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BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR BIOLOGY

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

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Abstract—The Fura-2AM fluorescent Ca^{2+} probe was used to study the possibility that the Arp2/3 complex and WASP proteins are involved in the effects of glutoxim and molixan on the intracellular Ca^{2+} concentration in macrophages. It has been demonstrated that preincubation of macrophages with inhibitors of the Arp2/3 complex or WASP proteins (CK-0944666 or wiskostatin, respectively) results in a significant suppression of Ca^{2+}-responses induced by glutoxim or molixan. This suggests that polymerization of actin filaments is a process involved in the effect of glutoxim or molixan on intracellular Ca^{2+} concentration in macrophages.

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Pharmacological analogues of the oxidized glutathione (GSSG), the preparations glutoxim (G, disodium salt of GSSG with d-metal in nanoconcentration, PHARMA-VAM, Russia) and molixan (M, a complex of glutoxim and nucleoside inosine, PHARMA-VAM, Russia) belong to a group of thiopoietins, the drugs having an effect on regulation of redox processes in cells. G and M are used in clinical practice as immunomodulators and hemostimulating agents with wide-spectrum effects [1].

We have earlier demonstrated that GSSG, G, or M can increase intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, due to a release of Ca^{2+} from the thapsigargin-sensitive Ca^{2+} stores and subsequent store-dependent Ca^{2+}-entry in the peritoneal rat macrophages [2, 3]. Using a wide spectrum of agents that influence cellular signaling systems, we have demonstrated that tyrosine kinases, tyrosine phosphatases [2], phosphatidylinositol kinases [4], phospholipase C, and protein kinase C [5] are the key participants of the GSSG- and G-triggered cascade leading to an increase of $[Ca^{2+}]$ in macrophages. The actin cytoskeleton [6], microtubules [7], Ras proteins, and vesicular transport are also involved in the effect of $[G]_i$ and M on $[Ca^{2+}]$ in macrophages [8].

The Arp2/3 complex (Actin-Related Proteins) and the proteins of the WASP family (Wiskott–Aldrich Syndrome proteins) play an important role in formation of the novel actin filaments [9, 10]. WASP proteins are activated upon binding with GTPase Cdc42, and after that can interact with Arp2/3 proteins and actin monomers. When bound to WASP, the Arp2/3 complex is activated to trigger actin polymerization [10].

To study in detail the involvement of microfilaments and actin-binding proteins, as well as of vesicular transport and exocytosis in the G- and M-triggered signaling cascade, we have analyzed the role of Arp2/3 and WASP complexes in the effect of glutoxim and molixan on $[Ca^{2+}]_i$ in macrophages. The results are described in this report.

Cultures of resident peritoneal macrophages from Wistar rats were preincubated for 1–2 days at the room temperature of 20–22°C. The cultivation procedure and $[Ca^{2+}]_i$ measurement using a fluorescent microscope Leica DM 4000B (Leica Microsystems, Germany) have been described in detail earlier [6]. The fluorescent probe Fura-2AM (Sigma-Aldrich, United States) was used for measuring of $[Ca^{2+}]_i$. After excitation of fluorescence of the object at wavelengths of 340 and 380 nm, emission was recorded at 510 nm. To avoid photobleaching, measurements were made every 20 s after irradiation of the object for 2 s. The $[Ca^{2+}]_i$ values were calculated according to the Grynkiewicz equation [11].

The results were processed statistically using Student's test. Figures 1 and 2 show typical results.

To reveal involvement of the Arp2/3 complex in the effects of G and M on $[Ca^{2+}]_i$ in macrophages, CK-0944666 compound, an inhibitor of Arp2/3, was used in our experiments [12]. To reveal participation of WASP proteins, we used wiskostatin, an agent stabilizing inactive conformation of these proteins [13].

In control experiments, macrophage incubation for 20 min with 100 μg/mL M or G in Ca-free medium (Figs. 1a, 2a) resulted in a slow increase in $[Ca^{2+}]_i$ concentration to 123 ± 18 and 167 ± 20 nM for M and G,

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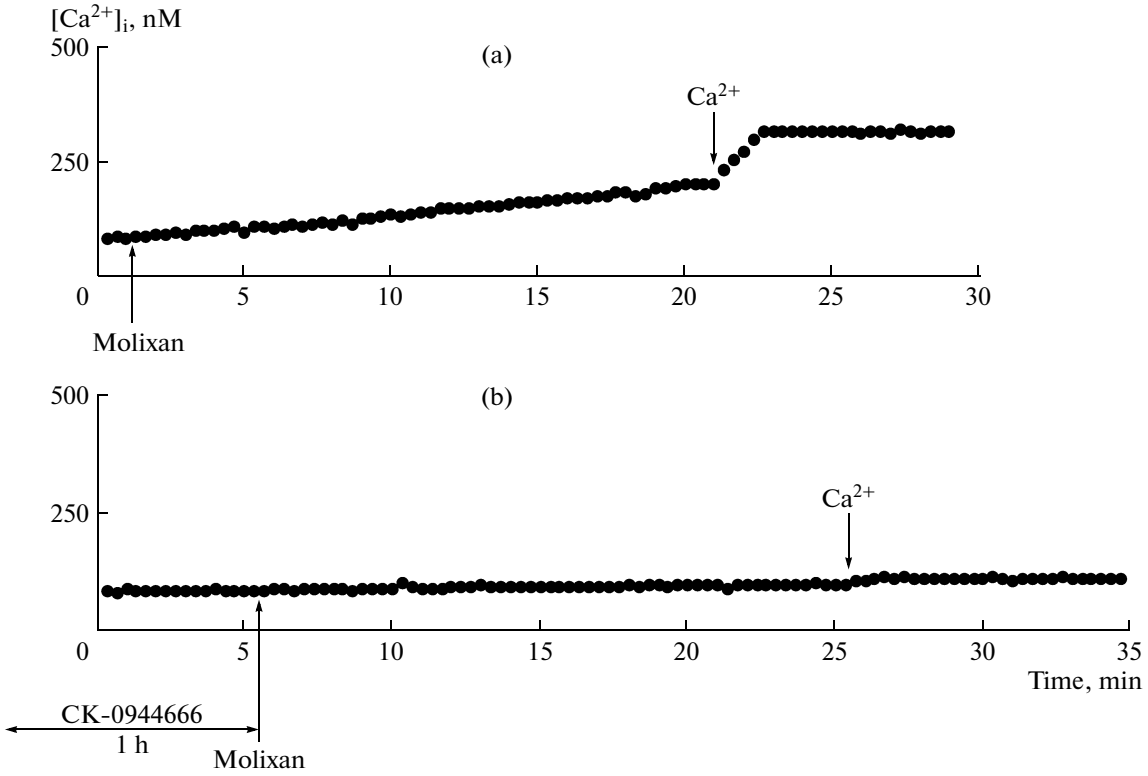

Fig. 1. The influence of CK-0944666 on the effect of molixan on $[Ca^{2+}]_i$ in macrophages. Y axis, $[Ca^{2+}]_i$; X axis, time. (a) The cells were incubated for 20 min in the presence of 100 μg/mL molixan in the nominally Ca-free medium and afterwards 2 mM Ca^{2+} was added into the external medium to induce Ca^{2+} entry into a cell; (b), the cells were incubated for an hour with 100 μM CK-0944666 in the Ca-free medium and 20 min after that, Ca^{2+}-entry was initiated by addition of Ca^{2+} into the external medium. Here and in Fig. 2, each registration was obtained for a group of 40–50 cells and represents a typical variant out of six-seven independent experiments.

respectively (the mean value for six experiments in each case). This process reflects Ca^{2+} mobilization from the intracellular Ca^{2+} stores. When 2 mM Ca^{2+} was added into the external medium of a culture, further increase in $[Ca^{2+}]_i$ reached 315 ± 20 and 393 ± 18 nM for M and G, respectively, (according to data of six experiments in each case), which reflects Ca^{2+} entry into cytosol (Figs. 1a, 2a).

Preincubation of macrophages for 1 h with 100 μM CK-0944666 before 100 μg/mL M administration (Fig. 1b) led to an almost complete inhibition (by 88.9 ± 5.2%) of both Ca^{2+} mobilization from the stores (seven experiments) and Ca^{2+} entry into the cytosol (by 91.1 ± 6.7%). The experiments with 100 μg/mL G yielded similar results (data not shown).

Preincubation of macrophages for 15 min with 30 μM wiskostatin before G administration (100 μg/mL) also resulted in a significant inhibition of both Ca^{2+} mobilization from the stores (by 91.5 ± 7.1%) and Ca^{2+} entry into cytosol by 71.2 ± 6.9% (Fig. 2b, seven experiments in each case). Administration of 40 μM wiskostatin when Ca^{2+} entry in response to glutoxim was already developed, led to a significant inhibition of Ca^{2+} entry (by 42.3 ± 5.7%, the mean value for six experiments, Fig. 2a). Similar results were obtained in experiments with 100 μg/mL M (data not shown).

Thus, we have demonstrated that Arp2/3 and WASP complexes are involved in the effects of G and M on $[Ca^{2+}]_i$ in macrophages.

These results are in accordance with our data on participation of the actin cytoskeleton in the effects of G and M on $[Ca^{2+}]_i$, as well as with our data suggesting that G and M directly induce rearrangement of the actin cytoskeleton in macrophages [6]. In intact macrophages, the actin cytoskeleton is localized under the plasmalemma and forms a clearly distinguished cortical layer. In cells treated with G or M, the cortical layer becomes broader and "looser," and actin accumulation is seen in the cytosol [6]. Thus, actin rearrangement in response to G or M action is probably necessary for signaling from plasmalemma to the intracellular Ca^{2+} stores to mobilize Ca^{2+} from the stores. Hence, Arp2/3 and WASP complexes are probably readily involved in actin rearrangement in response to the G and M. When Arp2/3 or WASP are inhibited by CK-0944666 or wiskostatin, microfilament branching is impossible and Ca^{2+} responses to G or M are prevented. This suggests that formation of a ramified framework of microfilaments is required to induce a

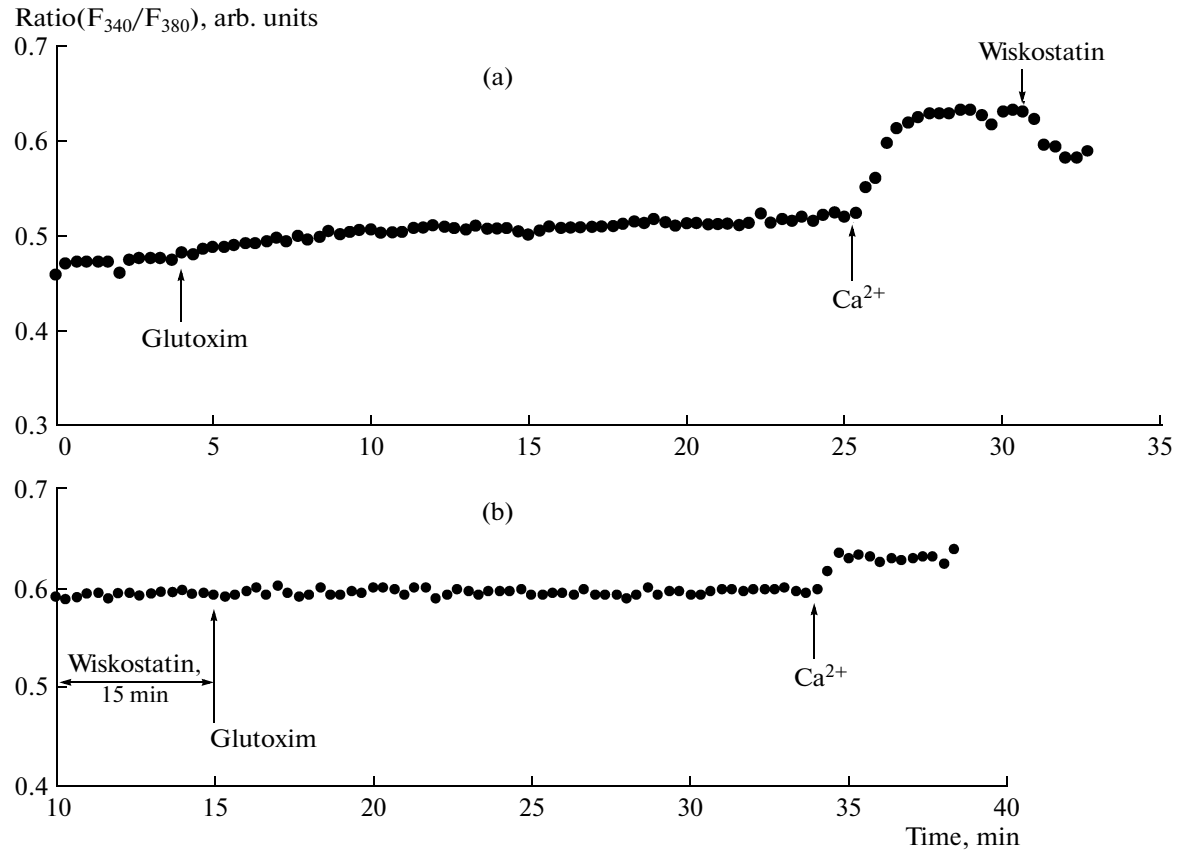

Fig. 2. The influence of wiskostatin on the effect of glutoxim on $[Ca^{2+}]_i$ in macrophages. Y axis, the ratio between intensity of fluorescence excited by a radiation of 340 nm wavelength and intensity of fluorescence excited by a radiation of 380 nm wavelength (Ratio(F_{340}/F_{380}) expressed in arbitrary units (arb. un.); X axis, time. (a) The cells were incubated for 20 min with 100 μg/mL glutoxim in the calcium-free medium; afterwards, 2 mM Ca^{2+} was added into the external medium to induce Ca^{2+} entry into a cell. Against the background of the developed Ca^{2+} entry into a cell, 40 μM wiskostatin was added; (b) the cells were incubated in calcium-free medium with 30 μM wiskostatin for 15 min before addition of 100 μg/mL glutoxim; 20 min after glutoxim administration, Ca^{2+} entry into a cell was initiated by addition of 2 mM Ca^{2+} into the external medium.

signaling cascade which is triggered by G or M in macrophages to increase $[Ca^{2+}]_i$.

The mechanism of Ca^{2+} entry into a cell in response to G or M is a store-dependent mechanism [2]. We have earlier demonstrated, using ATP, UTP, thapsigargin, and cyclopiazonic acid, that the store-dependent Ca^{2+} entry into the peritoneal macrophages occurs according to the "secretion-like coupling" model, which suggests a reversible translocation of Ca^{2+} stores towards plasmalemma with involvement of filaments [14, 15]. Thus, the data obtained in this study testify to participation of the Arp2/3 complex and WASP proteins in regulation of the store-dependent Ca^{2+} entry induced by G or M and, thereby, supports the Ca^{2+} entry via "secretion-like coupling" in the rat peritoneal macrophages.

Thus, according to this study and our earlier data [2–8], the signaling proteins and their complexes participating in exocytosis are also involved in the effects of M and G on $[Ca^{2+}]_i$ in macrophages. These are tyrosine kinases, small G-proteins, vesicular transport, actin and tubulin cytoskeleton, as well as the Arp2/3 complex and WASP proteins, which mediate rearrangements of the actin cytoskeleton. Reorganization of the actin cytoskeleton that occurs in macrophages under the influence of G and M, may mediate macrophage activation and facilitate the processes of endo- and exocytosis.

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