EXPERIMENTAL WORKS

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THE EFFECT OF AMIXIN AND AGMATINE ON CYTOCHROME *C* RELEASE FROM ISOLATED MITOCHONDRIA

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Mitochondrial nicotinic acetylcholine receptors (nAChRs) control permeability transition pore formation and cytochrome c release in the presence of apoptogenic factors. This study demonstrates that pharmacological agents amixin and agmatine affect mitochondrial nAChR functioning: they slightly suppress cytochrome c release from mouse brain and liver mitochondria stimulated with apoptogenic dose of Ca^{2+} and prevent the effect of α7 nAChR agonist PNU282987. We conclude that mitochondria may be one of therapeutic targets of amixin and agmatine.

K e y w o r d s: mitochondria, nicotinic acetylcholine receptor, amixin, agmatine.

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate fast synaptic transmission in neuromuscular junctions and autonomic ganglia, regulate other receptor activity and mediator release in the brain [1]. The nAChR dysfunction or alteration of its synthesis result in nervous and muscular system diseases, such as myasthenia, gangliopathy or neurodegenerative disorders [2]. The nAChRs expression is described in many non-excitable mammalian cells: respiratory tract epithelium, vascular endothelium, keratinocytes, T- and B-lymphocytes etc. In non-excitable cells, the nAChR activation does not evoke electrical excitation, but changes the vital cellular functions like proliferation, viability, adhesion, motility [3, 4]. Structurally, the nAChRs are homo- or heteropentamers composed of different subunits: α1–α10 and β2–β4; muscle-type nAChRs contain also γ, δ and ε subunits. The subunit composition determines pharmacological sensitivity and kinetic characteristics of the nAChR ion channels [5]. In recent studies of the Laboratory of Cell Receptors Immunology, the presence of α7(β2), α3β2 and α4β2 nAChR subtypes in the mitochondria outer membrane and their role in regulating mitochondrial pathway of apoptosis have been demonstrated [6]. Particularly, it was found that the nAChR activation prevented mitochondrial permeability transition pore opening and cytochrome *c* (cyt *c*) release in the presence of apoptogenic factors, such as Ca^{2+} or H_2O_2. However, mitochondrial nAChR signaling was not associated with the nAChR ion-channel opening and could be initiated with either nAChR agonists or antagonists [7].

Amixin and agmatine are widely used in pharmacology nowadays. Amixin (tilorone) is an interferon inductor, which also has antimicrobial, antifungal and anti-inflammatory effects [8]. Agmatine (4-aminobutyl-guanidine) is a product of arginine decarboxylation and is used for prevention of consequences of neuronal ischemia, chronic pain attenuation, as antidepressant, and as a dietary supplement for sportsmen [9]. According to the literature data, both these agents are able to affect nAChRs: amixin is an α7 nAChR agonist [10-11] and agmatine is an antagonist and a weak channel blocker of different nAChR subtypes [12].

The aim of this study was to reveal whether agmatine and amixin affect mitochondrial nAChRs, particularly, if they influence the cyt *c* release stimulated by apoptogenic dose of Ca^{2+} in isolated mitochondria.

Materials and Methods

Amixin was kindly provided to us by Dr. S. Lyakhov from A. V. Bogatsky Institute of Physics and Chemistry of the National Academy of Sciences of Ukraine. Agmatine (Sigma, USA) was a

kind gift of Dr. I. Ferenz from Ivan Franko National University of Lviv. All other reagents were of chemical grade and were purchased from Sigma, USA.

Mitochondria purification and characterization. Mitochondria isolation from the liver and brain of C57BL/6J mice was performed by differential ultracentrifugation according to standard procedure described [5, 12]. The obtained fractions were characterized by flow cytometry [5] using COULTER EPICS-XL™ device (Beckman Coulter, USA) and SYSTEM II™ Software. The purity of gated mitochondria was assessed using 0.1 μM acridine orange 10-nonyl bromide (NAO) ($\lambda_{excitation}$ = 488 nm, $\lambda_{emission}$ = 525 nm), which binds selectively to cardiolipine, phospholipid specific for mitochondrial inner membranes. In our experiments, 95-98% of registered events were "NAO-positive", i.e. they were of mitochondrial origin. The size of mitochondria was examined by forward scattering, and granularity – by side scattering.

Cyt c release. The isolated mitochondria were resuspended in the incubation medium containing 0.01 M HEPES, 0.125 M KCl, 0.025 M NaCl, 0.005 M sodium succinate, 0.1 mM P_i(K) (pH 7.4). Cyt *c* release was stimulated by addition of 0.5 mM H_2O_2 or different doses of $CaCl_2$. Agmatine (10 μM, 100 μM or 1 mM) or amixin (0.05 μM, 2.5 μM or 250 μM) were added in the presence or absence of α7 nAChR agonist PNU282987 (30 nM). After incubation during 2-5 min at room temperature, the samples were centrifuged (10 min, 8000 g) at 4 °C. The supernatants were collected and tested by sandwich ELISA assay as previously described [5].

Statistical analysis. Each experiment was reproduced with 3 to 4 repeats. The data are presented as mean ± S. E. Statistical analysis was performed according to Student's test using OriginPro 8.6 software.

Results and Discussion

According to the flow cytometry data, the brain mitochondria were of similar size with the liver mitochondria, but possessed larger granularity (Fig. 1).

In the functional test, the brain mitochondria were more resistant to apoptogenic stimulation than the liver ones: they released less cyt *c* in response to Ca^{2+}, and the cyt *c* release was suppressed more effectively by α7 nAChR agonist PNU282987 (Fig. 2).

Taking into account this functional difference, to compare the effects of amixin and agmatine, the data of following experiments were normalized, the maximum level of cyt *c* released being taken as 100%.

Fig. 3 shows that amixin reduced the amount of cyt *c* released at high Ca^{2+}, but much weaker than did PNU282987; however, it apparently prevented the effect of PNU282987. The brain mitochondria were slightly more sensitive to amixin than liver mitochondria: amixin affected the brain mitochondria at the minimal dose (0.05 μM) and significantly attenuated the effect of PNU282987 (Fig. 3, *A*).

Agmatine also prevented the cyt *c* release from mitochondria at high Ca^{2+} concentration. Similarly to amixin, this effect was more pronounced in the brain mitochondria where a strong effect of agmatine was already observed with the minimal dose. Simultaneous addition of PNU282987 and agmatine resulted in a dose-dependent reduction of PNU282987 effect observed in mitochondria of both the brain and liver (Fig. 4).

The data presented in Fig. 3 and 4 indicate that both amixin and agmatine produced a weak anti-apoptotic effect reducing the amount of cyt *c* released from mitochondria at high Ca^{2+} concentration. Both agents were more effective with the brain than with the liver mitochondria and prevented the effect of α7 nAChR agonist PNU282987. This indirectly suggests that the effect of amixin and agmatine on mitochondria was due to their interaction with α7 nAChR.

Obviously, the brain and liver mitochondria are not identical. According to our data, they were different in their physical parameters (granularity) and sensitivity to apoptogenic effect of Ca^{2+} and hydrogen peroxide. This is in accord with the data of Grancara et al. [13], who showed that the brain mitochondria were more resistant to oxidative stress, and the mechanism of permeability transition pore formation was different from that in the liver mitochondria.

Agmatine, as an endogenous metabolite of arginine, is transported into the mitochondria and, therefore, is able to influence them under physiological conditions. The protective effect of agmatine on the brain mitochondria has been already demonstrated. In particular, it was found that agmatine prevented the mitochondrial membrane potential drop under Ca^{2+} effect [14] and reduced the production of pro-apoptotic Bcl-2 family proteins and caspase-3 in the whole cell [15]. The authors suggested that agmatine neutralized free oxygen radicals formed under the effect of Ca^{2+} to prevent mitochondrial permeability transition pore formation. According to our data, agmatine can affect mitochondria in another

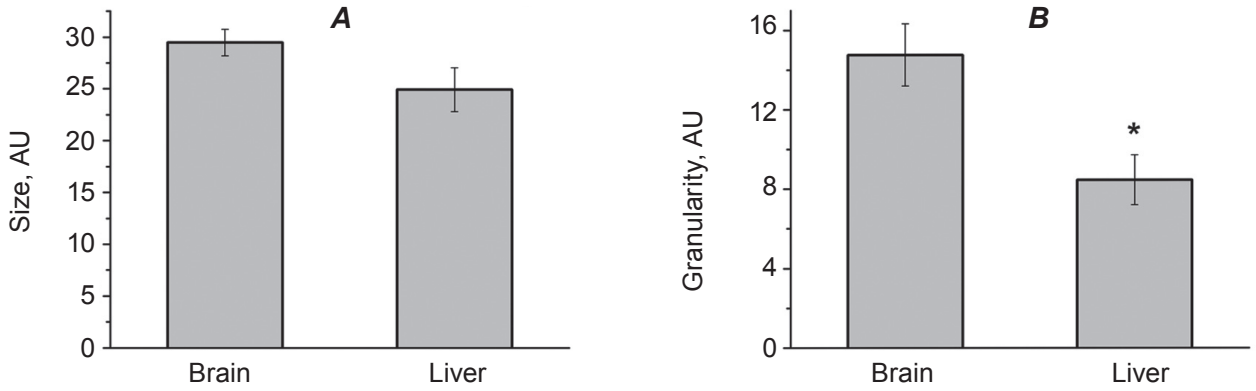

*Fig. 1. Size (A, forward scatter) and granularity (B, side scatter) of mitochondria isolated from either the brain or liver of mice. AU – arbitrary units. Each column corresponds to mean ± S.E. (n = 3), * P < 0.05*

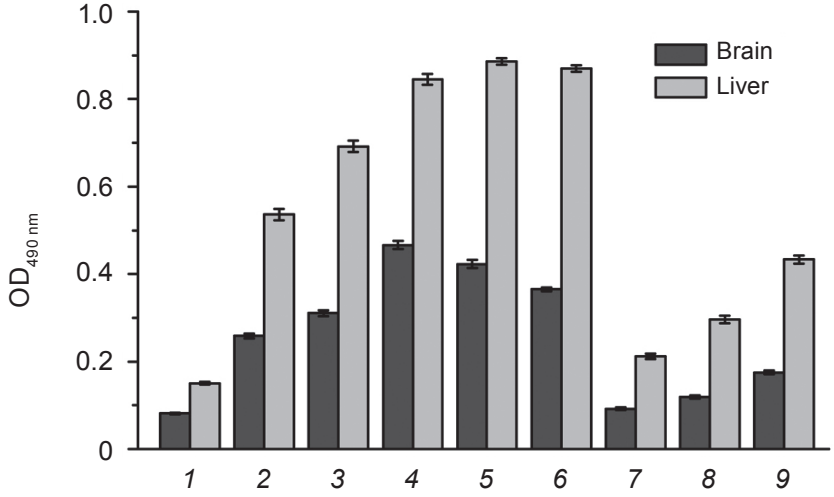

Fig. 2. Cyt c released from isolated mitochondria of the brain or liver under the effect of different Ca^{2+} doses or 0.5 mM H_2O_2 in the presence or absence of α7 nAChR agonist PNU282987 (30 nM): 1 – Control; 2 – 0.09 μM Ca^{2+}; 3 – 0.9 μM Ca^{2+}; 4 – 9 μM Ca^{2+}; 5 – 45 μM Ca^{2+}; 6 – H_2O_2; 7 – H_2O_2 + PNU; 8 – 0.09 μM Ca^{2+} + PNU; 9 – 9 μM Ca^{2+} + PNU. Each column corresponds to $OD_{490\ nm}$ mean ± S.E. (n = 3) defined by sandwich ELISA

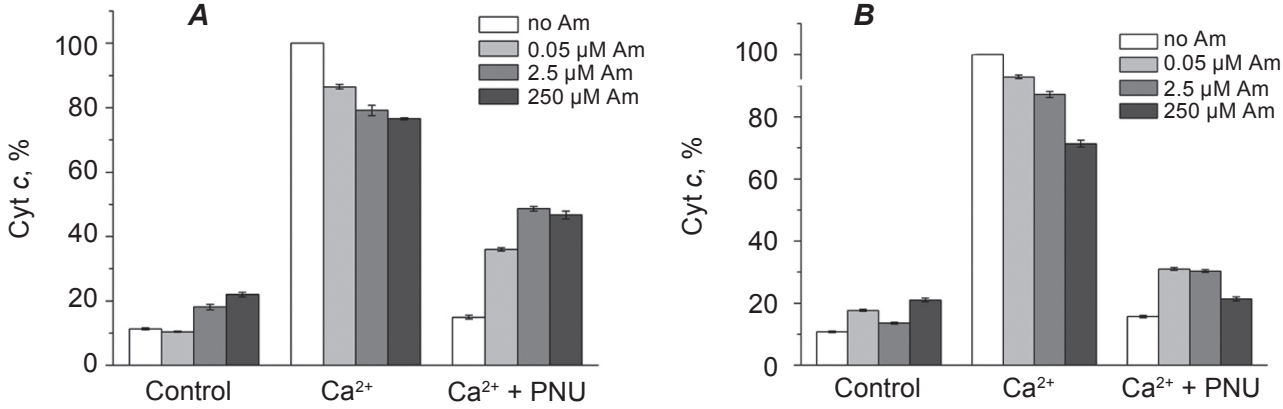

Fig.3. The effect of amixin (Am) on cyt c release from isolated mitochondria of the brain (A) and liver (B) at high Ca^{2+} concentration (9 μM) in the presence or absence of PNU282987 (30 nM). Each column corresponds to mean ± S.E. (n = 3), the data is normalized to maximal cyt c release level (without amixin) taken as 100%

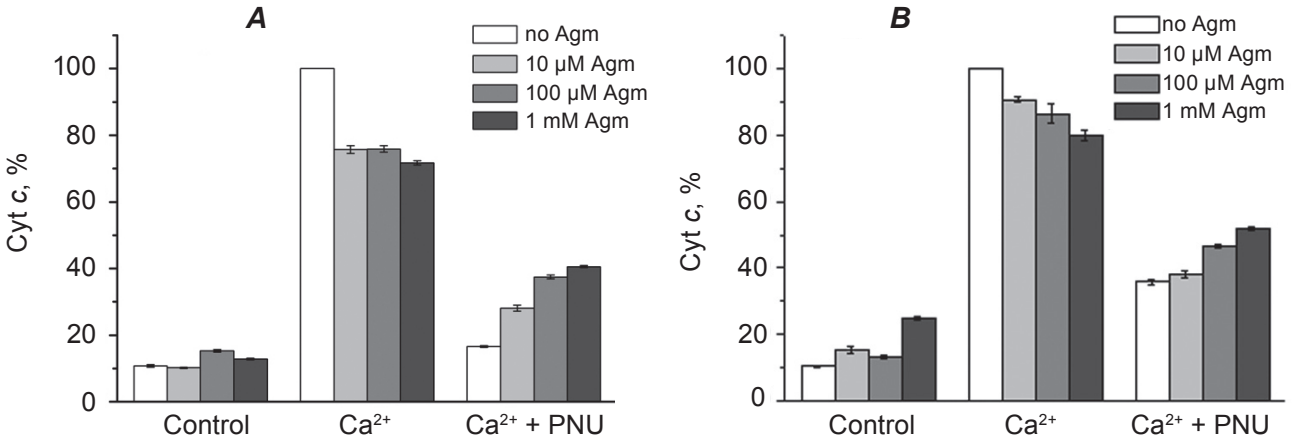

Fig. 4. The effect of agmatine (Agm) on cyt c release from isolated mitochondria of the brain (A) and liver (B) at high Ca^{2+} concentration (9μM) in the presence or absence of PNU282987 (30 nM). Each column corresponds to mean ± S.E. (n = 3), the data is normalized to maximal cyt c release level (without amixin) taken as 100%

way: it interacts with mitochondrial nAChRs, which regulate the pore formation. It was shown that agmatine bound to the brain mitochondria better than to the liver ones [16]. According to our data, this could be due to non-similar subunit composition of nAChRs expressed in the brain or liver. Previously we found that the liver mitochondria contain mostly α7 nAChRs, while the brain mitochondria possess also a considerable part of α4β2 nAChRs [6]. Accordingly, agmatine, as a non-selective nAChR antagonist, affected the brain mitochondria stronger than the liver ones.

In contrast to agmatine, the protective effect of amixin on mitochondria has been studied much less extensively. One study shows radioprotective effect of tilorone that might be regarded as cell protection from apoptosis stimulated by radiation [17]. Other authors have shown that derivatives of tilorone increased mitochondrial membrane potential and mitochondrial resistance to damaging agents [18] that match our results.

Our data suggest that pharmacological effects of amixin and agmatine, beside other activities, may include the influence on mitochondria functioning, in particular, the mitochondrial pathway of apoptosis. Taking into account the competitive effects of these drugs with α7 nAChR agonist PNU 282987 shown in our experiments, this influence is likely to depend on the presence of natural mitochondrial nAChR agonists, such as choline. Upon choline deficiency, amixin and agmatine maintain the integrity of mitochondria and support the cell survival. In contrast, in the presence (excess) of choline, they can prevent its effect, facilitating apoptosis. Indirect evidence in favor of such a mechanism is provided by the reported antitumor activity of amixin [19] and agmatine [20]: the published data indicate that malignantly transformed cells possess elevated levels of choline [21]. On the other hand, the effectiveness of amixin and agmatine mitochondria-targeted activity obviously depends on their availability in tissues and inside the cells. According to the literature, amixin injected intraperitoneally was accumulated mainly in the liver and spleen [22], but was able to penetrate the brain to affect intracellular processes [23]. Agmatine is naturally synthesized inside the cell; exogenous agmatine (introduced as a drug) can penetrate the cell through putrescine active transport system [24]. The data presented indicate that under physiological conditions amixin and agmatine can interact with mitochondrial nAChRs, which might be one of their therapeutic targets.

ВПЛИВ АМІКСИНУ ТА АГМАТИНУ НА ВИВІЛЬНЕННЯ ЦИТОХРОМУ *С* З ІЗОЛЬОВАНИХ МІТОХОНДРІЙ

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Нікотинові ацетилхолінові рецептори (нАХР) мітохондрій контролюють утворення пори перехідної провідності і вивільнення ци-

тохрому *c* за дії апоптогенних чинників. У цій роботі показано, що фармакологічні препарати аміксин і агматин, які, поряд з іншими властивостями, здатні впливати на функціонування нАХР, пригнічують вивільнення цитохрому *c* ізольованими мітохондріями мозку і печінки мишей за дії апоптогенної дози Ca^{2+} і запобігають дії агоніста α7 субтипу нАХР PNU282987. Дійшли висновку про те, що мітохондрії можуть бути однією із терапевтичних мішеней аміксину і агматину.

Ключові слова: мітохондрії, никотиновий ацетилхоліновий рецептор, аміксин, агматин.

ВЛИЯНИЕ АМИКСИНА И АГМАТИНА НА ВЫХОД ЦИТОХРОМА *C* ИЗ ИЗОЛИРОВАННЫХ МИТОХОНДРИЙ

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Никотиновые ацетилхолиновые рецепторы (нАХР) митохондрий контролируют образование поры переходной проводимости и высвобождение цитохрома *c* под действием апоптогенных факторов. В данной работе показано, что фармакологические препараты амиксин и агматин, которые, помимо других свойств, могут влиять на функционирование нАХР, ингибируют высвобождение цитохрома *c* из изолированных митохондрий мозга и печени мышей под действием апоптогенной дозы Ca^{2+} и ослабляют действие агониста α7 субтипа нАХР PNU 282987. Сделан вывод, что митохондрии могут быть одной из терапевтических мишеней амиксина и агматина.

Ключевые слова: митохондрии, никотиновый ацетилхолиновый рецептор, амиксин, агматин.

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