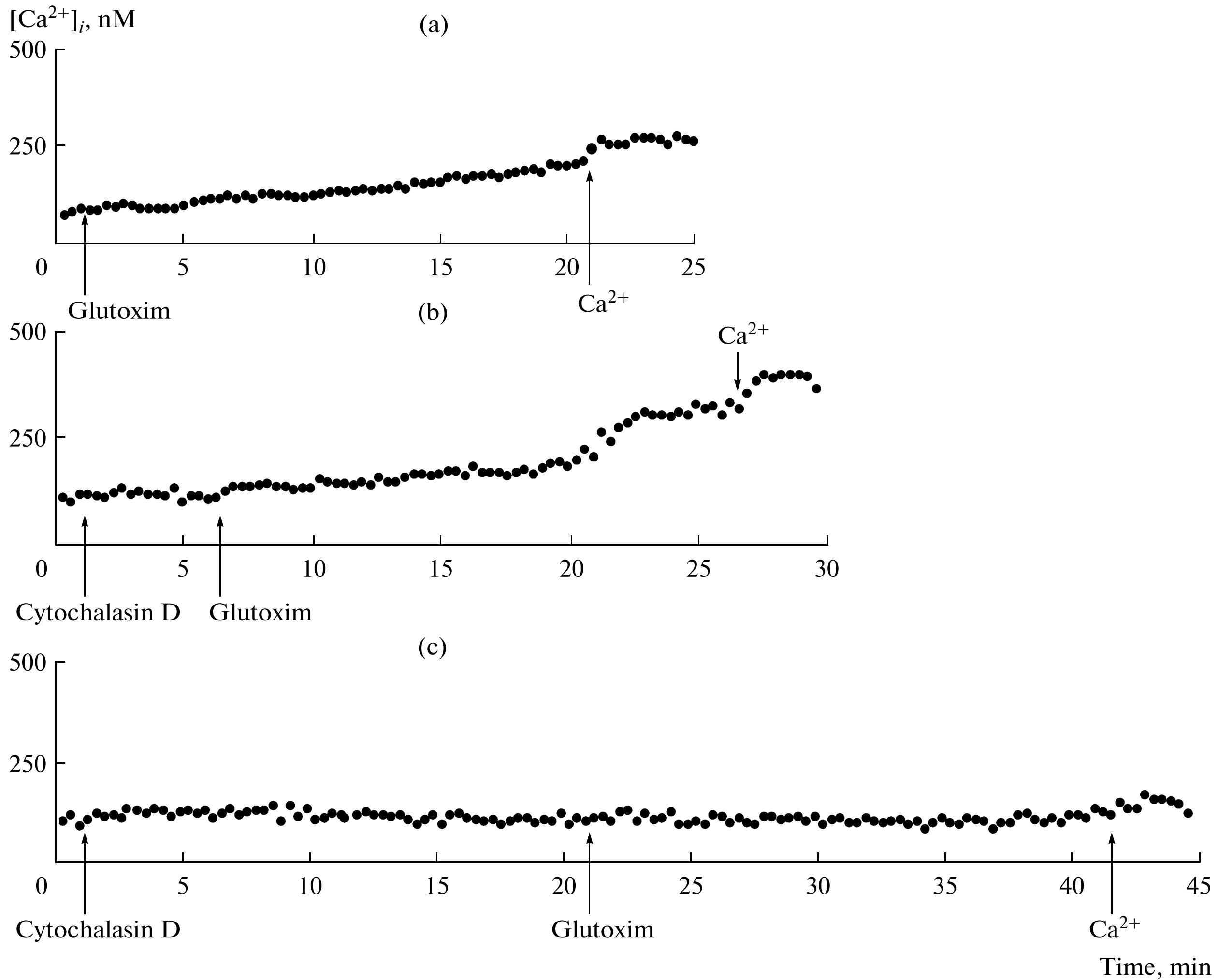

Fig. 1. The influence of cytochalasin D on $[Ca^{2+}]_i$ increase induced by glutoxim in macrophages.
Here and in Figs. 2–4: abscissa—time, min; ordinate—intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, nM.
(a) cells were incubated for 20 min with 100 μg/mL glutoxim in Ca^{2+}-free solution, then Ca^{2+}-entry was initiated by the addition of 2 mM Ca^{2+} to the external medium; (b) cells were preincubated for 5 min with 10 μg/ml cytochalasin D in Ca^{2+}-free solution, then 100 μg/mL glutoxim was applied, 20 min later Ca^{2+}-entry was initiated by the addition of 2 mM Ca^{2+} to the external medium; (c) cells were incubated for 20 min with 10 μg/mL cytochalasin D in Ca^{2+}-free solution, then 100 μg/mL glutoxim was applicated, 20 min later 2 mM Ca^{2+} was added to the external medium. Here and on Figs. 2–4 each recording is obtained for 40–50 cells and is typical of three to seven experiments.

Ca^{2+}-mobilization. Long-term cell exposure to latrunculin B or cytochalasin D leads to the complete disassembly of both submembrane and cytoplasmic actin filaments and results in inhibition of Ca^{2+}-release from the store.

Glutoxim- or molixan-induced Ca^{2+}-entry in the cells occurs, presumably, by store-dependent mechanism (Krutetskaya et al., 2007a, 2008). Earlier, using purinergic agonists ATP and UTP and endoplasmic Ca^{2+}-ATPase inhibitors (thapsigargin and cyclopiazonic acid) we demonstrated that store-dependent Ca^{2+}-entry in rat peritoneal macrophages occurred according to the "secretion-like coupling model" of store-dependent Ca^{2+}-entry (Patterson et al., 1999; Rosado and Sage, 2000) which supposed the reversible translocation of Ca^{2+}-store to plasmalemma provided by the actin filaments. Short-term cell exposure to latrunculin B or cytochalasin D induces partial depolymerization of submembrane actin filaments facilitating Ca^{2+}-store binding with plasmalemma. Conversely, prolonged cell treatment with latrunculin B or cytochlasin D produces complete actin filament disassembly that abolishes actin cytoskeleton assistance in Ca^{2+}-store interaction with store-dependent Ca^{2+} channels in the plasmalemma. Thus, our results on latrunculin B and cytochalasin D effect on Ca^{2+}-entry induced by glutoxim support the "secretion-like coupling model" for rat peritoneal macrophages. These data are in agreement with latrunculin A and cytocha-

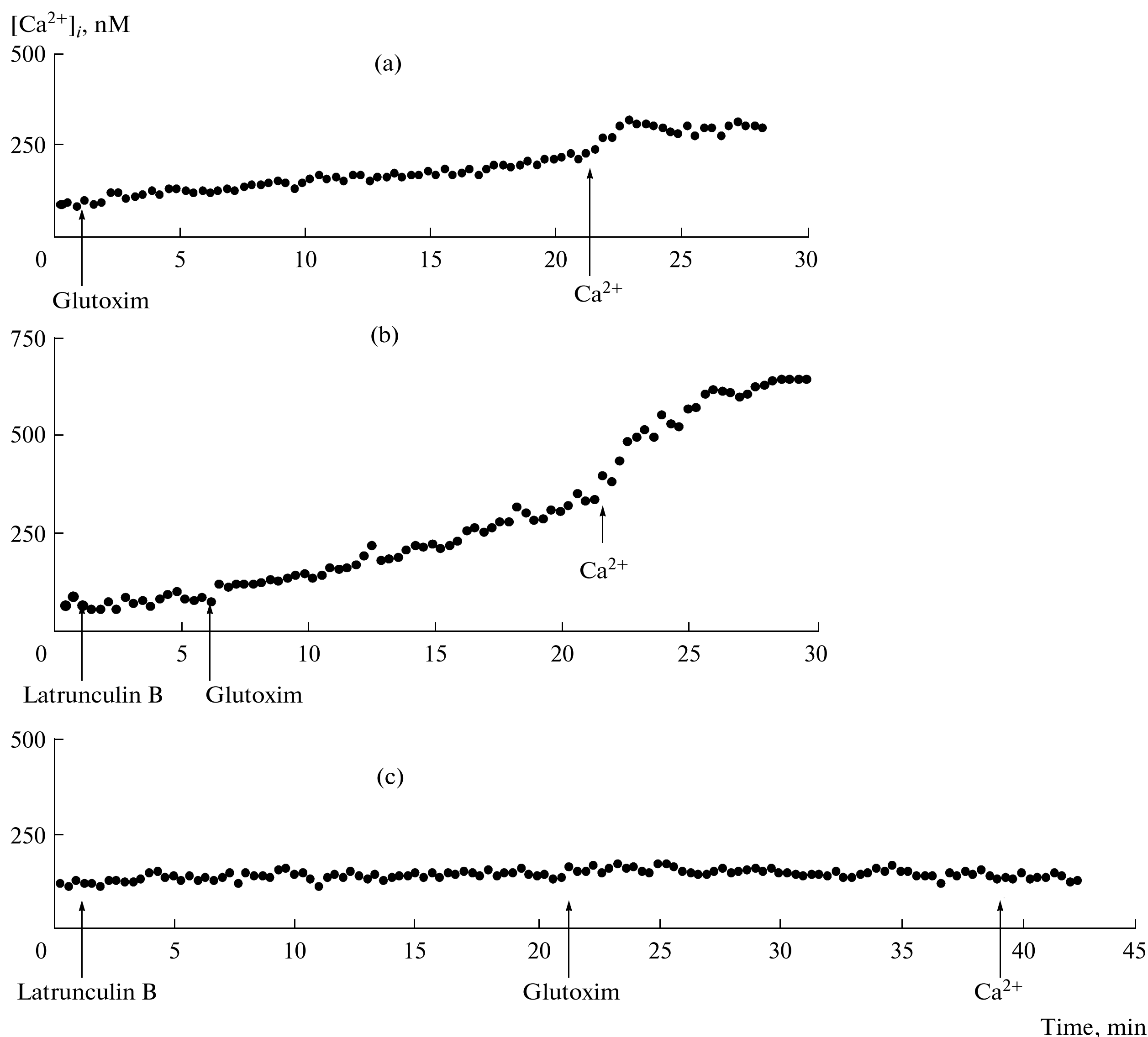

Fig. 2. The influence of latrunculin B on $[Ca^{2+}]_i$ increase induced by glutoxim in macrophages.

(a) cells were incubated for 20 min with 100 μg/mL glutoxim in Ca^{2+}-free solution, then Ca^{2+}-entry was initiated by the addition of 2 mM Ca^{2+} to the external medium; (b) cells were preincubated for 5 min with 5 μM latrunculin B in Ca^{2+}-free solution, then 100 μg/mL glutoxim was applied, 15 min later Ca^{2+}-entry was initiated by the addition of 2 mM Ca^{2+} to the external medium; (c) cells were incubated for 20 min with 5 μM latrunculin B in Ca^{2+}-free solution, then 100 μg/mL glutoxim was applied, 17 min later 2 mM Ca^{2+} was added to the external medium.

lasin D influence on store-mediated Ca^{2+}-entry in platelets (Rosado and Sage, 2000) and our results on lantrunculin B effect on Ca^{2+}-signals in rat peritoneal macrophages (Kurilova et al., 2006).

Effect of calyculin A, inducing microfilament condensation under plasmalemma. To verify further the microfilament involvement in glutoxim and molixan action on $[Ca^{2+}]_i$ in macrophages we examined whether the dense actin cortical layer formation affected glutoxim or molixan induced $[Ca^{2+}]_i$ increase. For this purpose, we used calyculin A. Calyculin A isolated from sea sponge *Discodermia calyx* is a highly effective and specific inhibitor of serine/threonine phosphatases PP1 and PP2A. In many cells, including platelets, calyculin A (up to 100 nM) induces actin filaments reorganization facilitating phosphorylation of actin-binding ERM proteins (ezrin, radixin, moesin) (Patterson et al., 1999). Phosphorylation of ERM-proteins promotes the interaction of actin filaments with plasmalemma (Kreienbuhl et al., 1992; Matsui et al., 1998). In cells treated with calyculin A actin filaments condense near the plasmalemma (Patterson et al., 1999) but actin polymerization does not increase (Rosado et al., 2000).

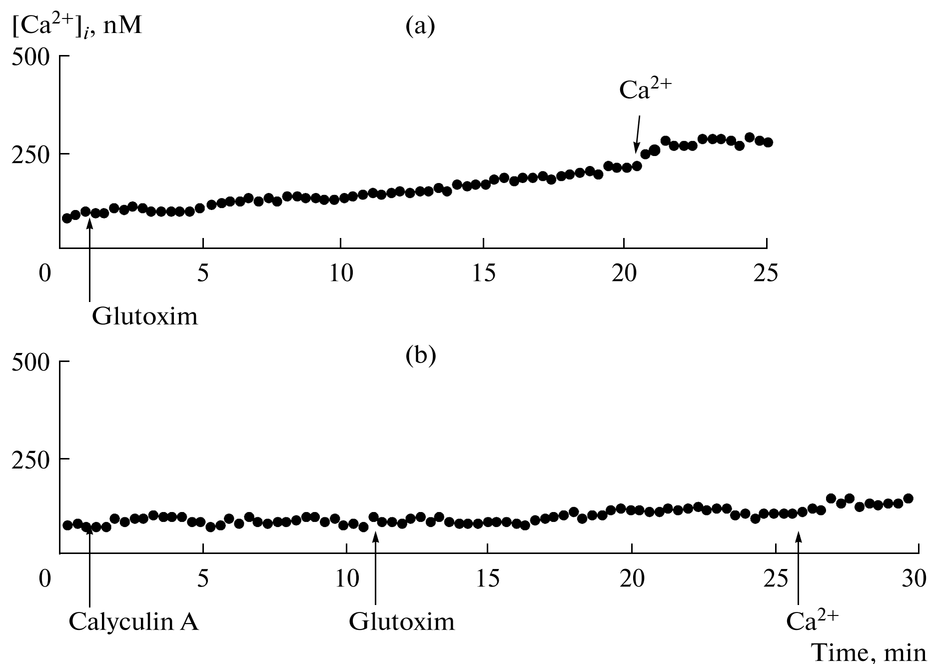

Fig. 3. The influence of calyculin A on $[Ca^{2+}]_i$ increase induced by glutoxim in macrophages.

(a) cells were incubated for 20 min with 100 μg/mL glutoxim in Ca^{2+}-free solution, then Ca^{2+}-entry was initiated by the addition of 2 mM Ca^{2+} to the external medium; (b) cells were preincubated for 10 min with 100 nM calyculin A in Ca^{2+}-free solution, then 100 μg/mL glutoxim was applied, 15 min later Ca^{2+}-entry was initiated by the addition of 2 mM Ca^{2+} to the external medium.

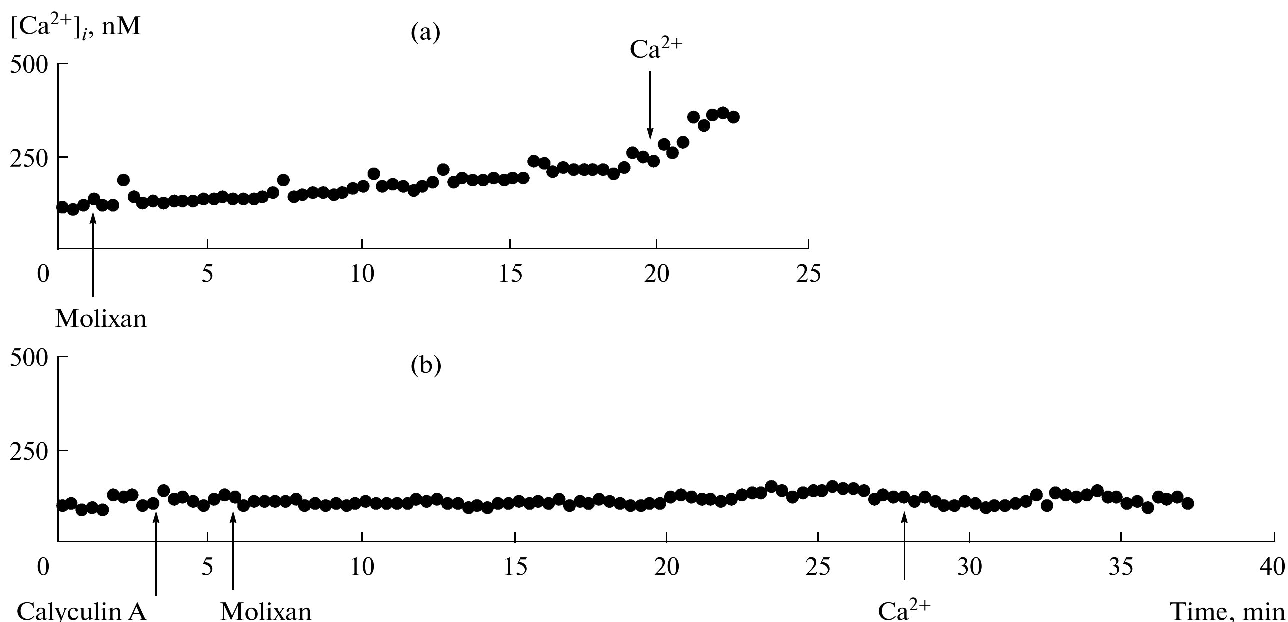

Fig. 4. The influence of calyculin A on $[Ca^{2+}]_i$ increase induced by molixan in macrophages.

(a) cells were incubated for 19 min with 100 μg/mL molixan in Ca^{2+}-free solution, then Ca^{2+}-entry was initiated by the addition of 2 mM Ca^{2+} to the external medium; (b) cells were preincubated for 2 min with 100 nM calyculin A in Ca^{2+}-free solution, then 100 μg/mL molixan was applied, 22 min later Ca^{2+}-entry was initiated by the addition of 2 mM Ca^{2+} to the external medium.

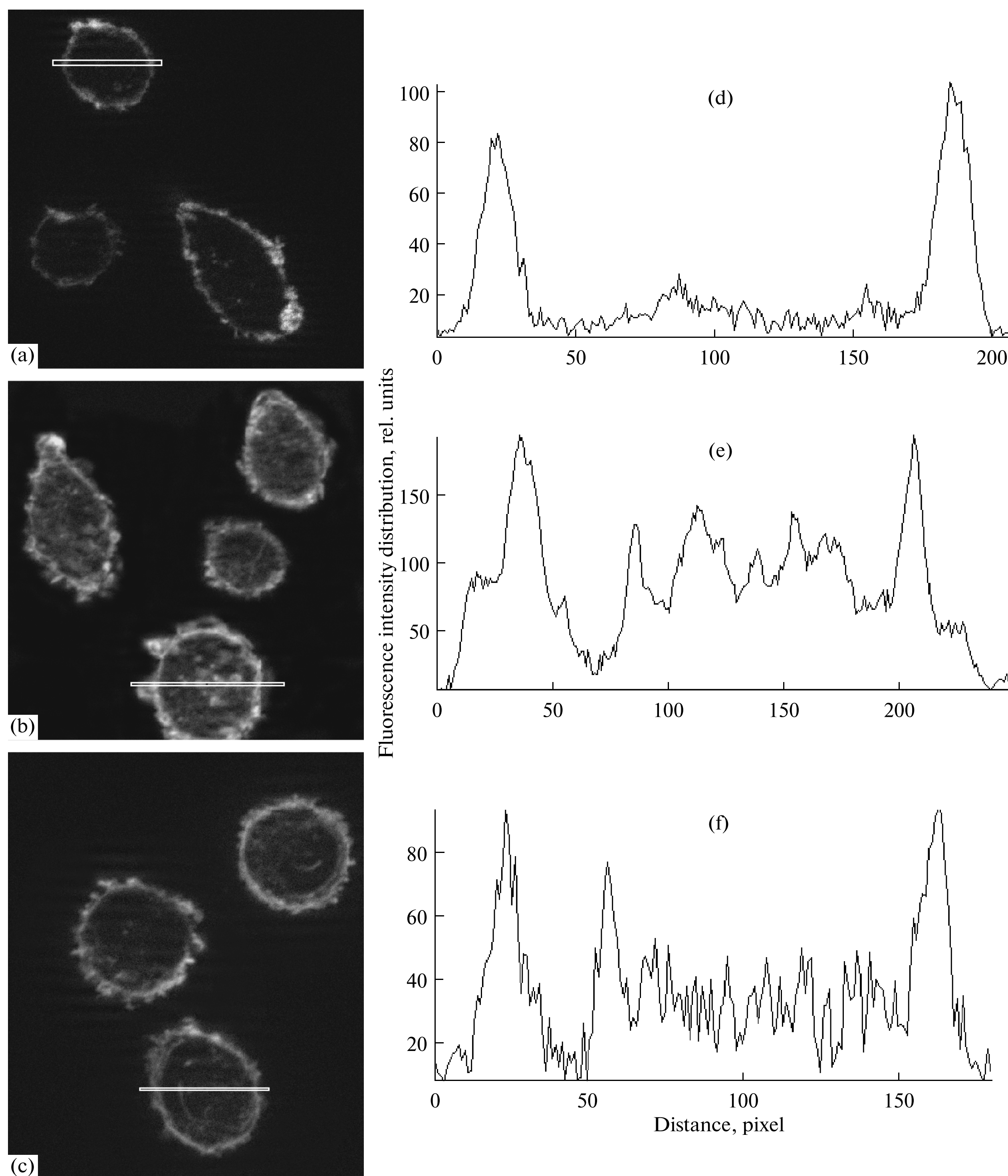

Fig. 5. Actin cytoskeleton organization in native macrophages (a, d) and macrophages, treated with glutoxim (b, e) or molixan (c, f).

(a–c) fluorescent microphotographs of actin, stained by rhodamine–phalloidin in control cells (a) and in cells, treated for 20 min with glutoxim (100 μg/mL) (b) or molixan (100 μg/mL) (c). Obj.: 100×. (d–f) fluorescence intensity distribution (rel. units) along the specified line in the control cells (d) and in cells, treated with glutoxim (e) or molixan (f).

It was shown that macrophage incubation with 100 nM calyculin A for 10 min before 100 μg/mL glutoxim addition almost completely inhibited glutoxim-induced increase in $[Ca^{2+}]_i$ and subsequent Ca^{2+}-entry(Fig. 3b). These results suggest actin filament involvement in the signaling cascade induced by glutoxim and leading to $[Ca^{2+}]_i$ increase in macrophages. Similar results were obtained with 100 μg/mL molixan (Fig. 4b).

Thus, calyculin A-induced dense cortical actin layer formation under plasmalemma prevents $[Ca^{2+}]_i$ increase caused by glutoxim or molixan.

The results on calyculin A inhibition of store-dependent Ca^{2+}-entry induced by glutoxim or molixan are in agreement with the data on store-operated Ca^{2+}-entry in human platelets (Rosado et al., 2000; Rosado and Sage, 2000) and our data on calyculin A influence on Ca^{2+} signals induced by thapsigargin (endoplasmic Ca^{2+}-ATPase inhibitor) or purinergic agonist ATP in rat macrophages (Kurilova et al., 2009). According to the "secretion-like coupling model" of Ca^{2+}-entry cortical F-actin prevents the activation of store-mediated Ca^{2+}-entry like submembrane actin averts translocation of secretory granules to the membrane (Muallem et al., 1995). Therefore, calyculin A inhibits store-mediated Ca^{+2}-entry producing the dense layer of submembrane actin which forces out organelles from this area and prevents interaction between plasmalemma and intracellular organelles. Thus, our experiments on calyculin A effect on store-operated Ca^{2+}-entry induced by glutoxim or molixan in rat peritoneal macrophages support the "secretion-like coupling model" of Ca^{2+}-entry in these cells. Inhibition of glutoxim- or molixan- induced Ca^{2+}-mobilization from the store may be explained by the prevention of signal transduction induced by glutoxim or molixan from plasmalemma to Ca^{2+}-store due to formed dense layer of cortical actin.

Actin cytoskeleton involvement in glutoxim or molixan effect on $[Ca^{2+}]_i$ in macrophages was supported by morphological observations on its reorganization in cells treated with the drugs.

Actin cytoskeleton reorganization in cells exposed to glutoxim or molixan. It was shown that microfilament network is drastically changed in macrophages treated with glutoxim or molixan. Fig. 5 shows actin stained with rhodamine-phalloidin in intact cells (Fig. 5a) and cells treated with glutoxim (Fig. 5b) or molixan (Fig. 5c). It is seen that in intact cells actin cytoskeleton is localized under the plasma membrane and forms well-defined cortical layer (Fig. 5a). In macrophages treated with glutoxim actin cytoskeleton is reorganized: actin clusters appeared in cytosol, cortical layer is wider and loose (Fig. 5b). Molixan exerts similar effect on the actin distribution in macrophages (Fig. 5c). Fluorescence intensity measured in control cells (Fig. 5d) and cells treated with glutoxim (Fig. 5e) or molixan (Fig. 5f) also indicates the reorganization of actin cytoskeleton induced by these disulfide-containing drugs.

Thus, we demonstrated that glutoxim and molixan induce reorganization of actin cytoskeleton in rat peritoneal macrophages. These results are consistent with those on actin cytoskeleton rearrangements produced by NOV-002 drug, another synthetic GSSG analogue (Townsend, 2007; Townsend et al., 2008). Actin cytoskeleton reorganization was also observed in mouse embryonic fibroblasts and mouse hepatoma 22-a cells exposed to N-acetylcysteine, a mucolytic and antioxidant SH-compound (Gamaley et al., 2010).

Wide and loose actin cortical layer formation and the appearance of actin clusters in cytosol in cells exposed to glutoxim or molixan may facilitate the signal transduction from the plasmalemma to intracellular Ca^{2+}-stores triggered by these drugs. Latrunculin B and cytochalasin D, inducing actin filament disassembly, presumably, prevent glutoxim-induced actin cytoskeleton rearrangements and inhibit the signaling cascade, initiated by these drugs in macrophages. Calyculin A, inducing formation of dense microfilament layer under plasmalemma, may attenuate glutoxim or molixan effect, inhibiting the signal transduction. Probably, the intact actin cytoskeleton structure is required for the signal transduction because both actin depolymerization and dense microfilament layer formation under the cell membrane abolished glutoxim- or molixan-induced $[Ca^{2+}]_i$ increase.

In conclusion, we found that any modification in the actin cytoskeleton structure in macrophages modulates glutoxim or molixan effect on $[Ca^{2+}]_i$. The drugs also induce actin cytoskeleton reorganization in these cells. It may be concluded that actin cytoskeleton is an important player in the glutoxim- or molixan-triggered signaling cascade, which leads to increase of $[Ca^{2+}]_i$ in rat peritoneal macrophages.

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