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Inhibitors of the Metabolism of Arachidonic Acid Suppress Ca^{2+} Responses Induced by Trifluoperazine in Macrophages

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Abstract—The influence of the neuroleptic trifluoperazine on the intracellular concentration of Ca^{2+} in macrophages of rats was studied using a Fura-2AM fluorescent Ca^{2+} probe. It was found that trifluoperazine causes a dose-dependent increase in the intracellular Ca^{2+} concentration associated with Ca^{2+} mobilization from intracellular Ca^{2+} stores and subsequent entry of Ca^{2+} into peritoneal macrophages of rats. It was also shown that inhibitors of phospholipase A_2 (4-bromophenacyl bromide, prednisolone, and dexamethasone), cyclooxygenases (aspirin and indomethacin), and lipoxygenases (caffeic acid, zileuton, and baicalein) suppress Ca^{2+} responses induced by trifluoperazine in macrophages. The data obtained indicate the participation of enzymes and/or products of the cascade of arachidonic acid metabolism in the influence of trifluoperazine on the intracellular concentration of Ca^{2+} in peritoneal macrophages.

Keywords: intracellular Ca^{2+} concentration, peritoneal macrophages, trifluoperazine, arachidonic acid, phospholipase A_2, cyclooxygenase, lipoxygenase

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INTRODUCTION

Trifluoperazine (triftazin, stelazine) (TFP) is a representative of the first generation of typical antipsychotics (antipsychotic agents) of the phenothiazine series, which are widely used in the treatment of schizophrenia and other mental diseases (Dilsaver, 1993). A multifaceted influence of TFP on intracellular processes has been revealed (Sudeshna and Parimal, 2010).

The multiplicity of the effects of TFP, as well as of other phenothiazines, may be related to its amphiphilic nature. Being an amphiphilic compound, it penetrates well through the membranes. A mechanism of incorporation of amphiphilic antipsychotic agents, including phenothiazine antipsychotics, into the inner monolayer of the membrane, in which anionic phospholipids, primarily phosphoinositides are localized has been proposed (Oruch et al., 2010). Due to this, TFP can modulate intracellular processes, such as signaling and intracellular transport.

It is known that TFP is an antagonist of the Ca^{2+}-binding protein calmodulin, which plays a key role in the regulation of Ca^{2+} signaling processes. Thus, binding of TFP to the complex of Ca^{2+}-calmodulin leads to the formation of a compact globular structure that is incapable of interacting with target proteins (Vandonselaar et al., 1994; Feldkamp et al., 2010). In addition, TFP inhibits the assembly and subsequent aggregation of the calsequestrin protein with an increase in the Ca^{2+} concentration in the lumen of Ca^{2+} store (He et al., 1993).

It was also found that phenothiazines (TFP, chlorpromazine) affect Ca^{2+}-dependent activation of macrophages and exert an immunomodulating effect on peritoneal macrophages of rats (Hadjimitova et al., 2003).

Previously, we showed that another neuroleptic of the phenothiazine series—chlorpromazine—increases the intracellular concentration of Ca^{2+}, $[Ca^{2+}]_i$, causing mobilization of Ca^{2+} from the Ca^{2+} store and the subsequent entry of Ca^{2+} into peritoneal macrophages of rats (Naumova et al., 2016; Krutetskaya et al., 2017). However, the mechanisms by which phenothiazines cause an increase in $[Ca^{2+}]_i$ in macrophages are not fully understood.

In the activation and functioning of immune cells, including macrophages, the cascade of the metabo-

Abbreviations: AA—arachidonic acid, 4-BPB—4-bromophenacyl bromide, $[Ca^{2+}]_i$—intracellular concentration of Ca^{2+}, PLA_2—phospholipase A_2, TFP—trifluoperazine.

lism of polyunsaturated arachidonic acid (AA) plays an important role (Needleman et al., 1986). AA is released from membrane lipids under the action of phospholipase A_2 (PLA_2) and then is oxidized in the cell along three main enzymatic pathways to form biologically active products, eicosanoids (Needleman et al., 1986). In macrophages, AA is oxidized predominantly with the participation of cyclooxygenases and lipoxygenases (Brown et al., 1988).

On human platelets, it was previously established that psychotropic compounds, including TFP, can modulate the activity of PLA_2 and the production of metabolites of the cyclooxygenase and lipoxygenase oxidation pathways of AA (Walenga et al., 1981; Oruch et al., 2008).

Thus, the goal of this work was to study the effect of TFP on $[Ca^{2+}]_i$ in macrophages and to investigate the possible participation of key enzymes in the cascade of AA metabolism in the effect of TFP on $[Ca^{2+}]_i$ in macrophages of rats.

MATERIALS AND METHODS

Cells. The experiments were performed on cultured resident peritoneal macrophages of Wistar rats. Resident macrophages were isolated from the peritoneal cavity of rats weighing 180–250 g in accordance with a method described previously (Conrad, 1981; Randriamampita and Trautmann, 1987). Immediately after isolation, the cells had a spherical shape (10–20 μm in diameter). The cell suspension was placed in culture dishes with 10 × 10 mm quartz glasses and cultivated for 1–3 days in 199 medium (pH 7.2) with 20% bovine serum, glutamine (3%), penicillin (100 U/mL) and streptomycin (100 mg/mL). The test for α-naphthyl esterase (Monahan et al., 1981) showed that at least 96% of the cells in the monolayers were macrophages.

The experiments were carried out at room temperature (22–24°C) from 1 to 2 days after the beginning of cell culture. Quartz glasses with cells were placed in experimental chamber filled with a physiological solution of the following ionic composition (mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES-NaOH, pH 7.3–7.4. The calcium-free medium was characterized by containing 0 mM $CaCl_2$ and 1 mM EGTA. The procedure for culturing macrophages was described in detail earlier (Krutetskaya et al., 1997).

A Fura-2AM fluorescent probe (Sigma-Aldrich, United States) was used to measure $[Ca^{2+}]_i$. The macrophages were incubated for 45 min in physiological saline containing 2 μM Fura-2AM at 22–24°C. Glasses with stained cells were washed with physiological saline and transferred to the experimental chamber fixed on the table of a Leica DM 4000B fluorescence microscope (Leica Microsystems, Germany). The fluorescence of the object was excited at the wavelengths from 340 and 380 nm through a microscope objective. Narrowband optical filters were used to isolate the corresponding portions of the spectrum. The emission was recorded at a wavelength of 510 nm using a specialized Leica DFC340FX camera. ImageJ processing system (Micro-Manager 1.4 plug-in) was used to control the experiment.

The measurements resulted in determination of the ratio of the fluorescence intensity of Fura-2AM irradiated with light at a wavelength of 340 nm to the fluorescence intensity when irradiated with light at 380 nm, F_{340}/F_{380}, where the F_{340} and F_{380} are the fluorescence intensity of Fura-2AM bound and not bound to Ca^{2+} respectively. The ratio reflects changes in $[Ca^{2+}]_i$ in cells during measurements (Bruce and Elliott, 2000; Xie et al., 2002). To avoid photobleaching, measurements were taken every 20 s, exposing the object for 2 s.

In experiments, a 10× lens with an aperture of 8 mm was used. The $[Ca^{2+}]_i$ values were calculated using the Grynkiewicz equation (Grynkiewicz et al., 1985). Statistical analysis was performed using Student's *t*-test. The data are presented as an average and standard deviation. Differences were considered reliable at $p \le 0.05$. The figures show the results of typical experiments.

Sigma-Aldrich reagents (United States) were used. Stock solutions of thapsigargin (0.5 mM), zileuton (1 mM), 4-bromophenacyl bromide (4-BPB, 10 mM), trifluoperazine (2 mg/mL), and Fura-2AM (1 mM) were prepared in dimethyl sulfoxide. Stock solutions of indomethacin (10 mM), acetylsalicylic acid (aspirin) (50 mM), caffeic acid (5 mM), baicalein (5 mM), and dexamethasone (4 mg/mL) were prepared in ethyl alcohol. The prednisolone (25 mg/mL) stock solution was prepared in water.

RESULTS AND DISCUSSION

The Effect of Trifluoperazine on the Intracellular Concentration of Ca^{2+}

The effect of TFP at concentrations of 2, 4, 6, and 10 μg/mL on $[Ca^{2+}]_i$ in macrophages was investigated. It has been shown that, in a medium containing Ca^{2+} ions, TFP causes a dose-dependent increase in $[Ca^{2+}]_i$ (Fig. 1). When TFP is applied, $[Ca^{2+}]_i$ is rapidly increased from the basal level of 92 ± 16 nM, after which a prolonged plateau phase of the $[Ca^{2+}]_i$ response is observed. $[Ca^{2+}]_i$ in the plateau phase is 161 ± 17, 200 ± 15, 241 ± 18, and 265 ± 19 nM after addition of 2, 4, 6, and 10 μg/mL TFP, respectively. For each concentration studied, eight independent experiments were carried out.

The increase in $[Ca^{2+}]_i$ after the exposure to TFP may be associated with mobilization of Ca^{2+} from intracellular Ca^{2+} stores and with an influx of Ca^{2+}

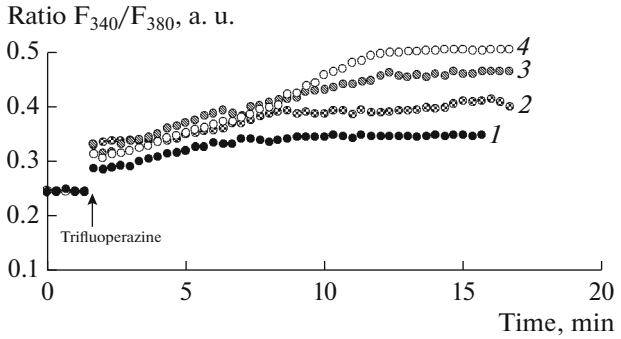

Fig. 1. Effect of trifluoperazine (TFP) on $[Ca^{2+}]_i$ in peritoneal macrophages of rat. Here and in Figs. 2–5: along the abscissa—time, min; along the ordinate axis—the ratio of Fura-2AM fluorescence intensities at 340 and 380 nm wavelengths of the exciting radiation (F_{340}/F_{380}), arbitrary units. TFP was added at a concentration of 2 (curve *1*), 4 (curve *2*), 6 (curve *3*), or 10 (curve *4*) μg/mL to the macrophages in a normal physiological solution containing Ca^{2+} ions. Here and in Figs. 2–5, each registration is obtained for a group of 40–50 cells and is a typical repeatable version of six or seven independent experiments.

from the external medium. To elucidate the mechanism of the $[Ca^{2+}]_i$ increase under the action of TFP, experiments were conducted in a nominally calcium-free medium. It was shown that the addition of TFP (2 μg/mL) to macrophages in a calcium-free medium causes a significant (up to 205 ± 19 nM, $n = 7$) increase in $[Ca^{2+}]_i$ associated with mobilization of Ca^{2+} from intracellular stores. When 2 mM Ca^{2+} is introduced into the external medium, a further increase in $[Ca^{2+}]_i$ up to 274 ± 20 nM is observed (Fig. 2a). This increase lasts for the whole time of the presence of Ca^{2+} in the medium and is connected with the Ca^{2+} entry from the external medium. The Ca^{2+} entry activated by TFP is apparently caused by the depletion of the Ca^{2+} stores.

To elucidate the nature of Ca^{2+} stores from which Ca^{2+} is mobilized under the action of TFP, experiments using a specific inhibitor (Thastrup et al., 1989) of endoplasmic Ca^{2+}-ATPases thapsigargin were conducted. The macrophages were first stimulated with 0.5 μM thapsigargin in a nominally calcium-free medium. After the phase of Ca^{2+} mobilization from the stores caused by thapsigargin was completed, the cells were incubated with 2 μg/mL TFP for 15 min before the introduction of 2 mM Ca^{2+} (Fig. 2b). Under these conditions, there is a significant decrease in the mobilization of Ca^{2+} from the stores (by 43.7 ± 11.2%, $n = 7$) induced by TFP. This indicates that the mobilization of Ca^{2+} from the stores under the action of TFP occurs both from thapsigargin-sensitive Ca^{2+} stores, such as the endoplasmic reticulum, and from other intracellular Ca^{2+} stores, possibly mitochondria.

The results obtained are consistent with the data presented in the literature. Thus, TFP causes an increase in $[Ca^{2+}]_i$ in glioblastoma cells associated with the release of Ca^{2+} from intracellular Ca^{2+} stores sensitive to inositol-1,4,5-triphosphate (IP_3) (Kang et al., 2017). This effect of TFP is associated with the inhibition of calmodulin subtype 2 (CaM2) and, as a consequence, irreversible activation of the IP_3 receptors (Kang et al., 2017). Moreover, TFP causes activa-

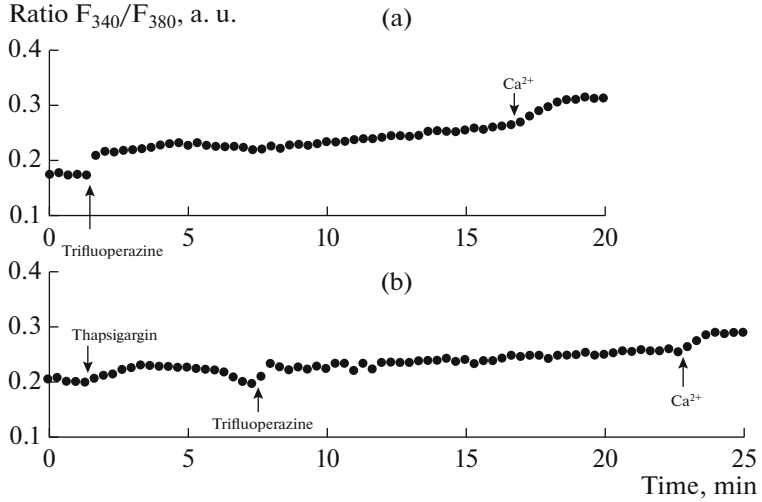

Fig. 2. Influence of TFP on $[Ca^{2+}]_i$ in peritoneal macrophages of rat in nominally calcium-free medium. (a) Macrophages were incubated for 15 min with 2 μg/mL of TFP in a calcium-free medium; then, 2 mM of Ca^{2+} was introduced into the external medium. (b) Cells were stimulated with 0.5 μM thapsigargin in a calcium-free medium; after the completion of the Ca^{2+} mobilization from the stores caused by thapsigargin, 2 μg/mL of TFP was added, and, after 15 min, 2 mM Ca^{2+} was introduced into the external medium.

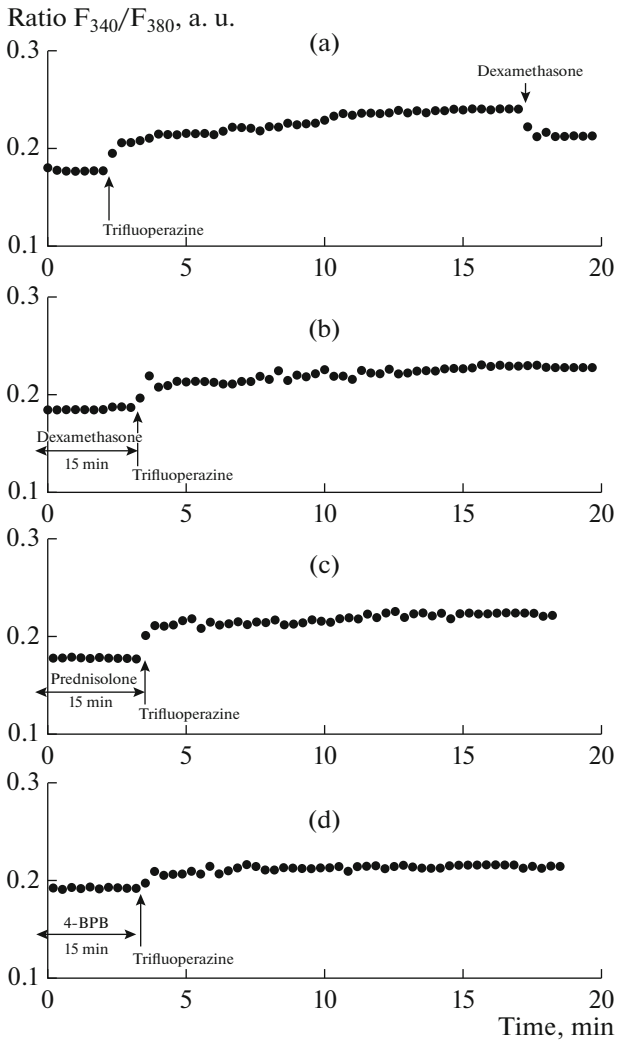

Fig. 3. Influence of phospholipase A_2 inhibitors on Ca^{2+} responses induced by TFP in rat peritoneal macrophages. (a) 4 μg/mL TFP was added to macrophages in a normal physiological solution containing Ca^{2+} ions; on the background of the developed Ca^{2+} response, 16 μg/mL of dexamethasone was introduced. (b–d) Macrophages in normal saline were incubated for 15 min with (b) 8 μg/mL dexamethasone, (c) 25 μg/mL prednisolone, or (d) 20 μM 4-BPB; then, 4 μg/mL of TFP was introduced.

tion of ryanodine receptors and mobilization of Ca^{2+} from the stores in cat and rabbit cardiomyocytes (Qin et al., 2009). In in vitro experiments, it was found that TFP can directly activate ryanodine receptors, increasing the probability of an open-channel condition (Qin et al., 2009).

Thus, we have shown for the first time that TFP causes an increase in $[Ca^{2+}]_i$ associated with the mobilization of Ca^{2+} from the Ca^{2+} stores and the subsequent Ca^{2+} entry into peritoneal macrophages of rats. The mechanism by which TFP triggers the mobilization of Ca^{2+} from the stores requires further research. The relatively fast kinetics of Ca^{2+} release from the stores under the action of TFP suggests the activation of IP_3-sensitive channels of Ca^{2+} release in the Ca^{2+}-store membrane. In addition, the mobilization of Ca^{2+} from the stores caused by TFP has a stationary phase (plateau phase). This may indicate the prevention of Ca^{2+} extrusion from the cytosol. It can be supposed that TFP inhibits Ca^{2+} ATPase in the Ca^{2+}-store membrane and in the macrophage plasmalemma. This suggestion is supported by evidence that TFP and other phenothiazine derivatives inhibit sarco(endo)plasmatic Ca^{2+} ATPases in the cells of the cerebellum and skeletal muscles (Khan et al., 2000).

The effect of TFP on Ca^{2+} signaling in cells can underlie the side effects of antipsychotic agents observed in cardiology, such as lengthening of the QT interval, tachycardia, and arrhythmia (Buckley and Sanders, 2000).

To identify the participation of the main pathways of the AA metabolism in the effect of TFP on $[Ca^{2+}]_i$ in macrophages, experiments in a medium containing Ca^{2+} ions were performed. In control experiments, it was shown that incubation of cells with 4 μg/mL of TFP caused an increase in $[Ca^{2+}]_i$ from a basal level of 92 ± 17 to 221 ± 25 nM (n = 14) (Figs. 3a, 4a, 5a).

The Effect of Phospholipase A_2 Inhibitors

Structurally different inhibitors of PLA_2: 4-BPB (Irvine, 1982) and synthetic glucocorticoids dexamethasone and prednisolone (Gewert and Sundler, 1995) were used to detect the involvement of PLA_2 in the effect of TFP on $[Ca^{2+}]_i$ in rat peritoneal macrophages.

It was found that preincubation of macrophages with 8 μg/mL of dexamethasone for 15 min before administration of 4 μg/mL of TFP leads to a significant suppression of Ca^{2+} responses (by 37.6 ± 9.3%, n = 7) caused by TFP (Fig. 3b). Similar results were obtained with 25 μg/mL prednisolone. The suppression of Ca^{2+} responses induced by TFP under the influence of prednisolone was 54.3 ± 13.5% (n = 7) (Fig. 3c). Preincubation of cells with 20 μM of 4-BPB for 15 min before administration of 4 μg/mL of TFP also resulted in significant suppression of Ca^{2+} responses (by 37.6 ± 8.8%, n = 7) caused by TFP (Fig. 3d).

It has also been shown that the administration of 16 μg/mL dexamethasone (Fig. 3a) or 25 μg/mL of prednisolone (not shown) against the background of the developed plateau of Ca^{2+} response induced by TFP causes a decrease in $[Ca^{2+}]_i$ by 35.0 ± 9.7 or 46.4 ± 9.2%, respectively. When 40 μM 4-BPB is added against the background of the plateau phase of the Ca^{2+}-response caused by TFP, there is also a sig-

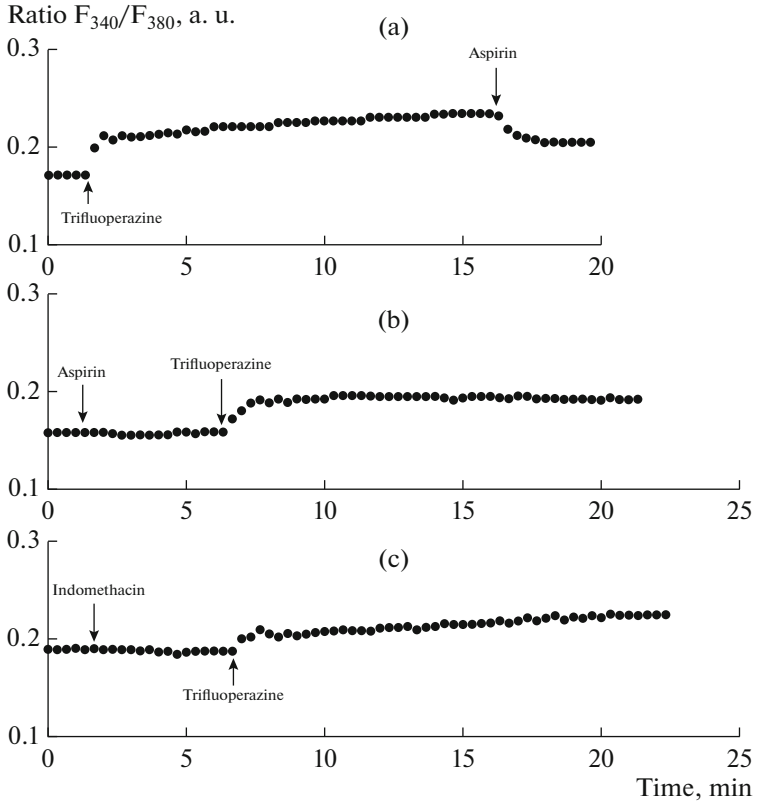

Fig. 4. Effect of cyclooxygenase inhibitors on the increase in $[Ca^{2+}]_i$ caused by TFP in rat peritoneal macrophages. (a) 4 μg/mL of TFP was added to macrophages in a normal physiological solution containing Ca^{2+} ions; on the background of the developed Ca^{2+} response, 100 μM of aspirin was introduced. (b, c) Macrophages in normal saline were incubated for 5 min with (b) 100 μM aspirin or (c) 10 μM indomethacin, followed by 4 μg/mL TFP.

nificant decrease in $[Ca^{2+}]_i$ (by 33.8 ± 11.4%, $n = 7$) in macrophages.

The results obtained indicate the participation of PLA_2, the key enzyme of the cascade of AA metabolism, in the effect of TFP on $[Ca^{2+}]_i$ in peritoneal macrophages.

Glucocorticoids are widely used as steroidal anti-inflammatory, antiallergic, and antishock medications (Becker, 2013). Our results suggest the undesirability of the combined use of antipsychotic TFP and dexamethasone or prednisolone glucocorticoids in the clinical practice.

The Effect of Cyclooxygenase Inhibitors

Structurally various inhibitors of cyclooxygenases, acetylsalicylic acid (aspirin) and indomethacin (de Witt et al., 1990; Mitchell et al., 1994) were used to identify the participation of the cyclooxygenase pathway of AA oxidation in the effect of TFP on $[Ca^{2+}]_i$ in macrophages.

It was shown that preincubation of cells with 100 μM aspirin (Fig. 4b) or 10 μM indomethacin (Fig. 4c) for 5 min before administration of 4 μg/mL TFP leads to suppression of the increase in $[Ca^{2+}]_i$ caused by TFP in comparison with control experiments. The suppression of Ca^{2+} responses (induced by TFP) by aspirin and indomethacin was 44.8 ± 10.6 and 44.4 ± 10.2%, respectively ($n = 9$ for each of the pharmacological agents).

It was also found that, when 100 μM aspirin (Fig. 4a) or 40 μM indomethacin (not shown) are introduced during the developed plateau of the Ca^{2+} response caused by TFP, $[Ca^{2+}]_i$ decreases by 60.1 ± 13.7% ($n = 8$) and 43.0 ± 8.5% ($n = 6$), respectively.

The data obtained suggest that cyclooxygenases and/or their products are involved in the formation of Ca^{2+} responses caused by TFP in peritoneal macrophages.

The cyclooxygenase inhibitors indomethacin and aspirin are nonsteroidal anti-inflammatory agents that have anti-inflammatory, analgesic, and antipyretic effects (Dubois et al., 1998). The results that have been

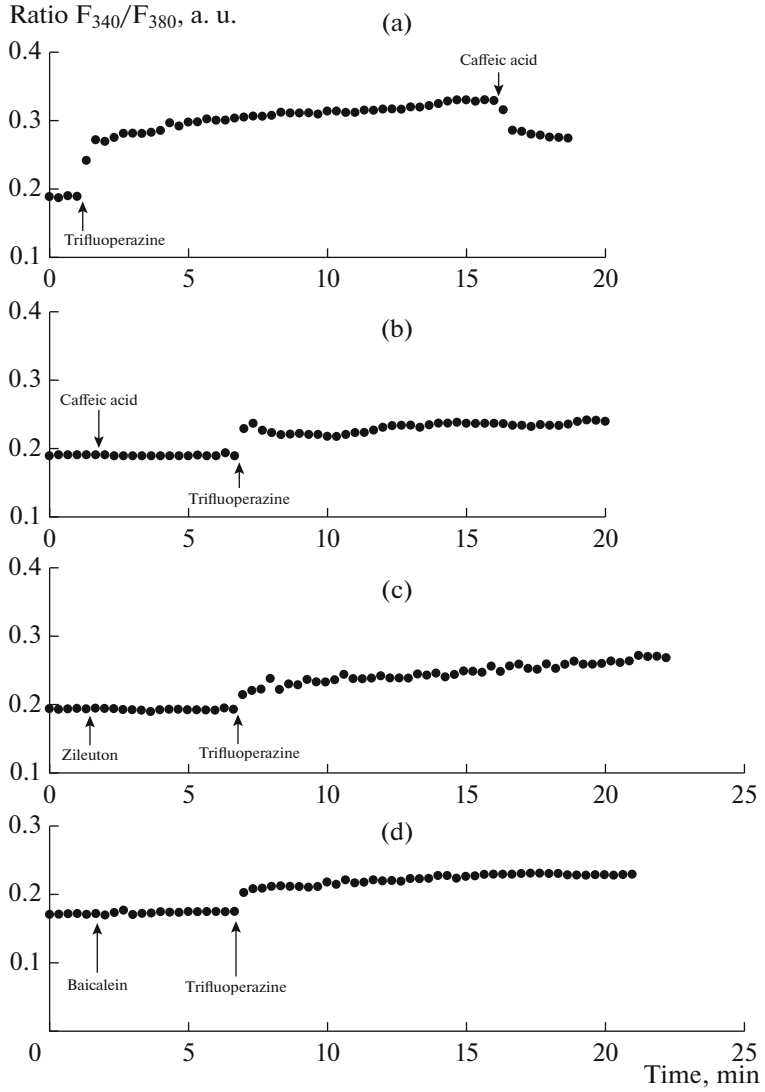

Fig. 5. Effect of lipoxygenase inhibitors on Ca^{2+} responses caused by TFP in rat peritoneal macrophages. (a) 4 μg/mL TFP was added to macrophages in normal saline; on the background of the developed Ca^{2+}-response, 20 μM of caffeic acid (CA) was introduced. (b–d) Macrophages in normal saline were incubated for 5 min with (b) 10 μM CA, (c) 2 μM zileuton , or (d) 10 μM baicalein; then, 4 μg/mL TFP were added.

obtained indicate that combined clinical use of TFP with medications based on indomethacin or aspirin is not desirable.

The Effect of Lipoxygenase Inhibitors

Selective inhibitors of 5-lipoxygenases, such as caffeic (3,4-dihydroxycinnamic) acid (CC) (Chung et al., 2004) and the anti-asthmatic agent zileuton (*N*-[1-(1-benzothien-2-yl)ethyl]-*N*-hydroxyurea, Zyflo®) (Wenzel and Kamada, 1996), as well as the selective inhibitor of 12/15-lipoxygenase flavonoid baicalein (van Leyen et al., 2006), were used in experiments.

It was shown that preincubation of macrophages with 10 μM of caffeic acid for 5 min before administration of 4 μg/mL of TFP resulted in a significant (by 61.4 ± 14.2%, $n = 7$) suppression of Ca^{2+} responses caused by TFP (Fig. 5b). Similar results were obtained with 2 μM zileuton (Fig. 5c) or 10 μM baicalein (Fig. 5d). The suppression of Ca^{2+} responses caused by TFP under the influence of zileuton was 34.6 ± 10.5% ($n = 7$), and under the influence of baicalein it was 58.8 ± 7.6% ($n = 6$).

It was also found that the introduction of 20 μM caffeic acid (Fig. 5a), 4 μM zileuton or 20 μM baicalein (not shown) against the background of the developed plateau of the Ca^{2+} response induced by TFP causes $[Ca^{2+}]_i$ to decrease by 46.3 ± 12.4, 33.5 ± 8.2, or 52.9 ± 3.0%, respectively ($n = 7$ for each of the agents).

The data obtained show the participation of 5- and 12/15-lipoxygenases and/or oxidation products of AA with the participation of these enzymes in the effect of TFP on $[Ca^{2+}]_i$ in macrophages. In addition, the results indicate the undesirability of combined clinical use of the antipsychotic TFP and the anti-asthmatic agent zileuton.

Thus, in the present work, it has been shown for the first time using rat peritoneal macrophages that the enzymes and/or products of the cascade of the AA metabolism play an important role in the effect of TFP on the processes of Ca^{2+} signaling in peritoneal macrophages of rat.

The participation of the enzymes of the cascade of AA metabolism in the influence of TFP on $[Ca^{2+}]_i$ can be explained by the model of embedding of amphiphilic antipsychotic agents, including phenothiazine neuroleptics, in the inner monolayer of the membrane in which anionic phospholipids are localized. The tricyclic hydrophobic ring of the TFP molecule is embedded in the hydrophobic phase of the membrane, while the alkyl moiety with the terminal amino group interacts with the polar heads of acid lipids (Oruch et al., 2010; Jaszczyszyn et al., 2012). This can lead to a change in the fluidity of the membrane and the functioning of membrane-bound enzymes, such as phospholipase A_2, which triggers a cascade of AA metabolism.

In turn, the enzymes and/or the products of AA metabolism are involved in the formation of Ca^{2+} responses caused by TFP.

The results that we have concerning the effect of TFP on the processes of Ca^{2+} signaling in macrophages help to understand the molecular mechanisms of the pharmacological action of TFP.

In addition, the obtained data indicate the undesirability of combined clinical use of the antipsychotic TFP with steroid and nonsteroidal anti-inflammatory agents or the anti-asthmatic agent zileuton.

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