CELL BIOPHYSICS

Haloperidol, a Sigma-1 Receptor Antagonist, Inhibits Ca²⁺ Responses in Rat Peritoneal Macrophages

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Abstract—Sigma-1 receptors are ubiquitous multifunctional ligand-regulated molecular chaperons in the membrane of the endoplasmic reticulum, having a unique history, structure and pharmacological profile. Sigma-1 receptors modulate a wide range of cellular processes in normal and pathological conditions, including Ca²⁺ signaling processes. Using the Fura-2AM fluorescent Ca²⁺ probe, we have shown that the sigma-1 neuroleptic receptor antagonist haloperidol significantly suppressed the mobilization of Ca²⁺ from intracellular Ca²⁺ depots and the subsequent depot-dependent inflow of Ca²⁺ into cells caused by endoplasmic Ca²⁺-ATPase inhibitors tapsigargin and cyclopyazonic acid, as well as immunomodulators glutoxim and molixan in rat peritoneal macrophages. The results indicated the participation of sigma-1 receptors in the complex signaling cascade caused by glutoxim or molixan and leading to an increase in intracellular Ca²⁺ concentration in macrophages, as well as the participation of sigma-1 receptors in the regulation of depot-dependent Ca²⁺ inflow in macrophages.

Keywords: haloperidol, sigma-1 receptors, intracellular Ca²⁺ concentration, macrophages

DOI: 10.1134/S0006350923040140

INTRODUCTION

Sigma-1 receptors, which have a unique history, structure and pharmacology, they modulate a wide range of cellular processes in normal and pathological conditions and are important participants in Ca²⁺ signaling processes in cells [1–5]. Sigma-1 receptors are unique multifunctional ligand-regulated molecular chaperones localized in the membrane of the endoplasmic reticulum, at the border with mitochondria (mitochondria-associated endoplasmic reticulum membrane, MAM) [1–5]. They can be translocated to the plasmalemma and interact with other receptors and ion channels; they were found in the nuclear envelope, where they participate in the regulation of transcription [1]. These receptors are expressed in various types of cells, including immune ones [2, 3, 5]. Sigma-1 receptors have a very wide pharmacological profile. Their ligands are compounds of various chemical structure and pharmacological effects: antidepressants (fluvoxamine, sertraline, and imipramine), neuroleptics (haloperidol and chlorpromazine), analgesics (pentazocin), anxiolytics (afobazole), anticonvul-

sants (phenytoin), antitussive (dextromethorphan and carbetapentan), antihistamines (chlorphenamine), narcotic drugs (methamphetamine and cocaine) and drugs used in the treatment of neurodegenerative diseases (amantadine, memantine and donepezil) [6–8]. Typical neuroleptics (haloperidol, flufenazine, chlorpromazine and trifluoperazine) have a high affinity for sigma-1 receptors [9].

Performing the functions of chaperones, sigma-1 receptors interact with target proteins (ion channels, receptors in the plasmalemma, etc.) and modulate many cellular processes, including Ca²⁺ signaling processes [1, 3, 10]. In the plasmalemma, they interact with potential-dependent Ca²⁺, Na⁺ and K⁺ channels, proton-activated ion channels, NMDA receptors, receptors associated with G-proteins (muscarinic acetylcholine receptors, μ-opioid and D1 and D2 dopamine receptors) and other proteins-targets [1, 3]. In the membrane of the endoplasmic reticulum, the sigma-1 receptor interacts with the inositol-1,4,5-triphosphate type 3 receptor, with another molecular chaperone protein BiP (binding immunoglobulin protein) [11] and Ca²⁺ sensor protein STIM1 [12]. It was found that by interacting with inositol-1,4,5-triphosphate receptors, sigma-1 receptors modulate Ca²⁺ sig-

naling processes in cells, namely, the mobilization of Ca^{2+} from the depot and the inflow of Ca^{2+} from the external medium [10, 11]. Their participation in the regulation of depot-dependent Ca^{2+} inflow in cells has been shown [12–14].

We have previously shown for the first time that sigma-1 receptor ligands, phenothiazine neuroleptics chlorpromazine and trifluoperazine, significantly suppressed both phases of Ca^{2+} responses caused by disulfide-containing immunomodulators glutoxim® (disodium salt of oxidized glutathione with d-metal in nano concentration) and molixan® (a complex of glutoxim and inosine nucleoside) and inhibitors of endoplasmic Ca^{2+}-ATPase tapsigargin and cyclopyazonic acid (CPA) in rat peritoneal macrophages [15, 16].

To confirm the participation of sigma-1 receptors in the regulation of Ca^{2+} signaling processes in macrophages, it seemed appropriate to investigate the effect of other structurally different sigma-1 receptor ligands on Ca^{2+} responses induced by glutoxim and molixan, as well as tapsigargin and CPA, in rat peritoneal macrophages; this was the subject of this study.

The sigma-1 receptor antagonist, the neuroleptic haloperidol (a derivative of butyrophenone) [17], was used in experiments; it belongs to the first generation of typical neuroleptics (antipsychotic agents), and has a long history of use in the clinic for the treatment of schizophrenia and other mental diseases [18].

MATERIALS AND METHODS

Isolation and Cultivation of Rat Peritoneal Macrophages

The experiments were carried out on cultured resident peritoneal macrophages of Wistar rats. Resident macrophages were isolated from the peritoneal cavity of rats with mass 180–250 g according to the method described earlier [19]. Immediately after isolation, the cells had a spherical shape (diameter 10–20 μm). The suspension of cells was placed in Count Flexi Plate containing quartz glasses with a size of 10 × 10 mm. The cells on the glasses were cultured for 1–3 days at 37°C in a medium 199 (pH 7.2) containing 20% bovine serum, glutamine (3%), penicillin (100 units/mL) and streptomycin (100 mg/mL). A test for α-naphthylesterase [20] showed that at least 96% of the cells in the monolayers were macrophages.

The experiments were carried out at room temperature (22–24°C) one to two days after the start of cell culture. Quartz glasses with cells were placed in an experimental chamber filled with a saline solution of the following composition: 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES-NaOH, pH 7.3–7.4. The calcium-free medium differed in that it contained 0 mM $CaCl_2$ and 1 mM EGTA.

Reagents

Reagents from Sigma-Aldrich (United States) were used in the work. Batch solutions of Fura-2AM (1 mM), cyclopiazonic acid (10 mM) and tapsigargin (0.5 mM) were prepared in dimethyl sulfoxide. The preparations of glutoxim and molixan were provided by PHARMA-VAM (Saint Petersburg). Batch solutions of haloperidol (5 mg/mL), glutoxim (50 mg/mL) and molixan (50 mg/mL) were prepared in water.

Measurement of Intracellular Ca^{2+} Concentration

To measure the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), a Fura-2AM fluorescent probe (Sigma-Aldrich, United States) was used. Macrophages were incubated for 45 min in a saline solution containing 2 μM of Fura-2AM at 22–24°C. Glasses with stained cells were washed with saline solution and transferred to the experimental chamber of the DM 4000B fluorescence microscope (Leica Microsystems, Germany). The fluorescence of the object was excited at 340 and 380 nm through a microscope lens. Narrow-band optical filters were used to isolate the corresponding sections of the spectrum. The emission was recorded at 510 nm using a specialized DFC340FX video camera (Leica Microsystems, Germany). The ImageJ image processing system (Micro-Manager 1.4 plugin) was used to control the experiment.

The results of the measurements were the ratio of Fura-2AM fluorescence intensities when irradiated at 340 nm to the fluorescence intensity when irradiated at 380 nm (F_{340}/F_{380}), where F_{340} was the intensity of Fura-2AM fluorescence associated with Ca^{2+}, and F_{380} was the intensity of Fura-2AM fluorescence not associated with Ca^{2+}. This ratio reflected changes in $[Ca^{2+}]_i$ in cells during measurements [21]. To avoid photobleaching, measurements were carried out every 20 s, irradiating the object for 2 s. In the experiments, a 10× lens with an aperture of 8 mm was used. The values of $[Ca^{2+}]_i$ were calculated using the Grinkevich equation [22].

Statistical Data Processing

Statistical analysis was performed using the Student's t-test. The data were presented in the form of mean and standard deviation. Each recording was obtained for a group of 40–50 cells. The figures show the results of the same type of experiments from six to eight independent ones. The differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The Effect of Haloperidol on Ca²⁺ Responses Induced by Disulfide-Containing Immunomodulators in Macrophages

Pharmacological analogues of oxidized glutathione (glutoxim and molixan) are used as immunomodulators and cytoprotectors in the complex therapy of bacterial, viral and oncological diseases [23–26]. These drugs have a complex effect on the processes of redox regulation in cells, however, the exact biophysical mechanisms of their action are far from being fully understood. Clinical studies have shown that molixan was effective in the prevention and treatment of COVID-19 coronavirus infection. It led to a more rapid regression of the severity of the disease into a lighter form [27]. Earlier we showed for the first time that glutoxim and molixan increased $[Ca^{2+}]_i$, causing the mobilization of Ca^{2+} from tapsigargine-sensitive Ca^{2+} depots and the subsequent depot-dependent inflow of Ca^{2+} into rat peritoneal macrophages [28, 29].

In this study, control experiments showed that incubation of macrophages for 20 min with 100 μg/mL of glutoxim (Fig. 1a) or 100 μg/mL of molixan (Fig. 2a) in a calcium-free medium caused a slow increase in $[Ca^{2+}]_i$, reflecting the mobilization of Ca^{2+} from intracellular Ca^{2+} depots. After 20 min of the addition of agents, $[Ca^{2+}]_i$ increased on average from a basal level of 90 ± 18 to 135 ± 18 nM ($n = 7, p < 0.05$) for glutoxim and 134 ± 20 nM ($n = 6, p < 0.05$) for molixan. When 2 mM Ca^{2+} was added to the external medium, a further increase in $[Ca^{2+}]_i$ was observed, reflecting the depot-dependent inflow of Ca^{2+} into the cytosol (Figs. 1a and 2a). Average increase in $[Ca^{2+}]_i$ during inflow of Ca^{2+} was 223 ± 22 nM ($n = 7, p < 0.05$) and 202 ± 20 nM ($n = 6, p < 0.05$) for glutoxim and molixan, respectively.

It was found that the preincubation of macrophages with 30 μg/mL of haloperidol for 10 min before the administration of 100 μg/mL of glutoxim led to a significant suppression of both the mobilization of Ca^{2+} from the depot (by $50.3 \pm 8.4\%, n = 7, p < 0.05$) and the subsequent depot-dependent inflow of Ca^{2+} into the cells (by $54.5 \pm 9.5\%, n = 7, p < 0.05$) induced by glutoxim (Fig. 1b). Similar data were obtained on the effect of 30 μg/mL of haloperidol on Ca^{2+} responses caused by 100 μg/mL of molixan (Fig. 2b). Haloperidol caused suppression of Ca^{2+} mobilization from the depot by $49.3 \pm 8.1\%$ ($n = 7, p < 0.05$) and suppression of Ca^{2+} inflow into cells by $47.6 \pm 9.7\%$ ($n = 7, p < 0.05$) induced by molixan. This indicated the involvement of sigma-1 receptors in the activation of the depot-dependent Ca^{2+} inflow induced by glutoxim and molixan in macrophages.

It was also found that the addition of 30 μg/mL of haloperidol under the developed Ca^{2+} inflow induced by glutoxim (Fig. 1a) or molixan (Fig. 2b) caused a significant (by $51.4 \pm 9.0\%, n = 12, p < 0.05$) suppression of the depot-dependent Ca^{2+} inflow into macrophages.

The Effect of Haloperidol on Ca²⁺ Responses Induced by Endoplasmic Ca²⁺-ATPase Inhibitors

It was found in control experiments that the addition of 0.5 μM of tapsigargin to macrophages in a calcium-free medium caused a slight increase in $[Ca^{2+}]_i$, reflecting the mobilization of Ca^{2+} from intracellular Ca^{2+} depots (Fig. 3a). On average, the increase in $[Ca^{2+}]_i$ during the mobilization phase was 31 ± 9 nM ($n = 7, p < 0.05$). When 2 mM Ca^{2+} was subsequently injected into the external medium, a depot-dependent Ca^{2+} inflow into the cytosol was observed (Fig. 3a). On average, the increase in $[Ca^{2+}]_i$ during Ca^{2+} inflow was 152 ± 20 nM ($n = 7, p < 0.05$). We obtained similar results when 10 μM of CPA was used (Fig. 4a). On average, the increase in $[Ca^{2+}]_i$ during the phase of Ca^{2+} mobilization from the depot caused by CPA was 26 ± 9 nM ($n = 7, p < 0.05$), and during the inflow of Ca^{2+} into macrophages it was 141 ± 22 nM ($n = 7, p < 0.05$).

It was shown that the preincubation of macrophages with 30 μg/mL of haloperidol for 10 min before the introduction of 0.5 μM of tapsigargin caused the suppression of both phases of the Ca^{2+} response induced by tapsigargin (Fig. 3b). On average, haloperidol suppressed the phase of Ca^{2+} mobilization from the depot by $23.2 \pm 7.9\%$ ($n = 7, p < 0.05$), and the subsequent depot-dependent inflow of Ca^{2+} into macrophages by $42.3 \pm 13.6\%$ ($n = 7, p < 0.05$). Similar results were obtained in experiments with 10 μM of CPA (fig. 4b). On average, haloperidol caused suppression of Ca^{2+} mobilization from the depot by $25.9 \pm 8.0\%$ ($n = 7, p < 0.05$) and suppression of Ca^{2+} inflow by $43.8 \pm 12.5\%$ ($n = 7, p < 0.05$) induced by CPA. This indicated the involvement of sigma-1 receptors in the activation of the depot-dependent Ca^{2+} inflow induced by tapsigargin and CPA in macrophages.

It was also found that the addition of 30 μg/mL of haloperidol under the developed Ca^{2+} inflow induced by tapsigargin (Fig. 3a) or CPA (Fig. 4a) caused a significant suppression of the depot-dependent Ca^{2+} inflow into macrophages. The suppression of Ca^{2+} inflow was $48.5 \pm 17.1\%$ ($n = 7, p < 0.05$) for tapsigargin and $48.1 \pm 16.9\%$ ($n = 7, p < 0.05$) for CPA. This indicated the involvement of sigma-1 receptors not only in activation, but also in maintaining the depot-dependent inflow of Ca^{2+} into macrophages.

Thus, in this study, we showed for the first time on rat peritoneal macrophages that the neuroleptic haloperidol, a sigma-1 receptor antagonist, suppressed
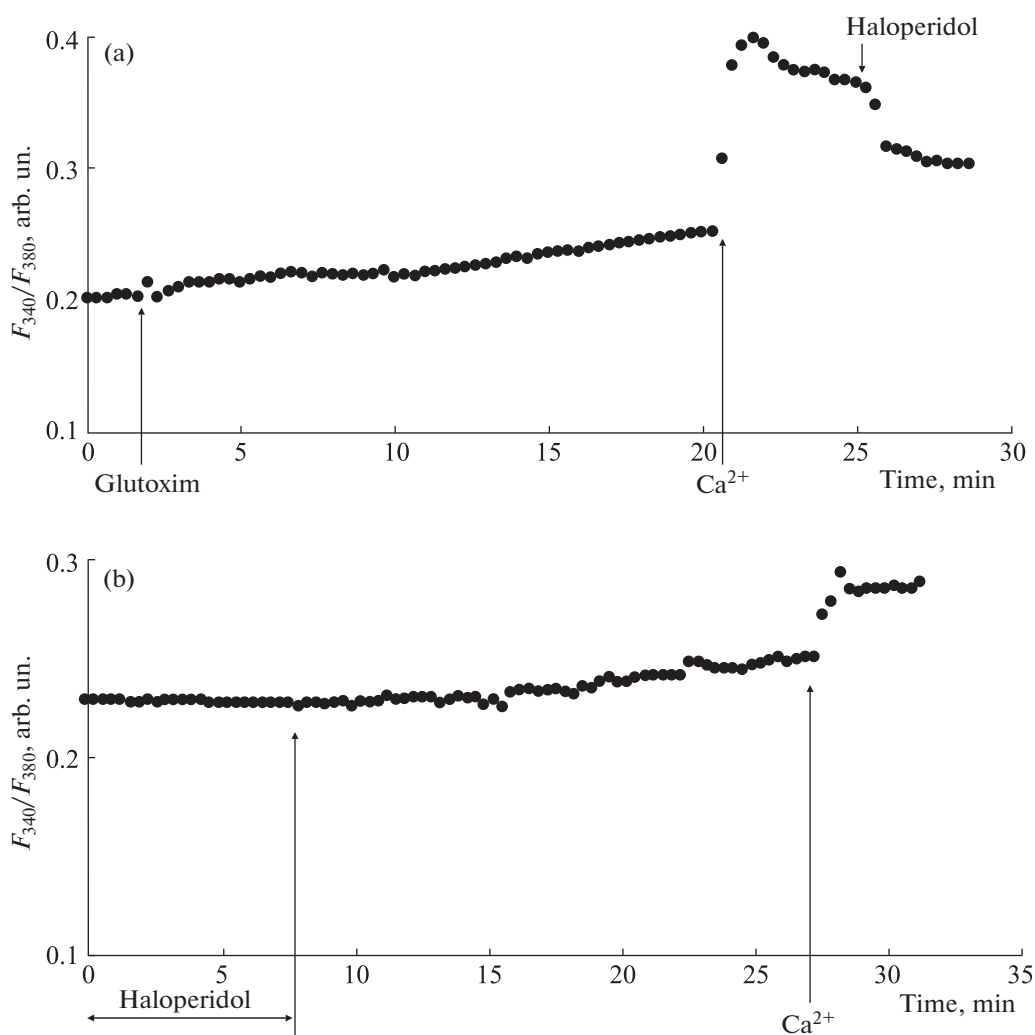
Fig. 1. The effect of haloperidol on the increase of $[Ca^{2+}]_i$ in rat macrophages caused by glutoxim: (a) cells were incubated for 20 min in the presence of 100 µg/mL of glutoxim in a nominally calcium-free medium, Ca^{2+} inflow was initiated by the addition of 2 mM Ca^{2+} into the external medium, 30 µg/mL of haloperidol was added under the developed Ca^{2+} inflow; (b) macrophages were preincubated for 10 min with 30 µg/mL of haloperidol in a calcium-free medium, 100 µg/mL of glutoxim was added after 20 min, Ca^{2+} inflow was initiated by adding 2 mM Ca^{2+} into the external medium. Here and further in Figs. 2–4: the ordinate axis is the ratio of fluorescence intensities of Fura-2AM at excitation wavelengths 340 and 380 nm (F_{340}/F_{380}, arb. un.); the abscissa axis is time (min). Each recording was obtained for a group of 40–50 cells and represented a typical variant of six to eight independent experiments.

both phases of Ca^{2+} responses in macrophages caused by glutoxim or molixan, as well as by tapsigargin and CPA. The results were consistent with the data of other authors who found that the ligands of the sigma-1 receptors of the phenothiazine neuroleptics chlorpromazine and trifluoperazine inhibited the mobilization of Ca^{2+} from the depot and the subsequent depot-dependent inflow of Ca^{2+} caused by ATP or tapsigargin in human leukemia cells (HL-60 line) [30, 31]. Chlorpromazine has also been shown to inhibit the depot-dependent Ca^{2+} inflow induced by bradykinin or tapsigargin in rat pheochromocytoma cells (PC12 line) [32]; preincubation of the cells with trifluopera-zine led to a significant suppression of the depot-dependent Ca^{2+} inflow caused by tapsigargin in human embryonic kidney cells (NEK-293 line) [33]. Sigma-1 receptor antagonists (substances BD1063 and BD1047) have been found to inhibit the histamine-induced Ca^{2+} depot-dependent inflow in human saphenous vein endothelial cells [34]; BD1063 significantly suppressed the tapsigargin-induced Ca^{2+} depot-dependent inflow in human breast adenocarcinoma cells (MCF7 line) [35].

Haloperidol is known to inhibit potential-dependent Ca^{2+} channels in cells of different types. Haloperidol and chlorpromazine blocked potential-dependent Ca^{2+} channels in rabbit aortic smooth muscle cells
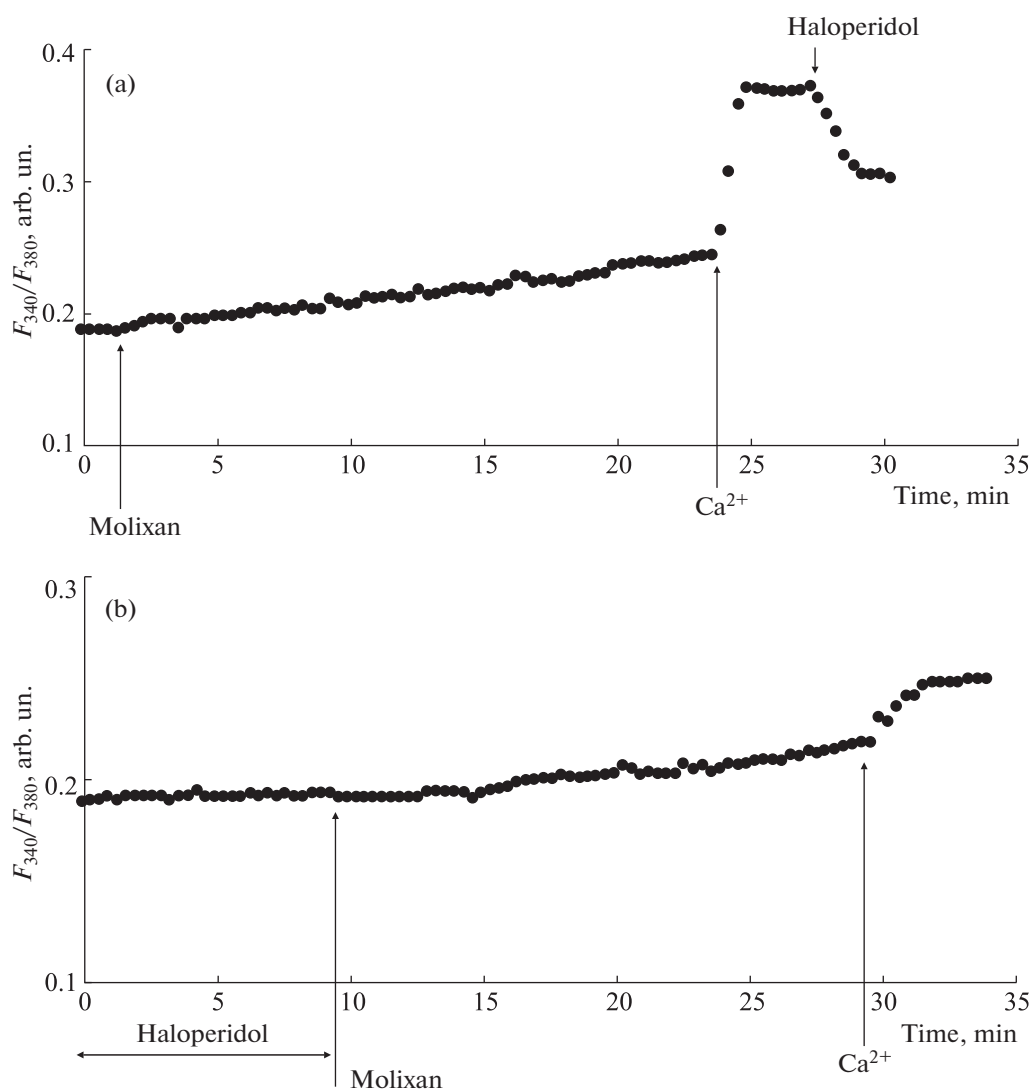
Fig. 2. The effect of haloperidol on the increase of $[Ca^{2+}]_i$ in rat macrophages caused by molixan: (a) cells were incubated for 20 min in the presence of 100 μg/mL of molixan in a nominally calcium-free medium, Ca^{2+} inflow was initiated by the addition of 2 mM Ca^{2+} into the external medium, 30 μg/mL of haloperidol was added under the developed inflow of Ca^{2+}; (b) macrophages were preincubated for 10 min with 30 μg/mL of haloperidol in a calcium-free medium, 100 μg/mL of molixan was added, and after 20 min, the Ca^{2+} inflow was initiated by adding 2 mM Ca^{2+} into the external medium.

[36]. Haloperidol inhibited L-type Ca^{2+} channels in mouse hippocampal neurons [37], rat cardiomyocytes [38], N-, L-, P/Q- and R-type Ca^{2+} channels in sympathetic and parasympathetic rat neurons [39] and T-type Ca^{2+} channels in human embryonic kidney cells (HEK293 line) [40].

The results of this study on the suppression of the sigma-1 receptor antagonist haloperidol Ca^{2+} responses caused by glutoxim and molixan in macrophages indicate the possible participation of sigma-1 receptors in a complex signaling cascade triggered by glutoxim or molixan and leading to an increase in $[Ca^{2+}]_i$ in rat peritoneal macrophages. The results also indicate that the combined use in clinical practice of the drugs glutoxim or

molixan and the neuroleptic haloperidol is not desirable.

Our data also showed the participation of sigma-1 receptors in the regulation of depot-dependent Ca^{2+} inflow induced by disulfide-containing immunomodulators and inhibitors of endoplasmic Ca^{2+}-ATPases in rat peritoneal macrophages; this allowed us to consider sigma-1 receptors as a new regulatory component of the signal complex of depot-dependent Ca^{2+} inflow in macrophages. Sigma-1 receptors can influence the depot-dependent Ca^{2+} inflow by modulating the binding between the main components of the depot-dependent Ca^{2+} inflow protein complex,
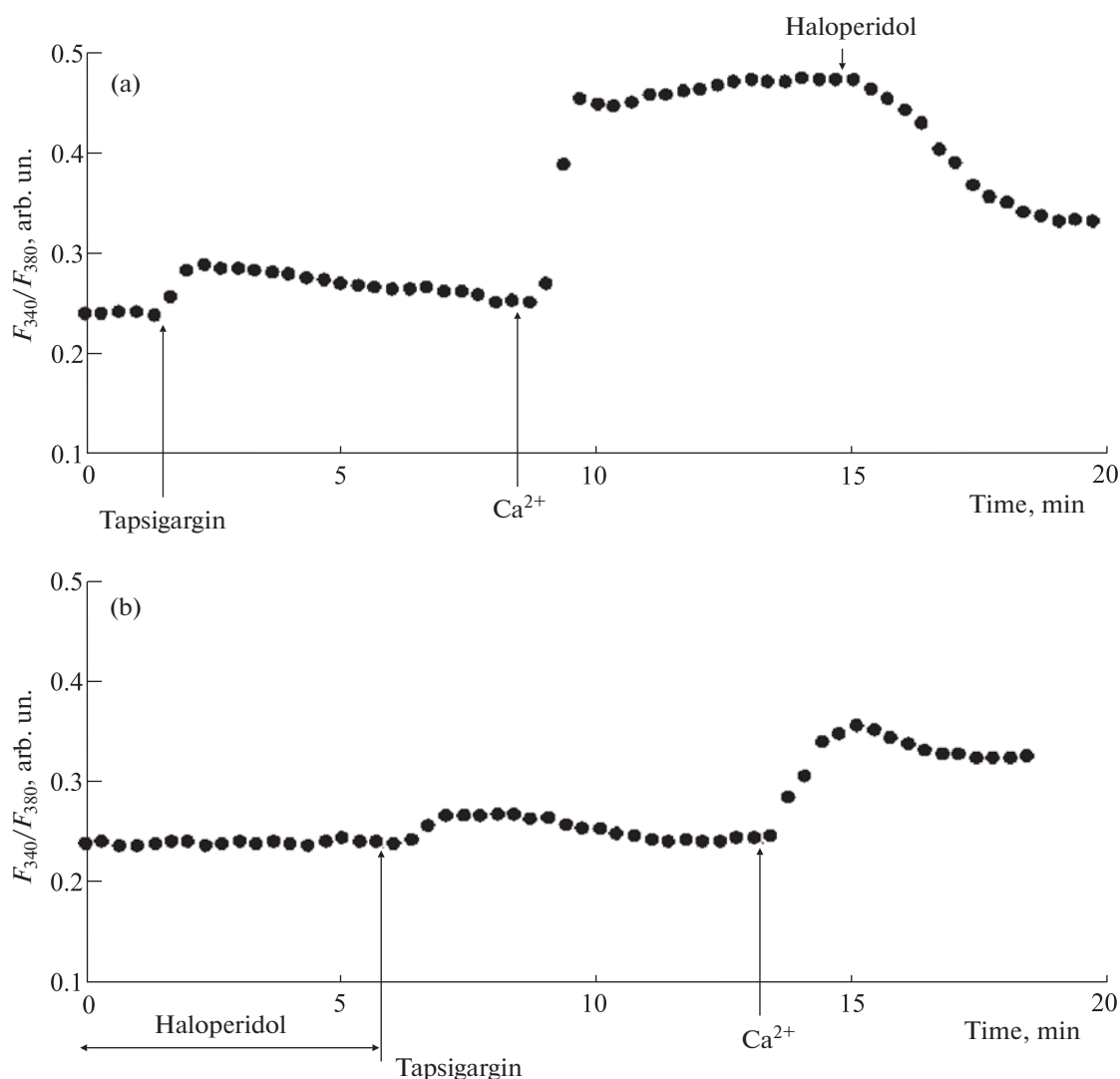
Fig. 3. The effect of haloperidol on Ca^{2+} responses induced by tapsigargin in rat peritoneal macrophages: (a) macrophages were stimulated with 0.5 μM of tapsigargin in a nominally calcium-free medium, Ca^{2+} inflow was initiated by the addition of 2 mM Ca^{2+} into the external medium, 30 μg/mL of haloperidol was added under the developed depot-dependent Ca^{2+} inflow; (b) macrophages were preincubated for 10 min with 30 μg/mL of haloperidol in a calcium-free medium, 0.5 μM of tapsigargin were added, after which the Ca^{2+} inflow was initiated by adding 2 mm Ca^{2+} into the external medium.

STIM1 proteins in the membrane of the endoplasmic reticulum and Orai1 in the plasmalemma [12].

The results obtained may also contribute to a more detailed understanding of the molecular mechanisms of pharmacological effects of first-generation neuroleptics. In addition, these data may be relevant for the treatment of diseases mediated by impaired functioning of sigma-1 receptors. Indeed, it is known that changes in subcellular localization, expression and signaling functions of sigma-1 receptors led to the development of a wide range of human diseases [2–5]. The involvement of these receptors in the pathophysiology of neuropsychiatric (schizophrenia, anxiety disorders, depressive states and dementia), neurodegen-

erative (Alzheimer's, Huntington's and Parkinson's diseases, amyotrophic lateral sclerosis), oncological and cardiovascular diseases, pain syndromes and retinopathy was revealed [2–5, 10, 41, 42]. This makes it possible to consider sigma-1 receptors as promising pharmacological targets for the treatment of these diseases.

Recently, the possible role of sigma-1 receptors in the pathophysiology of coronavirus infection (COVID-19) has been studied. There is evidence that sigma-1 receptors may be a promising therapeutic target in the treatment of patients with COVID-19. It is believed that sigma-1 receptors regulate the key mechanisms of adaptive stress response of host cells and
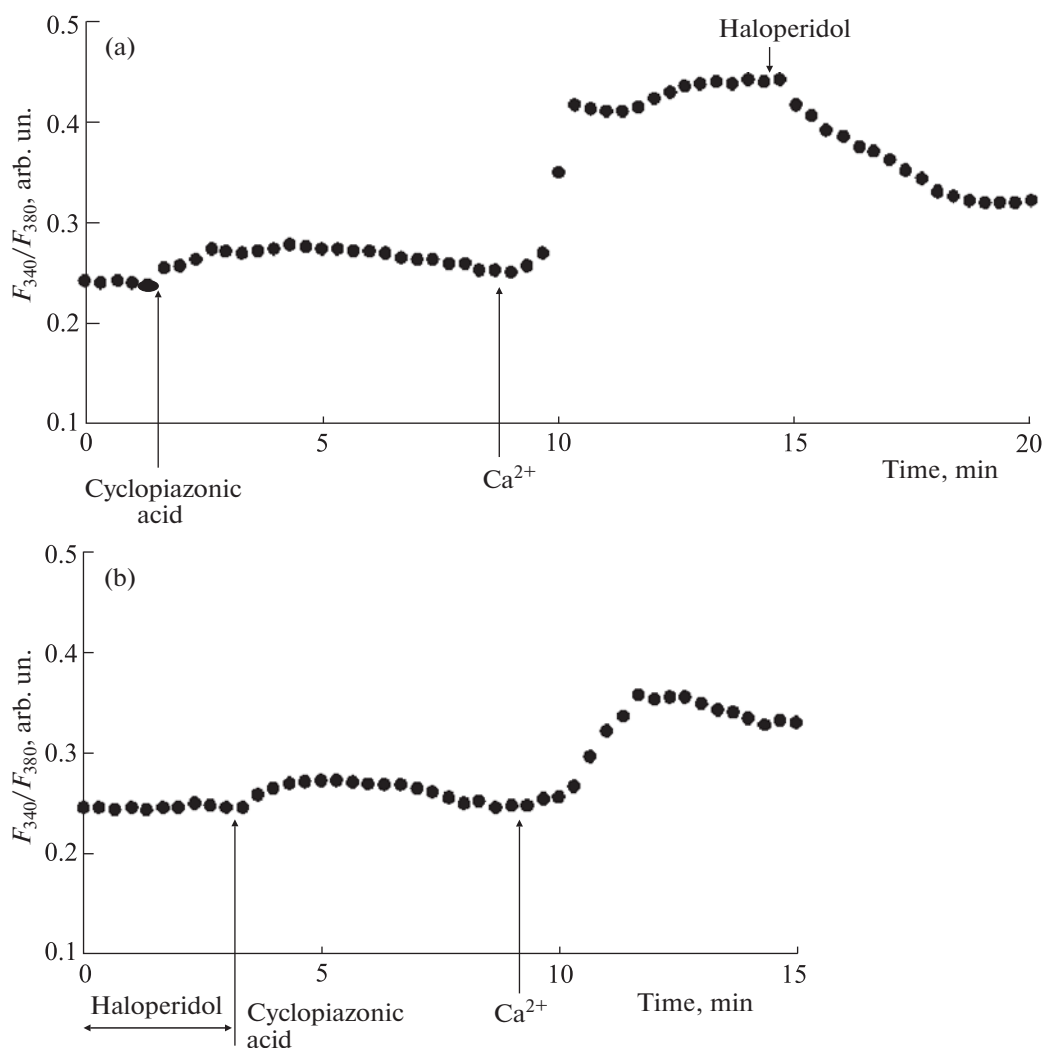
Fig. 4. The effect of haloperidol on Ca^{2+} responses induced by cyclopiazonic acid in rat peritoneal macrophages: (a) macrophages were stimulated with 10 µM of cyclopiazonic acid in a nominally calcium free medium, Ca^{2+} inflow was initiated by adding 2 mM Ca^{2+} into the external medium, 30 µg/mL of haloperidol was added under the developed depot-dependent Ca^{2+} inflow; (b) macrophages were preincubated for 10 min with 30 µg/mL of haloperidol in a calcium-free medium, 10 µM of cyclopiazonic acid were added, after which the inflow of Ca^{2+} was initiated by the addition of 2 mM Ca^{2+} into the external medium.

participate in the early stages of virus replication [43, 44]. It was found that the NSP6 protein of the SARS-CoV-2 virus interacts with sigma-1 receptors, which play an important role in the regulation of endoplasmic reticulum stress [45].

Many repurposed drugs included in the complex therapy regimens of patients with COVID-19 were identified as sigma-1 receptor ligands. These include the neuroleptics haloperidol, chlorpromazine and trifluoperazine [43, 46]. They have been shown to effectively inhibit the replication and growth of the SARS-CoV-2 virus [45]. Haloperidol had significant antiviral activity, it could bind strongly to the NSP6 protein of the SARS-CoV-2 virus [47] and inhibited the replication of SARS-CoV-2 in human lung epithelial cells (Calu-3 line) and monkey cells (VeroE6 line) [45, 48].

Sigma-1 receptor ligands haloperidol [49] and chlorpromazine [50] have already been clinically tested as drugs for the treatment of patients with COVID-19.

It is also known that viruses have developed mechanisms of disruption of Ca^{2+} homeostasis of host cells and increase $[Ca^{2+}]_i$, since Ca^{2+} is necessary for the virus to enter the cell, for replication, maturation and release of the virus [51, 52]. In this regard, blocking the virus-induced increase in $[Ca^{2+}]_i$ by inhibiting calcium release channels in the membrane of the endoplasmic reticulum (inositol-1,4,5-triphosphate receptors and ryanodine receptors) or Ca^{2+} inflow channels in the plasmalemma (potential-dependent and depot-dependent Ca^{2+} channels) is one of the approaches in the therapy of viral infections [52]. It was found that

blockers of potential-dependent Ca^{2+} channels nifedipine and amlodipine reduced mortality and decreased the risk of the need for artificial lung ventilation in elderly patients with COVID-19 and hypertension [53, 54]. It has also been shown that intravenous administration of the depot-dependent Ca^{2+} channel blocker Auxora to patients with severe pneumonia with COVID-19 stabilized the lung endothelium and inhibited the release of proinflammatory cytokines, thereby significantly facilitating and accelerating the recovery of patients [55, 56].

Thus, our data on the suppression of the sigma-1 receptor antagonist haloperidol of both phases of Ca^{2+} responses induced by disulfide-containing immunomodulators and inhibitors of endoplasmic Ca^{2+}-ATPases in rat peritoneal macrophages additionally confirm the versatility of the effects of neuroleptics and indicate in favor of their therapeutic potential as sigma-1 receptor ligands.

FUNDING

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

COMPLIANCE WITH ETHICAL STANDARDS

The animals were kept and all manipulations with them were carried out in accordance with regulatory documents and the requirements of the Order of the Ministry of Health of the Russian Federation No. 267 dated 06/19/03 "On approval of the rules of laboratory practice in the Russian Federation".

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Translated by E. Puchkov

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