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The effect of systemic injection of cyclosporin A on the phosphorylation of the PKC substrates MARCKS and GAP43 in the rat hippocampus

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ABSTRACT

Cyclosporin A (CsA) is an inhibitor of calcineurin, a calcium/calmodulin dependent serine/threonine phosphatase. Protein kinase C (PKC) is a family of serine/threonine kinases. Both calcineurin and PKC are implicated in psychiatric diseases and the therapeutic mechanisms of treatment agents. It has been reported that calcineurin interacts with components of PKC signaling pathways. We administrated 50 mg/kg CsA into rats by intraperitoneal injection and examined the acute effect of single systemic CsA on the locomotor activity of rats and the phosphorylation of PKC and its substrates GAP43 and MARCKS. Systemic CsA increased locomotor activity beginning 1 h after injection. The immunoreactivity of p-MARCKS(S152/156) was higher in the CsA group 1 h after injection, whereas p-GAP43(S41) immunoreactivity was increased by CsA after 5 h. The immunoreactivity of p-PKC pan was increased by CsA at both 1 and 5 h after administration. Our data suggest that activation of the PKC pathway might be related to CsA-induced hyperlocomotion.

Cyclosporin A (CsA) is a calcineurin inhibitor prescribed as an immunosuppressant. Calcineurin, also known as protein phosphatase2B (PP2B), is a serine/threonine phosphatase activated by calcium and calmodulin and is a heterodimer of catalytic subunit A (CN A) and regulatory subunit B (CN B). In mammals, there are three isoforms of CN A (α, β, γ) and two of CN B (1, 2) [14,36,37,46]. Calcineurin is present at notably high levels in the brain, and some studies have indicated that it may account for 1% of the total protein content of the brain [36]. Calcineurin regulates basic neuronal functions including excitability [46], G protein-mediated inhibition of calcium channels [47], and glutamatergic neurotransmission [43], which in turn functionally modulates synaptic plasticity.

There is accumulating evidence that calcineurin activity is altered in psychiatric diseases. The knockout of forebrain-specific conditional CN B1 in mice resulted in behavioral abnormalities similar to symptoms of schizophrenia, such as increased open field locomotor activity and reduced social interaction [24]. Expression of CN A subunit mRNA was found to be lower in the postmortem brains of patients who suffered from schizophrenia [13]. It has also been reported that schizophrenia is associated with the genetic variation of CN Aγ [14]. Furthermore, antipsychotics and antidepressants can alter the expression and activity of calcineurin [31,40].

It had been thought that CsA could not cross the blood–brain barrier [20]. However, patients who received CsA suffered from neuropsychological side effects such as hallucination, and delusion [4,15,44]. It has also been reported that systemic CsA administration can alter the behavior of normal animals [2,7,32]. Previously, we showed that the systemic administration of 50 mg/kg CsA altered the phosphorylation status of amphiphysin II, a known substrate of CN, in the cerebellum 5 h after injection [19]. Moreover, Sato et al. [32] as well as Serkova et al. [35] directly demonstrated that a single intraperitoneal (i.p.) injection of CsA increased the concentration of CsA in mouse brain tissue in both time- and dose-dependent manners.

Protein kinase C (PKC) is a family of serine/threonine kinases that is highly expressed in the brain and controls neurotransmitter release, synaptic plasticity, learning, and memory [1,17,26,28]. It has been suggested that PKC is involved in the pathophysiology of psychiatric disorders [9,12,23,28,38,45]. The activity of PKC is altered by treatments for various psychiatric diseases, including lithium, fluoxetine, imipramine, haloperidol, and clozapine, as well as psychostimulants such as amphetamine [8,11,18,29].

It has been reported that calcineurin can reverse the action of PKC. PKC phosphorylates growth-associated protein43 (GAP43) at

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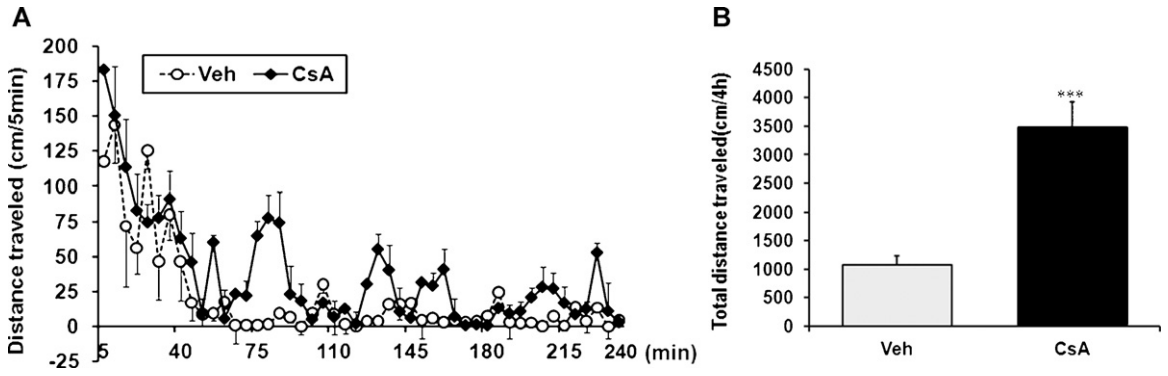

Fig. 1. Effect of CsA administration on the locomotor activity of rats. A single i.p. injection of 50 mg/kg CsA induced hyperlocomotion in rats. Locomotor activity was measured for 4 h after injection. The locomotor activity of CsA-injected rats started to differ from that of vehicle-treated rats beginning 1 h after injection. (A) Time course graph. Each dot represents the distance traveled every 5 min. (B) Total distance traveled after 4 h. Data are shown as means ± standard error. *N* for each group = 10. ***$P < 0.001$. Veh: vehicle, CsA: cyclosporin A.

Ser41 and myristoylated alanine-rich C kinase substrate (MARCKS) at Ser152/156 [25,28,41,42]. It has been shown that calcineurin dephosphorylates GAP43 and MARCKS at PKC target residues. [34] Furthermore, clozapine, which affects PKC signaling pathways [18], can attenuate CsA-induced behavioral abnormalities [32].

It is believed that primary function of hippocampus is learning and memory. However, there is accumulating evidence demonstrating that hippocampus is involved in behaviors as well. It has been shown that either chemical stimulation or lesion to the hippocampus could alter locomotor activity of animals [22,30,48]. Furthermore, abnormalities in hippocampal activity have been implied in schizophrenia [16,39].

Though it has been implied that the reduction of calcineurin activity underlies psychotic disorders [13,14], the molecular mechanism of CsA-induced behavioral changes in the *in vivo* brain remains to be clarified. Based on previous reports, we hypothesized that a single systemic administration of CsA might affect the PKC signaling pathway in rat brains. In this study, we measured the locomotor activity and studied the kinase activity of PKC in rats by examining the phosphorylation of PKC, GAP43, and MARCKS in the rat hippocampus at 1 and 5 h after the systemic injection of CsA.

Male Sprague-Dawley rats were purchased at the age of 7 weeks. Rats were housed under a 12 h:12 h light:dark cycle (lights on at 8:00 A.M.) with food and water freely available. Rats were allowed to habituate to the housing environment for at least 5 days. Experiments were performed at the age of 8 weeks. Animals were treated according to the NIH Guide for the Care and Use of Laboratory Animals and all experiments were approved by the Animal Subject Review Board of the Seoul National University Hospital.

The CsA dose and administration method were based on previous reports involving biochemical and behavioral experiments [19,32,33]. Intraperitoneal injections of 50 mg/kg CsA (Sigma–Aldrich Chemie, Steinheim, Germany) were administered. CsA was dissolved in a Cremophor EL (Sigma–Aldrich Chemie):EtOH (Merck, Darmstadt, Germany) solution with a ratio of 1:1 and then diluted in DW to yield a final ratio of Cremophor:EtOH:DW of 1:1:8. All animals received the same volume (1 mL) of solution. 50 mg/kg of CsA is higher than the dose used in chronic drug treatment scheme [2]. However, it was shown that repeated administration of 100 mg/kg/day CsA did not cause structural abnormalities in brain [5].

Locomotor activity was measured with a video tracking system and software (Activity monitor Ver5.0: MED Associates, St. Albans, VT, USA). Thirty minutes before the injection, rats were placed and habituated in a measurement chamber. Locomotor activity was measured for 4 h after injection during the light cycle (between 10 A.M. and 6 P.M.).

At 1 and 5 h after drug administration, rats were decapitated and hippocampus was isolated on ice. Hippocampus were homogenized in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.2% NP 40, 4 mM EGTA (pH 8.0), 10 mM EDTA (pH 8.0), 15 mM $Na_4P_2O_7$, 40 mM NaF, 4 mM Na_3VO_4, 100 mM β-glycerophosphate, 1 mM PMSF, 1 mM DTT, and protease inhibitor cocktail (Roche). The homogenate was centrifuged at 4 °C and 20,000 × *g* for 20 min and the supernatant was analyzed as a whole cell lysate.

Proteins were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman International Limited, Dassel, Germany). Membranes were incubated with primary antibodies against GAP43 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MARCKS (Calbiochem, Darmstadt, Germany), p-GAP43 (S41) (Phosphosolutions, Aurora, CO, USA), p-MARCKS (S152/156), p-PKC pan (Cell signaling technology, Beverly, MA, USA) and β-actin (Sigma–Aldrich, Saint Louis, MO, USA) at 4 °C overnight then with anti-rabbit or mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence substrate mixture (Pierce, Rockford, IL, USA) on X-ray film (Agfa Curix RPI, Rockford, IL, USA). Immunoblot signals were quantified with TINA version 2.10G (Raytest, Straubenhardt, Germany).

Immunoblot results are expressed as relative optical density (OD) and shown as means standard error. To analyze the biochemical and behavioral effects of CsA, Student's *t* tests were performed using Prism. A *P* value < 0.05 was considered significant.

We observed increased locomotor activity in the CsA-treated group compared to the vehicle group. There was no difference in distance traveled between the vehicle and CsA treatment groups until about 1 h after injection. After that time, the locomotor activity of vehicle-treated rats decreased drastically while that of the CsA injected group showed several peaks instead of a continuously high level of movement (Fig. 1A). The total distance traveled for CsA-treated rats at 4 h was higher than that of the vehicle group by about 3.2-fold, and this difference was statistically significant ($t = 5.120$, $P < 0.001$) (Fig. 1B).

To investigate the effect of CsA on the kinase activity of PKC, we performed western blots using antibodies against p-GAP43 and p-MARCKS at the residues where they are known to be phosphorylated by PKC (at S41 and S152/156, respectively). As shown in Fig. 2, at 1 h after injection, the immunoreactivity of p-MARCKS (S152/156) was higher in the CsA group than in the vehicle-treated rats ($t = 4.043$, $P < 0.001$), while that of p-GAP43 (S41) was increased by CsA 5 h after injection ($t = 3.318$, $P < 0.010$) (Fig. 2A and B).

We also examined whether CsA injection could affect the phosphorylation level of PKC, a value that is indicative of its activity. We