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Identification of 2',3'-cGMP as an Intermediate of RNA Catalytic Cleavage by Binase and Evaluation of Its Biological Action

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Abstract—Binase, the RNAse from *Bacillus pumilus*, is an endonuclease that cleaves the phosphodiester bond between the 3'-guanyl residue and 5'-OH residue of an adjacent nucleotide, with the formation of a corresponding intermediate, 2',3'-cGMP. Subsequent hydrolysis of 2',3'-cGMP into a 3'-phosphate is highly specific and proceeds slowly. Thus, a question arises in respect to the time interval that this positional isomer exists during catalytic cleavage of RNA by binase, and whether it may contribute to antitumor activity of the enzyme. In this study, we found, by implementing an enzyme-linked immunosorbent assay, that during catalytic cleavage of RNA by binase, 2',3'-cGMP is maintained in the reaction mixture for about one hour. Activation of phosphodiesterases did not lead to a complete elimination of 2',3'-cGMP. The highest amount of 2',3'-cGMP was detected at pH 8.5, underat which conditions, it reached nanomolar levels. The Iinitial RNA concentration in the reactions was in the range of 100–1000 μg/mL. We found that exogenous 2',3'-cGMP, as well as its positional isomer, 3',5'-cGMP, do not induce apoptosis of human lung adenocarcinoma A549 cells, which are sensitive to binase apoptogenic activity. Taking into consideration the data on binase internalization, and on the role of 2',3'-cGMP in the activation of opening of mitochondrial pores, we propose that 2',3'-cyclic guanosine phosphates contributes to apoptotic processes, induced by binase, only when generated intracellularly.

Keywords: cycle-forming guanyl-specific RNases, binase, 2',3'-cGMP, human lung carcinoma, A549

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INTRODUCTION

The family of T1 ribonucleases (RNases) (EC 3.1.27.3) includes about 25 low-molecular-weight extracellular proteins from bacteria and fungi that are similar in primary amino acid sequence and tertiary structure [1]. These enzymes depolymerize polyribonucleotides (or deaminated RNA) by cleaving the 3',5'-phosphodiester bond between guanosine 3'-phosphate (or 3'-phosphates of inosine or xanthosine) and the 5'-OH group of the adjacent nucleotide, forming a 2',3'-cyclic guanosine phosphate in the first stage of a catalytic reaction. This stage is reversible, and is much faster than the second, in which the cyclic intermediate is hydrolyzed to a corresponding 3'-phosphate [2]. RNases T1 are guanyl-specific, and only 2',3'-cGMP can be cleaved in the second step of catalysis [3]. A similar mechanism of enzyme action is characteristic for the family of pancreatic RNase A (EC 3.1.27.5). However, unlike for RNase T1, the second step of RNase A catalytic activity is specific for pyrimidine nucleotides, with terminal 2',3'-cyclic guanosine phosphatesphospahates [4].

Binase, RNase from *Bacillus pumilus* (formerly known as *B. intermedius* species) [5] belongs to the ribonucleases T1 family. It preferentially binds guanosine and its phosphorylated derivatives (GMP, GDP, GTP) in its active center [3]. One of its catalytic residues, His-101, is localized on a movable hinge, and another, Glu-72, is located in the beta-structure of the protein. In turn, residues Phe-55 and Tyr-102 are part of the binase substrate recognition site, which binds guanine bases [6]. Mutant forms of binase, with histidine substitutions in the active site (His101Asn or His101Thr), retain the ability for trans-esterification reaction, but are not able to hydrolyze the resulting cyclic-guanosine-phosphate [7]. Although, most known RNases can perform both stages of the catalytic reaction, two enzymes have been identified in *B. subtilis* that can only catalyze degradation of RNA (YurI), or 2',3'-cyclic nucleotides (YfkN), formed as a product of the YurI cleavage reaction [8].

The first stage of RNA hydrolysis is a reaction of low specificity, and can be performed by RNases, which do not possess the ability to cleave 2',3'-cyclic

Abbreviations: cGMP, cyclic guanosine monophospahate; PDE, phosphodiesterase; cNPasa, 2',3'-cyclic nucleotide-3'-phosphodiesterase.

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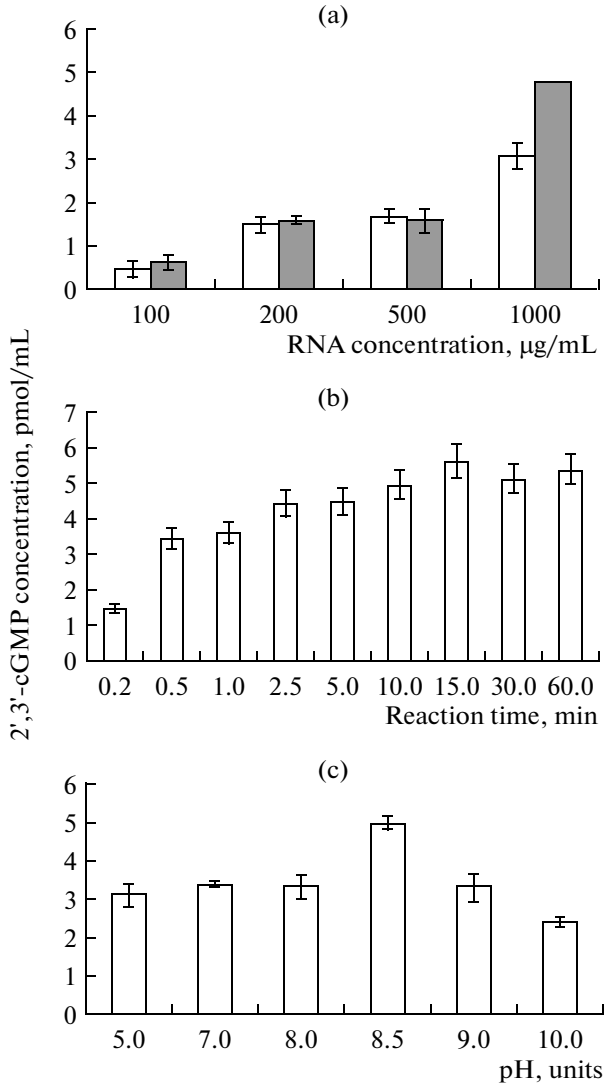

Fig. 1. Production of 2',3'-cGMP by binase at 37°C, depending on: the concentration of RNA in reaction mixture (pH = 8.5, incubation time 15 min) (a); pH (RNA at 1000 μg/mL, incubation time 15 min) (b); the incubation time (RNA at 1000 μg/mL, pH 8.5 (c). Concentration of binase: 10 μg/mL (light columns) and 100 μg/mL (dark columns).

nucleotides. In addition, the rate of the cyclization reaction is higher than that of the cleavage reaction cycle [2]. This points to a presence in the reaction mixture of a certain quantity of 2',3'-cyclic nucleotides, part of which, in the case of cleavage of RNA sequences with repetitive bases, will be free, not bound to 3'-ends of resulting oligonucleotide fragments. Chromatographic analysis of alpha-sarcin hydrolysis of polyadenylate revealed the presence of polyadenylic oligonucleotides in the reaction mixture, adenine, 3'-AMP and cAMP [9], and the latter compound is represented by a 2',3'-cyclic isomer, as is the case for all T1-RNases. It is likely, that binase can form a certain amount of free 2',3'-cGMP in the course of RNA hydrolysis. Yet, the potential effects of this positional isomer of the classical 3',5'-cGMP messenger molecule on cells functional activity have not been examined.

To understand the biological functions of exogenous guanyl-specific RNases, it is important to know, how long the 2',3'-cyclic nucleotides are retained in the reaction mixture, with RNA as a substrate, and how cells respond to their actions. Microbial RNases are well known for their anticancer potential [10–14]. Therefore, we sought to confirm a long term retention of 2',3'-cGMP in binase RNA hydrolysis reactions, and evaluate the effects of this cyclic nucleotide on lung adenocarcinoma cells, known to be sensitive to binase activity [15].

RESULTS AND DISCUSSION

First, we found that the immunodetection method using specific antibodies, allows the detection of 2',3'-cGMP production from RNA, and its levels increase with an increase in RNA concentration (Fig. 1a). We found that 2',3'-cGMP is already formed in the reaction mixture already in the first minutes of incubation, and its amount remained essentially constant for over one hour (Fig. 1b). Maximal 2',3'-cGMP levels were obtained, when the reaction was carried out at pH 8.5, which corresponds to the optimum conditions for RNA hydrolysis by binase (Fig. 1c). Figure 1a shows that about 1 nM of 2',3'-cGMP were produced by binase in a reaction mixture containing RNA at concentration 100 μg/mL. In reactions with RNA content at 1000 μg/mL, production of 2',3'-cGMP increased five-fold.

We have shown previously that binase is internalized by A549 cells [15]. Hence, its catalytic activity should be manifest inside cells and directed towards available RNA substrates. Intracellular phosphodiesterases (PDE) can degrade the 2',3'-cGMP product, formed during the first stage of binase catalytic reaction. Thus, we determined the extent of possible 2',3'-cGMP reduction due to PDE activity.

PDEs have been identified, practically, in all tissues: in brain cells, cardiac myocytes, endothelial cells, and others. As a known source of active PDE [16], we used cytosolic and membrane fractions from rat brain. We found that in the membrane rat brain fraction, PDE was more active, compared to the cytosolic fraction (Fig. 2a). The rate of 2',3'-cGMP cleavage in fractions correlated with the content of 2',3'-cyclic-nucleotide-3'-phosphodiesterase (cNPase), an enzyme found predominantly in adult rat brain (Fig. 2b). Notably, after 30 min of incubation, 2',3'-cGMP was still present in the reaction mixture (Fig. 3a). This is consistent with the presence of nanomolar concentrations of the first stage of the catalytic reaction product in cells, treated by binase, and characteristic for cyclizing RNases of the T1 family.

Cellular functions of classic second messengers, 3',5'-cAMP and 3',5'-cGMP, especially the latter, are

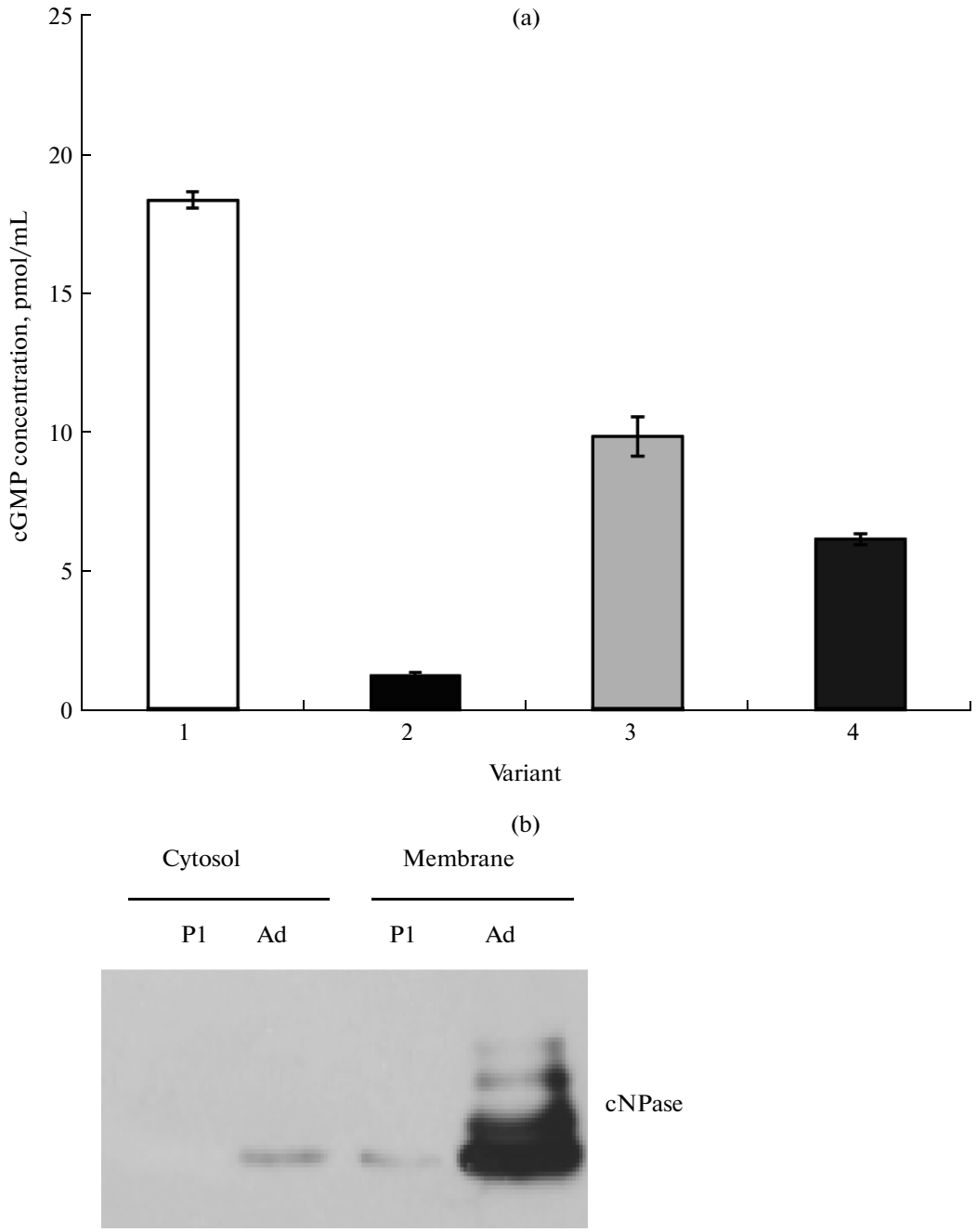

Fig. 2. Activity of nonspecific rat brain phosphodiesterases, degrading 2',3'-cGMP (a), and their content in the membrane and cytosolic fractions of newborn and adult rat brain, revealed by immunoblotting with antibodies to 2',3'-cyclonucleotide-3'-phosphodiesterase (b). *1*, No fractions added; *2*, membrane fraction with 50 μg protein content; *3*, cytosolic fraction with 50 μg protein content; *4*, cytosolic fraction with 100 μg protein content; P1, newborn rats, aged 1 day; Ad, adult rats, aged 3 months.

not fully understood. Furthermore, there are very few published reports on the roles of positional isomers of 2',3'-cyclic-guanosine-phosphates in the regulation of cellular processes. For instance, it is known that 3',5'-cGMP is involved in the induction of apoptosis in breast cancer cells, MCF-7 and MDA-MB-468 [17], in prostate cancer PC3 cells, in pancreatic PANC-1 and stomach MKN45 cancer cells [18]; and in blocking the progression of aggressive glioma [19]. Increased 3',5'-cGMP activity, due to blocking of intracellular PDE, causes a cell toxic effect. A similar effect was observed for its analogues, with enhanced ability to penetrate into the cell [20]. Notably, the entry of cyclic nucleotides and their analogues into cells does not correlate with their lipophilicity, and occurs by facilitated transport. In particular, the penetration rate

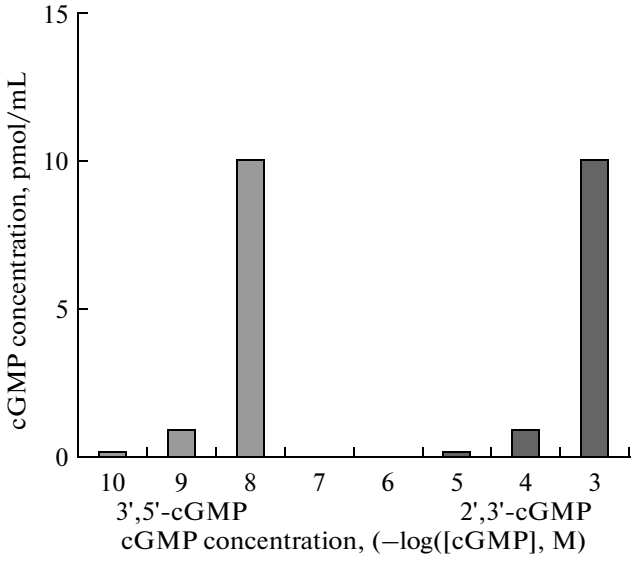

Fig. 3. Measurements of quantitieamounts of 3',5'-cGMP and 2',3'-cGMP, using 3',5'-cGMP–specific antibodies (ELISA) in a concentration range: for 3',5'-cGMP, from 0.1 nM to 10 nM; for 2',3'-cGMP, from 0.1 μM to 1 mM.

for cAMP in liver cells is 0.464 μM/min mL of cell volume [21].

2',3'-cGMP can promote thymidine incorporation in the DNA of lymphocytes [22]. This is probably due to the induction of DNA repair synthesis in response to the toxic effects of this nucleotide. 2',3'-cyclic nucleotides were implicated in the regulation of permeability of mitochondria in brain cells [23]. Increased extracellular levels of 2',3'-cAMP induce formation of pores in membranes of mitochondria in smooth muscle cells and in glomerular kidney cells, resulting in necrosis and apoptosis [24]. We also reported the opening of mitochondrial pores and induction of apoptosis under the action of binase in leukemia Kasumi-1 cells [25]. Given the probability of 2',3'-cGMP formation in tumor cells treated by binase, we propose that this compound can contribute to the biological effects of binase.

Several experimental studies reported a protective effect of exogenous cGMP [19]. To confirm the multidirectional role of cGMP, present inside and outside cells, we examined the effects of exogenous cGMP isomers on A549 lung carcinoma cells. Our previous experiments in these cells, uncovered apoptotic effects of binase, accompanied by an increase in cell permeability to macromolecules [15, 26]. We found that neither of two isomers induced apoptosis in A549 cells during a 48 h treatment (table). Therefore, the cytotoxic component of the binase effect is caused by its catalysis of 2',3'-cGMP, precisely, during intracellular RNA cleavage, as we have shown previously in binase treated cells [27].

Recent screening studies in mice, established that 2',3'-cGMP is present in virtually all tissues, with the concentration in the heart five times higher than that of 3',5'-cGMP, and only 2',3'-cGMP has been detected in the spleen and pancreas [28]. Thus, further studies are needed to investigate its roles in the regulation of cell signaling systems under the action of antitumor RNases T1.

Content of apoptotic cells in A549 cell population under the effect of 2',3'-cGMP and 3',5'-cGMP

Concentration of cGMP, μg/mL	Portion of apoptotic cells*, %	
	2',3'cGMP	3',5' cGMP
10	1.93 ± 0.47	1.07 ± 24
100	1.63 ± 0.73	1.33 ± 0.13
300	1.15 ± 0.4	1.57 ± 0.62
Without cGMP	1.02 ± 0.34	
Camptothecin, 50 mM	36 ± 4.3	

* $p \le 0.05$.

EXPERIMENTAL

Materials. We used guanyl-specific RNase, binase (M 12.3 kDa 109 amino acid residues, pI = 9.5), isolated as a homogeneous protein with catalytic activity, from a culture broth of a recombinant strain of *Escherichia coli* BL21, carrying pGEMGX1/ent/Bi plasmid. Binase catalytic activity towards yeast RNA is 14000000 U/mg at pH 8.5 [29]. Positional isomers of cyclic nucleotides, 3',5'-cGMP and 2',3'-cGMP, and rabbit anti-2',3'-cyclonucleotide 3'-phosphodiesterase (cNPase) were purchased from Sigma-Aldrich (Germany).

Cell culture. A549 lung adenocarcinoma cells (ACCC Collection, United States) were cultured at 37°C in standard DMEM medium (Invitrogen, United States), supplemented with penicillin/streptomycin (100 units each) and 10% calf serum, in a 5% CO_2 atmosphere. Cells were seeded at a density of 5–10 thousand/mL and grown to form a 50% confluent monolayer. Then, the medium was replaced by a similar medium, containing 3',5'-cGMP or 2',3'-cGMP (10–300 μg/mL). After incubation for 48 h, cytometric analysis was performed to detect guanosine phosphate-dependent induction of apoptosis.

Flow cytometric analysis. Changes in the cell characteristics were detected using a flow cytometer FACSCanto II (BD Biosciences, United States), as described previously [12]. Apoptogenic effects of the test compounds on A549 cells were measured using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, United States). As a positive control, we used

a classical inducer of apoptosis, camptothecin, at concentration 50 mM.

Reaction mixture for determination of binase cyclizing activity. Binase was incubated in a total volume of 200 μL in 0.5 M Tris-HCl buffer (at pH 5.0, pH 7.4 or pH 8.5) with yeast RNA (Sigma, United States) at concentrations, defined forin specific experiments, at 37°C, for a time from 10 s to 1 h. The catalytic reaction was stopped by adding 40 μL of cold 7% $HClO_4$. Then, 100 μL EPBS (0.1 M Na_2HPO_4, 0.15 M NaCl, 0.005 M EDTA, 0.2% bovine serum albumin (BSA), 0.01% thiomersal) were added, the reaction thoroughly mixed and centrifuged for 2 min at 12000 *g*, the supernatant decanted into a tube containing 60 μL EPBS and frozen at −80°C. The formation of the 2',3'-cGMP product was determined by enzyme immunoassay (ELISA), using commercial antibodies against 3',5'-cGMP.

ELISA-test for measurement of 2',3'-cGMP quantities. We used commercial 96-well plates with immobilized goat anti-rabbit antibody (secondary antibody), primary rabbit anti-3',5'-cGMP antibodies (Sigma-Aldrich, United States), and appropriate concentrations of reagents in accordance with manufacturer's instructions (IHF, Hamburg, Germany). Plates were thoroughly washed with 0.01 M phosphate buffer (pH 7.4). Then, 50 μL of test sample, 50 μL of biotin solution, and 100 μL of rabbit primary 3',5'-cGMP antibodies were added to each well. Plates were closed with lids and incubated overnight at 4°C for the binding of secondary antibodies, immobilized in the wells, to biotinylated primary antibody-antigen complexes. Then, 200 μL of freshly prepared solution of streptavidin–horseradish peroxidase conjugate (HRP-STREPT-reagent) was added to the wells, and plates were placed for 30 min in a refrigerator at 4°C to achieve binding between streptavidin and biotin, within the composition of the immunocomplex. Thereafter, plates were washed three times with buffer, left for 5 min at room temperature, filled with 250 μL of 3',3,5',5'-tetramethylbenzidine solution, and maintained at 4°C for 40 min. During this time, peroxidase converts colorless 3',3,5',5-tetramethylbenzidine into a blue derivative. Then, 50 μL of 2 M H_2SO_4 was added, solutions incubated for 5 min until the color changed from blue to yellow, and plates analyzed on a colorimeter at 450 nm. The color intensity is inversely proportional to the amount of cyclic nucleotides, bound to the primary antibody. Concentrations of cyclic nucleotides were calculated from the calibration curve.

Reaction of rabbit antibodies with 2',3'-cGMP, a positional isomer of 3',5'-cGMP. Due to a lack of commercially available antibodies to 2',3'-cGMP, we decided to determine the intensity of the cross reaction of antibodies to 3',5'-cGMP with 2',3'-cGMP. Since in reactions of catalytic RNA hydrolysis by guanyl-specific microbial RNases, 3',5'-cGMP is not formed [30], we tested the possibility of detection of 2',3'-cGMP, by a cross reaction with a commercial antibody to 3',5'-cGMP. We found that the sensitivity of this cross reaction was five orders of magnitude lower than binding to 3',5'-cGMP. Nevertheless, as is evident from Fig. 3, it is possible to use this antibody to detect 2',3'-cGMP at concentrations exceeding 10−5 M (from 10 nmol/mL or higher). Thus, we further used this technique to detect 2',3'-cGMP, the cyclic intermediate under investigation in this study.

Preparation of cytosolic and membrane fractions from rat brain cells. We used Wistar rat model (Charles River Laboratories, Sulzbach, Germany). All animal work was performed in accordance with the principles of state regulation of experimental animals (Resolution No G8151/591-00.33, Germany). Fractions were prepared as described previously [16]. Homogenized brain tissue was centrifuged at 3000 g to precipitate nuclei and cell debris. Then, proteins were separated into cytosolic and membrane fractions by centrifugation at 100000 *g*. Protein concentration was determined using a commercial Bio-Rad kit (Munich, Germany) with bovine serum albumin (Sigma, United States), as standard.

Activity of nonspecific phosphodiesterases in rat brain. To determine the potential extent of hydrolysis of 2',3'-cGMP by nonspecific PDE, 100 μL of 4 mM 2',3'-cGMP solutions were added to 20 μL of cytosolic or membrane fractions of rat brain of one of two ages: infants, aged 1 day, or adults, aged 3 months; and incubated for 30 min at 37°C. Then, samples were cooled to −80°C. As control, we used samples without addition of fractions, incubated for 30 min at 37°C. All samples were analyzed, using ELISA assay with antibodies against 3',5'-cGMP, to quantify non-hydrolyzed 2',3'-cGMP.

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