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Characterization of calcium, phosphate and peroxide interactions in activation of mitochondrial swelling using derivative of the swelling curves

Zdeněk Drahota · René Endlicher · Pavla Staňková · David Rychtrmoc · Marie Milerová · Zuzana Červinková

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Abstract We describe a new method for the analysis of mitochondrial swelling curves. Using classical swelling curves, only the maximum extent of the swelling can be calculated in a numerical form. However, taking the derivative of the classical swelling curves enables the evaluation of two additional parameters of the swelling process in a numerical form, namely, the maximum swelling rate after the addition of the swelling inducer (as dA_{520}/10 s) and the time (in sec) at which the maximum swelling rate after the addition of the swelling inducer is obtained. The use of these three parameters enables the better characterization of the swelling process as demonstrated by the evaluation of calcium and phosphate interactions in the opening of the mitochondrial permeability transition pore and by the characterization of the peroxide potentiating action.

Z. Drahota
Institute of Physiology and Center for Applied Genomics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Z. Drahota · R. Endlicher · P. Staňková · D. Rychtrmoc · Z. Červinková (✉)
Department of Physiology, Charles University in Prague, Faculty of Medicine in Hradec Králové, Šimkova 870, 500 38 Hradec Králové, Czech Republic
e-mail: wolff@lfhk.cuni.cz

R. Endlicher
Department of Anatomy Charles University in Prague, Faculty of Medicine in Hradec Králové, Hradec Králové, Czech Republic

M. Milerová
Institute of Physiology and Center for Cardiovascular Research, Academy of Sciences of the Czech Republic, Prague, Czech Republic

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Introduction

Changes in the turbidity of mitochondrial suspensions have been used as a very simple method to evaluate the mitochondrial swelling for more than 50 years (Carafoli 2010). After its introduction, this method was a very important tool in studies of the transport of inorganic ions and various organic substrates across the inner mitochondrial membrane (Passarella et al. 2003; Carafoli 2010). Much attention was concentrated on the accumulation and maintenance of calcium ions in mitochondria and on the role of mitochondria in cell calcium homeostasis (Drahota et al. 1965; Gunter and Pfeiffer 1990; Carafoli 2010). The assessment of mitochondrial swelling was one of the most popular methods used in these studies.

After the Ca^{2+}-dependent non-specific mitochondrial permeability transition pore (MPTP) was discovered, measurement of mitochondrial swelling was also used in the elucidation of its functional activity (Crofts and Chappel 1965; Hunter and Haworth 1979; Haworth and Hunter 1979; Crompton et al. 1987; Crompton et al. 1988; Halestrap 2009) and for the detection of a wide spectrum of endogenous and exogenous factors that modulate its function, among which oxygen radicals are very important (Bernardi et al. 1992; Petronilli et al. 1993; Walter et al. 2000; Halestrap et al. 2002). Later, the opening of MPTP demonstrated to be involved in apoptotic and necrotic processes (Crompton and Costi 1988; Broekemeier et al. 1992; Pastorino et al.

1993; Kim et al. 2006; Rasola and Bernardi 2011) and in the pathogenesis of many diseases such as cardiomyopathies, neuropathies, liver diseases and diabetes. Data also indicate that that modulation of MPTP function could help in the treatment of these diseases (Bernardi et al. 2006; Rasola and Bernardi 2011). The measurement of mitochondrial swelling as an indicator of MPTP opening was and still is one of the most important methods in studies.

However, in most studies in which mitochondrial swelling was used, this parameter was recorded as a graphical curve, and the swelling process was evaluated on the basis of the changes in the shape of this curve. The resulting findings are rather qualitative and cannot produce exact quantitative numerical data for the rate of the swelling process. A method based on the measurement of the calcium retention capacity of mitochondria, which is indicative of the Ca^{2+} concentration required for the induction of pore opening, by using fluorophore Calcium green has been developed (Fontaine et al. 1998); however, the majority of the studies still use classical swelling curves at least for the preliminary analysis.

We present data indicating that the evaluation of mitochondrial swelling may be improved by taking the derivative of the classical swelling curves that are used, e.g., for the evaluation of the mitochondrial respiration rate (Gnaiger et al. 1995). After taking the derivative, we obtain a modified swelling curve from which two additional parameters can be determined in a numerical form, which helps to better characterize the kinetics of the swelling process. The maximum extent of mitochondrial swelling can be obtained in a numerical form from the classical swelling curve, corresponding to an absorbance decrease during the measured period (dA520/time). The two additional parameters obtained from the modified swelling curve in a quantitative form are the maximum swelling rate, expressed as the change in the absorbance per unit of time (e.g., dA520/10 s), and the time (in sec) at which the peak of the maximum swelling rate reaches its maximum value after the addition of the swelling inducer. The maximum swelling rate and the time of its peak cannot be obtained as numerical values from classical swelling curves. Using these three parameters, it is possible to better characterize the kinetics of the swelling process and the effects of various factors that modulate mitochondrial swelling.

Material and methods

Animals

Male Wistar rats (Biotest Konarovice, Czech Republic) with body weights of 220–250 g were used. The animals had free access to a standard laboratory diet (DOS 2B Velaz, Czech Republic) and tap water. Animals were starved overnight before the end of experiment and were sacrificed by exsanguination from the aortic bifurcation under ether narcosis. All animals received care according to the guidelines set out by the Institutional Animal Use and Care Committee of Charles University, Prague, Czech Republic.

Chemicals

All chemicals were of the highest commercially available purity and were purchased from Sigma (Sigma Aldrich Co., Germany).

Isolation of rat liver mitochondria

Rat liver mitochondria were prepared by differential centrifugation as described in (Bustamante et al. 1977) with some modifications. Minced liver tissue was homogenized as a 10 % homogenate (w/v) at 0 °C in a glass-Teflon homogenizer. The isolation medium contained 220 mM mannitol, 75 mM sucrose, and 1 mM HEPES, pH 7.2. To the homogenization medium, 0.5 $g.L^{-1}$ fatty-acid-free bovine serum albumin (BSA) and 1 mM EGTA were added. The homogenate was centrifuged for 10 min at 800 g. The supernatant was filtered through nylon mesh and centrifuged for 10 min at 8,000 g. The sedimented mitochondria were washed in isolation medium without EGTA, and the final mitochondrial pellet was re-suspended to a concentration of 20–30 mg protein/mL. The protein content was determined according to the method of Bradford (Bradford 1976) using BSA as a standard.

Results

Measurement of three parameters of mitochondrial swelling

Mitochondrial swelling was estimated from the decrease in the absorbance of the mitochondrial suspension absorbance at 520 nm measured at 30 °C using a Shimadzu spectrophotometer. The swelling medium contained 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 5 mM succinate, and 0.01–1 mM K-PO_4, pH 7.2 (Castilho et al. 1998), and other additives as indicated in the figures. Mitochondria were added to yield an absorbance of approximately 1. This value was obtained by the addition of approximately 0.4 mg of mitochondrial protein per millilitre. After 1 min of preincubation of the mitochondria, $CaCl_2$ solution was added. The decrease in the absorbance was determined at 10-s intervals. From these swelling curves the extent of swelling was calculated as the change in the optical density during the measurement period. For most measurements, 5 and

10 min intervals after $CaCl_2$ addition were used. At the 5 min time period, the extent of swelling induced by 0.1–0.2 mM $CaCl_2$ represented approximately 95 % of the maximum swelling that can be obtained during 10 min of incubation. When a liver mitochondrial suspension with an A_{520} of approximately 1 was used, this value represented 30–40 % of the initial absorbance. The classical swelling curve thus provides in a numerical form the first parameter of the swelling process—the extent of swelling. This value induced by 0.1 mM $CaCl_2$ in the presence of 0.1 mM phosphate was 0.386 dA520/10 min (Fig. 1a).

From the classical swelling curve, data for the swelling rate cannot be obtained in a quantitative form. Therefore, for the exact determination of the swelling rate, we used the curve obtained after taking the derivative of the original data. Using the swelling recorded in 10 s intervals, we can obtain numeral data for the swelling rate for specific 10 s intervals. From these values, the maximum swelling rate can be determined. Figure 1b shows that the maximum value of the swelling rate under the experimental conditions used was 0.064 (dA_{520}/10 s). From this swelling curve, we can also obtain in a numeral form the third parameter of the swelling process—the time at which the maximum swelling rate was reached after calcium addition. Under our experimental conditions (Fig. 1b), the time of the peak was 70 s after the addition of $CaCl_2$. These three parameters can characterize the kinetics of the swelling process and the effects of various substances that modulate the mitochondrial swelling better than classical swelling curves can.

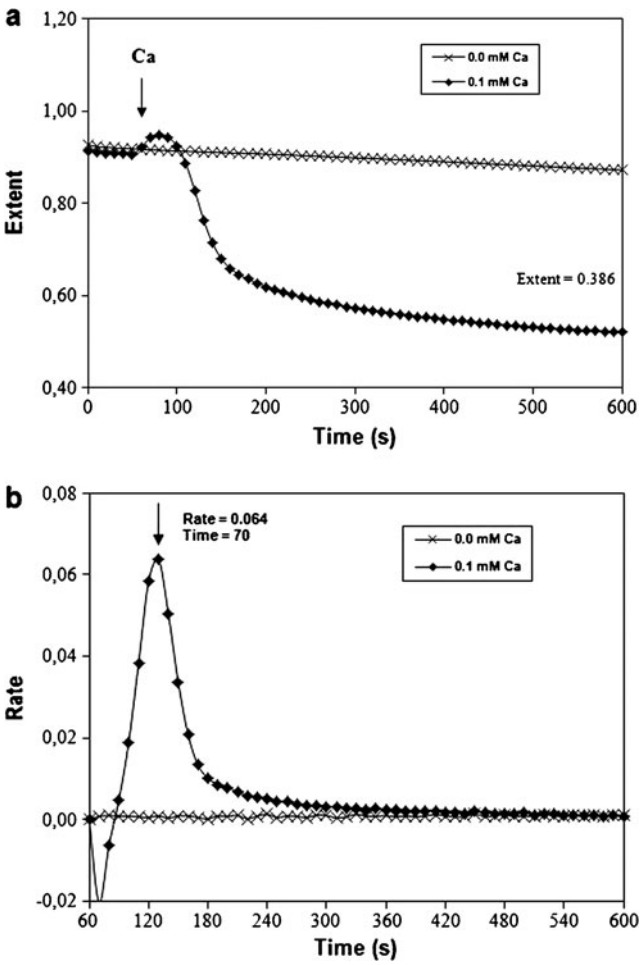

Fig. 1 Swelling of rat liver mitochondria. The classical swelling curve (**a**) and the curve obtained after taking the derivative of the original curve (**b**). Mitochondria (0.4 mg protein/mL) were incubated in the swelling medium at 30 °C. $CaCl_2$ (0.1 mM) was added after 60 s. The extent of swelling (decrease in the absorbance at 520 nm over 9 min, Fig. 1a), the rate (decrease in the absorbance over 10 s) and the time of the peak after $CaCl_2$ addition were calculated (Fig 1b)

Evaluation of the activating effects of calcium and phosphate on mitochondrial swelling using the derivatives of the swelling curves

Phosphate ions activate Ca^{2+}-induced swelling. Classical curves clearly document that the swelling extent induced

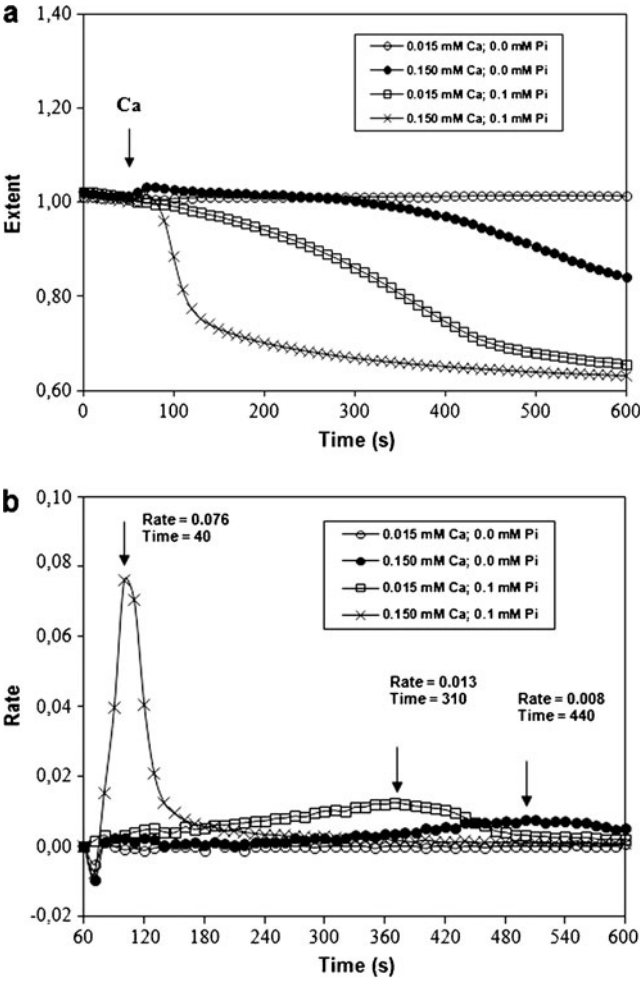

Fig. 2 Activation of calcium-induced swelling by phosphate. The experimental conditions are the same as in Fig. 1

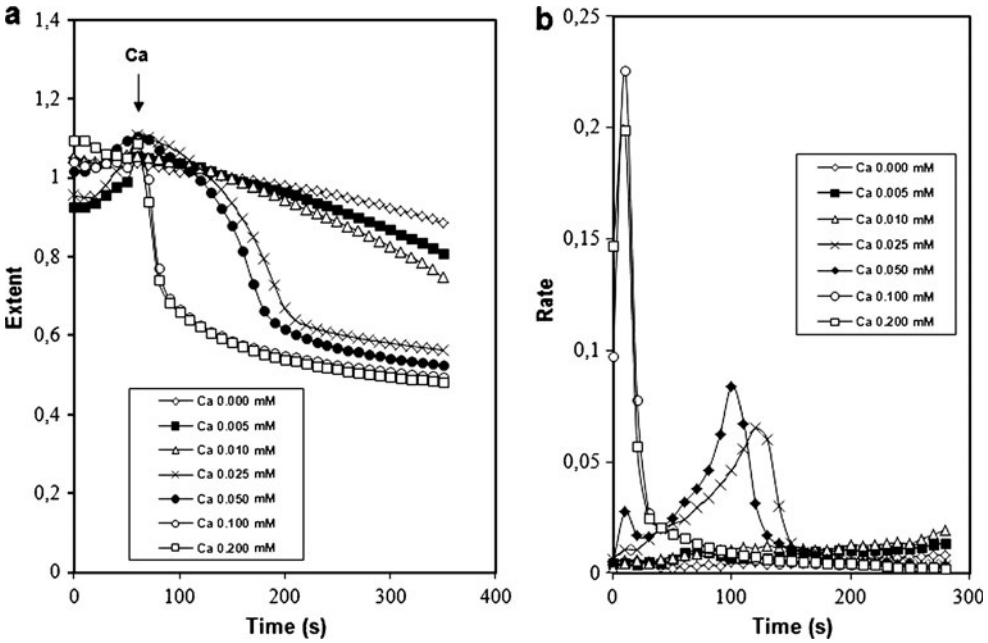

Fig. 3 $CaCl_2$ concentration dependence of rat liver mitochondria swelling. Mitochondria (0.4 mg protein/mL) were incubated in the swelling medium in the presence of 1.0 mM phosphate Different calcium concentrations were added after 60 s of preincubation

by 0.15 mM $CaCl_2$ is quite different during the 10 min incubation period in the presence and absence of phosphate. These curves indicate that the swelling rate is much lower in the absence of phosphate (Fig. 2a), but the curves obtained after taking the derivative reveal quantitative information about the maximum swelling rate induced by 0.15 mM $CaCl_2$; this rate was increased from 0.008 dA_{520}/10s to 0.076, i.e., by 9.5-fold, in the presence of phosphate. The time at which the maximum swelling was reached decreased from 440 s to 40 s, i.e., by 11-fold, which indicates a good correlation between the rate and time values. When we compare the induction by low and high calcium concentrations in the presence of phosphate, we can see that in the absence of phosphate low calcium concentrations cannot induce the swelling during the incubation period tested; however, in the presence of 0.1 mM phosphate, low calcium concentrations can induce the same extent of swelling as high calcium concentrations, though at much slower rate and after a much longer time (Fig. 2b). All these parameters are obtained in a digital form.

Higher concentrations of phosphate (1 mM) further increased the rate of calcium-induced swelling from 0.076 to 0.230, which represents a three-fold increase (Figs. 2b and 3b). We therefore tested the calcium-activating effect in the

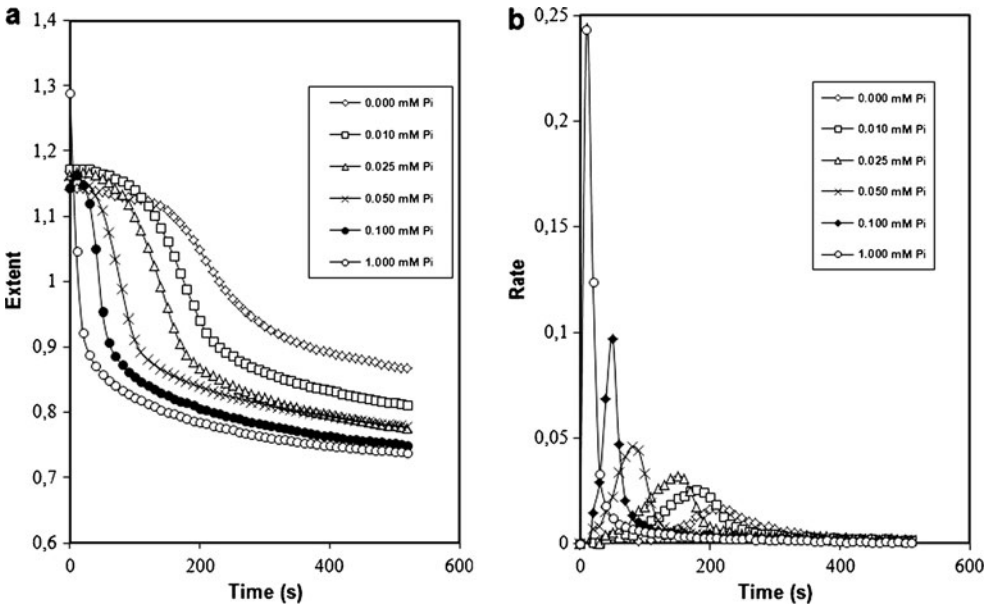

Fig. 4 Calcium-induced swelling activated by phosphate: Extent - change in absorbance over 5 min (**a**), Rate - change in absorbance over 10 s (**b**). Mitochondria (0.4 mg protein/mL) were incubated in the swelling medium in the presence of 0.01 and 1.0 mM phosphate. Swelling was induced by 0.15 mM $CaCl_2$

range from 0 to 0.200 mM $CaCl_2$. When the extent of swelling was evaluated in the range of 0.025 to 0.200 mM $CaCl_2$, similar values close to the maximum values were obtained (Fig. 3a). However, the swelling rate was greater, and the time to the maximum peak was less at higher $CaCl_2$ concentrations. In the range between 0.000 and 0.010 mM $CaCl_2$, the extent of swelling during the period tested is very low, and no peak could be detected during the period measured. From Fig. 3, we can also see that the kinetic parameters of the swelling process dramatically change between 0.010 and 0.025 and between 0.050 and 0.100 mM $CaCl_2$. Moreover, the swelling was approaching its limits and could not be further increased by further increases in the $CaCl_2$ concentration. With 1.0 mM phosphate, a very low swelling could also be detected in the absence of added $CaCl_2$. However, this swelling was evidently due to the presence of residual $CaCl_2$ because this swelling was completely inhibited by the addition of 0.25 mM EGTA (not shown).

These three parameters of the swelling process at different calcium concentrations were compared at increasing phosphate concentrations (0–1 mM). Both the extent and the rate induced by 0.15 mM $CaCl_2$ increase with an increasing phosphate concentration (Fig. 4a, b). The changes detected for all three parameters are most pronounced in the concentration range of 0–0.1 mM phosphate (Fig. 5). The changes between 0.1 and 1 mM phosphate are the most pronounced for the rate parameter (Fig. 5a) and were less pronounced for the time and extent parameters (Fig. 5b, c).

When we compare the values of the extent and the rate of swelling at the highest calcium concentrations in the presence of 1.0 mM phosphate with those obtained after the addition of the channel forming antibiotic alamecitin (Gostimskaya et al. 2003), we found that the maximum swelling rate is approximately 30 % lower (not shown). Evidently, at 0.15 mM $CaCl_2$ and 1 mM phosphate, the swelling of the whole mitochondrial population is approaching its maximum limiting value.

Activation of Ca^{2+}-induced swelling by tBHP

As another example of the validity of this method, we evaluated the effect of tBHP effect on the opening of the mitochondrial permeability transition pore (Fig. 6). These findings indicate that the activating effect of tBHP can be detected only at low calcium concentrations. After the addition of 0.005 mM $CaCl_2$, the swelling rate was increased 5-fold in the presence of 0.75 mM tBHP from 0.005 to 0.024. However, when the swelling induced by Ca^{2+} is approaching its limiting value, no activating effect of tBHP can be detected. These data indicate that peroxidative activation of the swelling process has an important role, especially in the acceleration of low swelling rates, thus increasing the sensitivity of MPTP to Ca^{2+}.

We may thus conclude from our data that the determination of numerical values for more parameters of the swelling process allows us to better characterize and better understand effects of many endogenous and exogenous factors that modulate the functional activity of a very complicated multiprotein complex that can open and close the pores in the inner mitochondrial membrane.

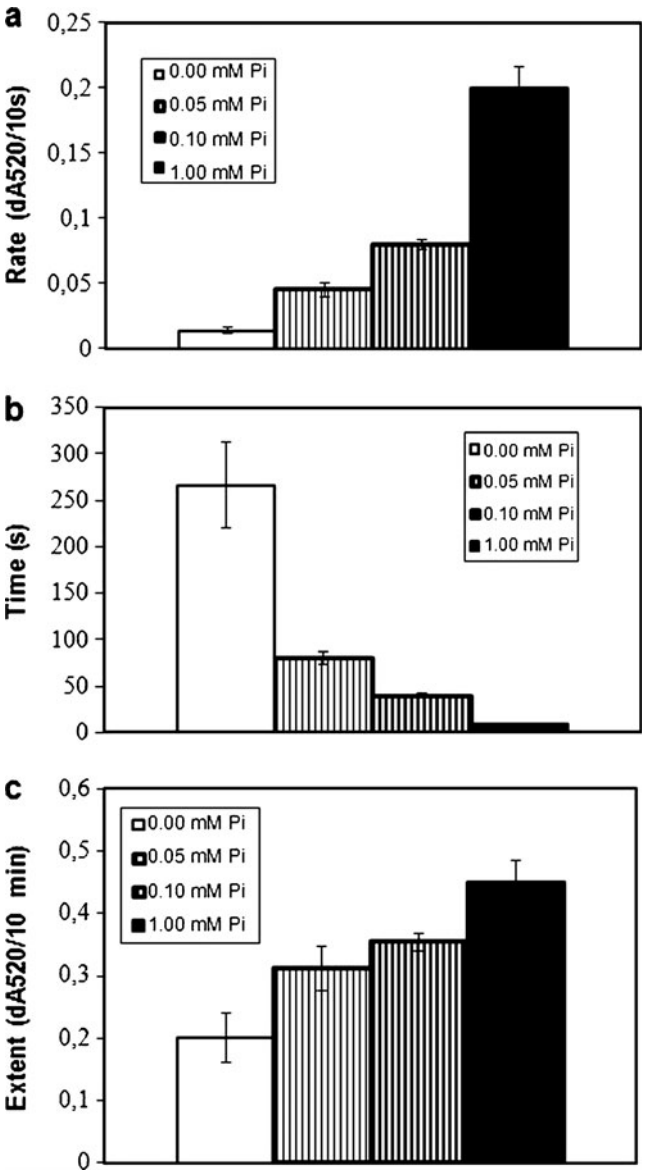

Fig. 5 Calcium-induced swelling at different phosphate concentrations. **a** Maximum values of the swelling rate during the 10 s intervals. **b** Time (s) when the maximum swelling rate was detected. **c** Extent of absorbance change over 9 min of incubation after the addition of 0.15 mM $CaCl_2$. $CaCl_2$ was added after 60 s of preincubation. Data represent averages ± SEM from 4 animals

Discussion

Calcium is a very important regulating factor in many cell metabolic pathways, and mitochondria have a very important role, in concert with the plasma membrane and the endoplasmic reticulum, in the maintenance of the cell's calcium homeostasis (Carafoli 2010). Recently, there has been increasing interest in elucidating the structural organisation and functional activity of one important mitochondrial device—the mitochondrial permeability transitions pore. The molecular structure of this pore is very complicated; it is composed of proteins localized to the on outer and inner mitochondrial membranes, and to date, there are still discussions which of these proteins are really necessary for the function of this pore (Bernardi et al. 1992; Walter et al. 2000; Carafoli 2010). In addition, the mechanisms involved in the regulation of the opening and closing the pore have not been fully elucidated.

One of the factors limiting our ability to answer these many questions is evidently the fact that the primary analytical method is 50 years old and cannot give exact data characterising the swelling process in a numerical form. We present in this communication a simple way to improve this method by taking a derivative.

Using this evaluation of the swelling curves we could determine calcium concentration range when the swelling induction starts to be dangerous for the cell. Also the extent and time parameters showed that above 0.01 mM calcium concentration the extent of swelling is reaching its maximum values, however, at different time period. Because all these three parameters are obtained in a digital form we could calculate significance of measured changes and conclude (Fig. 5) that contrary to calcium, phosphate activating effect is increasing in the concentration range 0–0.1 mM almost linearly. We can also calculate, that the activating effect between 0.1 and 1 mM phosphate is high and significant for rate and time parameters and not significant for the extent parameter. We also obtained better information about peroxide potentiating effect of calcium induced swelling. Data in Fig. 6 show that peroxide potentiating effect can be observed only at low swelling rate.

Using three parameters of the swelling process in combination with other parameters, such as the calcium retention value (Fontaine et al. 1998), could help to improve the analytical basis for answering many of the questions concerning the mitochondrial permeability transition pore that have not been solved. There are still many unanswered questions concerning this pore's structural arrangement, functional activity and regulation of opening and closing. There are also indications that the swelling process may differ among heterogeneous mitochondrial populations (Cossarizza et al. 1996), among mitochondria from various tissues, and among mitochondria from various species (Ricchelli et al. 2005; Panov et al. 2007); moreover, a developmental- and ageing-specific function may exist (Mather and Rottenberg 2000; Milerova et al. 2010). In addition, it will be important to answer the many remaining questions concerning the interactions of many factors that can positively and negatively modify the swelling process, such as factors related to physiological and pathological conditions. All of these situations can be better evaluated when we have numerical data characterising the kinetics of the swelling process.

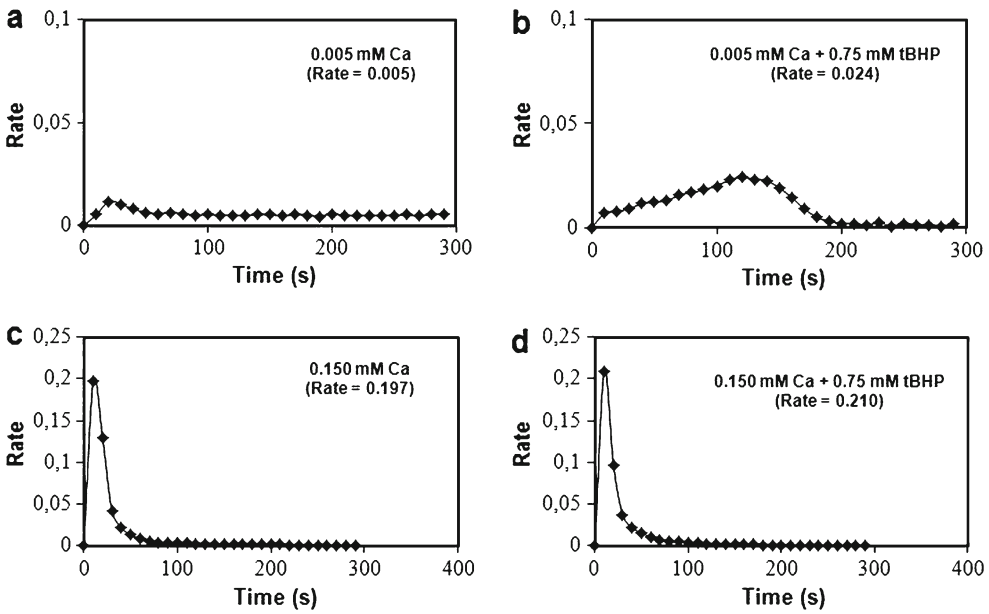

Fig. 6 Activation of calcium-induced swelling by tBHP. Mitochondria (0.4 mg protein/mL) were incubated in the swelling medium with 1 mM phosphate. tBHP (0.75 mM) was added to the incubation medium, and the swelling of mitochondria was induced by the addition of $CaCl_2$ (0.005 and 0.150 mM) after 60 s of preincubation

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Conflict of interest The authors declare that they have no conflict of interest.

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