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Influence of ATP on Ehrlich Ascites Carcinoma Cell Free Cytoplasmic Calcium Concentration in the Course of Tumor Growth

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Abstract—The changes in free cytoplasmic calcium concentration ($[Ca^{2+}]_{in}$) and the effects of extracellular ATP on $[Ca^{2+}]_{in}$ have been studied in Ehrlich ascites carcinoma cells in the dynamics of their growth. The basal level of $[Ca^{2+}]_{in}$ and the effects of ATP on the ascites cells were determined by the stage of tumor growth and depended on the content of reactive oxygen species (ROS). The sharp increase in basal and ATP-induced elevation of $[Ca^{2+}]_{in}$ levels were observed at the 12th day of ascites cell growth. Inhibition of ROS formation by N-acetyl-L-cysteine decreased $[Ca^{2+}]_{in}$ and suppressed the cell reaction to ATP. We suggest that the increased sensitivity of the ascites cells to ATP observed on the 12th day may be also attributed to a decrease in ecto-ATPase activity.

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Key words: Ehrlich ascites adenocarcinoma, Ca^{2+}, ATP, reactive oxygen species, ecto-ATPase

Intracellular calcium is one of the most important regulators of cell growth and differentiation. Its level is under complex multiple controls by various biologically active substances [1-3]. The increase in cytosolic Ca^{2+} induced by various cytokines and hormones can initiate mitotic processes in cells [1, 4]. Calcium ions also regulate other cellular processes, which sometimes are opposite to proliferation; these include apoptosis and necrosis [5]. The increase in cytosolic Ca^{2+} can involve its influx from extracellular space or mobilization from intracellular stores (in dependence on regulatory signal and functional state of the cell) [6]. Impairments of mechanisms responsible for intracellular Ca^{2+} level may result in the development of pathological states and possibly neoplastic transformation of tissue [6, 7].

Extracellular ATP is an important factor involved in regulation of intracellular Ca^{2+} [8-12]. ATP is secreted by many cells including Ehrlich ascites carcinoma cells. It influences cell growth and differentiation via interaction with plasma membrane purinergic receptors [8] and activation of extracellular regulated tyrosine kinase [9]. In ascites cells, ATP increases Ca^{2+} concentration via interaction with P2Y-purinergic receptors [10]. The interaction of ATP with these receptors results in activation of G-proteins followed by subsequent activation of phospholipase C, the increase in inositol triphosphate stimulating Ca^{2+} mobilization from endoplasmic reticulum [11-13]. The ATP-induced increase in Ca^{2+} causes activation of Na^+/H^+-exchange, expression of *c-myc* oncogene, increase in mitochondrial Ca^{2+}, plasma membrane hyperpolarization, activation of Ca^{2+}-dependent K^+-channels, and reduction in cell volume [9].

At various stages of malignant growth, Ehrlich carcinoma is characterized by different mitotic activity [14], and so the study of regulatory effect of ATP on ascites cells in the time-course of their development may reveal some possible reasons responsible for malignant growth stimulation. Such study of the ATP effect on ascites cells may have practical importance because ATP is used in medicine as a pharmacological agent inhibiting development of some pathological processes [8]. For example, high concentrations of ATP are used for blockade of tumor growth [15]; however, some experimental data suggest that it may cause the opposite effect [16]. Anyway, there is evidence that ATP may cause not only cell activation but also cell death [17]. It is possible that opposite

Abbreviations: $[Ca^{2+}]_{in}$) cytoplasmic concentration of free calcium ions; ROS) reactive oxygen species.

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effects of ATP on cells may be determined by their different functional states determining the rate of tumor growth. Validation of this hypothesis requires comparison of tumor growth rate and sensitivity of ascites cells to ATP in certain periods of malignant growth. So, in this study we have investigated the effect of ATP on Ca^{2+} content in Ehrlich ascites carcinoma cells at various stages of the development of this tumor.

MATERIALS AND METHODS

The following reagents were used in this study: Fura-2AM (Serva, USA); ATP, Hepes, EGTA, N-acetyl-L-cysteine (Sigma, USA). Other chemicals were from Reakhim (Russia).

The study employed Ehrlich carcinoma ascites cells isolated on the 5-16 days after their intraperitoneal transplantation to ICR albino mice ($3 \cdot 10^6$ cells per animal).

The whole ascites liquid was collected from a peritoneal cavity using an automatic pipette; for removal of all cells, the peritoneum was washed three times with saline. The collected ascites liquid was centrifuged at 600*g* for 5 min. Supernatant was removed, and cell pellet was washed three times using Hanks' medium containing 140 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mM Na_2HPO_4, 1 mM $NaHCO_3$, 6 mM glucose, 10 mM Hepes, pH 7.2. The total number of ascites cells was counted using a Goryaev chamber. Total number of ascites cells in the tumor bearing animals was calculated using total ascites volume and concentration of cells in it.

Intracellular calcium concentration was determined at 25°C using fluorescent probe Fura-2 [18] and an Aminco Bowman Series 2 spectrofluorimeter (Thermo Spectronic, USA). Loading of $5 \cdot 10^6$ cells with the probe was carried out in 100 µl of Hanks' medium containing 2.5 µM Fura-2AM at 37°C for 30 min. After this incubation, cell suspension was washed three times with Hanks' medium and the cell pellet was resuspended in 90 µl of the same solution. The spectrofluorimeter cuvette was filled with 2 ml of Hanks' medium and 20 µl of cell suspension loaded with Fura-2AM (10^6 cells). Precise cell concentration was determined in the Goryaev chamber after measurement of fluorescence. The fluorescence was measured during 2 min using excitation wavelength of 340 nm and emission wavelength 520 nm. Intracellular concentration of Ca^{2+} was calculated according to the formula:

$$[Ca^{2+}]_{in} = K_s(F - F_{min})/(F_{max} - F),$$

where F is fluorescence intensity of the probe in the sample (relative units), F_{max} is fluorescence intensity of the probe loaded with calcium and determined after addition of 6 µM digitonin, F_{min} is intrinsic fluorescence of Ca^{2+}-free probe measured after addition of 10 mM EGTA, K_s is the coefficient of probe binding to Ca^{2+} (135 nM at 25°C). Information on the final concentrations of ATP (2 mM), EGTA (10 mM), and N-acetyl-L-cysteine (10 mM) was taken from the literature [19-22].

Activity of ecto-ATPase was determined by inorganic phosphate formed during hydrolysis of 100 µM ATP by Ehrlich ascites carcinoma cells [8]. The enzyme activity was assayed at 37°C in medium containing 10 mM Hepes, 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM glucose, pH 7.4. Accumulation of inorganic phosphate in the medium was determined only by the ecto-ATPase, because addition of Na,K-ATPase inhibitor (ouabain) and Mg-ATPase inhibitor (azide) did not influence inorganic phosphate concentration in the sample. Inorganic phosphate concentration was determined as described in [23]. Protein was determined by the microbiuret method [24].

Statistical treatment of results was carried out using Origin 5.0.

RESULTS

In our experiments the time-course of the ascites tumor growth differed from the classic kinetic behavior of tumor process described by N. M. Emmanuel [14]: there was a decrease in the number of ascites cells on the 12th day after tumor transplantation, which then changed for subsequent augmentation (Fig. 1). During tumor growth intracellular Ca^{2+} concentration exhibited some oscillations, which depended on the growth stage; however, two maximums, on the 10th and 12th days, were detected (Fig. 2).

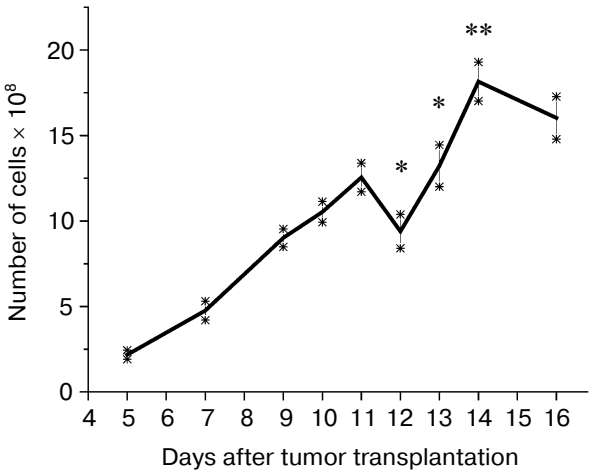

Fig. 1. Time-course of Ehrlich ascites carcinoma. Each point represents mean of 18-20 animals (±SEM). Asterisks indicate significance of differences compared with previous experimental point: * $P < 0.05$; ** $P < 0.01$.

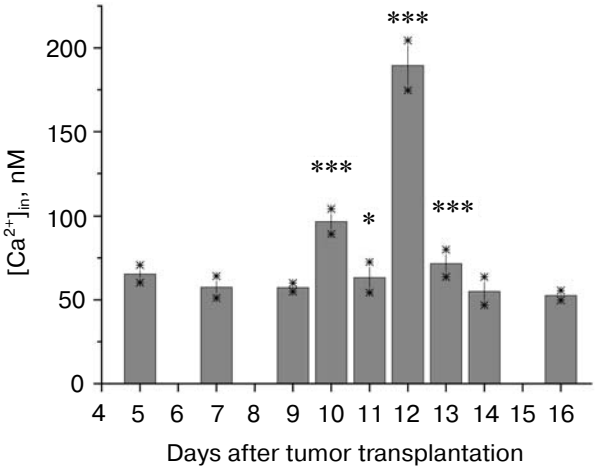

Fig. 2. Changes in Ca^{2+} content in ascites cells during tumor growth. Each point represents mean of 17-20 animals (±SEM). Asterisks indicate significance of differences compared with previous experimental point: * $P < 0.05$; *** $P < 0.001$.

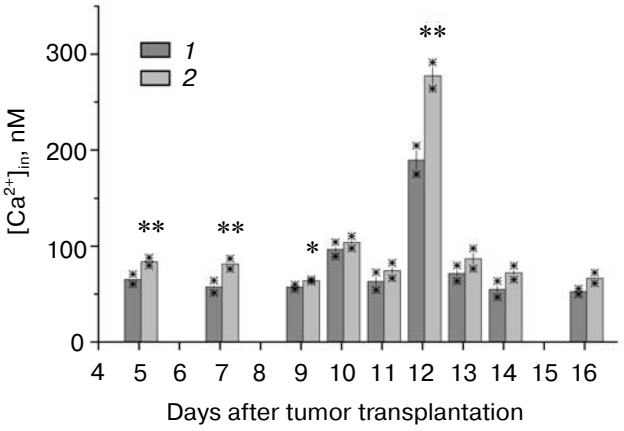

Fig. 3. Effect of ATP on ascites cell $[Ca^{2+}]_{in}$ on various phases of tumor growth. The measurements were carried out in Hanks' medium containing 1.3 mM Ca^{2+}. *1*) $[Ca^{2+}]_{in}$ without ATP addition; *2*) $[Ca^{2+}]_{in}$ after ATP addition. Asterisks indicate significance of differences between $[Ca^{2+}]_{in}$ in control and after addition of ATP: * $P < 0.05$; ** $P < 0.01$.

The effect of extracellular ATP on Ca^{2+} content in the ascites cells is also determined by a stage of tumor growth (Fig. 3). Maximal effect of ATP was noted on the 12th day.

The ATP-induced increase in Ca^{2+} concentrations in cells may occur due to increased Ca^{2+} influx from extracellular space or Ca^{2+} mobilization from intracellular stores. For evaluation of Ca^{2+} source responsible for the increase in this cation content in the ATP-activated ascites cells we have evaluated difference in the ATP-induced increase in intracellular Ca^{2+} concentrations assayed in the presence of free Ca^{2+} in the extracellular medium or in the presence of Ca^{2+} chelator, EGTA. These experiments revealed that the ATP-induced increase in Ca^{2+} observed on the 12th day after tumor transplantation occurred mainly due to Ca^{2+} influx from extracellular space: lack of Ca^{2+} in the extracellular medium caused about 80% inhibition of the ATP-induced increase in this cation in the ascites cells (Fig. 4). Thus, sensitivity of the ascites cells to ATP was determined by the stage of tumor growth.

Good evidence exists that the effects of many biologically active substances are mediated by reactive oxygen species (ROS) [25, 26], and inhibition of their formation is accompanied by suppression of cell growth and differentiation [25]. Agonist-induced increase in ROS often precedes the increase in cell Ca^{2+} [26] and possibly determines its biological effect. For elucidation of putative role of ROS in the regulation of Ca^{2+} content in the ascites cells, we determined Ca^{2+} concentrations after incubation of ascites cells with N-acetyl-L-cysteine, which neutralizes ROS [22, 27].

Experiments revealed that changes in Ca^{2+} in the ascites cells in response to ROS inhibition depended on the phase of the tumor development (Fig. 5). Lower initial Ca^{2+} concentration exhibited lower sensitivity to ROS neutralization. The dependence of intracellular Ca^{2+} on the presence of ROS was maximal on the 12th day after tumor transplantation.

The effect of ATP on the ascites cells also depended on the presence of ROS in them. The magnitude of this effect was determined by the phase of tumor growth (Fig. 6). The effect of ROS was minimal on the 5th and 9th days, but the ATP-induced increase in Ca^{2+} concentration on the 12th day almost totally depended on the presence of ROS.

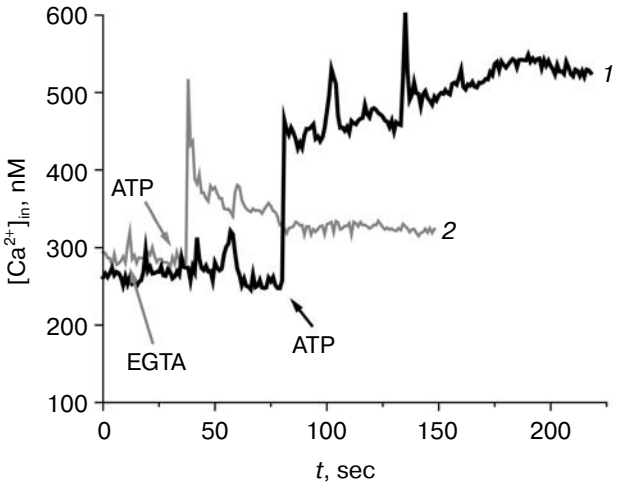

Fig. 4. Effect of EGTA on the change in $[Ca^{2+}]_{in}$ induced by ATP in ascites cells studied on the 12th day after tumor transplantation. Curves: *1*) increase in $[Ca^{2+}]_{in}$ induced by ATP in medium containing Ca^{2+}; *2*) increase in $[Ca^{2+}]_{in}$ induced by ATP in Ca^{2+}-free medium (Ca^{2+} chelation with EGTA).

For evaluation of one of the possible reasons underlying different cell responses of the ascites cells to exogenous ATP seen at different phases of the development of Ehrlich carcinoma, we analyzed activity of ecto-ATPase. Ecto-ATPase is a membrane enzyme responsible for hydrolysis of extracellular ATP [8]. Inhibition of ecto-ATPase induced spontaneous proliferation of B-lymphocytes [28]. So, it was reasonable to suggest that ecto-ATPase functioning may underlie the effect of ATP on the ascites cells as well. Experiments revealed insignificant variations of this enzyme activity during tumor growth with the exception of ecto-ATPase activity decrease observed on the 12th and 13th days (Fig. 7). Thus, it is possible that suppression of ecto-ATPase activity on the 12th day may be one of the reasons for the increase reactions in the ascites cells to ATP also observed in this period. At least it is reasonable to suggest that this enzyme exhibiting high catalytic activity in other periods of tumor growth prevented ATP-induced augmentation of cytosolic Ca^{2+} by local cleavage of ATP.

DISCUSSION

There are contradictory literature data on Ca^{2+} content in tumor cells [29-31]. In our experiments, cytosolic Ca^{2+} concentration of ascites cells and their sensitivity to ATP changed in dependence on the rate of tumor growth and the presence of ROS. Based on these data, we may suggest that contradiction of literature data about $[Ca^{2+}]_{in}$ in tumor cells may be attributed to different phases of the development of tumors used in experiments.

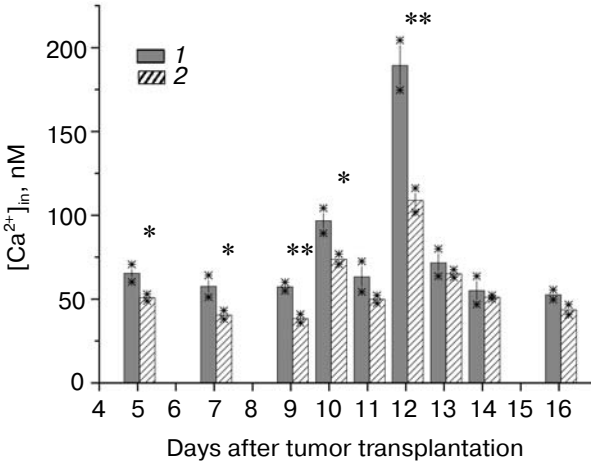

Fig. 5. Effect of N-acetyl-L-cysteine on the basal level of $[Ca^{2+}]_{in}$ in ascites cells. *1*) $[Ca^{2+}]_{in}$ without addition of N-acetyl-L-cysteine; *2*) $[Ca^{2+}]_{in}$ after incubation with 10 mM N-acetyl-L-cysteine at 37°C for 30 min. Asterisks indicate significance of differences between $[Ca^{2+}]_{in}$ in control and after incubation with N-acetyl-L-cysteine: * $P < 0.05$; ** $P < 0.01$.

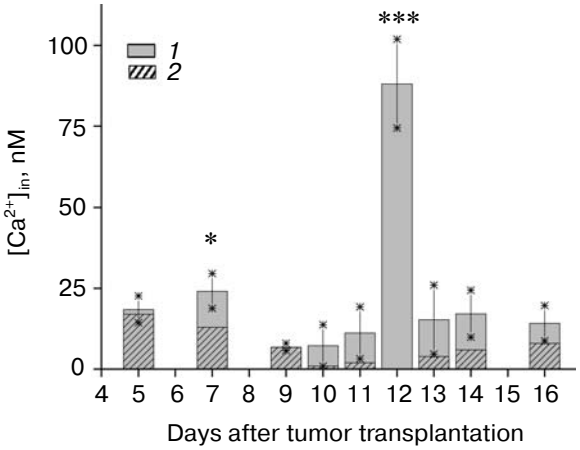

Fig. 6. Effect of N-acetyl-L-cysteine on the ATP-induced increase in $[Ca^{2+}]_{in}$ in ascites cells. *1*) Increase in $[Ca^{2+}]_{in}$ after addition of 2 mM ATP (final concentration); *2*) ROS-independent increase in $[Ca^{2+}]_{in}$ after addition of ATP (after preincubation with 10 mM N-acetyl-L-cysteine at 37°C for 30 min). Asterisks indicate significance of differences between $[Ca^{2+}]_{in}$ in control and after incubation with N-acetyl-L-cysteine: * $P < 0.05$; *** $P < 0.001$.

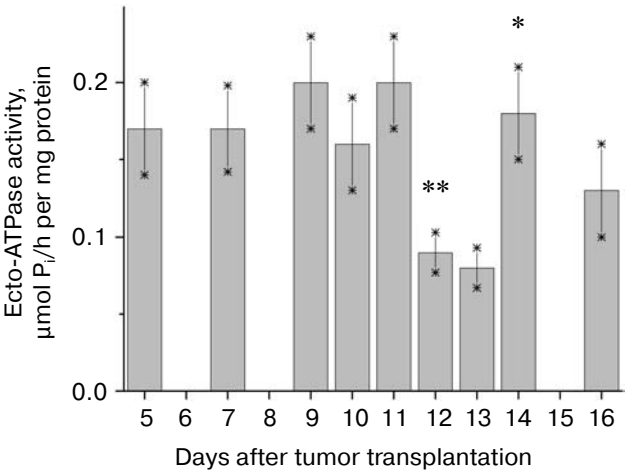

Fig. 7. Changes in ecto-ATPase activity in ascites cells during Ehrlich carcinoma growth. Asterisks indicate significance of differences compared with previous experimental point: * $P < 0.05$; ** $P < 0.01$.

Under our experimental conditions, ascites cells were characterized by high rate of growth on the 5-7th days after tumor transplantation. It is possible that such intensive growth provoked the development of hypoxia and relative deficit of energetic resources in the ascites tumor due to the increase in cell density in it. This might cause subsequent decrease in mitotic activity and consequently the decrease in tumor growth. Hypoxia reduces intracellular ATP content and inhibits tumor growth [32].

In our experiments, energy deficit developed due to increase in ascites cell density in the tumor caused transition of some cells into another functional state, the state of relative proliferation rest ("state of expectation"). Interestingly, the effect of ATP on the ascites cells was minimal during this period (9-11th days).

However, some proportion of the cells in the tumor died by the 12th day, and this caused a marked decrease in number of ascites cells. Reduction of cell number probably improved energy supply of remaining cells and thus caused a new increase in mitotic activity. This period was characterized by maximal Ca^{2+} concentrations and maximal responsiveness of the ascites cells to ATP and possibly by Ca^{2+} involvement in proliferative processes in the ascites cells. However, soon terminal phase developed; it was characterized by low rate of tumor growth accompanied by the decrease in cytosolic Ca^{2+} and reduced cell sensitivity to ATP. Thus, our experiments revealed that the effect of ATP on the ascites cells depended on their functional state.

There is convincing evidence that ROS are directly involved in the physiological response of cells to hormones and cytokines and that cell reaction is determined not only by a type of extracellular signal but also by ROS level [25, 26, 33]. It is also well documented that ROS formation in various cells is a very rapid transient post-receptor process determined by certain receptor agonists. Experimental evidence exists that ROS generation by cells precedes other intracellular signaling events including the increase in intracellular Ca^{2+} [34, 35]. ROS may also simulate effects of many hormones and neurotransmitters [36] because they regulate activity of proteins [37, 38]. Our studies revealed that not only cell response to the extracellular regulator but also basal level of cytosolic Ca^{2+} depended on the presence of ROS, and the higher Ca^{2+} concentration exhibited higher dependence on the presence of ROS. Elucidation of reasons underlying such dependence requires studies of physicochemical characteristics of membranes. We may only suggest that there are certain differences of plasma membrane properties at various phases of tumor growth characterized by different mitotic activity.

In our experiments, the manifestation of ATP effect on Ca^{2+} concentration also depended on the presence of ROS. In some phases of tumor development, the effect of ATP on the ascites cells was absent in the absence of ROS. This is consistent with data from other laboratories indicating that ROS are involved in signal transduction from receptors to intracellular targets [39], and that ROS determine degree of manifestation of receptor mediated biological response.

In conclusion we should emphasize that according to our data, the effect of ATP on the ascites cells varies in various phases of tumor growth, and so its pharmacological effect will differ at different stages of tumor growth. This may well explain opposite effect of ATP on tumor cells observed in different laboratories.

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