Transient activation of protein phosphatase 2A induced by electroconvulsive shock in the rat frontal cortex

Ung Gu Kang a, Won Je Jeon b, Yeni Kim c, Chun Kee Chung c, Joo Bae Park d, Yong Sung Juhnn e, Yong Sik Kim a, *

a Department of Psychiatry & Behavioral Science, Seoul National University College of Medicine, Seoul, South Korea
b Clinical Research Institute, Seoul National University Hospital, Seoul, South Korea
c Department of Neurosurgery, Institute of Human Behavioral Medicine, Seoul National University College of Medicine, Seoul, South Korea
d Department of Molecular Cell Biology and Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, South Korea
e Department of Biochemistry, Institute of Human Behavioral Medicine, Seoul National University College of Medicine, Seoul, South Korea

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Abstract

We have attempted to determine the effects of electroconvulsive shock (ECS) on protein phosphatase 2A (PP2A) in the frontal cortices of rats. PP2A exhibited a 30% increase in activity immediately after ECS treatment. Immunoblot analysis revealed that phosphorylation signals, including protein kinase B (Akt/PKB), glycogen synthase kinase-3β (GSK-3β), and cyclic adenosine monophosphate response element binding protein (CREB) were reduced immediately after ECS treatment. When an additional ECS was administered after the activation of these kinases, the immediate reactivation of PP2A overrode the kinase activity. ECS induces transient PP2A activation prior to kinase activation, and this pattern of activity may induce the biphasic phosphorylation of substrate proteins.

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Protein phosphorylation is one of the most important regulatory mechanisms in cellular signal transduction, and is known to be regulated by protein kinases and phosphatases. A host of studies have been conducted regarding the regulation of protein kinase activities within the brain. However, studies on phosphatases have been relatively overlooked. This bias reflects the conception that kinases do the active work, and that phosphatases function merely as “street sweepers and janitors” [19]. However, it has become apparent that phosphatases do, indeed, play active roles in the regulation of cellular functions in neurons, for example, in synaptic plasticity [19]. During a study of kinase activity and protein phosphorylation after ECS, using an animal electroconvulsive therapy model, we determined that certain phosphoproteins tend to be dephosphorylated immediately after the administration of ECS [11,14]. These findings caused us to suspect that the initial event in ECS-induced phosphorylation signaling may involve the activation of protein phosphatases, specifically the serine/threonine phosphatases.

Protein serine/threonine phosphatases are divided into two major classes. The PPP class phosphatases, which include PP1, PP2A, PP2B, PP4, PP5, PP6, and PP7, have been shown to share a common phosphatase domain, whereas the PPM class, which includes PP2C, exhibits only minor sequence homology with the members of the PPP class. Among these phosphatases, PP2A is the most abundant soluble serine/threonine phosphatase in animal cells. PP2A is known to affect primary signaling molecules, including mitogen-activated protein kinase (MAPK), β-catenin, and CREB [8]. PP2A is comprised of one core enzyme and a variety of associated regulatory subunits. The core enzyme itself is comprised of both catalytic and scaffolding subunits. The regulatory subunits are cell- and tissue-specific, and act as targeting molecules for the core enzyme. In the brain, three types of regulatory subunits are expressed, but...
their subcellular distributions differ from one another [17]. This would appear to suggest that they play roles in the specific trafficking of core subunits. The manner in which PP2A activity is regulated remains somewhat obscure. Although total cellular PP2A activity is not significantly altered upon physiological stimulation or cell-cycle progression [15], specific PP2A subpopulations may manifest activity regulation, according to the type of stimulus applied [16]. The tyrosine phosphorylation of the catalytic subunit C-terminal has been shown to reduce the activity of PP2A [2]. It has also been suggested that PP2A is locally regulated, upon mobilization with the regulatory subunits [5,7]. It can also be suggested that the signaling events which activate calcium signaling and kinase activation, including electroconvulsive shock (ECS), also affect the activity of serine/threonine phosphatases. In this report, we have evaluated the activity of PP2A after ECS treatment, and have attempted to determine whether PP2A activity after ECS treatment can be correlated with protein serine/threonine phosphorylation status, and with the phosphorylation status of Akt, GSK, and CREB, after ECS treatment.

Male Sprague-Dawley rats were used in this study. The individual time group contained either three or six animals. The animals were uniformly treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. ECS (Medcraft B 24-III, 130 V, 0.5 s) was administered via a set of ear-clip electrodes. Rats were decapitated 0–5 min after the administration of either a single or two consecutive ECS; in the latter case, the second ECS was administered 5 min after the first ECS. Some proteins were not visibly phosphorylated in the baseline state, and the phospho-antibodies used were applied either immediately after the administration of ECS or 5 min after the second ECS; sham levels. However, when an additional ECS was administered, 5 min after the first ECS, the PP2A activity increased more than 90% above sham levels. Therefore, our experimental paradigm involved two consecutive ECS administrations, separated by a period of 5 min. This paradigm also enabled us to determine whether the increased phosphatase activity induced by ECS can override the observed increases in kinase activity.

PP2A activity was determined with a molybdate dye-based phosphatase assay kit (Cat. No. V2-460, Promega, Madison, WI). However, because the substrate in this kit, RRAlpTVa, is a poor substrate for PP1, we presumed that the activity we measured had originated from the PP2 phosphatase. The specificity of the PP2A activity was evaluated via collagenous assays, using okadaic acid (5 μM) as a specific PP2A inhibitor. The IC50 for PP2A of okadaic acid was known to be 100 μM [3]. Sections of the frontal cortex were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, Complete Mini Protease Inhibitor Cocktail (Roche, Diagnostics, Switzerland)) on ice. These homogenates were applied to Sephadex G-25 columns, in order to remove free phosphate. The samples were then applied to a reaction premix which contained phosphoprotein substrate, 5 μM PP2A reaction buffer (250 mM imidazole (pH 7.2), 1 mM EGTA, 0.1% β-mercaptoethanol, 0.5 mg/ml BSA) and storage buffer, on 96-well plates. After 15 min of incubation at room temperature, the reactions were discontinued via the addition of a molybdate dye/additive mixture. The optical densities of the samples were assessed using a plate reader, fitted with a 630 nm filter. Statistical tests were then conducted using ANOVA, followed by a series of post hoc tests. The level of significance in this study was set at a p value of 0.05.

Rat frontal cortices were homogenized in 10 v/w of ice-cold homogenization buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.2% Nonidet P-40, 4 mM EGTA, 10 mM EDTA, 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, Complete Mini Protease Inhibitor Cocktail (Roche, Diagnostics, Switzerland), 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). These homogenates were then centrifuged, and the supernatants were boiled in Laemmli’s sample buffer. The samples were then separated via SDS-PAGE, and immunoblotted with antibodies against phospho-serine/threonine (BD, San Diego, CA), Akt, phospho-Akt (Ser473), GSK-3β, phospho-GSK-3β (Ser9), CREB, or phospho-CREB (Ser133) (Cell Signaling Technology, Beverly, MA) antibodies.

We observed immediate increase in the activity of PP2A after the administration of ECS. This increase in activity was detected in the samples harvested during the active seizures (E0, i.e. 0 min after ECS and E5/E0, i.e. 0 min after the second ECS), and our statistical analysis also revealed significant temporal effects (n = 6, ANOVA, F = 13.349, p = 0.000). We noted a 32% change from the sham condition at E0. At 2 min after ECS, approximately 90 s after the termination of the seizure, phosphatase activity had already begun to decrease, and at 5 min after ECS, the level of activity was approaching sham levels. However, when an additional ECS was administered, 5 min after the first ECS, the PP2A activity increased again immediately. Post hoc analysis revealed significant differences between the Sham and E0 levels, between E0 and E5 (5 min after the first ECS) levels, and between E5 and E5/E0 levels. Although not statistically significant, we did note a tendency toward lower peak activities after the second ECS treatment (Fig. 1A). In order to confirm the PP2A activity, we prepared a separate sample set (sham and E0; n = 3) and attempted to determine whether or not PP2A activity could be inhibited by addition of okadaic acid to the assay buffer. As is shown in Fig. 1B, phosphatase activity was again shown to have increased, immediately after the administration of ECS. The addition of 5 μM of okadaic acid also was shown to considerably attenuate phosphatase activity levels, and we noted no significant differences in OA-insensitive activity between the sham and E0 samples.

Immunoblot analysis, using phospho-specific serine/threonine antibodies indicated that, in the rat frontal cor-
The phosphorylation signals were affected by ECS. The most remarkable finding was an immediate drop in multiple signals, which occurred immediately after the first and the second ECS. This suggests that, although the observed ECS-induced phosphatase activations were transient and not so robust, they may have had significant functional consequences (Fig. 2). Among the many substrates in the PP2A phosphatase family, we selected Akt, GSK-3β, and CREB, and analyzed the phosphorylations of all of them. GSK-3β and CREB were determined to have been dephosphorylated under conditions in which PP2A activities were elevated (Fig. 3B and C), and subsequently the phosphorylations of these two were elevated above sham levels at 5 min after stimulation. The phosphorylation of Akt, which is located upstream of GSK-3β, was also affected by ECS. In our stimulation paradigm, Akt appeared to be a substrate of PP2A, and Akt phosphorylation levels fell when PP2A activity was elevated (Fig. 3A). However, Akt phosphorylation levels increased above sham levels thereafter, which suggests that Akt had been subsequently activated. Maximal phosphorylation levels (activation) were observed only after the phosphatases had achieved peak activity.

ECS elicited transient increases in PP2A activity, beginning immediately after stimulation. This effect was quite transient, and was followed soon thereafter by the activation of multi-purpose kinases. However, when we processed the samples immediately after the administration of ECS (animals were decapitated during active seizures), we noted an obvious increase in phosphatase activity. When a second ECS was administered 5 min after the initial ECS, we noted

**Fig. 1.** PP2A activity after ECS, and the effects of okadaic acid on PP2A activity in the rat frontal cortex. (A) PP2A activity was measured at 0–5 min after ECS, and at 0–5 min after a second ECS session, which was administered 5 min after the initial ECS. Transient and immediate PP2A activation was evident, and our statistical analyses revealed significant differences between Sham and E0, E0 and E5, and between E5 and E5/E0. The data are expressed as the mean ± S.E.M. of six rats. *p < 0.05 compared with the sham group, and **p < 0.05 compared with the E5 group (one-way ANOVA with post hoc test). (B) A separate set of samples was prepared for the sham and E0 conditions (n = 3), and our PP2A assay was conducted in both the absence and presence of okadaic acid (OA, 5 μM). We noted no differences in OA-insensitive activities between the sham and ECS groups. Sham, sham treated; E0, immediately after the first ECS; E2, 2 min after the first ECS; E5, 5 min after the first ECS; E5/E0, immediately after a second ECS administered 5 min after the first ECS; E5/E2, 2 min after a second ECS.

**Fig. 2.** The effects of ECS on the protein serine/threonine phosphorylation status of rat brain extracts. The frontal cortical homogenates were analyzed via SDS-PAGE, and immunoblotted with an antibody which detects phosphorylated serine/threonine protein residues. We noted multiple dephosphorylation signals immediately after the first and the second ECS (E0, E5/E0, respectively). Arrows indicate bands which were apparently regulated via ECS. The same membranes were reprobed with anti-β-actin, as a protein loading control. E5/E5, 5 min after a second ECS.

**Fig. 3.** The dephosphorylation of some key signaling molecules after the administration of ECS in the rat frontal cortex. The frontal cortical homogenates were analyzed via SDS-PAGE, and were immunoblotted with anti-phospho-Akt, anti-phospho-GSK-3β, or anti-phospho-CREB. Immediate dephosphorylation of both (A) Akt, (B) GSK-3β, and (C) CREB were observed after the administration of ECS. Phosphorylation status was shown to have recovered to basal level or above at 5 min after the administration of ECS, but after the administration of the second ECS, both proteins exhibited immediate dephosphorylation.
The inhibition of phosphatase inhibitors [13]. However, this late the activity of other phosphatases, including PP1, via known to activate calcium signaling. PP2B can also regu-

generally not well understood. Theoretically, it is possible by ECS remains somewhat unclear, and PP2A regulation is consequently activated kinases probably compensated for the returned to basal level within 5 min, and many of the sub-

an immediate phosphatase reactivation, which overrode the kinase activation and increased phosphorylation induced by the first ECS session (Figs. 1–3). This suggested that the phosphatases can act as “stronger” signals than the kinases. We also noted a comparable pattern of reduction in protein phosphorylation after pentylenetetrazol (PTZ)-induced brief seizures [4]. The increased phosphatase activity levels returned to basal level within 5 min, and many of the subsequently activated kinases probably compensated for the previous dephosphorylations.

The mechanism by which PP2A is immediately regulated by ECS remains somewhat unclear, and PP2A regulation is generally not well understood. Theoretically, it is possible that PP2B is activated by ECS, primarily because ECS is known to activate calcium signaling. PP2B can also regu-
late the activity of other phosphatases, including PP1, via the inhibition of phosphatase inhibitors [13]. However, this type of regulation has not established to occur in conjunction with PP2A, and in our experiment, we noted no increases in OA-insensitive phosphatase activity levels, even under conditions in which calmodulin and calcium were present (data not shown).

The patterns of PP2A and kinase activations described above should result in a biphasic phosphorylation profile for substrate proteins, as is diagrammatically shown in Fig. 4. In order to assess this hypothesis, we initially attempted to determine whether PP2A activity after ECS could be correlated with protein serine/threonine phosphorylation status in tissue extracts. We determined that phosphorylation did, indeed, exhibit a biphasic profile, as had been previously hypothesized (Fig. 2). We then attempted to evaluate the phosphorylation status of Akt, GSK and CREB, all potential PP2A substrates, and all of which are also considered important in the therapeutic mechanisms of ECS [9,10,14]. We found that the phosphorylation status of these molecules exhibited the same biphasic pattern, which was also corre-

lated with the patterns of change observed in association with PP2A activity (Fig. 3). An identical pattern of phospho-

phorylation after ECS was also reported for synapsin 1 [20]. Also, KCN-induced depolarization has been shown to induce an undulating pattern of PI3K/Akt and GSK-3β phosphory-

lation, and PP2A has been shown to be involved in GSK-3β (Ser9) dephosphorylation, after KCN-induced depolarization in the SH-SYSY human neuroblastoma cells [12].

For many functional proteins, activation is associated with an increase in phosphorylation, and transient dephosphoryla-

tion results in a transient “turning-off” of the signaling switch, which may not significantly alter the total amount of down-

stream signaling molecules generated within the time scale of cellular homeostatic response. However, in some proteins, such as GSK-3β [18] and synapsins [6], activities can be “turned on” as the result of dephosphorylation, and thus trans-

ient dephosphorylation may result in a generation of new downstream signaling molecules or events which did not exist prior to the administration of the dephosphorylation-eliciting stimuli. In this case, such a transient dephosphorylation would transmit signals downstream.

Considering the huge amounts of extant PP2A and its con-

stitutively high activity in the cells, phosphatase activity may require a transient deactivation in order that the phosphoryla-

tion signal can be effectively activated. It has been suggested that the transient inhibition of phosphatase might be a pre-

requisite for MAPK or p34cdc2 signaling [1] and that the activity of PP2A can be downregulated via phosphorylation [2]. According to these propositions, the sequence of events would be that, initially, kinase is activated in order to tran-

siently deactivate the PP2A, and this in turn amplifies the phosphorylation signal. However, in our ECS experiment, the factor which changed immediately was phosphatase activity, and the direction of that change was contrary to the above expectation. This suggests that the phosphatases do not func-

tion merely as “street sweepers and janitors”, but are, in fact, active protagonists [19].

In short, we have determined that ECS causes transient PP2A activation prior to the activation of protein kinase. This pattern of activity induces a biphasic regulation of substrate protein phosphorylation. However, the functional signifi-

cance of this early activation of phosphatases, and the mech-

anisms underlying its occurrence, should be investigated fur-

ther in future studies. Especially with regard to the therapeutic effects of electroconvulsive therapy, phosphatase activities in the chronic ECS paradigm clearly warrant further study.

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