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Glutamic Acid Signal Synchronizes Protein Synthesis Kinetics in Hepatocytes from Old Rats for the Following Several Days. Cell Metabolism Memory

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Abstract—The kinetics of protein synthesis was investigated in primary cultures of hepatocytes from old rats in serum-free medium. The rats were fed mixed fodder supplemented with glutamic acid and then transferred to a regular mixed fodder. The amplitude of protein synthesis rhythm in hepatocytes isolated from these rats increased on average 2-fold in comparison with the rats not receiving glutamic acid supplement. Based on this indicator reflecting the degree of cell–cell interactions, the cells from old rats were not different from those of young rats. The effect was preserved for 3-4 days. These results are discussed in connection with our previous data on preservation of the effect of single administration of gangliosides, noradrenaline, serotonin, and other synchronizers on various cell populations. In contrast to the other investigated factors, glutamic acid is capable of penetrating the blood–brain barrier, which makes its effect possible not only in the case of hepatocytes and other non-brain cells, but also in neurons.

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Glutamic acid introduced into the hepatocyte cell culture synchronizes the cell population and organizes the rhythm of protein synthesis [1]. Glutamic acid acts as a neurotransmitter, and blocking its metabotropic receptors eliminates the effect. The rhythm of protein synthesis, like other ultradian rhythms *in vitro*, is the result of direct cell–cell interactions [2]. The synthesis of special proteins in liver (blood plasma proteins, detoxification enzymes, and glycogen synthesis) is the most important function of the organ. Old animals exhibit the largest effect of glutamic acid on the kinetics of protein synthesis [3]. The rhythm of protein synthesis in hepatocytes from old rats is pronounced, but its amplitude is 2-fold lower than in the hepatocytes from young rats. The amplitude of any rhythm characterizes the intensity of cell–cell interactions. Hence, the connections between cells are significantly disrupted during aging; both the properties of cells and intercellular environment are changed [3]. Introduction of glutamic acid to the medium for hepatocytes isolated from old rats resulted in an increase in the protein synthesis rhythm amplitude to the level characteristic for young animals; the same effect was observed when the rats were fed glutamic acid. Disruptions of protein synthesis in the nervous system during neurodegenerative diseases are known [4]. Specific attention to the effect of glutamic acid is because, in contrast to blood transmitters organizing the rhythm of protein synthesis investigated in our previous works (serotonin and noradrenaline), glutamic acid penetrates the brain and, hence could affect protein synthesis not only in hepatocytes, but also in neurons.

For how long is the effect of glutamic acid maintained? The answer is interesting not only for physiological biochemistry, but also for gerontology, especially for sustaining the good state of aging people and for clinical practice. How long do cells remember the external organizing signal?

MATERIALS AND METHODS

Old Wistar rats (2-2.5 years old, body weight 500-600 g) were used in the experiments. The effect of glu-

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tamic acid on primary cultures of hepatocytes isolated from liver following feeding glutamic acid was investigated. A glutamic acid pill acquired from the pharmacy was powdered. The powder was transferred into warm physiological solution and centrifuged. The dose of glutamic acid for feeding was selected to be similar to the one used in medical practice with consideration of average weights of humans and rats, as well as respective blood volumes. The dose was 0.2 mg/ml (1.4 mM). The same effect was shown previously for the doses of 0.4 and 0.6 mg/ml [3]. In the experiments, after one day of fasting the rats were fed mixed fodder moistened with 62 mg of glutamic acid (this amount corresponds approximately to the abovementioned dose) followed by the regular mixed fodder.

The methods of hepatocyte isolation, their cultivation, and investigation of the protein synthesis kinetics were described previously in detail [1, 5]. Hepatocytes were isolated by perfusion of the liver with calcium-free Hanks' solution containing HEPES (Sigma, USA) and 0.5 mM EGTA, followed by HEPES with 0.05% collagenase (Sigma). Serum-free medium 199 (PanEko, Russia) was used for hepatocyte cultivation: the medium was supplemented with 0.2 mg/ml albumin for cell cultures (Sigma) and 0.5 μg/ml of insulin (Sigma); the gas mixture comprised 10% CO_2 and 90% air. To prepare dense cultures, suspension containing ~$1 \cdot 10^6$/ml of hepatocytes were poured into Petri dishes with glass slides coated with collagen. The rhythm of protein synthesis in such cultures is observed shortly after the medium change, hence, auto-synchronization of cells occurs [5]. After 2 h, the cultures were washed free from unattached cells and cell debris. After 24 h, the medium was exchanged for the normal fresh medium and the aliquots of the culture were sampled for 2 h with 10 min intervals from three culture replicates. Other details of the culture investigation are presented in the "Results and Discussion" section below.

Each culture sample was incubated simultaneously for 10 min at 37°C in leucine-free medium 199 (PanEko) supplemented with ^{3}H-labeled leucine (Institute of Molecular Genetics, Russian Academy of Sciences; 15-25 μCi/ml, specific molar activity 70-100 Ci/mmol). Then the cultures were washed with cold physiological solution and treated with 5% perchloric acid for 60 min followed by washing with alcohol; proteins were dissolved in hyamine (benzethonium chloride; Sigma). Incorporation of [^{3}H]leucine in proteins and radioactivity of free leucine (acid-soluble fraction) was measured with a Perkin Elmer 2810TR scintillation counter (Perkin Elmer, USA). For each culture the leucine incorporation into proteins was calculated and adjusted with respect to the acid-soluble fraction, I_{corr} [1, 5]. Such normalized relative values take into account number of cells in the sample and slight variability of temperature in the experiment.

RESULTS AND DISCUSSION

We have investigated previously both dense and sparse hepatocyte cultures. The protein synthesis rhythm – indicator of cell–cell interactions – was observed practically immediately in the dense cultures after washing and exchange of medium. Hence, the cells were auto-synchronized. Sparse cultures of hepatocytes from the same rat did not demonstrate rhythm, i.e. the cells were asynchronous. Thus, it was possible to detect unambiguously if the cells were interacting or not and to elucidate synchronization factors. There is no need for statistics in this case; each single case is sufficient for conclusions. The rhythm is observed in the dense hepatocyte cultures from old rats; based on the amplitude of the rhythm (one half on average), the cells are synchronized to a lesser degree than cells from young animals. Glutamic acid enhances synchronization. Examples of preservation of this effect of glutamic acid are presented below. All cases were investigated at least twice except for the last case (4 days), which was investigated once. Four examples of preservation of the signal were presented in our previous work [3]; the effect of glutamic acid on hepatocytes from old rats was demonstrated for five different animals.

The protein synthesis rhythm pattern in the dense hepatocyte culture from a rat (body weight 580 g) that was not given glutamic acid with its feed is presented in Fig. 1a. The rhythms of protein synthesis in hepatocytes from the rat (body weight 560 g) fed with mixed fodder supplemented with glutamic acid are presented in Fig. 1, b and c; hepatocytes were isolated from this rat 1 h after exposure to glutamic acid followed by their cultivation under conditions similar to those in the experiment presented in Fig. 1a. After one day, a significant (more than double) increase in the amplitude of protein synthesis was demonstrated in these cultures in comparison with the dense hepatocyte cultures from the rat that did not receive glutamic acid. The increased rhythm amplitudes were observed after 2-day cultivation of these cells, which is 3 days after administration of glutamic acid. For better visual representation, the values of relative incorporation of leucine into proteins (I_{corr}, see "Materials and Methods") are displayed as percent of the average level for each variant of the experiment.

The results of experiment with preservation of the effect of glutamic acid signal following hepatocyte isolation one day after the rat (body weight 550 g) was fed with glutamic acid are presented in Fig. 2. Then this rat was fed with regular mixed fodder. One day after glutamic acid administration (not immediately as in the experiment presented in Fig. 1) hepatocytes were isolated and dense cell cultures were prepared similar to those observed in the experiment presented in Fig. 1. The amplitudes of the protein synthesis rhythm in hepatocytes were increased (Fig. 2a), although the liver did not receive

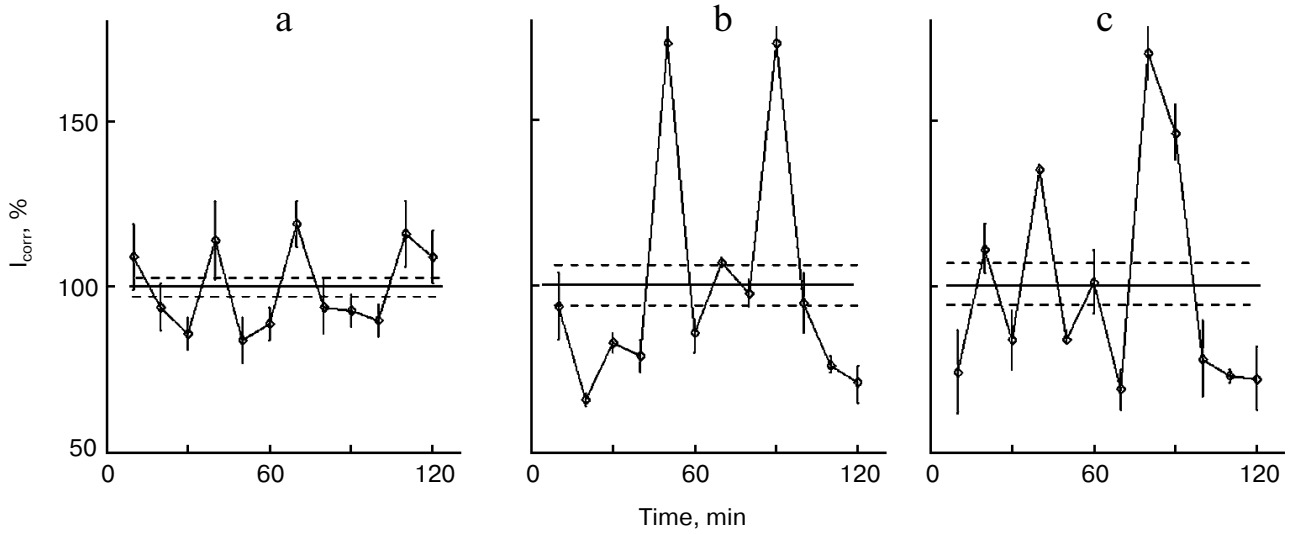

Fig. 1. Time-course of protein synthesis in dense cultures of hepatocytes from old rats. One rat (body weight 580 g) was fed with regular mixed fodder (Fig. 1a), and another (body weight 560 g) with mixed fodder moistened with 62 mg of glutamic acid (Fig. 1, b and c). In both cases, hepatocytes were isolated 1 h after feeding and cultivated in medium 199 supplemented with albumin and insulin (see "Materials and Methods"). After one day (b) or two days (c) the cultures were washed, and the time-course of protein synthesis was investigated in fresh medium. The values of relative incorporation of leucine into proteins (I_{corr}, see "Materials and Methods") are presented as percent of the average value for each variant of the experiment. Each point represents mean ± error for the sample. The average value for the cultures of this variant of the experiment (here 36 cultures) is presented as a straight line; dashed lines above and below the mean are errors of the mean. The measured average values of I_{corr} (cpm) were 464 ± 26 (Fig. 1a), 341 ± 29 (Fig. 1b), and 357 ± 28 (Fig. 1c).

glutamic acid signal for two days. Some of the cultures were incubated for one additional day. These cultures also demonstrated increased amplitudes of the protein synthesis rhythm (Fig. 2b).

In the next experiment (Fig. 3), hepatocytes were isolated on the 3rd day after feeding the rat (body weight 580 g) glutamic acid; the prepared dense cultures were examined after one more day. The rhythms of protein synthesis with increased amplitudes were not so well pronounced as in the cases of shorter periods after the glutamic acid signal; nevertheless, they were reliably detected. However, incubation for one additional day in the glutamic acid-free medium (Fig. 3b, in contrast to Figs. 1c and 2b) resulted in restoration of the initial rhythm observed for old rats as presented in Fig. 1a.

Hence, we demonstrated that the glutamic acid signal is remembered by hepatocytes for 3-4 days. Significant increase (2-fold on average) of the amplitude

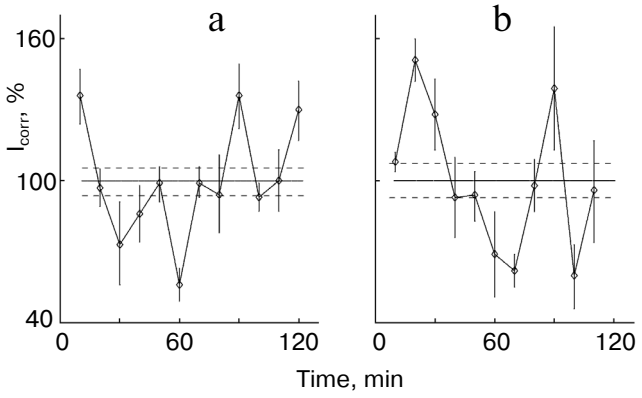

Fig. 2. Time-course of protein synthesis in dense cultures of hepatocytes isolated from a rat (body weight 550 g) one day after the rat was fed with mixed fodder moistened with glutamic acid followed by feeding with regular fodder. a) Cultures were investigated one day after start of cultivation; b) remaining cultures were investigated after one more day.

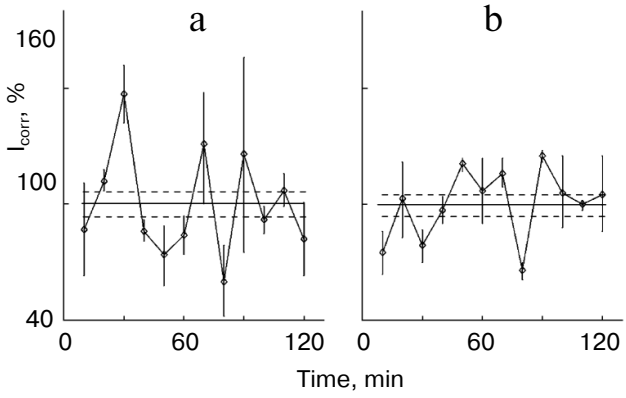

Fig. 3. Time-course of protein synthesis in dense cultures of hepatocytes from a rat (body weight 580 g) prepared three days after feeding the rat mixed fodder moistened with glutamic acid followed by feeding with regular mixed fodder. a) Cultures examined one day after the start of cultivation; b) remaining cultures examined after one more day.

of protein synthesis rhythm was observed in the primary hepatocyte cultures from the rats fed glutamic acid. It is clear that synchronization of the individual oscillations of the synthesis occurs in the liver following delivery of the effective dose of glutamic acid. At the time of preparation of cell cultures, the excess of transmitter no longer exists. But the cells "remember" the old signal. The value of amplitude of any ultradian rhythm characterizes the degree of synchronization of individual oscillations via direct cell–cell interactions. In the absence of synchronization, the resulting kinetics is linear; the higher is the amplitude of developing rhythm, the larger is the degree of synchronization of individual cells. During aging interactions are disrupted, which is manifested by the decreased amplitude of oscillations of protein synthesis rhythm in our case. Glutamic acid repaired the disruption, increasing the average amplitude (5 and 6 rats) from 34 ± 2 to 65 ± 4% [3]. In this case the transmitter *enhanced* synchronization, but in sparse cultures, where the rhythm was not observed initially, glutamic acid *initiated* synchronization [1]. Here we demonstrated that the reaction to the synchronization signal was maintained for 3-4 days.

Preservation of the effect of the rhythm of parotid gland cell dry weight developed during an experiment for approximately one day without support has been reported [6]. The same effect was demonstrated for protein synthesis kinetics in the gland explants. Even then, 10 years before the introduction of the fractal notion [7], we suggested the auto-oscillation nature of ultradian rhythms of protein kinetics. Special investigations justified the widespread presence of fractals in biology [8, 9]. The fractal nature of protein synthesis rhythm was also mentioned [2, 10].

We have shown previously [5], that 30-min exposure of asynchronous hepatocyte cultures to medium supplemented with 0.3 μM mixture of gangliosides (BBG) followed by the exchange of the medium with fresh normal medium resulted in synchronization of cells, and the rhythm of protein synthesis was organized in them. The rhythm was preserved for 2-3 following days of the culture incubation in normal medium [11]. The same effect was observed following 2-min exposure to medium containing 2 μM phenylephrine – a pharmacological analog of noradrenaline. In the control cultures (without BBG or without phenylephrine) the average amplitude of the rhythm was 25-34% of the mean level, while it increased to 63-65% following the exposure to the signal. We have also shown preservation of the melatonin signal, which synchronized protein synthesis oscillations for 1-2 days [12].

Preservation of the signal synchronizing a cell population was also shown in studies by Lloyd et al. [13, 14], who observed ultradian rhythm in respiration and other properties of yeasts without changes of the rhythm amplitude for three days after one-time cell synchronization. Gilbert, Hammond et al. [15, 16] also reported rhythms in activities of various enzymes following a one-time synchronization event mediated by the exposure of cell cultures to retinoic acid or insulin.

The notion of "memory" with respect to cells may not be entirely correct. Probably it is better to talk about the residual events in the cell behavior. But the main indicators of memory have been observed in various cells outside brain: the ability to sense an external signal via specific receptors, preservation of the effect for a certain time, and its reproduction without additional educating signals. The signal operates as a trigger organizing protein metabolism and other cellular properties for a certain period of time.

In a series of studies with stimulators and inhibitors of the processes of regulation of calcium flux and protein kinase activities we elucidated the key elements of a biochemical pathway for organization of the rhythm of protein synthesis [2]. For example, the chelator of cytoplasmic calcium BAPTA-AM eliminated the rhythm as well as blockers of protein kinase activities (mainly protein kinase C) H7 and H8. After the changes of calcium ion concentration and protein kinase activities were blocked, the signals did not affect the membrane receptors. It can be suggested that under normal conditions the one-time signal of glutamic acid, as well as of noradrenaline or serotonin, enhanced the process of calcium ion influx from a cytoplasmic depot. Rhythms of calcium ion concentration in cytoplasm of various cells are known [17]. These are rapid oscillations, but one can envisage generation of ultradian calcium rhythms based on them. The process of regular changes in calcium ion concentration continues for at least 1-2 days followed by the reaction of calcium-dependent protein kinases. Data are available on similar changes in adenylate cyclase and, respectively, cAMP and ATP, followed by ATP-dependent protein kinases (see references in a review by Brodsky [2]).

Hence, not the certain effector is memorized, but the signal from the receptor. Our observations could be compared with interesting results reported by Voronezhskaya et al. [18, 19], where the link between the serotonin content in blastomeres and the environment and the behavior of a new generation of mollusks was demonstrated. As in our case, the start of events was associated with activation of transmitter receptors, in this case on the surface of blastomeres; blocking of blastomere receptors eradicated the start of the changes in behavior of the mollusks. The processes of preservation were more complex, but the effect was sustained for one week. Some common stages of cell–cell interactions were revealed – dependence of the processes on concentration of calcium ions and activation of protein kinases. Participation of some protein kinases in brain memory was demonstrated by Balaban and coauthors [20].

Why is it important to study glutamic acid after the main factors were established already in the study of

noradrenaline, serotonin, and their derivatives? In contrast to the other transmitters, glutamic acid as a regular amino acid penetrates the blood–brain barrier, and hence can affect not only the cells outside the nervous system (liver, kidney, skin, and others), but also neurons. Protein synthesis suffers significantly in neurodegenerative diseases [4] and in the process of aging of healthy individuals. One can hope for improvement of their state with administration of glutamic acid; moreover, according the results of this work, the effect of the transmitter is maintained for 3-4 days after exposure to a single dose.

Preservation of the cell reaction to the signal organizing the kinetics of protein synthesis can be of interest to clinician also because some pharmaceuticals disrupt the kinetics. For example, dopamine exhibits such action via activation of its D2-receptors [21]. Dopamine is prescribed during acute cardiovascular failure and anaphylactic and cardiogenic shock. Its action is accompanied by the disruption of direct cell–cell interactions (protein synthesis rhythm is their indicator) in liver, kidney, and skin. Such unfavorable effect can be mitigated by administration of glutamic acid after 1-2 days.

Our data again attract attention to the fact that the ability to sense an external signal, respond to it, and reproduce the response during a certain period of time – memory – is characteristic not only to the complex of neurons, but also to other cell populations. Hepatocytes, keratinocytes, and other cells have specific receptors, possess the ability to transmit the signal to the cytoplasm, and have metabolic pathways in the cytoplasm that ensure cell cooperation for a certain period of time. The difference between memory (as a form of higher function of the nervous system) and preservation of metabolic signal lies the duration of the "memory". According to the available data, metabolic signals are memorized for days. The effect of brain signals is active for months and many years.

Acknowledgments

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