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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Methyl-β-cyclodextrin Inhibits Ca^{2+}-Responses Induced by Glutoxim and Molixan in Macrophages

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Abstract—Using Fura-2AM microfluorimetry, we have shown for the first time that methyl-β-cyclodextrin, inducing cholesterol extraction from membranes and raft disruption, significantly inhibits glutoxim- and molixan-induced Ca^{2+}-responses in rat peritoneal macrophages. The results suggest that intact rafts are necessary for signaling cascade induced by glutoxim or molixan and leading to intracellular Ca^{2+} concentration increase in macrophages.

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Glutoxim® (G, disodium salt of oxidized glutathione (GSSG) with d-metal at a nanoconcentration; PHARMA-VAM, Russia) and molixan® (M, complex of glutoxim with nucleoside inosine, PHARMA-VAM) belong to the group of thiopoietins, substances affecting the redox-regulation processes in cells. For example, G is used as an immunomodulator and hemostimulant in therapy of bacterial and viral diseases, psoriasis, as well as radiation and chemotherapy in oncology [1]. Drug M has an antiviral, immunomodulatory, and hepatoprotective effect and is used in therapy of acute and viral hepatitis B and C, mixed-hepatitis, and cirrhosis of the liver [1]. However, the cellular and molecular mechanisms of action of these drugs are far from fully understood.

Earlier [2, 3], we discovered that G and M increased the intracellular concentration of Ca^{2+}, $[Ca^{2+}]_i$, causing Ca^{2+} mobilization from thapsigargin-sensitive Ca^{2+}-stores and subsequent store-dependent entry of Ca^{2+} into rat peritoneal macrophages. In addition, we have shown that the signaling cascade triggered by G and M and leading to an increase in $[Ca^{2+}]_i$ in macrophages involves tyrosine kinases and tyrosine phosphatases, phosphatidylinositol kinases, enzymes of the phosphoinositide signaling pathway phospholipase C and protein kinase C, heterotrimeric and small G proteins, enzymes and/or products of the arachidonic acid cascade, as well as elements of the actin cytoskeleton, microtubules, and vesicular transport components [4].

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It is known that the key proteins involved in intracellular signaling, including the Ca^{2+} signaling, are located in specialized lipid microdomains—rafts. Rafts are ordered liquid membrane domains rich in cholesterol and sphingolipids [5].

In view of above, the aim of this study was to investigate the possible involvement of rafts in the effect of G and M on $[Ca^{2+}]_i$ in macrophages.

One of the main approaches to identify the role of rafts in the intracellular signaling is to reduce the membrane cholesterol level, i.e., to perform experiments under conditions of destruction of rafts or disturbance of their integrity. Previously, [6], it was found that partial extraction of cholesterol leads to dissociation of the majority of membrane proteins associated with rafts. Cholesterol is usually extracted with cyclodextrins—cyclic oligosaccharides composed of glucose units linked together through glycosidic bonds. The hydroxyl groups of cyclodextrins are located on the outer surface of the molecule, whereas their internal cavity is hydrophobic. The most effective and commonly used cholesterol acceptor is methyl-β-cyclodextrin (MBCD). Numerous data show that incubation of cells with MBCD leads to the extraction of cholesterol from model and cellular membranes. The incubation of cells with MBCD at a high concentration (5–10 mM) for 1 h can reduce the content of cholesterol by 80–90%. For example, the incubation of THP-1 human macrophages with 10 mM MBCD for 60 min resulted in a 85% extraction of cholesterol from rafts [6, 7].

Experiments were performed on cultured resident peritoneal macrophages of Wistar rats at room temperature (20–22°C) 1–2 days after the beginning of cell culturing. The macrophage cultivation procedure and the description of the automated system for mea-

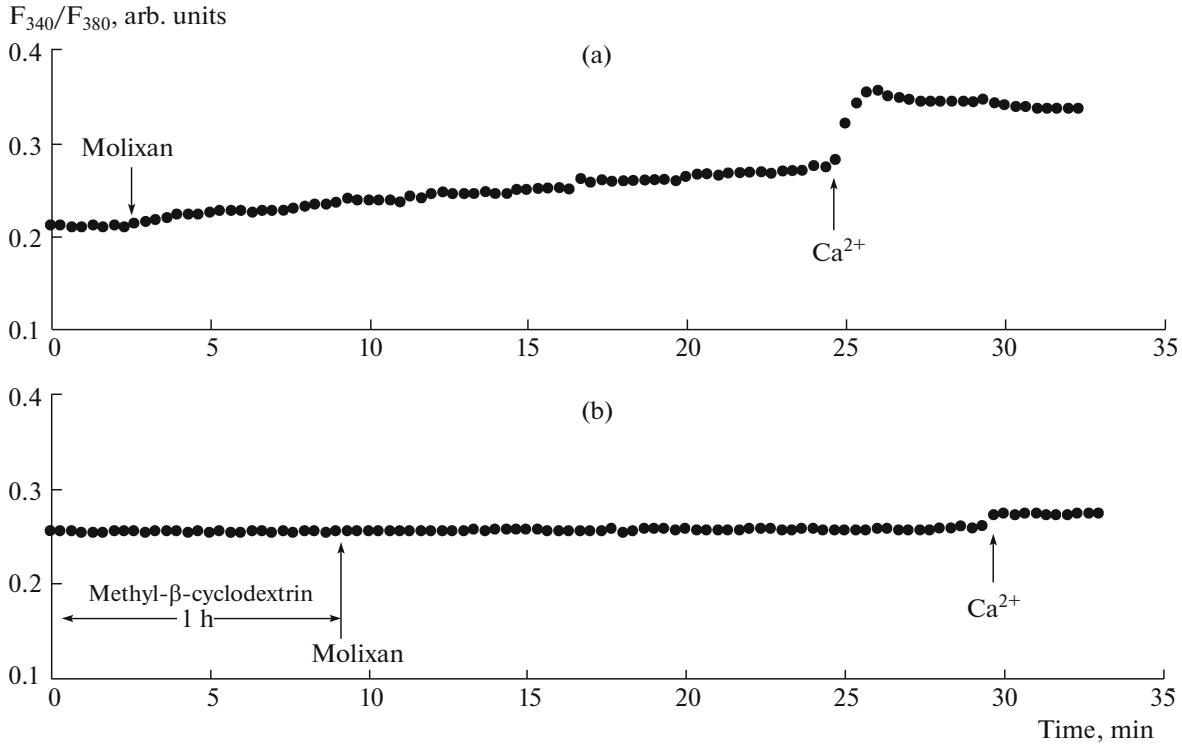

Fig. 1. Effect of methyl-β-cyclodextrin on the molixan-induced increase in $[Ca^{2+}]_i$ in rat macrophages. Here and in Fig. 2, the ordinate axis shows the ratio of Fura-2AM fluorescence intensities F_{340}/F_{380} at excitation wavelengths 340 and 380 nm, respectively (arb. units). The abscissa axis shows time. (a) Cells were incubated with 100 μg/mL molixan for 21 min in a nominally calcium-free medium, after which Ca^{2+} entry was initiated by the addition of 2 mM Ca^{2+} to the external medium. (b) Macrophages were preincubated for 1 h with 10 mM methyl-β-cyclodextrin in a calcium-free medium, followed by the addition of 100 μg/mL molixan and incubation for another 20 min, after which Ca^{2+} entry was initiated by the addition of 2 mM Ca^{2+} to the external medium. Each record was obtained for a group of 40–50 cells and is a typical variant of six or seven independent experiments.

suring $[Ca^{2+}]_i$ on the basis of a Leica DM 4000B fluorescent microscope (Leica Microsystems, Germany) were published earlier [8]. $[Ca^{2+}]_i$ was measured using the fluorescent probe Fura-2AM (Sigma-Aldrich, United States). Fluorescence of the object was excited at wavelengths 340 and 380 nm, and emission was detected at 510 nm. To prevent photobleaching, measurements were performed every 20 s, irradiating the object for 2 s. $[Ca^{2+}]_i$ was calculated using the Grynkiewicz equation [9]. Statistical analysis was performed using Student's *t* test. The figures show the results of typical experiments. Data are represented as plots showing the changes in the ratio of Fura-2AM fluorescence intensities at excitation wavelengths 340 and 380 nm (F_{340}/F_{380} ratio) over time, reflecting the dynamics of changes in $[Ca^{2+}]_i$ in cells depending on the measurement time [10].

The control experiments showed that the incubation of macrophages for 20 min with 100 μg/(mL M) (Fig. 1a) or 100 μg/(mL G) (Fig. 2a) in a calcium-free medium caused a slowly developing increase in $[Ca^{2+}]_i$, reflecting the mobilization of Ca^{2+} from the intracellular stores. On average (according to the results of six experiments for each drug), 20 min after the addition of these agents, $[Ca^{2+}]_i$ increased from the basal level (91 ± 17 nM) to 139 ± 19 nM for M and 141 ± 17 nM for G. The addition of 2 mM Ca^{2+} to the external medium caused a further increase in $[Ca^{2+}]_i$, reflecting the Ca^{2+} entry to the cytosol (Figs. 1a, 2a). On average (according to the results of six experiments for each drug), the increase in $[Ca^{2+}]_i$ during the entry of Ca^{2+} was 203 ± 18 and 209 ± 19 nM for M and G, respectively.

Our experiments demonstrated that the preincubation of macrophages with 10 mM MBCD for 1 h before the addition of 100 μg/(mL M) led to a nearly complete suppression of both Ca^{2+} mobilization from the stores (on average, by 77.6 ± 9.2% according to the results of seven experiments) and subsequent Ca^{2+} entry into the cell (on average, by 82.3 ± 10.5% according to the results of seven experiments), induced by M (Fig. 1b). Similar results were obtained in the experiments on the effect of 10 mM MBCD on the Ca^{2+} responses induced by 100 μg/(mL G) (Fig. 2b). On average, according to the results of seven experi-

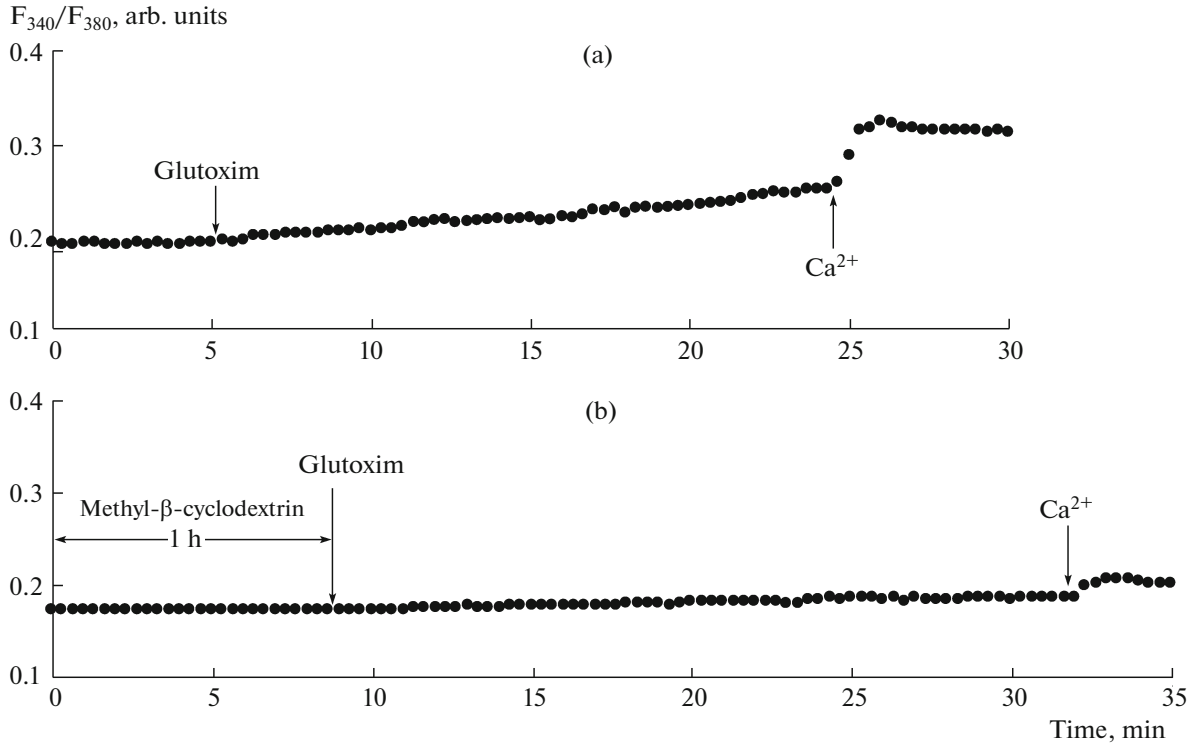

Fig. 2. Effect of methyl-β-cyclodextrin on the glutoxim-induced increase in $[Ca^{2+}]_i$ in rat macrophages. (a) Cells were incubated with 100 μg/mL glutoxim for 20 min in a nominally calcium-free medium, after which Ca^{2+} entry was initiated by the addition of 2 mM Ca^{2+} to the external medium. (b) Macrophages were preincubated for 1 h with 10 mM methyl-β-cyclodextrin in a calcium-free medium, followed by the addition of 100 μg/mL glutoxim and incubation for another 23 min, after which Ca^{2+} entry was initiated by the addition of 2 mM Ca^{2+} to the external medium.

ments, MBCD suppressed Ca^{2+} mobilization from the stores by 72.2% and Ca^{2+} entry into the cell by 69.9%, induced by G.

Thus, we have shown for the first time that MBCD inhibits both phases of Ca^{2+} response induced by G and M in macrophages. The results indicate that intact rafts are required for the development of a complex signaling cascade induced by G or M and leading to an increase in $[Ca^{2+}]_i$ in macrophages.

Data on the ability of MBCD to suppress the store-dependent Ca^{2+} entry induced by G or M indicate the involvement of rafts in the activation of store-dependent Ca^{2+} entry into macrophages. This is consistent with the fact that the preincubation with MBCD modulates the activation of the store-dependent Ca^{2+} entry induced by thapsigargin in human platelets [11], HEK293 human embryonic kidney cells [12, 13], and HSG human salivary gland cells [12].

REFERENCES

1. Borisov, A.E., Kozhemyakin, L.A., Antushevich, A.E., et al., *Vestnik Khirurgii im. I.I. Grekova*, 2001, vol. 4, pp. 32–38.
2. Kurilova, L.S., Krutetskaya, Z.I., Lebedev, O.E., et al., *Cell Tissue Biol.*, 2008, vol. 2, pp. 322–332.
3. Kurilova, L.S., Krutetskaya, Z.I., Lebedev, O.E., et al., *Tsitologiya*, 2011, vol. 53, no. 9, p. 708.
4. Krutetskaya, Z.I., Milenina, L.S., Melnitskaya, A.V., et al., *Redox Modulation of Ca^{2+} and Na^{+} Transport in Nonexcitable Cells*, St. Petersburg: St. Petersburg State Polytech. Univ. Publ. House, 2014, 171 p.
5. Pani, B. and Singh, B.B., *Cell Calcium*, 2009, vol. 45, pp. 625–633.
6. Zidovetzki, R. and Levitan, I., *Biochim. Biophys. Acta*, 2007, vol. 1768, pp. 1311–1324.
7. Gaus, K., Rodriguez, M., Ruberu, K.R., et al., *J. Lipid Res.*, 2005, vol. 46, pp. 1526–1538.
8. Milenina, L.S., Krutetskaya, Z.I., Naumova, A.A., et al., *Tsitologiya,* 2015, vol. 57, no. 7, pp. 518–525.
9. Grynkiewicz, G., Poenie, M., and Tsien, R.Y., *J. Biol. Chem.*, 1985, vol. 260, pp. 3440–3450.
10. Xie, Q., Zhang, Y., Zhai, C., et al., *J. Biol. Chem.*, 2002, vol. 277, pp. 16559 – 16566.
11. Dionisio, N., Galan, C., Jardin, I., et al., *Biochim. Biophys. Acta*, 2011, vol. 1813, pp. 431–437.
12. Pani, B., Ong, H.L., Liu, X., et al., *J. Biol. Chem.*, 2008, vol. 283, pp. 17333–17340.
13. Galan, C., Woodard, G.E., Dionisio, N., et al., *Biochim. Biophys. Acta*, 2010, vol. 1803, pp. 1083–1093.

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