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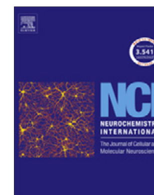

PKCδ inhibition enhances tyrosine hydroxylase phosphorylation in mice after methamphetamine treatment

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ABSTRACT

The present study was designed to evaluate the specific role of protein kinase C (PKC) δ in methamphetamine (MA)-induced dopaminergic toxicity. A multiple-dose administration regimen of MA significantly increases PKCδ expression, while rottlerin, a PKCδ inhibitor, significantly attenuates MA-induced hyperthermia and behavioral deficits. These behavioral effects were not significantly observed in PKCδ antisense oligonucleotide (ASO)-treated- or PKCδ knockout (−/−)-mice. There were no MA-induced significant decreases of dopamine (DA) content or tyrosine hydroxylase (TH) expression in the striatum in rottlerin-treated-, ASO-treated- or PKCδ (−/−)-mice. The administration of MA also results in a significant decrease of TH phosphorylation at ser 40, but not ser 31, while the inhibition of PKCδ consistently and significantly attenuates MA-induced reduction in the phosphorylation of TH at ser 40. Therefore, these results suggest that the MA-induced enhancement of PKCδ expression is a critical factor in the impairment of TH phosphorylation at ser 40 and that pharmacological or genetic inhibition of PKCδ may be protective against MA-induced dopaminergic neurotoxicity *in vivo*.

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1. Introduction

Tyrosine hydroxylase (TH; tyrosine 3-monooxygenase, EC 1.14.16.2) catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylanine (L-DOPA), which is the initial and rate-limiting step in the biosynthesis of catecholamines (CA; dopamine (DA), norepinephrine, and epinephrine; Nagatsu et al., 1964). TH also plays a central role in catecholaminergic neurotransmission (Nakashima et al., 2009) and may be related to various neuropsychiatric disorders such as Parkinson's disease (PD; Nagatsu, 1993), depressive illness (Meloni et al., 1995) and schizophrenia (Thibaut et al., 1997). Postmortem analyses of TH protein levels in nigrostriatal DA neurons have shown a marked decrease in the brains of those with PD but not those with schizophrenia (Ichinose et al., 1994). A number of phosphorylation sites have been identified on TH that influence the activity of this enzyme (Lee et al., 1989). It has been reported that TH is phosphorylated at the N-terminal serine (ser) amino sites at ser 8, ser 19, ser 31, and ser 40 by a variety of protein kinases (Dunkley et al., 2004; Nakashima et al., 2009). Thus, the phosphorylation of serine residues at the N-terminus appears to regulate the catalytic activity of TH *in vivo* (Dunkley et al., 2004; Hufton et al., 1995). Of the phosphorylation sites at the N-terminus of TH only ser 31 and ser 40 are readily phosphorylated and activate TH *in vitro* (Haycock and Wakade, 1992; Sutherland et al., 1993).

The protein kinase C (PKC) family consists of serine/threonine kinases and is broadly classified into three subgroups based on sensitivity to important cofactors, including phospholipids and Ca^{2+} (Dempsey et al., 2000; Gschwendt, 1999). The conventional

Abbreviations: MA, methamphetamine; PKC, protein kinase C; ASO, PKCδ antisense oligonucleotide; TH, tyrosine hydroxylase; PKA, protein kinase A; i.c.v., intracerebroventricular; CaMK, Ca^{2+}/calmodulin-dependent protein kinases; DA, dopamine; IR, immunoreactivity; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenylpyridinium; PD, Parkinson's disease.

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PKC isoforms (α, βI, βII, γ) are sensitive to Ca^{2+} and diacylglycerol and the novel isoforms (δ, ε, η, θ, μ) are Ca^{2+} independent but require diacylglycerol for activation. The atypical isoforms (ζ, ι/λ) require neither Ca^{2+} nor diacylglycerol for activation. PKC isoforms are differentially distributed in tissues and play key roles in various cellular biological processes, including cell differentiation and growth, apoptosis, tumor suppression, and carcinogenesis. In most studies, PKC inhibitors are used to demonstrate the anti-apoptotic role of the PKC family. Of the novel isoforms, PKCδ was the first member found to be functionally modulated by tyrosine phosphorylation upon H_2O_2 treatment (Konishi et al., 1997; Steinberg, 2004). A number of studies have found that the proteolytic activation of PKCδ plays a key role in apoptotic cell death of dopaminergic neurons (Kaul et al., 2003; Yang et al., 2004; Kitazawa et al., 2003; Latchoumycandane et al., 2005; Kanthasamy et al., 2006).

However, little is known concerning the role of PKCδ during *in vivo* dopaminergic toxicity induced by an amphetamine analog. Thus, the involvement of PKCδ in methamphetamine (MA)-induced *in vivo* dopaminergic toxicity is examined here. It was observed that PKCδ is critically involved in MA-induced dopaminergic toxicity and that PKCδ inhibition using the PKCδ inhibitor rottlerin or a PKCδ gene knockout (−/−) mouse model attenuates MA-induced dopaminergic toxicity through the upregulation of TH phosphorylation at ser 40. As recent reports indicate that rottlerin-mediated pharmacological effects as a PKCδ inhibitor are somewhat controversial (Soltoff, 2007; Susarla and Robinson, 2003; Tapia et al., 2006), an additional experiment using a PKCδ antisense oligonucleotide was performed.

2. Materials and methods

2.1. Animals

All mice were treated in accordance with the NIH Guide for the Humane Care and Use of Laboratory Animals. They were maintained on a 12/12-h light/dark cycle and fed *ad libitum*. They were adapted to these conditions for 2 weeks before the experiment. The experimental schedules are shown in Fig. 1.

2.2. Development and characterization of PKCδ (−/−) mice

A breeding pair of PKCδ (+/−) mice, originally bred into a C57BL/6 background, was a gift from Dr. K.I. Nakayama (Dept. of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan) (Miyamoto et al., 2002). These mice were subsequently maintained and bred into the C57BL/6 background for three to six generations in a SPF mice facility before use with wild-type mice from the same litter in our experiments. Tail DNA was evaluated and typed using polymerase chain reaction (PCR) and primers for PKCδ obtained from Bioneer Corporation (Daejeon, South Korea). PCR Primers for genotyping were as follows; 5′-GGAAGAATAAGAAACTGCATCACC-3′ and 5′-GAAGGAGCCAGAACCGAAAG-3′ for endogenous detection, and 5′-GGAAGAATAAGAAACTGCATCACC-3′ and 5′-TGGGGTGGGATTAGATAAATG-3′ for mutant detection. Brain tissue from PKCδ (−/−) mice was examined by Western blot analyses using antibodies for PKCδ and other isoforms (α, βI, βII, ζ; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to confirm that PKCδ protein was selectively absent in PKCδ (−/−) mice and that expression of the other isoforms was normal.

2.3. Guide cannula implantation and intracerebroventricular (i.c.v.) infusion

Mice were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). A stainless steel guide cannula (AG-4; Eicom, Kyoto, Japan) was implanted into the right lateral ventricle [stereotaxic coordinates: 0.5 mm posterior to bregma, 1 mm right to the midline, and 2 mm ventral to the dura, according to the atlas of Franklin and Paxinos (2008) and Shin et al. (2009)]. No histological or mechanical disruption was produced by implantation of the infusion cannula (data not shown). Microinfusion into the lateral ventricle was performed through a microinfusion cannula (AMI-4, Eicom, Kyoto, Japan) at a rate of 1 μL/min using a microinjection pump (CMA/100, CMA, Solna, Sweden). The microinfusion cannula was kept in place for 1 min after infusion to avoid backflow.

Guide cannula implantation did not affect the behavior of the subjects. Subsequent to guide cannula implantation, each animal was housed in a single cage in order to safely maintain the integrity of the implantation during the experimental period.

2.4. Intracerebroventricular infusion of rottlerin and treatment with methamphetamine (MA)

Rottlerin (PKCδ inhibitor; Biomol Research Laboratories Inc., Plymouth, PA, USA) was dissolved in DMSO as a stock solution and then stored at −20 °C. Rottlerin was diluted in sterile saline immediately before use at a concentration of 1.0 μg/μL. The final DMSO concentration was 10% (v/v). After 2 days of recovery from the guide cannula implantation, mice were microinfused into the lateral ventricle with rottlerin (1.5 or 3.0 μg) once a day for 5 days. On the next day, mice received four doses of MA (8 mg/kg, i.p.) or saline at 2-h intervals. Additional microinfusion of rottlerin was performed 4 and 0.5 h before the first MA injection (Fig. 1A). The dose of rottlerin was determined based on previous studies (Smith et al., 2007, 2006) and our pilot study (Shin et al., 2010).

2.5. Application of PKCδ antisense oligonucleotide (ASO) or PKCδ sense oligonucleotide (SO) and treatment with MA

ASO was complementary to the transition initiation region of mouse PKCδ mRNA (5′-AGGGTGCCATGATGGA) (Liedtke and Cole, 1997). As a control, SO was used (5′-TCGATCATGGCACCCT). SO and ASO used here were phosphorothioated on the two terminal bases of the 5′-end and three terminal bases of the 3′-end (Bioneer Corporation). Each oligonucleotide was dissolved in sterile saline immediately before use. After 2 days of recovery from the guide cannula implantation, PKCδ (+/+) mice received four doses of MA (8 mg/kg, i.p.) or saline at 2-h intervals. Then, 30 min before the first and third injections of MA and 30 min after the final injection of MA, ASO (2.5 μg/μL) or SO (2.5 μg/μL) was microinfused into the lateral ventricle (Fig. 1B). To confirm the decrease in PKCδ protein expression by ASO treatment, Western blot analysis was performed using an antibody against PKCδ (Santa Cruz Biotechnology, Inc.; Fig. 3).

2.6. Measure of rectal temperature

Rectal temperature was measured in the MA- and saline-treated mice. Measurement was performed at constant daytime intervals starting at 9:00 AM to avoid any influence of circadian variation. Rectal temperature (under ambient temperature: 21 ± 1 °C) was measured by inserting a thermometer probe lubricated with oil at least 3 cm into the rectum of the mice. To prevent sudden movements, especially in MA-treated mice, animals were gently handled with a wool glove while their tail was moved to allow probe insertion. This was done to reduce any effect of restraint stress on rectal temperature. When the attempt to insert probe was not successful (i.e., sudden movements of the animal or the need to restrain the mouse), the animal was excluded from the group.

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