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BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR BIOLOGY

Phospholipase A_2 Inhibitors Modulate the Effects of Glutoxim and Molixan on the Intracellular Ca^{2+} Level in Macrophages

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Abstract—Using the fluorescent Ca^{2+} probe Fura-2AM, the possible involvement of phospholipase A_2, the key enzyme in the arachidonic acid cascade, in the effect of drugs glutoxim and molixan on the intracellular Ca^{2+} concentration in macrophages was studied. It was shown for the first time that preincubation of macrophages with the classical phospholipase A_2 inhibitor, 4-bromophenacyl bromide, as well as with glucocorticosteroids prednisolone and dexamethasone significantly inhibits Ca^{2+} responses induced by glutoxim or molixan in macrophages. These results indicate the involvement of phospholipase A_2 and arachidonic acid cascade in glutoxim- and molixan-induced signaling in macrophages.

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Today, a large number of disulfide-containing drugs that alter the redox balance of the cell and have a physiologically significant effect on it have been developed and introduced into clinical practice. For example, the drug glutoxim® (a complex of disodium salt of oxidized glutathione (GSSG) with d-metal at nanoconcentration, PHARMA-VAM, Russia) is used as an immunomodulator and hemostimulator in complex therapy of bacterial and viral diseases, psoriasis, and radio- and chemotherapy in oncology [1, 2]. Another drug, molixan® (a complex of glutoxim and nucleoside inosine, PHARMA-VAM) exhibits antiviral, immunomodulatory, and hepatoprotective activity and is used in therapy of acute and viral hepatitis B and C, mixed hepatitis, and liver cirrhosis [1]. However, the cellular and molecular mechanisms of action of these drugs are not understood completely.

Earlier [3, 4], we have shown for the first time that GSSG, glutoxim, or molixan induce biphasic Ca^{2+} response: a gradual increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), reflecting the mobilization of Ca^{2+} from thapsigargin-sensitive Ca^{2+} stores, and subsequent store-dependent Ca^{2+} entry into rat peritoneal macrophages.

Macrophages respond to agonists by rapidly hydrolysing membrane phospholipids, which leads to the generation of a large number of intracellular and extracellular messengers [5]. For example, activated phagocytes produce a large amount of arachidonic acid (AA), which is released from membrane phospholipids as a result of hydrolysis by phospholipase A_2 (PLA_2) [6]. Free AA is readily oxidized to form biologically active eicosanoids: prostaglandins, thromboxanes, leukotrienes, and hydroxy acids [7]. There are three main pathways of enzymatic oxidation of AA: with the involvement of cyclooxygenases, lipoxygenases, and CYP2J2 epoxygenases [7].

The enzymes involved in AA metabolism are highly sensitive to oxidizing and reducing agents [8]. Using cyclooxygenase and lipoxygenase inhibitors, we have earlier shown for the first time that the cyclooxygenase and lipoxygenase pathways of AA oxidation are involved in the effect of glutoxim and molixan on $[Ca^{2+}]_i$ in macrophages [9, 10]. Since the triggering of the AA cascade is one of the key events in the activation of macrophages, it seemed appropriate to investigate the possible involvement of PLA_2 in the effect of glutoxim and molixan on $[Ca^{2+}]_i$ in macrophages. This was the subject of this study.

Experiments were performed on cultured resident peritoneal macrophages derived from Wistar rats at room temperature (20–22°C) 1–2 days after the beginning of culturing. The procedures of macrophage culturing and the automated system for measuring $[Ca^{2+}]_i$, based on a Leica DM 4000B fluorescence microscope (Leica Microsystems, Germany), was described in detail in [11]. $[Ca^{2+}]_i$ was measured using the fluorescent probe Fura-2AM (Sigma-Aldrich, United States). Fluorescence was excited at wavelengths of 340 and 380 nm, and emission was detected at 510 nm. To prevent photobleaching, measurements were performed every 20 s by irradiating the object for 2 s. The $[Ca^{2+}]_i$ values were calculated using Gryn-

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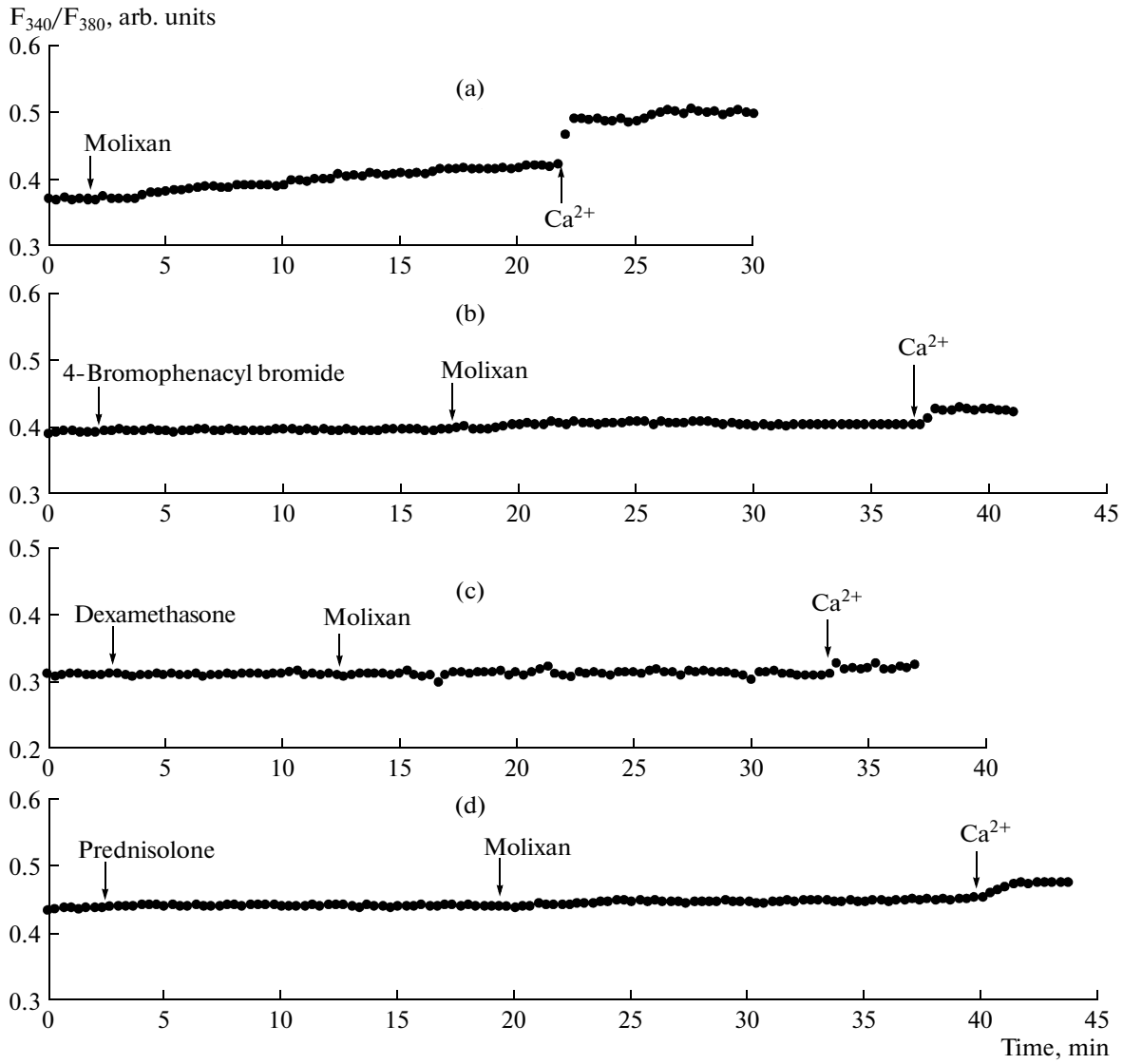

Fig. 1. Effect of phospholipase A_2 inhibitors on the molixan-induced increase in $[Ca^{2+}]_i$ in rat peritoneal macrophages. The ordinate axis shows the ratio of Fura-2AM fluorescence intensities F_{340}/F_{380} at excitation wavelengths of 340 and 380 nm, respectively (arb. units). The abscissa axis shows time. (a) Cells were incubated for 20 min in the presence of 100 μg/mL molixan in a nominally calcium-free medium; then, Ca^{2+} entry was induced by the addition of 2 mM Ca^{2+} to the incubation medium. (b–d) Cells were preincubated with (b) 20 μM 4-bromophenacyl bromide for 15 min, (c) 8 μg/mL dexamethasone for 10 min, or (d) 25 μg/mL prednisolone for 16 min in a calcium-free medium, after which 100 μg/mL molixan was added; 20 min after the addition of molixan, Ca^{2+} entry was induced by adding 2 mM Ca^{2+} to the incubation medium. Each recording was obtained for a group of 40–50 cells and is a typical variant of six or seven independent experiments.

kiewicz equation [12]. Statistical analysis was performed using the Student's *t* test.

Figure 1 shows the results of typical experiments. Data are represented as the time dependence of the F_{340}/F_{380} index (the ratio of the fluorescence intensity of Fura-2AM at excitation wavelengths of 340 nm and 380 nm, which reflects the dynamics of changes in $[Ca^{2+}]_i$ in cells [13]).

To detect the involvement of PLA_2 in the effect of glutoxim and molixan on $[Ca^{2+}]_i$ in macrophages, three PLA_2 inhibitors were used: 4-bromophenacyl bromide [14] and synthetic glucocorticosteroid drugs dexamethasone and prednisolone [15].

In control experiments it was shown that the incubation of macrophages for 20 min with 100 μg/mL molixan (Fig. 1a) or 100 μg/mL glutoxim (data not shown) in a calcium-free medium caused a slowly growing increase in $[Ca^{2+}]_i$, reflecting the mobilization of Ca^{2+} from the intracellular stores. According to the results of six experiments performed for each drug, 20 min after the addition of the agents, $[Ca^{2+}]_i$ increased from the basal level (91 ± 16 nM) to, on average, 153 ± 19 nM for molixan and 161 ± 20 nM for glutoxim. The

addition of 2 mM Ca^{2+} to the incubation medium was accompanied by further increase in $[Ca^{2+}]_i$, reflecting the entry of Ca^{2+} into the cytosol (Fig. 1a). 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 255 ± 21 and 259 ± 18 nM for molixan and glutoxim, respectively.

In our experiments, it was shown for the first time that preincubation of cells with 20 μM 4-bromophenacyl bromide for 15 min before the addition of 100 μg/mL molixan inhibited both the mobilization of Ca^{2+} from the stores (on average, by 71.7 ± 4.7%, according to the results of seven experiments) and subsequent entry of Ca^{2+} into the cell (on average, by 72.6 ± 5.1%, according to the results of seven experiments), induced by molixan (Fig. 1b). Similar data were obtained in the experiments on the effect of 4-bromophenacyl bromide on Ca^{2+} responses induced by 100 μg/mL glutoxim (data not shown).

In addition, we have also shown for the first time that the preincubation of cells with 8 μg/mL dexamethasone for 10 min before the addition of 100 μg/mL molixan almost completely inhibited the molixan-induced Ca^{2+} mobilization from the stores (on average, by 85.4 ± 5.9%, according to the results of seven experiments) and subsequent Ca^{2+} entry into the cytosol (on average, by 89.6 ± 6.2%, according to the results of seven experiments; Fig. 1c). The preincubation of macrophages with 25 μg/mL prednisolone for 16 min before the addition of 100 μg/mL molixan also inhibited the molixan-induced Ca^{2+} mobilization from the stores (on average, by 73.8 ± 4.8%, according to the results of seven experiments) and Ca^{2+} entry (on average, by 71.4 ± 5.3%, according to the results of seven experiments) (Fig. 1d). Similar data were obtained for 100 μg/mL glutoxim (data not shown).

Thus, we have shown for the first time that PLA_2 inhibitors cause a significant inhibition of both phases of Ca^{2+} response induced by glutoxim or molixan in macrophages. The results of this study testify to the involvement of PLA_2 and AA cascade in the effect of glutoxim and molixan on $[Ca^{2+}]_i$ in these cells. The results also indicate the inadvisability of a combined use of glutoxim or molixan and steroidal antiinflammatory drugs based on dexamethasone and prednisolone.

The data obtained by us earlier [3, 4, 9–11] and the results of this study indicate that glutoxim and molixan, unable to penetrate the plasma membrane in macrophages, induce a signaling cascade in macrophages, which leads to an increase in $[Ca^{2+}]_i$ and activation of macrophages, one of the key events in which is the triggering of AA cascade.

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