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PHYSIOLOGY

# The Possible Involvement of Calcium Ions in the Regulatory Effect of Oxidized Glutathione on Macrophages

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Oxidized glutathione (GSSG) is a low-molecular-weight thiol found in all cells and extracellular space. The GSSG content inside and outside of the cells is small and strictly regulated relative to the content of reduced glutathione (GSH) ($10^{-4}$ to $10^{-5}$ M versus $10^{-2}$ to $10^{-1}$ M GSH) [1]. The role of GSSG in the physiological processes is mainly considered in the aspect of cellular GSH reactions. The GSSG content is increased in cells during different disturbances in cellular functions; therefore, GSSG was originally considered a biologically aggressive molecule [2]. However, studies on the GSSG effect on cells at concentrations close to or higher than the concentrations determined outside the cells showed that GSSG could have a receptor-mediated effect on cellular processes [3–5]. In addition, a synthetic analogue of GSSG, the drug Glutoxim®, is used in clinics as an immunomodulator and hemostimulator for integrated treatment for bacterial and viral deseases [6], psoriasis [7, 8], and radio- and chemotherapy in oncology [9].

The role of $Ca^{2+}$ ions, a ubiquitous secondary messenger, in the regulatory action of GSSG on cells had not been studied. Hence, in this work, we studied the effects of oxidized glutathione and its synthetic analogue Glutoxim® on the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and $Ca^{2+}$ signals induced by the purinergic agonist ATP or the inhibitor of endoplasmic $Ca^{2+}$-ATPases thapsigargin in the native resident peritoneal macrophages in rats. The cultivation of macrophages and automated installation for $[Ca^{2+}]_i$ measurements with the use of the fluorescent probe Fura-2AM was described earlier [10]. The experiments were performed at a room temperature of 20–22°C on the second or third day of cultivation.

We used two experimental approaches. First, we studied the effect of the agents on the $Ca^{2+}$ response induced by ATP or thapsigargin in the macrophages placed into a normal physiological solution. The agents were introduced either before the agonist application or after it, during the plateau phase of the $Ca^{2+}$ signal, which reflects the $Ca^{2+}$ entry from the external medium. In the second variant of experiments, to detect and enhance the $Ca^{2+}$ entry into the cells, we used the following experimental procedure. The macrophages were incubated in a nominally calcium-free medium (0 mM $Ca^{2+}$, 1 mM EGTA) and then treated with one of the agonists to induce $Ca^{2+}$ mobilization from the intracellular stores. Addition of 2-mM $Ca^{2+}$ to the external medium and restoration of the physiological gradient of $Ca^{2+}$ concentration caused a fast $[Ca^{2+}]_i$ increase, which reflected $Ca^{2+}$ entry into the cell. We studied the effect of drugs added prior to the agonist application, before $Ca^{2+}$ application, or during the developed $Ca^{2+}$ entry from the external medium. Glutoxim® or GSSG was introduced to the incubation medium of macrophages at concentrations of 10, 100, 200, 300, 400, 500, and 600 μg/ml.

The addition of ATP or thapsigargin to peritoneal macrophages in a normal physiological solution induced a biphasic $Ca^{2+}$ signal: a relatively fast peak caused by $Ca^{2+}$ mobilization from the store and a long phase reflecting the $Ca^{2+}$ entry from the external medium [10, 11]. Application of 200 μM ATP induced $[Ca^{2+}]_i$ increase from a basal level of 75 ± 18 nM to a peak of 910 ± 105 nM. Afterwards, we observed a slow of decreasing plateau phase, which, 4 min after the ATP addition, corresponded to an average $[Ca^{2+}]_i$ of 460 ± 115 nM. The treatment with 0.5 μM thaspigargin induced a $[Ca^{2+}]_i$ increase to 450 ± 90 nM.

Figure 1 shows the effect of Glutoxim® (100 μg/ml) on the $[Ca^{2+}]_i$ in resting cells and $Ca^{2+}$ signals induced by 200 μM ATP (Figs. 1a, 1b) and 0.5 μM thapsigargin (Fig. 1c) in the macrophages incubated in a normal physiological solution (Fig. 1a) or nominally calcium-free medium (Figs. 1b, 1c). It can be seen in Fig. 1a that preincubation of the cells with Glutoxim® in the $Ca^{2+}$-

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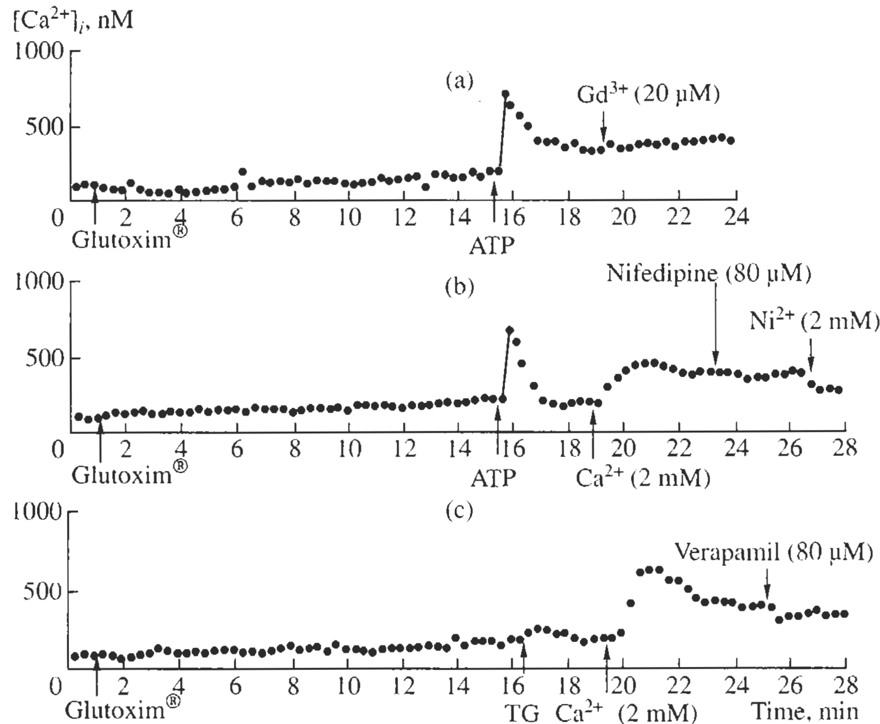


**Fig. 1.** The effect of Glutoxim® (100 μg/ml) on $[Ca^{2+}]_i$ in resting cells and $Ca^{2+}$ signals induced by (a, b) 200 μM ATP or (c) 0.5 μM thapsigargin (TG) in macrophages. Here and in Figs. 2 and 3, each recording was performed for a group of 40–50 cells and represents a typical variant of 3–7 experiments.

containing medium 15 min before ATP application resulted in a certain increase in the basal $Ca^{2+}$ level and a decrease (by 20–30%) in the ATP-induced phase of $Ca^{2+}$ mobilization from the store. The increase in the $[Ca^{2+}]_i$ after the application of Glutoxim® may have been determined by both the $Ca^{2+}$ mobilization from the intracellular $Ca^{2+}$ store and $Ca^{2+}$ entry from the external medium. To establish the mechanism of $[Ca^{2+}]_i$ increase, we performed experiments in the nominally calcium-free medium (Figs. 1b, 1c). We found that, under these conditions, Glutoxim® brought about a $[Ca^{2+}]_i$ increase and a subsequent decrease (by 20–30%) in the phase of $Ca^{2+}$ mobilization from the store induced by ATP (Fig. 1b) or thapsigargin (Fig. 1c). This indicates that $[Ca^{2+}]_i$ increase during glutoxim action is determined by $Ca^{2+}$ mobilization from the intracellular $Ca^{2+}$ store. Similar data were obtained with the use of GSSG (Fig. 2).

Note that we did not find any dose dependence of the effect of glutoxim or GSSG on $[Ca^{2+}]_i$. The agents concentrations studied had practically the same effect on $[Ca^{2+}]_i$ in resting cells and $Ca^{2+}$-signals induced by ATP or thapsigargin in the macrophages. However, more prolonged (for 30 min) incubation of the cells in the presence of glutoxim or GSSG induced a larger $[Ca^{2+}]_i$ increase.

The fact that the $[Ca^{2+}]_i$ increase evoked by Glutoxim® or GSSG is determined by $Ca^{2+}$ mobilization from the store is confirmed by the data shown in Fig. 3. Addition of 2-mM $Ca^{2+}$ to the external medium of the cells preincubated for 15 min in the presence of Glutoxim® (Fig. 3a) or GSSG (Fig. 3b) induced $Ca^{2+}$ entry caused, presumably, by $Ca^{2+}$ release from the intracellular store. After emptying the $Ca^{2+}$ store by 0.5 μM thapsigargin, Glutoxim® induced no $[Ca^{2+}]_i$ increase (Fig. 3c), which suggests that Glutoxim® or GSSG induced $Ca^{2+}$ mobilization from the thapsigargin-sensitive $Ca^{2+}$ store.

Earlier, we described the pharmacological properties of the store-operated $Ca^{2+}$ entry into the rat peritoneal macrophages [12, 13]. It was shown that the store-operated $Ca^{2+}$ entry induced by emptying of the $Ca^{2+}$ store by 0.5 μM thapsigargin or 200 μM UTP was blocked by the following pharmacological agents: two structurally different inhibitors of voltage-dependent $Ca^{2+}$ channels (20 μM nifedipine and 40 μM verapamil); the ions $Ni^{2+}$ (1 mM), $La^{3+}$ (1 mM), and $Gd^{3+}$ (10 μM); and the blocker of nonselective cation channels niflumic acid (100 μM).

We also studied the effect of classic organic (nifedipine and verapamil) and inorganic ($Ni^{2+}$, $La^{3+}$, and $Gd^{3+}$) blockers of $Ca^{2+}$ channels on the $Ca^{2+}$ entry induced by ATP or thapsigargin after treatment of the

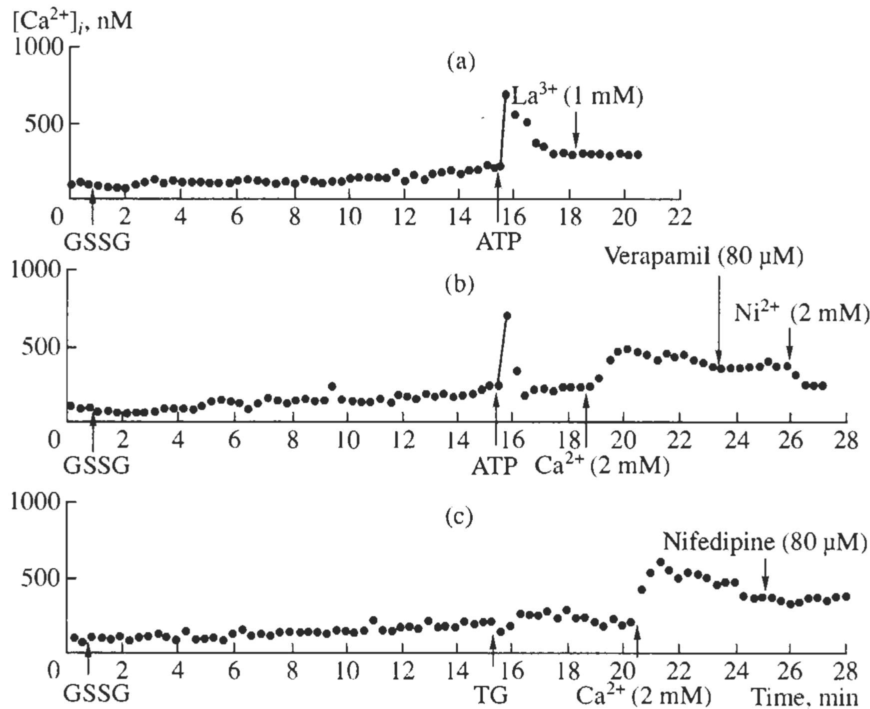


**Fig. 2.** The effect of oxidized glutathione (GSSG) on $[Ca^{2+}]_i$ in resting cells and $Ca^{2+}$ signals induced by (a, b) 200 μM ATP or (c) 0.5 μM thapsigargin in peritoneal macrophages.

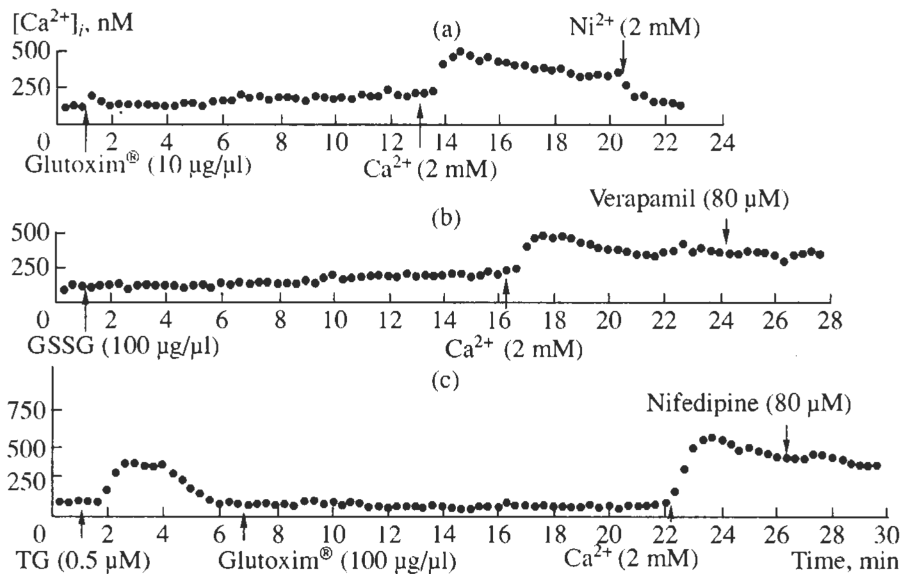


**Fig. 3.** The effects of Glutoxim® and GSSG on $[Ca^{2+}]_i$ in macrophages.

cells with Glutoxim® or GSSG. The data obtained suggest that the agents studied change the pharmacological properties of $Ca^{2+}$ channels in macrophages. The channels become less sensitive to the blocking effect of $Ca^{2+}$ antagonists, such as nifedipine and verapamil, as well as the inorganic inhibitors $La^{3+}$ and $Gd^{3+}$. The only effective blocker was $Ni^{2+}$ ions. Nifedipine and verapamil were ineffective even at a concentration of 80 μM. In many cases, after the application of nifedipine, we observed a paradoxical enhancement of $Ca^{2+}$ entry.

The results obtained demonstrate the effect of extracellular oxidized glutathione and Glutoxim® on the intracellular $Ca^{2+}$ concentration in peritoneal macroph-

ages. However, the mechanism of this effect and the physiological significance of the GSSG-induced effect require further studies. In addition, it is necessary to study the role of $Ca^{2+}$ to explain the pharmacological effectiveness of Glutoxim®.

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