Effect of caffeine and coffee diets on calcium signalling in rat hippocampal neurons

V.M. Shkryl¹, T.G. Turytska², V.A. Yavorsky¹, V.P. Lyashenko², S.M. Lukashov³, E.A. Lukyanetz¹

¹Bogomoletz Institute of Physiology NASU, Kyiv, Ukraine, e-mail: elena@biph.kiev.ua; ²Dnipro National Oles Honchar University, Dnipro, Ukraine; ³Medical center “Headache”, Regional Clinical Hospital N.A. Mechnikov, Dnipro, Ukraine

UDC 577.352.465

37

The effects of long-lasting high concentration coffee and caffeine diets on calcium mobilization in rat hippocampal neurons were studied. Changes in the basal calcium level in the hippocampal neurons of control and experimental rats kept on a coffee or caffeine diet were measured. We also recorded the changes in the Ca²⁺ transients’ amplitude evoked by membrane depolarization or emptying the Ca²⁺ depot of the endoplasmic reticulum (ER) induced by caffeine activator of the ryanodine receptors. In rats on a coffee or caffeine diet, the basal Ca²⁺ level was increased by 7.4% and 11%, respectively, compared to control animals. In these groups, the amplitude of Ca²⁺ transients increased by 70% and 90%, respectively, of the basal level in response to the membrane depolarization. In the same groups, the amount of Ca²⁺ released from the ER was increased by two and three times, respectively, compared to the control after activation of ryanodine receptors. We concluded that long-term coffee and caffeine diets in rats cause a significant disruption of the hippocampal neurons’ endoplasmic reticulum function. The diets evoke an increase in Ca²⁺ concentration in the neurons and an excessive release of Ca²⁺ in response to excitation. The latter can lead to increased excitability of neurons and their further death from excessive Ca²⁺ levels.

Key words: hippocampal neurons, coffee, caffeine, calcium, ryanodine receptor, endoplasmic reticulum, diet.

INTRODUCTION

Coffee is one of the most consumed beverages globally, with a total consumption of over 5 million tons per year. Consumers prefer coffee because of their taste and stimulating effects [1]. The main coffee component is caffeine, but coffee also includes other compounds such as trigonelline that can affect the nerve function (growth of neurites) [2] or flavonoids [3]. Furthermore, such biologically active substances as chlorogenic acid, catechin, N-methyl pyridine, hydroxytryptamines, pyrogallol, and others were found in the coffee [4]. Thus, coffee and caffeine can cause different effects on the body, taking into account the content of coffee in other biologically active substances.

It has been shown that coffee and caffeine can have both positive and negative effects on human health. On the one hand, caffeine is used to treat premature bronchopulmonary dysplasia, it may have a protective effect in some diseases such as Parkinson’s disease [5], Alzheimer’s disease (but not yet fully proven) [6], and for some types of tumors (hepatocyte, endometrial, prostate) [7]. Caffeine can prevent the death of pancreatic cells in alcohol poisoning [8]. Caffeine, also at low doses, reduces cardiovascular diseases, such as coronary artery disease and heart attack. On the other hand, at high doses, it increases the risk of these diseases [9]. Increased coffee/caffeine doses can also cause states such as caffeine addiction, hypertension, insomnia, anxiety states, tachycardia, arrhythmia, migraine, effects on the secretion of gastric juice, and loss of bone tissue, increased intraocular pressure (as a consequence of glaucoma), etc. [10]. The toxic dose is 10 g of caffeine per day for adults (one coffee cup contains 80-175 mg

© V.M. Shkryl, T.G. Turytska, V.A. Yavorsky, V.P. Lyashenko, S.M. Lukashov, E.A. Lukyanetz

ISSN 0201-8489 Фізіол. журн., 2021, Т. 67, № 4 37
of caffeine, depending on the preparation and type of grains). *Coffea arabica* contains twice as much caffeine as *Coffea robusta*. The highest content of caffeine is in “Express” coffee (100 mg 50 ml). The use of pure caffeine is more toxic than coffee. Coffee, caffeine, and other coffee ingredients are known to affect brain activity [11]. The coffee component of caffeine is often used as a separate component for stimulating brain activity.

It has been shown that it improves mental activity at a concentration of 3.3 mg/kg [12]. It is known that caffeine is an activator of ryanodine ER receptors, and it induces Ca$^{2+}$ release from calcium depots [13]. And thus, coffee and caffeine can affect intracellular calcium homeostasis in neurons and induce different Ca$^{2+}$-dependent processes, including vesicular trafficking as well they can induce Ca$^{2+}$-independent processes [14]. In our experiments, we studied the effects of long-lasting coffee and its component caffeine diets on Ca$^{2+}$ mobilization in rat’s hippocampal neurons.

**METHODS**

*Keeping the Rats on Coffee and Caffeine Diet.*

The research has been carried out following existing international and Bogomoletz Institute requirements and humane attitude norms towards animals. Experiments were performed on non-linear white male rats, which at the beginning of the study, weighed 0.14 kg. Animals were kept at common sanitary and hygienic conditions with a standard diet. The first group consisted of control animals (n = 6), which lived under standard conditions throughout the experiment. The second group (n = 10) was represented by animals that were kept with food “Caffeine sodium benzoate” (Darnytsya, UA) in the amount of 150 mg/kg/day (LD50). Animals of the third group (n = 9) daily received a mix of fried coffee beans with food, in 150 mg/kg/day of pure caffeine weight. The lyophilized powder of coffee was obtained from a commercial blend of coffee beans. According to the International Institute of Tasting Coffee’s recommendation, the mixture was 80% made up of coffee beans of the *Arabica* variety and 20% of the *Robusta* variety (the mass fraction of caffeine in the mix was 1.48%, which corresponds to GOST 6805-97). In acute experiments, intracellular calcium was measured in the hippocampal neurons in the subgroups of animals, which were taken from the study groups 39 weeks after the beginning of the diets.

Isolation of the neurons of the CA1 region of the hippocampus

The method of obtaining isolated neurons of the hippocampus of rats in general corresponded to that described in our previous works [15-18]. Animals (mature rats) decapitated after anesthesia with ether. The brain was quickly removed and transferred to a cold (4 °C) solution A. The sections of the hippocampus 0.4-0.5 mm thick were cut using a blade and held for 60 minutes in solution B at room temperature (21-25 °C), placed them on a nylon mesh in a chamber; Aeration of the medium was provided by carbogen. Enzymatic treatment in solution B of 0.1% protease (type 23) and 0.1% trypsin (Sigma-Aldrich, USA) lasted 20-35 minutes without changing the medium’s temperature. This sequence of processing allowed for the dispersion of sections to receive isolated neurons of the desired zone, which preserved small portions of apical and basal dendrites and had a soma with a diameter of 15 ÷ 20 μm and a length of 30 ÷ 50 μm.

Solution A contains (in mmol / l): NaCl-120, KCl-5, HEPES - 10, MgCl$_2$ -1, CaCl$_2$ -2, glucose - 25. Solution B: NaCl - 125, KCl-5, NaH$_2$PO$_4$ – 1.25, NaHCO$_3$ - 25, MgCl$_2$ -1, CaCl$_2$ -2, glucose - 10. All substances obtained from Sigma-Aldrich Company, USA.

Measurement of intracellular calcium

Intracellular calcium was measured as it was described previously [19-22]. Briefly, immediately before the experiment, the neurons’ staining in a solution of fluorescent dye Fura-
2AM (5μM) was performed for 20 minutes. Then the glass coverslips with the neurons were placed in the experimental chamber. The experimental chamber was installed on a fluorescence microscope and a solution flow system, washing the cells with the solution and changing it to apply the factors. Depolarization of the membrane was carried out using 5 s application of 50 mM KCl solution, and 5 s application of 20 mM caffeine was used to release calcium from the endoplasmic depot.

Changes in the level of fluorescence intensity in neurons were recorded with an experimental digital video camera at excitation wavelength 340 and 380 nm. Using computer software Cell M software (Olympus, Japan) and IDL programming environment (ITT Visual Information Solutions), further data analysis was performed, and the ratio of fluorescence intensity in the range of 340 nm fluorescence to 380 nm (R= F340/F380) was calculated. Dynamic changes in this indicator evaluated changes in the level of free calcium in the cytosol of neurons.

**Experimental solutions**

Basic solution used as a perfusion solution in the experimental chamber: NaCl - 140 mM; KCl - 2 mM; CaCl2 - 2 mM; MgCl2-2.0 mM; HEPES-10 mM, pH 7.4. To depolarize the neuronal membrane and induction of calcium transitions in this way, a high-potassium solution (50 mM KCl) was used: NaCl - 82 mM; KCl - 50 mM; CaCl2 - 2mM; MgCl2-2.0 mM; HEPES-10 mM, pH 7.4.

**Statistical analysis**

Numerical data were subjected to statistical processing (Investigator criterion) using Origin software (OriginLab Corporation, USA). Inter-sample differences with P <0.05 were considered statistically significant.

**RESULTS**

In all neurons, measurements of free calcium levels were performed during short-term cellular stimulation (5 s) with the high-potassium solution. After calcium level recovery to the basal level, stimulation was repeated. Then, after the basal level’s restoring, we made a short-term application of solution with 20 mM caffeine (5 s).

The application of high-potassium solution causes depolarization of the neuronal membrane, which leads to the influx of calcium ions through voltage-sensitive calcium channels in the cell. Also, this process contributes to the filling of intracellular calcium depots in the neurons. Caffeine is an agonist of the ryanodine receptor calcium channel of the endoplasmic reticulum (ER), and therefore its application causes the release of calcium from ER. It was found that 53.6% of the neurons from the control group (n = 28) responded to stimulation with the high-potassium solution.

In the neurons of the control group, the basal calcium level measured in relative units (F340/F380) was 1.36 ± 0.04 (n = 28; all investigated neurons) and 1.30 ± 0.07 (n = 15, corresponding to high-potassium solution). An example of changes in the intracellular level of Ca^{2+} that occurred due to these stimuli in the neuron in control animals is shown in Fig. 1. 54.2% of the neurons from the coffee group animals (n = 24) and 30.4% of the caffeine group (n = 23) responded to stimulation by a high-potassium solution. In the neurons of the caffeine group, the level of free basal calcium was 1.46 ± 0.07 (n = 23; all studied) and 1.20 ± 0.06 (n = 7; responding to high-potassium solution). In the coffee group’s neurons, the level of free basal calcium was 1.51 ± 0.09 (n = 24; all investigated) and 1.45 ± 0.09 (n = 7; responding to high-potassium solution). Changes in the intracellular level of calcium in animals of the coffee group, in response to stimulation of the high-potassium solution and caffeine, are shown in Fig. 2.

It was also found that the amplitude of calcium transient (ΔR, ratio F340 / F380) in response to high-potassium solution was 0.10 ± 0.02 (n = 15); 0.17 ± 0.06 (n = 7) and 0.19 ±
0.05 (n = 13) from the basal level, respectively, in the control group, caffeine and coffee groups, as shown in the diagram in Fig. 3

In Fig. 4 is a graph of statistical data on the amount of calcium release from the endoplasmic depot. The amount of calcium released from the ER was evaluated as the amplitude of the calcium transient from the basal level (ΔR = F340 / F380; when applying a solution of 20 mM of caffeine). In the control group, this value was 0.06 ± 0.01 (n = 5). In the caffeine and coffee groups’ animals, the amount of calcium released from the ER was increased compared to the control and amounted to 0.12 ± 0.04 (n = 5) and 0.19 ± 0.06 (n = 8), respectively.

**DISCUSSION**

Coffee, caffeine, and other coffee ingredients are known to affect brain activity [11]. I.P. Pavlov and his collaborators have yet investigated the physiological features of caffeine’s action on the central nervous system. They showed that caffeine enhances and regulates the processes of excitation in the cerebral cortex. They demonstrated that caffeine enhances and regulates the processes of excitation in
the cerebral cortex. In corresponding doses, it enhances positive conditioned reflexes and enhances motor activity. Stimulating action leads to increased mental and physical efficiency, reducing fatigue and drowsiness. However, large doses can lead to exhaustion of nerve cell state.

Recent data indicate that coffee (Coffea arabica or Coffea canephora var. robusta) caused an increase in the excitatory neurotransmitter effect on the accumulation of calcium and the release of dopamine in PC12 cells [4]. The release of neurotransmitters or exocytosis is regulated by the concentration of Ca$^{2+}$ ions, which play an essential role in the outflow of neurotransmitters during synaptic transmission [23-25]. Besides, the release of Ca$^{2+}$ from the intracellular depot also takes part in synaptic transmission regulation. Thus, the presence of ryanodine receptors in the hippocampal neurons’ presynaptic endings was demonstrated [26-29].

Caffeine, which belongs to the group of methylxanthines, has a chemical name: 1,3,7-trimethyl-xanthine. Like methylxanthines, it can be an antagonist of adenosine receptors [30], causing the release of neurotransmitters [4, 31]. It was shown that caffeine is an antagonist of all types of adenosine receptors - A1, A2A, A3, and A2B [32]. Caffeine also inhibits phosphodiesterases (PDE1, PDE4, PGE5), interferes with GABA-A receptors, and has an influence on several brain diseases, including Alzheimer’s disease [32]. Nevertheless, many of these processes are associated with the ER’s functioning - for example, the receptor-activated release of neurotransmitters and other calcium-dependent processes that occur in the cell. The latter is due to the ability of caffeine also to affect directly intracellular Ca$^{2+}$ depots of the ER. Calcium processes associated with ER are also influenced by other active components isolated from other plants, such as Taraxacum officinale [33]. This plant is also used to make tipping drinks. Also, other natural products can act on the ER and interfere with ER stress [3].

It is known that two types of ER are inositol trisphosphate (IP3R) and ryanodine receptors (RyR). These receptors cause a rapid Ca$^{2+}$ leakage from SR/ER, increasing Ca$^{2+}$ in the cytosol, and triggering a series of signaling processes. Caffeine is an activator of ryanodine ER receptors, and its activation causes Ca$^{2+}$ release from ER [34]. In turn, it is known that ER disfunction can promote a number of disorders of nervous system [35]. In our studies, we aimed to investigate the only one aspect of caffeine and coffee action through corresponding diet – their prolong effect on RyR-receptors of ER of hippocampal neurons.

Our experiments showed that basal Ca$^{2+}$ levels in neurons of rats with long-term coffee or caffeine diet increased by 7.4% and 11%, respectively, compared with control animals. In animals of the caffeine and coffee groups, the amplitude of calcium transient in response to high-potassium solution increased by 70% and 90%, respectively, of the basal level. It turned out that in the same groups, the amount of calcium released from ER after activation of the ryanodine receptors was increased two and three times, respectively, in comparison with the control animals. Thus, it is concluded that long-term caffeine and coffee diets in rats cause a significant violation of ER’s activity in the hippocampal neurons, namely, an increase in the basal level of Ca$^{2+}$ in the cell and excessive calcium release in response to irritation. The latter can lead to increased excitability of neurons and their death from excessive calcium levels.

Summing up, our results point that long-term caffeine and coffee diets in rats cause a significant violation of ER’s activity in the hippocampal neurons, namely, an increase in the basal level of Ca$^{2+}$ in the cell and excessive calcium release in response to irritation. The latter can lead to increased excitability of neurons and their death from excessive calcium levels.

**Acknowledgements**

This study was supported by National Acade-
The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

Ethical Approval
The experiments were approved by Local Ethics Committee at Bogomoletz Institute of Physiology, NAS of Ukraine.

REFERENCES
1. Geel L, Kinnear M, De Kock R. Relating consumer preferences to sensory attributes of instant coffee. Food Quality and Preference. 2005;16:237-44.
2. Tohda C, Nakamura N, Komatsu K, Hattori M. Trigonelline-induced neurite outgrowth in human neuroblastoma SK-N-SH cells. Biological & pharmaceutical bulletin. 1999;22(7):679-82.
3. Martucciello S, Masullo M, Cerulli A, Piacente S. Natural Products Targeting ER Stress, and the Functional Link to Mitochondria. Int J Mol Sci. 2020;21(6):1905.
4. Walker J, Rohm B, Lang R, Pariza MW, Hofmann T, Som-
20. Lukyanetz IA, Lukyanetz EA. Calcium signalling during hypoxia in fish Carassius gibelio. Fiziol Zh. 2009;55(6):135.

21. Rozunna NM, Shkryl VM, Ganzha VV, Lukyanetz EA. Effects of Modeling of Hypercalcemia and β-Amyloid on Cultured Hippocampal Neurons of Rats. Neurophysiology. 2020;52(5):348-57.

22. Shkryl VM. Error correction due to background subtraction in ratiometric calcium measurements with CCD camera. Heliyon. 2020;6(6):e04180.

23. Lukyanetz EA, Neher E. Different types of calcium channels and secretion from bovine chromaffin cells. European Journal of Neuroscience. 1999;11(8):2865-73.

24. Lukyanetz EA. Different secretory vesicles can be involved in depolarization-evoked exocytosis. Biochemical and Biophysical Research Communications. 2001;288(4):844-8.

25. Lukyanetz EA. Calcium signaling in secretion of catecholamines in chromaffin cells. Fiziol Zh. 2009;55(6):110-1.

26. Lauri SE, Bortolotto ZA, Nistico R, Bleakman D, Ornstein PL, Lodge D, et al. A role for Ca$^{2+}$ stores in kainate receptor-dependent synaptic facilitation and LTP at mossy fiber synapses in the hippocampus. Neuron. 2003;39(2):327-41.

27. Liang Y, Yuan LL, Johnston D, Gray R. Calcium signaling at single mossy fiber presynaptic terminals in the rat hippocampus. Journal of neurophysiology. 2002;87(2):1132-7.

28. Sato I, Kamiya H. Assessing the roles of presynaptic ryanodine receptors and adenosine receptors in caffeine-induced enhancement of hippocampal mossy fiber transmission. Neuroscience research. 2011;71(2):183-7.

29. Shimizu H, Fukaya M, Yamasaki M, Watanabe M, Manabe T, Kamiya H. Use-dependent amplification of presynaptic Ca$^{2+}$ signaling by axonal ryanodine receptors at the hippocampal mossy fiber synapse. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(33):11998-2003.

30. Wood PL, Kim HS, Boyar WC, Hutchison A. Inhibition of nigo striatal release of dopamine in the rat by adenosine receptor agonists: A1 receptor mediation. Neuropsychopharmacology. 1989;28(1):21-5.

31. Solinas M, Ferré S, You ZB, Karcz-Kubicha M, Popoli P, Goldberg SR. Caffeine induces dopamine and glutamate release in the shell of the nucleus accumbens. The Journal of Neuroscience. 2002;22(15):6321-4.

32. Ribeiro JA, Sebastião AM. Caffeine and adenosine. Journal of Alzheimer’s disease : JAD. 2010;20 Suppl 1:S3-15.

33. Gerbino A, Russo D, Colella M, Procino G, Svelto M, Milella L, et al. Dandelion Root Extract Induces Intracellular Ca$^{2+}$ Increases in HEK293 Cells. Int J Mol Sci. 2018;19(4):1112.

34. Thomas RC. Calcium content of the endoplasmic reticulum of snail neurones releasable by caffeine. Cell Calcium. 2013;53(2):120-4.

35. Stutzmann GE, Mattson MP. Endoplasmic reticulum Ca$^{2+}$ handling in excitable cells in health and disease. Pharmacol Rev. 2011;63(3):700-27.