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CELL BIOPHYSICS

Arp2/3 Complex is Involved in the Effect of Glutoxim and Molixan on Intracellular Ca^{2+} Concentration in Macrophages

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Abstract—The involvement of Arp2/3 complex, which causes actin filament branching, in the effect of drugs glutoxim and molixan on intracellular Ca^{2+} concentration was investigated. Using Fura-2AM microfluorimetry it was shown for the first time that Arp2/3 complex inhibitor CK-0944666 almost completely prevents the increase in intracellular Ca^{2+} concentration, induced by glutoxim or molixan in macrophages. The data suggest the involvement of Arp2/3 complex in the glutoxim and molixan effect on the Ca^{2+} signaling processes in macrophages.

Keywords: Ca^{2+} signalization, glutoxim, molixan, Arp2/3 complex, actin filaments

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INTRODUCTION

Presently developed and introduced into clinical practice are a significant number of disulfide-containing drugs changing the redox state and exerting a physiologically significant effect on cells. Thus a synthetic analog of oxidized glutathione (GSSG), pharmacological drug Glutoxim® (disodium salt of GSSG with nanoaddition of platinum, "FARMA VAM," Moscow) is used as immunomodulator and hemostimulator in complex therapy of bacterial and viral infections, psoriasis, radio- and chemotherapy in oncology [1]. Another drug, Molixan ("FARMA VAM," Moscow), a complex of glutoxim and nucleoside inosine, has antiviral, immunomodulating and hepatoprotective effects, is applied in therapy of acute and viral hepatitis B and C, mixed hepatitis and liver cirrhosis [1]. Glutoxim and molixan belongs to the pharmacological group of thiopoietins, influencing the process of redox regulation in cells. However, the cellular and molecular mechanisms of action of these drugs are not clearly understood.

Earlier we have shown for the first time that glutoxim and molixan increase the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), causing mobilization of Ca^{2+} from thapsigargin-sensitive Ca^{2+} stores and subsequent Ca^{2+} entry into rat peritoneal macrophages [2–4]. Using a wide range of agents affecting components of intracellular signaling systems in cells, we have shown for the first time that key players in a signal cascade triggered by GSSG and glutoxim and leading to $[Ca^{2+}]_i$ increase in macrophages are tyrosine kinases and tyrosine phosphatases [3, 5], phosphatidylinositol kinases [6], key enzymes of the phosphoinositide signal pathway – phospholipase C and protein kinase C [7]. It was also found that the effect of glutoxim and molixan on $[Ca^{2+}]_i$ in macrophages was mediated by actin cytoskeleton [8] and microtubules [9].

Apart from that, it has been disclosed that glutoxim or molixan themselves cause reorganization of the actin cytoskeleton in macrophages: the cortical layer becomes broader and "looser" and there appear clusters of actin in the cytosol [8]. Also we have shown the involvement of G-proteins of small molecular mass of the Ras superfamily and processes of vesicular transport in the effect of glutoxim or molixan on $[Ca^{2+}]_i$ in macrophages [10, 11].

One of the key participants of the process of formation of stable filaments from G-actin monomers comes to be Arp2/3 (Actin-Related Proteins) complex consisting of seven evolutionarily conserved proteins. Arp2/3 stabilizes intermediated consisting of two actin monomers, stimulating the branching of F-actin filaments [12]. In this way, Arp2/3 complex is a factor enhancing the nucleation of actin filaments. Arp2/3 complex consists of two subunits – Arp2 and Arp3 having close structural similarity with actin monomers and five additional subunits [12]. After assembly the complex heads toward the points of polymerization of new actin filaments, binds with an existing filament and aligns in the same way as actin is disposed in a dimer. In this way, Arp2/3 complex serves as a key element for formation of new filaments, which stretch at an angle of 70° to the already existing filaments and form a dense network of F-actin [12].

Arp2/3 complex takes part in various cellular processes requiring reorganization of actin filaments, such as rearrangement of cortical actin, formation of fillopodia, regulation of endosome shape and trans-

port, processes of endo- and exocytosis [13]. Arp2/3 complex is activated upon activation of receptors with intrinsic tyrosine kinase activity, G-protein-coupled receptors, and also integrin receptors. In macrophages the Arp2/3cx participates in processes of migration and chemotaxis [14].

Therefore, for further investigation of the involvement of actin filaments and actin-binding proteins, and also processes of vesicular transport and exocytosis in signal cascade triggered by glutoxim and molixan, it appeared reasonable to investigate the possible involvement of Arp2/3 complex in the effect of glutoxim or molixan on $[Ca^{2+}]_i$ in macrophages.

EXPERIMENTAL

Cells. Experiments were performed on culture of resident peritoneal macrophages of Wistar line rats. Resident macrophages were isolated from the peritoneal cavity of rats of 180–250 g mass. Right after isolation the cells had a spherical shape and diameter of 10–20 μm. Cell suspension was placed into culture dishes containing 10 × 10 mm quartz glasses. Cells on glasses were cultivated for 1–3 days at 37°C in 199 medium (pH 7.2) containing 20% bovine blood serum, glutamine (3%), penicillin (100 un./mL) and streptomycin (100 mg/mL). Test for α-naphthyl esterase has shown that at least 96% cells in monolayers were macrophages. Experiments were conducted at room temperature 22–24°C at 1–2 days after the beginning of cell cultivation. Quartz glasses with cells were placed in an experimental chamber filled with physiological solution of the following ionic composition (mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES-NaOH, pH 7.3–7.4. Calcium-free medium differed in that it contained 1 mM EGTA and did not contain $CaCl_2$. In detail the procedure of cultivating macrophages was described earlier [8].

Used reagents. For revealing participation of Arp2/3 complex in the action of glutoxim or molixan on $[Ca^{2+}]_i$ in macrophages we used a new effective low-molecular weight inhibitor of Arp2/3 complex – compound CK-0944666 [15, 16]. A stock solution of glutoxim (50 mg/mL) and molixan (50 mg/mL) ("FARMA VAM," Moscow) was prepared in water, a stock solution of compound CK-0944666 (100 mM) (Sigma-Aldrich, USA) –in dimethyl sulfoxide.

For measuring of $[Ca^{2+}]_i$ fluorescent probe Fura-2AM (Sigma-Aldrich, USA) was used. Macrophages were incubated for 45 min in physiological solution containing 2 μM Fura-2AM, at room temperature. Glasses with stained cells were washed with physiological solution and transferred into the experimental chamber positioned on the table of a fluorescent microscope Leica DM 4000B (Leica Microsystems, Germany). For excitation of Fura-2AM fluorescence Leica DM 4000B lighting (with xenon lamp with 75 Wt wattage as the issue of light) was used. Excitation of object fluorescence was performed at wavelengths 340 and 380 nm via the microscope objective. For isolation of corresponding regions of the spectrum a narrow-band optical filter was used. Emission was registered at a wavelength of 510 nm with the aid of a photoelectronic multiplier (FEU 85) (PEM) inbuilt into the optical system of the microscope. The signal from PEM was amplified with a specially constructed amplifier. For digitization of the signal from PEM and control of the microscope functions we used a specialized microcontroller on the basis of ATMEGA 168 microprocessor connected to a computer via a USB interface. For managing the experiment original software was used. In experiments we applied a 10× objective with 8 mm aperture. To avoid photobleaching the measurements were conducted every 20 s, irradiating the object for 2 s. The values of $[Ca^{2+}]_i$ were calculated according to the Grynkiewicz equation [17]. Statistical analysis was conducted with the use of Student's *t*-test. Data are presented as $x \pm s_x$. In the figures we present the results of typical experiments.

For revealing and amplifying of the Ca^{2+} entry into cells we used a classical scheme of experiment (Ca^{2+}-free/Ca^{2+}-reintroduction protocol). Macrophages were incubated in nominally calcium-free medium, then glutoxim or molixan was applied, causing mobilization of Ca^{2+} from intracellular stores. After addition into the external medium of 2 mM Ca^{2+} and restoration of a physiological gradient of Ca^{2+} concentration we observed rapid elevation of $[Ca^{2+}]_i$, reflecting Ca^{2+} entry into cell. The Arp2/3-complex inhibitor was added 1 h before adding glutoxim or molixan.

RESULTS AND DISCUSSION

In control experiments it is shown that incubation of macrophages for 20 min with 100 μg/mL molixan (Fig. 1a) or 100 μg/mL glutoxim (Fig. 2a) in nominally calcium-free medium causes a slowly growing increase in $[Ca^{2+}]_i$, reflecting mobilization of Ca^{2+} from intracellular Ca^{2+} stores. On average (by data of six experiments for each of the drugs) in 20 min after adding agents $[Ca^{2+}]_i$ increases from a basal level of 82 ± 17 nM to 205 ± 18 nM for molixan and 249 ± 20 nM for glutoxim. Upon introduction into external medium of 2 mM Ca^{2+} we observe further elevation of $[Ca^{2+}]_i$, reflecting Ca^{2+} entry into cytosol (Figs. 1a, 2a). On average (by data of six experiments for each of the agents) the $[Ca^{2+}]_i$ increase during the phase of Ca^{2+} entry constitutes 315 ± 20 nM and 393 ± 18 nM for molixan and glutoxim respectively.

It is for the first time shown that preliminary incubation of macrophages with 100 μM CK-0944666 for 1 h before addition of 100 μg/mL molixan (Fig. 1b) or 100 μg/mL glutoxim (Fig. 2b) causes practically complete suppression of both phases of Ca^{2+} responses induced by these agents. On average (by data of seven experiments for each of the preparations) the suppression of Ca^{2+} mobilization from stores constitutes

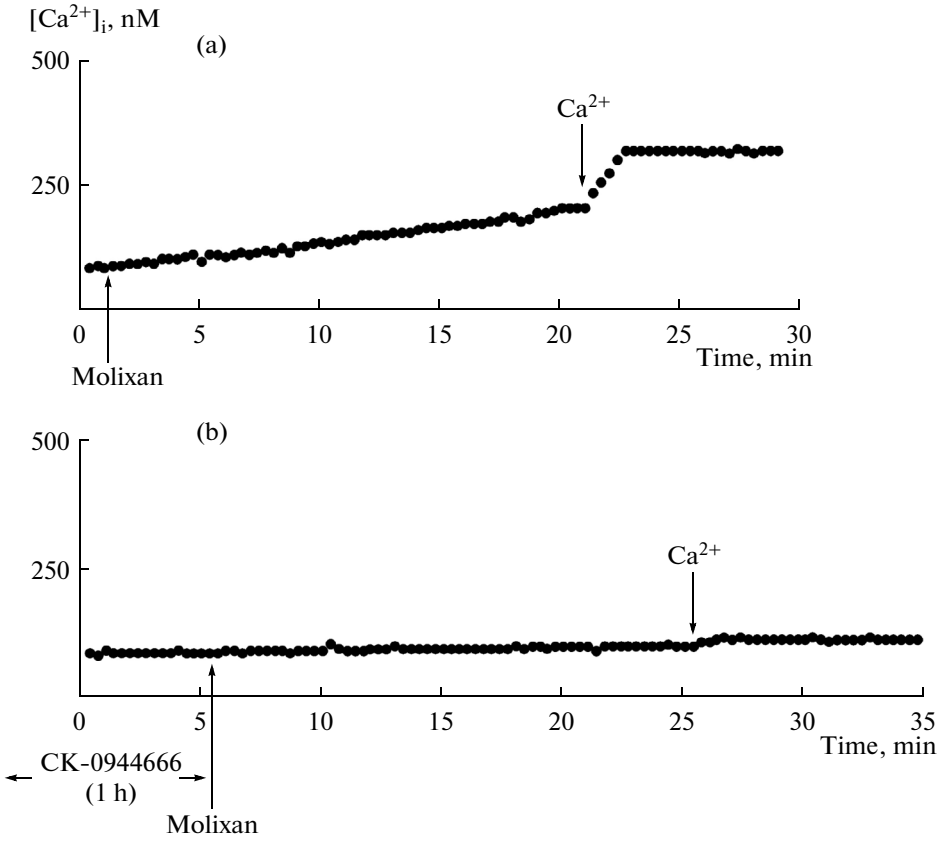

Fig. 1. Influence of compound CK-0944666 on the effect of molixan on $[Ca^{2+}]_i$ in macrophages. (a) Cells were incubated for 20 min in the presence of 100 μg/mL molixan in nominally calcium-free medium, then Ca^{2+} entry was initiated by introduction into external medium of 2 mM Ca^{2+}; (b) cells were preliminarily incubated for 1 h with 100 μM CK-094666 in calcium-free medium, then 100 μg/mL molixan was added, in 20 min Ca^{2+} entry was initiated by introduction into external medium of 2 mM Ca^{2+}. Here and in Fig. 2 each registration was obtained for a group of 40–50 cells and represents a typical variant of six–seven independent experiments.

88.9 ± 5.2% and 86.7 ± 5.0%, while of Ca^{2+} entry – 91.1 ± 6.7% and 89.3 ± 6.2% for molixan and glutoxim respectively.

In this way, we have for the first time revealed participation of Arp2/3 complex, one of the key regulators of actin polymerization, in the action of glutoxim or molixan on $[Ca^{2+}]_i$ in macrophages. It suggests that the Arp2/3 complex and the actin cytoskeleton appeares to be active participants of the processes of Ca^{2+} signaling in macrophages. Results are in agreement with data obtained on other immune cells. Thus it has been disclosed that in T lymphocytes upon activation of T-cell receptor a signaling cascade is triggered leading to $[Ca^{2+}]_i$ increase. In this cascade, Arp2/3 complex and actin cytoskeleton are engaged [18, 19].

The results of the present work are in agreement with our data on participation of the actin cytoskeleton in the action of glutoxim or molixan on $[Ca^{2+}]_i$ and data that glutoxim and molixan themselves cause reorganization of the actin cytoskeleton in macrophages [8]. In intact macrophages the elements of actin cytoskeleton are localized under the plasma membrane and form a distinctly distinguished cortical layer, while in cells treated with glutoxim or molixan, the cortical layer becomes broader and "looser" and there appeared clusters of actin in the cytosol [8]. Thus, reorganization of the actin cytoskeleton upon the action of glutoxim or molixan appears to be a necessary condition for signal transduction from plasmalemma to intracellular Ca^{2+} stores and development of Ca^{2+} mobilization from stores. Arp2/3 complex, as it is known, plays an important role in formation of a branching network of actin filaments [12]. Consequently, it may be supposed that upon action of glutoxim or molixan the Arp2/3 complex actively participates in reorganization of the actin cytoskeleton in macrophages. In its turn, inhibition of Arp2/3 complex upon action of compound CK-0944666 makes impossible the branching of microfilaments and prevents Ca^{2+} responses induced by glutoxim or molixan in macrophages. It suggests that formation of a branched network of microfilaments is necessary for development of a signaling cascade induced by glutoxim and molixan in macrophages and leading to an increase of intracellular Ca^{2+} concentration. The

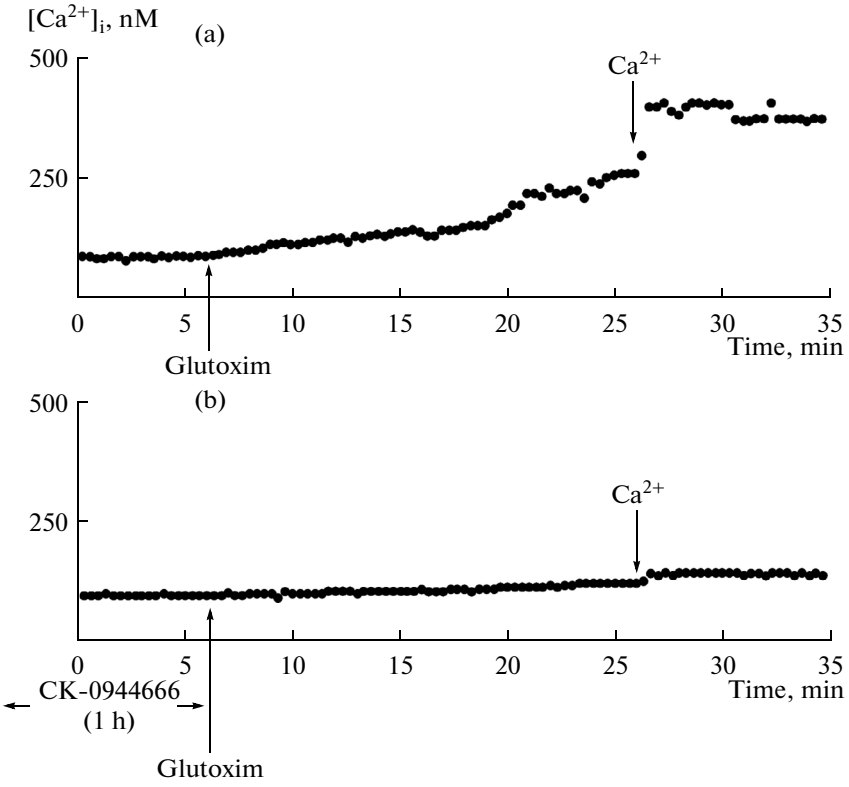

Fig. 2. Influence of compound CK-0944666 on the effect of glutoxim on $[Ca^{2+}]_i$ in macrophages. (a) Cells were incubated for 20 min in the presence of 100 μg/mL glutoxim in nominally calcium-free medium, then Ca^{2+} entry was initiated by introduction into external medium of 2 mM Ca^{2+}; (b) cells were preliminarily incubated for 1 h with 100 μM CK-094666 in calcium-free medium, then 100 μg/mL glutoxim was added, in 20 min Ca^{2+} entry was initiated by introduction into external medium of 2 mM Ca^{2+}.

results about suppression by compound CK0944666 of Ca^{2+} entry induced by glutoxim and molixan in macrophages agree with our data about participation of elements of cytoskeleton in regulation of Ca^{2+} entry evoked by glutoxim or molixan [8] and also purinergic agonists ATP and UTP and inhibitors of endoplasmic Ca^{2+}-ATPases thapsigargin and cyclopiazonic acid in rat peritoneal macrophages [20]. The glutoxim- or molixan-evoked Ca^{2+} entry into cell occurs by a store-dependent mechanism [3]. Earlier [20] in the experiments with the use of ATP, UTP, thapsigargin and cyclopiazonic acid we have shown that store-dependent Ca^{2+} entry in rat peritoneal macrophages occurs according to a store-dependent Ca^{2+} entry model named "secretion-like coupling," implying reversible translocation of the Ca^{2+} store to the plasmalemma, including active participation of actin filaments [21]. In this way, the data obtained in the present work about participation of Arp2/3 complex in regulation of store-dependent Ca^{2+} entry induced by glutoxim or molixan confirm the store-dependent Ca^{2+} entry model known as "secretion-like coupling" for rat peritoneal macrophages. The results about suppression by compound CK-0944666 of store-dependent Ca^{2+} entry induced by glutoxim and molixan correlate also with data obtained on T cells. Thus in T lymphocytes with defective protein WAVE2 activating the Arp2/3 complex one observes suppression of store-dependent Ca^{2+} entry activated by thapsigargin [22]. The above results, along with our previous findings [2–11] testify about that in the action of glutoxim or molixan on $[Ca^{2+}]_i$ in macrophages the same signal proteins and their complexes as in the process of exocytosis are involved: tyrosine kinases, G-proteins of small molecular mass, mechanisms of vesicular transport; actin and tubulin cytoskeleton, and also Arp2/3 complex mediating rearrangements of the actin cytoskeleton. Reorganization of the actin cytoskeleton induced in macrophages upon action of glutoxim or molixan may also mediate activation of macrophages and alleviate processes of endo- and exocytosis.

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