Original Article

Hyperthermia and Plasma Membrane Ca ATPase: Is There Any Effect of HT on PMCA?

Hipertermi ve Plazma membranı Ca ATPaz: HT'nin PMCA Üzerinde Herhangi Bir Etkisi Var mı?

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Çalışmamız, 1. uluslararası Kanser ve İyon Kanalları (IONCC2017) kongresinde, 21-23 Ekim 2017 tarihinde sunumu kabul edilmiştir.

Geliş tarihi / Received: 23.10.2017 **Kabul tarihi / Accepted:** 26.12.2017

Abstract:

Introduction and Aim: The idea of treating cancer cells with hyperthermia (HT) is based on at least 5000 years back. Past technologies in cancer research could not deliver effective and homogenous heating to all cites, especially in deep-seeded tumors. This is the main reason for lack of interest in modern cancer research. Also, HT is known to induce apoptosis in cancer cells. On the other hand, Plasma membrane Ca ATPase (PMCA) is an important pump in apoptosis pathways. PMCA pumps Ca ions in the cell to the outside of the cell. The aim of the present study is to evaluate the effect of HT in the kinetic parameters of PMCA.

Materials and Methods: To evaluate the effect of HT in PMCA, we used red blood cells plasma membrane. Human red blood cells which isolated from the healthy donor were heated at control, 37oC, 44oC, 50oC during 30min, 60min, 90min, 120min by using thermomixer. 1,7ml Lysis solution were added to Erythrocytes (39ul) and incubated for 15 minutes. During the incubation, the mix was gently stirred. The mix was centrifuged at 17000xg for 20 minutes at 0oC. They were resuspended in 1,7ml KCl buffer and centrifuged again. Then, hemoglobin removed from erythrocytes. After that, 0.4ml of this empty erythrocytes suspension was mixed with 0.1ml buffer with Indo 1FF-AM. This suspension was incubated for 15min on ice. These empty erythrocytes were resealed by incubation at 37°C for 45 min and stored on ice until use.

Results: According to calcium concentration levels, there are significant differences between 0oC and the other temperatures. Also, there is a significant difference between 37oC and the other temperatures. PMCA did not work very well at the temperatures over 37oC. So we measured high concentration levels of calcium in the erythrocytes.

Conclusion: If the pump worked properly, we had to see decreasing concentration levels of calcium with time. On the contrary, we measured growing concentration levels of calcium in the erythrocytes. Then, HT effects to PMCA and when PMCA does not work, Ca ions cannot go out from the cells. As a result, calcium overloading is one of the consequences of apoptosis.

Key Words: PMCA, Ca imaging, Hyperthermia

Öz.

Giriş ve Amaç: Hipertermi (HT) ile kanser hücrelerini tedavi etme fikri en az 5000 yıl öncesine dayanır. Kanser araştırmalarındaki geçmiş teknolojiler, özellikle derin çekirdekli tümörlerde, tam olarak etkili ve homojen bir ısıtma sağlayamadığından dolayı modern kanser araştırmalarında HT'ye ilgi eksikliğine sebep oldu. Ayrıca, HT'nin kanser hücrelerinde apoptozu indüklediği bilinmektedir. Öte yandan, plazma membranı Ca ATPaz (PMCA), apoptoz yolaklarında önemli bir pompadır. PMCA, hücredeki Ca iyonlarını hücrenin dışına pompalar. Bu çalışmanın

amacı HT'nin PMCA kinetik parametrelerine etkisini değerlendirmektir.
Yöntem:PMCA üzerinde HT'nin etkisini değerlendirmek için, kırmızı kan hücrelerinin plazma membranı kullandık. Sağlıklı donörden izole edilen insan kırmızı kan hücreleri, termomikser kullanılarak 30 dakika, 60 dakika, 90 dakika, 120 dakika boyunca 37oC, 44oC, 50oC'de ısıtıldı. Eritrositler (39ul) lizis çözeltisi 1,7ml eklendi ve 15 dakika inkübe edildi. İnkübasyon sırasında karışım hafifçe karıştırıldı. Karışım 0°C'de 20 dakika için 17000 xg'de santrifüje tabi tutuldu. Bunlar, 1,7 ml KCl tampon içinde yeniden süspansiyona alınmış ve tekrar santrifüje tabi tutulmuştur. Daha sonra, eritrositlerden hemoglobin çıkarılır. Bundan sonra, 0.4 ml'lik bu boş eritrosit süspansiyonu, Indo 1FF-AM ile 0.1 ml tampon ile karıştırıldı. Bu süspansiyon, buz üzerinde 15 dakika inkübe edildi. Bu boş eritrositler 37°C'de 45 dakika inkübe edilerek tekrar kapatılmış ve kullanıma kadar buz üzerinde depolanmıştır.
Sonuçlar:Kalsiyum konsantrasyon seviyelerine göre kontrol ile diğer sıcaklık arasında belirgin farklar vardır. Ayrıca, 37oC ile diğer sıcaklık arasında belirgin bir fark vardır. PMCA 37oC üzerindeki sıcaklıklarda çok iyi çalışmadı. Bu nedenle, yüksek kalsiyum konsantrasyonu ölçüldü.
Pompa düzgün bir şekilde çalışıyorsa, kalsiyum konsantrasyon seviyesi zamanla azalacaktır. Aksine, eritrosit keseciklerinde artan kalsiyum konsantrasyon seviyeleri ölçtük. HT'ye maruz kalan PMCA, yüksek sıcaklıktan etkilenir ve PMCA çalışmaz, böylece Ca iyonları hücrelerden dışarı çıkamaz. Hücre ölümü ısının etkisi ile bozulan PMCA kinetiğinden dolayı tetiklenmektedir.
Anahtar Kelimeler: PMCA, Ca görüntüleme, Hipertermi

INTRODUCTION

One of the factors which induce apoptosis in the cancer cells is Hyperthermia (HT). The history of HT which treats cancer cells is at least 5000 years back. HT could not deliver effective and homogeneous heating to all sites, particularly deep-seeded tumors in the past technologies. This is the main reason about lack of interest in modern cancer research. Recently new techniques are developed (e.g., nanotechnology, computer modeling, and non-invasive thermometry) that controlled and directly applying heat to tissue has stimulated interest in HT again. There is not any significant difference in HT sensitivity between tumor and normal cells. In an in-vivo study, tumor killing effect is achieved at temperatures between 40oC and 44°C. This is related to a characteristic difference in physiology between normal and tumor cells. The vasculature in solid tumors is complex and includes hypoxic and low-pH regions. These cases are not found in normal tissues under undisturbed conditions (1). If normal tissues were treated for 1 hour at a temperature of up to 44°C, generally they were not damaged in this case (2). HT induces many changes in cells. For example, an increase in temperature causes protein unfolding and aggregation. HT also alters the internal organization of cells including disruption of the cytoskeleton, fragmentation of the Golgi system and ER, and a decrease in the number of mitochondria and lysosomes (1). HT also affects nuclear processes and the cell membrane (3).

The plasma membrane Ca2+-ATPase (PMCA) is an ATP-driven Ca2+ pump that expressed in the plasma membrane of all eukaryotic cells. Four separate genes (PMCA1-4) and numerous splice variants that give rise to specific tissue distribution, cellular localization, and functional diversity encode PMCA (4). The PMCA is important for preserving cytosolic Ca2+ concentration ([Ca2+]i) below 300 nM (~100 nM), due to its high affinity for Ca2+ (Kd, ~0.2 μM) and is the major Ca2+ efflux pathway in non-excitable cells. For many years the PMCA was thought to have a housekeeping role in preserving low resting [Ca2+]i. However, the importance of PMCA in the spatiotemporal shaping of cytosolic Ca2+ signaling has steadily increased. PMCA displays memory of past [Ca2+]i increases, suggesting an important role in regulating the frequency of Ca2+ oscillations. Moreover, the different PMCA isoforms, and numerous splice variants of PMCA, can be differentially expressed in specific regions of cells and can also be differentially regulated by a sophisticated repertoire of additional signalling pathways (4). Despite the emerging role of the PMCA in dynamic Ca2+ signaling, the importance of the housekeeping role of the PMCA should not be underestimated, especially when one considers how important maintaining low resting [Ca2+]i is for cell survival and the prevention of Ca2+-dependent cell death. In this regard the PMCA can be regarded as the "last gatekeeper" for the maintenance of low resting [Ca2+]i; an essential "linchpin" for the delicate balance between cell survival and cell death. Moreover, the PMCA is inextricably linked to the specific nature of cell death. Not only does PMCA prevent Ca2+ overload associated apoptosis, but the PMCA is an ATP-driven pump and since ATP depletion induces necrosis, a decline in PMCA activity will accompany and exacerbate necrosis. Therefore, PMCA activity may act as an important switch between apoptosis and necrotic cell death, a key determinant of numerous disease processes. Thus the maintenance of PMCA activity is critical for cell survival, particularly in the face of modest-to-severe global ATP depletion, whereas inhibition of PMCA even when global ATP is maintained will facilitate Ca2+-dependent apoptosis (4). First, PMCA is worked in red blood cells (5). Erythrocyte has not got any organelles and nucleus so plasma membrane is worked by erythrocytes.

There is not any study which searches relationship between HT and PMCA. In this paper we search whether there is relation between temperature and the kinetic of PMCA. According to our hypothesis, if HT can kill tumor cells, then it can affect PMCA too. Therefore, we incubated erythrocytes with several incubation times at 37oC, 44oC and 50oC temperatures. After incubation, we removed hemoglobin from erythrocytes and loaded Ca2+ with Ca2+ indicator. We measured fluorescence intensity. Ca2+ concentration was calculated by fluorescence intensity. The kinetic parameters of PMCA were determined from Ca2+ concentration.

2. Material and Methods

2.1. Blood, solutions and chemicals

Freshly drawn blood from healthy human donor was used for the experiments. The erythrocytes were washed three times by centrifugation (2000×g, 5 min) at room temperature in physiological salt solution with pH 7.4. Plasma and buffy coat were removed by aspiration. Lysis solution contains 5 mM MgSO4 and 0.4 mM acetic acid, pH 4.2. Loading buffer consist of 1mM ATP, 2,5mM Fosfokeratin, 500U/l Fosfokeratin kinaz, 220µM CaCl, 20mM Hepes, 160mM KCl and 10µM Indo1FF-AM.

2.2. Preparetion of erythrocyt ghost

After separation of erythrocytes from white blood cells by centrifugation, the erythrocyte suspension was adjusted to a hematocrit of 50%. They were separated four group according to incubation temperatures. Group 1 is control with no incubation, group 2 is incubated at 37oC, group 3 is incubated 44oC and group 4 is incubated 50oC. These temperatures also were seperated 4 subgroup according to incubation times, each groups were incubated 30min, 60min, 90min, and 120min respectively. After carefully pre-cooling of all media and materials to 0°C, lysis was performed on ice. Erythrocytes (0.7 ml) were added to 30 ml of the lysis solution and incubated for 15 min with gentle stirring. Ghosts were then sedimented (16,000 X g for 20 min at 0°C), resuspended in 30 ml of KCI buffer, again sedimented, and finally resuspended in 1.75 ml of buffer. Then, 0.4 ml of this suspension was mixed with 0.1 ml of loading buffer and incubated for 15 min on ice. Erythrocyte ghosts were resealed by incubation at 37°C for 45 min and stored on ice until use.

2.3. Measurement of intracellular fluorescence intensities by fluorescence microscopy

Before microscopic studies, erythrocyte ghosts were washed twice with KCI buffer. The ghost suspension was added to glass slide surface. The glass slide was scanned under the microscope. A good single erythrocyte ghost was selected. The Ca2+ indicator indo-1 FF shows a shift and an increase in the peak of its emission spectra when Ca2+ binds, whereas the excitation spectra remain unaltered. Thus, the dye is excited at a single wavelength between 338nm and 350nm and emission is monitored at 400nm and 450nm, the respective peaks of the Ca2+-bound and Ca2+-free spectra. Therefore, An erythrocyte ghost was monitored by DAPI filter for Indo 1 FF with

Ca2+-bound and shortly after it was monitored by FITC filter for Indo 1 FF with Ca2+-free.

Determination of Km and Vmax

The kinetics of the calcium pump can be approximated by the Michaelis-Menten equation:

$$v = \frac{V\max[Ca^{+2}]}{Km + [Ca^{+2}]} \quad (1)$$

[Ca2+] was detected time dependent from fluorescence intensity.

$$[Ca^{+2}] = K_d \frac{(R - R_{\min})}{(R_{\max} - R)} \frac{S_{f2}}{S_{b2}} \quad (2)$$

$$R_{\min} = \frac{S_{f1}}{S_{f2}} \text{ and } R_{\max} = \frac{S_{b1}}{S_{b2}}$$

In the equation 2, Sf1 is the concentration of free Ca2+ in λ1, Sf2, is the concentration of free Ca2+ in λ2, Sb1 is the concentration of binding Ca2+ in λ1, Sb2, is the concentration of binding Ca2+ in λ2. Km and Vmax were calculated by the GraphPad software. Also, all graphs were drawn by GraphPad software. For the statistical analysis, the statistical software of GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used. We used ANOVA to evaluate the differences among groups.

3. RESULTS

3.1 The graph of reaction rate versus [Ca2+]

Erythrocytes were incubated 30 minutes, 60 minutes, 90 minutes and 120 minutes at 37oC, 44oC and 50oC temperatures separately. [Ca2+]-reaction rates of PMCA were measured and their graphs were shown in fig. 3.1-4. These graphs show the kinetic of the pump. Vmax and Km were calculated according to these graphs. Control group erythrocytes were not incubated at any temperature. If the kinetic of the pump is neglected, there is a linear relationship between the rate of Ca2+ and the concentration of Ca2+.

In the fig 3.1-4, the graphs are linear at 30min, 60min 90min 120min of 44oC and 50oC temperatures. PMCA of erythrocyte ghosts which were incubated at these temperatures during these times doesn’t have correct Vmax and Km values. According to linear graphs, reaction rate reaches Vmax at higher values than the control group. But there is usually a hyperbolic relationship between the reaction rate and the concentration of Ca2+. In the control group, the graph is hyperbolic. The graphs of all incubation times of 37oC temperature and the graphs of 30min incubation of 44oC and 50oC temperatures are hyperbolic as the control. But the graphs of 60min, 90min and 120min incubations of 44oC and 50oC temperatures are not hyperbolic. Their graphs are linear.

3.2. Km and Vmax between temperatures and incubation time

According to temperatures, the graphs of Km and Vmax were shown in fig. 3.5-7. The Km is the inverse of the Ca2+ affinity of the pump. It changes according to Vmax. For Km and Vmax values, there is not any significant difference between 37oC temperature and the control group. Also, there is not any significant difference among incubation times of 37oC temperature. There is a significant difference between control group and 90min and 120 min incubation times of 44oC temperature ($p<0,01$). When the incubation times of 44oC temperature are compared, there are significant differences between 30min and 90min, 30min and 120min, 60min and 90min and 60min and 120min ($p<0,01$).

There is a significant difference between control group and 90min incubation of 50oC temperature in Vmax values ($p<0,05$). Also there are significant differences between 30, 60, 120 min and 90min incubation times of 50oC in Vmax values ($p<0,05$).

3.3. The Relation Of Temperature Between Each Other

The Km and Vmax graphs which show the relationship between temperatures is shown in fig. 3.8-9. When these temperatures are compared with each other, for Km and Vmax there are significant differences between 90-120min incubation times of 37oC temperature and 90-120min incubation times of 44oC temperature. There are significant differences between 90-120min incubation times of 37oC temperature and 90-120min incubation times of 50oC temperature.

4. Discussion

First of all, the important point that we need to emphasize, is that erythrocytes incubated at temperatures before lysis. So while we were evaluating the results, we thought whole erythrocytes. Because hemoglobin and other proteins were affected by incubation temperatures. Some studies show that the tumor cells are killed by temperature when they are incubated a long time at 40oC - 44oC temperatures (1). Erythrocytes which were not lysis were incubated at 37oC, 44oC, and 50oC temperatures. These temperatures affected hemoglobin, proteins and other things inside the erythrocytes. The functions of PMCA were determined by intracellular ion imaging. Km and Vmax which are the kinetic parameters of PMCA mean to the Ca2+ capacity of PMCA (6). There is not any direct information about a relation between HT and PMCA. Our data shows the relation between HT and PMCA.

PMCA has a critical role in cell death. If PMCA is inhibited, Ca2+ accumulates and cell undergoes apoptosis. But, if PMCA is induced, more Ca2+ can come out from cell than normal situation. In this case, the cell undergoes necrosis (4).

Vmax of PMCA is higher at the temperatures of 44oC and above. Vmax means that the reaction rate of PMCA is maxed and stable. Ca2+

concentration can change but Vmax remains at the stable value. Km value of PMCA is the Ca2+ concentration in the half of Vmax. The higher Km value of the pump, the lower the Ca2+ removal capacity. In our results, there are significant differences between incubation times 90min-120min at 44oC and control group in Km and Vmax of PMCA. Also, there are significant differences between incubation times 90min-120min at 44oC and 37oC in Km and Vmax of PMCA. According to these results, PMCA may cause cell death with temperature.

When a cell is exposed to high temperatures for a long time, intracellular proteins accumulate and fold in the cell. These proteins lose their biological activities. At the same time, they damage plasma membrane because the balance of protein decays (1). In this case, Hemoglobin may be affected by temperature because hemoglobin is a protein in the red blood cells. Also, it binds to the plasma membrane with a weak binding (7).

Structurally, PMCA consists of ten transmembrane domains, two cytosolic loops with both N- and C-terminal cytosolic tails. The most functionally important structural domain is the C-terminal tail which contains the autoinhibitory calmodulin (CaM)-binding motif (8). At low resting [Ca2+]i, the autoinhibitory CaM-binding motif interacts with the catalytic site (first and second cytosolic loops) thereby inhibiting the PMCA (4). In another study, there is a relation between CaM and hemoglobin. CaM may be affected by temperature so CaM-binding motif of PMCA may not work as before (8).

Consequently, this study shows that PMCA is affected by temperature. Incubation times of temperatures are very important. HT causes cell death. A great possibility, the one reason of cell death associated with HT is that the kinetic parameters of PMCA change more than normal situation. The function of PMCA damaged with temperature. The Ca2+ affinity of PMCA is changed. We showed that Km and Vmax values of PMCA are different with 44oC and 50oC temperatures. For more clear results, more molecular studies have to be performed.

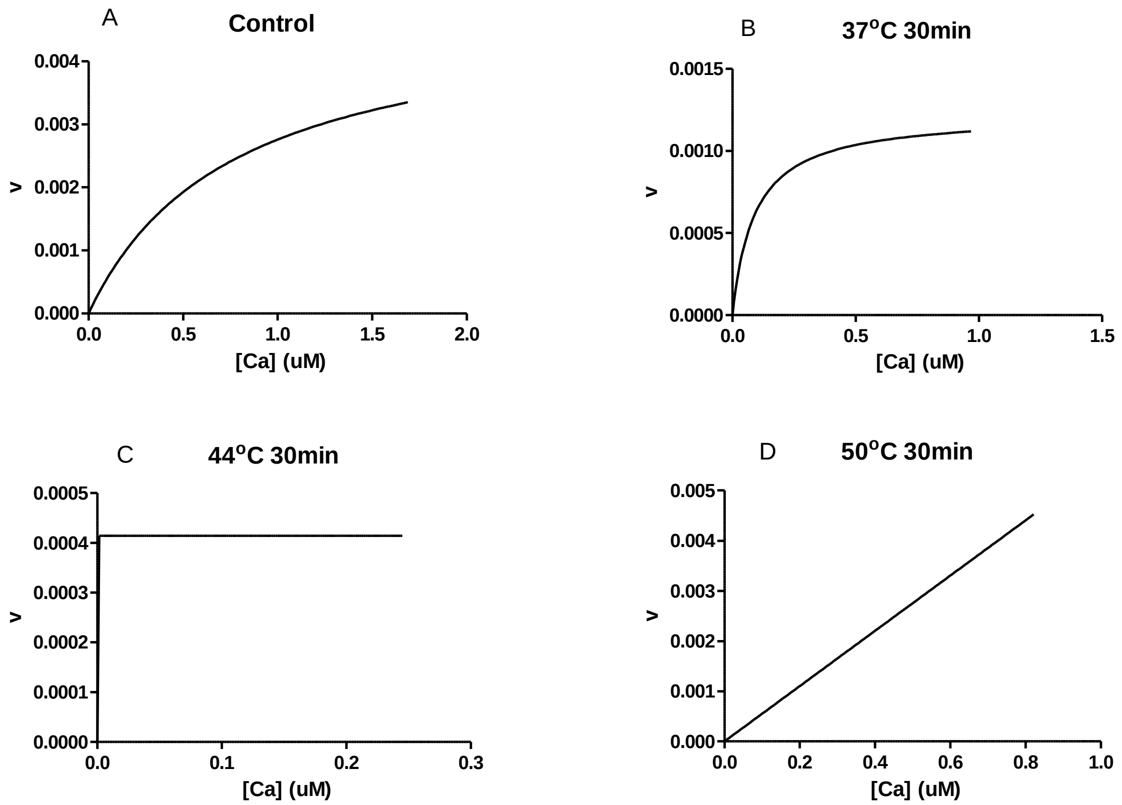

Figure3.1. The graph of reaction rate versus [Ca^{2+}]. A. Erythrocytes were not incubated at any temperatures. B. Erythrocytes were incubated 30min at 37°C temperature. C. Erythrocytes were incubated 30min at 44°C temperature. D. Erythrocytes were incubated 30min at 50°C temperature.

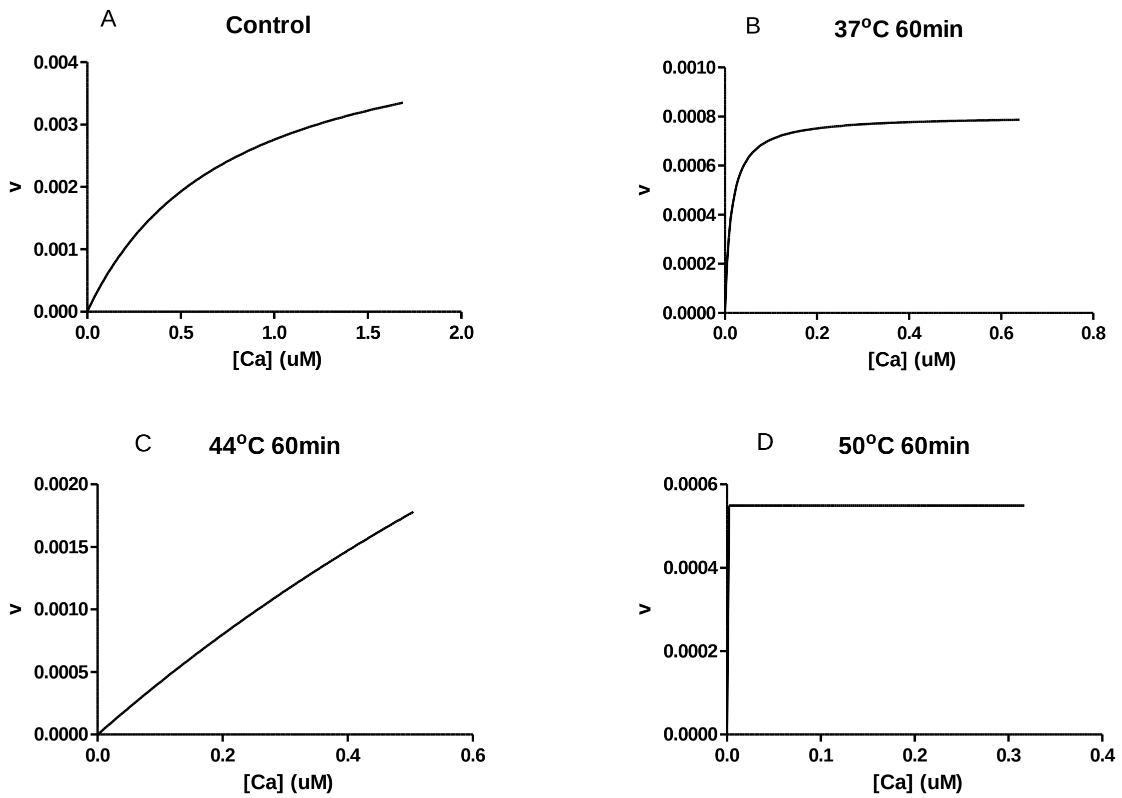

Figure3.2. The graph of reaction rate versus [Ca^{2+}]. A. Erythrocytes were not incubated at any temperatures. B. Erythrocytes were incubated 60min at 37°C temperature. C. Erythrocytes were incubated 60min at 44°C temperature. D. Erythrocytes were incubated 60min at 50°C temperature.

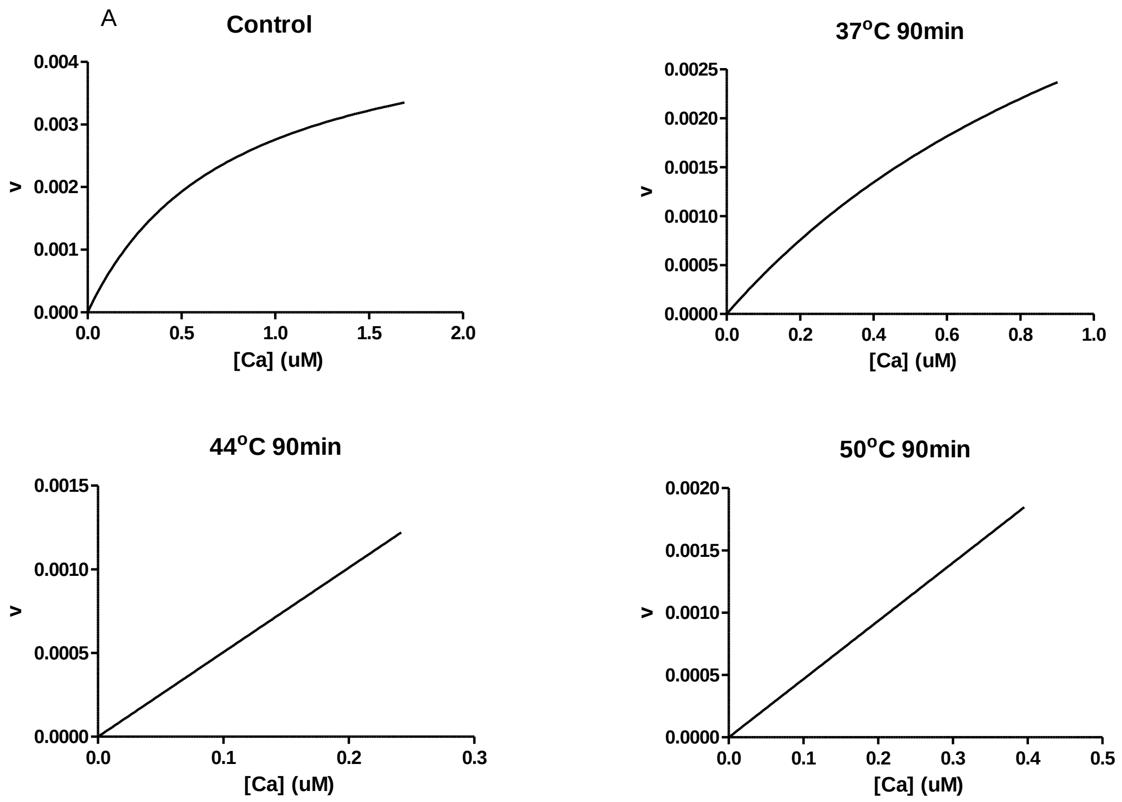

Figure3.3. The graph of reaction rate versus [Ca^{2+}]. A. Erythrocytes were not incubated at any temperatures. B. Erythrocytes were incubated 90min at 37°C temperature. C. Erythrocytes were incubated 90min at 44°C temperature. D. Erythrocytes were incubated 90min at 50°C temperature.

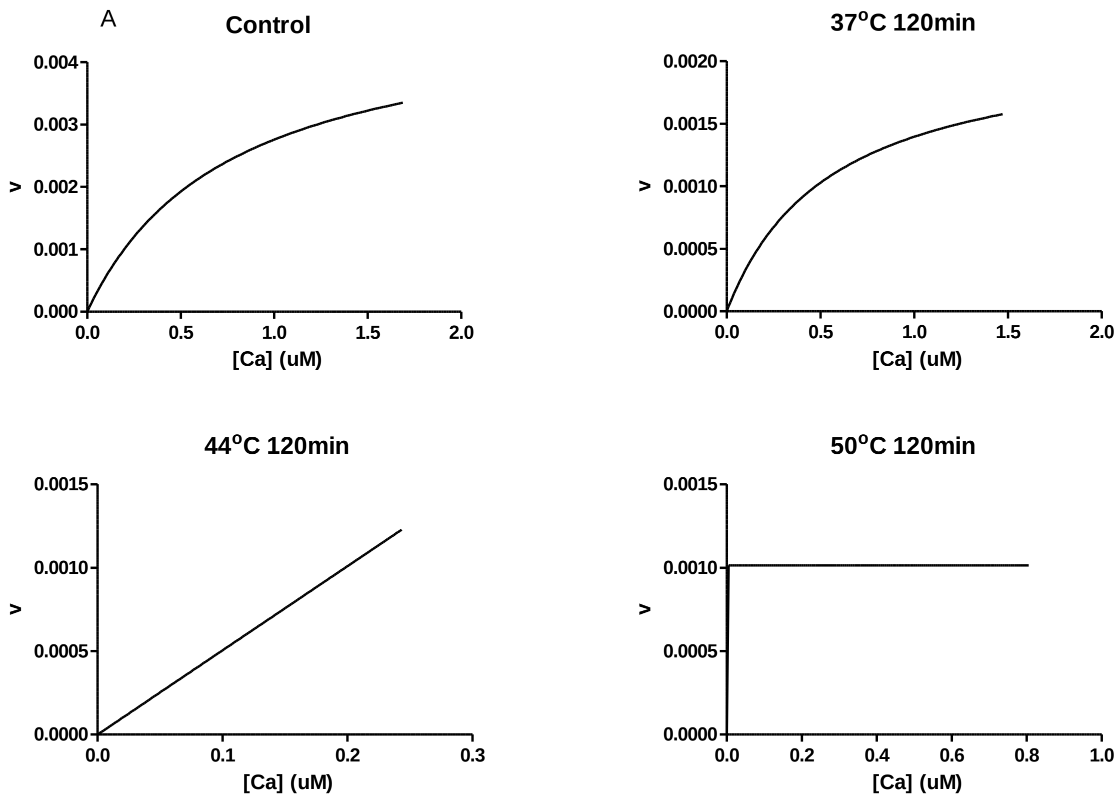

Figure3.4. The graph of reaction rate versus [Ca^{2+}]. A. Erythrocytes were not incubated at any temperatures. B. Erythrocytes were incubated 120min at 37°C temperature. C. Erythrocytes were incubated 120min at 44°C temperature. D. Erythrocytes were incubated 120min at 50°C temperature.

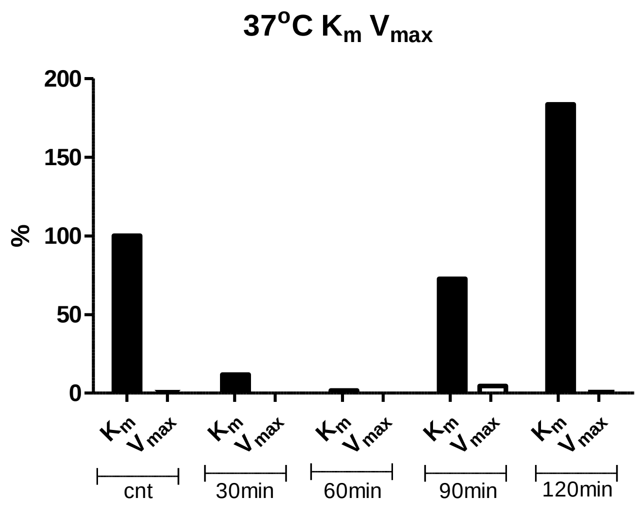

Figure3.5. K_m and V_{max} of PMCA in 37^{o}C temperature

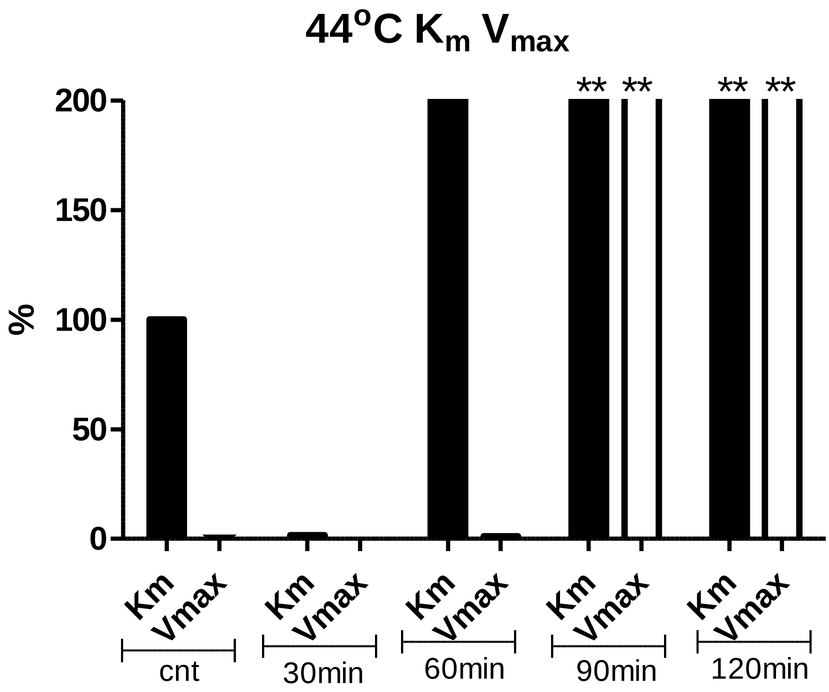

Figure3.6. K_m and V_{max} of PMCA in 44^{o}C temperature

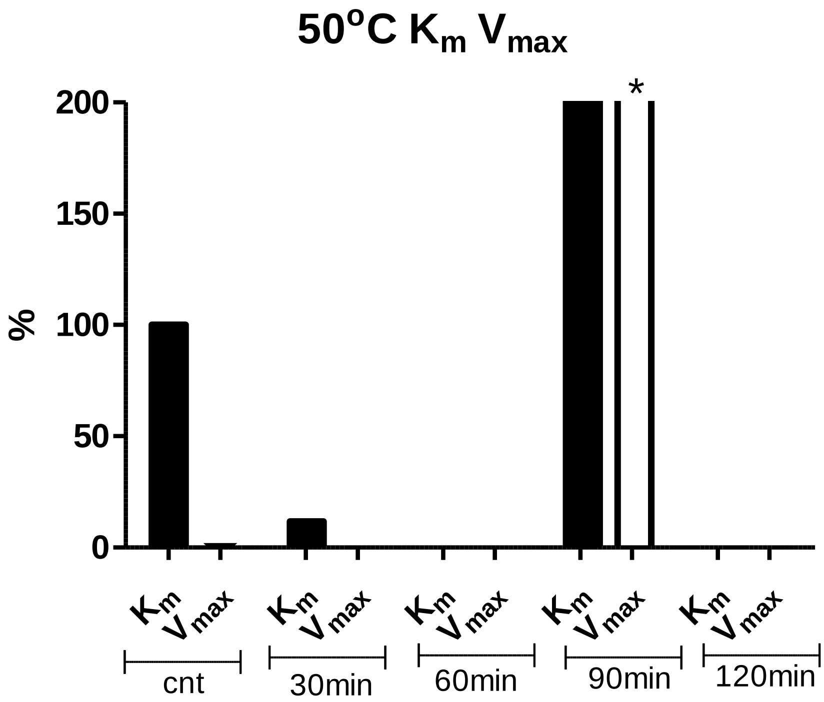

Figure3.7. K_m and V_{max} of PMCA in 50°C temperature

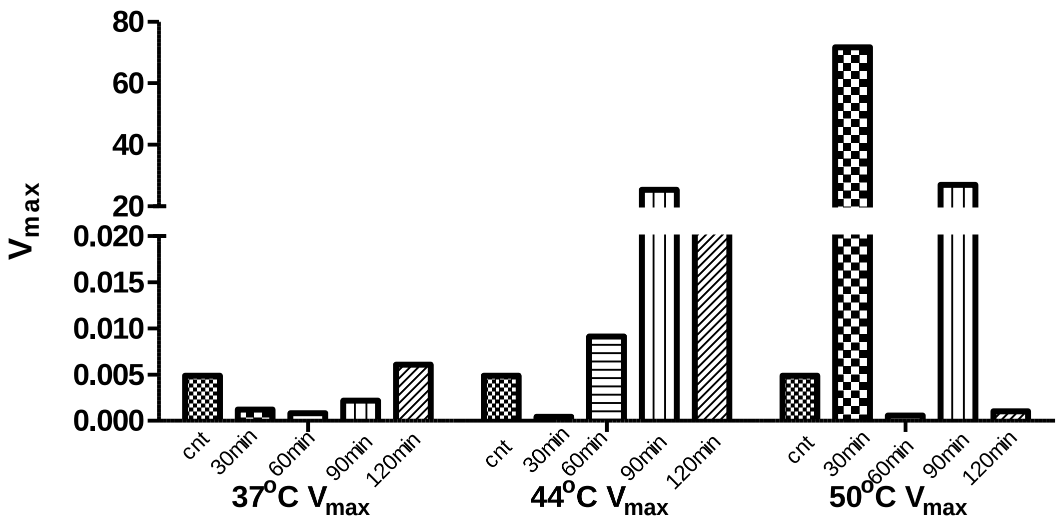

Figure3.8. The graphs of Vmax of 37oC, 44oC and 50oC

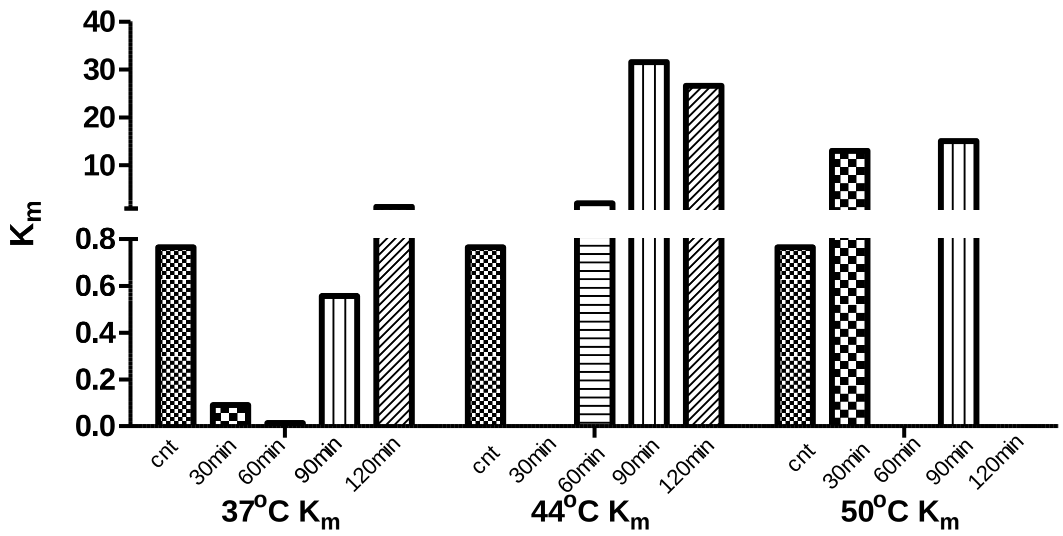

Figure3.9. The graph of K_m of 37ºC, 44ºC and 50ºC

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