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CELL BIOLOGY

Involvement of Small G Proteins and Vesicle Traffic in the Glutoxim and Molixan Effects on the Intracellular Ca^{2+} Concentration in Macrophages

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Glutoxim (bisodium salt of oxidized glutathione (GSSG) containing nanoconcentrations of cis-platinum; PharmaVAM, Russia) is a pharmacological GSSG analogue used as an immunomodulating and hematopoesis-stimulating agent in the complex therapy of bacterial and viral infections, psoriasis, and in radio- and chemotherapy of malignant tumors [1]. Molixan (PharmaVAM) is a complex of glutoxim and inosine with an antiviral, immunomodulating, and hepatoprotective action used in the therapy of acute viral hepatitis B and C, mixed hepatitis, and liver cirrhosis [1]. Glutoxim and molixan belong to the pharmacological group of thiopoetines, which affect intracellular redox regulation. However, the cellular and molecular mechanisms of their action are insufficiently understood.

In our previous studies, it was first shown that GSSG, glutoxim, and molixan increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat peritoneal macrophages by mobilizing calcium ions from thapsigargin-sensitive Ca^{2+} stores and subsequently stimulating the Ca^{2+} uptake [2–4].

Using a wide range of agents affecting different components of intracellular signaling systems, we first identified the principal elements of the signal cascade triggered by GSSG and glutoxim and resulting in a $[Ca^{2+}]_i$ increase in macrophages, namely, tyrosine kinases and tyrosine phosphatases [3, 5], phosphatidylinositol kinases [6], and the key enzymes of the phosphoinositide signaling system, phospholipase C and protein kinase C [7]. It was also found that the effects of glutoxim and molixan on $[Ca^{2+}]_i$ in macrophages were mediated by actin cytoskeleton elements [8] and microtubules [9].

The involvement of microtubules and the actin cytoskeleton in the glutoxim and molixan action on $[Ca^{2+}]_i$ in macrophages invites the assumption that macrophage activation induced by these agents is mediated by vesicle traffic. It is known that intracellular trafficking of secretory vesicles depends on microtubules, which regulate the transport efficiency and organize the vesicle traffic by acting like cellular highways. Agents causing microtubule disintegration have been shown to inhibit secretion in different types of cells [10]. In addition, it was reported that glutoxim could induce vesicle exocytosis in macrophages containing *M. tuberculosis* [11]. Based on these data, we considered it worthwhile to investigate the possible involvement of vesicle transport and small G proteins, important components of the exocytosis signaling pathway, in mediating the glutoxim and molixan effects on the $[Ca^{2+}]_i$ level in macrophages.

Experiments were performed on a culture of residential peritoneal macrophages of Wistar rats at room temperature (20–22°C) 24–48 h after the beginning of cell culture. The procedures of macrophage culturing and the automated device for $[Ca^{2+}]_i$ measurements based on a Leica DM 4000B fluorescent microscope (Leica Microsystems, Germany) were previously described in detail [8]. The levels of $[Ca^{2+}]_i$ were measured using a Fura-2AM fluorescent probe (Sigma-Aldrich, United States). Fluorescence was induced at wavelengths of 340 and 380 nm, and emission was recorded at 510 nm. To avoid photobleaching, measurements were performed at 20 s intervals, using 2-s irradiation. $[Ca^{2+}]_i$ values were calculated using the Grynkiewicz equation [12]. Statistical analysis was performed using Student's *t*-test. Data were presented as $\bar{x} \pm sd$. Figures 1 and 2 show typical experimental results.

The involvement of small G proteins of the Ras superfamily in the effects of glutoxim and molixan on $[Ca^{2+}]_i$ was analyzed using a farnesylcysteine analogue, N-acetyl-S-farnesyl-L-cystein (AFC). AFC inhibits farnesylmethyl transferases and prevents methylation,

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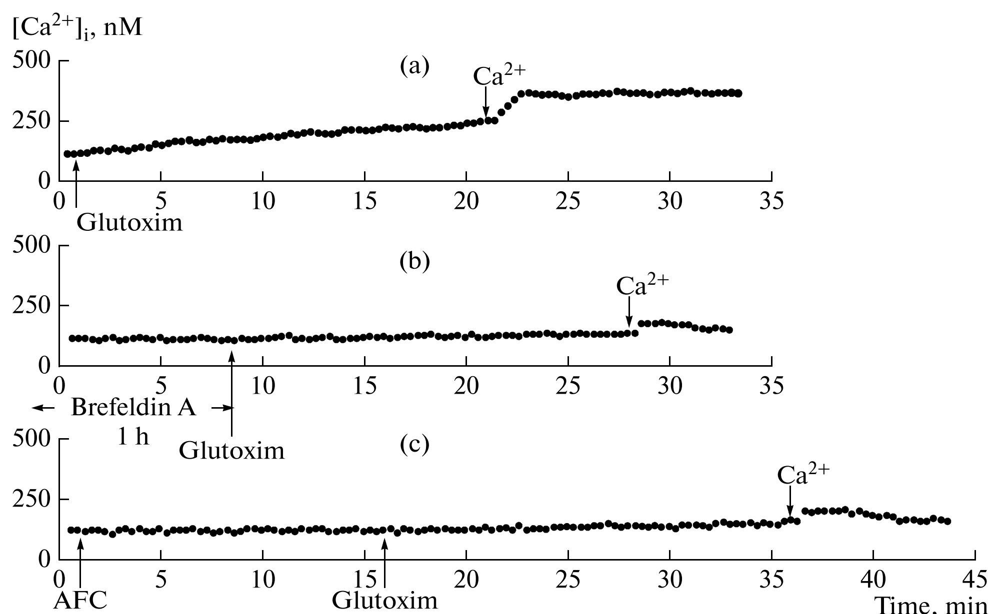

Fig. 1. Influence of brefeldin A and a farnesylcysteine analogue, N-acetyl-S-farnesyl-L-cysteine (AFC), on the glutoxim effect on $[Ca^{2+}]_i$ level in macrophages. (a) Cells were incubated for 20 min in a nominally calcium-free medium in the presence of 100 μg/mL glutoxim; next, Ca^{2+} uptake was activated by supplementing the culture medium with 2 mM Ca^{2+}; (b, c) cells were preincubated (b) with 100 μM brefeldin A for 1 h or (c) with 50 μM AFC for 15 min in a calcium-free medium; then, 100 μg/mL glutoxim was added and, within 20 min, Ca^{2+} uptake was activated by adding 2 mM Ca^{2+} to the medium. Each recording was obtained for a group of 40–50 cells and represents a typical result observed in six to ten independent experiments.

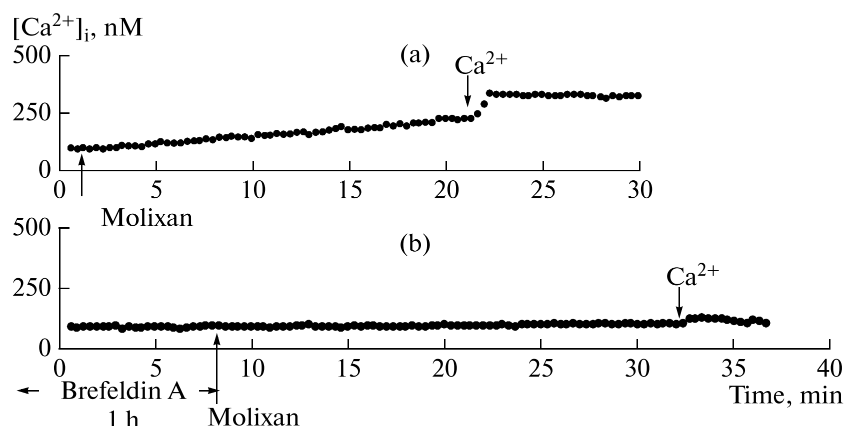

Fig. 2. Influence of brefeldin A on the molixan effect on the $[Ca^{2+}]_i$ level in macrophages. (a) Cells were incubated for 20 min in a calcium-free medium in the presence of 100 μg/mL molixan; then, Ca^{2+} uptake was activated by supplementing the culture medium with 2 mM Ca^{2+}; (b) cells were preincubated with 100 μM brefeldin A for 1 h in a calcium-free medium; then, 100 μg/mL molixan was added and, within 25 min, Ca^{2+} uptake was activated by adding 2 mM Ca^{2+} to the medium. Each recording was obtained for a group of 40–50 cells and represents a typical result observed in six to ten independent experiments.

membrane binding, and activation of Ras proteins [13]. The role of vesicle transport in the glutoxim or molixan effects on $[Ca^{2+}]_i$ was studied using brefeldin A, an inhibitor of vesicle transport. Brefeldin A inactivates small G proteins of the Arf subfamily that are central to the regulation of vesicle transport [14]. GDP complexes of Arf proteins are located in the cytosol, while their GTP-associated forms are strongly bound to the cellular membrane via their N-terminal domains. Activated Arf proteins capture vesicle coat proteins and induce vesicle formation. Thus, activation of Arf proteins is an important mechanism of activating vesicle transport [14].

Control experiments showed that the $[Ca^{2+}]_i$ levels in macrophages incubated for 20 min with 100 μg/mL glutoxim (Fig. 1a) or 100 μg/mL molixan (Fig. 2a) in nominally calcium-free medium were slowly increasing due to Ca^{2+} mobilization from intracellular Ca^{2+} stores. Based on six experiments for either agent, it was found that a 20-min incubation with either drug

resulted in a $[Ca^{2+}]_i$ increase from the basal level of 85 ± 18 nM to 219 ± 20 nM for glutoxim and to 217 ± 19 nM for molixan. In the medium supplemented with 2 mM Ca^{2+}, the levels of $[Ca^{2+}]_i$ were observed to increase further, reflecting the Ca^{2+} uptake in the cytosol (Figs. 1a, 2a). During the Ca^{2+} uptake phase, the $[Ca^{2+}]_i$ levels increased to 327 ± 19 and to 325 ± 17 nM for glutoxim and molixan, respectively (data from six experiment for either agent).

This study was the first to demonstrate that a preliminary 1-h incubation of macrophages with 100 μM brefeldin A before adding 100 μg/mL glutoxim (Fig. 1b) or 100 μg/mL molixan (Fig. 2b) resulted in an efficient suppression of both phases of the drug-induced Ca^{2+} response. Based on the data from seven experiments for either agent, mobilization of deposited Ca^{2+} was reduced by 87.1 ± 7.0% and 91.0 ± 6.5%, and Ca^{2+} uptake, by 69.1 ± 6.2% and 65.3 ± 5.2% for glutoxim and molixan, respectively.

Similarly, it was found that a 15-min preincubation with 50 μM AFC prior to adding 100 μg/mL glutoxim or 100 μg/mL molixan suppressed considerably the Ca^{2+} mobilization from cell stores, as well as Ca^{2+} uptake induced by glutoxim (Fig. 1c) or molixan (data not shown). According to the data from eight experiments for either drug, Ca^{2+} mobilization from cell stores was reduced by 71.0 ± 5.1% and 76.2 ± 6.3%, and Ca^{2+} uptake, by 65.0 ± 3.0% and 62.1 ± 4.0%, for glutoxim and molixan, respectively.

These results suggest that the Ca^{2+} responses induced by glutoxim and molixan in macrophages depend critically on small G proteins of the Ras superfamily, as well as on vesicle traffic.

The above results, along with our previous findings [2–9], also indicate that the action of glutoxim and molixan on $[Ca^{2+}]_i$ levels in macrophages involves the same signal proteins and complexes that participate in exocytosis: tyrosine kinases and tyrosine phosphatases, phosphatidylinositol-3- and -4 kinases, protein kinase C, small G proteins of the Ras superfamily, vesicle trafficking, and actin and tubulin cytoskeleton [15]. In addition, we have shown that glutoxim and molixan themselves induce reorganization of the actin cytoskeleton [8], which mediates macrophage activation and facilitates endo- and exocytosis.

Presumably, glutoxim and molixan not only cause an increase in $[Ca^{2+}]_i$ levels, but can also stimulate Ca^{2+}-dependent exocytosis in macrophages. The signal cascades triggered by glutoxim and molixan and inducing $[Ca^{2+}]_i$ increase and exocytosis are apparently in a close interaction and crosstalk with each other.

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