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CELL BIOPHYSICS

Estimation of the Mitochondrial Calcium Pool in Rat Brain Synaptosomes Using Rhod-2 AM Fluorescent Dye

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Abstract—The literature data on the role of synaptic mitochondria in the regulation of the cytosolic calcium level are contradictory. In the present paper calcium storage by mitochondria in rat brain synaptosomes using the fluorescent dye Rhod-2 has been investigated. The addition of 60 mM KCl increases Rhod-2 fluorescence. This effect is completely abolished by replacing K^+ with Na^+ or withdrawing Ca^{2+} from the incubation medium. A proton ionophore, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone, and a mixture of rotenone/oligomycin mitochondrial toxins cause a two-fold decrease in Rhod-2 fluorescence. Thapsigargin, an inhibitor of endoplasmic reticulum ATPase (1 μM), but not bafilomycin, an inhibitor of ATPase in synaptic vesicles (500 nM) also leads to a mitochondrial calcium influx. The addition of calcium to synaptosomes with the retained plasma membrane potential increased Rhod-2 fluorescence; however, this effect is insensitive to carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone. We have shown that mitochondria can serve as a calcium store in synaptosomes only in the case of a high cytosolic concentration of calcium.

Keywords: synaptosomes, Rhod-2, calcium, mitochondria, synapse, endoplasmic reticulum

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INTRODUCTION

Mitochondria are not only bioenergetic stations of the cell but also participate in intracellular signaling [1–3]. Calcium deposition is an important signaling function of mitochondria [1, 2, 4].

The presynaptic terminals of neurons contain mitochondria; moreover, they occupy approximately one-third of the total volume [5]. It has been shown using the Rhod-2 fluorescent probe that mitochondria in synapses of the calyx of Held are able to store calcium [6]. This conclusion has been further supported using hippocampal neurons and the genetically encoded mito-GCaMP2 sensor [7]. Calcium is transported to mitochondria via a uniporter [4, 8]. This transporter has low affinity [1, 3]. The kinetic properties of the calcium uniporter indicate that mitochondria perform the calcium store function only at the excited state of the cell and, therefore, a high level of this ion in the cytosol [1, 3]. Experimental results confirm this point of view [6]. It has also been shown that calcium in synaptic mitochondria cannot initiate the release of neuromediators but regulates endocytosis [7].

Along with this, it has been shown in experiments with synapsotomes that calcium release from mitochondria via the Na^+/Ca^{2+} exchanger plays the key role in the induction of neurotransmitter release upon potassium depolarization [9, 10]. This supposes the presence of calcium in intrasynaptosomal mitochondria and its low concentration in the cytosol. However, the level of intramitochondrial calcium has not been measured in these works. It is known that not only average calcium concentrations in the cytosol but also microdomains that occur near the sites of Ca^{2+} influx or release from stores for a relatively short period are important for calcium signaling [4, 11]. The synapses of the calyx of Held are giant synapses, which makes them very convenient for study. However, their calcium microdomains may remarkably differ from the microdomains in presynaptic terminals, which have a size of approximately 600 nm [5, 12]. In this work, we studied calcium deposition by mitochondria in rat brain synaptosomes with the fluorescent probe 9-[4-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-3-[2[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino] phenoxy]ethoxy]phenyl]-3,6-bis(dimethylamino)xanthylium bromide (Rhod-2 AM).

Rhod-2 is a positively charged fluorescent probe. It can bind calcium but has lower affinity compared to the common Fura-2 and Fluo-3 probes. The major part of Rhod-2 localizes in mitochondria upon the incubation of cells with its acetoxymethyl ester; changes in its fluorescence can be used to estimate the calcium accumulation in mitochondria [13].

As a major object, we used synaptosomes, that is, isolated presynaptic terminals of neurons. These are capable of exocytosis and neurotransmitter release in

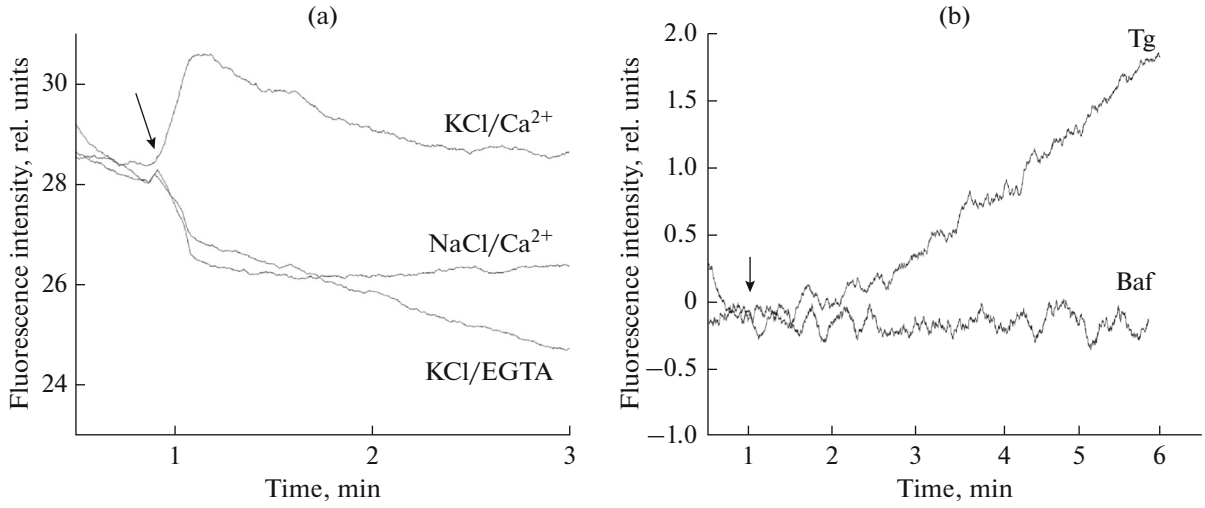

Fig. 1. The effect of (a) potassium depolarization and (b) thapsigargin and bafilomycin on fluorescence of the Rhod-2 probe in synaptosomes. The arrow marks the moment of the addition of supplements into a cuvette: KCl/Ca, 60 mM of KCl were added, incubation mixture contained 2 mM of Ca^{2+}; KCl/EGTA, 60 mM of KCl were added, incubation mixture without Ca^{2+} but with the addition of 10 μM of EGTA; NaCl/Ca, 60 mM of NaCl were added, incubation mixture contained 2 mM of Ca^{2+}; Tg, 1 μM of thapsigargin was added; Baf, 500 nM of bafilomycin A1 were added. Curves represent the results of eight independent measurements.

response to an increase in the cytosolic calcium concentration, have intrasynaptosomal mitochondria, and can maintain the potential of the plasma and mitochondrial membrane [5, 14–17].

MATERIALS AND METHODS

This work was performed using the following reagents: 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) (Merck, Germany); carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and rotenone (Calbiochem, United States); thapsigargin, bafilomycin, oligomycin, Rhod-2 AM, and EGTA (Sigma, United States). All other reagents were of analytical grade.

Synaptosome isolation. Synaptosomes were isolated from rat brain hemispheres according to the Hajos method [18]. The obtained pellet was suspended in medium A with the following ionic composition (in mM): NaCl, 132; KCl, 5; $MgCl_2$, 1.3; NaH_2PO_4, 1.2; glucose, 10; HEPES, 10; pH 7.4. Prior to the experiments the suspension of synaptosomes (protein concentration of 10–20 mg/mL) was preincubated for 10 min at 37°C, placed on ice, and used for experiments for 3 h.

Determination of the calcium concentration in intrasynaptosomal mitochondria. The calcium content in intrasynaptosomal mitochondria was determined with the Rhod-2 fluorescent probe according to [13].

Synaptosomes were additionally washed. The suspension of synaptosomes was incubated for 30 min at 37°C in the presence of 10 μM of the probe. The extracellular probe was eliminated by triple centrifugation. The pellet was suspended in incubation medium A.

In order to measure mitochondrial calcium, 200 μL of synaptosomes were preliminarily loaded with the probe and were added to a cuvette containing 1.8 mL of incubation medium A. Fluorescence was measured in a Cary Eclipse spectrofluorometer (Varian, United States) in a thermostat-controlled cuvette (37°C) at λ_{ex} = 550 nm and λ_{reg} = 571 nm, a width of the slits of 5 nm, and constant mixing. FCCP, rotenone, and oligomycin were added directly into the cuvette.

The protein concentration was determined by the Lowry method [19] with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

At the first stage we studied the effect of a calcium influx to synaptosomes via voltage-dependent calcium channels. Figure 1a shows that potassium depolarization in the presence of calcium resulted in an increase in Rhod-2 fluorescence. The analogous additions of sodium or potassium depolarization in the absence of calcium were inefficient (Fig. 1a).

Apart from calcium entering from the environment, mitochondria can also capture calcium released from intracellular stores [11]. It has been shown that the endoplasmic reticulum can perform the role of a calcium store in synaptosomes [20]. Moreover, this role can be possibly played by synaptic vesicles in presynaptic terminals of neurons [21, 22]. We studied the effect of thapsigargin, an inhibitor of Ca ATPase, which is localized on the membrane of the endoplasmic reticulum, and bafilomycin, an inhibitor of H^+

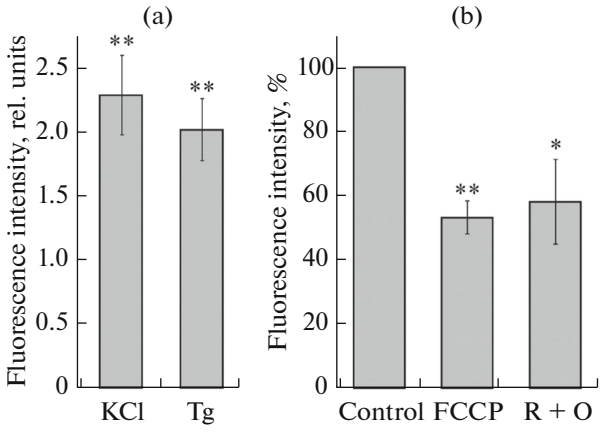

Fig. 2. The effect of (a) thapsigargin and (b) mitochondrial toxins on the increase in Rhod-2 fluorescence in synaptosomes induced by potassium depolarization: KCl, 5 min after the addition of 60 mM KCl; Tg, 5 min after the addition of 1 μM thapsigargin; C, control, 5 min after the addition of 60 mM KCl; FCCP, 4 min after the addition of 60 mM KCl, incubation mixture contains 10 μM FCCP; R , 4 min after the addition of 60 mM KCl, incubation mixture contains 10 μM of rotenone and 5 μg/mL of oligomycin. An increase in fluorescence 5 min after the addition of 60 mM KCl is taken as 100%. $*P \le 0.05$ in respect to the control (response to KCl without mitochondrial toxins); ** $P \le 0.01$ in respect to (a) zero or (b) the control. The response to KCl without mitochondrial toxins is taken as the control in (b). The data represent the mean values of eight experiments ± *Sx*.

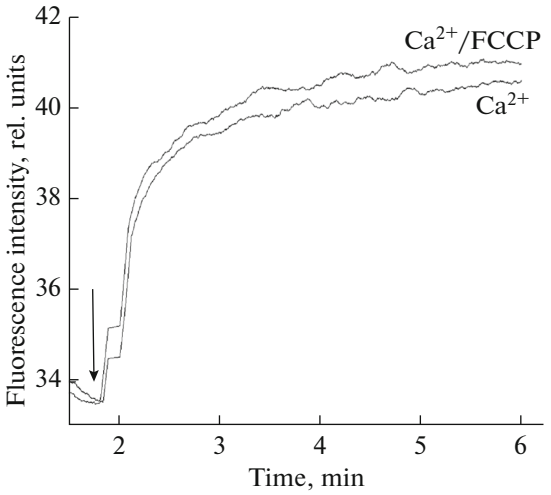

Fig. 3. The effect of calcium on Rhod-2 fluorescence in polarized synaptosomes. The arrow marks the moment of the addition of 2 mM $CaCl_2$ into the cuvette. Ca/FCCP, 10 μM FCCP was additionally added to the incubation mixture. The curves represent the results of five independent measurements.

ATPase, which is located on the membranes of synaptic vesicles. It can be seen in Fig. 1b that thapsigargin but not bafilomycin causes an increase in Rhod-2 fluorescence. The bafilomycin concentration (500 nM) was sufficient for complete inhibition of the proton ATPase [17]. The increase in fluorescence was statistically significant; moreover, the effect of thapsigargin was comparable with the effect of potassium depolarization (Fig. 2a).

We then studied the effect of the proton ionophore FCCP and a mixture of mitochondrial toxins rotenone and oligomycin on the increase in the probe fluorescence induced by potassium depolarization. These substances lower the membrane potential of intrasynaptosomal mitochondria and practically "switch them off" [16, 23]. It can be seen from Fig. 2b that the preliminary treatment of synaptosomes with mitochondrial poisons partially blocked the effect of high potassium concentrations. Consequently, at least a part of the signal is of mitochondrial nature.

At the following stage we studied the effect of calcium added to synaptosomes that retained the potential of the plasma membrane. In this case, synaptosomes were in the standard incubation medium containing 5 mM of K^+. It can be seen in Fig. 3 that in this case calcium caused an increase in Rhod-2 fluorescence; however, it was not inhibited by the proton ionophore FCCP. We can suppose that in this case the signal is connected with the probe in the cytosol or even the external medium but not in intrasynaptosomal mitochondria.

Consequently, we showed that the fluorescent probe Rhod-2 AM can be used for the estimation of the calcium mitochondrial pool in synaptosomes. However, we should note that the probe may not completely localize in mitochondria and the cytosolic form may have an input to the increase in fluorescence (Fig. 2b). A similar distribution of the probe has also been described by other authors [24].

We showed that mitochondria are calcium stores in synaptosomes upon the excessive influx of calcium from the external medium via voltage-dependent channels or release of this ion from the endoplasmic reticulum (Figs. 1 and 2). We were not able to prove that mitochondria can be calcium stores upon its release from synaptic vesicles or a normal concentration of this ion in the cytosol (Figs. 1b and 3).

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