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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

5-Lipoxygenase Inhibitor Zileuton 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 5-lipoxygenase specific inhibitor antiasthmatic agent zileuton significantly inhibits Ca^{2+}-responses induced by glutoxim and molixan in macrophages. The results support 5-lipoxygenase involvement in the effect of glutoxim and molixan on intracellular Ca^{2+} concentration in macrophages and indicate the inadvisability of a combined use of drugs glutoxim and molixan and antiasthmatic agent zileuton.

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Disulfide-containing drugs 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) are used as broad-spectrum immunomodulators and hemostimulants in complex therapy of bacterial and viral diseases and psoriasis as well as in radiation and chemotherapy in oncology [1].

Previously we have first discovered that G and M increase the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), causing Ca^{2+} mobilization from the thapsigargin-sensitive Ca^{2+} stores and subsequent store-dependent Ca^{2+} entry into rat peritoneal macrophages [2, 3]. In addition, we have previously shown that the complex signaling cascade triggered by G or M in macrophages involves phospholipase A2 (the key enzyme of arachidonic acid (AA) metabolism) [4] as well as the enzymes and/or products of the cyclooxygenase and lipoxygenase [5] and epoxygenase [6] pathways of AA oxidation.

The enzymes involved in the AA metabolism are the targets of a wide range of natural and synthetic pharmaceuticals. Such compounds are an important tool to study the role of AA itself and its enzymatic oxidation products in intracellular signaling processes. In addition, many pharmaceuticals that inhibit AA metabolism are currently widely used in medical practice for treatment of many inflammatory, allergic, and infectious diseases [7]. For example, it is long known that the products of the 5-lipoxygenase pathway of AA oxidation (leukotrienes) play an important role in the pathogenesis of asthma [8]. The first specific 5-lipoxygenase inhibitor used for chronic asthma therapy was the drug zileuton (*N*-[1-(1-benzothien-2-yl)ethyl]-*N*-hydroxyurea, Zyflo®) [9]. Zileuton reduces the formation of sulfodipeptide leukotrienes and leukotriene B_4, has a bronchodilator effect, and prevents the development of bronchospasms caused by cold air and aspirin [10]. In addition, there is evidence of the effectiveness of this antileukotriene drug in the treatment of acne [11].

Since the triggering of the lipoxygenase pathway of AA oxidation plays an important role in macrophage activation, it seemed relevant to study the effect of the specific 5-lipoxygenase inhibitor zileuton on the Ca^{2+}-response induced by G and M in macrophages. This was the subject of this paper.

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 automated device for measuring $[Ca^{2+}]_i$ on the basis of Leica DM 4000B fluorescent microscope (Leica Microsystems, Germany) were described in detail previously [6]. To measure $[Ca^{2+}]_i$ we used the fluorescent probe Fura-2AM (Sigma-Aldrich, United States). Fluorescence of the object was exited at wavelengths of 340 and 380 nm, and emission was detected at 510 nm. To prevent photobleaching, measurements were performed every 20 s, irradiating the objects for 2 s. The $[Ca^{2+}]_i$ values were calculated from the Grynkiewicz equation [12]. Data were statistically processed using Student's *t* test. The figures show the results of typical experiments. Data are presented as the plots showing the changes in the ratio of Fura-2AM fluorescence intensities at excitation wavelengths of 340 and 380 nm (F_{340}/F_{380} ratio) over time,

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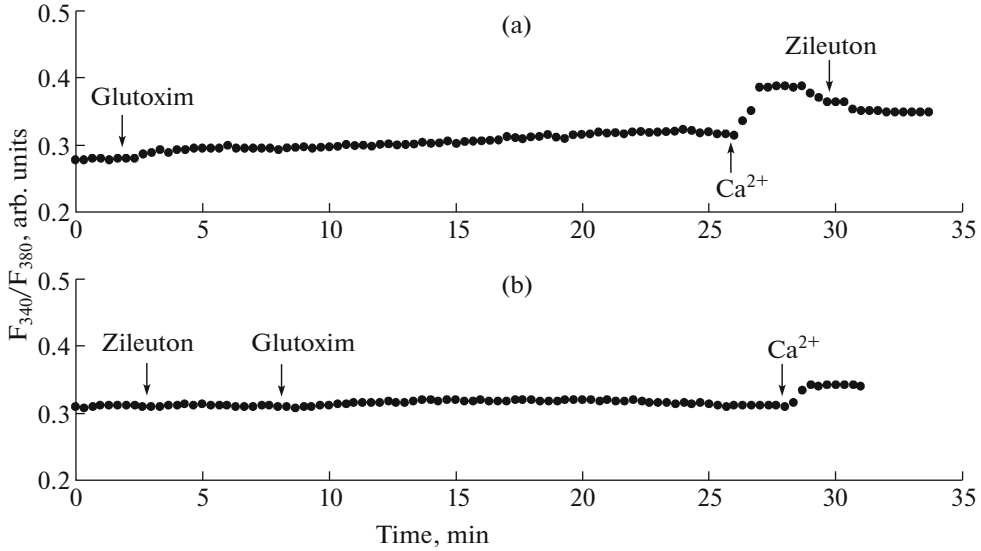

Fig. 1. Effect of zileuton on the glutoxim-induced $[Ca^{2+}]_i$ increase in rat macrophages. Here and in Fig. 2, the ordinate axis shows the ratio of the fluorescence intensities of Fura-2AM F_{340}/F_{380} at excitation wavelengths of 340 and 380 nm, respectively (arb. units). The abscissa axis shows time. (a) Cells were incubated for 25 min in the presence of 100 μg/mL glutoxim in nominally calcium-free medium, after which Ca^{2+} entry was initiated by adding 2 mM Ca^{2+} to the incubation medium; zileuton (4 μM) was added against the background of developing Ca^{2+} entry. (b) Macrophages were preincubated for 5 min with 1 μM zileuton in a calcium-free medium, after which 100 μm/mL glutoxim was added, and 20 min later Ca^{2+} entry was initiated 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.

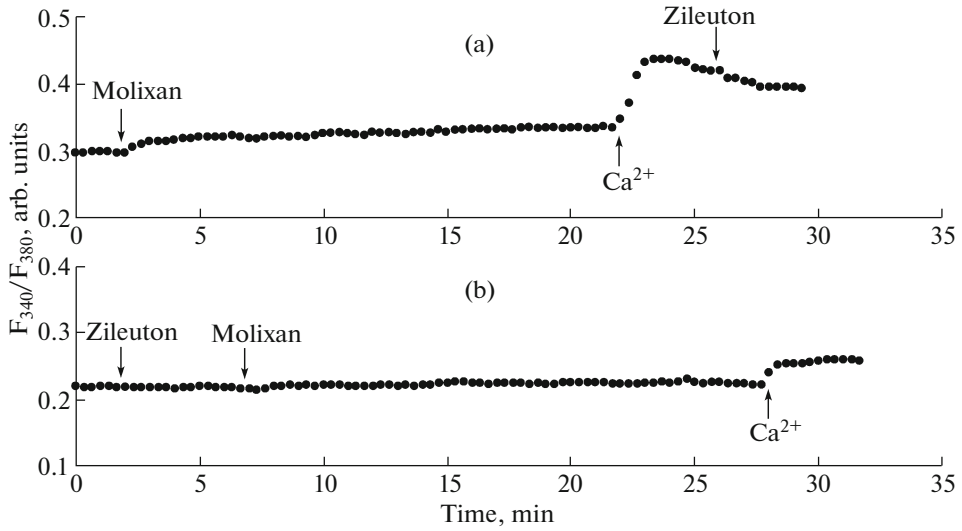

Fig. 2. Effect of zileuton on the molixan-induced $[Ca^{2+}]_i$ increase in rat macrophages. (a) Macrophages were incubated for 25 min in the presence of 100 μg/mL molixan in a calcium-free medium, after which Ca^{2+} entry was initiated by adding 2 mM Ca^{2+} to the culture medium; zileuton (4 μM) was added against the background of developing Ca^{2+} entry. (b) Cells were preincubated for 5 min with 1 μM zileuton in a calcium-free medium, after which 100 μg/mL molixan was added, and 20 min later Ca^{2+} entry was initiated by adding 2 mM Ca^{2+} to the incubation medium.

reflecting the dynamics of changes in $[Ca^{2+}]_i$ in cells depending on the measurement time [13].

In the control experiments, it was shown that the incubation of macrophages for 25 min in the presence of 100 μg/mL of G (Fig. 1a) or 100 μg /mL of M (Fig. 2a) in a calcium-free medium caused a slow increase in $[Ca^{2+}]_i$, reflecting the mobilization of Ca^{2+} from the intracellular stores. On average, judging by

the results of six experiments for each drug, 20 min after the addition of agents, $[Ca^{2+}]_i$ increased from the basal level (92 ± 15 nM) to 142 ± 18 nM for G and 138 ± 19 nM for M. The addition of 2 mM Ca^{2+} to the incubation medium caused a further increase in $[Ca^{2+}]_i$, reflecting the entry of Ca^{2+} into 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 Ca^{2+} entry was 228 ± 23 and 229 ± 21 nM for G and M, respectively.

In our experiments, it was shown for the first time that preincubation of macrophages with 1 μM zileuton for 5 min before the addition of 100 μg/mL G caused an almost complete (on average, according to the results of seven experiments, by 79.2 ± 9.1%) inhibition of Ca^{2+} mobilization from the stores and a significant (on average, according to the results of seven experiments, by 63.4 ±8.7%) inhibition of subsequent Ca^{2+} entry into the cell induced by G (Fig. 1b). Similar results were obtained in experiments on the effect of 1 μM zileuton on Ca^{2+} responses induced by 100 μg/mL M (Fig. 2b). On average, according to the results of seven experiments, zileuton suppressed Ca^{2+} mobilization from the stores by 67.5% and inhibited Ca^{2+} entry into the cell by 70.81%.

We also showed that the addition of 4 μM zileuton against the background of developing Ca^{2+} entry induced by G (Fig. 1a) or M (Fig. 2a) caused a significant (on average, according to the results of 11 experiments, by 45.9 ± 9.7%) inhibition of Ca^{2+} entry into macrophages.

Thus, we have shown for the first time that zileuton inhibits both phases of Ca^{2+}-response induced by G or M in macrophages. This is consistent with the suppression of Ca^{2+}-response in rat mast cells by 1 μM zileuton [14, 15].

The results presented in this study confirm our earlier data on the involvement of 5-lipoxygenase in the complex signaling cascade triggered by G or M and leading to an increase in $[Ca^{2+}]_i$ in macrophages. Data on the inhibition of developing Ca^{2+} entry by zileuton indicate that 5-lipoxygenase is involved not only in generation but also in maintaining the store-dependent Ca^{2+} entry in macrophages.

The results also point to the inadvisability of combined use of drugs G or M and the antiasthmatic agent zileuton in clinical practice.

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