Modification of calcium retention capacity of rat liver mitochondria by phosphate and tert-butyl hydroperoxide

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Short title:

Calcium retention capacity and rat liver mitochondria

Summary

By determining the calcium retention capacity (CRC) of rat liver mitochondria, we confirmed and extended previous observations describing the activation of mitochondrial swelling by phosphate and tert-butyl hydroperoxide (t-BHP). Using CRC measurements, we showed that both phosphate and t-BHP decrease the extent of calcium accumulation required for the full mitochondrial permeability transition pore (MPTP) opening to 35% of control values and to only 15% when both phosphate and t-BHP are present in the medium. When changes in fluorescence were evaluated at higher resolution, we observed that in the presence of cyclosporine A fluorescence values return after each Ca^{2+} addition to basal values obtained before the Ca^{2+} addition. This indicates that the MPTP remains closed. However, in the absence of cyclosporine A, the basal fluorescence after each Ca^{2+} addition continuously increased. This increase was potentiated both by phosphate and t-BHP until the moment when the concentration of intramitochondrial calcium required for the full opening of the MPTP was reached. We conclude that in the absence of cyclosporine A, the MPTP is slowly opened after each Ca^{2+} addition and that this rate of opening can be modified by various factors such as the composition of the media and the experimental protocol used.

Key words

Liver mitochondria · Calcium Retention Capacity · Mitochondrial permeability transition pore · Phosphate · Tert-Butyl Hydroperoxide

Introduction

The mitochondrial permeability transition pore (MPTP) is a non-specific channel on the inner mitochondrial membrane that enables the transfer of substances smaller than 1.5 kD between the intramitochondrial matrix and the cytosol (Haworth and Hunter 1979, Hunter and Haworth 1979a, Hunter and Haworth 1979b, Hurst *et al.* 2017). Opening of the MPTP results in the dissipation of the mitochondrial membrane potential and in the penetration of water from the cytosol into the mitochondrial matrix followed by mitochondrial swelling. Despite the fact that this phenomenon was discovered 65 years ago (Raaflaub 1953), its molecular mechanism is not yet fully clear. This phenomenon has been studied and confirmed in many laboratories (Lehninger 1962, Crofts and Chappell 1965, Bernardi *et al.* 1999). It was found that calcium-induced swelling is strongly inhibited by the immunosuppressive agent cyclosporine A (Broekemeier *et al.* 1989, Halestrap *et al.* 1997). This inhibition is due to the interaction of cyclosporine A with cyclophilin D, a soluble isomerase in the mitochondrial matrix. Cyclosporine A prevents binding of cyclophilin D to the inner mitochondrial membrane (Bernardi *et al.* 1994) which is required for activating effect of calcium. MPTP is thus characterized as calcium-induced and cyclosporine A sensitive pore. This phenomenon has been used from the beginning as a method for the evaluation of pore function and its regulation, and this method is still in use.

It was proposed that the MPTP is composed of porin, adenine nucleotide translocator, phosphate translocator, hexokinase, creatine kinase and the Bax/Bak proteins (Halestrap 2009). Recently, it was also proposed that the MPTP is formed on the boundary between two ATP synthase molecules (Giorgio *et al.* 2013, Alavian *et al.* 2014). However, based on data obtained by molecular genetic studies all these molecules were excluded as structural components of the MPTP (Kokoszka *et al.* 2004, He *et al.* 2017), and it was suggested that these molecules are involved in MPTP regulation.

Using the swelling method many endogenous and exogenous factors were described that can affect opening of the MPTP. The effect of oxygen radicals on the MPTP opening using prooxidant t-BHP was very extensively studied. Its effect was explained as peroxidative damage of mitochondrial membrane proteins resulting in metabolic disturbances. The effect of phosphate on the pore opening was not yet fully elucidated, especially when it was found that intramitochondrial calcium and phosphate may form calcium phosphate granules in the matrix (Carafoli 2010). Inorganic phosphate plays essential role in oxidative phosphorylation and is important for calcium homeostasis in mitochondria by buffering vast amount of calcium that mitochondria can accumulate (Wei *et al.* 2015). Besides modification of membrane permeability features by various endogenous and exogenous factors, mitochondrial membrane properties may differ depending on organ type, gender and age (Panov *et al*. 2007, Drahota *et al*. 2012b, Milerová *et al*. 2016).

When the intramitochondrial calcium concentration is decreased, the MPTP can close again. Short, transient MPTP openings also have a positive effect of decreasing ROS generation by the mitochondrial respiratory chain (Hunter *et al.* 1976). However, when the MPTP remains open for a longer period due to an increased calcium concentration inside the mitochondria, the pore cannot be closed, and the processes of cell death are triggered (Bernardi *et al.* 1999). It is thus evident that this phenomenon plays a very important role in the development of necrosis in all organs and that various factors, both endogenous and exogenous, involved in its regulation are intensively studied in an effort to find out pharmacological substances that could help in the treatment of and protection from necrotic processes in which MPTP is involved (Smith *et al.* 2012). .

Therefore, it is necessary to obtain more data on both the function and the regulation of the MPTP. For evaluating MPTP function and its regulation, there are two accessible

methodological approaches: (a) measurement of mitochondrial swelling that gives us information about the rate of pore opening after calcium addition and about the maximum extent of mitochondrial swelling (Drahota *et al.* 2012), (b) measurement of the calcium retention capacity of the mitochondria that informs us about amount of calcium necessary for pore opening (Szabo and Zoratti 2014). These methods are based on different principles. We attempted in this study to demonstrate that measurement of calcium retention capacity gives the same results about the effect of phosphate and tert-butylhydroperoxide on the MPTP function as we obtained in our previous paper (Drahota *et al*.2012a) using mitochondrial swelling, and that using more sensitive fluorescent probe provides additional information that cannot be obtained by the swelling method.

Methods

Chemicals

All chemicals, unless otherwise stated, were of analytical grade and obtained from Sigma-Aldrich (Darmstadt, Germany). Calcium Green-5N was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Animals

Male Wistar rats (220±20 g) were obtained from Velaz (Lysa nad Labem, Czech Republic). The rats were housed at 23±1 °C, 55±10% humidity, with air exchange 12-14 times/h and a 12 h light-dark cycle period. The animals had free access to a standard laboratory diet (ST-1, Velaz, Czech Republic) and tap water. All animals received care according to the guidelines set by the Animal-Welfare Body of Charles University, Czech Republic and the International Guiding Principles for Biomedical Research Involving Animals. The animals were sacrificed under general anesthesia by exsanguination from the aortic bifurcation. The livers were removed, washed in a cold isolation medium, and cut into small pieces.

Isolation of mitochondria

Liver mitochondria were isolated as described previously (Bustamante *et al.* 1977). The cut and washed tissue was homogenized at 0 °C by a Teflon-glass homogenizer in an isolation medium containing 220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, 0.2 mM EGTA, and 0.5 g of fatty acid free bovine serum albumin (BSA) per liter, at a pH of 7.2. The 10% homogenate was centrifuged for 4 min at 830 g, and the resulting supernatant was centrifuged for 15 min at 5 200 g. The mitochondrial sediment was washed twice (10 min at 11 200 and at 13 000 g) in isolation medium lacking EGTA and resuspended in the same medium to a final

volume of 3 ml. Isolated mitochondria were stored at 0 °C. Calcium retention capacity was measured immediately after isolation; we did not use mitochondria for more than five hours after isolation.

Determination of mitochondrial proteins

The mitochondrial protein concentration was determined using the Bradford method with bovine serum albumin as a standard (Bradford 1976).

Measurement of calcium retention capacity

The mitochondrial retention capacity for calcium was evaluated using the fluorescent probe Calcium Green-5N on an AMINCO-Bowman Series 2 spectrofluorometer (Thermo Electron Corporation) using an excitation wavelength of 506 nm and emission wavelength of 592 nm; measurements were performed at room temperature. Briefly, 1 μM Calcium Green-5N, 10 mM succinate, 0.5 μM rotenone, and mitochondria (0.4 mg protein per ml) were added to 1 ml of swelling medium (125 mM sucrose, 65 mM KCl, 10 mM HEPES, and pH 7.2). Succinate a substrate of Complex II was added because calcium uptake by mitochondria is energy-dependent process and rotenone an inhibitor of Complex I was included to eliminate oxidation of endogenous NADH-dependent substrates present in mitochondrial matrix. Afterwards, calcium chloride ($CaCl_2$) was added at various concentrations. All other substances (phosphate, t-BHP, cyclosporine A) were always added to the medium prior to calcium at the concentrations indicated in the figures and table. The probe reversibly binds to calcium ions. If mitochondria accumulate calcium, the fluorescent signal in the medium decreases. When the retention capacity is exceeded mitochondria release the accumulated calcium, and the fluorescence rises dramatically (Ichas *et al.* 1997, Fontaine *et al.* 1998).

Statistical analysis

The experiments were performed at least three times; the representative results are shown. Values are depicted as the means ± SD; $P < 0.05$ was set as the threshold for statistical significance. Statistical evaluation was performed using GraphPad Prism 6.01 software (La Jolla, CA, USA). The data were first tested for normality by means of the Kolmogorov-Smirnoff test. The data with a Gaussian distribution (Table 1) were further analyzed by a parametric ANOVA and the Dunnett posttest, and the data with a non-Gaussian distribution (Table 1) were analyzed by the nonparametric Kruskal-Wallis test and the Dunn posttest.

Results

The goals of our study were to confirm and extend our previous data concerning the role of phosphate ions and t-BHP in the regulation of MPTP function, obtained by the measurement of mitochondrial swelling (Drahota *et al.* 2012) by using a novel method for the evaluation of MPTP function, i.e., the measurement of mitochondrial calcium retention capacity (Obame *et al.* 2008). This method, based on a different principle, can give us additional information about the process of pore opening that cannot be obtained by the swelling method.

Fig. 1 demonstrates the experimental conditions that we selected for our measurements of calcium retention capacity (CRC) of rat liver mitochondria. We used low amounts of added calcium ions (1.25 μM) to allow us to better evaluate the effects of phosphate and t-BHP on the sensitivity of the MPTP to calcium. Under our experimental conditions, the opening of the pore was sensitive to cyclosporine A.

Fig. 1 confirms our previous findings obtained by the swelling method: both phosphate ions and t-BHP activate the opening of the MPTP. From the measurements of calcium

retention capacity under our experimental conditions, additional data about the opening of the MPTP were obtained. We calculated the amount of calcium that must accumulate in the mitochondria for induction of the pore opening. The values of CRC without the addition of phosphate were 24.1 nmol Ca^{2+}/mg protein (Table 1). CsA increased this CRC value to 40.6 nmol Ca^{2+}/mg protein. 1 mM phosphate without CsA decreased this value to 8.9 nmol Ca^{2+}/mg protein. Similar data were obtained also for the decreasing effect of t-BHP (Table 1). Phosphate and t-BHP decreased the CRC values to 3.1 nmol Ca^{2+}/mg protein of those required for the MPTP to open in the absence of phosphate and t-BHP (Table 1). These data cannot be obtained from the swelling curves. These data also show that both methods give the same information when the effects of various substances on MPTP function are tested.

When the curves presented in Fig. 1A, C, D were evaluated at higher resolution (as in Fig. 1B), the values of basal fluorescence after each calcium addition could be evaluated (Fig. 2A, B). We could see that in the presence of CsA, the MPTP remains closed during the period tested; after each calcium addition, fluorescence values returned again to the baseline (Fig. 2A). However, in the absence of CsA, after each calcium addition the fluorescence value did not return to the baseline and continuously increased. This increase observed in the absence of CsA was higher when phosphate or t-BHP was added into the incubation medium (Fig. 2A, B). These data indicate that the opening of the MPTP occurs in two phases. When low amounts of calcium accumulate in the absence of CsA, the MPTP is slowly opened. Complete opening of the MPTP, however, requires the accumulation of critical amounts of intramitochondrial calcium, and this critical amount is highly dependent on phosphate and t-BHP concentrations (see Fig. 1A and 1C). From Table 1 and Fig. 1D, it is also evident that the rise of MPTP sensitivity to calcium ions by phosphate and t-BHP is further enhanced when both are present in the incubation medium.

Fig. 3 illustrates that not only the composition of the incubation medium but also the addition of various quantities of $CaCl_2$ may greatly affect the values of the calcium retention capacity detected with the same amount of mitochondrial protein. When low concentrations (1.25 μM) were used similarly as in Table 1, 25 nmol Ca^{2+}/mg protein was required for pore opening. This amount of calcium was added in 8 portions. When the amount of calcium added by each portion was increased, the pore is opened sooner, i.e., after 6, 5 and 3 additions (Fig. 3). However, the total sum of accumulated calcium was increased, which means that under these various experimental conditions, the determined values of CRC can increase up to 300%. This indicates that when mitochondria are exposed to higher calcium concentrations, the sensitivity of the MPTP to calcium ions is modified.

Discussion

Measurement of CRC, due to the very high sensitivity of fluorophore Calcium Green-5N, can help us better understand the mechanism of the swelling process. Our data presented here give us new information about the processes of calcium movement between the cytosol and the mitochondrial matrix. We can see that when the concentration of calcium ions in the medium is increased and when calcium is accumulated in the mitochondria, the pore is slowly opened. When the calcium concentration required for the full opening of the pore is reached, the pore is fully opened, and accumulated calcium is released. This value of accumulated calcium required for full opening can be markedly decreased both by phosphate and t-BHP. The effect of t-BHP is explained as the result of peroxidative damage of mitochondrial membrane, the effect of phosphate was not yet fully elucidated. Such information about the particular calcium concentration values cannot be obtained by the swelling method. Evidently, the partial opening of the pore, before it is fully opened, represents the intramitochondrial calcium concentration range and can be considered a positive effect by decreasing ROS

production by the respiratory chain (Hunter *et al.* 1976). At this concentration range, the pore may be closed again. The critical calcium concentration at which the pore is fully opened indicates the “death point”, i.e., the start of cell death. Our data also have shown that CRC values should be considered as arbitrary values because they are highly dependent on various factors present in the medium and on the experimental protocol used when different ratios between mitochondrial protein and the quantity of added calcium are used.

Mechanisms of the function and the regulation of the MPTP are easier to study by the swelling methods then by measurement of the calcium retention capacity; however, understanding its molecular structure is more difficult, and new methods will be required. The main problem is to recognize whether the membrane permeability transition pore is a classical pore composed of various proteins or whether it is just a hole in the inner membrane. Such speculations were presented already four years ago by Szabo and Zoratti (Szabo and Zoratti 2014). They suggested that the MPTP is not a classical pore and that “MPTP is a many faceted multifactorial phenomenon with no absolute dependence on any one parameter. In the absence of definitive genetic evidence on its molecular composition, the MPTP must be defined operationally on the basis of its pharmaceutical and biophysical properties. Inner mitochondrial membrane permeabilization may conceivably result from the activation or formation of more than one kind of channel, that is, the possible existence of more than one MPTP must be kept in mind”. One pore probably does not exist but many different pores could be formed when cyclophilin D is bound to various inner membrane components, which then participates in forming the hole in the inner mitochondrial membrane: the pore. New biophysical methods will be required to understand how the hole in the inner membrane of the mitochondria is formed, how it can be opened and closed and how its diameter can be regulated.

Table 1. Values of calcium retention capacity of rat liver mitochondria modified by phosphate (Pi), tert-butyl hydroperoxide (t-BHP) and cyclosporine A (CsA).

Additions to medium	**No. of Ca^{2+} additions (1.25 μM Ca^{2+})**	**Total Ca^{2+} added (nmol Ca^{2+})**	**CRC (nmol Ca^{2+}/mg protein)**	**% CRC**
control (no additions)	7.7 ± 1.6	9.6 ± 2.0	24.1 ± 5.0	100.0 ± 0.0
0.1 mM Pi	6.2 ± 1.5	7.8 ± 1.9	19.4 ± 4.6	75.1 ± 10.8 ***
1 mM Pi	2.8 ± 0.8 ***	3.5 ± 0.9 ***	8.9 ± 2.4 ***	38.0 ± 11.7 ***
0.1 mM t-BHP	2.5 ± 0.6 *	3.1 ± 0.7 *	7.8 ± 1.8 *	35.9 ± 5.0 *
0.5 mM t-BHP	1.7 ± 0.6 **	2.1 ± 0.7 **	5.2 ± 1.8 **	21.4 ± 6.2 ***
1 mM Pi+0.1 mM t-BHP	1.0 ± 0.0 ***	1.3 ± 0.0 ***	3.1 ± 0.0 ***	15.6 ± 3.9 ***

n = 3 – 7; *p<0.05; **p<0.01; ***p<0.001 versus control

TEXT OF FIGURES

Fig. Representative curves of the mitochondrial calcium retention capacity of rat liver mitochondria: Fig. 1A shows the effect of phosphate and cyclosporine A with phosphate. Fig. 1B presents the evaluation of the measured curves (Fig. 1A) at a higher resolution. Fig. 1C shows the effect of t-BHP and Fig. 1D shows the combined effect of phosphate and t-BHP. Mitochondria (0.4 mg protein/ml) were incubated in media with different concentrations of phosphate and t-BHP. Where indicated, 5 μM CsA was added.

Fig. 2A, B. Calcium retention capacity of rat liver mitochondria. Fluorescence curves from Fig. 1C and 1D were evaluated at a higher resolution, as in Fig. 1B. This enables the detection of fluorescence values obtained after each calcium addition when added calcium was accumulated in the mitochondria.

Fig. 3. Values of calcium retention capacity of rat liver mitochondria obtained after the addition of different amounts of $CaCl_2$. Curve 1: 1.25 μM calcium was added. Curve 2: 2.5 μM $CaCl_2$ was added. Curve 3: 5 μM $CaCl_2$ was added. Curve 4: 10 μM $CaCl_2$ was added. Mitochondria were incubated in the presence of 10 mM succinate and 0.5 μM rotenone.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments:

This work was supported by the Program PROGRES Q40/02 and InterCOST LTC17044.

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FIGURES

Figure 1

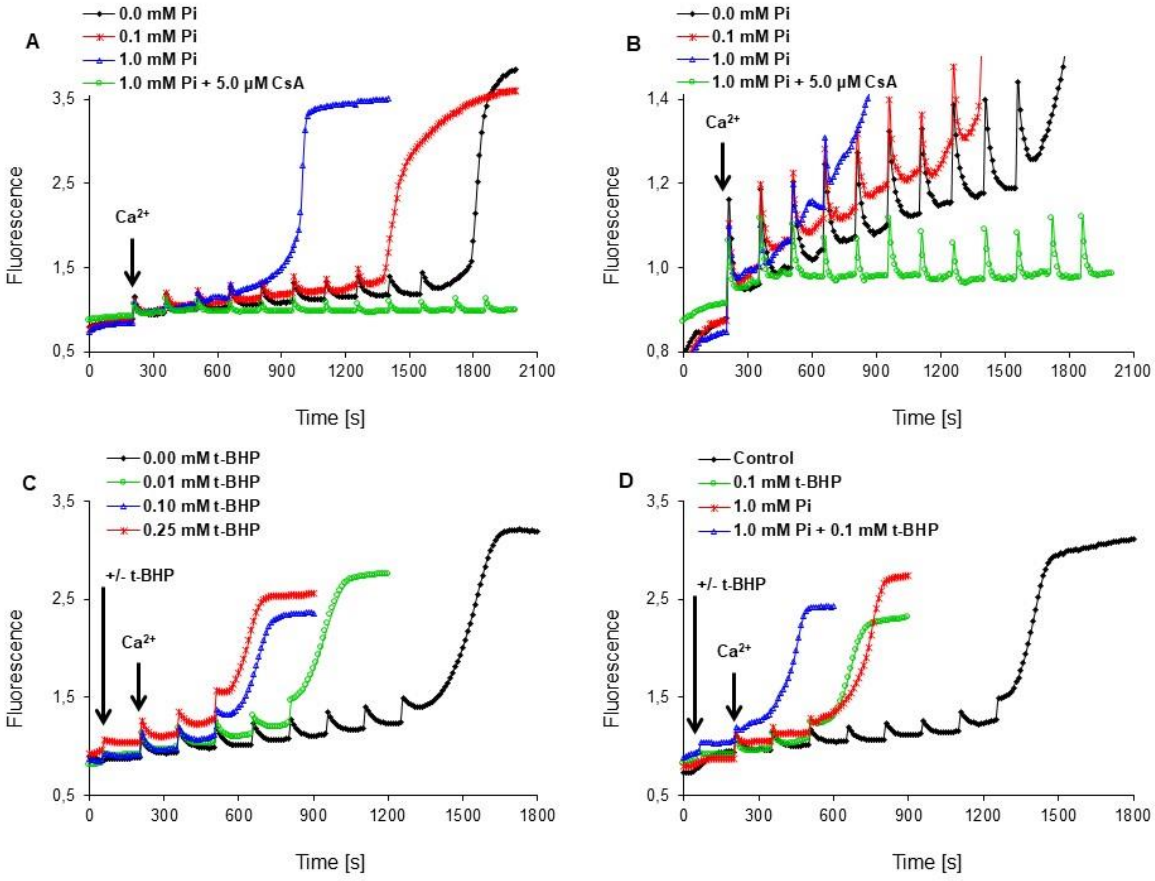


Figure 2

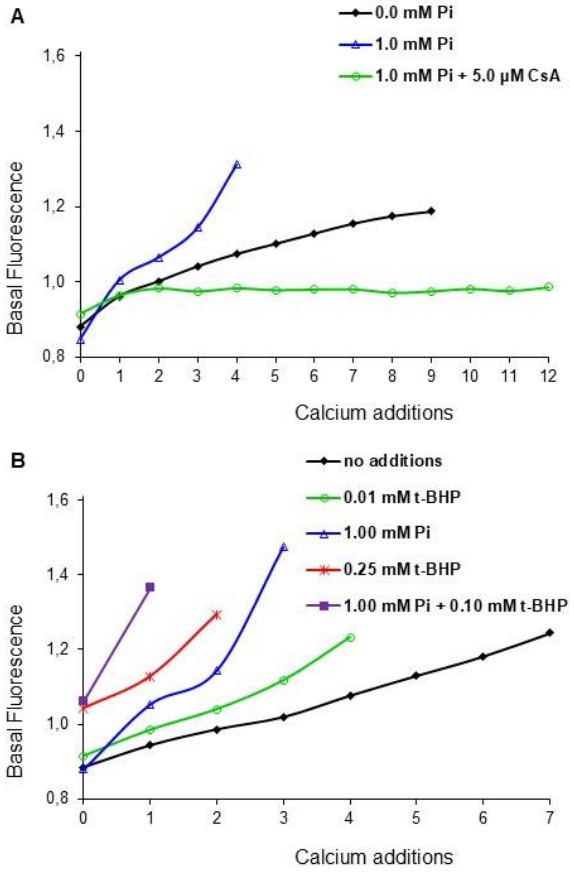
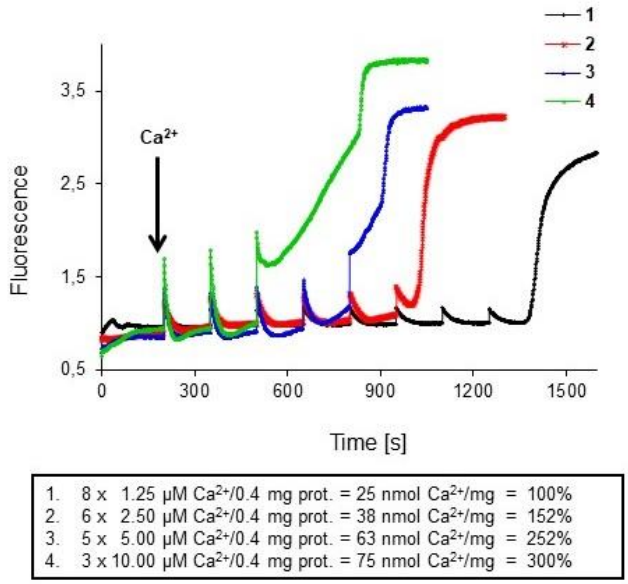


Figure 3



1. 8 x 1.25 µM Ca^{2+}/0.4 mg prot. = 25 nmol Ca^{2+}/mg = 100%
2. 6 x 2.50 µM Ca^{2+}/0.4 mg prot. = 38 nmol Ca^{2+}/mg = 152%
3. 5 x 5.00 µM Ca^{2+}/0.4 mg prot. = 63 nmol Ca^{2+}/mg = 252%
4. 3 x 10.00 µM Ca^{2+}/0.4 mg prot. = 75 nmol Ca^{2+}/mg = 300%