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Available calcium levels in central nervous system for Arzenazo III method

Rogério de Freitas Lacerda [1,*] and Isabela Cristina Sena Romano [2]

[1] *Biological and Nature Sciences Center, Federal University of Acre, Rio Branco, AC, Brazil.*
[2] *Molecular and Behavioral Neurosciences Laboratory, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.*

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Abstract

The measurement and evaluation of changes in calcium levels between cellular compartments can provide important information for the understanding of biochemical processes, both physiological and pathological. The objective of this study was to validate the use of the calcium diagnostic kit (Arzenazo III) to tissue samples from the Central Nervous System. We used 96-well, flat-bottomed microplates with an internal diameter of 6.7 mm, total height of 12 mm and actual height of 10.705 mm, bottom thickness 1.295 mm, to carry out the tests and calculation parameters, therefore, the path optic was 5.729 mm with a total volume of 202 µL per well. The microplates were incubated at 37 °C for 2 minutes. Optical densities were measured at 630 nm on the Elx800 plate reader, (BioTek, VT, USA). According to the Arzenazo III Bioclin© calcium diagnostic kit. the absorbance of the tests/samples was normalized with the absorbance of the blank (colorimetric reagent 2 - Arsenazo III – Arzenazo III Bioclin© calcium diagnostic kit). PFC, Thalamus and Hippocampus were normalized by blank and presented respectively 0.1782 ± 0.0013; 0.1296 ± 0.0102; 0.0231 ± 0.0053; 0.0170 ± 0.0051; 0.0136 ± 0.0026. The calculation µg calcium / mg tissue obtained 0.4670 ± 0.1056 to sample PFC; 0.4322 ± 0.1228 to sample Thalamus and 0.1027 ± 0.0203 to sample Hippocampus. Although the limitations of the technique, the measurement of free calcium in the CNS tissues (PFC, Thalamus and Hippocampus) showed positive and satisfactory results, as an alternative and viable means for the quantification of calcium levels.

Keywords: Calcium; Arzenazo-III; SNC; Microplates

1. Introduction

It is known that the calcium ion plays a fundamental role in cellular homeostasis and that an imbalance in its concentrations can induce different types of cell death, such as caspase-dependent apoptosis (1–4). It is important to highlight that this imbalance intensifies the production of oxygen radicals (5,6), activation of proteases (7), alterations in the regulation of kinases (8,9) and activation of ion channel proteins (10). These concomitant events represent a set of factors that involved in cell death process (3,4).

The Increased in the calcium level in the cytosol can activate calcium-dependent proteases, such as the calpain family, which cleave pro-caspase 9 into caspase 9, triggering the apoptotic cascade (11,12). Calpain I can also act on protein constituents of Na^+ / Ca^{2+} exchange channels, intensifying intracellular calcium accumulation. These channels may function as an alternative source to the role of NMDA-type channels, which also increase calcium flux across the plasma membrane (13,14).

This excess intracellular calcium stimulates its own uptake by organelles such as mitochondria, generating a destructive cascade of cellular functions (8,15–17). This mitochondrial dysfunction triggers the intrinsic pathway of apoptosis with concomitant release of cytochrome C into the cytosol. This, together with the apoptotic protease activated by factor 1

* Corresponding author: Rogério de Freitas Lacerda; Orcid ID : 0000-0002-1249-7988; E-mail: rogerio.lacerda@ufac.br

(APAF-1) and the active caspase 9, form the apoptosome that activates caspase 3 and, with consequent cell death (13,18,19).

The occurrence of an imbalance in molecular systems involved in these processes can cause damage that characterizes a series of diseases known as neurodegenerative diseases. Some of the mechanisms that lead to cell death, understood independently of one another, have characteristics in common and can co-participate in many health or disease situations (4,20).

In cerebellar granular neurons submitted to Thiamine Deficiency (TD), for example, an increase in protein levels of the CaV1.2 component, a VGCC (Voltage-gated calcium channels) of type L, and an increase in current density were observed MOREIRA-LOBO et al. (2016). This type of channel regulates intracellular calcium concentrations, for example, through the influx of this ion as a result of a change in membrane potential (21,22).

Furthermore, it has been shown that the influx of calcium into the intracellular environment can stimulate the expression of **transcriptional factors**, such as the CREB protein (cAMP response element-binding protein). These factors have a pleiotropic role in the central nervous system, that is, acting on several processes (23,24). The increase in the influx of calcium into the cytosol also allows the dephosphorylation of a coactivator of neuronal circuits CRTC1 (CREB-regulated transcription coactivator) by the action of calcineurin, which together with CREB favors the transcription of the protein / neurotrophic factor, BDNF (brain-derived neutrophic factor), (24).

The fundamental role of ion channels, especially those responsible for cellular calcium ion homeostasis, is known in several cellular processes and, therefore, in cellular viability.

In this sense, the reliable, simple and easily accessible evaluation to basic research, by a test commonly known and used with other biological fluids, would allow answering several scientific questions in addtion to reducing the costs of analyses. Thus, the objective of this study was to validate the use of the calcium diagnostic kit (Arzenazo III) already used in biological fluids, such as blood and liquor, in tissue samples from the Central Nervous System.

2. Material and methods

2.1. Experimental animals

For the experimental protocol, rats from the central vivarium of UFMG were used. All animals received chow containing all nutrients (commercial chow) and water ad libitum. The animals were kept separate (2-3 rats/cage) in polypropylene cages (22x30x40 cm^3). As inclusion criteria in the study, we used the parameters of average weight equal to 250 g and/or 8 weeks of life, considered young, according to MENEZES; NEVES; FILHO, 2013 (25).

All experiments were approved by the Ethics Committee for the Use of Animals (CEUA/UFMG) under number: 288/2016

After decapitation of the animals, samples from three brain regions (Pre-frontal córtex (PFC), hippocampus and thalamus) were dissected from the right or left hemispheres, frozen in liquid nitrogen and stored in an ultrafreezer at -71 ºC, until the time of biochemical analysis (Calcium levels measured by the kit commercial Calcium Arsenazo III – Bioclin©). Descriptions of methods for these biochemical assays are found below. Biochemical analyzes were performed within a maximum period of 20 days after dissection.

2.2. Dosage of free calcium levels

Adjustment of optimal conditions for calcium measurement in brain tissue samples, using microplate and commercial kit

The processing of the samples involved the use of the denaturing agent TCA at 10% and, it is known that the use of this chemical reagent releases and solubilizes calcium ions associated with proteins, allowing their quantification in aqueous solution. The proposal was to use samples processed according to NUNES et al., (2017) (26), lyophilized overnight and resuspended in 100 mM HCl, to measure calcium.

Samples from each of the three brain regions were processed by pooling tissue obtained from the hemispheres of two animals. Therefore, each point used to calculate the mean corresponds to the data obtained from a pool of samples from two animals. Tissue samples were homogenized in 10% trichloroacetic acid (TCA) at a rate of 15 times the tissue weight,

shaken for 1 minute, kept on ice for 15 minutes and shaken again for 1 minute. The samples were then centrifuged at 10,000 x rcf (Relative Centrifugal Force = g) (Sorval RC 5B Centrifuge – Sorval SS-34 rotor) for 15 minutes at 4°C. The supernatant was separated and filtered through a 0.45 µm membrane (Millipore Durapore). The filtered supernatant was washed twice (2x) in water saturated ethyl ether (5:1 v/v), 5x volume of the supernatant and lyophilized overnight.

The lyophilized samples were resuspended in 0.1 M hydrochloric acid (HCl). Therefore, the first step to establish the optimal conditions for calcium measurement in the CPF, thalamus and hippocampus samples, kept in 100 mM HCl, was to verify the interference of HCl in the determination of this ion using the commercial Calcium Arsenazo III – Bioclin© kit.

2.2.1. Calibration curve

Calibration Curve: We used 96-well, flat-bottomed microplates with an internal diameter of 6.7 mm, total height of 12 mm and actual height of 10.705 mm, bottom thickness 1.295 mm, to carry out the tests and calculation parameters, therefore, the path optic was 5.729 mm with a total volume of 202 µL per well. The microplates were incubated at 37°C for 2 minutes. Optical densities were measured at 630 nm on the Elx800 plate reader, (BioTek, VT, USA). According to the Arzenazo III Bioclin© calcium diagnostic kit. the absorbance of the tests/samples was normalized with the absorbance of the blank (colorimetric reagent 2 - Arsenazo III – Arzenazo III Bioclin© calcium diagnostic kit).

To obtain the Calibration Curve, we used calcium carbonate 10 mg/dL (Calcium Arsenazo III – Bioclin©) as a calcium standard, at concentrations of 0; 0.312; 0.625; 1,250; 2,500; 5,000;10,000 mg.

2.2.2. Optimal HCl concentration for calcium measurement

The reaction was started with 2 µL of lyophilized control serum, containing 6 mg/dL calcium (Biocontrol N control – Bioclin© - lot 0057) and 0.9% sodium azide to which 200 µL of Arsenazo III were added. The following steps were the same as described above to obtain the Calibration Curve. Data were also expressed in mg calcium/dL.

We used aliquots of Biocontrol N – Bioclin© (lot 0057) containing HCl in serial dilutions from 0.1M (0.1; 0.05; 0.025; 0.0125; 0.00625; 0.00313; 0.00156; 0.00078), which were compared with aliquots undiluted in acid.

2.2.3. Determinations of calcium levels in biological samples

Samples obtained from animals, resuspended in HCl, and used to determine calcium levels. As mentioned above, the dosage was performed using the Calcium Arsenazo III – Bioclin© *kit*, adapted and validated for a 96-well microplate. Results were expressed in µg calcium / mg tissue (PFC, thalamus or hippocampus).

2.3. Statistical analysis

All analyzes were performed using the GraphPad Prism version 6.00 program (*GraphPad Software*, La Jolla California USA). We used the factorial ANOVA 2 statistical test (treatment x tissue) to analyze calcium levels. All ANOVAs were followed by post hoc tests using multiple comparisons using Tukey and/or Sidak tests. Data were expressed as mean ± SEM, adopting α=0.05. That is, effects or differences with values of $p<0.05$ were considered significant. SEM is the abbreviation for standard error of the mean.

3. Results and discussion

3.1. Dosage of free calcium levels

3.1.1. Calibration curve

According to the Beer-Lambert law and adopting the formula C=Abs/εL, wich C (concentrationo), Abs (absorbance ε (absorption coefficient) e L (total volume) proced to read the calcium standart (Cálcio Arsenazo III – Bioclin©) 10 mg in confort kit wavelenght (*λ=630) on microplate, as described above. The average values found for the absorption coeficiente* (**ε)** of the arsenazo III / cálcium complex was approximately 105.80±4.31 (Beer-Lambert law), wich was compatible that found bay MICHAYLOVA; ILKOVA, 1971; MICHAYLOVA; KOULEVA, 1974 (27, 28).

Through serial dilution, starting from 10 mg (2.5 mM) of calcium standard (Calcium Arsenazo III - Bioclin©) as described (2.5, 1.25, 0.625, 0313, 0.156, 0.078 and 0 mM) versus absorbance, we obtained a linear curve with R2 values above 0.99 (Figure 1).

After checking the linearity conditions / Abs ratio and concentration (Calcium Arsenazo III – Bioclin©) (Beer-Lambert Law), a new calibration factor was calculated in a 96-well flat-bottom microplate. In this case, we obtained mean values of 70.57 ± 4.68.

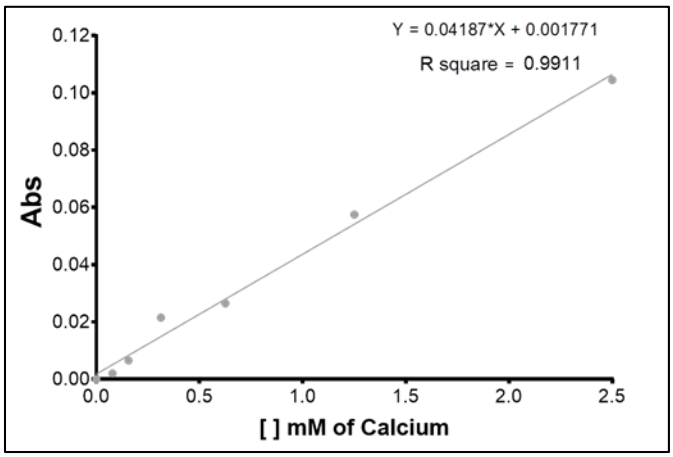

Figure 1 Calibration curve of calcium level

3.1.2. Optimal HCl concentration for calcium measurement

Using aliquots of Biocontrol N – Bioclin© (lot 0057) which contained in serial dilutions on average 6.00; 3.00; 1.50 mg of calcium, performed verification with parameters found, (**ε** = 105.80 ± 4.31), volume of 202 µL (0.573 L) and collected absorbance data applied to formula C = Abs / Ɛ. L. Where found based on collected absorbance values 6.19; 2.88; 1.43 mg, ± 2.54% variation from true average. Values below concentration 1.5 (0.75; 0.37 and 0.19) did not show an approximate correlation.

We observed that there is no significant effect of HCl on the calcium concentration in aliquots of Biocontrol N – Bioclin© (lot 0057) diluted (3.66 ± 0.074 mg/dL), $F_{(8.18)} = 2{,}651$(p=0.041) **Figure 2A** . The aliquots diluted in 6.25 mM HCl, as can be seen in **Figure 2B**, showed a smaller difference in relation to the mean, when compared with the aliquots of Biocontrol diluted in water (Mean diff. = - 0.072 and p=0.999). Thus, the concentration of 6.25 mM of HCl for dilution was established as the optimal condition for processing the samples.

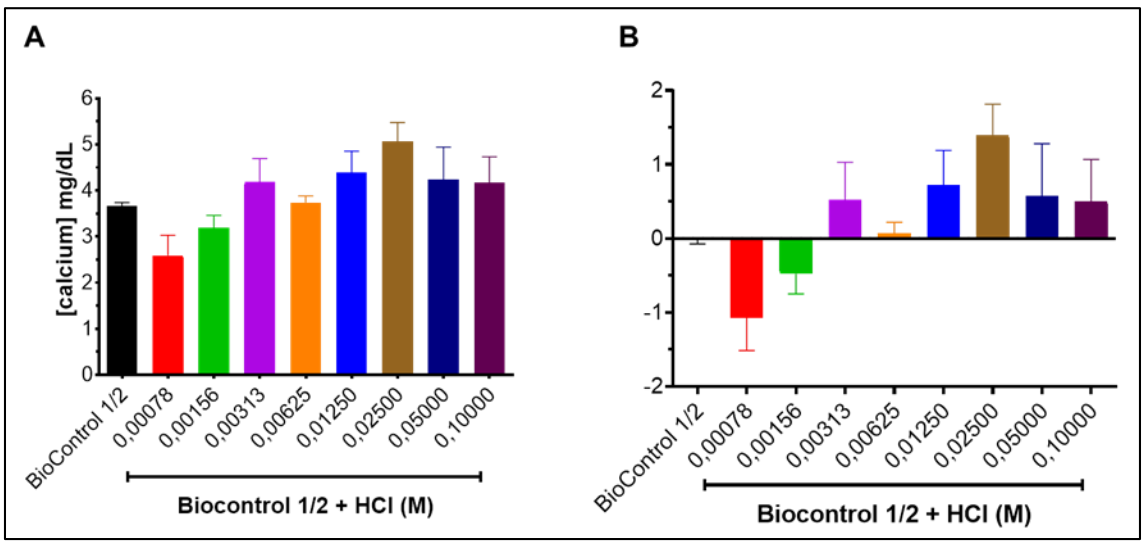

Figure 2 Optimal HCl concentration for calcium measurement

3.1.3. Determinations of calcium levels in biological samples

As shown, the samples were then diluted to a concentration of 6.25 mM for processing. The use of HCl during the processing of the samples was due to the use of the same protocol using for (29,30). The processed samples of PFC, thalamus and Hippocampus were dissected and optimized for two biochemical assays. The use of 10% TCA as a denaturing agent, chemical reagent, releases and solubilizes calcium ions associated with proteins, allowing their quantification in aqueous solution. However, it makes it impossible to measure the protein content of the dissected samples. Thus, the results were expressed in µg of calcium/mg of tissue (PFC, thalamus and Hippocampus). Using wavelength (*λ=630)*, color reagent Arsenazo III 0,2 mmol/L, 8-Hidroxiquinoleína < 20 mmol/L, Bioclin Kit©, observed optical density 0.4320 ± 0.0018, adopted as blank. All values obtained STD (10 mg calcium – Bioclin kit©), Control $^{+}$ (6.0 ± 0.6 mg/dL calcium - Biocontrol N – Bioclin© - lote 0057), PFC, Thalamus and Hippocampus were normalized by blank and presented respectively 0.1782 ± 0.0013; 0.1296 ± 0.0102; 0.0231 ± 0.0053; 0.0170 ± 0.0051; 0.0136 ± 0.0026. $F_{(5.18)}$ = 116.9 (P <0.0001)(One way – ANOVA). *Post hoc* test (Fisher) showed difference between STD and Control^{+} *vs* blank (p <0,0001); PFC *vs* blank (p=0.0160); tendency to Thalamus *vs* blank (p=0.0661); and no statistical difference for Hippocampus vs blank (p=0.1365) **Figure 3A**. Data on free calcium concentrations, analyzed by a multicomparative method, ANOVA-one way were 0.4670 ± 0.1056 ug Ca / mg Tissue to sample PFC; 0.4322 ± 0.1228 ug Ca / mg Tissue to sample Thalamus and 0.1027 ± 0.0203 ug Ca / mg Tissue to sample Hippocampus **Figure 3B**. The changes in free calcium levels determined in the present study cannot be considered an increase in the cytosolic pool, due to limitations of the technique used, as discussed below. Despite the limitations of this method, making it impossible to distinguish between cytosolic and extracellular calcium levels, it was possible to observe changes in the calcium levels of the tissues analyzed in the samples that were collected.

Knowledge of the levels of this ion in different tissues can contribute to the understanding of previous knowledge about the involvement of calcium in one of the cell death pathways. It is known that in the Central Nervous System (CNS) we can distinguish two types of voltage-dependent calcium, CaV-1.2 e CaV-1.3, located on the postsynaptic membrane, both in the cell body of the neuron and in the dendrites and terminals of the neuron (31–34). These channels, as one of the modulating components of calcium flux, may play a crucial role in cell physiology or cell death processes in regions of the CNS (35–37) and, possibly, could explain the differences in calcium levels.

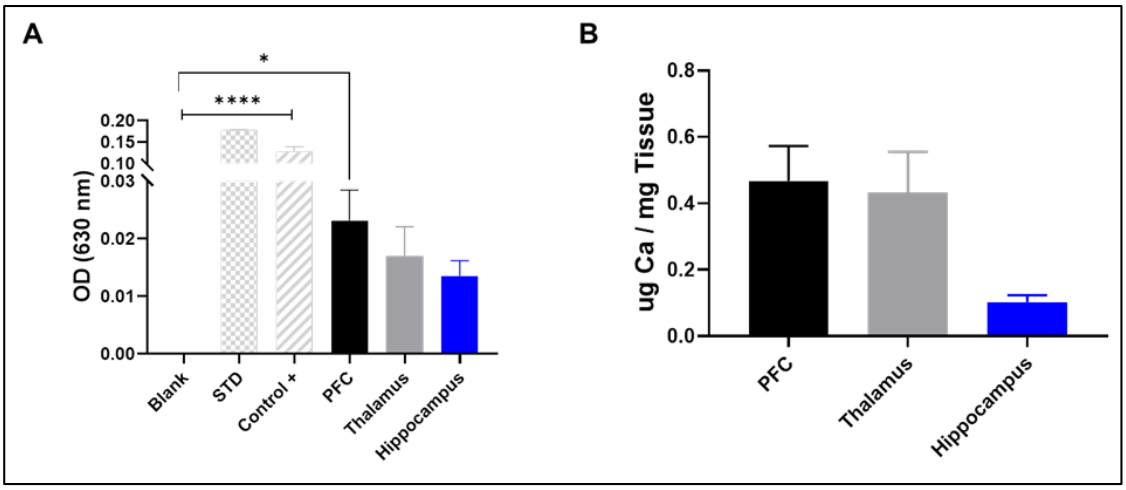

Figure 3 Intracellular calcium levels in brain structures. Panel A shows the absorbance values found under the different conditions: Blank (reagent only); STD (standard calcium 10mg); Control (6mg/dl calcium Biocontrol N - Bioclin© - lot 0057); PF (prefrontal cortex); Thalamus and Hippocampus. Panel B shows the calcium concentration per mg of tissue

Abbreviations

- PFC - prefrontal cortex;
- H - hippocampus;
- T - thalamus;
- CNS - central nervous system;

4. Conclusion

Despite the limitations of the technique, the measurement of free calcium in the CNS tissues (PFC, Thalamus and Hippocampus) showed positive and satisfactory results, as an alternative and viable means for the quantification of calcium levels. It was still possible to show the sensitivity of the technique for this type of dosage, using the arzenazo III reagent and perhaps the use in fractional derivations of these tissues, such as the possibility of dosing in a pool of organelles with mitochondria.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors have no other conflicts of interest.

Statement of ethical approval

All experiments were performed according to the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA/UFMG), approved on 08/05/2017, Protocol no. 288 / 2016.

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