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ALTERED CALCIUM DYNAMICS IN HYPERPARATHYROIDISM: CALCULATION OF PMCA PUMP KINETICS IN ERYTHROCYTE GHOSTS

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Indo 1 FF AM,
Michaelis-Menten,
PMCA

ABSTRACT

Calcium is an important chemical for the metabolic functions of the cell. It is very important in the cellular process that the amount of calcium is balanced both inside and outside the cell. Calcium balance within the cell is achieved through channels and ATP-dependent pumps in the cell membrane. The plasma membrane calcium pump is the most important ATP-dependent pump that maintains calcium balance within the cell by removing calcium from the cell. The maximum calcium removal rate of PMCA (V_{max}) and the amount of calcium released from the pump (K_m) are the parameters that are effective in maintaining the calcium balance within the cell. In this study, the kinetic parameters of the PMCA pump of Hyperparathyroid patients were calculated. For this purpose, erythrocytes isolated from the blood of Hyperparathyroid patients were studied. The hemoglobins in the erythrocytes were emptied. These erythrocytes were closed again and erythrocyte ghosts with only cell membranes were obtained. In this study, erythrocyte ghosts were loaded with a buffer containing Indo 1 FF AM calcium indicator and calcium before being closed again. Calcium concentrations in erythrocyte ghosts were calculated from fluorescence intensities under a fluorescent microscope. From the calcium concentrations measured over time, the kinetic parameters of PMCA were calculated from the Michaelis-Menten equation. In Hyperparathyroid patients, K_m = 0.45 uM, V_{max} = 0.21 uM. According to these results, the PMCA pumice of Hyperparathyroid patients works faster and pumps more Ca out of the cell compared to the control group.

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INTRODUCTION

Calcium (Ca^{2+}), which is responsible for cellular events such as muscle contraction, memory, learning, hormone release, gene transcription, fertilization, differentiation and development of cells, necrosis and apoptosis, is seen in most cellular reactions as an intracellular second messenger. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is 100 nM when the cell is at rest state. When cells are stimulated in various ways, $[Ca^{2+}]_i$ can increase to 1–3 μM (1000–3000 nM). $Ca^{2+}{}_i$ signaling consists of a transient increase in $[Ca^{2+}]_i$. The signal production function is directly proportional to the increase in cytosolic Ca^{2+} [1]. The increase in Ca^{2+} in the cytosol occurs due to the leakage of Ca^{2+} from organs that store Ca^{2+} within the cell or from the cell nucleus into the cytosol [2].

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As Ca^{2+} increases in the blood, parathyroid hormone (PTH) secretion from the parathyroid glands decreases. Conversely, when the calcium concentration decreases in the extracellular, these pathways are suppressed and PTH secretion is stimulated. Hyperparathyroidism is caused by excessive activity of the parathyroid glands. The parathyroid glands produce excessive amounts of hormones independent of Ca^{2+} in the blood[3].

Plasma Membrane Ca^{2+} ATPases (PMCAs) belong to the type IIB subfamily of P-type ATPases[4]. It is a high-affinity pump responsible for Ca^{2+} export across the cell membrane. The pump contains 10 transmembrane domains, two major cytosolic loops, and a long cytosolic C-terminal tail. The C-terminal tail of PMCAs is crucial for pump regulation[5]. Along with the PMCA pump, the Na/Ca converter is also present in the plasma membrane to ensure calcium balance within the cell. Although the two appear to work together, PMCA activity was found to be sufficient to maintain Ca^{2+} homeostasis in cardiac-specific Na/Ca exchanger knockout mice[6]. Since there is no Na/Ca converter in the erythrocyte membranes, Ca ions leave the erythrocyte vesicles via PMCA pumps. In other words, in our study, there is no other mechanism that can remove Ca ions from erythrocyte vesicles. The most important protein involved in pumping Ca ions out of the PMCA pump in cells is the Calmodulin (CaM) protein[7–9]. The CaM protein complex, which binds with Ca ions, regulates the operation of the PMCA pump by binding to the CaM binding region located at the C terminus of the PMCA pump[10]. The ATP-binding region is located between segments 4 and 5 of the 10-segment structure of the PMCA pump. Terminal C, in the rest state of the PMCA pump, this terminal is connected between segments 2 and 3 of the PMCA pump. Due to this connection, the region where ATP binds is closed. However, when Ca ions bind with CaM, the C terminal opens and ATP can be bound[11].

Red blood cells are the simplest, most common and well-defined cells in which PMCA has been studied extensively. PMCA activity was best determined in studies with erythrocyte membranes[12]. Erythrocyte ghosts are residues left after hemolysis of red blood cells. It is generally assumed that these residues lack intracellular structure and mainly contain cell membrane. For this reason, ghosts are widely used in the study of red blood cell membrane composition, structure and function[13].

In this study, the kinetic parameters of PMCA pumps of hyperparathyroid patients were measured in the erythrocyte ghosts of them. For this purpose, the erythrocytes of the blood of patients were emptied and Ca^{2+} and the Ca^{2+} indicator Indo 1 FF AM were loaded and sealed again. Kinetic parameters of the PMCA pump were calculated by monitoring Ca^{2+} outputs.

METHOD

Ethics approval

Ethics Committee of Erciyes University School of Medicine. Ethics committee number: 2010/70

Erythrocyte Isolation

In this study, 66 erythrocytes from the blood of untreated patients diagnosed with hyperparathyroidism who came to Erciyes University Faculty of Medicine Hospitals Endocrinology Metabolism outpatient clinics between 2016 and 2017, and 33 erythrocytes from healthy volunteer bloods were studied. As soon as 5 ml peripheral blood samples were taken into EDTA biochemistry tubes from patients diagnosed with hyperparathyroidism and control subjects without any health problems, they were centrifuged at 2000 rpm, 4°C for 5 minutes for erythrocyte isolation. The serum remaining on top was discarded, the remaining portion at the bottom was mixed with 5 ml NaCl solution and centrifuged at 2000 rpm, 4°C for 5 minutes. The supernatant was discarded and the pellet was washed 3 times with NaCl. 2.5 ml erythrocyte suspension was prepared.

Release of Hemoglobin from Erythrocytes

To remove the hemoglobin in the erythrocytes, 2 ul lysis solution ($MgSO_4$ 5 mM, Acetic acid 0.4 mM, pH 4.2) was added to 50 ul erythrocyte suspension. It was gently mixed every 5 minutes and kept for 15 minutes in ice. At the end of 15 minutes, it was centrifuged at 17000xg at 4°C for 20 minutes. The remaining supernatant was discarded. The pellet was resuspended in 1 ml KCl buffer (KCl 160 mM, Hepes 20 mM, pH 7.2). It was centrifuged again at 17000xg at 4°C for 20 minutes. The supernatant was discarded and the pellet was resuspended with 1 ml KCl buffer.

Loading of Erythrocyte Ghost with Ca^{2+} Indicator

Hemoglobin-depleted erythrocytes were loaded with loading solution containing Ca^{2+} indicator (DMN 200 µM, ATP 1 mM, Phosphokeratin 2.5 mM, Phosphokeratin kinase 500 U/l, CaCl 220 µM, Hepes 20 mM, KCl 160 mM, Indo1FF-AM 10 µM). For this, 400 µl of empty erythrocyte solution was taken and resuspended in ice with 100 µl of loading solution. After incubation on ice for 15 minutes, it was incubated at 37°C for 1 hour. After 1 hour, it was centrifuged at 5000 rpm for 10 minutes at room temperature. The upper part was discarded without touching the pellet and 500 µl KCl buffer was added.

Measurement of Fluorescence Intensity Inside Erythrocyte Vesicles

Fluorescence intensity from erythrocyte ghosts was measured by a Nikon Ti Eclipse fluorescence microscope. The erythrocyte ghost suspension was mixed slowly and gently. In a dark environment, 10 µl of this suspension was taken and placed on a clean slide. It was excited with a single wavelength every 5 minutes for 30 minutes and imaged at two emission wavelengths. Excited at approximately 350 nm. The decrease in fluorescence intensity was detected by taking photographs at the emission wavelength of Indo1 FF at ~405 nm when bound to Ca^{2+} and at the emission wavelength of Indo-1 FF at ~485 nm when Ca^{2+} was free.

Calculation of Fluorescence Intensity

Fluorescence intensity was measured with the Fiji 64 version of the Image J program from photographs taken every 5 minutes. A single erythrocyte was selected for each measurement. Fluorescence intensity was calculated from the area values and background values of the selected erythrocytes with the following formula:

Fluorescence intensity=Integrated intensity – (Area x Background average gray value) (1)

Calculation of Ca^{2+} Concentration in Erythrocyte Ghosts

Analyzes using fluorescent ion indicators can be performed using ratiometric or non-ratiometric methods. Ca^{2+} concentration in cells loaded with indicators that can be both stimulated and measured with a single wavelength is calculated by non-ratiometric methods. The ratio between two fluorescence intensities is calculated by ratiometric measurements. In ratiometric measurements there is either an emission shift or an excitation spectrum shift. Since Indo1 FF is a ratiometric Ca2+ indicator, the Ca2+ concentration in erythrocyte ghosts was calculated with the ratiometric calculations of Tsien et al[14].

What all ratiometric methods have in common is that the intensity of the emitted light is measured twice and a ratio (R) of these intensities is calculated. A ratio value is calculated for each pixel of two simultaneously acquired images. Calcium concentration is calculated from formula 2.

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

$$R_{max} = \frac{S_{b1}}{S_{b2}} \qquad R_{min} = \frac{S_{f1}}{S_{f2}} \qquad R = \frac{F_1}{F_2}$$

Sf1, measured concentration of free Ca^{2+} indicators in λ1, S_{f2}, measured concentration of free Ca^{2+} indicators in λ2, S_{b1}, measured concentration of Ca^{2+} indicators bound with Ca^{2+} ions in λ1, S_{b2}, measured Ca^{2+} indicators bound with Ca^{2+} ions in λ2 is the measured concentration of indicators. Kd=Indo1 FF is the dissociation constant of the FF indicator. Indo1 FF dissociation constant is 230 nM.

Calculation of K_m and V_{max} of PMCA from Ca^{2+} Concentration

After calculating the Ca^{2+} concentration, the kinetic parameters of PMCA were calculated from these values. In these calculations, the enzyme-substrate relationship was taken as a model in the working principle of PMCA. The kinetics of PMCA were adapted according to the principles of enzyme kinetics. In enzyme kinetics, reaction rate expresses the change in concentration

of a product per unit time. The reaction rate in PMCA kinetics expresses the change in Ca^{2+} concentration per unit time.

Reaction Rate:

$$v = \frac{\Delta[Ca^{2+}]}{\Delta t} \quad (3)$$

Its unit is mole/litre.time

$$v = \frac{V\,max[Ca^{2+}]}{Km+[Ca^{2+}]} \quad (4)$$

Vmax refers to the maximum outflow rate of Ca^{2+} ion and K^m refers to the Ca^{2+} concentration corresponding to half the rate of V^{max}.

Statistical Analysis

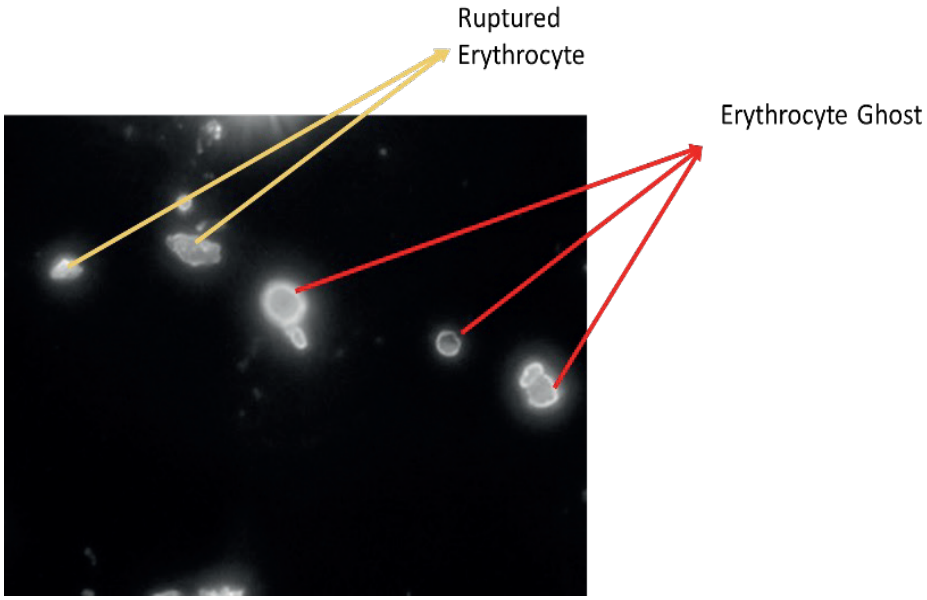
Michaelis Menten graphs were drawn and V^{max} and Km values were calculated using Graph Path 8 software. T-student test was applied for statistical analysis of PMCA K^m and V^{max} values between the patient group and the control group. $p<0.05$ was considered statistically significant.

FINDINGS

Calibration of Erythrocyte Ghosts

Erythrocytes were isolated from the blood and hemoglobin was removed from erythrocytes. The important thing here was that the bursting erythrocytes closed after loading. Various experiments were made for temperature and incubation time and the most suitable time was determined. When the hemoglobin in the erythrocyte ghosts is emptied and incubated in the loading solution at 37°C for 1 hour, a complete closure of the erythrocytes is observed as in Figure 1.

Figure 1. *Erythrocyte ghosts. During the preparation phase, some erythrocytes could not reseal after being discharged. Under the microscope, lysed erythrocytes are easily distinguished from others.*

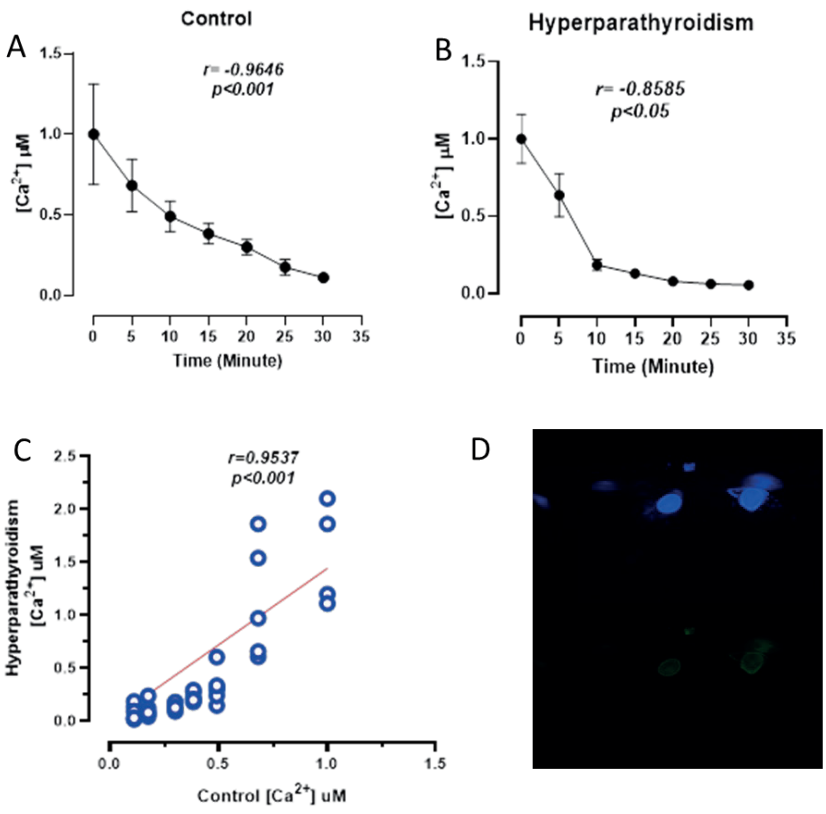


Changes in Ca Concentrations From Fluorescence Intensities Over Time in Erythrocyte Ghosts

The loaded erythrocyte ghosts were photographed under a fluorescent microscope on DAPI and FITC filters at 5-minute intervals for 30 minutes. Accordingly, 924 images obtained from 66 erythrocyte ghosts of Hyperparathyroid patients and 464 images of healthy people's erythrocyte ghosts were analyzed in Image J software. Fluorescence intensities of erythrocyte ghosts were calculated over time in photographs taken with DAPI and FITC filters. Erythrocyte ghosts were evaluated one by one. The area and integrated density of the selected erythrocyte ghost were measured. A region was selected in the background area and the area and mean gray value of this region were measured. These values are the values measured in Image-J software for the erythrocyte ghost we selected. With these values, fluorescence intensity values were calculated over time from DAPI and FTIC images of erythrocyte ghosts from Formula 1.

Since Indo1-FF is a ratiometric indicator, ratio values (R) are required to calculate the $[Ca^{2+}]_i$ in erythrocyte ghosts. The R value was calculated by dividing the fluorescence intensity of erythrocyte ghosts measured at 405 nm wavelength by the fluorescence intensity measured at 485 nm. $[Ca^{2+}]_i$ change over time was measured from formula 2. It was observed that the $[Ca^{2+}]_i$ of the control group decreased statistically significantly over time ($p<0.001$) and had a perfectly negative decrease over time ($r=0.9646$) (Figure 2.A). Likewise, $[Ca^{2+}]_i$ in the ghosts of Hyperparathyroid patients has a negative correlation with time ($r=0.8585$) and decreased statistically significantly ($p<0.05$) (Figure 2.B). The change in Ca^{2+} concentration in the Hyperparathyroid group over time was found to be blunt with the change in Ca^{2+} concentration in the control group and was significant ($r=0.9537$, $p<0.001$). According to this result, the Ca^{2+} level of the patient group decreases together with that of the control group (Figure 2.C).

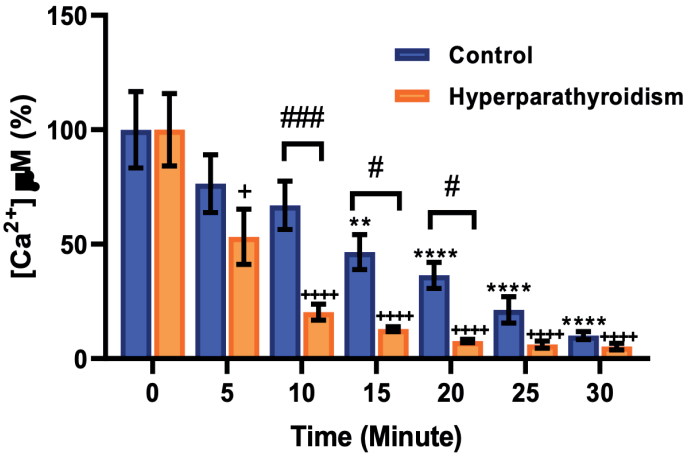
Figure 2. *Change of Ca^{2+} concentration in erythrocyte ghosts over time*



Ca^{2+} concentrations were calculated from the fluorescence intensity of the Indo1 FF AM Ca^{2+} indicator loaded on the erythrocyte ghosts of the control (A) and patient (B) groups. C). Graph showing the correlation of Ca2+ over time in the Hyperparathyroid and control groups. The Hyperparathyroid patient group has a strong positive correlation with the control group. D) Image of erythrocyte ghosts loaded with Indo 1 FF AM Ca^{2+} indicator under fluorescence microscope. Ca^{2+} bound to the indicator (top, blue) Ca^{2+} not bound to the indicator (bottom, green).

Ca^{2+} was calculated as a percentage based on the concentration at minute 0 in both groups (Figure 3). While Ca^{2+} decreased to 76.4% after 5 minutes in the control group, it decreased to 56.2% in the patient group. There was a statistically significant decrease between the 0 minute and the 5 minutes of the patient group ($p<0.05$).

Figure 3. *The percentage of Ca^{2+} concentration values between the Control and the Patient groups*

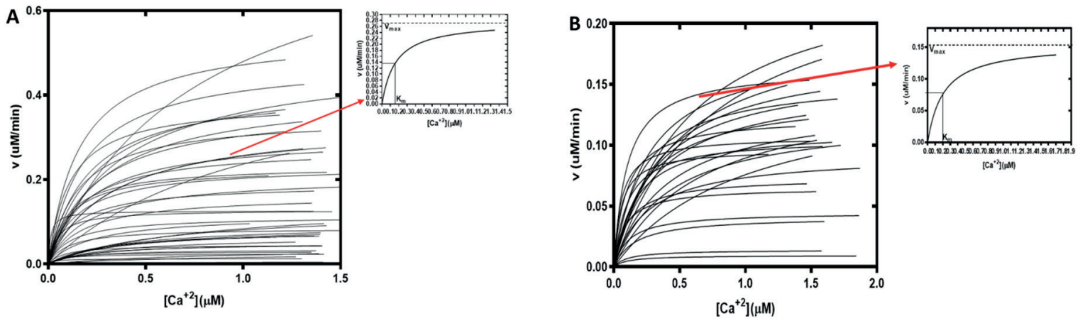


Percentage concentrations were calculated according to the Ca^{2+} concentration values measured at minute 0 of the control and patient groups over time, and the control group and patient groups were evaluated statistically according to the decrease amounts. ****,++++ $p<0.0001$, ### $p<0.001$, ** $p<0.01$, +,# $p<0.05$.

Kinetic Parameters of PMCA

To calculate the amount (K_m) and removal rate (V_{max}) of calcium ions in the erythrocyte ghosts of PMCA of Hyperparathyroid patients, a speed graph was drawn according to the calcium concentration in all erythrocytes (Figure 4). From these graphs, the calcium removal rate of the pump and the released [Ca^{2+}] were calculated. K_m is [Ca2+], which is half of V_{max}.

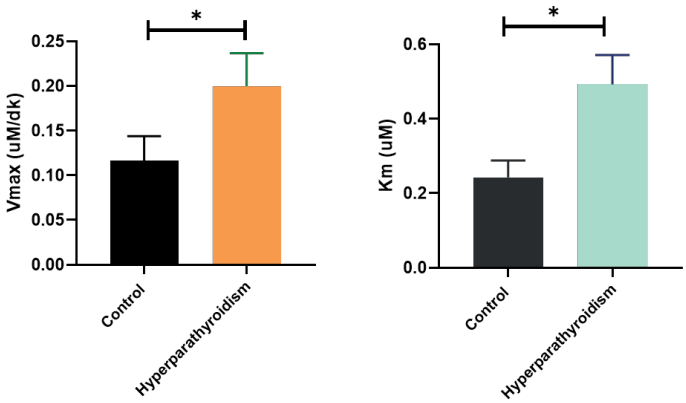
Figure 4. *Michaelis-Menten graphs of the Hyperparathyroid patient group and the control group*



In Figure 4; A. Michaelis-Menten graphs of Hyperparathyroid patient groups. From the velocity graph relative to [Ca^{2+}], V_{max}, the rate of calcium ions leaving the pump, and K_m, the amount of calcium ions leaving the pump, were calculated. $K_m=V_{max}/2$. B. Control group Michaelis-Menten plots. Velocity curves relative to [Ca^{2+}] in control group erythrocytes.

When the K_m values of the Hyperparathyroid and control groups were compared with each other, the average K_m value of the PMCA pumps of the Hyperparathyroid patients was found to be 0.4541±0.069 uM, and the average K_m value of the PMCA pumps of the control group was found to be 0.2428±0.045 uM. A statistically significant difference was found between the amount of calcium released from the PMCA pump of Hyperparathyroid patients and the amount of calcium released from the PMCA pump of the control group ($p<0.05$).

Figure 5. *The difference between the K_m and V_{max} mean values of PMCA in the Hyperparathyroid and control groups*



The V_{max} value calculated from the Michaelis-Menten graphs is higher and statistically significant in the Hyperparathyroid patient group than in the control group ($p<0.05$). The calcium concentration rising at $V_{max}/2$ rate is higher and statistically significant in the patient group than in the control group ($p<0.05$).

DISCUSSION

Ca^{2+} were labeled with a fluorescent marker and visualized in erythrocyte ghosts under a fluorescence microscope. A ratiometric Ca^{2+} indicator was used for this. In fluorescence measurements, side effects such as quenching of fluorescence and optical denstiy differences that affect the accuracy of the measurement are not taken into account in such ratiometric indicators. Because ratiometric Ca^{2+} indicators, when bound to Ca^{2+}, their fluorescence properties change and they are excited or radiate at different wavelengths. Therefore, they become normalized within themselves[15]. The fact that the PMCA pump can be best studied in erythrocyte membranes was proven by the first discovery of PMCA pumps in erythrocytes[16]. Generally, loading a Ca^{2+} marker into a cell is achieved by making the cell membrane permeable to the Ca^{2+} marker[17]. The Ca^{2+} marker was loaded into the erythrocytes by evacuating the hemoglobin and other proteins in the erythrocyte. The resealing erythrocytes without anything other than the chemicals loaded inside them means that the cell membrane will be activated only according to these chemicals[16].

There was any question of whether keeping it at low temperature for a long time or at high temperature for a short time during the closing phase would be more suitable for resealing. After the loading phase of erythrocyte ghosts, it was kept at 4°C overnight, as Teorell et al. tried. It was determined that the erythrocyte membranes were deformed and the resealable erythrocyte membranes were few. Schwoch et al. reported in their publication that resealing the erythrocyte membranes incubated at 37°C for 1 hour gave

better results[13]. Thereupon, after the erythrocyte ghosts were loaded, they were incubated for 1 hour at 37°C. Resealed erythrocytes appeared to retain their vesicle state without being damaged or reopened during subsequent washing steps.

Fluorescence intensity differed in images taken through two different filters. The DAPI filter revealed Ca^{2+} markers bound with Ca^{2+} within the erythrocyte ghosts. When PMCA takes Ca^{2+} out of the erythrocyte ghost, it cannot be detected with the DAPI filter because it releases Ca^{2+} markers. Therefore, the fluorescence intensities determined on the DAPI filter decreased over time. Since the Ca^{2+} marker remaining in the ghost was not bound with the Ca^{2+}, the emission wavelengths changed and were viewed with a FITC filter on the fluorescence microscope. Therefore, the measured fluorescence intensities of erythrocyte ghosts imaged with the FITC filter increased with time. Intracellular Ca^{2+} imaging studies performed in different tissues were also used[15,18–20]. After measuring the fluorescence intensities with two different filters, the Ca^{2+} concentration was calculated by ratiometric analysis[21–23].

Time-dependent Ca^{2+} concentration was evaluated between the Hyperparathyroid patient group and the control group. The amount of Ca^{2+} that decreased over time was found to be different between the two groups. While the Ca^{2+} concentration in the control group did not decrease significantly during the 5-minute period, the Ca^{2+} concentration in the Hyperparathyroid patient group decreased significantly in the first 10 minutes of the 5-minute time period. In healthy individuals, the decrease in Ca^{2+} inside and outside the cell over time varies from cell to cell and from person to person[1,24]. Since it is very important that the intracellular Ca^{2+} concentration is in balance, the decrease in the intracellular Ca^{2+} concentration over time should be in a way that does not disrupt this balance[25].

The kinetics of the active PMCA pump may be altered in Hyperparathyroid patients. Therefore, by calculating the kinetic parameters of the PMCA pump, it was determined numerically that this pump, located in the cell membrane, was affected by the calcium increase caused by hyperparathyroidism. The PMCA pump, which works with ATP phosphorylated by the enzyme, is suitable for the enzyme model, and this model has been supported by various studies[12,26]. In this model, the Ca^{2+} in the erythrocyte ghosts was assumed to be the substrate. The PMCA pump acts as an enzyme in this case. Therefore, the pump has a K_m constant and V_{max} speed depending on the enzyme kinetics[27]. Here, although K_m is a fixed value for each enzyme, since it varies depending on the substrate, the K_m values of the PMCA pumps of the erythrocyte ghosts obtained in the study were found to be different for each erythrocyte ghosts. K_m value was found to be different between the patient group and the control group compared to the control. K_m value is the substrate concentration required for the enzyme to perform its function. If K_m has a high value, the bond between the enzyme and the substrate is weak. In this case, the enzyme needs a large amount of substrate to reach its maximum speed[28].

In our study, the K_m value of PMCA pumps of Hyperparathyroid patients was found to be higher than the control group. This means that the PMCA pump needs to extract more calcium to activate. Only then does the pump remove Ca^{2+} at an increasing rate until it reaches a certain maximum value. When the maximum speed reaches V_{max}, the removal rate remains constant even if the Ca^{2+} removed increases. At high K_m value, it reaches this maximum speed at very high Ca^{2+} concentration[29]. In the study, a significant difference was found between the V_{max} values calculated in the patient and control groups compared to the control. The rate of removal of Ca^{2+} by PMCA pumps in Hyperparathyroid patients was found to be higher than in the control group. The fact that K_m and V_{max} values were different in Hyperparathyroid patients compared to the control group proved numerically that the PMCA pump works kinetically differently in patient tissues.

The V_{max} and K_m values of the normal human PMCA pump, calculated by Delgado et al. using erythrocyte ghosts, are 33 nmol/min and 2μM, respectively [30]. In studies conducted on liver cells, V_{max} values were found to be between 33 and 1,300 nmol/min, and K_m was between 25 nM and 1.6 μM[30]. In another study similar in method to our study, it was conducted with normal human erythrocytes and K_m for the PMCA pump was found to be 24±14 μM and V_{max} was 1.0±0.6 μM/s[27]. In our study, the K_m value of the PMCA pumps of the normal human group was found to be 0.2428±0.045 μM and the Vmax value was 0.1037±0.025 μM/min.

CONCLUSION

As a result, PMCA pumps, which are responsible for maintaining intracellular calcium balance, have important intracellular functions. It is at a key point in the survival of the cell and in the apoptosis of tumor cells. Therefore, disruption of the kinetics of the PMCA pump means disruption of the calcium balance of a healthy cell. In our study, it was shown that the kinetics of PMCA pumps are different in Hyperparathyroid cells, a type of disease in which the calcium balance is disrupted, compared to healthy cells. This difference may be due to various reasons. One of these reasons is that the calcium concentration circulating in the plasma is high in Hyperparathyroid patients, and therefore the amount of calcium entering the cells will be high. The PMCA pump will work faster to remove this calcium within the cell. Another reason may be that the PMCA pump in Hyperparathyroid patients is expressed more than normal.

Conflict of interest statement

The authors declare that they have no conflicts of interests.

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