cAMP-Dependent Phosphorylation of Microtubule-Associated Protein-2 during Treatment of Sodium Valproate and Audiogenic Kindling

T. A. Savina* and T. G. Shchipakina*,**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 7, pp. 60-64, July, 2011
Original article submitted February 16, 2010

Administration of anticonvulsant sodium valproate alleviated audiogenic seizures in Krushinskii–Molodkina rats, which was accompanied by a decrease in cAMP-dependent phosphorylation of microtubule-associated protein MAP2 in the hippocampus *ex vivo*. In contrast, audiogenic kindling resulted in a marked increase in MAP2 phosphorylation at cAMP-dependent protein kinase-specific sites. These changes in the state of MAP2 phosphorylation providing restructuring of dendrites in response to specific influences modulate neuronal activity and are the important mechanisms of neuronal plasticity.

Key Words: *sodium valproate; neuronal hyperexcitability; MAP2, phosphorylation*

Valproic acid and its salts are traditionally used in neurological practice for more than 40 years are highly effective in the therapy of generalized and partial epilepsy, bipolar disorder, and migraines [4,10]. It was accepted for a long time that anticonvulsant sodium valproate increases the content of inhibitory neurotransmitter GABA in the brain and inhibits generation of action potentials by neurons upon binding with potential-dependent Na^+-channels [9]. Over the last decade, new neurochemical targets of this drug were identified: extracellular signal-regulated protein kinase (ERK) cascade, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, metabolic pathways of arachidonic acid and inositol [12,15]. Valproic acid and its metabolites also interact with tubulin and microtubule-associated MAP proteins, thereby inhibiting processes of microtubule formation *in vitro* [5].

Microtubule-associated protein 2 (MAP2) is a cytoskeletal phosphoprotein. Its macromolecular isoforms (MAP2A and MAP2B, 270-280 kDa) are localized in the body and dendrites of neurons. About a third of cytosolic cAMP-dependent protein kinase (PKA) is anchored to MAP2 and phosphorylates from 8-10 to 20 (according to different sources) serine and threonine residues in the protein molecule [14]. MAP2 phosphorylation in PKA-specific sites protects the protein from calpain proteolysis, reduces its binding to tubulin and neurofilaments, and increases the rate of tubulin polymer dissociation [14]. Thus, modification of MAP2 phosphorylation state can cause significant morphological changes in neuronal cytoskeleton, remodeling of dendrites and, consequently, functional changes in synaptic transmission. We hypothesized that epileptogenesis and, on the contrary, administration of anticonvulsant sodium valproate elevating the seizure threshold will have different impacts on cAMP-dependent endogenous phosphorylation of MAP2 in the brain.

MATERIALS AND METHODS

Experiments were performed on 3-month-old Krushinskii–Molodkina (KM) rats with hereditary predisposition to audiogenic seizures. The animals were maintained under standard conditions at 12:12-h light/dark cycle and had free access to water and food. In experi-

*Laboratory of Experimental Neurobiology, Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences; **Pushchino State University, Russia. ***Address for correspondence:*** neurochem@mail.ru. T. A. Savina

0007-4888/11/1521-0053

mental series I, the rats were injected intraperitoneally with sodium valproate (200 mg/kg) controls received 0.9% NaCl. One hour after injection, seizures were evoked by acoustic stimulation (80 dB, 12-15 kHz). Seizures severity was scored using a conditional scale: 0 – no response; 1 – clonic running; 2 – clonic seizures in a prone position; 3 – clonic seizures of limbs on one side; 4 – tonic seizures of forelimbs, hindlimb clonus; 5 – tonic seizure of fore- and hind limbs. In experimental series II, the rats were injected with sodium valproate (controls received 0.9% NaCl); after 1 h, the animals were decapitated without acoustic stimulation. In series III, the rats of the experimental group were subjected to chronic 20-fold acoustic stimulation (audiogenic kindling, seizures 1 time per day, 6 days per week). Control group consisted of intact animals. Biochemical studies were performed 2 days after the last seizure. All the animals were decapitated under light ether anesthesia. Sensorimotor cortex and hippocampus were isolated and stored in liquid nitrogen until use. In series I and II, each group consisted of 5 rats and in series III of 7 rats.

The tissue was homogenized at 4°C in a buffer containing 50 mM Hepes-NaOH pH 7.0, 10 mM EGTA, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 100 mM β-glycerophosphate, and a mixture of protease inhibitors (10 mM benzamidin, 100 ng/ml aprotinin, and 100 ng/ml leupeptin). MAP2 phosphorylation and PKA activity were determined in the same protein sample. Phosphorylation of MAP2 was carried out at 30°C in a final volume of 50 μl containing 50 μg protein, 50 mM Hepes-NaOH pH 7.0, 1 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM $Na_4P_2O_7$, 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 μM okadaic acid, 2.5 mM NaF, 10 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, specified protease inhibitors, 10 mM $MgCl_2$, 20 μM ATP (3 μCi γ[^{32}P]ATP/sample), and 100 μM cAMP. Phosphoproteins were separated in 6-12% SDS-PAGE followed by autoradiography of gels on Kodak X-OMAT/AR5 film. Incorporation of ^{32}P into MAP2 was estimated by densitometry. PKA activity was measured using peptide substrate of protein kinase kemptide in a final volume of 25 μl containing 2 μg protein, 50 mM Hepes-NaOH pH 7.0, 1 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM $Na_4P_2O_7$, 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 μM okadaic acid, 2.5 mM NaF, 10 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, protease inhibitors, 1 mg/ml BSA, 10 mM $MgCl_2$, 120 μM ATP (1.4 μCi γ[^{32}P]ATP/sample), 100 μM cAMP, and 100 μM kemptide. After 3-min incubation at 30°C, the reaction was stopped by adding 10 μl solution containing 450 mM H_3PO_4, and 1.8 mM ATP. Aliquots from each sample applied to a Whatman P-81 phosphocellulose filters and washed in 75 mM H_3PO_4 at room temperature (2×40 min, 20 ml/filter). Incorporation of ^{32}P incorporation in kemptide ^{32}P was determined by the method of Cherenkov [2] using Beckman LS-650 liquid scintillation counter (Beckman Instruments). PKA activity was expressed in pmol ^{32}P/min×μg protein.

The significance of the results was assessed by ANOVA and Student's *t* test. Differences between the groups were considered significant at $p<0.05$. The data are presented as mean±standard error of the mean. γ[^{32}P]ATP was from V. A. Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, all other reagents were from Sigma-Aldrich.

RESULTS

MAP2 is phosphorylated in neurons by cAMP-dependent protein kinase at sites localized in tubulin-binding and projection domains of the protein. The degree of MAP2 phosphorylation is determined by the balance between activities of protein kinase and cytosolic protein phosphatases (PP1, and PP2A, and PP2B), which is regulated through activation of metabotropic and NMDA-glutamate receptor subtypes [11].

A single systemic administration of sodium valproate (200 mg/kg) to rats significantly reduced the severity of audiogenic seizures 60 min after injection (Table 1). However, 60% experimental animals exhibited two clonic running phases in the convulsive seizure, which indicates activation of the inhibitory processes in the hippocampus [3,13]. In the control group, biphasic running was not detected. Seizure facilitation after administration of sodium valproate was accompanied by the reduction of cAMP-dependent MAP2 phosphorylation *ex vivo* only in the hippocampus of the experimental group animals (100±8.92 and 67.83±8.40% relatively, $p<0.01$; Fig. 1, *a*). Structure-dependent effect of single injection of this anticonvulsant was previously reported. The level of GABA in rat midbrain significantly increased 5 minutes after

TABLE 1. Seizure Parameters in Krushinskii–Molodkina Rats 1 h after Intraperitoneal Injection of Sodium Valproate in a Dose of 200 mg/kg (*M±m*)

Parameter of seizures	Control (*n*=5)	Injection of sodium valproate (*n*=5)
Latency of stage 1, sec	1.2±0.2	4.4±0.5**
Latency of stage 2, sec	4.6±0.3	32.6±7.4**
Severity score	5.0±0.0	2.2±0.2**

Note. **$p<0.01$ in comparison with the control.

administration of sodium valproate [9]. The content of phosphorylated forms of the transcription factor CREB selectively increased in rat amygdala and nucleus accumbens 60 min after administration of the anticonvulsant [6]. Changes in phosphorylation state of MAP2 caused by audiogenic kindling inducing limbic epileptogenesis differed from those caused by sodium valproate administration (Fig. 1, *b*). A significant increase in MAP2 phosphorylation both in the hippocampus (100±5.19% in controls and 271.23±22.97% after kindling, $p<0.01$) and in the cortex of KM rats (100±6.28 and 184.73±14.74%, respectively, $p<0.01$) was shown. Based on published data, the observed changes in MAP2 phosphorylation state *ex vivo* can be determined by changes in brain MAP2 content and number of sites on the MAP2 molecule available for phosphorylation, which depends on the balance of PKA and protein phosphatase activities.

Audiogenic kindling does not change MAP2 content in the hippocampus of KM rats [1]. The study of intracellular signaling pathways regulating gene expression showed that not only chronic, but also single injection of valproate modulates the levels of transcription factors [6,7]. However, there are no published reports on modulation of MAP2 synthesis in neurons under these conditions. The observed changes in cAMP-dependent endogenous MAP2 phosphorylation could be explained by changes in PKA activity: decrease after administration of sodium valproate or increase as a result of audiogenic kindling. *In vitro* sodium valproate in concentrations 10^{-7}-10^{-3} M had no effect on activity of protein kinase (Fig. 2, *a*). Measurements of PKA activity *ex vivo* in the presence of exogenous cAMP revealed no effects of sodium valproate or kindling on enzyme activity in the studied brain structures (Fig. 2, *b, c*), although the degree of

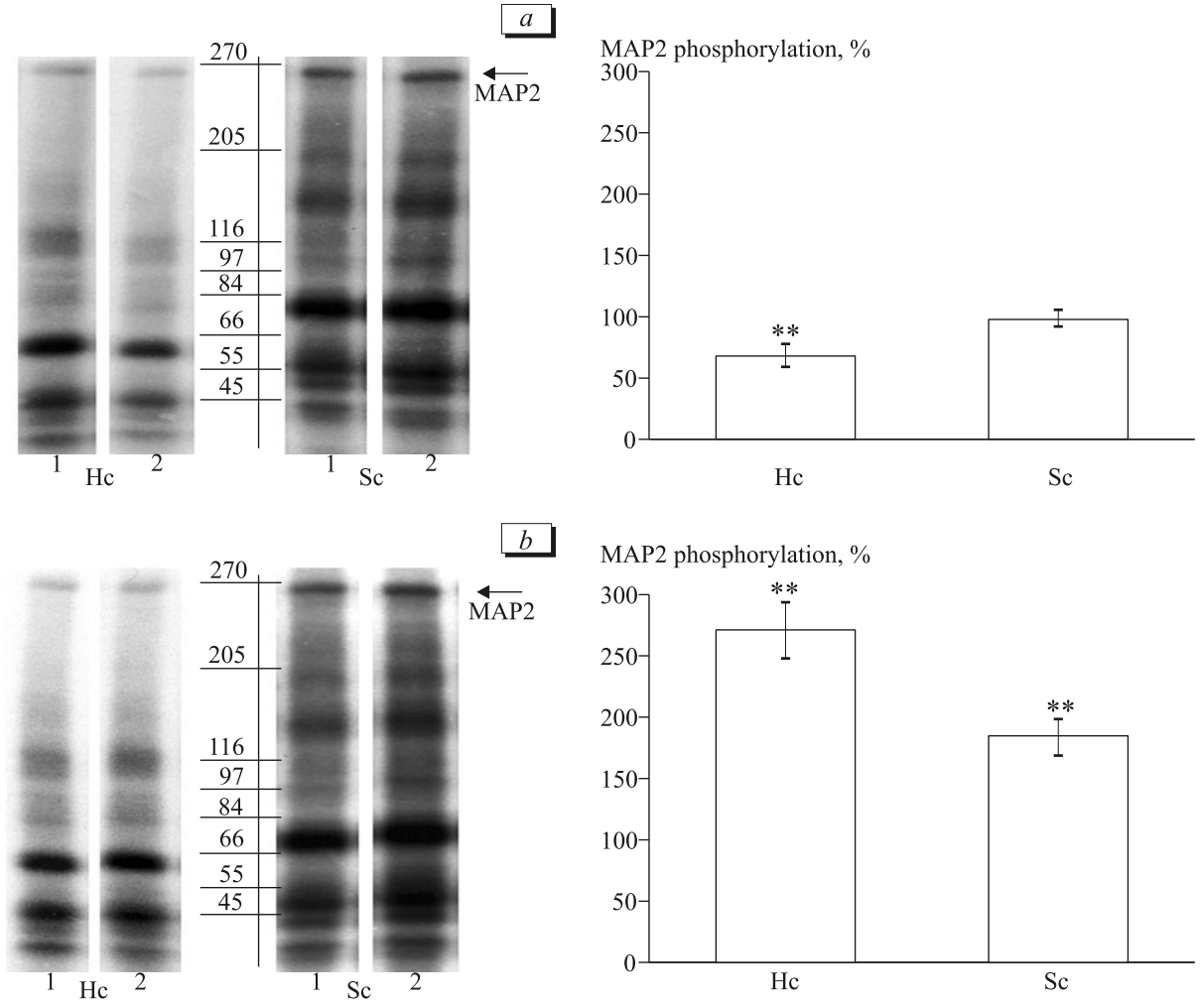

Fig. 1. cAMP-dependent MAP2 phosphorylation in the hippocampus and cortex of KM rats. *a*) MAP2 phosphorylation 1 h after administration of sodium valproate; *b*) day 2 after audiogenic kindling. Control was considered as 100%. Radioautograph of gels after separation of proteins from rat hippocampus and cortex homogenates in 6-12% SDS-PAGE and position of marker proteins in the gel are shown. Hc, hippocampus, Sc, sensorimotor cortex. *1*) control, *2*) experiment (administration of sodium valproate or audiogenic kindling, respectively). **$p<0.01$ in comparison with the control.

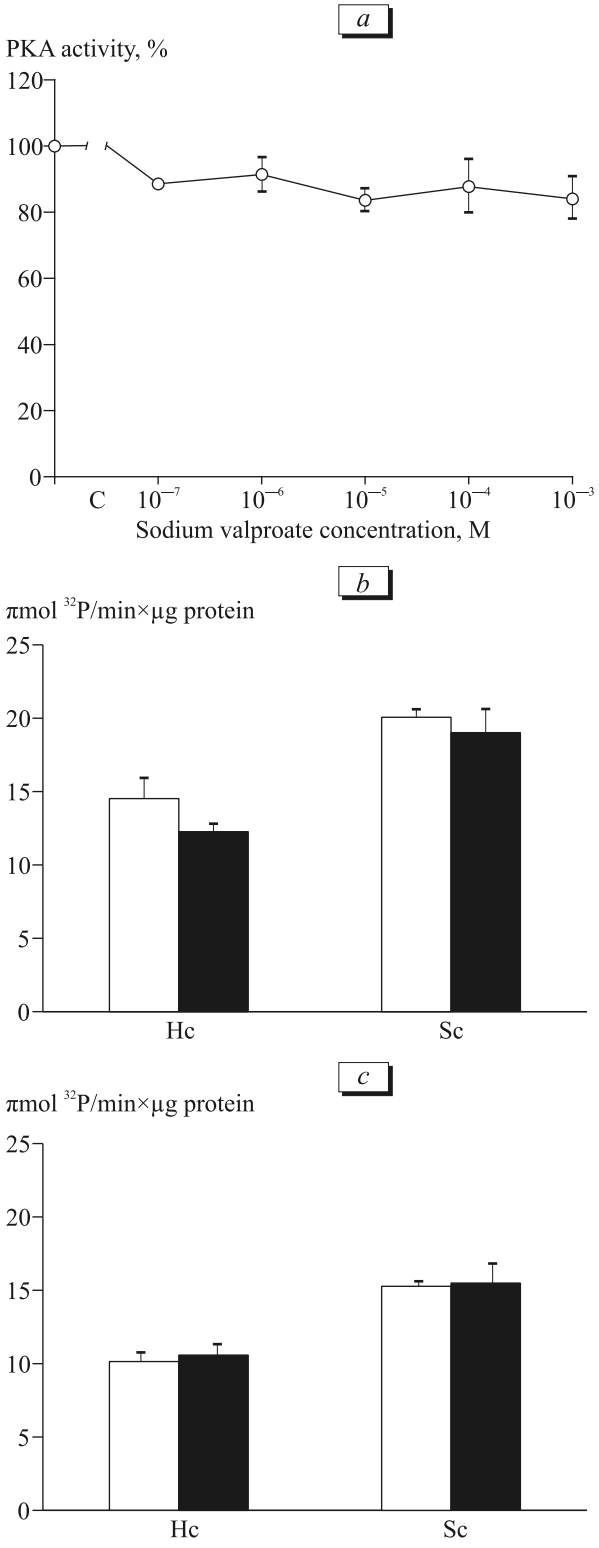

Fig. 2. Effect of sodium valproate and audiogenic kindling on activity of cAMP-dependent protein kinase. *a*) effect of sodium valproate on PKA activity *in vitro*. Control was considered as 100%. Sodium valproate was added in concentrations of 10^{-7}-10^{-3} M during incubation before adding cAMP, ATP and kemptide. Reaction medium contained no BSA. *b*) PKA activity *ex vivo* 1 h after administration of sodium valproate (black bars); *c*) PKA activity 2 days after audiogenic kidling (black bars). Light bars, control group.

PKA activation *in vivo* could vary depending on cAMP level in cells. It is known that PKA molecule is an inactive tetramer consisting of two catalytic and two regulatory subunits; cAMP binding to enzyme regulatory subunits activates catalytic subunits of protein kinase. It was shown that amygdala kindling caused long-term increase in the content of RNA for type II adenylate cyclase in the hippocampus, an enzyme regulating the level of cAMP in the cell [8].

Thus, seizure parameters in KM rats decreased after administration of sodium valproate, which was accompanied by attenuation of cAMP-dependent MAP2 phosphorylation in the hippocampus *ex vivo*. This allows us to consider the neuronal cytoskeleton as a potential therapeutic target for psychotropic drugs. In contrast, audiogenic kindling resulted in a marked increase in MAP2 phosphorylation at PKA specific sites. We assume that administration of sodium valproate and audiogenic kindling set a new balance between activities of PKA and phosphatases in the microtubular compartments of the hippocampal and cortical dendrites *in vivo*, which determines the state of MAP2 phosphorylation characteristic of reduced neuronal activity or neuronal hyperexcitability, respectively. It can be concluded that the change in MAP2 phosphorylation state providing restructuring of dendrites in response to specific impact modulates neuronal activity and is one of the major mechanisms of neuronal plasticity.

This work was supported by Russian Foundation for Basic Research (grand No. 09-04-01254), analytical departmental special-purpose program "Development of Scientific Potential of Higher Education" Ministry of Education and Science (Project No. 2.1.1/3876) and Federal Program "Scientific and Scientific-Pedagogical Cadres of Innovative Russia" 2009-2013 (contract No. P610).

REFERENCES

1. E. N. Bezgina, D. A. Moshkov, and S. N. Echikov, *Tsitologia*, **45**, No. 10, 1005-1012 (2003).
2. L. A. Osterman, *The Study of Biological Macromolecules Electrofocussing, Immunoelectrophoresis, and Radioisotope Method* [in Russian], Moscow (1983).
3. A. F. Semiokhina, I. B. Fedotova, and I. I. Poletaeva, *Zh. Vyssh. Nervn. Dyeyat.*, **56**, No. 3, 298-316 (2006).
4. G. G. Shanko, E. H. Ivashina, and N. M. Charuhina, *Med. Novosti*, **11**, 522-526 (2001).
5. G. R. Cannell, M. J. Bailey, and R. G. Dickinson, *Life Sci.*, **71**, No. 22, 2633-2643 (2002).
6. M. A. Casu, A. Sanna, G. P. Spada, *et al.*, *Brain Res.*, **1141**, 15-24 (2007).
7. M. Fukuchi, T. Nii, N. Ishimaru, *et al.*, *Neurosci. Res.* **65**, No. 1, 35-43 (2009).
8. H. Iwasa, S. Kikuchi, S. Mine, *et al.*, *Neurosci. Lett.*, **282**, No. 3, 173-176 (2000).

9. C. U. Johannessen, *Neurochem Int*., **37**, Nos. 2-3, 103-110 (2000).
10. G. M. Peterson and M. Naunton, *J. Clin. Pharm. Ther*., **30**, No. 5, 417-421 (2005).
11. E. M. Quinlan and S. Halpain, *Neuron*, **16**, No. 2, 357-368 (1996).
12. G. Rosenberg, *Cell. Mol. Life Sci*., **64**, No. 16, 2090-2103 (2007).
13. K. C. Ross and J. R. Coleman, *Neurosci. Biobehav. Rev*., **24**, No. 6, 639-653 (2000).
14. C. Sanchez, J. Diaz-Nido, and J. Avila, *Prog. Neurobiol*., **61**, No. 2, 133-168 (2000).
15. T. A. Savina, O. A. Balashova, and T. G. Shchipakina, *Neurosci. Behav. Physiol*., **38**, No. 1, 99-102 (2008).