Ca\textsuperscript{2+}-dependent permeability transition regulation in rat brain mitochondria by 2',3'-cyclic nucleotides and 2',3'-cyclic nucleotide 3'- phosphodiesterase

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Azarashvili T, Krestina O, Galvita A, Grachev D, Baburina Y, Stricker R, Evtodienko Y, Reiser G. Ca\textsuperscript{2+}-dependent permeability transition regulation in rat brain mitochondria by 2',3'-cyclic nucleotides and 2',3'-cyclic nucleotide 3'- phosphodiesterase. Am J Physiol Cell Physiol 296: C1428–C1439, 2009. First published April 8, 2009; doi:10.1152/ajpcell.00006.2009.—Recent evidence indicates that 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), a marker enzyme of myelin and oligodendrocytes, is also present in neural and nonneural mitochondria. However, its role in mitochondria is still completely unclear. We found CNP in rat brain mitochondria and studied the effects of CNP substrates, 2',3'-cyclic nucleotides, on functional parameters of rat brain mitochondria. 2',3'-cAMP and 2',3',3'-cNADP stimulated Ca\textsuperscript{2+} overload-induced Ca\textsuperscript{2+} release from mitochondrial matrix. This Ca\textsuperscript{2+} release under threshold Ca\textsuperscript{2+} load correlated with membrane potential dissipation and mitochondrial swelling. The effects of 2',3'-cyclic nucleotides were suppressed by cyclosporin A, a potent inhibitor of permeability transition (PT). PT development is a key stage in initiation of apoptotic mitochondria-induced cell death. 2',3'-cAMP effects were observed on the functions of rat brain mitochondria only when PT was developed. This demonstrates involvement of 2',3'-cAMP in PT regulation in rat brain mitochondria. We also discovered that, under PT development, the specific enzymatic activity of CNP was reduced. Thus we hypothesize that suppression of CNP activity under threshold Ca\textsuperscript{2+} load leads to elevation of 2',3'-cAMP levels that, in turn, promote PT development in rat brain mitochondria. Similar effects of 2',3'-cyclic nucleotides were observed in rat liver mitochondria. Involvement of CNP in PT regulation was confirmed in experiments using mitochondria from CNP-knockdown oligodendrocytes (OLN93 cells). CNP reduction in these mitochondria correlated with lowering the threshold for Ca\textsuperscript{2+} overload-induced Ca\textsuperscript{2+} release. Thus our results reveal a new function for CNP and 2',3'-cAMP in mitochondria, being a regulator/promotor of mitochondrial PT.

oligodendrocyte mitochondria; 2',3'-cyclic nucleotide 3'- phosphodiesterase; permeability transition; calcium transport

THE ENZYME 2',3'-CYCLIC NUCLEOTIDE 3'-PHOSPHODIESTERASE (CNP, EC 3.1.4.37) accounts for ~2–5% of the total protein in the central nervous system myelin and 0.5–1% of peripheral nervous system myelin (36). CNP catalyzes the hydrolysis of 2',3'-cyclic nucleotides to form the corresponding 2'-monophosphates (3). CNP was shown to be an integral protein of myelin of oligodendrocytes in the central nervous system and of peripheral myelin in Schwann cells (36, 39). The majority of studies investigating the role of CNP were focused exclusively on the expression of CNP in oligodendrocytes and Schwann cells and the involvement of CNP in myelinogenesis. However, there is increasing evidence showing that this enzyme is present in a variety of other cell types. CNP-like enzyme activity was found in nonmyelin membrane preparations derived from spleen, liver, thymus, adrenal glands, kidney, heart, and skeletal muscle. The levels of the enzymatic activity, however, are significantly lower than those in the central nervous system (12, 15, 40). CNP activity was revealed in rat liver mitochondria, specifically in the outer and inner mitochondrial membranes (12). In cultured adrenal medullary chromaffin cells, which are not myelin associated, CNP was found to be located in mitochondria by means of immunofluorescence staining (27).

The in vivo biological roles of CNP are still largely unknown, and the possible function of CNP in mitochondria is even more enigmatic. Two CNP isoforms, CNP1 (46 kDa) and CNP2 (48 kDa), were found (16, 23) that are encoded by a single gene. The two isoforms are due to the presence of two alternative translation start sites (28). Several posttranslational modifications of CNP, such as phosphorylation, isoprenylation, and acylation, with unclear functional consequences are known (1, 2, 10, 36, 38, 39). A recent study of transfected oligodendrocyte cells (OLN93), overexpressing CNP1 and CNP2, showed that CNP2 can be translocated to mitochondria due to the presence of a mitochondrial targeting signal at the NH\textsubscript{2} terminus, which is cleaved on import into mitochondria (24). The translocation of CNP2 is regulated via phosphorylation of the targeting signal by protein kinase C. However, there are no data on the function of CNP in mitochondria.

CNP hydrolyzes not only 2',3'-cyclic nucleotides, but also oligonucleotides containing a 2',3'-cyclic terminus, showing base preference for purine over pyrimidine (23). 2',3'-Cyclic NADP is also known to be a substrate for CNP, and the \( K_m \) values for cyclic NADP and for 2',3'-cAMP were found to be the same (35). 2',3'-Cyclic NADP is usually used for detection of CNP activity. It should be noted that the enzymatic activity of CNP was established in vitro, and, up to now, there are no data on the effect of the CNP substrates on mitochondrial function. However, association of CNP with the inner segments of photoreceptors in the retina, which contain mitochondria, suggested that the electrochemical gradient maintained by photoreceptors may be regulated, in part, by CNP utilizing 2',3'-cyclic nucleotides (14). 2',3'-Cyclic phosphate-containing products could be a result of RNA damage. It was recently shown that CNP-like 2',3'-cyclic phosphodiesterase activity of clostridium thermocellum polynucleotide kinase-phosphatase might take part in RNA repair (22).

In the present study, the CNP substrates 2',3'-cAMP and 2',3'-cNADP were shown to be able to stimulate cyclosporin A...
(CsA)-sensitive, Ca$^{2+}$ overload-induced Ca$^{2+}$ release in rat brain mitochondria. 2',3'-cAMP and 2',3'-cNADP shortened the lag time before Ca$^{2+}$ release, increased the rate of Ca$^{2+}$ efflux from the mitochondrial matrix, and activated Ca$^{2+}$-induced, CsA-sensitive swelling of mitochondria. Similar effects of the 2',3'-cyclic nucleotides were observed in rat liver mitochondria. These findings indicate involvement of 2',3'-cyclic nucleotides in regulation of mitochondrial permeability transition (PT). The oligodendrocyte cell line OLN93 endogenously expresses CNP. We generated CNP knockout OLN93 cells. Interestingly, in mitochondria isolated from CNP knockout OLN93 cells, the reduced CNP level correlated with lowered threshold of Ca$^{2+}$ concentration to stimulate Ca$^{2+}$ efflux. Thus the present results suggest that CNP might participate in regulation of Ca$^{2+}$ fluxes in mitochondria and probably in regulation of PT via hydrolysis of 2',3'-cyclic nucleotides.

**MATERIALS AND METHODS**

**Isolation of rat brain mitochondria.** Adult male Wistar rats (230 – 250 g), which were used for obtaining tissues, were purchased from Harlan Winkelman (Borchen, Germany). All animal procedures have been approved by the ethics committee of the German federal state of Sachsen-Anhalt and are in accordance with the European Communities Council Directive (86/609/EEC). Rat brains were rapidly resected and homogenized in a glass homogenizer; the ratio of brain tissue to isolation medium was 1:10 (wt/vol). The homogenate was washed with phosphate-buffered saline, collected by scraping in a glass homogenizer; the ratio of brain tissue was 1:10 (wt/vol). The homogenate was centrifuged twice at 500 g for 5 min. Cells were resuspended in the same ice-cold solution, containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 0.02% Triton X-100, pH 7.4, and pelleted at 500 g for 10 min. The mitochondrial pellet was obtained by centrifugation of the 2,000 g supernatant at 12,500 g for 10 min. At the next step, in representative experiments, mitochondria were purified on Percoll gradient (15%:23%:40%), according to published procedures (34). Rat brain mitochondria were suspended in ice-cold solution containing 0.32 M sucrose and 10 mM Tris-HCl (pH 7.4). All solutions used were ice cold; all manipulations were carried out at +4°C. The tissue was homogenized in a glass homogenizer; the ratio of brain tissue to isolation medium was 1:10 (wt/vol). The homogenate was centrifuged twice at 500 g for 5 min, the pellet of mitochondria was obtained by centrifugation of the 2,000 g supernatant at 12,500 g for 10 min. At the next step, in representative experiments, mitochondria were purified on Percoll gradient (15%:23%:40%), according to published procedures (34). Rat brain mitochondria were suspended in ice-cold solution containing 0.32 M sucrose and 10 mM Tris-HCl (pH 7.4), and pelleted at 500 g for 5 min. Cells were resuspended in the same ice-cold buffer and disrupted with a Dounce homogenizer. The cell homogenate was centrifuged twice at 500 g for 5 min to remove unbroken cells and nuclei, and twice at 3,000 g for 4 min to remove trapped peroxisomes and other organelles. The supernatant obtained was centrifuged at 12,000 g for 10 min. The mitochondrial pellet was suspended in ice-cold medium containing 0.32 M sucrose and 10 mM Tris-HCl (pH 7.4). The mitochondrial matrix was collected by centrifugation at 500 g for 10 min. The isolated mitochondria were resuspended in the same ice-cold medium.

**Evaluation of mitochondrial functions.** The mitochondrial membrane potential was measured as described earlier (4) by determining the distribution of tetrabutylphosphonium (TBP) into the incubation medium with a TPP$^-$-selective electrode, and Ca$^{2+}$ transport was determined with a Ca$^{2+}$-sensitive electrode (Nico). Oxygen consumption rate was detected with a Clark-type O$_2$ electrode in the 2-ml cell volume. Mitochondria (1.0 mg protein/ml) were incubated in the medium containing 100 mM KCl, 100 mM sucrose, 10 mM Tris-HCl, 0.4 mM K$_2$HPO$_4$, and 2.0 mM rotenone, pH 7.4, at 37°C. Succinate (5 mM potassium succinate) was used as mitochondrial respiratory substrate.

PT opening in rat brain mitochondria was induced by threshold (0.32 M sucrose and 10 mM Tris-HCl, 0.4 mM K$_2$HPO$_4$, and 2.0 mM rotenone, pH 7.4, at 37°C. Succinate (5 mM potassium succinate) was used as mitochondrial respiratory substrate.)

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**Detection of CNP activity in isolated mitochondria.** CNP activity was detected according to published procedures (9). Aliquots of the mitochondrial suspension (rat brain mitochondria or OLN93 cells) containing 40 µg of protein were taken from the chamber under control conditions and after PT opening and were then solubilized in Laemmli buffer. Soluteitisolized mitochondria were separated by electrophoresis in a 12.5% polyacrylamide gel and were transferred to nitrocellulose membranes. CNP activity was detected on membrane after blotting. The membrane was preincubated for 1 h in 50 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer, pH 6.1, containing 30 mM MgCl$_2$, 0.1% Triton X-100, 3 M guanidinium chloride, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol and then for 1 h in the same buffer without guanidinium chloride. The preincubation stage is essential for recovery of enzyme activity after SDS electrophoresis. The blot was then stained for activity by using nitro blue tetrazolium (3,3',3'-dimetoxy-4,4'-diphenylene)2,2'-di-p-nitrophenyl-5,5'-diphenilditerazolium) chloride and phenazine methosulfate to detect NADPH produced in enzyme-linked reaction for CNP. The colored reaction product was immobilized with diluted agarose. Equal volumes of 1% agarose in water at 55°C and reaction medium (200 mM MES buffer, pH 6.1, containing 60 mM MgCl$_2$, 0.2% Triton X-100, 0.1 M 2',3'-cyclic NADP, and 4 mg/ml glucose-6-phosphate, 0.4 mg/ml nitro blue tetrazolium, 0.04 mg/ml phenazine methosulfate, and 0.7 units of glucose-6-phosphate dehydrogenase) were rapidly mixed and poured on the nitrocellulose membrane. Colored reaction was visible within 15 min and was stopped with 10% acetic acid. CNP activity in the bands (intensity of colored band) on the blot was evaluated by determining the optical density of the colored product.

**Electrophoresis and immunoblotting of mitochondrial proteins.** For immunoblotting, mitochondrial proteins solubilized in Laemmli buffer were separated under denaturing conditions on 10% or 12.5% SDS-PAGE gels and transferred to nitrocellulose membranes. Precision Plus Pre-stained Standards from Bio-Rad Laboratories (Hercules, CA) were used as markers. After overnight blocking, the membrane was incubated with the appropriate primary antibody. Monoclonal anti-CNP antibody was obtained, as described (30). Anti-SOD2 (Mn-superoxide dismutase 2, antibody 13533, Abcam, 1:10,000 dilution), anti-VDAC (voltage-dependent anion channel) antibody, (Ab-5, Calbiochem, Schwabach, Germany, 1:2,000 dilution), and β-tubulin I antibody (Sigma, 1:20,000 dilution) were used for immunoblotting.

**Scanning mitophotential parameter.** The mitochondrial membrane potential was measured as described earlier (4) by determining the measurement of tetrabutylphosphonium (TBP$^-$) in the incubation medium with a TPP$^-$-selective electrode, and Ca$^{2+}$ transport was determined with a Ca$^{2+}$-sensitive electrode (Nico). Oxygen consumption rate was detected with a Clark-type O$_2$ electrode in the 2-ml cell volume. Mitochondria (1.0 mg protein/ml) were incubated in the medium containing 100 mM KCl, 100 mM sucrose, 10 mM Tris-HCl, 0.4 mM K$_2$HPO$_4$, and 2.0 µM rotenone, pH 7.4, at 37°C. Succinate (5 mM potassium succinate) was used as mitochondrial respiratory substrate.
Immunoreactivity was detected using the appropriate secondary antibody conjugated to horseradish peroxidase. Peroxidase activity was detected with ECL chemiluminescence reagents (Pierce Chemical).

Cell culture and transfection with small interfering RNA. OLN93 cells, an oligodendrocyte cell line (32), kindly provided by C. Richter-Landsberg (University of Oldenburg, Germany), were grown in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), containing 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. To knock down the endogenous CNP, OLN93 cells were transiently transfected with the chemically synthesized pool of small interfering RNAs (siRNAs) targeting CNP (Dharmacon, Chicago, IL), or with the nonsilencing siRNA served as a scrambled control (Qiagen). Cells (50% confluent) were transfected with rat CNP siRNA by using magnet-assisted transfection, according to the manufacturer’s protocol (IBA). CNP knockdown was assessed by Western blot at 48 h after transfection.

Statistical analysis. For statistical analysis, relative levels of protein density were expressed as means±SD from at least three to four independent experiments with samples of mitochondria isolated at different days. Several replica experiments were done with each sample of mitochondria. The total number of observations or sample size was 10 for rat brain mitochondria and 12 for rat liver mitochondria. Statistical significance was evaluated using the Student’s t-test and ANOVA with Bonferroni post hoc comparison. A value of P < 0.05 was accepted as significant.

RESULTS

Effects of 2′,3′-cAMP on mitochondrial functions in rat brain mitochondria. The effect of the CNP substrate 2′,3′-cAMP on mitochondrial functions is still unclear. We assayed the influence of 2′,3′-cAMP on functions of rat brain mitochondria by simultaneous registration of Ca2+ flux, membrane potential change (ΔΨM), and O2 consumption, using selective Ca2+, TPP+, and oxygen electrodes. The recordings for the three parameters, Ca2+ influx/efflux, ΔΨM measured by TPP+ distribution, and respiration, under threshold Ca2+-load, are presented separately, in Fig. 1, A–C, and average data in D. For average data of control values, we obtained the following means and standard deviations: Ca2+ influx rates after first Ca2+ pulse (VCa2+in, 224 ± 32 nmol·min⁻¹·mg protein⁻¹); Ca2+ efflux rate (VCa2+out, 117 ± 24 nmol·min⁻¹·mg protein⁻¹); lag time for Ca2+ efflux (lag time, 230 ± 61 s); oxygen consumption rate in state 2 (V0, 12.9 ± 3.2 ng-atom O·min⁻¹·mg protein⁻¹); oxygen consumption rate in state 3 after Ca2+ addition (V0, 53.6.9 ± 4.9 ng-atom O·min⁻¹·mg protein⁻¹); and ATP-induced membrane repolarization, calculated as TPP+ influx rate (VTPP+, 0.24 ± 0.04 μM/min).

Figure 1, A–C, demonstrates typical results with rat brain mitochondria in the absence and in the presence of 2′,3′-cAMP (5

![Figure 1: Effect of 2′,3′-cAMP on mitochondrial functions in rat brain mitochondria. Isolated rat brain mitochondria (1.0 mg protein/ml) were incubated in the electrode chamber under conditions described in MATERIALS AND METHODS. Oxygen consumption rate, Ca2+ concentration in the incubation medium, and changes in mitochondrial membrane potential were recorded simultaneously in an open chamber. The time scale represents the time after rat brain mitochondria were added to the chamber. Two additions of Ca2+ (80 μM each) were applied. Arrows show the times at which CaCl2 and ATP were added. 2′,3′-cAMP (5 μM) was applied 20 s after addition of rat brain mitochondria to the incubation medium. Recordings are shown for the same samples for Ca2+ fluxes (A), tetraphenylphosphonium (TPP+) concentration (membrane potential) (B), and oxygen consumption (C). In all panels, the solid trace shows control rat brain mitochondria, and the dashed trace shows rat brain mitochondria treated with 2′,3′-cAMP. D: the summary of effects of 2′,3′-cAMP on rat brain mitochondria functions. The following parameters of Ca2+-induced permeability transition (PT) pore opening were calculated from the curves, for which examples are given in A–C: oxygen consumption rate in state 2 (V0, ng-atom O·min⁻¹·mg protein⁻¹), Ca2+ influx rates (VCa2+in, nmol·min⁻¹·mg protein⁻¹), after first (VCa2+1out, ng-atom O·min⁻¹·mg protein⁻¹) and after second calcium pulse (VCa2+2out), lag time for Ca2+ efflux (lag time, s), Ca2+ efflux rate (VCa2+out, nmol·min⁻¹·mg protein⁻¹), and ATP-induced membrane repolarization, calculated as TPP+ influx rate (VTPP+, μM/min). Values are given relative to the control value of 100 and represent the means ± SD from three independent experiments. *P < 0.05 vs. control. A detailed description of the parameter analysis is given in our laboratory’s previous publications (4, 5). VTPP+, TPP+ efflux rate.}
µM. As Ca²⁺ fluxes in Fig. 1A show, rat brain mitochondria rapidly accumulated Ca²⁺ after the first addition of 80 nmol Ca²⁺/mg protein. The rate of uptake (Vuptake) was similar in the absence and in the presence of 2',3'-cAMP. The second Ca²⁺ addition in this case exceeds the threshold Ca²⁺ concentration. After the second Ca²⁺ pulse, reduced Ca²⁺ accumulation with the following spontaneous Ca²⁺ efflux from the rat brain mitochondria matrix was observed. In the 2',3'-cAMP-treated rat brain mitochondria, this Ca²⁺ uptake rate (Vuptake) was decreased compared with control rat brain mitochondria.

Moreover, the ability to retain Ca²⁺ at the threshold Ca²⁺-induced Ca²⁺ release differed significantly in control and in 2',3'-cAMP-treated rat brain mitochondria. Therefore, we measured the lag time (see explanation in Fig. 1A). The lag time was ~4 min for control and ~1.4 min for 2',3'-cAMP-treated rat brain mitochondria. Thus, in 2',3'-cAMP-treated rat brain mitochondria, the Ca²⁺ loading-induced Ca²⁺ release was accelerated with a three times shorter lag time. In addition, the rate of the Ca²⁺-stimulated Ca²⁺ efflux was higher in 2',3'-cAMP-treated rat brain mitochondria, as shown by the parameter Vout in Fig. 1. Simultaneous registration of ΔΨM in the same preparations is shown in Fig. 1B. After the first addition of Ca²⁺, a transient depolarization of the inner membrane took place. After the second Ca²⁺ addition, a sustained depolarization occurred. Inhibition of Ca²⁺ uptake after the second Ca²⁺ addition (see Fig. 1A) was accompanied by Ca²⁺-induced decline in ΔΨM (see Fig. 1B) in both control and 2',3'-cAMP-treated rat brain mitochondria.

The ability of ATP to initiate repolarization of the inner membrane of rat brain mitochondria in the absence and in the presence of 2',3'-cAMP was also checked. In control rat brain mitochondria, we observed rapid Ca²⁺ reaccumulation and restoration of the membrane potential, when ATP was added after PT induction. However, reaccumulation of Ca²⁺ was slower in the presence of 2',3'-cAMP (Fig. 1A). The rate of ATP-induced repolarization of rat brain mitochondria was decreased in the presence of 5 µM 2',3'-cAMP (Fig. 1B). Thus 2',3'-cAMP was able to accelerate threshold Ca²⁺-induced Ca²⁺ efflux and to slow down both ATP-induced restoration of ΔΨM and the rate of ATP-induced Ca²⁺ influx.

The respiration of rat brain mitochondria was also measured under the same conditions. The results are shown in Fig. 1C. No significant changes in oxygen consumption rates were observed in the presence and in the absence of 2',3'-cAMP before Ca²⁺ addition. However, after the threshold Ca²⁺ load, oxygen consumption was increased in the presence of 2',3'-cAMP. The latter demonstrates an uncoupling-like effect, which is related to promotion of the PT development.

The bar graph in Fig. 1D gives a quantitative analysis of the parameters exemplified in the traces in Fig. 1, A–C. These mean data obtained from several experiments demonstrate the relative effects of the nucleotide 2',3'-cAMP on mitochondrial functions (100% corresponds to control). 2',3'-cAMP did not affect rat brain mitochondria functions in normal conditions before the threshold Ca²⁺ load, that is, the Vout and the Vin in. However, 2',3'-cAMP clearly affected the rat brain mitochondria functions at the second Ca²⁺ addition (threshold Ca²⁺ load). Vin in was decreased. The time of Ca²⁺ retention (lag time) was decreased twofold, and the Vout was increased by 30%. In addition, the ATP-induced ΔΨM restoration (TPP⁺ reaccumulation determined by VTPP⁺) was diminished twofold in the presence of 2',3'-cAMP, demonstrating a lowered repolarization ability of the inner membrane. Thus 2',3'-cAMP was able to promote the threshold Ca²⁺-induced PT pore opening and to resist the ATP-induced PT pore closing.

Efficiency of 2',3'-cAMP action and effects of 2',3'-cNADP on mitochondrial functions in rat brain mitochondria. Next we evaluated the effects of different concentrations of 2',3'-cAMP, and the effect of combined application of 2',3'-cAMP and CsA, as well as of 2',3'-cNADP and CsA. As shown in Fig. 2A, with addition of CsA, a specific inhibitor of PT, no Ca²⁺-induced Ca²⁺ efflux was seen in the presence of 2',3'-cAMP. This inhibition of Ca²⁺ efflux by CsA supports the conclusion of participation of PT. The concentration dependence of the efficiency of 2',3'-cAMP to accelerate Ca²⁺ release was also checked. The maximal efficiency of 2',3'-cAMP was observed at a concentration of ~5 µM, as measured by Vout and lag time of Ca²⁺ efflux. However, a marked change was seen already at 0.5 µM 2',3'-cAMP. The concentrations of 2',3'-cAMP examined were in the range from 0.1 to 50 µM (Fig. 2B). At 10 µM 2',3'-cAMP, the Vout reached a plateau. After further increasing the 2',3'-cAMP concentration up to 50 µM, a slight increase of the Vout and decrease of the lag time of Ca²⁺ efflux were observed. This could be due to a superposition of an unspecific Ca²⁺ binding effect of 2',3'-cAMP.

We also checked whether 2',3'-cNADP (5 µM) and 2',3'-cGMP (5 µM) were able to affect the threshold Ca²⁺-induced Ca²⁺ release. Figure 2 demonstrates the effects of 2',3'-cNADP and 2',3'-cGMP used at the same concentration as 2',3'-cAMP (5 µM). In Fig. 2C, trace 1 demonstrates Ca²⁺ fluxes in control rat brain mitochondria at threshold Ca²⁺ load. In the presence of 2',3'-cNADP, after the threshold Ca²⁺ load, a decreased rate of Ca²⁺ influx (Vin in) and a shortened lag time of Ca²⁺ efflux were seen (Fig. 2C, trace 2). The lag time in 2',3'-cNADP-treated rat brain mitochondria was about two times shorter than in control rat brain mitochondria (compare traces 1 and 2). No acceleration of Ca²⁺ efflux was observed in the presence of 2',3'-cGMP (trace 3). CsA was found to prevent also the 2',3'-cNADP-induced acceleration of Ca²⁺ release in the presence of threshold Ca²⁺ load (trace 4).

The average data obtained with 5 µM 2',3'-cNADP, 2',3'-cAMP, and 2',3'-cGMP are shown in Fig. 2D, which presents lag time and Ca²⁺ efflux under Ca²⁺ overload (Vout). There was no significant effect of 2',3'-cGMP on Ca²⁺ efflux in the presence of threshold Ca²⁺ concentrations; also, similarly, 2',3'-cGMP was not effective (data not shown).

Effects of 2',3'-cAMP and 2',3'-cNADP on mitochondrial functions in rat liver mitochondria. The paper reporting CNP activity in rat liver mitochondria appeared already 27 years ago, where the CNP activity was detected using 2',3'-cNADP as substrate (12). However, so far, there are no data yet on the effect of CNP substrates on rat liver mitochondria functions. Therefore, we next used rat liver mitochondria to study the effect of 2',3'-cNADP and 2',3'-cAMP on stimulation of Ca²⁺ release after addition of threshold Ca²⁺ concentration. This helped to evaluate the effect of the cyclic nucleotides on another type of mitochondria, besides rat brain mitochondria.

Figure 3A shows that, in control rat liver mitochondria as well as in 2',3'-cAMP- and 2',3'-cNADP (5 µM)-treated rat liver mitochondria, after the first and the second Ca²⁺ addition, mitochondria were able to accumulate Ca²⁺, but after a lag time (the time of calcium retention), spontaneous Ca²⁺ efflux
took place. The lag time was approximately 2.5 min for control rat liver mitochondria (Fig. 3A, trace 1). 2',3'-cAMP shortened the lag time of Ca\(^{2+}\) release to 1 min, while the lag time with 2',3'-cNADP was 0.5 min (Fig. 3A, traces 2 and 3). 2',3'-cNADP was found to be more effective than 2',3'-cAMP in accelerating the Ca\(^{2+}\) efflux.

Just after the second pulse of Ca\(^{2+}\), dissipation of ΔΨ\(_M\) was observed as TPP\(^{+}\) efflux (Fig. 3B). The rate of TPP\(^{+}\) efflux (V\(_{\text{TPP}}\)) was used to measure the rate of depolarization of the inner membrane. This gave the following order of potency: 2',3'-cNADP-treated rat liver mitochondria > 2',3'-cAMP-treated rat liver mitochondria > control rat liver mitochondria. This depolarization of the inner membrane of rat liver mitochondria correlated with the corresponding rates of Ca\(^{2+}\) efflux after the second addition of calcium (compare Fig. 3, A and B). After the ΔΨ\(_M\) dissipation was almost completed, a significant increase in V\(_{\text{out}}\) was observed that indicated PT opening. The Ca\(^{2+}\) efflux and ΔΨ\(_M\) dissipation were not observed in the absence of CsA (data not shown). Respiratory activity of the rat liver mitochondria was increased after the second Ca\(^{2+}\) pulse (Fig. 3C, traces 1–3) showing uncoupling-like effect.

The average data obtained with 2',3'-cNADP and 2',3'-cAMP (5 μM) on the parameters obtained under Ca\(^{2+}\) overload, that is, V\(_{\text{in}}\), lag time, V\(_{\text{out}}\), and ΔΨ\(_M\) decrease (V\(_{\text{out}}\)), in rat liver mitochondria, are shown in Fig. 3D. For average data control values, we obtained the following means and standard deviations: V\(_{\text{in}}\), 263 ± 39 nmol·min\(^{-1}\)·mg protein\(^{-1}\); V\(_{\text{out}}\), 164 ± 35 nmol·min\(^{-1}\)·mg protein\(^{-1}\); lag time for Ca\(^{2+}\) efflux (lag time), 192 ± 26 s; V\(_{\text{C}\text{O}_{2}}\), 19.9 ± 3.8 ng-atom O·min\(^{-1}\)·mg protein\(^{-1}\); V\(_{\text{ATP}}\), 113.7 ± 22.7 ng-atom O·min\(^{-1}\)·mg protein\(^{-1}\); and ATP-induced membrane repolarization, calculated as V\(_{\text{TPP}}\), 0.35 ± 0.04 μM/min.

The data presented in Fig. 3D show that 2',3'-cNADP clearly stimulated the rate of ΔΨ\(_M\) dissipation and shortened the lag time for the Ca\(^{2+}\)-induced Ca\(^{2+}\) efflux in Ca\(^{2+}\)-overloaded rat liver mitochondria, while, in rat brain mitochondria, 2',3'-cAMP was more effective.

Effects of 2',3'-cAMP and 2',3'-cNADP on mitochondrial swelling in rat brain and liver mitochondria. Induction of mitochondrial swelling is a prominent feature of PT; therefore, we examined the influence of 2',3'-cAMP and 2',3'-cNADP on Ca\(^{2+}\)-induced swelling of isolated rat brain mitochondria and rat liver mitochondria to corroborate our hypothesis that the cyclic nucleotides are involved in PT regulation. Swelling was initiated by addition of Ca\(^{2+}\) to mitochondria incubated in standard medium (see MATERIALS AND METHODS). The decrease of light scattering at 540 nm that indicates swelling was accelerated in the presence of 5 μM 2',3'-cAMP or 5 μM 2',3'-cNADP. The swelling was more obvious in rat liver mitochondria than in rat brain mitochondria.

The data in Fig. 4 (A, typical curves, and B, average data) show that the relatively small swelling of rat brain mitochondria is significantly enhanced by 2',3'-cAMP and 2',3'-cNADP. We used the half time for reaching the maximum of the rat brain mitochondria swelling as characteristic parameter for quantification.

In isolated rat liver mitochondria, the effects of 2',3'-cAMP and 2',3'-cNADP on swelling were more prominent, as shown in Fig. 4, C and D. In the presence of 500 nM 2',3'-cNADP (trace 2) or 500 nM 2',3'-cAMP (trace 3), the rate of swelling was greatly accelerated. 2',3'-cAMP and 2',3'-cNADP induced rapid, large amplitude swelling of rat liver mitochondria within 5 min. In rat liver mitochondria, the cyclic nucleotides accelerated the
mitochondrial swelling ~3.5 times compared with control Ca$^{2+}$-induced swelling (Fig. 4D). Mitochondrial swelling elicited by 2',3'-cAMP and 2',3'-cNADP was prevented by CsA (traces 4 and 5 in Fig. 4C), confirming the participation of PT.

**CNP localization in sub mitochondrial fractions and influence of threshold Ca$^{2+}$ on CNP activity in rat brain mitochondria.** Here, we also determined the enzymatic CNP activity in isolated rat brain mitochondria after SDS-PAGE and Western blot on nitrocellulose membrane. This method is used according to the procedure published before (9). A suspension of purified rat brain mitochondria was added to the incubation medium in the chamber with installed electrodes to measure PT development. From there, the samples were taken for CNP enzyme activity assay. The CNP activity was measured before and after Ca$^{2+}$ threshold loading that leads to PT development. The enzymatic activity was decreased in the presence of threshold Ca$^{2+}$ load, as shown in Fig. 5, A and B. Equal levels of CNP in rat brain mitochondria were found both in control condition and after Ca$^{2+}$ threshold loading (Fig. 5, C and D).

We then examined the localization of CNP in fractions of rat brain mitochondria, such as mitoplasts (the inner membrane with matrix) and outer membranes. As shown in Fig. 5E, CNP was found in both fractions. The fractions tested were also stained for SOD2 (marker for mitoplasts) and for VDAC (marker for outer membranes). The faint staining of mitoplasts with anti-VDAC antibody revealed small amounts of VDAC in the mitoplast fraction. This can be explained by the presence of contact sites in the mitoplasts that usually contain VDAC. SOD2 was found mainly in mitoplasts, and only a negligible trace amount of SOD2 was detected in the outer membranes. We also checked for the presence of CNP in mitochondria isolated from several other tissues, like rat liver, heart, and pancreas, as well as mitochondria isolated from C6 glioma cells. In all mitochondria isolated from these tissues and cells, CNP was detected by immunoblotting (data not shown).

**Ca$^{2+}$-dependent PT in mitochondria isolated from CNP siRNA-treated oligodendrocytes.** Next, we studied the question of whether the CNP content was related to the induction of PT. Therefore, RNA interference studies were carried out. After testing the CNP expression in different types of cultured cells (HEK293 human embryonic kidney; N2A mouse neuroblastoma, and OLN93, a rat oligodendrocyte line; Fig. 6A), we found that endogenous expression of CNP detected with the anti-CNP-antibody was highest in the OLN93 cells. OLN93 cells were chosen for transfection with siRNA targeting CNP. Nontargeting scrambled siRNA served as a control. Forty-eight hours after transfection, the level of CNP expression was assayed by Western blot. Transfection with CNP-targeting siRNA significantly reduced the CNP protein expression, as measured in total OLN93 cell lysate (Fig. 6B). Mitochondria were isolated from OLN93 wild-type cells, as well as from CNP knockdown OLN93 cells. Scrambled siRNA did not affect CNP expression. In mitochondria isolated from CNP knockdown OLN93 cells, the level of CNP was reduced to ~20% of control level (Fig. 6C). Reduction of CNP expression in mitochondria correlated with decreased enzymatic CNP activity, which was not affected in mitochondria isolated from OLN93 cells transfected with scrambled siRNA (Fig. 6D).
We also investigated whether the reduction in CNP level in mitochondria was correlated with the Ca\(^{2+}\) release ability. We measured the threshold Ca\(^{2+}\), which is the Ca\(^{2+}\) concentration determined as the amount of added Ca\(^{2+}\), leading to Ca\(^{2+}\) efflux from the matrix of mitochondria. Figure 7A shows that threshold Ca\(^{2+}\) concentration in mitochondria isolated from wild-type OLN93 cells was achieved after four additions of Ca\(^{2+}\) (70 nmol Ca\(^{2+}\)/mg protein each), giving a threshold Ca\(^{2+}\) for OLN93 mitochondria of 280 nmol/mg protein in the given experiment. A similar threshold was found in mitochondria isolated from scrambled siRNA-treated OLN93 cells (data not shown). The calcium threshold was evaluated and is presented on the basis of multiple Ca\(^{2+}\) additions. For the calculation, we used three additions (4 \times 70 nmol) or four additions (4 \times 70 nmol), and then, for the last addition by interpolation, we determined the exact amount of Ca\(^{2+}\) load that was accumulated before Ca\(^{2+}\) efflux developed. That allowed presentation of the statistical data.

Mitochondria isolated from CNP knockdown OLN93 cells released Ca\(^{2+}\) after the third addition (also 70 nmol Ca\(^{2+}\)/mg protein each), having a lower threshold calcium concentration equal to 210 nmol Ca\(^{2+}\)/mg protein (Fig. 7B). The average data on Ca\(^{2+}\)-induced Ca\(^{2+}\) efflux in mitochondria isolated from wild-type and knockdown cells are shown in Fig. 7E. No noticeable changes in \(V_{\text{Ca}}\) were observed in all kinds of isolated mitochondria. However, a reduced Ca\(^{2+}\) threshold to stimulate Ca\(^{2+}\)-induced Ca\(^{2+}\) release was found for CNP knockdown mitochondria. These results demonstrate a correlation between reduced CNP content and lowered Ca\(^{2+}\) capacity.

Then we tested the ability of 2',3'-cAMP to affect the Ca\(^{2+}\) retention in mitochondria. The effect of 2',3'-cAMP on PT induction in mitochondria isolated from wild-type OLN93 cells is demonstrated by comparison of Fig. 7, A and C, where Ca\(^{2+}\) efflux occurred already after the third Ca\(^{2+}\) addition. The presence of 2',3'-cAMP leads to further stimulation of Ca\(^{2+}\) release after Ca\(^{2+}\) overloading in CNP knockdown mitochondria, when we compare Fig. 7, B and D.

### DISCUSSION

The physiological role of 2',3'-cyclic nucleotides in biological systems is scarcely understood. 2',3'-Cyclic nucleotides and oligonucleotides containing a 2',3'-cyclic terminus are hydrolyzed by CNP to produce 2'-nucleotides. CNP was shown to be able to hydrolyze the terminal cyclic phosphate of RNA without influencing the internucleotide linkages (36, 39). Besides being a major protein in the central nervous system and highly expressed in oligodendrocytes and Schwann cells, CNP was also found outside myelin. Earlier, CNP activity was discovered in rat liver mitochondria (12) and later in mitochondria in cultured adrenal cells (27). Nevertheless, the CNP function in mitochondria has not yet been determined, and the influence of the CNP substrates on the mitochondrial function has not yet been investigated.

We found CNP in mitochondria isolated from brain, liver, heart, and from C6 glioma cells. In all mitochondrial samples tested here, CNP was visualized on Western blots stained by the highly specific monoclonal anti-CNP antibody. In our
experiments, we, for the first time, studied the effect of 2',3'-cAMP and 2',3'-cNADP and other 2',3'-cyclic nucleotides on mitochondrial functions. We found that the cyclic nucleotides did not affect Ca^{2+} transport and other functions in mitochondria having the PT pore closed, before threshold Ca^{2+} loading. However, 2',3'-cAMP and 2',3'-cNADP enhanced PT development, as seen on Ca^{2+} transport, membrane potential dissipation, and swelling of both rat brain and liver mitochondria in the presence of threshold Ca^{2+} concentrations. The cyclic nucleotides were found to be effective in the low micromolar concentration range. The effects of 2',3'-cAMP on PT development were observed even at 0.5 μM, and maximal efficiency was seen at 5 μM. 2',3'-Cyclic NADP is also a substrate for 2',3'-cyclic nucleotide 3'-phosphohydrolase (20). 2',3'-cNADP was able to shorten the lag phase before Ca^{2+} efflux and to increase the rate of Ca^{2+} release from both kinds of mitochondria, rat brain and liver mitochondria, when they were loaded with threshold Ca^{2+} concentrations. 2',3'-cGMP (Fig. 2D) and 2',3'-cCMP (not shown) were not effective in induction of threshold Ca^{2+}-induced Ca^{2+} efflux. The potency sequence in rat brain mitochondria is as follows: 2',3'-cAMP > 2',3'-cNADP >> 2',3'-cGMP, 2',3'-cCMP. Interestingly, the weak capacity of 2',3'-cCMP to activate Ca^{2+}-induced Ca^{2+} release in Ca^{2+} overloaded mitochondria is in parallel with the earlier reported lower capacity of CNP to hydrolyze 2',3'-cCMP compared with the cAMP analog (13).

The 2',3'-cAMP- and 2',3'-cNADP-induced stimulation of Ca^{2+} efflux from mitochondria and collapse of ΔΨ_{m} were CsA sensitive, confirming involvement of PT in the process. Mitochondrial PT is manifested as a sudden opening of nonelective megachannel (pore) in the inner mitochondrial membrane, which increases the permeability to solutes with molecular mass up to 1,500 Da in response to mitochondrial Ca^{2+} overload and/or oxidative stress (7, 18). PT is a complex process with many known inducers, modulators, and inhibitors. The Ca^{2+}-induced, CsA-sensitive PT system is a multiprotein complex formed in the contact sites between outer and inner membranes of mitochondria to increase permeability of the inner membrane. Main events of PT opening are collapse of the membrane potential, Ca^{2+}-induced Ca^{2+} efflux, induction of mitochondrial swelling, and release of apoptotic factors (7, 19, 43). We observed that even 500 nM 2',3'-cAMP or 2',3'-cNADP was able to accelerate the Ca^{2+}-stimulated, large-amplitude swelling of liver mitochondria that was prevented by CsA. Moreover, the CNP level was not changed in Ca^{2+}-loaded mitochondria, but the enzymatic CNP activity was decreased after Ca^{2+} threshold loading, which prevented hydrolysis of 2',3'-cAMP. Consequently, this increases the effi-
ciency of the action of 2',3'-cyclic AMP in a feedback cycle. Therefore, we propose that, in living cells, inhibition of CNP activity under threshold Ca²⁺ accumulation in mitochondria contributes to elevating the 2',3'-cAMP level. 2',3'-cAMP, in turn, seems to work as a second messenger by promoting the mitochondrial Ca²⁺ efflux and ΔΨₘ dissipation.

Functional importance of CNP in mitochondria was obtained by RNA interference experiments in OLN93 cells, which contain a high endogenous level of CNP. OLN93 cells were transfected with siRNA targeting CNP. As a result, the endogenous CNP protein expression level was reduced. Mitochondria isolated from CNP knockdown OLN93 cells possessed reduced level of CNP and decreased enzymatic CNP activity compared with mitochondria isolated from wild-type OLN93 cells. Mitochondria isolated from scrambled nontargeting siRNA OLN93 cells served as a control. Lowered level of CNP and reduced CNP activity correlated with facilitation of Ca²⁺ efflux from mitochondria. That process was further increased in the presence of added 2',3'-cAMP. These results allow us to suppose that the CNP level and activity in mitochondria are important for the regulation of PT development.

Localization of CNP in the inner and outer membranes of rat brain mitochondria was found, which is in agreement with the distribution of CNP activity in liver mitochondria (12). Such CNP localization in mitochondria indicates its possible concentration in contact sites, similar to the PT pore localization. It is difficult to determine which components of the PT pore can be targets for CNP and its substrates, since the exact protein composition of the pore is not established so far. The PT pore complex was previously considered as a complex formed by the association of adenine nucleotide translocase of the inner membrane and cyclophilin D of the matrix, the VDAC and peripheral benzodiazepine receptor localized in the outer membrane, and additional proteins, such as proteins of the Bcl-2 family, creatine kinase, and hexokinase. Recent experiments with mitochondria from adenine nucleotide translocator- and VDAC-deficient mice demonstrated that both proteins are not indispensable structural elements of the unselective pore, but they could still be considered as regulators or modulators of PT (6, 21). Since facilitation of PT development by CNP substrates in the presence of threshold Ca²⁺ concentrations was observed, and reduced CNP activity correlated with PT initiation, a regulatory role of CNP in PT pore opening might be suggested.

An important question is still, what are the sources of the CNP substrates, 2',3'-cAMP, and other cyclic nucleotides in mitochondria? Oligonucleotides containing a 2',3'-cyclic ter-

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Fig. 6. CNP level and enzymatic activity in oligodendrocyte OLN93 cells and OLN93 mitochondria. A: endogeneous CNP protein expression in different types of cultured cells was evaluated by Western blotting, as described in MATERIALS AND METHODS. Experiments were repeated three times with similar results. Twenty-five micrograms of total cell lysates were loaded. B: OLN93 cells were transfected with either 50 nM scrambled small interfering RNA (siRNA) (control siRNA) or CNP siRNAs. At 48 h after transfection, CNP in total cell lysates was determined by Western blot analysis. Representative data from three independent experiments are given, and β-tubulin I served as control for specificity of knockdown. C: OLN93 cells were transfected with either CNP siRNAs (50 nM) or scrambled siRNA (50 nM); after 48 h, mitochondria were isolated. The reduction of CNP expression in mitochondria was evaluated by Western blotting. CNP and SOD2 protein bands were quantified by densitometry. The histogram shows relative units as ratio of CNP to SOD2. Mitochondria isolated from nontransfected OLN93 cells were used as reference and assigned the value of 100%. Values represent the means ± SD from three independent experiments. *P < 0.05. D: CNP activity in mitochondria isolated from OLN93 cells, transfected with siRNA or nontransfected, was detected, as described in MATERIALS AND METHODS. The activity of CNP in mitochondria isolated from nontransfected OLN93 cells was taken as 100%. Values represent the means ± SD from three independent experiments. *P < 0.05.
minus could be generated as 2',3'-cyclic intermediate in the enzymatic degradation of RNA, as well as in processing and splicing reactions for mammalian RNA. In this relation, it is interesting to mention that RNA could be a substrate for the mitochondrial unspecific endonuclease G, which is released from the inner mitochondrial membrane during the early stage of apoptosis (26). RNA may be a natural substrate for CNP (37). Moreover, recently it was found that mammalian CNP could function as a tRNA splicing enzyme (33). In addition, it was reported that CNP is an RNA binding protein that inhibits protein synthesis (17).

On the other hand, cAMP can be synthesized in mitochondria by adenylyl cyclases. The bicarbonate-regulated, Ca²⁺-dependent soluble adenylyl cyclase was detected in isolated mitochondria (42). Although the substrate selectivity of the mitochondrial soluble adenylyl cyclase is not completely clear,

![Graphs](image-url)
we can suppose that, at the conditions of our experiments, under threshold Ca\textsuperscript{2+} load, the soluble adenyllyl cyclase was activated by Ca\textsuperscript{2+} and 3',5'-cAMP was produced. 2',3'-cAMP could be created by transformation of the 3',5'-cyclic terminus to the 2',3'-cyclic one that can occur in alkaline conditions. This conversion was shown for the 3',5'-cNADP to 2',3'-cNADP conversion, with 2',3'-cyclic NADP as substrate for 2',3'-cyclic nucleotide 3'-phosphodiesterase (35). It is well known that highly alkaline conditions are prevalent in mitochondria under threshold Ca\textsuperscript{2+} load.

In summary, the results obtained here suggest that 2',3'-cyclic nucleotides might be able to modulate PT in mitochondria, a key process in initiation of apoptotic cell death. In particular, 2',3'-cAMP might probably act as a second messenger. In addition, the intramitochondrial 2',3'-cAMP level might be elevated due to inhibition of CNP activity under threshold Ca\textsuperscript{2+} load.

**REFERENCES**


