ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXI, N 2, 69, 2008 SECTIO DDD DOI: 10.2478/v10080-008-0147-1

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Flow cytometric analysis of Ca^{2+}-induced changes of membrane potential in smooth muscles mitochondria

Analiza indukowanych jonami wapnia zmian potencjału błonowego mitochondriów mięśni gładkich z zastosowaniem cytometrii przepływowej

Mitochondrial calcium transport is of fundamental importance in controlling intracellular calcium transients and correspondingly in calcium signaling in the cytosol [1–3]. Accumulation of Ca^{2+} in these organelles is provided by the functioning of Ca^{2+} uniporter, whose activity depends on the value of Ca^{2+} gradient and membrane potential. Release of Ca^{2+} from mitochondria is realized by the cation exchange through the mechanism of Ca^{2+}-H^+ and/or Ca^{2+}-Na^+ antiporter. Recently an important effect of the mitochondrial permeability transition pore was found, which can control the transmembrane Ca^{2+}-exchange, being sensible to the blocking influence of cyclosporin A in mitochondria [2, 3]. As is known, dysfunction of mitochondrial transmembrane Ca^{2+}-exchange is very dangerous both in cellular bioenergetics and intracellular signaling; it could cause harmful pathology [3]. Therefore, in clinical and research practice, it is very important to have express and sensitive methods for diagnostics of possible mitochondrial dysfunctions and corresponding corrections by pharmaceutical manipulations [4, 5].

The purpose of this study was to develop a flow cytometric protocol for the analysis of membrane potential and Ca^{2+} level in isolated myometrium mitochondria.

MATERIAL AND METHODS

Mitochondria were isolated from nonpregnant rat myometrium by the method of differential centrifugation [6]. The content of protein in mitochondrial fractions was determined by the Bradford method [7]. The concentration of mitochondrial protein was 50μg/ml. In experiments, the flow cytometer COULTER EPICS XL™ (Beckmam Coulter, USA) and the SYSTEM II™ Software (Beckmam Coulter, USA) were used.

Registration of the membrane potential was done using the potential-sensitive fluorescent probe TMRM (tetramethylrhodamine-methyl-ester, λ_{ex}=488 nm, λ_{fl}=590 nm; 100 nM) in the incubation medium containing (mM): 20 HEPES (pH= 7.4), 250 sucrose, 0.1 P_i (K^+-phosphate buffer, pH=7.4), 0.5 $MgCl_2$, and 5 sodium succinate. The data are presented as an increase of the TMRM fluorescence intensity relative to the basal level at the presence of 1 μM CCCP. Free Ca^{2+} level registration was done using the fluorescent probe fluo-3AM (λ_{ex}=488 nm, λ_{fl}=520 nm) in the incubation medium containing (mM): 20 HEPES (pH=7.4), 250 sucrose, 2 P_i (K^+-phosphate buffer, pH=7.4), 3 $MgCl_2$, 3 ATP, and 5 sodium succinate. The loading of fluo-3AM into mitochondria was conducted during

30 min at 37^{0}C. The data are presented as an increase of the fluo-3AM fluorescence intensity relative to the basal level at the presence of 1 mM EGTA and 5 μM A23187.

RESULTS AND DISCUSSION

In order to determine the purity, isolated mitochondria preparations were stained with the fluorescent mitochondrial marker NAO (λ_m=525 nm). In all studied preparations, more than 95% of the events were NAO-positive.

Using the potential-sensitive fluorescent probe TMRM, the changes of membrane potential of isolated mitochondria were studied. As shown in Fig, 1, the mitochondrial membranes were polarized, and an addition of protonophore CCCP (1 μM) into the incubation medium produced depolarization of mitochondrial membranes (curves 1 and 3, respectively). The addition of Ca^{2+} (100 μM) also induced the mitochondrial membrane depolarization (curve 2) if compared with the control (curve 1).

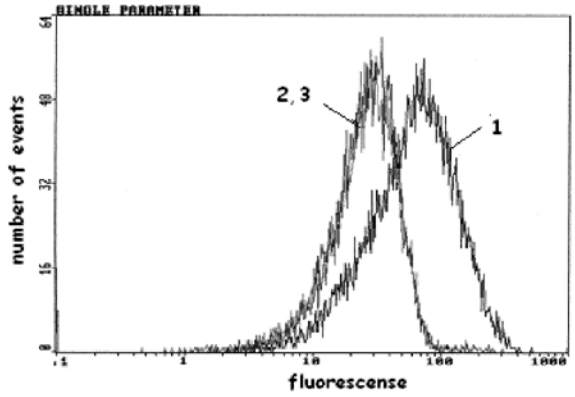

Fig. 1. Registration of mitochondrial membrane potential using fluorescent probe TMRM (100 nM), 1 – control, 2 – Ca^{2+} (100 μM), 3 – CCCP (1 μM)

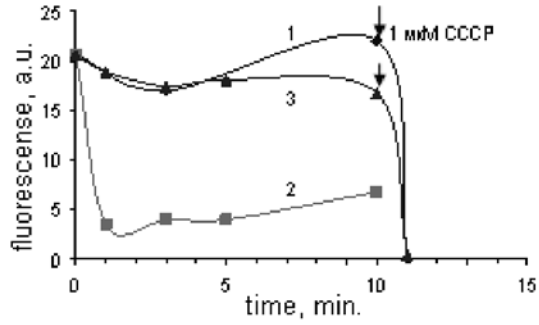

Fig. 2. Kinetics of mitochondrial membrane potential changes with TMRM probe (100 nM); 1 – control, 2 – Ca^{2+} (100 μM), 3 – Ca^{2+} (100 μM), Mg^{2+} (3 mM) and ATP (3 mM)

The analysis of the TMRM fluorescence revealed the stability of the mitochondrial membrane potential in time (Fig. 2, curve 1). The addition of Ca^{2+} (100 μM) caused the membrane depolarization

that culminated within three minutes of incubation (curve 2). At the initial presence of Mg^{2+} (3 mM) and ATP (3 mM), the Ca^{2+} (100 μM) addition did not cause the membrane depolarization (curve 3). Using the fluorescent probe fluo-3AM, the free Ca^{2+} level in isolated mitochondria was studied. As shown in Fig. 3, the Ca^{2+} (1 mM) addition at the presence of Ca^{2+}-ionophore A23187 (5 μM) led to increase of the fluo-3AM fluorescence (curve 3) relative to the control (curve 1). At the presence of A23187 (5 μM) only, no changes of fluorescence intensity were seen in comparison with the control (curve 2).

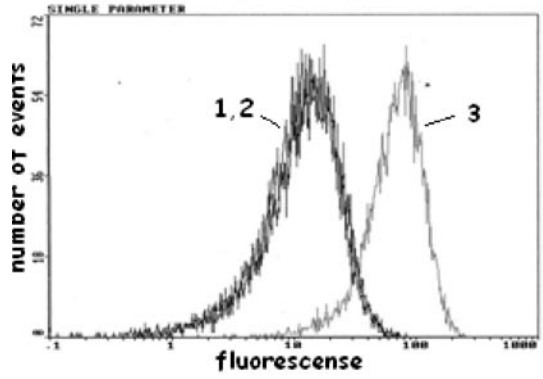

Fig. 3. Registration of free Ca^{2+} level using fluorescent probe fluo-3AM (3 μM);
1 – control, 2 – A23187 (5 μM), 3 – A23187 (5 μM) plus Ca^{2+} (1 mM)

According to the data in Fig. 4, accumulation of Ca^{2+} in mitochondria was fairly rapid and reached the plateau during five-minutes initial period (curve 1). The addition of Ca^{2+}-ionophore A23187 together with EGTA after 10 minutes of incubation led to the rapid Ca^{2+} release from mitochondria (curve 1). At the presence of protonophore CCCP, no changes were detected as compared to the control (curves 2 and 3, correspondingly).

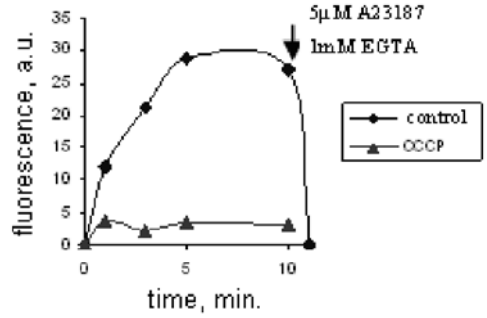

Fig. 4. Kinetics of Ca^{2+} level changes with fluo-3AM probe (3 μM); 1 – control, 2 – CCCP (1 μM)

CONCLUSIONS

In this work we proved the importance of express and sensitive methods for monitoring of Ca^{2+} transport in intact mitochondria of smooth muscles with the aim of diagnostics of possible mitochondrial dysfunctions and corresponding corrections by pharmaceutical manipulations. We demonstrated that the changes of membrane potential and Ca^{2+} level in isolated mitochondria can be

registered by flow cytometry using fluorescent potential-sensitive TMRM and Ca^{2+}-sensitive fluo-3AM probes.

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SUMMARY

A flow cytometric protocol for the analysis of membrane potential and Ca^{2+} level in isolated myometrium mitochondria was developed. Ca^{2+} addition induced the mitochondrial membrane depolarization. At the presence of Mg^{2+} and ATP, the Ca^{2+} addition did not cause the membrane potential dissipation. It was demonstrated that the flow cytometry is a useful tool to analyze Ca^{2+} level and membrane potential in isolated myometrium mitochondria.

STRESZCZENIE

Stworzono metodę analizy potencjału błonowego i poziomu wapnia w wyizolowanych mitochondriach mięśniówki macicy na bazie cytometrii przepływowej. Dodatek wapnia wywoływał depolaryzację błony mitochondrialnej. W obecności magnezu i ATP dodatek wapnia nie powodował rozproszenia się potencjału błonowego. Wykazano, że cytometria przepływowa jest użytecznym narzędziem w analizie poziomów wapnia w wyizolowanych mitochondriach mięśniówki macicy.