FEBS Letters 348 (1994) 211–215

FEBS 14229

Mitochondrial calcium spiking: a transduction mechanism based on calcium-induced permeability transition involved in cell calcium signalling

François Ichas[a,*], Laurence S. Jouaville[a], Sergueï S. Sidash[a,b], Jean-Pierre Mazat[a], Ekhson L. Holmuhamedov[b,c]

[a]*Groupe d'Étude des Systèmes Biologiques Intégrés, D(BM)$_2$, Université Bordeaux II, 33076 Bordeaux Cedex, France*
[b]*Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences, 142292 Pushchino, Russian Federation*
[c]*The Upjohn Laboratories, Kalamazoo, Michigan 49007, USA*

Received 25 May 1994

Abstract

We report reversible Ca^{2+}-induced Ca^{2+} release from mitochondria, which takes the form of Ca^{2+} spikes. Mitochondrial Ca^{2+} spiking is an all-or-none process with a threshold dependence on both the frequency and the amplitude of the Ca^{2+} pulses used as stimuli. This spiking relies on the transient operation of the mitochondrial permeability transition pore, and is initiated – in a threshold-dependent manner – with inositol-triphosphate-mediated Ca^{2+} responses in permeabilized cells. Evidence that mitochondrial Ca^{2+}-induced Ca^{2+} release contributes to inositol-triphosphate-mediated Ca^{2+} responses in intact cells is also reported.

Key words: Calcium-induced calcium release; Mitochondrion; Permeability transition pore; Cyclosporin A; Cell calcium signalling; Inositol triphosphate

1. Introduction

Intracellular Ca^{2+} release is a convergence point for many receptor-mediated cell signals [1]. Ca^{2+} oscillations and waves are considered important events in cell signalling and may encode information relevant to the cell [2]. In these processes Ca^{2+}-induced Ca^{2+} release (CICR) appears to play a fundamental role [3]. Mitochondria are capable of CICR; however, mitochondrial Ca^{2+} release upon Ca^{2+} loading in vitro has historically been characterized as a non-specific and irreversible harmful process. Moreover, the free Ca^{2+} concentrations required to load mitochondria were thought to be unreachable in cells, except in lethal situations [4]. Contrary to this view, Rizzuto and co-workers have shown that mitochondria accumulate Ca^{2+} in cells during activation of the Ca^{2+}-linked transduction pathways [5]. In addition, studies concerning the divalent cation excitability of mitochondria have shown a possible self-reversal of the mitochondrial permeability induced by strontium [6] and oscillating operation of the mitochondrial permeability transition pore (mPTP) after Ca^{2+} stimulation [7]. These results address the question of whether mitochondria can act as a CICR pool during cell calcium signalling. Thus, we have examined isolated mitochondria for a self-reversible CICR behaviour, and determined whether this behaviour could be observed in cells during agonist-induced Ca^{2+} responses.

2. Materials and methods

2.1. Mitochondria

Rat liver mitochondria were isolated according to a standard method [8]. For each experiment, mitochondria were suspended (2 mg/ml protein) in a medium containing 30 mM sucrose, 10 mM Trizma base (pH 7.5), 5 mM succinate, 0.1 mM ATP and 25 μM $CaCl_2$. Low osmolarity medium and ATP were found to promote steady mitochondrial Ca^{2+} spiking. However, a comparable behaviour can be observed in media without ATP or of higher osmolarity (up to 150 mOsM) (unpublished data). Measurements were made at 30°C under stirring on 2 ml aliquots in the multi-channel chamber of an ESON-6ch computerized analyzer (Mutual Data Inc., Moscow). Free Ca^{2+} and K^+ were measured with calibrated mini-electrodes (NICO, Moscow) and the mitochondrial membrane electric potential either with a tetraphenylphosphonium (TPP^+)-sensitive electrode (NICO) in the presence of 2 μM TPP^+ (data not shown) or with the fluorimetric channel of the analyzer using rhodamine 123 (5 μg/ml) fluorescence at the 490–535 nm excitation–emission (*x-m*) pair. These 3 parameters were monitored simultaneously. Specific conditions are described in the corresponding figure legends (Figs. 1 and 2).

2.2. Permeabilized cells

Erhlich ascites tumor cells were cultured, harvested and digitonin-permeabilized according to [9]. Cells were resuspended at high concentration ($20 \cdot 10^6$/ml) in a medium containing 100 mM KCl, 30 mM NaCl, 5 mM PPi, 1 mM NaH_2PO_4, 10 mM succinate, 30 mM HEPES (pH 7.3), 1 mM ATP, 25 mM phosphocreatine and 25 $U \cdot ml^{-1}$ creatine phosphokinase. Measurement of free Ca^{2+} was performed at 37°C on 2 ml aliquots under stirring using Fluo 3 (2 μM) fluorescence (490–535 nm *x-m* pair) with the ESON-6ch. Calibration was performed by calculation from F_{max} and F_{min} values. Specific conditions are described in the corresponding figure legend (Fig. 3).

*Corresponding author. Fax: (33) 56 99 03 80.
E-mail: iuchas@hippocrate.u-bordeaux2.fr

Abbreviations: CICR, calcium-induced calcium release; mCICR, mitochondrial calcium-induced calcium release; mPTP, mitochondrial permeability transition pore; $InsP_3$, inositol triphosphate; ER, endoplasmic reticulum; Cys A, cyclosporin A; PSC 833, SDZ PSC 833; TPP^+, tetraphenylphosphonium; Ant A, antimycin A.

SSDI 0014-5793(94)00615-3

2.3. Intact cells

Intact Fura-2AM-loaded Erlich cells were prepared as in [9]. Fura-2AM was prefered to Fluo-3AM due to a better cell retention of the dye. Cells were suspended ($7 \cdot 10^6$/ml) in Dulbecco's PBS (Gibco) supplemented with 5 mM Na-pyruvate (pH 7.4) and fluorescence was measured at the 340–510 nm *x-m* pair (according to [10]) at 37°C under stirring in a SFM25 (Kontron) fluorimeter on 1 ml aliquots. Calibration was performed by calculation from F_{max} and F_{min} values. Specific conditions are described in the corresponding figure legends (Figs. 3 and 4).

2.4. General

Traces presented are representative of at least 3 experiments performed on at least 2 different batches. All chemicals used were from Sigma, except TPP^+ (Aldrich), cyclosporin A (Cys A) and SDZ PSC 833 (PSC 833) (gifts from Sandoz, Basel, Switzerland). Cys A and PSC 833 (bases) were dissolved in absolute ethanol. The Ehrlich cell line was generously provided by Dr. F. Lavelle (Rhône-Poulenc Rorer).

3. Results and discussion

Free Ca^{2+} concentration was monitored continuously in mitochondrial suspensions, while Ca^{2+} stimulations were performed using Ca^{2+} bolus additions (pulses) at regular time intervals. Ca^{2+} added during each pulse is rapidly accumulated by mitochondria and [Ca^{2+}] steadily returns to the basal resting level (Fig. 1a–c) until a certain degree of Ca^{2+} loading is reached; at this time (Fig. 1d) a large release of Ca^{2+} occurs which is immediately followed by a reaccumulation. We refer to this large Ca^{2+} transient as a mitochondrial Ca^{2+} spike. This spike appears as an all-or-none and threshold-dependent response of mitochondria towards Ca^{2+} stimuli. By varying the amount of Ca^{2+} added during each pulse and the time interval between pulses, we found that the threshold of the mitochondrial Ca^{2+}-induced Ca^{2+} release (mCICR) depends on both the total amount of Ca^{2+} added during the stimulation sequence and the frequency of the pulses (Fig. 1e,f). At higher frequency, fewer pulses (Fig. 1e) and thus, a smaller Ca^{2+} loading (Fig. 1f), were needed to trigger mCICR. This suggests that mitochondria could respond differently depending on the speed and the

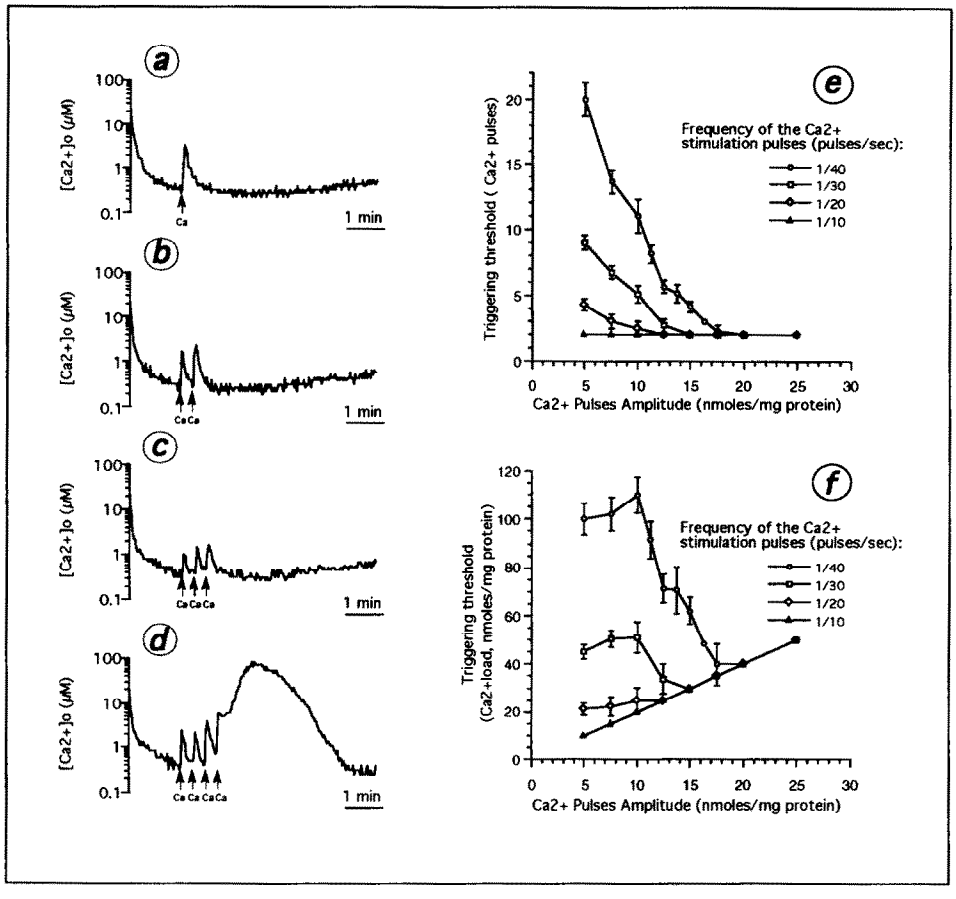

Fig. 1. Calcium spiking of isolated mitochondria is an all-or-none and threshold-dependent response towards Ca^{2+} Stimuli. (a–d) Mitochondrial suspensions were stimulated with exogenous Ca^{2+} pulses (arrows) of 24 nmol Ca^{2+} at 20 s time intervals, and extramitochondrial free Ca^{2+} concentration was monitored. One (a), two (b), or three (c) pulses are quickly buffered by mitochondrial uptake as shown by a steady return to the basal Ca^{2+} concentration. In panel d, the 4th pulse triggers a massive calcium release, spontaneously followed by a reaccumulation phase leading to the restoration of the resting Ca^{2+} level. In panel e, the threshold number of pulses triggering a spike is plotted against the amplitude of the pulses. Each curve corresponds to a defined frequency of stimulation (see key). Panel f shows that the threshold Ca^{2+} loading of mitochondria is not constant, but varies with the frequency and the amplitude of stimulation (see key). Data plotted in panels e and f, are means ± S.D. corresponding to at least 5 measurements.

amplitude of cytosolic Ca^{2+} increases, either by buffering the cytosolic [Ca^{2+}] rise without further variation (sub-threshold), or by generating a Ca^{2+} spike (supra-threshold). This feature is equivalent to a gate-controlling transducing process capable of transforming continuous Ca^{2+} signals into discrete ones (mCICR units), or of transforming the frequency of periodical signals.

Fig. 2 shows that the occurence of a mitochondrial Ca^{2+} spike is dependent on the transient operation of the mPTP [4,11]. The mPTP involvement is evidenced by: (i) a transient, non-specific permeability of the inner mitochondrial membrane which occurs during the spike, i.e. permeability increases for Ca^{2+}, K^+ and protons (Fig. 2a), and (ii) the mCICR inhibition by Cys A (4 μM), a specific inhibitor of the mPTP [12], concomitant with the inhibition of the K^+ efflux and H^+ influx (Fig. 2b). Further, PSC 833 (a Cys A structural analog: [(3′-keto-Bmt1)-(Val2)-cyclosporin]) gives a similar inhibition at a 10-times smaller concentration (0.4 μM) (Fig. 2b).

We also tested the involvement of mCICR in agonist-induced Ca^{2+} responses in Ehrlich ascites tumor cells using Cys A and PSC 833. These cells exhibit a Ca^{2+} homeostasis comparable with that of normal cells [13]. Data obtained using digitonin-permeabilized cells are shown in Fig. 3. Permeabilized cells were suspended in a standard cytosol-like medium supplemented for optimal function of the mitochondria and endoplasmic reticulum (ER) (see section 2). Permeabilized cells were stimulated with an inositol-triphosphate ($InsP_3$) bolus. Depending on the amount of $InsP_3$ used, the Ca^{2+} responses elicited were qualitatively and quantitatively different. Fig. 3a shows that under our conditions 10 μM $InsP_3$ induces a slow Ca^{2+} release of small amplitude (nanomolar range). A much faster and larger rise in [Ca^{2+}] is observed after 20 μM $InsP_3$ (micromolar range) (Fig. 3b). Thus, mechanisms must exist which significantly increase the Ca^{2+} release when $InsP_3$ stimulation is strong enough (in this case 20 μM $InsP_3$). We hypothetized that this is due to the threshold-dependence of mCICR described above. Supporting this view, we show that the speed of the response elicited by 20 μM $InsP_3$ is diminished twofold by 4 μM Cys A and almost abolished by 4 μM PSC 833 (Fig. 3c). Increasing the concentration of PSC 833 to 30 μM did not cause further inhibition of the response to $InsP_3$ (Fig. 3c). The residual response that is insensitive to PSC 833 is similar to the trace observed in Fig. 3a and appears to correspond to $InsP_3$-induced Ca^{2+} release from the ER. Since $InsP_3$ has no direct effect on mitochondria, we propose that, depending on the speed and/or on the amplitude of the

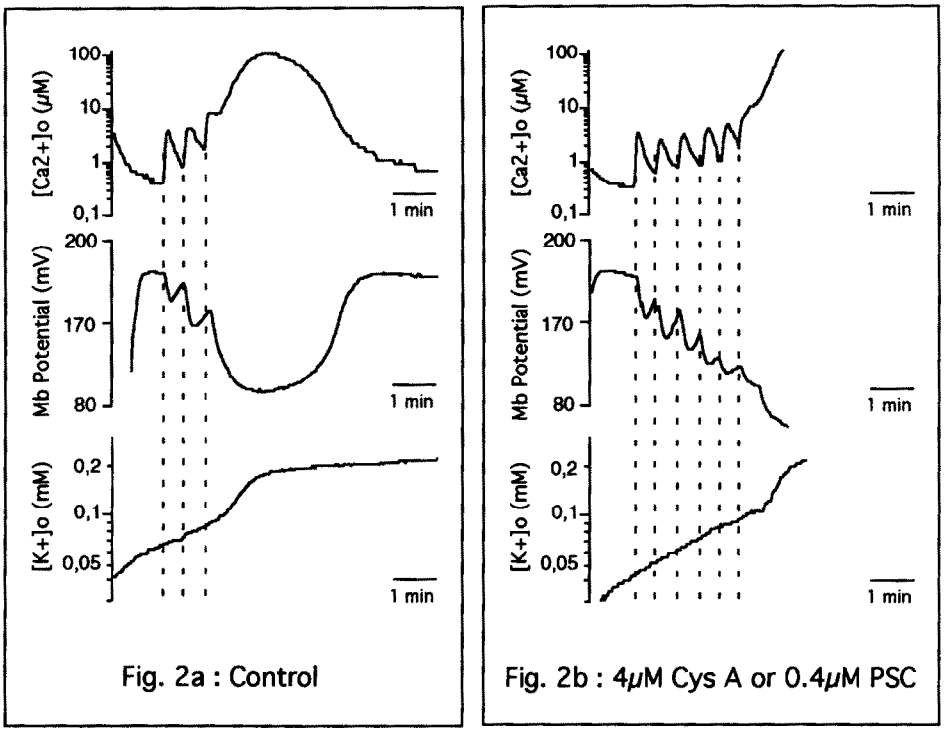

Fig. 2. Transient operation of the permeability transition pore underlies the Ca^{2+} release observed during mitochondrial Ca^{2+} spiking. (a) The dynamics of the medium free Ca^{2+} and K^+ concentrations as well as of the mitochondrial inner membrane potential were monitored simultaneously on a mitochondrial suspension, while a Ca^{2+} stimulation sequence was performed. 50 nmol of Ca^{2+} were added every 30 s (dotted lines). After 3 pulses, a typical mitochondrial Ca^{2+} spike is triggered (upper panel). (b) The antagonist effect of 4 μM Cys A or 0.4 μM PSC 833. However, if the Ca^{2+} stimulation sequence is extended an irreversible mPTP opening is observed. The potential traces presented were recorded using rhodamine 123, and calibration was estimated from identical experiments done in the presence of TPP^+ and monitored with a TPP^+-sensitive electrode. Calculations were performed assuming 1 μl/mg protein as mitochondrial volume.

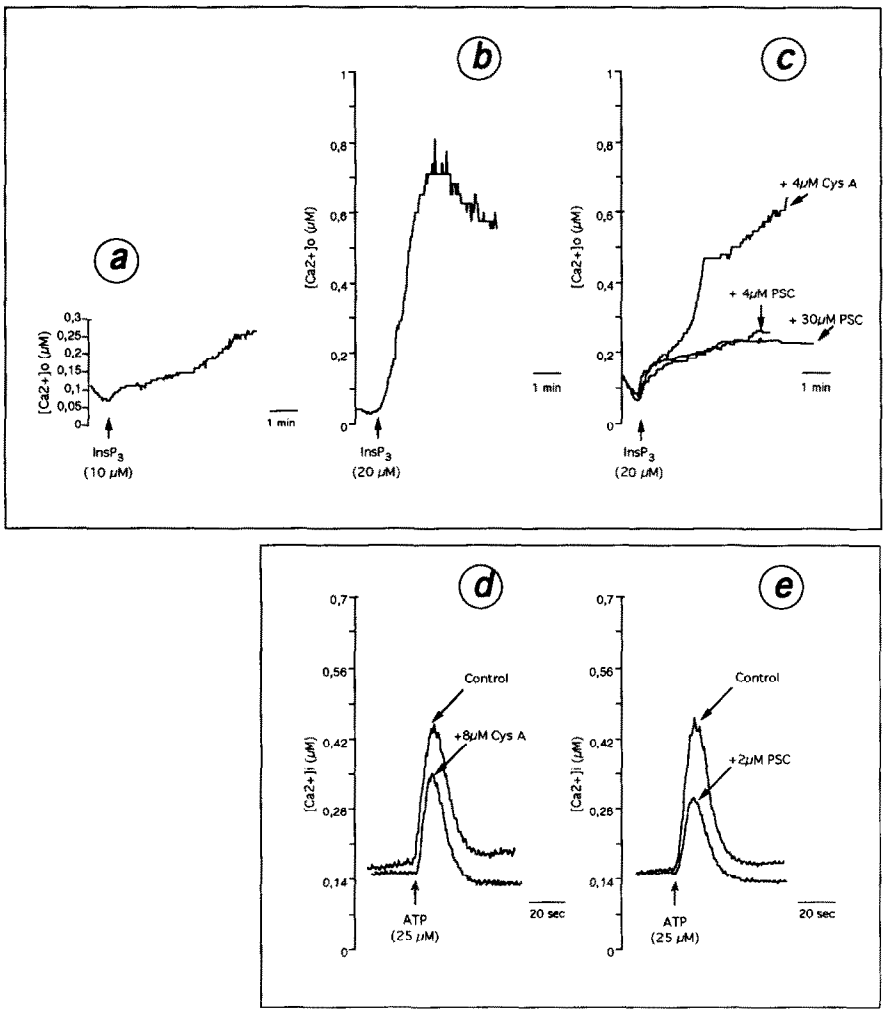

Fig. 3. Cyclosporin A and PSC 833 inhibit agonist-induced Ca^{2+} responses in cells. Panels a, b and c show Ca^{2+} releases obtained in suspensions of permeabilized Erhlich cells under $InsP_3$ stimulation (arrows). Panels a and b, show a threshold-dependent mechanism located between 10 μM (a) and 20 μM (b) $InsP_3$. The response observed in panel b is sensitive to Cys A and PSC 833 (c), a residual response similar to this observed in panel a is insensitive to PSC 833 (c). Panels d and e show intracellular Ca^{2+} spikes observed in suspensions of intact Fura-2AM-loaded Ehrlich cells stimulated with 25 μM ATP. Panel d shows the inhibitory action of Cys A, and panel e that of PSC 833.

primary $InsP_3$-induced Ca^{2+} release, mCICR may be triggered, thus accounting for the near-micromolar response observed in our assay.

We investigated the effect of Cys A and PSC 833 on the Ca^{2+} spikes generated by external ATP in intact cells. The ATP-induced Ca^{2+} responses in Ehrlich cells depend on the operation of the $InsP_3$ pathway [9]. Fig. 3d shows a normal Ca^{2+} spike induced by raising the external ATP concentration to 25 μM, and the inhibition of this response when cells were incubated with 8 μM Cys A. Fig. 3e shows that at a lower concentration (2 μM), PSC 833 has a more pronounced inhibitory action on the ATP-induced spike than Cys A. Taken together, the concordance of the respective inhibitory abilities of Cys A and PSC 833 observed on isolated mitochondria, on permeabilized cells and on intact cells, seems to imply that mCICR plays an active role in cell Ca^{2+} signalling following stimulation of the $InsP_3$ pathway.

Action of antimycin A (Ant A) on the ATP-induced Ca^{2+} spikes in intact cells gives further support to this view. Ant A – which induces collapse of the mitochondrial membrane potential, empties the mitochondrial Ca^{2+} pool, and prevents mCICR (not shown) – blocks the occurence of a normal ATP-induced Ca^{2+} response in intact cells (Fig. 4).

We conclude that mitochondria are threshold-dependent Ca^{2+}-excitable organelles involved in the transduction of cell Ca^{2+} signals as part of the CICR pool. We propose that the role of mCICR is to perform a gate-control towards primary Ca^{2+} signals originating from $InsP_3$-sensitive stores, thus participating to the encoding of signals relevant to the cell.

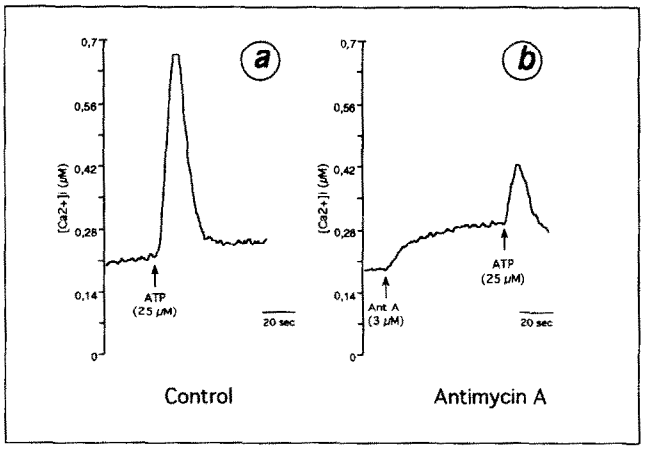

Fig. 4. Emptying the mitochondrial Ca^{2+} pool with antimycin a inhibits agonist-induced Ca^{2+} responses in intact cells. Panel a shows the control trace of an ATP-induced Ca^{2+} spike in Fura-2AM-loaded Ehrlich cells. Panel b shows that 3 μM antimycin A (Ant A) induces a Ca^{2+} release due to emptying of the mitochondrial Ca^{2+} pool (see text). After addition of Ant A, Ca^{2+} response of the cells to ATP stimulation is decreased more than twofold (b).

Acknowledgements: We are deeply indebted to Drs. J.D. Lechleiter and J. Robert for help and support during all the steps of this work. E.L.H. expresses his gratitude to Drs. A.M. Zhabotinsky, A.N. Zaikin, and Yu.V. Evtodienko. This work was funded by la Ligue Contre le Cancer, l'Association Française contre les Myopathies (AFM), l'Université Bordeaux II, and la Région Aquitaine.

References

[1] Berridge, M.J. and Irvine, R.F. (1989) Nature 341, 197–205.
[2] Berridge, M.J. (1993) Nature 361, 315–325.
[3] Lechleiter, J.D. and Clapham, D.E. (1992) Cell 69, 283–294.
[4] Gunter, T.E. and Pfeiffer, D.R. (1990) Am. J. Physiol. 258, C755–C786.
[5] Rizzuto, R., Brini, M., Murgia, M. and Pozzan, T. (1993) Science 262, 744–747.
[6] Holmuhamedov, E.L., Teplova, V.V. and Chukhlova, E.A. (1991) Biologicheskie Membrany (Russian) 6, 612–620.
[7] Evtodienko, Yu. V., Teplova, V.V., Khawaja, J. and Saris, N.-E.L. (1994) Cell Calcium 15, 143–152.
[8] Schneider, W.C. (1948) J. Biol. Chem 176, 259–267.
[9] Dubyak, G.R. (1986) Arch. Biochem. Biophys. 245, 84–97.
[10] Bygrave, F.L., Gamberucci, A., Fulceri, R. and Benedetti, A. (1993) Biochem. J. 292, 19–22.
[11] Petronilli, V., Cola, C. and Bernardi, P. (1993) J. Biol. Chem. 268, 1011–1016.
[12] Szabo, I. and Zoratti, M. (1992) J. Bioenerg. Biomembr. 24, 111–117.
[13] Arslan, P., Di Virgilio, F., Beltrame, M., Tsien, R.Y. and Pozzan, T. (1985) J. Biol. Chem. 260, 2719–2724.