Protein Kinase C-delta in dopaminergic system and experimental models of Parkinson's disease

Danhui Zhang
Iowa State University

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Protein Kinase C-delta in dopaminergic system and experimental models of Parkinson’s disease

by

Danhui Zhang

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Neuroscience

Program of Study Committee:
Anumantha G. Kanthasamy, Major Professor
Mark Ackermann
Vaclav Ourendnik
Etsuro Uemura
Srdija Jeftinija
Arthi Kanthasamy

Iowa State University
Ames, Iowa
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Parkinson’s disease (PD) is a major neurodegenerative disorder characterized by progressive and substantial loss of dopaminergic neurons in the substantia nigra compacta (SNc). Currently, no available drugs prevent the progressive loss of nigral dopaminergic neurons. The mechanisms underlying the dopaminergic degenerative process observed in PD are not well understood, which has hampered development of successful neuroprotective drugs. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine synthesis. Severely reduced TH positive neurons and TH fibers in dopaminergic terminal fields of PD patients and the successful application of L-DOPA therapy for Parkinson's disease suggest that this enzyme has a primary role in the progression of this disease. Previously, we found that the caspase-3 mediated proteolytic activation of Protein Kinase C δ (PKCδ), a member of the novel PKC isoform family, plays a critical role in oxidative stress-induced dopaminergic cell death in cell culture models of PD. In the present study, we report a novel interaction between PKCδ and TH, in which the kinase modulates dopamine synthesis by negatively regulating TH activity via protein phosphatase 2A (PP2A). We observed that PKCδ is highly expressed in nigral dopaminergic neurons and co-localizes with TH in the mouse brain. Interestingly, suppression of PKCδ activity with the kinase inhibitor rottlerin, PKCδ-siRNA, or with PKCδ dominant negative mutant effectively increased a number of key biochemical events in the dopamine pathway, including TH-ser40 phosphorylation, TH enzymatic activity, and dopamine synthesis in neuronal cell culture models. Additionally, we found that PKCδ not only physically associates with the PP2A catalytic subunit (PP2Ac) but also phosphorylates the phosphatase to increase its activity. Notably, inhibition of PKCδ reduced the dephosphorylation activity of PP2A and thereby increased TH-ser40 phosphorylation, TH activity, and dopamine synthesis. To further validate our findings, we used the PKCδ knockout (PKCδ -/-) mouse model. Consistent with other results, we found greater TH-ser40 phosphorylation and reduced PP2A activity in the substantia nigra of PKCδ -/- mice than wild-type mice. Importantly, this was accompanied by an increased dopamine level in the striatum of PKCδ -/- mice. Taken together, these results suggest that PKCδ phosphorylates PP2Ac to enhance its activity, and thereby reduces
Suppression of PKCδ activity with the kinase inhibitor rottlerin can increase dopamine synthesis in neuronal cell culture models. Thus, we treated C57 black mice with the PKCδ inhibitor rottlerin and found that rottlerin can effectively increase a number of key neurochemical events in the dopamine pathway, including TH phosphorylation, TH enzymatic activity, and striatal dopamine and DOPAC levels. Time course studies revealed that TH-ser31 and -ser40 phosphorylation and dopamine synthesis were increased within 1hr of rottlerin treatment. Increased dopamine synthesis was accompanied by stimulation of locomotor activity and stereotypic behavior. Consistent with the pharmacological effects of rottlerin, naïve PKCδ-knockout mice showed enhanced striatal dopamine levels and behavioral function as compared to wild-type mice. These results suggest that inhibition of PKCδ in the nigrostriatal dopaminergic system can enhance dopaminergic neurotransmission and neurobehavioral characteristics in animal models.

Previously, we discovered that proteolytic activation of PKCδ mediated by caspase-3 plays a critical role in oxidative stress-induced dopaminergic cell death in cell culture models of PD. Proteolytic cleavage of PKCδ (74 kDa) by caspase-3 results in a 41-kDa catalytic subunit and a 38-kDa regulatory subunit, leading to a persistent activation of the kinase (Kaul et al., 2003; Yang et al., 2004). Blockade of proteolytic activation of PKCδ by overexpression of the kinase-dominant negative PKCδ mutant, cleavage-resistant PKCδ mutant, or siRNA directed against PKCδ almost completely prevented the dopaminergic cell death. Therefore, in the present study, we examined the neuroprotective efficacy of the PKCδ inhibitor rottlerin in both primary cell culture and animal models. Herein, we report that rottlerin not only protects against MPP+-induced degeneration of TH-positive neurons in primary mesencephalic culture models but, most importantly, is clearly neuroprotective in an MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) animal model of Parkinson’s disease. Administration of rottlerin, either intraperitoneally or orally, to C57 black mice showed significant protection against MPTP-induced locomotor deficits and striatal depletion of dopamine and its metabolite DOPAC. Importantly, stereological analysis of nigral neurons revealed rottlerin treatment significantly protected against MPTP-induced TH-positive neuronal
loss in the substantia nigra compacta. Our findings demonstrate the neuroprotective effect of rottlerin in both cell culture and preclinical animal models of PD, and suggest that pharmacological modulation of PKCδ may offer a novel therapeutic strategy for treatment of PD.

Manganese (Mn) exposure causes manganism, a neurological disorder similar to PD. However, the cellular mechanism by which Mn induces dopaminergic neuronal cell death remains unclear. We found that caspase-3-dependent proteolytic activation of protein kinase Cδ (PKCδ) plays a key role in Mn-induced apoptotic cell death. In the present study, we report that chronic Mn treatment suppresses TH activity without causing any cytotoxicity, and is accompanied by an increase of PKCδ cleavage in the dopaminergic cells. The reduced TH activity by chronic Mn treatment can be attenuated by the PKCδ inhibitor rottlerin. Here we also showed that Mn treatment can increase PP2A activity, which may contribute to the decreased TH activity induced by MnCl₂. The increased PP2A activity induced by chronic MnCl₂ treatment is suppressed by the PKCδ inhibitor rottlerin, which may suggest that the activation of PKCδ induced by Mn exposure can increase PP2A activity, which might decrease TH activity induced by chronic Mn treatment. We suggest that Mn-mediated regulation of TH through PKCδ and PP2A may have important implications in neurological dopaminergic system disorders, such as Parkinson’s disease and Manganism.

Collectively, these results suggest that PKCδ phosphorylates PP2A to enhance its activity and thereby reduces TH-ser40 phosphorylation and TH activity and ultimately dopamine synthesis. Regulation of dopaminergic function through PKCδ and PP2A may be important for neurobehavioral function and metal-mediated neurological dopaminergic system disorders. Inhibition of PKCδ in the nigrostriatal dopaminergic system can offer dual benefits of neuroprotection and enhanced dopaminergic function in the development of therapeutic agents for treatment of Parkinson’s disease.
CHARPTER 1: GENERAL INTRODUCTION

Dissertation organization

Alternative thesis format is used for this thesis, which includes modified versions of several manuscripts either published or to be submitted for publication. The thesis contains a general introduction, four research papers, a general conclusion, and an acknowledgement. References for each individual section are listed at the end of corresponding chapters, except for the background and literature review: references for these are listed following the general conclusion. The general introduction (Chapter I) consistently provides the current knowledge of the etiopathogenesis of Parkinson’s disease and describes the overview of the research objective. Background and literature review I provide background information about the PKC protein in dopaminergic system, particularly focusing on the role of the PKCδ isoform in the survival of DA neurons and DA neurotransmission. Background and literature review II of the chapter summarizes the current available pharmacotherapeutic treatment options for the Parkinson’s animal models. Chapter II “Protein kinase Cδ negatively regulates tyrosine hydroxylase activity and dopamine synthesis by enhancing protein phosphatase-2A activity in dopaminergic neurons” is a research paper published in the Journal of Neuroscience (27: 5349-62, 2007). Chapter III “Neuroprotective Effect of PKCδ Inhibitor Rottlerin in Cell Culture and Animal Models of Parkinson’s Disease” has been published in Journal of Pharmacology and Experimental Therapeutics (322:913-22, 2007). Chapter IV “Inhibition of PKCdelta (PKCδ) isoform in the substantia nigra enhances dopamine synthesis and neurobehavioral alterations in mice: Dual neuroprotective strategies in Parkinson’s disease” and chapter V “Effects of Manganese on tyrosine hydroxylase activity and phosphorylation in dopaminergic neuronal cells” will be submitted for publication soon.

This dissertation contains the experimental results obtained by the author during her Ph.D study under the supervision of his major professor, Dr. Anumantha G. Kanthsamy.
Introduction

Parkinson’s disease (PD) is a major neurodegenerative disorder characterized by progressive and substantial loss of dopaminergic neurons in the substantia nigra compacta (SNc), resulting in debilitating motor signs including tremors, bradykinesia, and rigidity. The PKC family consists of more than 12 isoforms and is subdivided into three major subfamilies, which include conventional PKC (α, βI, βII, γ), novel PKC (δ, ε, μ, η, θ), and atypical PKC (τ, λ, ζ) (Gschwendt 1999; Dempsey et al., 2000; Maher et al., 2001; Kanthasamy et al., 2003). PKCδ, a key member of the novel PKC family, plays a role in a variety of cell functions including cell differentiation, proliferation, and secretion. Our recent studies demonstrate that PKCδ is an oxidative stress-sensitive kinase, and activation of this kinase via caspase-3 dependent proteolysis induces apoptotic cell death in cell culture models of Parkinson’s disease (Kanthasamy et al., 2003; Kaul et al., 2003; Yang et al., 2004; Latchoumycandane et al., 2005). Blockade of proteolytic activation of PKCδ by overexpression of the kinase-dominant negative PKCδ mutant, cleavage-resistant PKCδ mutant, or siRNA directed against PKCδ almost completely prevented the dopaminergic cell death (Kaul et al., 2003; Kitazawa et al., 2003; Anantharam et al., 2004; Yang et al., 2004; Latchoumycandane et al., 2005). We also observed a high level of PKCδ expression in the nigral dopaminergic neurons and a physical association of PKCδ with tyrosine hydroxylase (TH). TH is a rate-limiting enzyme in the biosynthesis of catecholamines and catalyzes the first step of a biochemical synthetic pathway in which L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-dopa). Severely reduced TH positive neurons and TH fibers in dopaminergic terminal fields and the successful application of L-DOPA therapy for Parkinson’s disease suggest that this enzyme has a primary role in progression of this disease. Phosphorylation and dephosphorylation of TH represent important post-translational regulatory mechanisms that mainly determine the amount of catecholamine synthesis. A number of phosphorylation sites have been identified in TH, and phosphorylation of TH
greatly influences the enzyme activity (Lee et al., 1989). General PKCs can phosphorylate TH-ser40 and TH-ser31 (Albert et al., 1984; McTigue et al., 1985; Tachikawa et al., 1987; Cahill et al., 1989; Haycock et al., 1992; Bunn and Saunders 1995; Bobrovskaya et al., 1998); however, direct phosphorylation of TH by PKA and not by PKC results in activation of the enzymatic activity (Funakoshi et al., 1991). The role of PKC isoforms in the regulation of TH activity is not well studied. The present studies investigate the physiological role of PKCδ in regulating dopamine synthesis and the development of therapeutic agents for treatment of Parkinson’s disease.

**Background and Literature Review I**

**Protein Kinase C delta (PKCδ) in dopaminergic system**

Dopamine (DA) is one of the major neurotransmitters within the basal ganglia system and is synthesized and secreted by dopaminergic neurons, which are localized in the substantia nigra. Alterations in DA neurotransmission have been implicated in a number of human neuropathologies ranging from Parkinson’s disease to attention deficit disorder, schizophrenia to drug abuse. The proteins that play a key role in DA synthesis and DA neurotransmission are tyrosine hydroxylase (TH), dopamine transporter (DAT), and dopamine receptors. In addition, several other proteins such as α-synuclein, parkin, MAP kinases, 14-3-3, and heat shock proteins have all been suggested to play crucial roles in DA synthesis and DA neurotransmission. Investigations into signal transduction mechanisms and potential involvement of these molecules in brain disorders have progressed at a breathtaking pace.

Phosphorylation-dephosphorylation is one of the key mechanisms by which the activity of several cell signaling molecules is regulated in vivo. Studies have shown the activities of TH, the rate limiting enzyme in DA synthesis, DAT, D1/D2-receptors, parkin, α-synuclein, and 14-3-3 can be regulated by some or all of various kinases such as PKA, PKC, MAP kinases, CAM kinases, and tyrosine kinases.
PKC is a multigene family involved in a plethora of cellular signaling cascades and divergent biologic functions. PKCs have been demonstrated to play a role in cell death, development, differentiation, homeostasis, migration, contraction, secretion, and immunity. PKCδ, a novel PKC isoform, has been implicated in the regulation of cell cycle progression and survival. PKCδ has also been implicated as a tumor suppressor and has been reported to positively and negatively regulate apoptotic cell death. Recently, we established that PKCδ plays a critical role in dopaminergic degeneration in cell culture and animal models of Parkinson’s disease. The specific emphasis of this chapter will be to review the role of PKC in the survival of DA neurons and in DA neurotransmission.

Protein Kinase C

Protein kinase C (PKC) is a ubiquitous enzyme originally described as a Ca$^{2+}$-activated, phospholipid-dependent serine-threonine protein kinase. PKCs are activated by GPCRs, tyrosine kinases, and by non-receptor-mediated signaling cascades. Molecular cloning and biochemical analysis have revealed the existence of 12 isoforms (Fig. 1.), which are classified into three distinct sub-family groups based on their activation patterns, namely, conventional or classical PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC) (Gschwendt, 1999; Dempsey et al., 2000; Maher, 2001; Kanthasamy et al., 2003).

![Diagram of PKC isoforms](image)

**Figure 1. Classification and primary structure of PKC isoforms**

(Kikkawa et al., 2002)
PKCα, βⅠ, βⅡ, and γ are members of the conventional PKC family and require calcium, phosphatidylserine, and diacylglycerol (DAG) for their activation, whereas members of the novel PKC family, PKCδ, ε, η, θ, and μ, are activated by phosphatidylserine and DAG and do not require calcium for activation. On the other hand, the members of the atypical family, PKCζ and λ/ι, require phosphatidylserine but not calcium or DAG for their activation. The conventional PKCs are made up of four conserved (C1–C4) and five variable regions (V1–V5). The C1 region contains cysteine-rich zinc finger-like motifs, is immediately preceded by an autoinhibitory pseudosubstrate sequence, and contains the recognition site for phosphatidylserine, DAG, and phorbol ester. The C2 region of some PKC isoforms is rich in acidic residues and binds Ca²⁺. The C3 and C4 regions contain the ATP and substrate-binding sites, respectively. The novel PKC isoforms lack the C2 region and therefore do not require Ca²⁺ for activation. The atypical PKCs (aPKC), ζ and λ/ι, have only one cysteine-rich zinc finger-like motif and are dependent on phosphatidylserine, but are unaffected by DAG, phorbol esters, or Ca²⁺.

Protein Kinase C-delta

Protein kinase Cδ (PKCδ), a member of the novel PKC family, is a widely expressed PKC isoform in mammalian tissues and was first isolated from the rat cDNA library (Kanthasamy et al., 2003). PKCδ is ubiquitously expressed in most tissues and cell types (Leibersperger et al., 1991; Wetsel et al., 1992). A high expression level of PKCδ was found in murine epidermis, placenta, uterus, brain, lung, and kidney (Leibersperger et al., 1991). PKCδ contains N-terminal regulatory and C-terminal catalytic fragments connected by a hinge region harboring the caspase-3 recognition and cleavage motif (Fig. 2.). The regulatory fragment contains the C1 domain that confers the binding for lipid molecules (diacylglycerol, DAG or phorbol 12-myristate 13-acetate, TPA) and the C2-like domain that lacks the Ca²⁺ binding capacity. The regulatory fragment also contains pseudosubstrate that binds to the catalytic site in the tertiary structure and keeps the kinase inactivated.
PKCδ activation mechanisms:

A variety of stimuli including reactive oxygen species (Konishi et al., 2001; Majumder et al., 2001), chemicals (Reyland et al., 1999; Anantharam et al., 2002), ultraviolet radiation (Chen et al., 1999), growth factors (Denning et al., 1996), and cytokines (Carpenter et al., 2002) can activate PKCδ. At least three distinct activation mechanisms have been reported for PKCδ to date: a) membrane translocation, b) proteolytic cleavage, and c) tyrosine phosphorylation.

Membrane translocation: PKCδ is primarily activated by translocation to the plasma membrane upon stimulation with the lipid signaling molecules phosphatidyl serine (PS), diacylglycerol (DAG), or phorbol ester (TPA). The lipid signaling molecules bind to the cysteine-rich zinc-finger like domain (C1) present in the regulatory subunit and expose the catalytic domain, allowing substrates to bind to the site. Subsequent to membrane translocation and upon exposure of the catalytic site, the PKCδ kinase activity is regulated by many upstream serine-threonine kinases, primarily via phosphorylation of Thr-505, Ser-643, and Ser-662 present in the catalytic subunit. These ser/thr residues have also been
demonstrated to be autophosphorylated, thus enhancing the catalytic activity when PKCδ is in a low activity form (Le Good et al., 1998). Furthermore, recent studies have shown that, in addition to the plasma membrane, PKCδ also translocates to the nucleus, mitochondria, cytosol, and other subcellular organelles.

**Tyrosine phosphorylation:** Tyrosine phosphorylation of PKCδ has been reported to either increase or decrease the kinase activity, depending on the type of cellular stimulation. Some of the non-receptor tyrosine kinases that phosphorylate PKCδ during H₂O₂ treatment are Src (Haendeler et al., 2003), Lck (Hardwick and Sefton, 1997) and Syk (Takada et al., 2003). Several studies have reported the phosphorylation of tyrosine residues Y52 and Y187 in the N-terminus of the protein, Y512 and Y523 in the C-terminus, and Y311 in the intermediate hinge region, which connects the regulatory and catalytic domain (Blake et al., 1999; Kanthasamy et al., 2003). Konishi et al. (2001) demonstrated that H₂O₂ treatment induces the phosphorylation of PKCδ at various tyrosine residues including Y311, Y332, and Y512, but phosphorylation at Y311 is critical for initiation of PKCδ catalytic activity in COS cells. Y311 and Y332 phosphorylated PKCδ recovered from H₂O₂-treated cells has been found to be constitutively active and independent of DAG (Konishi et al., 1997; Konishi et al., 2001). Prooxidant etoposide has also been shown to induce PKCδ tyrosine phosphorylation at Y311 in C6 glial cells. Recently, using pharmacological and genetic tools, we demonstrated that phosphorylation of PKCδ at Y311 precedes and is essential for caspase-3-dependent proteolytic activation. Since Y311 is in close proximity to the caspase-3 cleavage site 324DIPD327, Y311 phosphorylation may cause conformational change to expose the caspase cleavage site. In this study we demonstrated that the p⁶⁰src peptide inhibitor, tyrosine specific kinase inhibitor peptide (TSKI), significantly blocked H₂O₂-induced PKCδ Y311 phosphorylation, cleavage, and kinase activity, suggesting that p⁶⁰src may be an upstream kinase responsible for the Tyr-311 phosphorylation in dopaminergic cells. In addition, we also demonstrated dopaminergic neuronal cells expressing tyrosine phosphorylation defective PKCδ³³¹¹F mutant also prevented H₂O₂ and
MPP⁺-induced PKCδ cleavage and activation of PKCδ. Our studies clearly demonstrated that Y311 phosphorylation is essential for oxidative-stress mediated proteolytic activation of PKCδ and neuronal apoptosis. On the other hand, Okhrimenko et al. (2005) demonstrated that phosphorylation of PKCδ on Y155 was essential for TRIAL induced proteolytic cleavage of PKCδ and subsequent translocation to ER to exert its antiapoptotic effect in A172 glioma cells.

Caspase-3-dependent proteolytic cleavage of PKCδ: In addition to plasma membrane translocation, several studies have demonstrated a novel form of PKCδ activation in which the kinase is proteolytically cleaved by caspase-3. Caspase-3 cleaves PKCδ adjacent to the Asp 327 at the caspase-3 recognition site DIPD↓N to yield 41-kDa catalytically active and 38-kDa regulatory PKCδ fragments, resulting in permanent dissociation of the regulatory subunit from the catalytic subunit for persistent kinase activity. PKCδ mutants, in which the aspartate residue at position 327 has been mutated to an alanine residue, have been shown to be caspase-3 cleavage resistant. The proteolytic activation of PKCδ depends upon the type of cell and form of stimulation (Brodie and Blumberg, 2003; Kanthisamy et al., 2003). Some of the apoptotic inducers used in non-neuronal cells are etoposide, aplidin, mitomycin, and UV radiation. Recently, using pharmacological and genetic tools, we characterized the role of proteolytic activation of PKCδ in neuronal apoptosis.

Proapoptotic role of PKCδ in dopaminergic cell death:

PKCs play an important role in CNS function and pathology (Messing et al., 1991; Aronowski et al., 2000; Dempsey et al., 2000; Schechtman and Mochly-Rosen, 2001; Raval et al., 2003). PKCδ has been implicated in cell differentiation, secretion, and in programmed cell death (Cross et al., 2000; Kikkawa et al., 2002; Kaul et al., 2003). PKCδ is expressed in brain tissues and its level increases with age (Goldberg and Steinberg, 1996). The regional distribution of PKCδ in the CNS has not been studied in detail. Previous studies have shown PKCδ expression in the cerebellum, thalamus, septal nuclei, and purkinje cells in the posterior cerebellum (Barmack et al., 2000; Naik et al., 2000). We observed the highest level
of PKCδ expression in the substantia nigra as compared to other brain regions in both rat and mouse brain (Yang et al., 2004).

Recently, we showed that caspase-3 dependent proteolytic cleavage of PKCδ mediates apoptotic cell death induced by various dopaminergic neurotoxins (MMT, Mn, dieldrin, MPP⁺ and 6-OHDA) and proteasomal inhibitors (MG-132) in immortalized mesencephalic clonal neuronal cells (N27), primary mesencephalic cultures, and in intact animals. We also noted that the oxidative stress inducer H₂O₂ can directly activate PKCδ via proteolytic cleavage. Interestingly, neither translocation of PKCδ to the plasma membrane nor the cleavage of other isoforms including PKCα, PKCβ₁, and PKCβII were noted during dopaminergic toxins treatment, demonstrating the specificity of PKCδ proteolytic activation in the induction of apoptosis in dopaminergic neurons. Pharmacological inhibitors directed against ROS, MPT pore, caspase-9, caspase-3, and non-receptor tyrosine kinases significantly blocked toxin induced proteolytic activation of PKCδ and dopaminergic cell death. Subsequently, we also showed that overexpression of loss of function dominant negative mutant PKCδD327A (caspase-cleavage resistant), PKCδK376R (kinase inactive), and PKCδY311F (phosphorylation defective) proteins attenuate dopaminergic toxin induced apoptotic cell death (Kaul et al., 2003; Kitazawa et al., 2005; Anantharam et al., 2004; Kaul et al., 2005; Latchoumycandane et al., 2005). The unequivocal role of PKCδ in mediating neuronal apoptosis was demonstrated in RNAi studies, where suppression of PKCδ expression by small interfering RNA (siRNA) prevented MPTP and 6-OHDA induced dopaminergic degeneration in N27 cells, primary mesencephalic cultures, and in the substantia nigra of animals. Very recently, we demonstrated that overexpression of the mitochondrial antiapoptotic protein Bcl-2 attenuates neurotoxin-induced PKCδ cleavage, indicating that mitochondrial-dependent proapoptotic factors can influence the proteolytic activation of PKCδ (Kitazawa et al., 2005). In addition to the proapoptotic role, we also observed PKCδ amplified apoptotic signaling via positive feedback activation of the caspase cascade. Thus, the dual role of PKCδ as a mediator and amplifier of apoptosis may be
imported in dopaminergic degeneration observed in the pathogenesis of PD. Thus, identifying PKCδ-interacting proteins may help elucidate the role of PKCδ in dopaminergic neurotransmission.

**Diversity of PKC functions:**

Recent studies have demonstrated distinct functions among PKC isoforms. The similarity in enzymatic properties of the PKC catalytic domain alone cannot explain the diverse cellular functions of the different PKC isoforms. Apart from different activation, only the subcellular distribution of the activated PKCs can explain the diverse functions. Functional interactions between PKCs and a wide range of cell signaling proteins enable PKCs to carry out a wide variety of different cellular tasks. Studies have shown subcellular localization of PKCs in the cell changes dramatically upon cellular activation. Activated PKCs can translocate to the different subcellular organelles, such as mitochondria, nucleus, plasma membrane, and ER, which is mediated by scaffold proteins termed receptor for activated c kinase or RACKs. Translocation to different subcellular organelles enables PKCs to be in close proximity and regulate the activity of its substrates. Translocation to various subcellular organelles also enables PKC to be regulated by other signaling proteins. Among the various PKC isoforms, only PKCδ has the greatest flexibility to affect multiple cellular functions because its subcellular localization is finely regulated by phosphorylation and proteolytic activation and may explain in part both the protective and detrimental function of PKCδ. At a molecular level, the function of PKC interacting proteins can be classified into four different groups: a) Upstream activators; b) Intracellular compartments; c) Substrates; d) Signaling proteins. Some of the protein-protein interactions that occur between PKCδ and proteins associated DA metabolism, DA synthesis, and DA neurotransmission are described below.

**Protein kinase C regulates tyrosine hydroxylase activity and phosphorylation**

Tyrosine hydroxylase (TH) is the initial and rate-limiting step in the biosynthesis of catecholamines such as dopamine and norepinephrine. TH catalyzes the hydroxylation of
L-tyrosine to L-DOPA, which is then converted to dopamine (DA) and epinephrine by DOPA decarboxylase. In addition to regulation by feedback inhibition by catecholamines, allosteric activation by heparin, phospholipids, polyanions, and RNA, the activity of this rate-limiting enzyme is also regulated by the phosphorylation-dephosphorylation reaction. TH activation by phosphorylation is the primary mechanism responsible for the maintenance of DA levels in tissues after DA secretion. PKC can regulate TH activity at both transcriptional and post-translational levels in dopaminergic cell lines (Campbell et al., 1986). Studies have shown PKC activator TPA can induce increases in TH enzymatic activity at the enzyme level, and TPA treatment can increase TH gene transcription in PC12 cells (Wolf and Roth, 1990). Recently, Lopez-Toledano et al. (2004) reported that TPA treatment promoted the generation of TH-positive neurons in neural stem cells, and the generation of TH-positive cells was suppressed in the presence of the PKC inhibitor staurosporine (Lopez-Toledano et al., 2004). Phosphorylation/dephosphorylation of TH represents an important mechanism in the regulation of the enzymatic activity. Phosphorylation mediated by protein kinases results in

activation of TH. The regulation of TH activity by phosphorylation in vitro has been extensively investigated (Haycock and Haycock, 1991). TH can be phosphorylated at serine residues at positions 8, 19, 31 and 40 present in the N-terminal half by a variety of protein kinases (Fig. 3). Studies have shown that both PKC as well PKA can directly phosphorylate TH at ser31 and ser40 and that phosphorylation by PKA plays the predominant role in vivo (Haycock, 1990). Purified protein kinase C can only phosphorylate and activate partially

![Figure 3. TH phosphorylation by distinct protein kinases](image-url)
purified TH at ser40 (Albert et al., 1984; Haycock and Haycock, 1991; Haycock and Wakade, 1992). PKC as well as PKA phosphorylate TH at the same rate and similar kinetics.

Studies have also shown phosphorylation by PKA results in activation of the TH enzymatic activity, whereas phosphorylation by PKC resulted in a marginal increase in TH activity (Funakoshi et al., 1991). Recently, we observed high levels of the PKC isoform PKCδ in the substantia nigra (SN) in murine and human brain samples. Confocal microscopic analysis revealed co-localization of PKCδ and TH in the substantia nigra of rat brain and postmortem human brain (Zhang et al., 2007). These results suggest that PKC-TH interactions may play a critical role in dopaminergic function and are currently under investigation in our laboratory.

**Protein kinase C modulates phosphorylation and trafficking of dopamine transporter**

The dopamine transporter (DAT) plays a key role in terminating dopaminergic neurotransmission by regulating the availability of DA in the synapse. The primary function of DAT is to reuptake DA into the presynaptic neurons after its release and thus clear DA from the synaptic space. The activity of DAT and its ability to clear DA is acutely controlled by PKC. PKC mediated phosphorylation of DAT results in reduced transporter activity, suggesting PKC negatively modulates DAT activity (Huff et al., 1997; Zhang et al., 1998; Lin et al., 2003). DAT contains multiple potential ser/thr phosphorylation sites at the N terminus (Foster et al., 2002; Granas et al., 2003; Lin et al., 2003). The PKC activator TPA increased DAT phosphorylation and reduced its transport activity by affecting Vmax, as determined by [³H]DA uptake. Together, these data suggest that DAT phosphorylation through the PKC pathway can lead to decreased transporter function and expression (Huff et al., 1997; Zhu et al., 1997; Zhang et al., 1998; Lin et al., 2003). Regulation of DAT by PKC is assumed to be due to direct phosphorylation of DAT protein by PKC, according to the existence of consensus amino acid sequences for PKC phosphorylation. But when all putative PKC phosphorylation sites are eliminated, in PKC null mutant hDAT, PKC activator and inhibitor
still affected regulation of DAT function, which suggests that PKC may regulate DAT function in an indirect way through a mediator protein or activation of a clathrin-mediated pathway (Chang et al., 2001). To determine which PKC isoforms are capable of regulating DAT activity, Doolen and Zahniser studied the effect of activating or inhibiting different PKC isoforms on DAT function in *Xenopus laevis* oocytes expressing human (h)DAT. Activators of both conventional PKCs and novel PKCs significantly inhibited DAT-associated transport currents, and this effect was reversed by pharmacological inhibitors directed against PKCα, β, γ and δ but not by PKCε translocation inhibitor. Their data suggest that TPA regulated DAT activity is primarily mediated by conventional PKCs and PKCδ (Doolen and Zahniser, 2002). In another study, PKCβ(II) was physically associated with DAT and was responsible for increased DA uptake into the rat striatum (Johnson et al., 2005). In addition to negatively regulating transporter activity of DAT, PKC also induces down regulation of DAT via the clathrin mediated endocytic pathway. Since PKC can down regulate phosphorylation-defective DAT mutants, PKC mediated down-regulation and endocytosis occur independent of phosphorylation, possibly via other DAT associated proteins. Studies with PC12 cells stably transfected with human DAT cDNA revealed that TPA treatment decreased transporter capacity and the number of DAT molecules on the cell surface (Melikian and Buckley, 1999). Immunostaining studies showed that [3H]DA uptake was reduced by PKC activation because of rapid sequestration/internalization of hDAT protein from the cell surface, while the increased DA uptake by PKC inhibition was associated with the recruitment of internalized or intracellular transporters to the plasma membrane (Pristupa et al., 1998). Therefore, PKC activation accelerates DAT endocytosis, reduces recycling, and does not involve degradation (Melikian and Buckley, 1999; Loder and Melikian, 2003). Subsequently, a PKC-sensitive autonomous endocytic signal was identified in the C terminus of DAT. The C-terminal residues 587–596 in the FREKLAYAIA region are necessary and sufficient to drive constitutive and protein kinase C-regulated DAT internalization in dopaminergic cells (Holton et al., 2005). In COS7 cells expressing the truncated hDAT
lacking the first 22 N-terminal residues, PKC activation still induced transport function and internalization, suggesting that phosphorylation of hDAT was not critical for both receptor-mediated or PKC-mediated internalization of the hDAT (Granas et al., 2003). Thus, PKC can negatively regulate DAT function by direct phosphorylation as well as by a combination of increased internalization and decreased recycling (Fig. 4). Dysregulation of synaptic DA levels caused by altering DAT function is associated with many neurological disorders, including depression.

**Figure 4. PKC-regulated DAT trafficking in dopaminergic cells**

Although the cellular and molecular mechanisms leading to PKC-DAT interactions are still unclear, the regulation of DAT activity by PKC is presumed to function as a mechanism for fine-tuning DA levels under diverse physiological conditions.

**PKCs and Dopamine receptor cell signaling:**

Dopamine receptors are a family of G-protein coupled receptors that are activated by dopamine and are classified into two subfamilies: the D1-receptor group (D1 and D5) and the D2-receptor group (D2, D3, and D4). These receptors are highly expressed in the central nervous system and in the pituitary gland. Upon binding to dopamine, D1 receptors stimulate adenylate cyclase activation via Go₆s, which then activates PKA mediated signal transduction events. Studies have also shown D1 receptor subtype coupling to other
heterotrimeric G proteins to Gαo and Gαq and D5 receptor subtype to Gαz. D1 receptor activation has also been shown to result in the activation of MAP kinases such as ERK and JNK (Barbieri et al., 2004; Chen et al., 2004; Shishodia et al., 2004; Chan et al., 2005). In normotensive animals, dopamine, via D1-like receptors, mediates decreased sodium excretion transport in the renal proximal tubule via stimulation of PKC activity at 15 min; however, at 60 min there was a decrease in PKCδ and PKCζ activities associated with an increase in sodium excretion (Nowicki et al., 2000). In this study, dopamine induced translocation of PKCα and PKCδ to the plasma membrane in the renal epithelial cell line as early as within 20 seconds of stimulation. The translocation was suggested to be an important event in the dopamine induced PKC mediated regulation of ion pumps, channels, and other transporters in renal cells. The antioxidant effect of Tempol (superoxidedismutase mimetic) in Zuker rats was mediated by decreased D1 receptor phosphorylation and reduced PKC activity (Marwaha et al., 2006). Interaction between metabotropic glutamate receptors (mGluR1/5) and D1 receptor subclass via enhanced phosphorylation of ERK2 in striatal medium spiny neurons was mediated by PKC (Lania et al., 2003).

Unlike the D1-receptors, activation of D2-receptor subtypes inhibits adenlylcylase activity and cAMP production via Gαi/o. Ethanol and other substances of abuse have been shown to increase the dopamine levels in the nucleus accumbens (Inoue et al., 2007). Ethanol and NPA D2 receptor agonists caused translocation and activation of PKCδ and PKCδ in the Chinese hamster kidney cells and in NG 108-15 to the perinucleus and cytoplasm, respectively (Yao et al., 2008). Activation of PKC caused the switching of coupling of D2 receptors from inhibition of adenyl cyclase towards facilitation of arachidonic acid release by phosphorylating components of the receptor-Gαi/o protein complex. Therefore, PKC activation results in the amplification of D2 receptor signaling via the arachidonic acid release alongside the inhibition of adenylcyclase (Di Marzo et al., 1993).

In dopamine-producing PC12 cells, D2 receptor activation protects against H2O2-induced apoptotic cell death. The anti-apoptotic effect of D2-receptors was mediated
by PI-3 kinase/protein kinase B (Akt) via c-Src dependent transactivation of epidermal growth factor receptor (EGFR). In HeLa cells, D2 receptor mediated activation of NfκB via phosphorylation and activation of cSrc tyrosine kinase at Y418 (Yang et al., 2003). Further, TPA treatment resulted in a 3-fold increase in D2-receptor phosphorylation in D2-receptor transfected HEK293T cells. Phosphorylation of D2 receptor on multiple serine/threonine sites results in the internalization of the receptor (Morris et al., 2007). Humans have two isoforms of D2 receptors – D2L (long form) and D2S (short form). In D2L and D2S transfected Ltk-fibroblast cells, TPA treatment blocked D2S-mediated increases in cytosolic free Ca^{2+}, whereas D2L-mediated increases in cytosolic Ca^{2+} were only partially blocked, suggesting differential regulation by PKC (Morris et al., 2007). Therefore, PKCs can regulate the D1/D2 receptor activity, surface expression, and down-stream cell signaling.

**α-Synuclein**

α-Synuclein is a neuronal protein thought to be central in the pathogenesis of Parkinson's disease (PD) because it comprises the fibrillar core of Lewy bodies, one of the histologically defining lesions of PD, and because mutations in α-synuclein cause autosomal dominant PD. α-Synuclein is an abundant pre-synaptic protein belonging to the family of proteins that include α-, β, γ synuclein and synoretin (Jakes et al., 1994; Goedert, 2001). Although the physiological role of α-synuclein is still unclear, it has been suggested to play a role in synaptic plasticity, memory development, neurotransmitter release, and axonal transport (George et al., 1995; Abeliovich et al., 2000; Jensen and Gai, 2001; Chen et al., 2002). Because of its open random coiled structure, α-synuclein has been suggested to also function as a chaperone (Weinreb et al., 1996) and shares physical and functional homology with the 14-3-3 protein chaperone family (Ostrerova et al., 1999). Abnormal accumulation and aggregation of α-synuclein have been implicated in the dysfunction, degeneration, and ultimate death of dopaminergic neurons in PD (Spillantini and Goedert, 2000; Wakabayashi et al., 2002). Recent studies have shown physical and/or functional interactions between α-synuclein and several proteins associated with DA metabolism and survival of dopaminergic
neurons. These proteins include TH, 14-3-3, PP2A, PLD2, Fyn, Src, Parkin, DAT, ERK, PI3-Akt kinase, cytochrome C, cytochrome C oxidase (COX), BAD, calmodulin, histones, GSK3, and caspase-3.

Recently, we demonstrated that 1-methyl-4-phenylpyridinium (MPP+) induces caspase-3-dependent proteolytic activation of PKCδ, which subsequently contributes to neuronal apoptotic cell death in mesencephalic dopaminergic neuronal cells (Kaul et al., 2003; Yang et al., 2004). Subsequently, we also showed physical and functional interaction between PKCδ, α-synuclein, and BAD in an in vitro model of Parkinson’s disease (Kaul et al., 2003; Kaul et al., 2005; Yang et al., 2004). In that study, we explored whether the α-synuclein-PKCδ interaction modulates MPP+ -induced apoptotic cell death in mesencephalic dopaminergic neuronal cells (N27). Over-expression of wild-type human α-synuclein in N27 cells attenuated MPP+-induced cytotoxicity, mitochondrial cytochrome c release, caspase-3 activation, proteolytic activation of PKCδ, and DNA fragmentation without affecting ROS production. On the contrary, expression of the mutant human α-synuclein A53T did not attenuate, but rather exacerbated, the MPP+-induced PKCδ activation under similar conditions. Similarly, wild-type human α-synuclein expression also rescued mesencephalic dopaminergic neuronal cells from MPP+-induced apoptotic cell death, while α-synuclein A53T exacerbated the MPP+-induced DNA fragmentation. Co-immunoprecipitation studies revealed that α-synuclein interacted with the pro-apoptotic proteins PKCδ (PKC isozyme) and BAD (Bcl-2 related), and their association is further strengthened after stimulation with MPP+. Alternatively, no association was observed between α-synuclein and Bcl-2 or between α-synuclein and PKCα, suggesting that α-synuclein preferentially associates with the Bcl-2 or PKC family members. We also observed that the interaction between PKCδ and α-synuclein does not involve direct phosphorylation. On the other hand, physical association of α-synuclein with PKCδ prevents caspase-3 dependent proteolytic activation of PKCδ. The putative contributions of
α-synuclein-PKCδ interactions to neurodegenerative processes in PD merit further investigation.

**14-3-3 proteins:**

14-3-3 proteins (28-33 kDa) are abundantly expressed in the brain and participate in controlling cell cycle, cell growth, differentiation, survival, cell adhesion, transcription, apoptosis, migration, and spreading. 14-3-3 Proteins have been identified as phosphoserine/phosphothreonine-binding proteins and >200 binding partners have been identified to date. 14-3-3 targets are found in all subcellular compartments which include transcription factors, biosynthetic enzymes, ion channels, cytoskeletal proteins, signaling molecules, apoptosis factors, and tumor suppressors. 14-3-3 binding has been shown to alter the subcellular localization, stability, phosphorylation state, activity and/or molecular interactions, and function as adaptor molecules to stimulate protein–protein interaction of target proteins. There are at least seven known members of the 14-3-3 family. 14-3-3 proteins have recently been implicated in several neurodegenerative disorders including ALS, Prion associated diseases, Alzheimer's, Huntington, and Parkinson's diseases.

Immunohistochemical investigation of Lewy bodies showed intense positive 14-3-3 staining in PD and DLBD post-mortem brains (Kawamoto et al., 2002). Recent studies indicate that only ε, γ, ζ and θ, but not β, η and σ 14-3-3 isotypes, colocalized with Lewy bodies in PD. In DA neurons, 14-3-3 play a critical role in DA synthesis and metabolism by binding to phospho-TH. Binding of 14-3-3 proteins to TH is required for its optimal activation by phosphorylation, and prevention of TH dephosphorylation leads to a prolonged stimulation of TH activity (Ichimura et al., 1988). On the other hand, α-synuclein, one of the main components of Lewy bodies, binds to dephosphorylated TH and prevents phosphorylation, leading to prolonged inhibition of TH activity (Perez et al., 2002).

Several PKCs, including PKCa, PKCb, PKCe, PKCζ and PKCλ, have been demonstrated to bind to 14-3-3 proteins in an isoform-specific manner. Very recently, a sphingosine-dependent protein kinase was shown to phosphorylate β-, η- and ζ- isoforms of
14-3-3 at positions ser60, ser59, and ser 58, respectively (Megidish et al., 1998). The sphingosine-dependent protein kinase was later identified to be the proteolytically cleaved catalytic fragment of PKCδ. It is postulated that dimer formation of 14-3-3 proteins is required for binding to its target proteins. PKCδ phosphorylation of 14-3-3 inhibits 14-3-3 dimer formation and prevents it from binding to its partners. The 14-3-3 family of proteins has been shown to interact with several proteins associated with cell signaling and apoptotic cell death, including α-synuclein, BAD, BAX, histones, HDAC, PP2A, Stat, MAP kinases, heat shock proteins and ubiquitin proteasomal systems associated proteins. We postulate that dopaminergic degeneration during PD phosphorylation of 14-3-3 proteins by proteolytically activated PKCδ may prevent 14-3-3 binding to TH, resulting in decreased DA synthesis, and also prevent 14-3-3 binding to BAD/BAX, allowing them to freely translocate to mitochondria to promote their proapoptic effect. Although PKC-14-3-3 interactions have not been established in DA neurons in the nigro-striatal systems, the isotype-specific interaction of PKC isoforms with 14-3-3 isoforms may play a crucial role in the survival of dopaminergic neurons in healthy individuals as well as during dopaminergic degeneration observed in PD.

**Parkin:**

Mutations in the parkin gene were recently discovered to cause autosomal recessive juvenile Parkinsonism. Parkin mutations have been recognized as the most common cause of
of hereditary PD and a possible risk factor for idiopathic PD. The parkin gene comprises 12 exons, codes for a 465-amino acid 52-kDa protein, and is most prominently expressed in muscle and throughout the brain. Parkin functions in the ubiquitin-proteasome system as an E3 ubiquitin-protein ligase together with the E2 ubiquitin conjugating co-enzymes. Because this function appears to be defective in patients with parkin mutations, the exact molecular mechanisms by which parkin dysfunction causes PD remain to be elucidated. Immunohistochemical investigation of Lewy bodies showed intense positive parkin staining in PD and DLBD post-mortem brains (Nakamura et al., 2006). Recent studies indicate that parkin colocalized with α-synuclein in Lewy bodies (Choi et al., 2001). Parkin overexpression was also shown to rescue dopaminergic neurons from oxidative stress-induced cell death. Regulation of parkin activity by nitrosylation results in the inhibition of its ubiquitin ligase activity (Chung et al., 2004). Recently, parkin activity was also shown to be regulated by casein kinase-1, PKA, and PKC, resulting in decreased ligase activity (Sato et al., 2006). Unfolded protein stress mediated by proteasomal inhibition or ER stress, but not oxidative stress, reduced the overall phosphorylation of parkin. Therefore, regulation of parkin phosphorylation by PKC may contribute to the survival of dopaminergic neurons.

**Mitogen Activated Protein Kinases (MAPKs)**

Transduction pathways in eukaryotic cells integrate diverse extracellular signals, and regulate complex biological responses such as growth, differentiation, and death. One group of proline-directed Ser/Thr protein kinases, the mitogen-activated protein kinases (MAPKs), plays a central role in these signaling pathways. MAPKs are members of a three-kinase phosphorylated system composed of the MAPK, MAPK kinase (MKK), and MAPK kinase kinase (MKKK). MKKKs phosphorylate and activate MKKs, which in turn phosphorylate and activate MAPKs. The extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and the p38 kinases are MAPKs family members. Several MAP kinases have been implicated in neurodegenerative disorders, in particular the c-Jun N-terminal kinase
The JNK pathway in Parkinson's disease. In mice, adenoviral gene transfer of the JNK binding domain of JNK-interacting protein-1 (a scaffold protein and inhibitor of JNK) inhibited this cascade downstream of MKK4 phosphorylation, and blocked JNK, c-Jun, and caspase activation, as well as the death of dopaminergic neurons and the loss of catecholamines in the striatum (Xia et al., 2001). Direct blockade of JNK with specific inhibitor SP-600125 protects dopaminergic neurons both from MPP⁺-induced neuronal apoptosis in vitro and in the MPTP Parkinson's disease model. Immunohistochemical investigations revealed the presence of all three MAPKs, ERK, JNK and P38 α-synuclein, in Lewy bodies of PD and DLBD post-mortem brains (Ferrer et al., 2001). Some of the MAP kinase family members have also been shown to physically and functionally interact with α-synuclein. JNK inhibitors have been or are currently being tested in clinical trials to decrease the progression of PD.

PKC isoforms, including PKCα, PKCδ, and PKCɛ, regulate activity of ERK, JNK, and p38 kinases directly or via upstream MAP kinases, RAS, RAC and the RAF pathway. Inhibition or depletion of PKCδ protects cells from apoptotic cell death mediated via these MAP kinase pathways (García-Fernández et al., 2002). Some studies have shown a physical and functional interaction between PKCδ and several MAP kinase family members, including stress-activated protein kinase, RAC, MEKK1, and MEKK4 (Bogoyevitch et al., 1995; Clerk et al., 1998). Therefore, PKCδ-MAP kinase interactions may be critical to DA homeostasis and dopaminergic neurotransmission.

Non-receptor tyrosine kinases (NTKs)

Protein-tyrosine phosphorylation by receptor and non-receptor tyrosine kinases is thought to be important in regulating synaptic function and plasticity. Unlike receptor tyrosine kinases, non-receptor tyrosine kinases are ubiquitously expressed in all metazoan cells and are oncogenic once they are activated. Non-receptor tyrosine kinases were first discovered in Rous Sarcoma virus; therefore, they are also referred to as Src tyrosine kinases. Molecular cloning and biochemical analysis have revealed the existence of nine members:
Src, Fyn, Yes, Fgr, Lyn, Hck, Blk, and Yrk.. Src tyrosine kinases mediate cell signaling in cell growth, differentiation, and proliferation (Brown and Cooper, 1996). Several members of the Src tyrosine kinases (Src, Fyn, Lyn, c-Abl, PYK2, Lck, and growth factor receptors) have been shown to regulate the PKCδ activity (Li et al., 1994). Regulation of PKCδ activity by tyrosine phosphorylation is particularly important because PKCδ is most efficiently tyrosine-phosphorylated when compared to other PKC isoforms. Further, PKCδ has also been shown to phosphorylate Src and Fyn tyrosine kinases. Co-immunoprecipitation studies revealed PKCδ to physically associate with several Src tyrosine kinases and regulate their activity (Shanmugam et al., 1998). Immunohistochemical investigations also revealed colocalization PKCδ with Src tyrosine kinases (Betty et al., 2001). Studies have also shown Src kinase family members such as Fyn and Src to phosphorylate α-synuclein (Ellis et al., 2001). Recently, it was shown that activation of Pyk2/RAFTK1 and Pyk2/RAFTK, a non receptor protein-tyrosine kinase that is expressed to a high degree in human brain, inhibited α-synuclein phosphorylation in response to cell stress (Nakamura et al., 2002). In addition, Dunah et al. (2004) reported that dopamine receptor (D1)-dependent trafficking of striatal N-methyl-D-aspartate glutamate receptors required Fyn protein tyrosine kinase. Thus, functional interactions between PKCδ, TH, α-synuclein, NMDA receptors, and NTKs may be crucial for normal DA metabolism and neurotransmission.

**Heat Shock Proteins (HSP)**

HSPs are chaperone proteins which function to assist a nascent polypeptide chain to attain a functional conformation as a new protein and then to assist the protein’s arrival at the site in the cell where the protein carries out its functions. It has become increasingly clear that disruption of chaperoning mechanisms contributes to aging and disease. Deficiencies and defects in HSPs affect a wide range of neurological disorders including PD. HSPs are encoded in genes that are inducible by heat shock and are classified into high-molecular-mass...
Hsp (≥100 kD), Hsp90 (81 to 99 kD), Hsp70 (65 to 80 kD), Hsp60 (55 to 64 kD), Hsp40 (35 to 54 kD), and small Hsp (≤34 kD).

Immunohistochemical investigations revealed colocalization of Hsp70 and Hsp25 with α-synuclein in the Lewy bodies of PD and DLBD post-mortem brains (Uryu et al., 2002). PCR screening of DNA samples from 274 PD patients and 183 controls revealed an A to C mutation at position -110 in the 5' promoter region of Hsp70, suggesting this functional polymorphism may render susceptibility to PD (Wu et al., 2004). Recently, Hsp70 was shown to attenuate alpha-synuclein-induced apoptotic cell death in cell culture and animal models of Parkinson's disease (Dedmon et al., 2005). Adenoviral-mediated Hsp70 gene transfer inhibited MPTP-induced nigrostriatal degeneration in an animal model of PD (Dong et al., 2005). Recently, Hsp27 was shown to inhibit 6-OHDA-induced apoptosis in PC12 cells (Gorman et al., 2005). In this study, 6-OHDA induced the heat shock response, leading to increased levels of Hsp25 and Hsp70. Prior heat shock or overexpression of Hsp27 (human homologue of Hsp25) delayed cytochrome C release and caspase activation, and reduced the level of apoptosis caused by 6-OHDA, suggesting increased Hsp25 expression is associated with cell survival. Thus, both Hsp70 and Hsp25 may play a critical role in the survival of DA neurons.

Recently, Lee at al. (2005) demonstrated HSP25 inhibits PKCδ-mediated cell death through direct interaction. Direct binding of HSP25 to the PKCδ V5 region (aa 606-676) prevents PKCδ translocation and activation. To release this inhibitory effect, PKCδ phosphorylates Hsp25 at Ser-15 and Ser-86. Phosphorylation of Hsp25 at these residues releases it from bound PKCδ. In another study, PKCδ-Hsp70 interaction was shown to play a critical role in cardioprotection in the rat heart (Honma et al., 2002). PKCδ activation led to the phosphorylation and translocation of heat-shock factor 1 (HSF1), and thus promoted expression of Hsp72 protein, leading to cardioprotection. Since Hsp70 and Hsp25 are both present in Lewy bodies, PKCδ-HSP interactions may be critical to the pathophysiology observed in PD.
**RACKs:**

The newly discovered class of PKC anchoring proteins called Receptors for Activated C-Kinase (RACKs) and caveolins help target the PKC isozymes to the different organelles within the cell and facilitate the interactions between the individual PKCs and their substrates (Schechtman and Mochly-Rosen, 2001; Chen et al., 2002b). In 1995, Mochly-Rosen was first to propose the name RACK for PKC isozyme-selective anchoring proteins. Because RACK proteins can act as scaffolds to bring multiple proteins together in signaling complexes, they confer PKC isozymes the specificity of interaction with their substrates. RACKs were one of the first identified binding partners for PKCs, but many proteins have since been shown to associate physically with PKCs. These PKC-interacting proteins (C-KIPs) are classified into four categories. a) Proteins that target PKC to its upstream activators. A major function of C-KIPs is to enable the regulation of individual PKC isoforms by immediate upstream regulators such as DAG. b) Proteins that direct PKC to intracellular compartments. A feature of PKC signaling is that the kinases translocate between cellular compartments such as the cytosol, nucleus, mitochondria, ER and cytoskeleton. c) Substrates of PKC. Many C-KIPs are also substrates of PKC (termed substrates that interact with C-kinase). Scaffolding proteins such as RACKs and receptors for inactive C-kinase also fall into this category because they bring together PKC and its substrates. d) Other signaling proteins. A RACK protein that specifically interacts with PKCδ but not with other PKC isoforms has recently been identified (Chakraborti et al., 2005). Therefore, RACK-PKCδ interactions probably play a crucial role in the survival of dopaminergic neurons and thus merit further investigation.

**Summary:** PKCδ is a serine/threonine kinase that plays a key role in growth regulation and tissue remodeling. We observed that PKCδ is highly expressed in nigral dopaminergic neurons and co-localizes with TH (Zhang et al., 2007). PKCδ negatively regulates TH by the PKCδ-PP2A-TH pathway, PKCδ phosphorylates PP2Ac to enhance its activity and thereby reduces TH-ser40 phosphorylation and TH activity and ultimately
dopamine synthesis. The exact nature of the physical association and dynamic regulation of TH, PKCδ, and PP2A are yet to be characterized; however, recent literature provides some information regarding this interaction. PKCδ, TH, or PP2A have been recently shown to physically associate with each other, as well as with other putative chaperone proteins such as α-synuclein and 14-3-3 (Ostrerova et al., 1999; Kleppe et al., 2001; Srivastava et al., 2002; Kjarland et al., 2006). Recently, Peng et al. (Peng et al., 2005) demonstrated that a functional interaction between α-synuclein and PP2A can regulate TH phosphorylation and TH activity. Srivastava et al. reported a physical interaction between PKCδ and PP2A in NIH3T3 cells, and that dephosphorylation of PKCδ by PP2A results in its inactivation (Srivastava et al., 2002). In our recent study, we showed that α-synuclein interacts with PKCδ and regulates its activity following neurotoxic insults (Kaul et al., 2005). Therefore, in the dopaminergic system, the physical and functional association between PKCδ, TH, and PP2A could be facilitated and/or regulated by chaperone proteins such as α-synuclein (Kaul et al., 2005; Peng et al., 2005) and 14-3-3 (Ostrerova et al., 1999; Kleppe et al., 2001; Kjarland et al., 2006). Nevertheless, further studies are required in both in vitro and in vivo model systems to elucidate the dynamics of physical and functional regulation of PKCδ and PP2A in regulation of TH activity. Regulation of TH activity and DA levels is critical for normal dopaminergic neurotransmission in the CNS. Excessive DA production may not only alter neurotransmission, but may also contribute to neuronal cell death through increased oxidative stress (Hoyt et al., 1997; Luo et al., 1998). In this regard, we wish to point out that PKCδ can be activated by at least two independent mechanisms in neuronal cells. These include membrane translocation and caspase-3-dependent proteolytic cleavage (Kikkawa et al., 2002; Brodie and Blumberg, 2003; Kanthasamy et al., 2003). Of the two activation mechanisms, PKCδ activated by membrane translocation following tonic stimulation by lipid activators, contributes to cell survival and proliferation (Kikkawa et al., 2002; Kanthasamy et al., 2003; Jackson and Foster, 2004). As demonstrated in our recent studies, another form of activation is caspase-3-dependent proteolytic cleavage of native
PKCδ into regulatory and catalytic fragments, resulting in persistent activation during exposure to neurotoxic agents such as MPP⁺, MMT, manganese, or dieldrin (Anantharam et al., 2002; Kaul et al., 2003; Latchoumycandane et al., 2005). This form of proteolytic activation contributes to apoptotic cell death of dopaminergic neurons (Kanthasamy et al., 2003). The lipid activator TPA induced membrane translocation of native PKCδ, but did not induce apoptotic cell death in dopaminergic cell lines (unpublished observations). Furthermore, recent studies from our lab and others have shown that, unlike the native 74 kDa PKCδ, the 41 kDa catalytically active PKCδ fragment, upon proteolytic cleavage, is targeted to various sub-cellular organelles including the mitochondria (Majumder et al., 2000) and nucleus (DeVries et al., 2002) to activate apoptotic cell death signaling molecules. These results suggest that native and cleaved PKCδ have different substrate profiles.

Activation of PKC causes a decrease in DAT-mediated activity and cell surface expression. The activity of biogenic amine and amino acid neurotransmitters is limited by presynaptic and astrocytic Na⁺-dependent transport systems. Their functional importance is underscored by the observation that these transporters are the targets of broad classes of psychotherapeutic agents, including antidepressants and stimulants. The clearance of extracellular dopamine is apparently mediated by a single gene product. The activity and cell surface expression of the dopamine transporter (DAT) are decreased in response to PKC activation. In a series of studies, activation of PKC was shown to acutely decrease DAT activity and increase transporter trafficking of neurotransmitter transporters phosphorylation (Huff et al., 1997). Several different groups have reported similar effects of PKC on DAT activity and have provided compelling evidence that this effect is associated with internalization of the transporter protein in different cellular systems including *Xenopus* oocytes (Zhu et al., 1997; Pristupa et al., 1998; Daniels and Amara 1999; Melikian and Buckley 1999). The ultimate fate of internalized transporter, whether it is targeted for degradation or recycling to the plasma membrane, may be influenced by factors that have not yet been identified. PKC phosphorylates the D2 dopamine receptor (DAR) on multiple sites
within two domains of the third intracellular loop. This PKC-mediated phosphorylation of
the D2 DAR was demonstrated to promote both functional desensitization and internalization
of the receptor protein via a β-arrestin- and dynamin-dependent pathway. PKC can mediate
phosphorylation of the D2 DAR, producing novel functional effects. This regulatory event
appears to be specific for the βI isoform of PKC, although additional isoforms remain to be
tested.

**Puzzles and future directions:**

PKC-mediated regulation of TH, DAT, dopamine receptor, and other important
proteins in dopaminergic cells may have important implications in neurological dopaminergic
system disorders, such as Parkinson’s disease. But most studies investigating the protein
kinase C family have focused on general PKC, with little data generated on the different PKC
isoforms, such as PKCδ. A detailed understanding of the cellular actions of individual PKC
isoforms ultimately will require knowledge of their distinct cellular substrates. PKCδ
substrates traditionally have been identified using pharmacological strategies (with activators
or inhibitors) or molecular strategies (involving targeted deletion or overexpression of
individual PKC isoforms or translocation modifier peptides). Each of these approaches is
accompanied by its own distinct set of problems. For example, PMA may not strictly report
PKC actions, since high-affinity DAG/PMA-binding C1 domains have been identified in
proteins that lack kinase domains (and are unrelated to PKC). The chimaerins (a family of
Rac GTPase activating proteins), RasGRPs (Ras/Rap1 exchange factors), and Munc13
isoforms (scaffolding proteins involved in exocytosis) are examples of proteins that bind
PMA with nanomolar affinity and translocate to membranes in response to PMA. The
interpretation of studies with PMA can be confounded further by cross-talk between PKCs
and other members of the extended phorbol ester binding protein family. For example, recent
studies identified RasGRP3 (a guanine nucleotide exchange factor for Ras) as a binding
partner and substrate for PKCδ (Brodie et al., 2003). These proteins co-localize to similar
subcellular compartments and interact functionally (in an as yet poorly understood manner)
at the level of downstream signaling pathways such as ERK. Studies with PKC inhibitors can be even more problematic, since many commonly used PKC inhibitors lack the requisite specificity for PKC. For example, chelerythrine (which has been used widely as a general PKC inhibitor) induces apoptosis through a mitochondrial mechanism that is unrelated to PKC inhibition; chelerythrine is also reported to inhibit interactions of Bcl-XL with BH3 (Bcl-2 homology domain 3)-containing proteins such as Bax (Clerk et al., 2001; Chan et al., 2003; Yamamoto et al., 2001). Rottlerin (which has been touted to be a selective PKCδ inhibitor) uncouples mitochondrial respiration from oxidative phosphorylation and exerts inhibitory actions in PKCδ−/− cells; some studies even challenge the efficacy of rottlerin as an in vitro PKCδ inhibitor (Soltoff et al., 2001; Leitges et al., 2001). Finally, even Ro318220 and GF109203X (agents considered to be relatively selective PKC inhibitors) are reported to inhibit RSK (p90 ribosomal S6 kinase) and p70 S6 kinase (Alessi et al., 1997). These caveats emphasize that studies using PKC inhibitors as tools to investigate PKC function must be interpreted with caution, and that newer strategies to identify PKCδ targets are imperative. A novel chemical genetic approach developed by Shokat and colleagues (Shah et al., 2003) represents a very exciting methodological breakthrough that holds tremendous promise for the identification of endogenous PKC substrates in future studies. Their approach involves engineering the ATP-binding site in a kinase of interest so that it accepts a structurally modified γ-32P-labelled ATP analogue with a bulky substitution attached at the N6 position [for example N6-(benzyl)ATP or N6-(phenethyl)ATP]. Since the chemically modified γ-32P-labelled ATP analogue binds only the mutated enzyme’s active site (which carries an alanine or glycine in place of a conserved bulky residue), only unique substrates of the kinase of interest are labeled in in vivo cells. This strategy has been used to identify substrates of JNK, CDK2 (cyclin-dependent kinase 2), and v-Src. This viable strategy can identify the distinct substrates of allosterically activated PKCδ in membranes as well as tyrosine-phosphorylated PKCδ in the soluble fractions of cells exposed to oxidant stress.

Understanding the mechanisms that contribute to the regulation of dopamine function
by PKCδ and finding more PKCδ substrate protein has the potential to impact many different areas of neuroscience research and drug use. PKCδ may become a target of future drugs therapeutic for different brain diseases. As this relatively young field continues to mature, the mechanisms of this regulation and the impact of these diverse types of regulation on normal and abnormal brain function will be elucidated.

**Background and Literature Review II**

**Neuroprotective strategies in a Parkinson’s disease animal model**

Parkinson’s disease (PD) is a major neurodegenerative disorder characterized by substantial loss of dopaminergic neurons in the substantia nigra, resulting in irreversible motor symptoms consisting mainly of tremors, bradykinesia, and rigidity. The etiology of Parkinson’s disease is not known. Nevertheless, a significant body of biochemical data from human brain autopsy studies and from animal models points to an ongoing process of oxidative stress in the substantia nigra, which could initiate dopaminergic neurodegeneration. Although the pathology and clinical symptoms of PD are well defined, the cellular and molecular mechanisms underlying the selective degeneration of dopaminergic neurons remain elusive. Lack of such fundamental knowledge has severely hindered the development of neuroprotective strategies to circumvent the chronic progression of this debilitating neurodegenerative disorder. Much of our knowledge about dopaminergic neurodegeneration has come from studies employing two neurotoxins that produce animal models and Parkinsonism syndrome in rodents, primates, and other species. Both neurotoxins, namely 6-hydroxydopamine (6-OHDA) (Kostrzewa et al., 1974) and MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Burns et al., 1983; Chiueh et al., 1993) cause the degeneration of nigro-striatal dopaminergic neurons, with the subsequent loss of striatal dopamine (Table 1). Earlier studies with 6-OHDA indicate that this neurotoxin is a highly reactive substance, which is readily autooxidised and oxidatively deaminated by monoamine
oxidase to give rise to hydrogen peroxide and reactive oxygen species (ROS) (Cohen et al., 1974). This neurotoxin may exert its neurodegenerative action via OS (Glinka et al., 1996). The consequence of OS is the initiation of ROS generation, followed by brain membrane lipid peroxidation. The possibility that an endogenous toxin similar to a neurotoxin, such as 6-OHDA, is formed in the brain and involved in the neurodegeneration process has been envisioned (Kostrzewa et al., 1974).

Between 1979 and 1982, a severe and irreversible parkinsonian syndrome was diagnosed among young drug addicts in California. They have symptoms of bradykinesia, rigidity, and resting tremor closely resembled idiopathic Parkinson’s disease and these symptoms can be relieved by dopaminergic substitution therapy, as in typical Parkinson’s disease. Subsequently, MPTP, a meperidine analog, was recognized to be present as a by-product in designer drug preparations and to have caused the nigral degeneration underlying this new disorder. MPTP can cross the blood-brain barrier, and be converted to MPP⁺ in glial cells by monoamine oxidase B, and be taken up by catecholaminergic neurons via the dopamine reuptake system (dopamine transporter, DAT). Thus, specific MAO B inhibitors or catecholamine reuptake inhibitors are able to protect against MPTP toxicity. MPP⁺ is concentrated in the mitochondria according to the electrochemical gradient. Inside mitochondria, it inhibits the complex I of the respiratory chain, leading to ATP depletion and, eventually, to cell death of dopaminergic neurons (Zigmond and Stricker, 1989). It’s interesting that here also is a decrease in complex I activity in brain and other tissues in Parkinson’s disease. Apart from mitochondrial dysfunction, extramitochondrial damage by MPTP is suggested by the fact that cells without electron transport chain activity (Rho 0) still seem to be susceptible to MPP⁺ (Przedborski and Jackson-Lewis, 1998; Soldner et al., 1999). This finding was disputed, though, by others (Fall and Bennett, 1999). In the following years, it became evident that MPTP represents a highly useful tool to study dopaminergic degeneration modeling Parkinson’s disease in various species, including rodents and non-human primates (Burns et al., 1983; Heikkila et al., 1984).
Here are increasing controversies about the MPTP animal model. As shown in table 1, although MPTP model mimics PD patients, here are still some imperfections in MPTP model. First, in MPTP treated rodents the persistence of gross behavioral and motor features strongly depends on the dosing regimen. People found that it needs several years for the neurodegeneration happening in PD patients, but in MPTP animal model, this process occurs in several days. Second, In MPTP-treated animals, nigral cell death is paralleled by death in other catecholaminergic cell groups, such as the ventral tegmental area and locus coeruleus on a minor scale as in Parkinson’s disease (Seniuk et al., 1990). Third, lewy bodies, which are the characteristic of PD haven’t been observed in MPTP animal models (Forno et al., 1988). Safety considerations in handling MPTP are important (Przedborski et al., 2001).

Table 1 Deficiency of MPTP model

<table>
<thead>
<tr>
<th>Time for neurodegeneration occurring</th>
<th>PD</th>
<th>MPTP animal model</th>
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<tbody>
<tr>
<td><strong>Time for neurodegeneration occurring</strong></td>
<td>Slow progress, over several years</td>
<td>Within a few days after MPTP treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acute treatment (20mg/kg,i.p.,each 2h*4 doses)</td>
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<tr>
<td></td>
<td></td>
<td>Subchronic treatment(30mg/kg,i.p.,each 24h*5 days)</td>
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<tr>
<td></td>
<td></td>
<td>Chronic treatment (40mg/kg,s.c.,each 24h*30 days)</td>
</tr>
<tr>
<td><strong>Neurochemistry changes</strong></td>
<td>Dopamine deficiency</td>
<td>Dopamine deficiency</td>
</tr>
<tr>
<td><strong>Histology changes</strong></td>
<td>Cell degeneration mostly in substantia nigra</td>
<td>Cell degeneration in substantia nigra, ventral tegmental area and locus coeruleus</td>
</tr>
<tr>
<td></td>
<td>Lewy bodies found in substantia nigra</td>
<td>Lewy bodies not found</td>
</tr>
<tr>
<td><strong>Behavior changes</strong></td>
<td>Muscle rigidity, tremor, bradykinesia, akinesia</td>
<td>Tremor, bradykinesia only seen in acute MPTP treated animals</td>
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</table>

The discovery of MPTP and its neurodegenerative property gave the notion that an environmental toxin similar to it might be responsible for the onset of PD. However, no
similar toxin as MPTP has been identified as synthetic substance so far, either in the environment or in the brain. MPTP, similarly to 6-OHDA, is thought to initiate its dopaminergic neurotoxicity via metabolism catalyzed by monoamine oxidase (MAO), giving rise to its reactive metabolite MPP⁺. The latter is thought to begin the neurodegeneration process via ROS induced by OS and inhibition of mitochondrial complex I (Chiueh et al., 1992), as it produces sustained dopamine oxidation, hydroxyl radical formation, and membrane lipid peroxidation (Chiueh et al., 1992). Furthermore, both toxins induce an inflammatory process that results in proliferation of reactive microglia in the SNPC (McGeer et al., 1988).

**Table 2 Oxidative stress in 6-OHDA and MPTP-induced nigrostriatal degeneration**

<table>
<thead>
<tr>
<th>Biochemical variables which increase Reactive microglia proliferation</th>
<th>6-OHDA</th>
<th>MPTP</th>
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<tbody>
<tr>
<td>Lipid peroxidation</td>
<td>Kumar et al. 1995</td>
<td>Jennen 1998</td>
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<tr>
<td></td>
<td>Ogawa et al. 1994</td>
<td>Yousid et al. 1993</td>
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<tr>
<td>Biochemical indices which decrease Reduced glutathione (GSH) Ratio of GSH to oxidized</td>
<td>Perumal et al. 1989</td>
<td>Perumal et al. 1989</td>
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<td></td>
<td>Pearce et al. 1997</td>
<td>Tohgi et al. 1995</td>
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<tr>
<td>Calcium binding proteins</td>
<td>Gerfen et al. 1987</td>
<td>German et al. 1992</td>
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<td>Iacopino et al. 1992</td>
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<tr>
<td>Mitochondrial complex I (NADPH oxidase) activity</td>
<td>Glinka et al. 1995</td>
<td>Mizuno et al. 1988</td>
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It is assumed that the induction of the inflammatory process is related to reactive microglia because of its ability to generate substantial amounts of ROS. Similar in parkinsonian brains, where a considerable increase in the inflammatory cytokines interleukin (IL)-1b, IL-2, IL-6, and tumor necrosis factor (TNF)-a (Mogi et al., 1994) was observed. It is not known whether oxidative stress is a primary or secondary event. Oxidative stress induced
by MPTP has been used in animal models to investigate the neurodegenerative process to facilitate the development of antioxidant, neuroprotective drugs (Johannessen et al., 1986). It is apparent that radical scavengers, iron chelators, dopamine agonists, nitric oxide synthase inhibitors, and certain calcium channel antagonists provide neuroprotection against such toxins if given prior to the insult in these animal models. Furthermore, an inflammatory process was found from human and animal studies, which can express itself as proliferation of activated microglia in the substantia nigra, activation and translocation of transcription factors and neurotrophic factor (NF), and kappa-b and elevation of cytotoxic cytokines, tumor necrosis factor (TNF)-a, interleukin (IL)-1b, and IL-6 (McGeer et al., 1997; Kurkowska et al., 1999; Kim et al., 2000; Knott et al., 2000). Both radical scavengers and iron chelators prevent lipopolysaccharide (LPS) and iron-induced activation of NF kappa-b. If an inflammatory response is involved in Parkinson’s disease, antioxidants and the newly developed, non-steroidal, anti-inflammatory drugs such as cyclo-oxygenase (COX2) inhibitors would be considered as a form of treatment. However, there has been little or no success in the clinical treatment of neurodegenerative diseases (for example, Parkinson’s disease, ischemia, etc.) in which neurons die, while in animal models the same drugs provide neuroprotection. This may indicate that either the animal models employed do not reflect the events in neurodegenerative diseases, or that because neuronal death involves a cascade of events, a single neuroprotective drug is not effective. Thus, consideration should be given to multi-neuroprotective drug therapy in Parkinson’s disease, similar to the approach taken in AIDS and cancer therapy.

The treatment of Parkinson’s disease can be both rewarding and challenging. Unfortunately, no proven “neuroprotective” agents have been identified, and treatment remains symptomatic only. Treatment options for Parkinson’s disease have greatly expanded in recent years. Most PD treatment strategies are on pharmacologic agents, but exercise and lifestyle changes have been shown to be helpful as well. When medications are no longer able to provide adequate relief, neurosurgical options in the form of deep brain stimulation of
the subthalamic nucleus or the internal segment of the globus pallidus are playing an increasingly important role in symptomatic treatment. Pharmacological treatments, such as levodopa, dopamine receptor agonists, antioxidants, anti-oxidative stress, anticholinergic medications, monoamine oxidase B inhibitors, and the catechol-O-methyl transferase inhibitors, remain the mainstay of therapeutic intervention and are reviewed. Despite the great efficacy of levodopa, “levodopa-sparing strategies” in early Parkinson’s disease are emphasized in order to delay the development of difficult-to-manage motor fluctuations and dyskinesias. In the paragraphs that follow, a review of currently available pharmacotherapeutic treatment options for the Parkinson’s animal models will be presented.

**Dopamine receptor agonist**

It is generally believed that treatment with dopamine receptor agonists is symptomatic rather than curative, these drugs normally are used as adjunctive therapy for treatment of PD., and does not stop or delay the progression of neuronal degeneration. However, recent studies showed that several DA agonists of the DA D2–receptor family (including D2, D3 and D4-subtypes) possess neuroprotective properties in different *in vitro* and *in vivo* experimental PD models (Grilli et al., 1999; Le et al., 2000; Uberti D, Abramova et al., 2002; Piccioni et al., 2002; Uberti et al., 2004; Fujita et al., 2006).

Apomorphine has been introduced in the treatment of late-stage Parkinson's disease (PD). The disadvantage of a short half-life of apomorphine is now overcome by the use of a continuous subcutaneous (s.c.) self-delivering system. (R)-Apomorphine (5-10 mg/kg, s.c.) pretreatment in C57BL mice protects against MPTP (24 mg/kg, i.p.) induced loss of nigro-striatal dopamine neurons, as indicated by striatal dopamine content, tyrosine hydroxylase content, and tyrosine hydroxylase activity (Grünblatt et al., 1999). Apomorphine was continuously infused in mice by means of a s.c. minipump that delivered the drug at a rate of 3.15 mg/kg/day. At 40 h following a 30 mg/kg MPTP injection 30% DA loss was prevented and striatal dopaminergic terminals were rescued (Battaglia et al., 2002). Pramipexole (SND 919; 2-amino-4,5,6,7-tetrahydro-6-propyl- amino- benzthiazole-
dihydrochloride) is a potent and selective dopamine D2/D3 receptor agonist. Co-administration of 1.0 mg/kg Pramipexole (s.c.) and 30 mg/kg MPTP to mice, followed by 24 and 48 h of additional Pramipexole treatment, significantly attenuated MPTP-induced striatal DA loss, with a 50% protection (Ramirez et al., 2003). Pramipexole treatment also significantly attenuated the loss of tyrosine hydroxylase immunoreactive neurons (TH-IR) within the substantia nigra pars compacta (SNc) in both young and aged animals. Low dose Pramipexole (0.1 mg/kg/day*5days) down regulates the dopamine transporter via the D3 receptor (Joyce et al., 2004). Another dopamine agonist, bromocriptine(10 mg/kg i.p.), blocked ·OH formation caused by MPTP (30 mg/kg i.p.). Bromocriptine blocked MPTP as well as 6-OHDA-induced behavioral dysfunction as well as glutathione and dopamine depletion, indicating its potent neuroprotective action (Archer et al., 1994; Ogawa et al., 1994). One study checked the combined effect of bromocriptine (BRC) and Hypericum perforatum extract (HPE), which is a natural oxidant, and got more than 40% protection against MPTP induced dopamine loss (Mohanasundaria et al., 2006). SKF-38393 (10 mg/kg), a dopamine receptor agonist, blocked the MPTP-induced depletion of glutathione and attenuated MPTP-induced depletion of dopamine (a 40% protection) (Muralikrishnan et al., 2001).

**MAO inhibitor**

The antiparkinsonian drug l-selegiline (l-deprenyl), an irreversible MAO-B inhibitor, was the first neuroprotective agent to prevent MPTP-induced Parkinsonism in mice and non-human primates. The mechanism of this process has been explained as l-selegiline inhibition of MAO-B, to prevent metabolism of the neurotoxin to the reactive metabolite, MPP+, and the formation of hydrogen peroxide. This explanation appears to be too simple, as selegiline was shown to prevent, as well as to rescue, cultured nigral neurons from the induced oxidative damage caused by the reactive metabolite MPP+. Selegiline can rescue neurons after they have sustained lethal damage and the rescue is independent of MAO-B inhibition. C57BL mice were treated with MPTP and then treated with selegiline 72 h after
the MPTP treatment to allow for complete conversion of MPTP to MPP+ and for maximal
dSNn damage induced by MPP+. The delayed selegiline treatment rescued approximately
69% of the dSNns that had not died by the time the treatment began (the neurons died in the
saline control group) (Tatton et al., 1993). (R)-[(N-Propargyl-(3R) aminoindan-5-yl) ethyl
methyl carbamate] (TV3326) is a novel cholinesterase and brain-selective monoamine
oxidase (MAO)-A/-B inhibitor. Chronic treatment (150 mol/kg i.p) in mice gave 90%
protection against striatal dopamine depletion induced by MPTP and prevented the reduction
in striatal tyrosine hydroxylase activity, like selective B and non-selective MAO inhibitors.
TV3326 preferentially inhibits MAO-B in the striatum and hippocampus, and the degree of
MAO-B inhibition correlates with the prevention of MPTP-induced dopamine depletion
(Sagi et al., 2003). MAO-B inhibitors, such as L-deprenyl, prevent MPTP-toxicity in
different species, and have been used in Parkinson therapy (Wiener et al., 1989; Nakashima
et al., 1991; Othblat et al., 1998; Castagnoli et al., 1999; Tatton et al., 1999; RSteyn et al.,
2001). However, it is metabolized to (-)-methamphetamine, and new MAO-B inhibitors
without a structural amphetamine moiety are needed. (Perez et al., 2003) reported a novel
non-amphetamine-like MAO-B inhibitor, PF 9601N or N-(2-propynyl)-2-(5-benzyloxy-indolyl)
methylamine. This attenuates the MPTP-induced striatal dopamine
depletion in young-adult and adult-old C57/BL mice, using different schedules of
administration. The intraperitoneal (i.p.) co-administration to young-adult C57/BL6 mice of
MPTP (30 mg/kg) and different concentrations of PF 9601N or L-deprenyl (29.5-0.357
micromol/kg) showed a dose-dependent protective effect against striatal dopamine depletion.
Lower doses of PF 9601N (1.5 micromol/kg) were necessary to get almost total protection,
without any change in the DOPAC and HVA content, when administered 2 h before MPTP
(30 mg/kg), whereas partial protection (45%) against dopamine depletion was observed in the
case of L-deprenyl. When adult-old (8-10 months) C57/BL6 mice were used, MPTP (25
mg/kg) administration induced 25 days later irreversible dopamine depletion. Under these
conditions, chronic administration with 0.15 micromol/kg of PF 9601N, before the toxin,
every 24 h for 10 days, rendered almost total protection against dopamine depletion, whereas L-deprenyl yielded only 50% protection of the dopamine content, assayed under the same conditions. It is worth remarking that in both cases MAO-B was not affected. These results indicate that PF 9601N attenuates MPTP neurotoxicity in vivo better than L-deprenyl through different mechanisms, with special relevance to the protective effect, independent of MAO-B inhibition, observed in the irreversibly MPTP-lesioned adult-old mice.

**Anti-oxidative stress**

Recent investigations in animal models and in post-mortem human brain tissues have demonstrated that apoptosis is an important mode of cell death in nigral dopaminergic degeneration (Przedborski et al., 1998; Jellinger et al., 2000; Hartmann et al., 2001; Kanthasamy et al., 2003; Tatton et al., 2003). Oxidative damage has been unambiguously identified as contributing to dopaminergic cell death in Parkinson’s disease and in the MPTP model. Increases in markers of oxidative stress, such as malondialdehyde, protein carbonyls, 8-hydroxy-2-deoxyguanosine, and lipid peroxides were noted in PD substantia nigra, but also in other brain regions (Jenner et al., 1998). Morphological hallmarks encompass internucleosomal DNA cleavage, chromatin margination and condensation, preservation of membrane integrity and of cellular organelles (mitochondria, endoplasmic reticulum), membrane blebbing and cellular shrinkage as well as the formation of membrane-enclosed apoptotic bodies. Typically, there is a lack of inflammatory changes around apoptotic cells as opposed to necrosis (Mattson et al., 2000). A method of showing apoptotic cell death relies on the demonstration of caspase cleavage. Two major pathways leading to caspase activation have been characterized (Kaufmann and Hengartner, 2001). One involves the activation of death receptors on the cell surface, e.g., Fas/CD95 or TNFα. They share a common motif, the death domain, necessary for the binding of cytoplasmic adaptor molecules, e.g., FADD or TRADD. Through involvement of a common death effector domain, the prodomain of procaspase-8 is bound, bringing about the autocatalytic activation of the enzyme. This, in turn, leads to the activation of downstream effector caspases, directly or via a mitochondrial
pathway as an amplification step (Fig. 1). The mitochondrial pathway involves the release of cytochrome C from mitochondria, forming a ternary complex with procaspase-9 and with the scaffolding protein Apaf-1 and procaspase-9. Along with cytochrome C, other proapoptotic effectors, such as Smac/Diablo or apoptosis-inducing factor (AIF), are released from mitochondria into the cytosol. In the ternary complex, the so-called apoptosome, caspase-9 activation can take place. Again, in further steps effector caspases, such as caspase-3, are activated (Fig. 1) and are responsible for the cleavage of at least 200 protein substrates involved in the degenerative process.

Minocycline, a semisynthetic tetracycline, has neuroprotective effects in an MPTP mouse model of Parkinson's disease. The neuroprotective effect of minocycline is associated with marked reductions in inducible NO synthase (iNOS) and caspase 1 expression. Minocycline at 90 and 120 mg/kg significantly protects TH-positive neurons from death induced by MPTP exposure, and minocycline pretreatment had a similar “protective” effect on striatal dopamine levels (120mg/kg minocycline showed 60% protection) after acute MPTP administration (Du et al., 2001).

Figure 1. Pathways implicated in MPTP-mediated toxicity

(Eberhardt and Schulz, 2003)
Additional studies in primary mesencephalic cultures provided evidence that caspase inhibition by zVAD-fmk rescued the tyrosine hydroxylase-positive somata, but not their neurites and synapses from MPP+ and 6-hydroxydopamine-induced toxicity (Eberhardt et al., 2000). A novel peptidyl broad-spectrum caspase inhibitor, Q-VD-OPH, which offers improvements in potency, stability, and toxicity over zVAD-fmk, showed significant protection against MPTP, 3-nitropropionic acid (3NP), and malonate toxicities. 20 mg/kg daily Q-VD-OPH significantly reduced 30% dopamine depletion in striatum produced by MPTP administration and prevented 50% of MPTP-induced loss of dopaminergic neurons in the substantia nigra. Western blots performed on tissues from the midbrain showed that Q-VD-OPH inhibited increases of the active forms of caspase-9 and caspase-8, as well as the caspase-8-mediated proapoptotic protein Bid induced by MPTP (Yang et al., 2004). Reportedly neuronal nitric oxide synthase (nNOS) inhibitor, 7-nitroindazole, can protect against MPTP neurotoxicity in mice. 50mg/kg 7-nitroindazole almost completely protected against both dopamine depletions and tyrosine hydroxylase (TH) positive neuron decreases in the mouse brain in an acute MPTP model (i.p. four times with 10 mg/kg MPTP at 1-h intervals) (Watanabe et al., 2004). Bis-ethylthiomethyl analog of K-252a, CEP-1347/KT-7515, an inhibitor of c-jun N-terminal kinase activation, attenuates the MPTP-mediated loss of nigrostriatal dopaminergic neurons in vivo (Saporito et al., 1999). The neuronal survival properties of CEP-1347/KT-7515 may be related to its ability to inhibit the activation of c-jun N-terminal kinase, a key kinase in some forms of stress-induced neuronal death and perhaps apoptosis (Ham et al., 1995; Xia et al., 1995; Maroney et al., 1998). In the low dose MPTP model (20 mg/kg), CEP-1347/KT-7515 (0.3 mg/kg/day) attenuated the MPTP-mediated loss of striatal dopaminergic terminals by 50%. In the high MPTP dose model (40 mg/kg), CEP-1347/KT-7515 ameliorated the loss of dopaminergic cell bodies by 50% and partially preserved striatal dopaminergic terminals. CEP-1347/KT-7515 did not inhibit monoamine oxidase B or the dopamine transporter, suggesting that the neuroprotective effects of CEP-1347/KT-7515 occur downstream of the metabolic conversion
of MPTP to MPP+ and accumulation of MPP+ into dopaminergic neurons (Saporito et al., 1999).

Recent studies from our laboratory demonstrated that the protein kinase C (PKC) delta isoform is an oxidative stress-sensitive kinase and a key mediator of apoptotic cell death in PD models (Kaul et al., 2003; Yang et al., 2004). In an attempt to translate the mechanistic studies to a neuroprotective strategy targeting PKC delta, we systematically characterized the neuroprotective effect of the PKC delta inhibitor rottlerin. Administration of rottlerin, either intraperitoneally or orally, to C57 black mice showed significant protection against MPTP-induced locomotor deficits and striatal depletion of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid. Notably, rottlerin post-treatment was effective even when MPTP-induced depletion of dopamine and its metabolites was greater than 60%, demonstrating its neurorescue potential. Furthermore, the dose of rottlerin used in neuroprotective studies effectively attenuated the MPTP-induced PKC delta kinase activity. Importantly, stereological analysis of nigral neurons revealed rottlerin treatment significantly protected against MPTP-induced TH-positive neuronal loss in the substantia nigra compacta (Fig. 2) (Zhang et al., 2007).

Figure 2. Stereological evaluation of neuroprotective effect of rottlerin on number and morphology of SNpc neurons in brains of MPTP-treated mice

(Zhang et al., 2007)
Antioxidants, iron chelators and neurotrophic factors

Melatonin, N-acetyl-5-methoxytryptamine, is an effective free radical scavenger and antioxidant (Reiter et al., 2000). Moreover, melatonin significantly inhibits the generation of ·OH in the brain following hypoxic or ischemic stimulation *in vivo* and *in vitro* (Li et al., 1997). The neuroprotective role of melatonin is apparent in terms of its antioxidant effect as previously reported. Although melatonin prevents some pathological changes induced by MPTP in brain (Acuna-Castroviejo et al., 1997; Jin et al., 1998), studies investing protection against dopamine loss are not promising. Green tea containing high levels of (-)-epigallocatechin 3-gallate (EGCG) showed protective effects in a PD animal model (Levites et al., 2001; Choi et al., 2002; Mandel et al., 2004). Both tea and the oral administration of EGCG prevented the loss of tyrosine hydroxylase (TH)-positive cells in the substantia nigra (SN) and of TH activity in the striatum. 25 mg/kg of EGCG preserved 40% striatal levels of dopamine and its metabolites compared with the MPTP treatment group (20 mg/kg, twice/day *5days). Both tea and EGCG decreased expression of nNOS in the substantia nigra (Choi et al., 2002). Moderate elevation of brain VE is not adequate for protecting DA-containing neurons against the toxic actions of a high dose of MPTP (20 mg kg⁻¹, i.p. x 3, 2 h intervals) (Gong et al., 1991).

Iron has been shown to accumulate at sites where neurons degenerate in neurodegenerative diseases like Parkinson's disease. Iron is thought to participate or initiate oxidative stress via generation of reactive oxygen species (ROS), such as hydroxyl radical. Iron chelators are neuroprotective and prevent 6-hydroxydopamine and MPTP dopaminergic neurotoxicity in rats and mice (Youdim et al., 2004, Kooncumchoo et al., 2006, Youdim et al., 1999, Kaur et al., 2003). In an iron-loaded (carbonyl iron, 25 g iron/kg diet) MPTP (30mg/kg, i.p.)-treated mouse model, desferrioxamine inhibits the iron accumulation and thus reverses the increase in oxidized glutathione (GSSG), oxidized to reduced glutathione ratios, ·OH and lipid peroxidation levels. The striatal dopamine concentration was elevated to normal value. Pretreatment with the bioavailable metal chelator clioquinol (CQ) (30 mg/kg, p.o., 8 weeks)
protected against MPTP-mediated oxidative stress and dopaminergic cell loss in an acute MPTP treatment model, with 50% protection against dopamine loss (Kaur et al., 2003).

Several lines of evidence also suggest that steroids have neuroprotective properties. 17β-Estradiol was shown to protect neurons in vitro against death from a variety of stressors (Behl et al., 1995; Green et al., 1997). Hence, it could act as a free-radical scavenger or also have a direct modulatory effect on glutamate receptors (Weaver et al., 1997). Administration of 17β-estradiol to C57Bl/6 MPTP mice prevented depletion of striatal DA concentrations (Dluzen et al., 1996; Miller et al., 1998; Callier et al., 2000) and the glial fribillary acid protein elevation induced by the neurotoxin (Miller et al., 1998). In C57Bl/6 MPTP mice, treatments with 17β-estradiol, progesterone, or raloxifene, a selective estrogen receptor modulator (SERM), prevent the depletion of striatal DA concentrations induced by MPTP by more than 50%, whereas 17β-estradiol (the isomer with weak estrogenic activity on estrogen receptors) does not (Grandbois et al., 2000).

**Adenosine receptor antagonists**

Adenosine is a ubiquitous neuromodulator in the central nervous system. Its major role in the central nervous system is to modulate neurotransmitter release, the postsynaptic components, and also the nonsynaptic components such as glial cell signaling. Adenosine exerts these diverse physiological actions through activation of specific G protein-coupled receptors termed A1, A2A, A2B, and A3 (Fredholm et al., 1994). Adenosine A2A receptors are specifically localized in the striatum (Svenningsson et al., 1999), where they are coexpressed with dopamine D2 receptors in the GABAergic striatopallidal neurons; in contrast, there are no A2A receptors in the neurons projecting from the striatum to the substantia nigra that express D1 receptors (Ferre et al., 1997). Stimulation of adenosine A2A receptors decreases the binding affinity of D2 receptors (Ferré et al., 1991), and it elicits effects opposite of D2 receptor activation at the level of second-messenger systems and early gene expression (Svenningsson et al., 1999). These data suggest that antagonistic adenosine-dopamine interactions may regulate basal ganglia activity and that they could explain the depressant and
stimulating effects of adenosine A2A receptor agonists and antagonists on motor behavior (Ferre et al., 1997). Adenosine receptor antagonists, including caffeine and related methylxanthines, produce motor stimulant effects, which seem to be related to an action on A2A, rather than A1, receptors (Fredholm et al., 1999). Caffeine may attenuate MPTP neurotoxicity through dual actions of monoamine oxidase inhibition and A2A receptor antagonism (Chen et al., 2002; Xu et al., 2002; Schwarzschild et al., 2003). And estrogen can prevent neuroprotection by caffeine in the mouse MPTP model of Parkinson's disease (Xu et al., 2006). The A2A-selective antagonist KW-6002 (istradefylline) exhibits antiparkinsonian activities in experimental models of PD (Kanda et al., 1998; Shiozaki et al., 1999; Koga et al., 2000; Chen et al., 2001; Pierri et al., 2005), and is in clinical development. More recently, other adenosine A2A antagonists, SCH-420814 and BIIB014/V2006, have entered clinical development as antiparkinsonian drugs. These findings support a role for A2A receptors as neuromodulators of dopaminergic function, and they suggest that they may play an important role in movement disorders such as PD. In addition, antagonism of adenosine A2A receptors exerts dual actions on motor dysfunction and neurodegenerative processes in animal models of PD (Ikeda et al., 2002). Consistent with its pharmacology, A2A receptor knockout mice are resistant to both motor impairment and neurochemical changes relevant to neurodegenerative disorders such as PD (Ledent et al., 1997; Chen et al., 2001). Hence, adenosine A2A receptor antagonists may represent a novel therapeutic approach to pathologies characterized by neurodegenerative events, since they both reverse motor impairment and are neuroprotective. A novel adenosine A1 and A2A dual antagonist, 5-[5-amino-3-(4-fluorophenyl) pyrazin-2-yl]-1-isopropylpyridine-2(1H)-one (ASP5854), dose-dependently improved the reduction of striatal DA content by MPTP administration (Mihara et al., 2007). The percentages of recovery of DA content from the vehicle-treated control level were 7.09, 21.11, 38.78, and 60.41 at doses of 0.01, 0.032, 0.1, and 0.32 mg/kg, respectively, with statistical significance at doses of 0.1 mg/kg and higher. The specific adenosine A2A antagonist 8-(E)-2-(3,4-dimethoxyphenyl)
ethenyl)-1,3-diethyl-7-methyl-3,7-dihydro-1H-purine-2,6-dione (KW-6002; istradefylline) similarly restored the striatal DA content reduced by MPTP treatment in a dose-dependent manner. The percentages of recovery in DA contents at doses of 0.1, 0.32, 1, and 3.2 mg/kg were 22.91, 28.37, 38.79, and 42.45%, respectively (Mihara et al., 2007).

**Anticholinergics and dopamine uptake blockers**

MPTP in mice induces a decrease in dopamine and an increase in acetylcholine in the neostriatum. Both responses to MPTP can be blocked by prior treatment with atropine or trihexyphenidyl (Hadjiconstantinou et al., 1985). Pretreatment with 3-(10,11,-dihydro-5H-dibenzo-[a,d]-cycloheptan-5-ylidene)-1-ethyl- 2- methylpyrrolidine (piroheptine), an anticholinergic drug which also inhibits dopamine uptake, completely prevented loss of striatal dopamine in MPTP-treated mice. Trihexyphenidyl partially protected against the neurotoxicity of MPTP. However, clomipramine, a selective 5-hydroxytryptamine uptake inhibitor, did not prevent the loss of striatal dopamine. Piroheptine is another agent which was found to prevent MPTP neurotoxicity (Saitoh et al., 1988). Dopamine uptake blockers protect against the dopamine depleting effect of MPTP in the mouse striatum; a series of DA uptake blockers was tested for their ability to prevent this effect of MPTP. The agents tested (amfonelic acid, benztropine, bupropion and mazindol) completely protected against DA depletion in the mouse striatum when given before DA-depleting doses of MPTP were administered, whereas atropine and trihexyphenidyl (which were employed for comparative purposes) did not. DA uptake blocking agents appear to represent a second general class of compounds, monoamine oxidase inhibitors being the first, which protect against the biologic effects of MPTP in the mouse (Ricaurte et al., 1985).

**Anti-inflammatory aspirin, salicylic acid**

Anti-inflammatory drugs such as salicylic acid and acetylsalicylic acid (ASA) were protective against MPTP-induced striatal dopamine depletion in mice (Aubin et al., 1998; Ferger et al., 1999). Chuieh and others have demonstrated that MPTP results in the formation of the highly toxic hydroxyl radical *in vivo*. Hydroxyl radical formation was monitored in
dialysis studies by the salicylate detection method (Chiueh et al., 1993; Obata and Chiueh, 1992). Hydroxyl radicals react with salicylate to form 2,3- and 2,5-dihydroxybenzoic acid (DHBA). MPTP increases the formation of both 2,3- and 2,5-DHBA \textit{in vivo}. If hydroxyl radicals are interacting with salicylate, then of course salicylate is also mopping up hydroxyl radicals. If indeed these are the agents through which MPTP exerts its neurotoxicity, Aubin et al. (1998) found that salicylate totally protects against the neurotoxic effects of MPTP and that this property is shared by acetylsalicylate (aspirin) and a lysine salt formulation of aspirin, Aspegic, but not by other cyclooxygenase inhibitors. The neurotoxic effects of the dopamine-selective neurotoxin MPTP (15 mg/kg, s.c.), in mice, were totally prevented by systemic administration of salicylate (ED$_{50}$ = 40 mg/kg, i.p.), aspirin (ED$_{50}$ = 60 mg/kg, i.p.), or the soluble lysine salt of aspirin, Aspegic (ED$_{50}$ = 80 mg/kg, i.p.) (Aubin et al., 1998). As many pathophysiological conditions induce COX-2 (Masferrer et al., 1992; Herschman, 1996), the impact of COX-2 seems to be higher in PD than that of the constitutive isoenzyme COX-1. Overexpression of neuronal COX-2 in mice potentiated excitotoxicity, suggesting a possible role of COX-2 in neurodegenerative diseases (Kelley et al., 1999). Additionally, recent results show evidence of chronic inflammation in humans who were exposed to MPTP up to 14 years ago (Langston et al., 1999). Different pathways lead to the activation of COX-2. TNF-alpha activates COX-2 via the JNK pathway (Westwick et al., 1994; Adams et al., 1996) and via induction of NF-kB (Yamamoto et al., 1998). NF-kB is a transcription factor for COX-2 and iNOS (Baeuerle and Baichwal, 1997) and was found to be enhanced 70-fold in parkinsonian brains (Hunot et al., 1997). Inductors for NF-kB were, besides others, ROS and pro-inflammatory cytokines (Baeuerle and Baichwal, 1997). MPTP/ MPP1 administration enhanced ROS production (Adams et al., 1993; Sriram et al., 1997; Ferger et al., 2000) as well as iNOS expression (Liberatore et al., 1999), which is able to enhance COX-2 activity (Nogawa et al., 1998). Different mechanisms involved in MPTP toxicity and probably in the pathophysiology of PD could be able to activate COX-2. Teismann et al. showed that dopamine depletion induced by a single administration of MPTP (30 mg/kg) was significantly
attenuated to 37.1% and 38.6% of saline control values by acetylsalicylic acid (50 and 100 mg/kg) and to 36% and 40% by meloxicam (7.5 and 50 mg/kg), respectively (Teismann et al., 2001).

**Conclusion:** The principle of utilizing levodopa-sparing strategies to delay the development of motor fluctuations and minimize dyskinesias has had a major impact on maintaining long-term symptomatic control with medications in Parkinson’s disease. It is now well documented that, when appropriate, dopamine receptor agonists combined with MAO-B inhibitors, amantadine, and anticholinergic drugs provide excellent symptomatic relief for a number of years before the utilization of levodopa is required. The MPTP model still constitutes the best characterized toxin paradigm for Parkinson’s disease, faithfully replicating most of its clinical and pathological hallmarks. Many lines of evidence point to a significant contribution of apoptosis to cell death after application of MPP in cell culture or MPTP *in vivo*. This holds true for apoptotic DNA strand breaks, activation of the JNK pathway and caspases, induction of Par-4 protein, and the protection conferred by interference with p53, Apaf-1, or Bax signaling. In MPTP models, intervention in upstream events in apoptosis, e.g., inhibition of the JNK pathway, provides morphological and functional rescue. In contrast, inhibition of the propagation and execution phase of apoptosis, e.g., by inhibition of caspases, blocks or delays cell death but may not recover neuronal function. At this stage, the combination of an anti-apoptotic together with a neurorestorative therapy may be promising. Here is a list of neuroprotective reagents in table 3.

**Table 3  List of neuroprotective reagents**

<table>
<thead>
<tr>
<th>Properties</th>
<th>Drugs</th>
<th>Doses</th>
<th>% of DA recovery</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA receptor agonist</td>
<td>Apomorphine</td>
<td>3 mg/kg, s.c.</td>
<td>30%</td>
<td>Battaglia et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Pramipexole</td>
<td>1.0 mg/kg, s.c.</td>
<td>50%</td>
<td>Ramirez et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Bromocriptine</td>
<td>10 mg/kg, i.p.</td>
<td>40%</td>
<td>Mohanasundaria et al., 2006</td>
</tr>
<tr>
<td></td>
<td>SKF-38393</td>
<td>10 mg/kg, i.p.</td>
<td>40%</td>
<td>Muralikrishnan et al., 2001</td>
</tr>
</tbody>
</table>
Table 3 (continued)

<table>
<thead>
<tr>
<th>Properties</th>
<th>Drugs</th>
<th>Doses</th>
<th>% of DA recovery</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAO inhibitor</strong></td>
<td>Selegiline</td>
<td>10 mg/kg, i.p.</td>
<td>90%</td>
<td>Takahata et al., 2003</td>
</tr>
<tr>
<td></td>
<td>TV3326</td>
<td>150 μM/kg, i.p.</td>
<td>90%</td>
<td>Sagi et al., 2003</td>
</tr>
<tr>
<td></td>
<td>L-deprenyl</td>
<td>1.5 μM/kg, i.p.</td>
<td>45%</td>
<td>Perez et al., 2003</td>
</tr>
<tr>
<td></td>
<td>PF 9601N</td>
<td>0.15 μM/kg, i.p.</td>
<td>99%</td>
<td>Perez et al., 2003</td>
</tr>
<tr>
<td><strong>Anti-oxidative stress</strong></td>
<td>Minocycline</td>
<td>120 mg/kg, i.p.</td>
<td>60%</td>
<td>Du et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Q-VD-OPH</td>
<td>20 mg/kg, i.p.</td>
<td>30%</td>
<td>Yang et al., 2004</td>
</tr>
<tr>
<td></td>
<td>7-nitroindazole</td>
<td>50 mg/kg, i.p.</td>
<td>99%</td>
<td>Watanabe et al., 2004</td>
</tr>
<tr>
<td></td>
<td>CEP-1347</td>
<td>0.3 mg/kg, i.p.</td>
<td>50%</td>
<td>Saporito et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Melatonin</td>
<td>15 mg/kg, i.p.</td>
<td>25%</td>
<td>Li et al., 2002</td>
</tr>
<tr>
<td></td>
<td>17b-Estradiol</td>
<td>2 mg/daily, s.c.</td>
<td>90%</td>
<td>Callier et al., 2000</td>
</tr>
<tr>
<td><strong>Antioxidants</strong></td>
<td>EGCG</td>
<td>25 mg/kg, i.p.</td>
<td>40%</td>
<td>Choi et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Vitamine E</td>
<td>7 g/kg diet</td>
<td>100%</td>
<td>Lan et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Coenzyme Q</td>
<td>1600 mg/kg diet</td>
<td>45%</td>
<td>Cleren et al., 2008</td>
</tr>
<tr>
<td><strong>Iron chelators</strong></td>
<td>Clioquinol</td>
<td>30 mg/kg, p.o</td>
<td>50%</td>
<td>Kaur et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Cytisine</td>
<td>2.0 mg/kg, s.c.</td>
<td>50%</td>
<td>Ferger et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Desferrioxamine</td>
<td>250 mg/kg, i.p.</td>
<td>100%</td>
<td>Lan et al., 1997</td>
</tr>
<tr>
<td><strong>Neurotrophic</strong></td>
<td>3-Hydroxymorphinan</td>
<td>24 mg/kg, i.p.</td>
<td>90%</td>
<td>Zhang et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Caffeine</td>
<td>20 mg/kg, i.p.</td>
<td>40%</td>
<td>Xu et al., 2002</td>
</tr>
<tr>
<td></td>
<td>LY404187</td>
<td>0.5 mg/kg s.c.</td>
<td>25%</td>
<td>O'Neil et al., 2004</td>
</tr>
<tr>
<td><strong>Adenosine receptor antagonists</strong></td>
<td>ASP5854</td>
<td>0.32 mg/kg</td>
<td>60%</td>
<td>Mihara et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Istradefylline</td>
<td>3.2 mg/kg</td>
<td>42%</td>
<td>Mihara et al., 2007</td>
</tr>
<tr>
<td><strong>Anti-inflammatory</strong></td>
<td>salicylic acid</td>
<td>40 mg/kg, i.p.</td>
<td>99%</td>
<td>Aubin et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Meloxicam</td>
<td>50 mg/kg</td>
<td>40%</td>
<td>Teismann et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Aspirin</td>
<td>60 mg/kg, i.p.</td>
<td>99%</td>
<td>Aubin et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Dextromethorphan</td>
<td>25 mg/kg, i.p.</td>
<td>50%</td>
<td>Vaglini et al., 2003</td>
</tr>
</tbody>
</table>

**Puzzles and future directions:** The current therapeutic approach to the treatment of PD is symptomatic, and the most commonly used treatment is dopamine replacement therapy with levodopa or dopamine receptor agonists (bromocriptine, lisuride, pergolide and
apomorphine) or increasing the availability of brain dopamine by administering inhibitors of dopamine metabolic enzymes (MAO-A and -B and catechol O-methyltransferase). If neuroprotective therapy is to be realized in PD, a much better understanding of the biochemical pathology of the ongoing progressive dopaminergic degeneration is required.

Intriguingly, in humans a presymptomatic phase of 5 years duration before overt parkinsonian features appear has been estimated from functional imaging and pathological studies. In a normal population a nigral cell loss of 4.4% is assumed to occur, while in PD patients the loss may be up to 10-fold higher (Dunnett et al., 1999). The therapeutic challenge might lie in the identification of the proper time-point for treatment onset, and optimally at a presymptomatic stage. Toxin models of Parkinsonism will probably help clarify this point. Furthermore, future novel therapeutic targets might be tested in the MPTP model, such as telomerase (Mattson et al., 2000), Smac/Diablo overexpression, and inhibitors of proapoptotic members of the Bcl-2 family.
CHAPTER II: PROTEIN KINASE C-DELTA NEGATIVELY REGULATES TYROSINE HYDROXYLASE ACTIVITY AND DOPAMINE SYNTHESIS BY ENHANCING PROTEIN PHOSPHATASE-2A ACTIVITY IN DOPAMINERGIC NEURONS

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Danhui Zhang, Arthi Kanthasamy, Yongjie Yang, Vellareddy Anantharam, Anumantha G. Kanthasamy

Abstract

Tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, can be regulated by phosphorylation at multiple serine residues including serine-40. In the present study, we report a novel interaction between a key member of the novel PKC family, PKC-delta (PKCδ), and TH, in which the kinase modulates dopamine synthesis by negatively regulating TH activity via protein phosphatase 2A (PP2A). We observed that PKCδ is highly expressed in nigral dopaminergic neurons and co-localizes with TH. Interestingly, suppression of PKCδ activity with the kinase inhibitor rottlerin, PKCδ-siRNA or with PKCδ dominant negative mutant effectively increased a number of key biochemical events in the dopamine pathway, including TH-ser40 phosphorylation, TH enzymatic activity, and dopamine synthesis in neuronal cell culture models. Additionally, we found that PKCδ not only physically associates with the PP2A catalytic subunit (PP2Ac) but also phosphorylates the phosphatase to increase its activity. Notably, inhibition of PKCδ reduced the dephosphorylation activity of PP2A and thereby increased TH-ser40 phosphorylation, TH activity and dopamine synthesis. To further validate our findings, we used the PKCδ knockout (PKCδ -/-) mouse model. Consistent with other results, we found greater TH-ser40 phosphorylation and reduced PP2A activity in the substantia nigra of PKCδ -/-
mice than in wild-type mice. Importantly, this was accompanied by an increased dopamine level in the striatum of PKCδ -/- mice. Collectively, these results suggest that PKCδ phosphorylates PP2Ac to enhance its activity and thereby reduces TH-ser40 phosphorylation and TH activity and ultimately dopamine synthesis.

Introduction

Tyrosine hydroxylase (TH) is a rate-limiting enzyme in the biosynthesis of catecholamines and catalyzes the first step of a biochemical synthetic pathway in which L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-dopa). Phosphorylation and dephosphorylation of TH represent important post-translational regulatory mechanisms of the enzymatic activity that mainly determine the amount of catecholamine synthesis. A number of phosphorylation sites have been identified in TH, and phosphorylation of TH greatly influences the enzyme activity (Lee et al., 1989). Phosphorylation of TH at amino-terminal serine (Ser) amino sites at Ser8, Ser19, Ser31, and Ser40 leads to activation of TH. A number of protein kinases have been shown to phosphorylate these serine residues to varying degrees. For example, THser19 is phosphorylated by CaMKII, TH-ser40 by Protein kinase A (PKA), Protein kinase G, MSK, Protein kinase C (PKC), and TH-ser31 by ERK1/ERK2 kinases and indirectly by PKC (Haycock, 1990). Among these serine phosphorylation sites, TH-ser40 is a major residue that positively regulates the TH activity in vivo (Campbell et al., 1986; Wu et al., 1992). The phosphorylation state of TH can also be regulated by dephosphorylation reactions mediated by phosphatases. Haavik et al. demonstrated that phosphatase 2A (PP2A) is the major serine/threonine phosphatase that dephosphorylates TH, resulting in reduced TH activity (Haavik et al., 1989).

The PKC family consists of more than 12 isoforms and is subdivided into three major subfamilies, which include conventional PKC (α, βI, βII, γ), novel PKC (δ, ε, μ, η, θ), and atypical PKC (τ, λ, ζ) (Gschwendt, 1999; Dempsey et al., 2000; Maher, 2001; Kanthasamy et al., 2003). PKCδ, a key member of the novel PKC family, plays a role in a variety of cell
functions including cell differentiation, proliferation and secretion. Our recent studies demonstrate that PKC\(\delta\) is an oxidative stress-sensitive kinase, and activation of this kinase via caspase-3 dependent proteolysis induces apoptotic cell death in cell culture models of Parkinson's disease (Kanthasamy et al., 2003; Kaul et al., 2003; Yang et al., 2004; Latchoumycandane et al., 2005). General PKCs can phosphorylate TH-ser40 and TH-ser31 (Albert et al., 1984; McTigue et al., 1985; Tachikawa et al., 1987; Cahill et al., 1989; Haycock et al., 1992; Bunn and Saunders, 1995; Bobrovskaya et al., 1998), however, direct phosphorylation of TH by PKA and not by PKC results in activation of the enzymatic activity (Funakoshi et al., 1991). The role of PKC isoforms in the regulation of TH activity is not well studied. Following the characterization of a key proapoptotic role of PKC\(\delta\) in dopaminergic neuronal cell death during neurotoxic insults (Kanthasamy et al., 2003; Kaul et al., 2003; Yang et al., 2004; Kitazawa et al., 2005), we further examined in the present study whether PKC\(\delta\) has any physiological role in regulating dopamine synthesis. Herein, we report a novel functional interaction between PKC\(\delta\) and TH in which PKC\(\delta\) negatively regulates TH activity and dopamine synthesis by enhancing PP2A activity.

Materials and Methods

**Chemicals.** Rottlerin, recombinant PKC\(\delta\) protein, NSD-1015, okadaic acid, dibutyryl cAMP, protease cocktail, ATP, protein-A-sepharose, protein-G-sepharose and anti-\(\beta\)-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO); purified PP2A and PP2Ac enzyme was purchased from Upstate (Chicago, IL). Mouse tyrosine hydroxylase antibody, PhosphoTH-ser40 and ser31 antibodies were purchased from Chemicon (Temecula, CA); the rabbit polyclonal antibody for tyrosine hydroxylase was obtained from Calbiochem Bioscience, Inc. (King of Prussia, PA). Rabbit PKC\(\delta\) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse PKC\(\delta\) antibody and PP2Ac antibody were from BD Biosciences (San Jose, CA). Anti-rabbit and anti-mouse secondary antibodies and the ECL chemiluminescence kit were purchased from Amersham Pharmacia
Biotech (Piscataway, NJ). Alexa 488 conjugated anti-rabbit/mouse, Cy3 conjugated anti-rabbit/mouse antibody and Hoechst 33342 were purchased from Molecular Probes, Inc. (Eugene, OR). [γ-32P]ATP was purchased from Perkin Elmer Life Science Products (Boston, MA). Serine/Threonine phosphatase assay kit was purchased from Promega; AMAXA® Nucleofector™ kit was from AMAXA (AMAXA GmbH, Germany). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). RPMI, fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Invitrogen (Gaithersburg, MD).

Animal studies. Six to 8-week-old C57/BL/6 mice and PKCδ knockout mice weighing 25-30 g were housed in standard conditions: constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-h light/dark cycle with free access to food and water. PKCδ knockout animals were kindly provided by Dr. Keiichi Nakayama’s Laboratory (Division of Cell Biology, Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). We obtained a pair of male and female heterozygous PKCδ (+/-) C57 black mice from Dr. Nakayama’s laboratory and established a breeding colony in our animal facility. We then genotyped animals in our colony as per the protocol described previously (Miyamoto et al., 2002). Briefly, genomic DNA was isolated from tails of 3-4 week old mice and then subjected to PCR followed by electrophoresis. The genotype of the animals was confirmed using the molecular size of PCR products: PKCδ naïve (+/+): 900 bp; PKCδ (+/-): 900 and 600 bp; and PKCδ knockout (-/-): 600 bp. The animals and protocol procedures were approved and supervised by the Committee on Animal Care (COAC) at Iowa State University.

Cell culture models. PC12 and differentiated N27 cells were cultured as described earlier (Adams et al., 1996; Anantharam et al., 2002; Kaul et al., 2003). Briefly, cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units of
penicillin, and 50 µg/ml streptomycin. Cells were maintained in a humidified atmosphere of
5% CO₂ at 37°C. N27 cells were differentiated with 2 mM dibutyryl cAMP for 3-5 days
and then used for experiments described below. Primary mesencephalic neuronal cultures
were prepared from the ventral mesencephalon of gestational 16-18-day-old mice embryos as
described previously (Yang et al., 2004). Mesencephalic tissues were dissected and
maintained in ice-cold Ca²⁺-free HBSS and then dissociated in HBSS solution containing
trypsin-EDTA (0.25%) for 20 min at 37°C. The dissociated cells were then plated at equal
density (0.5 × 10⁶ cells) in 30-mm-diameter tissue culture wells precoated with poly-D-lysine
(1 mg/ml). Cultures were maintained in a chemically defined medium consisting of
neurobasal medium fortified with B-27 supplements, L-glutamine (500 µM), penicillin (100
IU/ml), and streptomycin (100 µg/ml) (Life Technologies). The cells were maintained in a
humidified CO₂ incubator (5% CO₂, 37°C) for 24 hr and then treated with cytosine
arabinoside (10 µM) for 24 hr to inhibit glial cell proliferation. Half of the culture medium
was replaced every 2 days. Approximately 6-7-day-old cultures were used for experiments.

Transfection of PKCδ²⁷⁶R gene in N27 cells and primary mesencephalic neurons.
Plasmid pPKCδ²⁷⁶R-V5 encodes the loss of function PKCδ-V5 epitope tagged mutant
protein; K376R refers to the mutation of the lysine residue at position 376 to arginine in the
catalytic site, resulting in inactivation of the kinase. Plasmid pLacZ-V5 encodes the
β-galactosidase protein alone with a V5-epitope and is used as a vector control. N27 cells
stably expressing PKCδ²⁷⁶R-V5 (herein referred to as PKCδ-DN cells) and LacZ
alone-expressing cells (LacZ cells) were cultured as described previously (Kitazawa et al.,
2005). PKCδ-DN or LacZ expressing N27 cells were identified by immunostaining of the
C-terminal V5 epitope on expressed proteins and were differentiated with 2 mM dibutyryl
cAMP prior to experiments. PKCδ-DN and LacZ were also transiently expressed in mouse
primary mesencephalic neurons using an AMAXA® Nucleofector™ kit (AMAXA GmbH,
Germany). As described above, primary mesencephalic neurons were prepared from the
midbrain of 16-18-day-old mice embryos. After digestion with trypsin-EDTA-HBSS, the primary neurons were homogenously resuspended with transfection buffer provided with the kit to a final concentration of 4-5 x 10^6 neurons/100 μl and mixed with 2 μg plasmid DNA encoding either PKCδ-DN-V5 or LacZ-V5. Electroporation was carried out with an AMAXA® Nucleofector™ instrument as per the manufacturer's protocol. The transfected neurons were then transferred to 24-well plates containing poly-D-lysine and laminin coated cover slips. After 24 hr, the primary neurons were fixed and used for immunocytochemistry. Transfection efficiency was >75% as determined by immunostaining of V5-expression.

**Design, synthesis and transfection of siRNA.** Small interfering RNAs (siRNAs) were prepared by an in vitro transcription method as described previously (Yang et al., 2004). Initially, siRNA target sites specific to rat PKCδ mRNA (gi: 18959249), as determined by blast analysis, were chosen. One nonspecific siRNA (NS-siRNA) was also chosen based on random sequence. For each siRNA, sense and antisense templates were designed based on each target sequence and partial T7 promoter sequence (Donze and Picard, 2002): for PKCδ-siRNA, sense, 5'-AACTGTTTGTGAATTTGCTTCTCTGTC-3'; antisense, 5'-AAAAGGACATCAACAGCCTGTCTCTGTC-3'; for NS-siRNA, sense, 5'-AATTCTCACACTTGAGAACCTGTCTCTGTC-3'; antisense, 5'-AAGTTCTCCGAAAGTGTGAGAACCTGTCTCTGTC-3'. All template oligonucleotides were chemically synthesized and PAGE purified. In vitro transcription, annealing, and purification of siRNA duplexes were performed using the protocol supplied with the silencer siRNA construction kit (Ambion). Briefly, approximately 2 μg of each single-strand (ss) transcription template was first annealed with the T7 promoter and filled in by Klenow DNA polymerase to form double-strand transcription templates. For preparation of each siRNA duplex, transcription reactions were first performed with separated antisense and sense templates using the T7 RNA polymerase provided with the kit and then annealed to form siRNA duplexes. Then, the siRNA duplex was treated with DNase and RNase to remove the extra nucleotides of
transcribed siRNA to meet the structural 3′UU overhang and 5′ phosphate requirement (Elbashir et al., 2001). N27 cells (50–70% confluence) and primary mesencephalic neurons were transfected with siRNA duplexes by using an AMAXA® Nucleofector™ kit (AMAXA GmbH, Germany) as described in our recent publication (Yang et al., 2004).

**Treatment paradigm.** PC12 cells, differentiated N27 dopaminergic cells and primary mesencephalic neurons were exposed to 1-10 μM rottlerin for the duration of the experiment. DMSO (0.01%) was used as vehicle control. PKCδ-DN- and Lac Z-expressing N27 cells were only treated with 0.01% DMSO. For measurement of TH activity in rottlerin-treated cultures, cells were exposed to 2 mM NSD-1015 for 1 hr prior to rottlerin treatment. Untreated or vehicle-treated cells were used as control samples. We derived the concentrations of rottlerin and okadaic acid used in this study based on previously published literature. Rottlerin inhibits PKCδ kinase activity with a Ki of 3-6 μM, while PKCa, β, γ, ε and λ Ki values are at least 5-10 times higher (Gschwendt, 1999; Davies et al., 2000; Way et al., 2000; Soltoff, 2001). In our previous study, we showed 3-10μM rottlerin dose-dependently attenuated kinase activity to a greater extent (Anantharam et al., 2002). We used 5 μM rottlerin in the study, which was lower than Ki values of other PKC isoforms. Additionally, we employed various genetic approaches such as RNAi, dominant negative mutant and knockout approaches to validate the results obtained with rottlerin treatment. We used 2 μM okadaic acid to completely inhibit PP2A activity in dopaminergic cells, since previous studies demonstrated IC50 ranges from 0.1-1 μM for PP2A activity in cell culture models (Favre et al., 1997; Schonthal, 1998).

**Western blotting.** Cell and brain lysates containing equal amounts of protein were loaded in each lane and separated on a 10-12% SDS-PAGE gel as described previously (Kaul et al., 2003). After the separation, proteins were transferred to nitrocellulose membrane, and nonspecific binding sites were blocked by treating with 5% nonfat dry milk powder. The
membranes were then treated with primary antibodies directed against PKCδ (rabbit polyclonal or mouse monoclonal for PKCδ knockout studies, 1:2000 dilution), TH (rabbit polyclonal or mouse monoclonal, 1:1000), phospho TH-ser40 (rabbit polyclonal, 1:1000), phospho TH-ser31 TH (rabbit polyclonal, 1:1000), or PP2Ac (mouse monoclonal, 1:1000). The primary antibody treatments were followed by treatment with secondary HRP-conjugated anti-rabbit or anti-mouse IgG (1:2000) for 1 hr at RT. Secondary antibody-bound proteins were detected using Amersham's ECL chemiluminescence kit. To confirm equal protein loading, blots were reprobed with a β-actin antibody (1:5000 dilution). Western blot images were captured with a Kodak 2000 MM imaging system and data were analyzed using 1D Kodak imaging analysis software.

**Co-immunoprecipitation.** Immunoprecipitation studies were conducted to determine the association properties between PKCδ, TH and PP2A and were performed as described in our recent publications (Kaul et al., 2005). Briefly, PC12, N27 cells or substantia nigral tissue from PKCδ (+/+ or PKCδ (-/-) mouse brain were washed with ice-cold Ca2+-free PBS saline and resuspended in a lysis buffer [25 mM HEPES (pH 7.5), 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 10 mM NaF, and protease inhibitor cocktail]. The suspension was kept on ice for 30 min and then centrifuged at 10,000 × g for 5 min. The supernatants were collected and used for immunoprecipitation. Extracts containing ~200 μg total protein were immunoprecipitated overnight at 4°C with 5-20 μg of anti-PKCδ (rabbit polyclonal), anti-TH (mouse monoclonal), anti-PP2Ac (mouse monoclonal) antibodies, rabbit IgG or mouse IgG. Mouse monoclonal anti-PKCδ antibody was used for PKCδ (-/-) brain tissue. 50μg PKCδ blocking peptide was used to neutralize PKCδ antibody by incubating for one hr and used as a negative control. The immunoprecipitates were then adsorbed onto Protein A or G sepharose for 1 hr at 4°C. The sepharose-bound antigen–antibody complexes were washed three times with PBS to remove unbound proteins. For association studies, samples were...
mixed with 2X SDS PAGE loading buffer, boiled for 5 min, and then proteins were separated on SDS-PAGE and subjected to Western blot as described earlier.

**32P-Phosphorylation assays.** To determine whether PKCδ can directly phosphorylate PP2A, we used recombinant PKCδ in in vitro phosphorylation assays. Immunoprecipitations were performed as described earlier (Kaul et al., 2003). Briefly, PP2Ac was immunoprecipitated from N27 cell lysates using mouse monoclonal PP2Ac antibody. The samples were then incubated with protein G sepharose, and the immunoprecipitates and recombinant PP2Ac were used in in vitro phosphorylation assays. Recombinant PKCδ was resuspended in 2X kinase buffer [40 mM Tris (pH 7.4), 20 mM MgCl₂, 20 μM ATP, 2.5 mM CaCl₂] and the reaction was started by adding 20 μl of reaction buffer containing immunoprecipitated or recombinant PP2Ac and 5 μCi of [γ-32P] ATP (4500 Ci/mM). To inhibit PKCδ, recombinant PKCδ protein was preincubated with 5μM rottlerin for 15 min before the reaction. After incubation for 10 min at 30°C, the reaction was terminated by addition of 2X SDS-gel loading buffer and separated by SDS-PAGE. PP2Ac and histone H1 phosphorylated bands were detected using a Personal Molecular Imager (FX model, BioRad Labs) and quantified with Quantity One 4.2.0 software. Histone H1 substrate was used as a positive control for PKCδ kinase activity.

**PP2A assay.** To determine PP2A phosphatase activity, we used the Serine/Threonine phosphatase assay kit from Promega. In cell free assay, 20 ng of recombinant PP2A enzyme in PP2A reaction buffer (250 mM imidazole, pH 7.2, 1 mM EGTA, 0.1% β-mercaptoethanol, 5 μl peptide substrate, 0.5 mg/ml BSA) was incubated with 20 ng of recombinant PKCδ protein in the presence or absence of 5 μM rottlerin. 2 μM okadaic acid was used as a positive control. Reaction was started by adding PKCδ reaction buffer containing 40 mM Tris, pH 7.4, 20 mM MgCl₂, 20 μM ATP, 2.5 mM CaCl₂, 50 μg/ml phosphatidylinserine, and 4.0 μM dioleoylglcerol. After 1 hr incubation, molybdate dye (2X vol) was added to the
reaction mixture. For in vivo PP2A activity measurement, N27 cells and substantia nigral tissue from mouse brain were homogenized in lysis buffer (25 mM Tris-HCl, 10 mM β-mercaptoethanol, 2 mM EDTA, protease inhibitor) supplied with the kit. After centrifugation, the supernatants were used for measurement of PP2 activity by incubating equal volumes of the substrate and PP2A reaction buffer for 1 hr. PP2A activity was determined by measuring the amount of free phosphate generated in a reaction by measuring the absorbance of a molybdate:malachite green: phosphate complex at 600 nm using a Spectramax plate reader (Molecular Devices). The effective range for the detection of phosphate released in this assay is 100–4,000 pmol of phosphate.

**Tyrosine hydroxylase activity.** TH enzyme activity was measured by the modified method of Hayashi et al (Hayashi et al., 1988) in which DOPA levels are quantified as an index of TH activity after inhibition of DOPA decarboxylase with the decarboxylase inhibitor NSD-1015 (Hayashi et al., 1988). Briefly, cells were incubated with Krebs-HEPES buffer (pH 7.4) containing 2 mM NSD-1015 at 37°C for 30 min, and then subjected to the treatment paradigm as described earlier. After treatment, cells were collected and resuspended in antioxidant solution, sonicated, centrifuged, and DOPA levels in the supernatants were measured by HPLC detection as described below.

**Measurements of DA and its metabolites.** DA and DOPAC levels in PC12, N27 cells and in brain striatal tissues were determined by high-performance liquid chromatography with electrochemical detection (HPLC-EC); samples were prepared as described previously (Kitazawa et al., 2001; Sun et al., 2006). Briefly, neurotransmitters were extracted from samples using 0.1 M perchloric acid containing 0.05% Na₂EDTA and 0.1% Na₂S₂O₅. The extracts were filtered in 0.22 micron spin tubes and 20 μl of the samples was loaded for analysis. DA and DOPAC were separated isocratically by a reversed-phase column with a flow rate of 0.7 ml/min. An HPLC system (ESA Inc., Bedford, MA) with an ESA
automatic sampler (model 542) was used for these experiments. The electrochemical
detection (EC) system consisted of an ESA coulochem model 5100A with a microanalysis
cell model 5014A and a guard cell model 5020 (ESA Inc., Bedford, MA). The peak areas of
standard DA and DOPAC were compared with that of samples. The DA and DOPAC levels
in the samples were measured and expressed as ng/mg protein, and retention times for DA
and DOPAC were 5.8-7.7 min and 4.7-5.5 min, respectively.

**Immunohistochemical staining of brain slices.** Mice were perfused with 4%
paraformaldehyde following anesthesia with ketamine, and then the brain was cut on a
microtome into 20-µm sections. Sections from SNpc were treated for immunofluorescence
staining. Brain sections were first blocked with 5% normal goat serum containing 0.4% BSA
and 0.2% Triton-X 100 in PBS for 20 min, then incubated with antibodies directed against
PKCδ (rabbit polyclonal, 1:500 dilution) and TH (mouse monoclonal, 1:500 dilution)
overnight at 4°C followed by incubation with either Alexa 488-conjugated (green, 1:1000) or
Cy3-conjugated (red, 1:1000) secondary antibody for 1 hr at RT. Secondary antibody
treatments were followed by incubation with Hoechst 33342 (10 µg/ml) for 3 min at room
temperature to stain the nucleus. Then the slices were mounted on a slide and viewed under a
Nikon inverted fluorescence microscope (Model TE-2000U); images were captured with a
SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

**Immunocytochemical staining in cell cultures.** Immunostaining of PKCδ, TH,
P-TH-ser40 and P-TH-ser31 were performed in PC12, N27 and primary mesencephalic
neurons. Cells were grown on poly-L-Lysine-coated glass cover slips. After treatment,
the cells were fixed with 4% paraformaldehyde and processed for immunocytochemical
staining. First, non-specific sites were blocked with 5% normal goat serum containing 0.4%
BSA and 0.2% Triton-X 100 in PBS for 20 min. Cells and primary neurons were then
incubated with antibodies directed against PKCδ (mouse monoclonal, 1:500 dilution), TH
(rabbit polyclonal, 1:500 dilution), and P-TH-ser40 (rabbit polyclonal, 1:500 dilution)
overnight at 4°C followed by incubation with either Alexa 488-conjugated (green, 1:1000) or
Cy3-conjugated (red, 1:1000) secondary antibody for 1 hr at RT. Secondary antibody treatments were followed by incubation with Hoechst 33342 (10 μg/ml) for 3 min at room temperature to stain the nucleus. Then the cover slips containing stained cells were washed with PBS, mounted on a slide, and viewed under a Nikon inverted fluorescence microscope (Model TE-2000U); images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

**Data analysis.** Data analysis was performed using Prism 4.0 software (GraphPad Software, San Diego, CA). Data were first analyzed using one-way ANOVA and then Bonferroni's post-test was performed to compare all treatment groups, and differences with p<0.05 were considered significant.

**Results**

**PKCδ physically associates with TH.** First we examined the level of PKCδ expression in nigral dopaminergic neurons. Double immunostaining of mouse nigral tissues with PKCδ and TH showed a strong co-localization of these proteins (Fig. 1A). To further confirm their possible interaction, we performed co-immunoprecipitation and reverse immunoprecipitation studies. As shown in Fig. 1B, immunoprecipitation with PKCδ antibody followed by immunoblotting with TH antibody showed a clear association of TH with PKCδ in mouse substantia nigral lysate (Fig. 1B). In reverse immunoprecipitation analysis, TH antibody was used for immunoprecipitation and PKCδ antibody was used for immunoblotting. The right panel of Fig. 1B shows a PKCδ band in TH immunoprecipitates from mouse brain, indicating an association between these two proteins. Rabbit IgG and mouse IgG immunoprecipitates were used as negative controls. These results clearly indicate that PKCδ physically associates with TH in the dopaminergic neuronal system.

To further determine the functional relationship between PKCδ and TH, we used a PC12 model, which has been used extensively for post-translational regulation of TH
(Haycock, 1989, 1990). First we performed immunoprecipitation studies to verify that PKCδ interacts with TH in this cell model. Similar to nigral tissue, immunoprecipitation and reverse immunoprecipitation studies showed a clear association and interaction between PKCδ and TH (Fig. 1C). These results not only confirmed the PKCδ and TH interaction in dopamine producing cells, but also indicated the usefulness of PC12 cells for examining the role of PKCδ in the regulation of TH function.

**PKCδ inhibition enhances TH activity.** Since PKCδ colocalized with TH, we examined whether PKCδ has any influence on TH activity. As a first step, we determined TH activity under conditions of PKCδ inhibition using various doses of the PKCδ specific inhibitor rottlerin. TH activity was measured by determining DOPA levels following inhibition of DOPA decarboxylase with the enzyme inhibitor NSD-1015, as described in the methods section. PC12 cells were exposed to 2 mM NSD-1015 for 1 hr prior to treatment with the PKCδ-specific inhibitor rottlerin for 3 hr. We used 1-10 μM rottlerin in the present study because we previously showed this dose range effectively inhibits PKCδ activity (Anantharam et al., 2002). As shown in Fig. 2, rottlerin treatment significantly increased intracellular DOPA levels, indicating increased TH activity following PKCδ inhibition. Treatment with 1 μM, 5 μM and 10 μM rottlerin increased TH activity to 2091.09±80, 3045±75 and 2067±44 pg DOPA/10^6 cells/hr, respectively, compared with the control level of 1519±91 pg DOPA/10^6 cells/hr. Together, these results suggest that inhibition of PKCδ activation results in increased TH activity.

**Effect of PKCδ inhibition on TH phosphorylation.** Since inhibition of PKCδ resulted in enhanced TH activity, we examined whether PKCδ has any effect on the phosphorylation status of TH. It is well established that TH activity can be regulated by phosphorylation of multiple serine residues (Campbell et al., 1986; Mitchell et al., 1990), and serine phosphorylation at positions 31 and 40 has been suggested to play a key role in TH
activation and increased dopamine biosynthesis (Haycock, 1990). We measured the extent of TH-ser31 and TH-ser40 phosphorylation in immunoblots using phosphospecific antibodies directed against TH-ser31 and TH-ser40. As shown in Fig. 3A, the level of TH-ser40 phosphorylation was significantly enhanced in rottlerin-treated cells (5 μM rottlerin for 3 hr), whereas the level of TH-ser31 phosphorylation was unaltered. Similarly, the level of total TH and PKCδ were unaltered in rottlerin-treated PC12 cells as compared to 0.01% DMSO control-treated cells, indicating that rottlerin treatment does not alter the expression of TH and PKCδ. Nitrocellulose membranes were reprobed with β-actin antibody and the density of the 43 kDa β-actin band was identical in all lanes, confirming equal protein loading. Densitometric analysis of the 60 kDa P-TH-ser40 band in Fig. 3A revealed a 3-fold increase in phosphorylation as compared to vehicle-treated cells, whereas there was no increase in protein levels of P-TH-ser31 and TH. TH-ser40 phosphorylation was also confirmed in immunofluorescence measurements. After treatment with 5 μM rottlerin for 3 hr, PC12 cells were fixed and processed for immunofluorescence staining of P-TH-ser40 using Alex 488 secondary antibody. An increase in bright green immunofluorescence positive cells was observed in rottlerin-treated cells (Fig. 3B), but only a weak staining was observed in vehicle-treated cells, indicating that PKCδ inhibition results in increased phosphorylation of TH-ser40. No staining was observed in cells stained with Alexa 488 alone. Cell count analysis of images using Metamorph image analysis revealed that rottlerin treatment induced the increase in P-TH-ser40 stained cells by 333% compared to vehicle-treated cells. These results demonstrate that PKCδ inhibition results in enhanced TH phosphorylation specifically at ser40.

Effect of loss of function PKCδ mutant on TH activity and DA synthesis in mesencephalic dopaminergic neuronal cells. Although PC12 cells are a good model to study TH function, they are non-neuronal cells derived from adrenal pheochromocytoma. Therefore, to further determine whether PKCδ alters TH activity in neuronal cells, we used
immortalized rat mesencephalic dopaminergic neuronal cells (N27 cells), which are a homogenous population of TH-positive neuronal cells that synthesize and release dopamine upon differentiation (Clarkson et al., 1999; Zhou et al., 2000). Also, the N27 dopaminergic cell line is easily transfectable and convenient for establishing stable cell lines compared to hard-to-transfect PC12 cells. In recent years, this neuronal cell line has been recognized by many investigators, including us, as a highly useful cell culture model for studying degenerative mechanisms in Parkinson’s disease (Clarkson et al., 1999; Kaul et al., 2003; Miranda et al., 2004; Kaul et al., 2005; Peng et al., 2005).

First, we examined whether PKCδ interacts with TH in N27 dopaminergic cells in a manner similar to that observed in PC12 cells and mouse nigral tissue (Fig. 4A). As shown in Fig. 4A, double immunostaining studies with differentiated N27 cells showed co-localization of PKCδ with TH and P-TH-ser40, further supporting the previous results of association of PKCδ with TH observed in PC12 cells. Next, we examined whether PKCδ inhibition would alter TH activity and dopamine in N27 cells. For these studies, we used genetic approaches in addition to PKCδ pharmacological inhibitor studies. We utilized N27 cells stably expressing a loss of function PKCδ dominant negative mutant (PKCδ-DN) established in our laboratory (Kaul et al., 2003; Kitazawa et al., 2005). N27 cells were exposed to 2 mM NSD-1015 for 1 hr prior to a 3 hr treatment with the PKCδ-specific inhibitor rottlerin (5 μM) or 0.01% DMSO, and then measured the DOPA levels as a measure of TH activity. We also measured DOPA levels in PKCδ-DN expressing N27 cells and Lac-Z-expressing N27 cells (vector control). As shown in Fig. 4B, DOPA levels were significantly higher in rottlerin-treated cells compared to vehicle-treated cells, and were in agreement with the data obtained in PC12 cells (see Fig. 2). Also, TH activity was significantly higher in PKCδ-DN mutant-expressing cells as compared to LacZ-expressing cells (Fig. 4B). The TH activity was 1231±120 and 3025±267 pg DOPA/10^6 cells/hr in vehicle- and rottlerin-treated cells, and 1483±146 and 2539±307 pg DOPA/10^6 cells/hr in LacZ- and PKCδ-DN-expressing cells, respectively (Fig. 4B).
Increase in TH activity should result in increase in dopamine synthesis and therefore, we measured the levels of cellular dopamine (DA) by HPLC in N27 cells expressing PKCδ-DN and in rottlerin-treated N27 cells. Vehicle-treated N27 cells and Lac-Z-expressing N27 cells were used as controls. Steady-state DA levels were significantly increased in rottlerin-treated and PKCδ-DN mutant-expressing N27 cells compared to vehicle-treated and LacZ-expressing cells, respectively (Fig. 4C). The DA level in rottlerin-treated cells was 2748±209 ng/mg protein compared to 754±73 ng/mg protein in vehicle-treated N27 cells, a 3.7-fold increase. Similarly, DA levels in PKCδ-DN mutant-expressing cells were 4806±373 ng/mg protein compared to 1015±73 ng/mg protein in LacZ-expressing cells, a 4.8-fold increase (Fig. 4C). Thus, both inhibition of baseline PKCδ activity with rottlerin and loss of function PKCδ-DN mutant increased DA levels in the dopaminergic cells. To determine whether increased DA levels in the rottlerin-treated and PKCδ-DN-expressing cells were due to enhanced TH activity or due to reduced degradation of DA to its major metabolite dihydroxyphenyl acetic acid (DOPAC) by monoamine oxidase (MAO), we measured DOPAC levels. As shown in Fig. 4D, DOPAC levels were also significantly increased by 4-fold in rottlerin-treated cells and by 3-fold in PKCδ-DN-expressing cells, compared to the vehicle-treated and LacZ-expressing N27 cells, indicating that the dopamine degradation pathway is not altered during rottlerin treatment. Together, these data suggest that PKCδ inhibition increases DA synthesis in dopaminergic neuronal cells as a result of increased TH activation.

**PKCδ negatively modulates TH-ser40 phosphorylation.** Since TH activity and dopamine synthesis were enhanced in N27 cells expressing PKCδ-DN and in rottlerin-treated N27 cells, we further examined whether PKCδ inhibition increases the phosphorylation status of TH in N27 dopaminergic neuronal cells. As shown in Fig. 5A, the levels of TH-ser40 phosphorylation were significantly enhanced in rottlerin-treated N27 cells, whereas the level of TH-ser31 phosphorylation was not altered compared to vehicle-treated cells.
Also, the level of total TH was unaltered in rottlerin-treated N27 cells. Similarly, the TH-ser40 phosphorylation level was also significantly enhanced in PKCδ-DN-expressing cells compared to LacZ-expressing cells, whereas TH-ser31 phosphorylation and total TH levels were unaltered. Nitrocellulose membranes were reprobed with β-actin antibody and the density of the 43 kDa β-actin band was identical in all lanes, confirming equal protein loading. Densitometric analysis of the P-TH-ser40 (60 kDa) band revealed a 3-4-fold increase in rottlerin-treated and PKCδ-DN-expressing cells, compared to DMSO and LacZ-expressing cells. We also confirmed TH-ser40 phosphorylation by immunofluorescence measurements in N27 cells. N27 cells stably expressing PKCδ-DN-V5 and LacZ-V5 were fixed and double immunostained for antibodies directed against P-TH-ser40 and V5-epitope in PKCδ-DN-V5 and LacZ-V5 constructs. Primary antibody staining was followed by Cy3-labeled secondary antibody against TH-ser40 and Alexa 488-labeled secondary antibody against the V5-epitopes. As shown in Fig. 5B, a bright red immunofluorescence staining was observed in PKCδ-DN expressing cells as compared to a very weak staining in Lac-Z expressing cells, suggesting that PKCδ inhibition resulted in enhanced phosphorylation of TH at ser40. Both the LacZ- and PKCδ-DN-expressing cells were stained for vector fusion protein V5 (Green), demonstrating the expression level of the constructs. Analysis of fluorescent intensity for TH-ser40 immunostaining revealed a 2.5-fold increase in PKCδ-DN-expressing cells as compared to Lac-Z-expressing cells (Fig. 5B). Collectively, these results demonstrate that PKCδ inhibition results in enhanced TH phosphorylation at ser40 in N27 dopaminergic neuronal cells.

To further confirm the regulatory role of PKCδ in TH phosphorylation in the dopaminergic system, we examined the effect of PKCδ inhibition on TH-ser40 phosphorylation in primary dopaminergic neurons obtained from the ventral mesencephalon of E16-18 mouse embryos. Primary mesencephalic cultures were transfected with plasmids coding for the loss of function PKCδ-DN mutant and LacZ using the Amaxa nucleofector system. 24 hr post-transfection, primary neurons were processed for immunohistochemical
Enhanced TH-ser40 phosphorylation and DA synthesis in PKCδ
siRNA-transfected dopaminergic neuronal cells. To further substantiate the regulatory role of TH activity and DA synthesis by PKCδ, we used an RNA interference (RNAi) approach. We recently developed PKCδ siRNAs that specifically suppress PKCδ expression, but not the expression of PKCε, a novel isoform most closely and phylogenetically related to PKCδ (Yang et al., 2004). The siRNAs also did not produce any cytotoxic effect in N27 dopaminergic cells. In this experiment we measured TH-ser40 phosphorylation, and DA and DOPAC levels following siRNA mediated suppression of PKCδ expression in N27 cells. Figure 6A shows a significant suppression of endogenous PKCδ expression in PKCδ siRNA-transfected cells as compared to non-specific siRNA (NS-siRNA) transfected N27 cells. A 60% reduction in PKCδ expression was observed in PKCδ-siRNA transfected cells as measured by Western blot analysis. Importantly, TH-ser40 phosphorylation levels were also significantly higher in PKCδ-siRNA-transfected cells as compared to NS-siRNA transfected N27 cells, whereas the total TH levels were similar in both PKCδ-siRNA and NS-siRNA transfected cells (Fig. 6A). Densitometric analysis of P-TH-ser40 (60 kDa band) revealed a 2-fold increase in PKCδ-siRNA transfected cells compared to NS-siRNA transfected N27 cells. Reprobing of the nitrocellulose membranes with β-actin antibody showed the density of the 43 kDa β-actin band to be identical in all lanes, confirming equal
protein loading. Next, we measured DA and DOPAC levels in siRNA transfected N27 cells by HPLC. As shown in Fig. 6B, DA and DOPAC levels were significantly higher in PKC-siRNA transfected cells compared to NS-siRNA-transfected N27 cells. A 45% increase in the DA level and a 2-fold increase in DOPAC were noted in PKCδ-siRNA-transfected cells as compared to NS-siRNA-transfected N27 cells. Together, these data further substantiate that PKCδ negatively regulates TH-ser40 phosphorylation and DA synthesis in dopaminergic neuronal cells.

**Increased TH-ser40 phosphorylation in primary mesencephalic dopaminergic neurons from PKCδ knockout (-/-) mice.** We also extended the TH phosphorylation studies to primary mesencephalic dopaminergic neurons derived from naïve and PKCδ knockout (-/-) E16-18 mouse embryos (Miyamoto et al., 2002). The level of TH-ser40 phosphorylation in nigral dopaminergic neurons in PKCδ (+/+) and PKCδ (-/-) mice was compared by immunostaining. The baseline TH-ser40 phosphorylation levels were significantly higher in untreated primary neurons obtained from PKCδ (-/-) mice compared to untreated PKCδ (+/+) mice (Fig. 7). Fluorescent intensity measurements revealed that the TH-ser40 level was 3-fold higher in PKCδ (-/-) dopaminergic neurons as compared to PKCδ (+/+) mesencephalic neurons. In addition to the knockout studies, we tested the effect of the PKCδ inhibitor, rottlerin, on TH-ser40 phosphorylation in the PKCδ (+/+) primary neuronal cultures. The cultures were treated with 5 μM rottlerin for 3 hr and then the level of TH-ser40 phosphorylation was measured. As shown in Fig. 7, rottlerin treatment increased TH-ser40 phosphorylation in PKCδ (+/+) primary dopaminergic neurons. The fluorescent intensity was increased 2-fold in rottlerin-treated dopaminergic neurons as compared to dopaminergic neurons. Together, these results confirm that suppression of PKCδ increases TH-ser40 phosphorylation in dopaminergic neurons.

**Effect of PP2A inhibition on TH activity and TH-ser40 phosphorylation.** The
increased TH-ser40 phosphorylation resulting from PKCδ inhibition suggested that PKCδ may affect dephosphorylation of TH under normal conditions. Previous studies have demonstrated that protein phosphatase 2A (PP2A) is a major serine phosphatase that mediates the dephosphorylation of TH, in particular TH-ser40, resulting in the inactivation of TH (Vrana and Roskoski, 1983; Haavik et al., 1989). Since both of these proteins negatively regulate TH, we hypothesize that PKCδ may work through PP2A to reduce dephosphorylation of TH-ser40 and TH activity. This would explain why inhibition of PKCδ increases TH-ser40 phosphorylation, TH activity and dopamine synthesis.

In order to test this novel hypothesis, we first examined whether PP2A regulates TH activity and TH-ser40 phosphorylation in our dopaminergic model system. N27 cells were incubated with the PP2A inhibitor okadaic acid (2μM) for 2 hr, and then TH-ser40 phosphorylation and TH activity were measured. As shown in Fig. 8A, Western blot analysis revealed an increase in baseline TH-ser40 phosphorylation levels in okadaic acid-treated cells as compared to untreated cells. The levels of total TH were similar in okadaic acid-treated and untreated cells, suggesting that increase in the TH-ser40 phosphorylation level is not due to increased TH expression. We also measured TH activity as well as dopamine content in okadaic acid-treated cells. HPLC measurement revealed a significant increase in TH activity (Fig. 8B) and DA level (Fig. 8C) in okadaic acid–treated cells. TH activity was increased from 826±64 DOPA pg/10^6 cells/hr in control cells to 1434±21 DOPA pg/10^6 cells/hr in okadaic acid-treated cells. The dopamine levels increased from 997±30 ng/mg protein in untreated cells to 1421±10 ng/mg protein in okadaic acid-treated cells. Together, these results suggest that PP2A regulates TH activity and dopamine synthesis in dopaminergic neurons.

**Association and phosphorylation of PP2A by PKCδ.** To determine the interaction between PKCδ and PP2A, we first examined whether PP2A is physically associated with PKCδ. Immunoprecipitation studies were conducted in cell culture as well as in mouse
substantia nigral tissue lysate to determine the possible association. Immunoprecipitation studies revealed PP2Ac immunoreactivity in PKCδ immunoprecipitates from PKCδ (+/+)
mouse substantia nigra lysates (Fig. 9A) and N27 cell lysates (Fig. 9C). Similarly, in reverse immunoprecipitation analysis, the PP2Ac immunoprecipitates showed PKCδ immunoreactivity. The nigral lysate from PKCδ (-/-) mice was used as a negative control. No PP2Ac immunoreactivity was observed in PKCδ immunoprecipitates from PKCδ (-/-) mouse substantia nigra lysates (Fig. 9B), demonstrating the specificity of immunoprecipitation studies. In addition, immunoprecipitated samples using preadsorbed PKCδ antibody with blocking peptide showed substantially reduced immunoreactivity to PP2Ac, indicating the specificity of PKCδ antibody used in the study. Rabbit IgG and mouse IgG immunoprecipitates were used as additional negative controls in these experiments. Collectively, these results indicate a physical association between PP2Ac and PKCδ in a dopaminergic cell line as well as in substantia nigra.

Next we determined whether physical association of PP2Ac with PKCδ involves direct phosphorylation of PP2Ac by PKCδ. We performed phosphorylation studies using immunoprecipitated PP2Ac from N27 cells and pure recombinant PP2Ac protein as substrate. As shown in Fig. 9D, both immunoprecipitated and recombinant PP2Ac were effectively phosphorylated by recombinant pure PKCδ as determined by 32P-in vitro kinase assays (Fig. 9D, lanes 1 and 3). Histone H1 was used as a positive control substrate in phosphorylation assays (Fig. 9D, lane 5). To further confirm that PKCδ directly phosphorylates PP2Ac, and not another PP2Ac-associated kinase, recombinant PKCδ was preincubated with rottlerin for 15 minutes prior to the addition of 32P-ATP in in vitro kinase assays. Rottlerin strongly reduced direct phosphorylation of immunoprecipitated and recombinant PP2Ac by PKCδ (Fig. 9D, lanes 2 and 4). In the positive control, incubation with rottlerin also blocked the direct phosphorylation of histone by PKCδ (Fig. 9D, lane 6). Immunoprecipitated and recombinant PP2Ac samples incubated with 32P-ATP without PKCδ were used as negative controls (Fig. 9D, lanes 7 and 8). A weak band was observed in PP2Ac immunoprecipitates
which may be attributed to the endogenously associated PKCδ (Fig. 9D, lane 7). Collectively, these data indicate that PKCδ associates with and phosphorylates PP2Ac.

Since PP2Ac can be phosphorylated by PKCδ, we examined whether PKCδ phosphorylation activates or inactivates PP2A enzyme activity. We first checked the possibility in a cell free system. Purified PP2A was incubated with recombinant PKCδ protein in the presence or absence of rottlerin, and PP2A phosphatase activity was determined. The PP2A inhibitor okadaic acid was used as a positive control. As shown in Fig. 10A, incubation of PP2A with PKCδ strongly enhanced PP2A activity, which was attenuated by rottlerin. For the in vivo studies, we determined PP2A phosphatase activity in rottlerin-treated N27 cells and in N27 cells expressing the loss of function PKCδ-DN mutant. PP2A activity was significantly reduced in rottlerin-treated cells as compared to vehicle-treated N27 cells (Fig. 10B). Similarly, PP2A activity was also significantly reduced in PKCδ-DN-mutant-expressing cells compared to LacZ-expressing N27 cells (Fig. 10B), indicating that suppression of PKCδ kinase activity reduces the enzymatic activity of endogenous PP2A. However, total PP2A level was unchanged in rottlerin-treated or PKCδ-DN cells (Fig. 10C), indicating that the decreased PP2A activity is not due to reduced PP2A protein levels. Taken together with the phosphorylation studies, these data suggest that PKCδ phosphorylates PP2Ac and enhances PP2A activity.

**Regulation of PP2A activity, TH-ser40 phosphorylation, and DA synthesis in PKCδ (-/-) knockout animals.** Further validation of the role of PKCδ in the regulation of TH activity and DA synthesis was sought by extending these studies to PKCδ (-/-) knockout animals. As shown in Fig. 11A, lack of PKCδ expression in PKCδ (-/-) animals was confirmed in Western blots; 74 kDa native PKCδ protein was present only in the brain lysates from PKCδ (+/+) mice, but not from the PKCδ (-/-) mice. Next we examined TH-ser40 phosphorylation status in PKCδ (-/-) animals. Determination of TH-ser40 phosphorylation in substantia nigra brain tissue revealed a significantly higher level in PKCδ (-/-) animals.
compared to PKCδ (+/+ ) animals, whereas the total TH levels were similar in the substantia nigra of these animals (Fig. 11A). The density of the 43 kDa β-actin band was identical in all lanes, indicating equal protein loading.

To determine whether PKCδ influences PP2A activity in vivo, we compared PP2A enzyme activity in the substantia nigra of PKCδ (+/+ ) and PKCδ (-/-) animals. PP2A activity in the substantia nigra lysates from PKCδ (-/-) mice was significantly lower than in PKCδ (+/+ ) mice (Fig. 11B), supporting our findings from cell culture studies that PKCδ positively influences PP2A activity in nigral neurons.

We also compared DA and DOPAC levels between PKCδ (-/-) animals and PKCδ (+/+ ) animals. HPLC analysis revealed that striatal DA and DOPAC levels were significantly higher in the striatum of PKCδ (-/-) animals compared to PKCδ (+/+ ) animals (Fig. 11C). DA levels were determined to be 311.7±24.88 ng/mg protein in PKCδ (-/-) compared to 190±14.24 ng/mg protein in PKCδ (+/+ ) animals, an increase of 60%. Similarly, striatal DOPAC levels were estimated to be 83.64±12.02 ng/mg protein in PKCδ (-/-) compared to 59.41±8.697 ng/mg protein in PKCδ (+/+ ) animals, an increase of 40%. Together, these in vivo data further demonstrate that PKCδ negatively regulates TH activity, resulting in reduced TH phosphorylation and DA synthesis.

**Discussion**

In the present study, we systematically characterized the regulation of TH by a member of the novel class of the protein kinase C family, PKCδ, and demonstrated that (i) PKCδ is highly expressed in nigral dopaminergic neurons, (ii) suppression of PKCδ activity by kinase inhibitors, dominant negative mutants or RNAi-mediated knockdown increases TH-ser40 phosphorylation, TH activity, and dopamine levels (iii) PKCδ phosphorylates PP2A to promote dephosphorylation of TH-ser40, and inhibition of PKCδ attenuates PP2A activity, resulting in elevated TH-ser40 phosphorylation, TH activity, and dopamine levels, and finally (iv) TH activity and dopamine levels are enhanced in PKCδ (-/-) knockout
animals. These results were obtained using both cellular and molecular biological approaches in multiple cell culture models including PC12 cells, N27 mesencephalic dopaminergic cells and primary mesencephalic neurons, as well as in animal models, including a knockout model. Collectively, to our knowledge, this is the first report describing a negative regulation of the rate-limiting enzyme of the dopamine synthetic pathway, TH, by PKCδ via modulation of PP2A activity.

Phosphorylation is a key post-translational mechanism to regulate TH activity. Phosphorylation of serine residues at 8, 19, 31 and 40 can activate TH, resulting in enhanced dopamine synthesis (Campbell et al., 1986; Haycock, 1990; Mitchell et al., 1990; Lindgren et al., 2001; McCulloch et al., 2001; Dunkley et al., 2004). A number of kinases, including PKC, PKA, CaMPK-II, and MAPkinase, have been shown to phosphorylate one or more of these sites to increase TH activity, depending on the cell type. Since we found a high expression of PKCδ in nigral dopaminergic neurons (Fig.1), we initially hypothesized that PKCδ might phosphorylate TH to increase its activity. In order to test this hypothesis, we used the PKCδ inhibitor rottlerin to inhibit the kinase and anticipated that inhibition of PKCδ would result in inhibition of TH activity. Surprisingly, we observed a dose-dependent increase in TH activity and dopamine levels in cells treated with the PKCδ inhibitor rottlerin. To further confirm this observation, PKCδ-dominant negative mutant and siRNAs were used for PKCδ inhibition, which also caused increased TH activity and dopamine levels in dopaminergic cells. Determination of TH-ser40 phosphorylation under conditions of PKCδ inhibition further revealed an enhanced TH-ser40 phosphorylation with no significant change in phosphorylation of TH-ser31. Of the different phosphorylation sites, Ser40 is the major regulatory site contributing to increased TH activity and dopamine synthesis in vivo (Meligeni et al., 1982; Waymire et al., 1991; Daubner et al., 1992; Wu et al., 1992; McCulloch et al., 2001). Studies using PC12 cells either responsive or non-responsive to cAMP stimuli demonstrated that PKA is an important kinase in TH-ser40 phosphorylation (Wilson et al., 1996; Salvatore et al., 2001). Recently, Kobori et al. reported that GDNF
increases TH-ser31 and TH-ser40 phosphorylation, which contributes to enhanced dopamine synthesis in mesencephalic cultures (Kobori et al., 2004). Taken together, phosphorylation of the serine residues also appears to depend on cell types and stimuli.

General PKC increases ser-40 phosphorylation and TH activity (Cahill et al., 1989; Haycock, 1990; Haycock and Haycock, 1991; Waymire et al., 1991; Haycock, 1993; Bobrovskaya et al., 1998), however, the effect of PKC subtypes has never been explored. At the present time, 12 different isoforms of PKC have been identified and grouped into three major classes (Gschwendt, 1999; Dempsey et al., 2000; Maher, 2001; Kanthasamy et al., 2003). The conventional class of PKC isoforms PKCα, βI, βII, and γ require DAG and Ca²⁺ for activation, the novel PKC isoforms, PKCδ, ε, μ, η, and θ require only DAG but not Ca²⁺ for activation, and the atypical PKCs, PKC τ, λ, and ζ require neither Ca²⁺ nor DAG for activation. The isoform-specific physiological functions of each subtype of PKCs are yet to be characterized in CNS. The recently available, more specific pharmacological inhibitors, genetic mutants and siRNAs specific for subtypes of isoforms are extremely useful in characterizing functional significance of PKC isoforms. Our results of enhanced TH activity by pharmacological inhibitors and siRNA in three different cell culture models clearly demonstrate that PKCδ can negatively regulate TH activity.

An increase in TH-ser40 phosphorylation is normally mediated by either activation of a kinase responsible for the serine phosphorylation, or by blocking the activity of the phosphatase responsible for dephosphorylation of ser40. Since we found an increase in TH-ser40 phosphorylation and TH activity under the condition of PKCδ inhibition (pharmacological inhibitor, dominant negative mutant, siRNA studies; Figs. 2-6), we hypothesized that PKCδ attenuates TH function by enhancing phosphatase activity. Haavik et al. demonstrated that PP2A is the major serine/threonine phosphatase that regulates greater than 90% of the dephosphorylation of TH at Ser40 (Haavik et al., 1989). In their study, okadaic acid treatment dramatically increased TH phosphorylation and TH activity, establishing PP2A as an important regulator of both TH activity and Ser40 phosphorylation.
Recently, Leal et al. (Leal et al., 2002) demonstrated that PP2A dephosphorylated TH-ser40 at twice the rate compared to TH-ser31 and TH-ser19, suggesting a preferential dephosphorylation of TH-ser40 by PP2A. Additionally, Lindgren et al. (Lindgren et al., 2001) showed that PP2A inhibitor treatment in rat striatal slices did not increase TH phosphorylation at Ser31, whereas TH-ser40 phosphorylation was readily increased, indicating that the TH-ser40 site may be highly regulated by phosphorylation/dephosphorylation reactions as compared to TH-ser31. Furthermore, among these three phosphorylated serine residues, TH-ser40 mainly contributed to tyrosine hydroxylase activation and dopamine synthesis in vivo (Ramsey et al., 1996). Thus, consistent with previous studies, our results indicate TH ser40 is predominantly regulated by phosphorylation/dephosphorylation.

An active PP2A enzyme consists of a heterotrimer of the structural A subunit, a catalytic C subunit, and a regulatory B subunit (Dobrowsky and Hannun, 1993; Sontag et al., 1995; McCright et al., 1996; Ruvolo et al., 2002). The exact nature of the physical association and dynamic regulation of TH, PKCδ and PP2A are yet to be characterized; however, recent literature provides some information regarding this interaction. PKCδ, TH or PP2A have been recently shown to physically associate with each other, as well as other putative chaperone proteins such as α-synuclein and 14-3-3 (Ostrerova et al., 1999; Kleppe et al., 2001; Srivastava et al., 2002; Kjarland et al., 2006). Recently, Peng et al. (Peng et al., 2005) demonstrated that a functional interaction between α-synuclein and PP2A can regulate TH phosphorylation and TH activity. Srivastava et al. reported a physical interaction between PKCδ and PP2A in NIH3T3 cells, and that dephosphorylation of PKCδ by PP2A results in its inactivation (Srivastava et al., 2002). In our recent study, we showed that α-synuclein interacts with PKCδ and regulates its activity following neurotoxic insults (Kaul et al., 2005). Therefore, in the dopaminergic system, the physical and functional association between PKCδ, TH and PP2A could be facilitated and/or regulated by chaperone proteins such as α-synuclein (Kaul et al., 2005; Peng et al., 2005) and 14-3-3 (Ostrerova et al., 1999;
Kleppe et al., 2001; Kjarland et al., 2006). Nevertheless, further studies are required in both in vitro and in vivo model systems to elucidate the dynamics of physical and functional regulation of PKCδ and PP2A in regulation of TH activity.

In the present study, we show that PKCδ and PP2Ac physically associate in dopaminergic neuronal cell lysates as well as in mouse brain substantia nigra lysates. This interaction of PKCδ with PP2Ac may stimulate PP2A activity. To determine whether the physical association is accompanied by a functional interaction, we measured PP2A activity under conditions where PKCδ activity was inhibited. We found that PP2A activity was significantly decreased, without altering the PP2A protein levels, by the PKCδ inhibitor rottlerin. Basal PP2A enzymatic activity was also significantly reduced in dopaminergic cells stably expressing loss of function kinase inactive PKCδ-DN mutant compared to LacZ cells. Furthermore, substantia nigra of PKCδ (-/-) mice showed significantly lower basal PP2A activity compared to naïve animals, indicating that PKCδ can augment PP2A activity. Additionally, in vitro kinase assays also revealed that PKCδ can phosphorylate PP2Ac. Taken together, these data suggest that physical association accompanied by PP2Ac phosphorylation by PKCδ, results in PP2A activation. PP2A activity can be effectively regulated by phosphorylation. Many studies have shown that phosphorylation of PP2A at Tyr307 reduces its activity (Chen et al., 1992), indicating that Tyr307 is a key negative regulatory phosphorylation site. PP2A could also be phosphorylated at serine/threonine sites (Guo and Damuni, 1993), but the specific PKC isoforms involved in the PP2A phosphorylation at various sites of PP2A are yet to be characterized. Following the observation that PKCδ can phosphorylate PP2Ac to increase activity, we further examined whether enhanced PP2A activity reduces TH-ser40 phosphorylation and TH activity. Treatment with the PP2A inhibitor okadaic acid increased the TH-ser40 level and enhanced TH activity, suggesting that PP2A effectively regulates TH activity and ser40 phosphorylation. Our results are in agreement with a recent study showing attenuation of TH activity and TH-ser40 phosphorylation by PP2A (Peng et al., 2005). Collectively, our data
on PKCδ and PP2A suggest that PKCδ negatively regulates TH-ser40 phosphorylation and TH activity via increased PP2A activity by direct phosphorylation of PP2A.

Regulation of TH activity and DA levels is critical for normal dopaminergic neurotransmission in the CNS. Excessive DA production may not only alter neurotransmission, but may also contribute to neuronal cell death through increased oxidative stress (Hoyt et al., 1997; Luo et al., 1998). In this regard, we wish to point out that PKCδ can be activated by at least two independent mechanisms in neuronal cells. These include membrane translocation and caspase-3-dependent proteolytic cleavage (Kikkawa et al., 2002; Brodie and Blumberg, 2003; Kanthasamy et al., 2003). Of the two activation mechanisms, PKCδ activated by membrane translocation following tonic stimulation by lipid activators contributes to cell survival and proliferation (Kikkawa et al., 2002; Kanthasamy et al., 2003; Jackson and Foster, 2004). As demonstrated in our recent studies, another form of activation is caspase-3-dependent proteolytic cleavage of native PKCδ into regulatory and catalytic fragments resulting in persistent activation during exposure to neurotoxic agents such as MPP+, MMT, manganese or dieldrin (Anantharam et al., 2002; Kaul et al., 2003; Latchoumycandane et al., 2005). This form of proteolytic activation contributes to apoptotic cell death of dopaminergic neurons (Kanthasamy et al., 2003). In the case of a lipid activator, TPA induced membrane translocation of native PKCδ, but did not induce apoptotic cell death in dopaminergic cell lines (unpublished observations). Furthermore, recent studies from our lab and others have shown that unlike the native 74 kDa PKCδ, the 41 kDa catalytically active PKCδ fragment, upon proteolytic cleavage, is targeted to various sub-cellular organelles including the mitochondria (Majumder et al., 2000) and nucleus (DeVries et al., 2002) to activate apoptotic cell death signaling molecules. These results suggest that native and cleaved PKCδ have different substrate profiles. In this study, we examined the effect of native intact PKCδ on PP2A activity and TH regulation under normal conditions, but we did not study the effect of neurotoxicant-induced proteolytically activated PKCδ. Apparently, a high expression of PKCδ in nigral dopaminergic neurons has dual
functions. In normal situations, PKCδ phosphorylates PP2A to increase the phosphatase activity, resulting in enhanced dephosphorylation of TH-ser40, which eventually leads to decreased TH activity and DA synthesis (summarized in Supplemental Fig. 1). This negative regulation may be important for maintaining optimal dopamine levels. In addition to the function of regulating dopamine synthesis, under the condition of enhanced oxidative stress induced by neurotoxic insults, PKCδ serves as a key downstream proapoptotic effector of caspase-3, resulting in proteolytic activation of the kinase which contributes to cell death (Anantharam et al., 2002; Kanthasamy et al., 2003; Kaul et al., 2003; Yang et al., 2004).

In conclusion, we provide novel evidence that PKCδ negatively regulates TH activity and DA synthesis via activation of PP2A, and we suggest that PKCδ-mediated regulation of TH may have important implications in neurological dopaminergic system disorders, such as Parkinson's disease.

References


Kitazawa M, Anantharam V, Kanthasamy AG (2001) Dieldrin-induced oxidative stress and


Figure 1. PKCδ associates with TH in mouse brain and PC12 cells. A, Immunohistochemical analysis. Mouse brain was cut to 20 μm thickness at the level of the substantia nigra and stained with PKCδ polyclonal Ab (1:500 dilution) and TH monoclonal Ab (1:500 dilution), followed by incubation with either Alexa 488-conjugated (green, 1:1000)
or Cy3-conjugated (red, 1:1000) secondary antibody. Hoechst 33342 (10 μg/ml) was used to stain the nucleus. TH as red, PKCδ as green, nucleus as blue are shown. **B, PKCδ (74 kDa) co-immunoprecipitated with TH in mouse substantia nigra.** PKCδ was immunoprecipitated (IP) from mouse substantia nigra lysates by using PKCδ polyclonal Ab (1:100) and immunoblotted (IB) with anti-TH (1:100). In reverse immunoprecipitation studies, TH was immunoprecipitated with mouse monoclonal anti-TH antibody (1:100) and immunoblotted with PKCδ. TH (60 kDa) co-immunoprecipitated with PKCδ in mouse substantia nigra. Similarly, PKCδ (74 kDa) co-immunoprecipitated with TH in mouse substantia nigra. **C, PKCδ also co-immunoprecipitated with TH in PC12 cells,** PKCδ was immunoprecipitated (IP) from PC12 cell lysates by using PKCδ polyclonal Ab (1:100) and immunoblotted (IB) with anti-TH (1:100). In reverse immunoprecipitation studies, TH was immunoprecipitated with mouse monoclonal anti-TH antibody (1:100) and immunoblotted with PKCδ. TH (60 kDa) co-immunoprecipitated with PKCδ in PC12 cell lysates. Similarly, PKCδ (74 kDa) co-immunoprecipitated with TH in PC12 cell lysates.

Rabbit IgG and mouse IgG were used as negative controls. IP: immunoprecipitated samples; IB: immunoblots.
Figure 2. **PKCδ inhibition enhances TH activity.** PC12 cells were incubated with the DOPA decarboxylase inhibitor NSD-1015 (2 mM) for 1 hr prior to treatment with the PKCδ inhibitor rottlerin (1-10 μM). 0.01% DMSO was used as vehicle control. After 3 hr rottlerin treatment, cells were lysed and extracts were used for determining L-DOPA levels by HPLC. Rottlerin treatment increased L-DOPA levels, indicating enhanced TH activity. The data represent mean ± SEM of six to eight individual measurements. Asterisks (*p<0.05 and **p<0.01) indicate significant differences between rottlerin-treated cells and control cells.
Figure 3. PKCδ inhibition enhances TH-ser40 phosphorylation. A, Western blot analysis, PC12 cells were exposed to rottlerin (5 μM) or 0.01% DMSO (vehicle control) for 3 hr. Cell extracts were prepared and separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Phospho-specific antibodies directed against P-TH-ser40 and P-TH-ser31, and antibodies directed against TH and PKCδ were used for immunoblotting. To confirm equal protein loading in each lane, the membranes were
reprobed with β-actin antibody. The immunoblots were visualized using the Amersham’s ECL detection agents. Densitometric analysis of 60 kDa TH and P-TH-ser40 and P-TH-ser31 bands represents the mean ± SEM from three separate experiments (**p < 0.01). **B, Immunocytochemistry:** PC12 cells were grown on poly-L-lysine coated cover slips and then exposed to either 5 μM rottlerin or 0.01% DMS0 for 3 hr. After treatment, the cells were fixed and immunostained for P-TH-ser40 followed by staining with Alexa 488-conjugated antibody (green), as described in the methods section. For a negative control, cells were also immunostained with Alexa 488 without primary antibody staining. Immunostained cells were mounted and viewed under a Nikon TE2000 fluorescence microscope and images were captured with a SPOT digital camera. For in situ quantitative analysis of P-TH-ser40 levels, fluorescence immunoreactivity in cells was measured from six different areas in each group using Metamorph image analysis software, and data were analyzed with Prism software. Experiments were repeated three times, and representative images are presented. Asterisks (**p < 0.01) indicate significant differences between rottlerin treated and control cells.
Figure 4. PKCδ inhibition regulates TH activity and DA synthesis in N27 dopaminergic neuronal cells. A, In situ co-localization of TH and PKCδ. Differentiated N27 cells were grown on poly-L-lysine coated cover slips and processed for double immunostaining with anti-PKCδ monoclonal and anti-TH polyclonal antibodies or anti-PKCδ monoclonal and anti-P-TH-ser40 polyclonal antibodies, as described in the
methods section. **B, TH activity in PKCδ inhibitor rottlerin-treated cells and PKCδ-dominant negative mutant (PKCδ-DN) cells.** Differentiated N27 cells stably expressing LacZ or PKCδ-DN were pretreated with 2 mM NSD-1015 for 1 hr and lysed, and extracts were used for determining L-DOPA levels by HPLC. Also, cell extracts from N27 cells incubated with 2 mM NSD-1015 for 1 hr prior to treatment with 0.01% DMSO (vehicle control) or rottlerin (5 μM for 3 hr) were also used for measuring DOPA levels. **C and D, DA synthesis:** cell extracts from N27 cells stably expressing LacZ and PKCδ-DN, or treated with rottlerin (5 μM for 3 hr) were used for determining DA and DOPAC levels by HPLC. The data represent a mean ± SEM of six to eight individual measurements. Asterisks (**p<0.01 and ***p<0.001) indicate significant differences between rottlerin-treated cells and vehicle control cells, or between LacZ- and PKCδ-DN-expressing cells.
Figure 5. Effect of PKCδ-DN and rottlerin on TH-ser40 phosphorylation. A, Western blot: Cell extracts from rottlerin treated, PKCδ-DN and LacZ expressing N27 cells were subjected to Western blot, as described in the methods section. P-TH-ser40, P-TH-ser31 and
TH levels were detected using appropriate phospho-specific and TH antibodies. Densitometric analysis of 60 kDa P-TH-ser40, P-TH-ser31 and TH bands are shown next to the Western blot image. The data represent the mean ± SEM from three separate experiments (***p<0.01). B, TH-ser40 phosphorylation in stably expressing PKCδ-DN and LacZ N27 cells and C, primary mesencephalic cultures transiently expressing PKCδ-DN and LacZ constructs. Briefly, N27 cells and primary mesencephalic neurons expressing PKCδ-DN and LacZ (these constructs express V5 fusion protein) were cultured and processed for double immunostaining of P-TH-ser40 and V5 using specific antibodies. Stained cells were mounted on slides and viewed under a Nikon TE2000 fluorescence microscope, and images were captured with a SPOT digital camera, as described in the methods section. P-TH-ser40 levels were quantitatively analyzed from six different areas in each group using Metamorph image analysis. Experiments were repeated three times and representative images are shown. Asterisks (***p<0.01) indicate significant differences between LacZ and PKCδ-DN expressing cells.
Figure 6. *Effect of RNAi-mediated knockdown of PKCδ on P-TH-ser40 levels and DA synthesis.* A, Western blot of TH-ser40 phosphorylation. Briefly, N27 cells were transfected with PKCδ-siRNA and non-specific siRNA (NS-siRNA) and then extracts from siRNA transfected N27 cells were subjected to Western blot analyses of PKCδ, P-TH-ser40 and total TH. Densitometric analysis of the 74 kDa native PKCδ band and 60 kDa P-TH-ser40 and TH bands were normalized to β-actin levels, and are plotted below their
respective Western blots. B, DA synthesis. Cell extracts from PKCδ-siRNA and NS-siRNA transfected N27 cells were used for determining DA and DOPAC levels by HPLC. The data represent a mean ± SEM of six to eight individual measurements. Asterisks (*p<0.05 and **p<0.01) indicate significant differences between PKCδ-siRNA and NS-siRNA transfected N27 cells.
Figure 7. P-TH-ser40 levels in primary mesencephalic cultures from PKCδ knockout (-/-) animals. Primary mesencephalic neurons were cultured on laminin coated cover slips from PKCδ (-/-) and PKCδ (+/+) E16-18 pups. Primary cultures from PKCδ (+/+) animals were also incubated with 5 μM rottlerin for 3 hr. Cells were fixed and processed for P-TH-ser40 immunocytochemistry. Cy3-conjugated secondary antibody was used for visualization of P-TH-ser40 and viewed under a Nikon TE2000 fluorescence microscope. P-TH-ser40 levels were quantitatively analyzed from six different areas in each group using Metamorph image analysis. Asterisks (**p<0.001) indicate significant differences from controls. Experiments were repeated three times and representative images are shown.
FIGURE 8.

A, Western blot analysis of P-TH-ser40. N27 cells were treated with 0.01% DMSO (vehicle control) or 2 μM okadaic acid for 2 hr and then cell lysates were subjected to immunoblot for total TH, P-TH-ser40 and β-actin.

B, Effect of okadaic acid on TH activity.

C, Effect of okadaic acid on dopamine levels.

Figure 8. Effect of PP2A inhibition on TH-ser40 phosphorylation, TH activity and dopamine levels. A, Western blot analysis of P-TH-ser40. N27 cells were treated with 0.01% DMSO (vehicle control) or 2 μM okadaic acid for 2 hr and then cell lysates were subjected to immunoblot for total TH, P-TH-ser40 and β-actin. B, Effect of okadaic acid on
TH activity and C, dopamine content. N27 cells were treated with 2 μM okadaic acid for 2 hr. To determine TH activity, N27 cells were incubated with a DOPA decarboxylase inhibitor (2 mM NSD-1015 for 1 hr) prior to okadaic acid treatment. After treatment, cells were lysed and neurochemicals were extracted for determining L-DOPA and dopamine levels by HPLC. The data represent the mean ± SEM from three separate experiments. Asterisks (*p<0.05 and **p<0.01) indicate significant differences between untreated and okadaic acid-treated N27 cells.
FIGURE 9.

A  PKC\(\delta\) (+/+ ) Substantia nigra

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Figure 9. PKCδ associates with PP2Ac and modulates PP2A activity. Immunoprecipitation assays: A, PKCδ (+/+ ) mouse substantia nigra; B, PKCδ (-/- ) mouse substantia nigra and C, N27 cells. Anti-PKCδ polyclonal antibody was used for immunoprecipitation and immunoblotted with mouse monoclonal PP2Ac antibody. In reverse immunoprecipitation studies, anti-PP2Ac mouse monoclonal antibody was used for immunoprecipitation followed by immunoblotting with rabbit polyclonal PKCδ antibody. Anti-PKCδ polyclonal antibody was co-incubated with antigenic blocking peptide in the immunoprecipitation studies as a negative control. Rabbit IgG and mouse IgG were also used as negative controls. D, In vitro kinase assay, PKCδ phosphorylates PP2Ac. The top panel is a representative autoradiography showing [32P] phosphorylation of the immunoprecipitated PP2Ac (IP-PP2Ac) and recombinant PP2Ac (R-PP2Ac) by recombinant PKCδ (R-PKCδ). PP2Ac immunoprecipitates from N27 cell homogenates represent endogenous PP2Ac. Recombinant PKCδ protein was purchased from Sigma. Immunoprecipitation and phosphorylation assays were performed as described in the methods section. The quantitative data in the bottom panel represent the mean ± SEM from three assays. Histone H1 was used as positive control substrate, and for negative controls, no substrate was added.
Figure 10. Effect of PKCδ inhibition on PP2A activity. A, Cell free system, Recombinant PP2A enzyme (R-PP2A) was incubated with recombinant PKCδ (R- PKCδ) in the presence or absence of 5 μM rottlerin for 1 hr. PP2A enzyme activity was measured by
using a serine/threonine phosphatase assay kit from Promega. 2 μM okadaic acid was used as a positive control to inhibit PP2A activity. B, N27 cells: N27 cells were treated with 0.01% DMSO (vehicle control) or 3 μM rottlerin for 3 hr, or expressed LacZ or PKCδ-DN. The data represent a mean ± SEM of four to six individual measurements. Asterisks (**p<0.01 and ***p<0.001) indicate significant differences between either rottlerin treated and vehicle control cells or LacZ and PKCδ-DN-expressing N27 cells. C, Effect of PKCδ inhibition on PP2A protein level. Cell lysates from control, rottlerin treated N27 cells, or LacZ and PKCδ-DN expressing N27 cells were used for determination of PP2A protein level by Western blot.
Figure 11. Increased TH-ser40 phosphorylation, dopamine levels and PP2A activity in PKCδ (-/-) knockout animals. A, Western blot analysis of P-TH-ser40. Substantia nigral lysates from PKCδ (-/-) knockout and PKCδ (+/+) naïve animals were subjected to immunoblotting of PKCδ, P-TH-ser40, and total TH. B, PP2A activity: PP2A activity was measured in substantia nigra homogenates obtained from PKCδ (+/+) and PKCδ (-/-) animals. The data represent a mean ± SEM from four to six animals. Asterisks (**p<0.01) indicate...
significant differences between PKCδ (+/+) and PKCδ (-/-) animals. C, Neurotransmitter levels: DA and DOPAC levels were determined in the striatal extracts of PKCδ (-/-) and PKCδ (+/+), animals by HPLC. The data represent a mean ± SEM from six to ten animals. Asterisks (*p<0.05) indicate significant differences between PKCδ (-/-) and PKCδ (+/+) animals.
Supplemental Figure 1. A schematic depiction of TH regulation by PKC\(\delta\) in dopaminergic cells. PKC\(\delta\) enhances phosphatase 2A (PP2A) activity by phosphorylation of PP2Ac. Increased PP2A activity decreases TH phosphorylation at site ser40, which leads to inactivation of TH and reduction of its activity and an ultimate decrease in dopamine synthesis. Inhibition of PKC\(\delta\) by rottlerin, PKC\(\delta\)-DN mutant, PKC\(\delta\) KO or PKC\(\delta\) siRNA suppresses PP2A activity due to a decreased phosphorylation of PP2A. The okadaic acid
inhibits the PP2A phosphatase activity, thereby preventing the dephosphorylation of P-TH-ser40 and enhancing the TH enzymatic activity. In conclusion, our data suggest that PKCδ negatively regulates TH via PP2A.
CHAPTER III: NEUROPROTECTIVE EFFECT OF PKCδ INHIBITOR ROTTLERIN IN CELL CULTURE AND ANIMAL MODELS OF PARKINSON’S DISEASE

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Danhui Zhang, Vellareddy Anantharam, Arthi Kanthasamy and Anumantha Kanthasamy

Abstract

Recent studies from our laboratory demonstrated that the protein kinase C delta (PKCδ) isoform is an oxidative stress-sensitive kinase and a key mediator of apoptotic cell death in PD models (Kaul et al., 2003; Yang et al., 2004). We showed that native PKCδ is proteolytically activated by caspase-3 and that suppression of PKCδ by dominant negative mutant or siRNA against the kinase can effectively block apoptotic cell death in cellular models of PD. In an attempt to translate the mechanistic studies to a neuroprotective strategy targeting PKCδ, we systematically characterized the neuroprotective effect of a PKCδ inhibitor, rottlerin, in MPP+-treated primary mesencephalic neuronal cultures, as well as in an MPTP animal model of PD. Rottlerin treatment in primary mesencephalic cultures significantly attenuated MPP+-induced TH-positive neuronal cell and neurite loss. Administration of rottlerin, either intraperitoneally or orally, to C57 black mice showed significant protection against MPTP-induced locomotor deficits and striatal depletion of dopamine and its metabolite DOPAC. Notably, rottlerin post-treatment was effective, even when dopamine/metabolite depletion by MPTP was greater than 60%, demonstrating its neurorescue potential. Additionally, the dose of rottlerin used in neuroprotective studies effectively attenuated the MPTP-induced PKCδ kinase activity. Importantly, stereological analysis of nigral neurons revealed rottlerin treatment significantly protected against
MPTP-induced TH-positive neuronal loss in the substantia nigra compacta. Collectively, our findings demonstrate the neuroprotective effect of rottlerin in both cell culture and preclinical animal models of PD, and suggest that pharmacological modulation of PKCδ may offer a novel therapeutic strategy for treatment of PD.

**Introduction**

Parkinson’s disease (PD) is a major neurodegenerative disorder characterized by progressive and substantial loss of dopaminergic neurons in the substantia nigra compacta (SNc), resulting in debilitating motor signs including tremors, bradykinesia, and rigidity. PD affects more than 1% of the population over the age of 60 in the US (Allam et al., 2005; West et al., 2005), ranking it as the second most common neurodegenerative disorder. Although the existing approaches to PD treatment alleviate the signs, they fail to prevent the progression of the neurodegenerative process. Currently, no available drugs prevent the progressive loss of nigral dopaminergic neurons. The mechanisms underlying the dopaminergic degenerative process observed in PD are not well understood, which has hampered development of successful neuroprotective drugs. Several clinical studies in post-mortem PD human brain tissues and experimental studies in animal PD models indicate that oxidative stress, mitochondrial and ubiquitin proteasomal dysfunction, and apoptosis all contribute to the dopaminergic degenerative process, primarily due to the higher vulnerability of nigral neurons to oxidative damage, compared to other brain regions (Zigmond et al., 2002; Dawson and Dawson, 2003; Greenamyre and Hastings, 2004; Maguire-Zeiss et al., 2005). However, identification of the key cellular target responsible for mediating the degenerative process following oxidative insult is being actively pursued by examining various apoptotic cell death signaling pathways (Santiago et al., 1997; Saporito et al., 1999; Du et al., 2001; Duan et al., 2002; Obata, 2006).

Recently, we discovered that the caspase-3 mediated proteolytic activation of PKCδ, a member of the novel PKC isoform family, plays a critical role in oxidative stress-induced
dopaminergic cell death in cell culture models of PD (Anantharam et al., 2002; Kaul et al., 2003; Yang et al., 2004). Proteolytic cleavage of PKCδ (74 kDa) by caspase-3 results in a 41-kDa catalytic subunit and a 38-kDa regulatory subunit, leading to a persistent activation of the kinase (Kaul et al., 2003; Yang et al., 2004). Blockage of proteolytic activation of PKCδ by overexpression of the kinase-dominant negative PKCδ mutant, cleavage-resistant PKCδ mutant, or siRNA directed against PKCδ almost completely prevented the dopaminergic cell death (Kaul et al., 2003; Yang et al., 2004). Therefore, in the present study, we examined the neuroprotective efficacy of the PKCδ inhibitor rottlerin in both primary cell culture and animal models. Herein, we report that rottlerin not only protects against MPP+-induced degeneration of TH-positive neurons in primary mesencephalic culture models but, most importantly, is clearly neuroprotective in an MPTP animal model of Parkinson’s disease.

**Materials and Methods**

**Chemicals and Biological Reagents.** Rottlerin, 1-methyl-4-phenylpyridinium, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, protease cocktail, ATP, Protein A-Sepharose®, protein-G-sepharose and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO); mouse tyrosine hydroxylase (TH) antibody was purchased from Chemicon (Temecula, CA); rabbit PKCδ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-rabbit and anti-mouse secondary antibodies and the ECL chemiluminescence kit were purchased from Amersham Biosciences (Piscataway, NJ). [γ-32P]ATP was purchased from Perkin Elmer Life Science (Boston, MA). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Neurobasal medium, B27 supplement, L-glutamine, penicillin, and streptomycin were purchased from Invitrogen (Gaithersburg, MD).

**Mesencephalic primary neuron cultures and treatment.** Primary mesencephalic neuronal
cultures were prepared from the ventral mesencephalon of gestational 16-18-day-old mice embryos as described in our previous publication (Yang et al., 2004). Mesencephalic tissues were dissected and maintained in ice-cold Ca^{2+}-free HBSS and then dissociated in HBSS solution containing trypsin-EDTA (0.25%) for 20 min at 37°C. The dissociated cells were then plated at equal density (0.5 × 10^6 cells) on 12 mm coverslips precoated with poly-D-lysine (1 mg/ml). Cultures were maintained in a chemically defined medium consisting of neurobasal medium fortified with B-27 supplements, L-glutamine (500 μM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Life Technologies). The cells were maintained in a humidified CO₂ incubator (5% CO₂, 37°C) for 24 hr and then treated with cytosine arabinoside (10 μM) for 24 hr to inhibit glial cell proliferation. Half of the culture medium was replaced every 2 days. Approximately 6–7-day-old cultures were used for experiments. Primary mesencephalic dopaminergic neuronal cells were exposed to 10 μM MPP⁺ in the presence or absence of rottlerin (0.3 and 1 μM) for 48 hrs. Then cells were fixed and stained for TH.

**Immunocytochemistry.** After treatment, the primary mesencephalic neurons were fixed with 4% paraformaldehyde and processed for immunocytochemical staining. First, non-specific sites were blocked with 5% normal goat serum containing 0.4% BSA and 0.2% Triton-X 100 in PBS for 20 min. Cells were then incubated with antibody directed against TH (1:500 dilution) at 4°C overnight, followed by incubation with Cy3-conjugated (red, 1:1000) secondary antibody for 1 hr at room temperature. Secondary antibody treatments were followed by incubation with Hoechst 33342 (10 μg/ml) for 3 min at room temperature to stain the nucleus. Then the coverslips containing stained cells were washed with PBS, mounted on a slide, and viewed under a Nikon inverted fluorescence microscope (Model TE-2000U); images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).
**Quantification of TH⁺ cell count and neuronal processes.** Metamorph software (Version 5.0, Molecular Devices Corp., Sunnyvale, CA) was used for measurement of TH⁺ cells and neuronal processes in primary neurons from each coverslip. For measurement of TH cell count, the images were first thresholded, and then neuronal count and volume were measured using the Integrated Morphometry Analysis (IMA) function. The data were logged to an Excel spreadsheet and analyzed. For measurement of neuronal processes, the lengths of the processes were marked by applying the region and length measurement function in the IMA. The data were exported to an Excel spreadsheet and analyzed. TH⁺ neurons and their processes were counted in at least six individual cultures for each treatment. This is a modified version of a method recently used for quantification of neuronal processes (Yang et al., 2004).

**Animal studies and rottlerin treatment.** Six- to 8-week-old 26/C57/bL mice weighing 25-30 g were housed in standard conditions: constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-h light/dark cycle. Mice were allowed free access to food and water. Use of the animals and protocol procedures were approved and supervised by the Committee on Animal Care (COAC) at Iowa State University. Rottlerin was dissolved in 1% dimethyl sulfoxide (DMSO) and administered either intraperitoneally or orally at various doses ranging from 3 mg/kg to 20 mg/kg. An equal volume of DMSO was given to controls. For rottlerin pre-treatment study, rottlerin and DMSO administrations were started 24 hr before the injections of MPTP, and continued for 5 days. In the subchronic regimens of MPTP administration, MPTP in phosphate-buffered saline (PBS) was injected intraperitoneally at doses of 30 mg/kg, once per day. For rottlerin post-treatment study, mice were treated with MPTP (20 mg/kg, i.p.) once a day for 3 days and then rottlerin was administered (20 mg/kg, p.o.) once a day for an additional 7 days. Mice were sacrificed 7 days after the MPTP injections, and the striata were dissected for catecholamine analysis. The mesencephalons were fixed in 4% paraformaldehyde for TH immunostaining.
**Immunohistological and stereological analysis of nigral sections.** Briefly, brains were harvested following cervical dislocation, post-fixed in paraformaldehyde, and used for TH immunolabelling and stereological analysis as described previously (Thiruchelvam et al., 2003; Thiruchelvam et al., 2004). Fixed brains were cut into 30-µm sections and collected in cryoprotectant. Sections were rinsed in PBS at room temperature before immunostaining, and then incubated with anti-TH antibody. The total number of TH+ and Nissl-stained neurons in the SNpc were counted using an optical fractionator with the criteria previously described by others (Thiruchelvam et al., 2003; Thiruchelvam et al., 2004). After delineation of the region at low magnification (4× objective), every fourth section from the entire substantia nigra was sampled at higher magnification (100× objective) using a StereoImager (Microbrightfield, VT).

**PKCδ kinase assay.** PKCδ enzymatic activity was assayed using an immunoprecipitation kinase assay as described by Reyland et al. (Reyland et al., 1999). Briefly, substantia nigra brain tissue from rottlerin, MPTP, MPTP+rottlerin treated mice, and control mice was washed once with PBS and resuspended in 1 ml of PKC lysis buffer (25 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 10 mM NaF, and 4 µg/ml each aprotinin and leupeptin). The lysates were cooled on ice for 30 min, and then centrifuged at 13,000 × g for 5 min, and the supernatants were collected as cytosolic fraction. Protein concentration was determined using a Bradford assay. Cytosolic protein (0.25-0.5 mg) was immunoprecipitated overnight at 4°C using 2 µg of anti-PKCδ antibody. The immunoprecipitates were then incubated with Protein A-Sepharose® (Sigma) for 1 hr at 4°C. The protein A-bound antigen-antibody complexes were then washed three times with 2× kinase buffer (40 mM Tris, pH 7.4, 20 mM MgCl₂, 20 µM ATP, and 2.5 mM CaCl₂), and resuspended in 20 µl of 2× kinase buffer. The reaction was started by adding 20 µl of reaction
buffer containing 0.4 mg histone H1, 50µg/ml phosphatidylserine, 4.1 µM dioleoylglycerol, and 5 µCi of [γ-32P] ATP (3000 Ci/mM) to the immunoprecipitated samples and incubated for 10 min at 30°C. SDS gel-loading buffer (2×) was added to terminate the reaction, the samples were boiled for 5 min, and the products were separated on a 12.5% SDS-PAGE gel. The H1 phosphorylated bands were detected using a Personal Molecular Imager (FX model; Bio-Rad), and quantified using Quantity One 4.2.0 software.

**HPLC analysis of neurotransmitters.** DA and DOPAC levels in brain striatal tissues were determined by high-performance liquid chromatography with electrochemical detection (HPLC-EC); samples were prepared as described previously (Kitazawa et al., 2001). Briefly, neurotransmitters were extracted from samples using 0.1 M perchloric acid containing 0.05% Na2EDTA and 0.1% Na2S2O5. The extracts were filtered in 0.22 µm spin tubes, and 20 µl of the samples was loaded for analysis. DA and DOPAC were separated isocratically by a reversed-phase column with a flow rate of 0.7 ml/min. An HPLC system (ESA Inc., Bedford, MA) with an automatic sampler (ESA model 542) was used for these experiments. The electrochemical detection (EC) system consisted of an ESA Coulotech model 5100A with a microanalysis cell (model 5014A) and a guard cell (model 5020) (ESA Inc., Bedford, MA). One mg DA and DOPAC standards were dissolved in 1 ml antioxidant solution separately to prepare standards for HPLC. Peaks representing DA and DOPAC in the samples were identified on the basis of their retention times and co-chromatography of the respective authentic standards in several different chromatographic conditions. The DA and DOPAC levels were measured as ng /mg protein.

**Western blot.** Brain lysates containing equal amounts of protein were loaded in each lane and separated on a 10-12% SDS-PAGE gel as described previously (Kaul et al., 2003). After the separation, proteins were transferred to a nitrocellulose membrane, and nonspecific binding sites were blocked by treating with 5% nonfat dry milk powder. The membranes
were then treated with primary antibodies directed against TH (mouse monoclonal, 1:1000). The primary antibody treatments were followed by treatment with secondary HRP-conjugated anti-mouse IgG (1:2000) for 1 hr at room temperature. Secondary antibody-bound proteins were detected using an ECL chemiluminescence kit (Amersham Biosciences). To confirm equal protein loading, blots were reprobed with a β-actin antibody (1:5000 dilution). Western blot images were captured with a Kodak 2000 MM imaging system and data were analyzed using 1D Kodak Imaging Analysis software.

**Locomotor activity.** Behavioral data were collected using VersaMax Animal Activity Monitors (AccuScan Model RXYZCM-16, Columbus, OH). Each chamber was 40×40×30.5 cm, made of clear Plexiglas and covered with a Plexiglas lid with holes for ventilation. Infrared monitoring sensors were located every 2.54 cm along the perimeter (16 infrared beams along each side) and 2.5 cm above the floor. Two additional sets of 16 sensors were located 8.0 cm above the floor on opposite sides. Data were collected and analyzed by a VersaMax Analyzer (AccuScan Model CDA-8, Columbus, OH) which in turn sent information to a computer where it was stored for future analyses. Locomotor activity was presented as horizontal movement and vertical movement. All data are expressed as percent of the vehicle-treated control group (mean ± SEM, n=6) and were obtained 1 day after MPTP or vehicle treatment in a 20-min test session.

**Data analysis.** Data analysis was performed using Prism 4.0 software (GraphPad Software, San Diego, CA). Data were first analyzed as raw by using one-way ANOVA and then Bonferroni’s post-test was performed to compare all treatment groups, and differences with $p < 0.05$ were considered significant.

**Results**

*Rottlerin rescues TH+ neuronal loss from MPP+ in primary mesencephalic*
cultures. First, we examined whether the PKCδ inhibitor rottlerin (Figure 1) can rescue TH⁺ neurons from MPP⁺ toxicity in primary mesencephalic cultures. Primary mesencephalic cultures were isolated from E16-18 C57 black mice embryos and exposed to 10 μM MPP⁺ in the presence or absence of rottlerin (0.3 and 1 μM) for 48 hr. After treatment, primary neurons were processed for TH immunocytochemistry. As shown in Fig. 2A, MPP⁺ treatment induced about 90% loss of both TH cell count and neurite processes. Quantitative analysis of TH⁺ cell counts showed 0.3 μM and 1 μM rottlerin treatment significantly protected against MPP⁺ neurotoxicity. Similarly, average lengths of TH⁺ neuronal processes in MPP⁺ plus rottlerin-treated primary neurons were significantly longer than the processes of neurons treated only with MPP⁺ (Fig. 2B). These data suggest that the PKCδ inhibitor rottlerin possesses neuroprotective properties in the MPP⁺-induced dopaminergic degenerative model.

PKCδ kinase activity is significantly suppressed in rottlerin-treated animals. We previously showed that pretreatment with rottlerin significantly attenuated oxidative stress-induced increases in PKCδ kinase activity in an immortalized mesencephalic dopaminergic neuronal cell line (Kaul et al., 2003). We also observed a high expression of PKCδ in TH positive nigral neurons (unpublished observation). In this experiment, we examined whether rottlerin effectively inhibits PKCδ kinase activity in mouse substantia nigra. Rottlerin was administered at doses of 3-20 mg/kg. As shown in Fig. 3, PKCδ kinase activity was significantly suppressed in the substantia nigra of rottlerin-treated animals in a dose-dependent manner compared to vehicle-treated control animals. Densitometric analysis of phosphorylated histone H1 bands revealed 48%, 37%, and 29% decreases in protein kinase activity in SN of mice treated with 20, 10, and 7 mg rottlerin, respectively. These results indicate that rottlerin significantly inhibits PKCδ activity, as observed in our previous cell culture experiments (Kaul et al., 2003).

Rottlerin attenuates striatal dopamine depletion induced by MPTP-treatment.
After characterizing the neuroprotective effect of rottlerin in cell culture models of PD, as well as verifying rottlerin’s ability to suppress PKCδ activity in the brain, we examined the neuroprotective efficacy of rottlerin in an MPTP-induced mouse model of PD. In order to determine the optimal route of rottlerin treatment, we chose the intraperitoneal route for low doses of rottlerin (3 and 7 mg/kg) and the oral route for high doses (10 and 20 mg/kg). Animals were administered rottlerin (3-20 mg/kg body weight) 24 hr prior to MPTP administration. Animals were then co-treated with rottlerin and MPTP (30 mg/kg, i.p.) daily for 5 days. Saline- and vehicle-injected animals were used as controls. To assess the protective effect of rottlerin, we first examined whether rottlerin could block MPTP-induced loss of striatal dopamine and its metabolites. As shown in Fig. 4, MPTP treatment induced loss of (A) dopamine (>94%), (B) DOPAC (>95%), and (C) HVA (>95%) in mouse striatum. The dopamine levels were determined to be 187.5 ± 7.4, 14.5 ± 3.8, and 112.8 ± 25.3 ng/mg protein in control-, MPTP-, and MPTP + rottlerin (20 mg/kg)-treated animals, respectively. Pretreatment with rottlerin (20 mg/kg) afforded nearly a 50% protection against MPTP-induced striatal dopamine loss. Similar results were found for DOPAC and HVA (Fig. 4). These data suggest that rottlerin treatment could afford protection against MPTP-induced striatal dopamine and dopamine metabolite loss in animal PD models.

**Rottlerin inhibits PKCδ kinase activity in MPTP-treated animals.** In a previous study, we showed that PKCδ activity was increased in MPP⁺-treated N27 dopaminergic cells as a result of proteolytic kinase activation (Kaul et al., 2003). Therefore, we examined whether 20 mg/kg rottlerin, the most effective dose against MPTP-induced dopamine depletion, suppressed MPTP-induced increases in PKCδ kinase activity in nigral tissue. In Fig. 5, MPTP-treated mice showed nearly a 2-fold increase in PKCδ activity compared with control mice, and rottlerin treatment almost completely suppressed PKCδ kinase activity induced by MPTP treatment. These results suggest rottlerin protects against PKCδ activation.
**Rottlerin prevents TH cell loss induced by MPTP toxicity in SNC.** Since rottlerin effectively attenuated MPTP-induced loss of dopamine and its metabolites in the striatum, we next examined whether rottlerin could protect the TH⁺ neurons from MPTP-induced toxicity in the SNC. MPTP treatment caused a 40% loss of TH⁺ neurons in the SNpc as compared to saline-treated animals. However, MPTP induced only a 13% loss of TH⁺ neurons in animals treated with 20 mg/kg (p.o) rottlerin (Fig. 6). Data analysis revealed that rottlerin treatment afforded a 37% protection against MPTP. In addition, there was a noticeable loss of TH⁺ fiber density in MPTP-treated animals, while fiber density in the rottlerin-treated animals was close to that of the vehicle-treated group (Fig. 6A).

**Rottlerin prevents MPTP-induced reduction of TH protein in SNC.** Based on our finding that rottlerin counteracted the MPTP-induced dopamine and TH⁺ neuronal loss, we further confirmed nigral TH protein levels by Western blot. MPTP treatment greatly reduced TH protein levels in mouse SN (Fig. 7), which may have resulted from the loss of TH⁺ neurons in this region. Treatment with rottlerin (3-20 mg/kg) restored the TH levels in MPTP-treated animals to those of control animals, further confirming the protective effect of rottlerin against TH-positive neuronal loss.

**Rottlerin attenuates MPTP-induced motor deficits.** With evidence suggesting that rottlerin protects against MPTP-induced dopamine and TH neuronal loss, we next determined if rottlerin treatment could also afford protection against MPTP-induced motor deficits. We compared the motor activity of vehicle, MPTP, and MPTP plus rottlerin-treated animals, using a Versamax computerized activity monitoring system (Accuscan, Columbus, OH). This system uses infrared sensors to measure repetitive movements both in the horizontal and vertical planes in real time, and provides color-coded output. Representative activity maps of the animals are presented in Fig. 8A. The cumulative horizontal and vertical activities are shown in Fig. 8B. All data are expressed as percent of the vehicle-treated group and
were obtained 1-2 hr before sacrificing the animals for neurochemical and histological studies. The data indicate that both the vertical and horizontal motor activity were significantly reduced (>45%) in MPTP-treated animals compared to the vehicle-treated group. However, administration of rottlerin partially restored both vertical and horizontal motor activity of MPTP-treated animals to the levels observed in control animals. These results demonstrate rottlerin treatment attenuates MPTP-induced locomotor deficits in mice.

Rottlerin post-treatment rescues striatal dopamine depletion induced by MPTP treatment. To determine whether rottlerin post-treatment can protect against MPTP-induced dopaminergic toxicity, we created a PD model that shows over 60% of striatal dopamine depletion by pretreating the mice with 20 mg/kg MPTP (i.p.) for 3 days. Following the MPTP treatment, animals were given 20 mg/kg rottlerin for 3 days. As shown in Fig. 9, post-treatment with rottlerin significantly attenuated the loss of dopamine induced by MPTP pretreatment. Post-treatment with rottlerin (20 mg/kg) afforded nearly a 33% protection against MPTP pretreatment-induced striatal dopamine loss. Similar results were found for DOPAC and HVA (Fig. 9). These data demonstrate that rottlerin has the potential to rescue MPTP-induced dopaminergic neurotoxicity in animal PD models.

Discussion

The present study demonstrates that the PKCδ inhibitor rottlerin protects against MPTP-induced motor deficits, striatal dopamine depletion, and nigral dopaminergic neuronal loss. The neuroprotective effect of rottlerin was also evident against dopaminergic neurodegeneration, as the inhibitor rescued TH⁺ neurons from MPP⁺-induced neurotoxicity in primary mesencephalic cultures. To our knowledge, this is the first demonstration of a neuroprotective strategy using a pharmacological inhibitor of PKCδ in an animal model of Parkinson’s disease.

The PKC family of kinases consists of 13 isoforms classified into three distinct
sub-families based on their activation profiles. Conventional isoforms of PKCs (cPKCs), PKCα, βI, βII, and γ, require both intracellular calcium and diacylglycerol (DAG) for their activation, whereas the novel PKC (nPKC) isoforms, PKCδ, ε, η, θ, and μ require only DAG. The atypical PKCs (aPKCs), PKCζ and λ(I), require neither calcium nor phospholipids for activation. The expression patterns and biological functions of these isoforms in CNS are only beginning to be recognized. For example, PKCγ is expressed predominantly in the brain, and has been implicated in neural plasticity, including long term potentiation (Saito and Shirai, 2002) and ischemic injury (Aronowski and Labiche, 2003). The levels of another novel PKC isoform, PKCε, are greatly reduced in Alzheimer’s brains, and are linked to increased production of amyloid β protein (Matsushima et al., 1996). In the CNS, PKCε is localized mainly in the presynaptic region and induces neurite outgrowth during differentiation and mediates apoptosis during embryonic development, among other functions (Matsushima et al., 1996).

Recent studies from our laboratory and others have demonstrated that PKCδ, a key member of nPKCs, plays a proapoptotic role in various cell types (Anantharam et al., 2002; Kanthasamy et al., 2003). We also showed that PKCδ is an oxidative stress kinase in CNS (Kanthasamy et al., 2003; Kaul et al., 2005). Oxidative stress and apoptotic cell death have been implicated in several neurodegenerative disorders including Parkinson’s disease (Allam et al., 2005; Maguire-Zeiss et al., 2005; West et al., 2005; Smith et al., 2006). We showed that oxidative stress, in dopaminergic neuronal models, persistently activates PKCδ by proteolysis via caspase-3 cleavage of the native kinase (72-74-kDa), which yields a 41-kDa catalytically active fragment and a 38-kDa regulatory fragment (Anantharam et al., 2002). We also demonstrated that the proteolytic activation of PKCδ contributes to apoptotic cell death in cell culture models of PD (Kaul et al., 2003; Yang et al., 2004). In addition to the proapoptotic role, PKCδ amplifies apoptotic signaling via positive feedback activation of the caspase cascade (Anantharam et al., 2002; Kaul et al., 2003), demonstrating the dual role of PKCδ as a mediator and amplifier of apoptosis. Overexpression of the dominant negative
mutants, PKC\(\delta^{D327A}\) (caspase-cleavage resistant), PKC\(\delta^{K376R}\) (kinase inactive), and PKC\(\delta^{Y311F}\) (phosphorylation defective) loss-of-function mutants, as well as suppression of PKC\(\delta\) by siRNA provided protection against apoptotic cell death induced by various dopaminergic toxins, including MPP\(^+\), in dopaminergic neuronal cells (Kaul et al., 2003; 2005; Yang et al., 2004). Recently, another study showed that glutamate-induced cell death was significantly attenuated in cells transfected with PKC\(\delta\) siRNA, suggesting that PKC\(\delta\) may play an important role in excitotoxicity (Choi et al., 2006). A common mechanism has been proposed in degenerative processes of PD (Dawson and Dawson, 2003; Greenamyre and Hastings, 2004). Together, these studies indicate that PKC\(\delta\) plays an important role as downstream effector of apoptotic cell death in PD models, and that the kinase is a valid pharmacological target for development of novel inhibitors against oxidative stress-induced dopaminergic degenerative processes in PD.

Small molecule inhibitors of protein kinases are being increasingly evaluated for therapeutic use for various human diseases. In this study, we show that the pharmacological inhibitor of PKC\(\delta\), rottlerin, is neuroprotective in an MPTP model of PD. Rottlerin is isolated from the seeds of Mallotus philippinensis and has been shown to be a potent inhibitor of PKC\(\delta\), with an IC\(_{50}\) of 3-6 \(\mu\)M, whereas the K\(_{i}\)s for other PKC isoforms (\(\alpha\), \(\beta\), \(\gamma\), \(\epsilon\) and \(\lambda\)) are at least 5-10 times higher (Gschwendt et al., 1994; Davies et al., 2000; Soltoff, 2001). Toxicity data for rottlerin indicate that the compound has a low toxicity profile (LD\(_{50}\) 750 mg/kg, rat oral); 120 mg/kg (oral 6-day rat study) is the lowest toxic dose (Varma et al., 1959). The relative safety, combined with its efficacy, make rottlerin quite attractive as a potential therapeutic agent.

Previous studies have shown rottlerin treatment rescues non-neuronal cells from apoptotic death induced by various stimuli (Reyland et al., 1999); in addition, rottlerin inhibits PKC\(\delta\) enzyme activity \textit{in vitro} (Anantharam et al., 2002). Recently, we showed MPP\(^-\)-induced increases in PKC\(\delta\) kinase activity were effectively blocked by rottlerin treatment in an immortalized dopaminergic mesencephalic neuronal cell line (Kaul et al.,
2003). In the present study, we provide evidence that rottlerin treatment can also rescue TH+ neurons from MPP+-induced neurotoxicity in primary mesencephalic cultures. Extension of these studies in animal models revealed rottlerin can effectively attenuate the MPTP-induced increase in PKCδ kinase activity in mouse SNc. Further, rottlerin administration attenuated neurochemical depletion and locomotor activity of MPTP-treated animals, demonstrating protection against both behavioral and neurochemical deficits. Rottlerin treatment also protected against MPTP-induced loss of TH neurons in the SNc, revealing the neuroprotective effect in a well-characterized preclinical model of PD. Taken together, the observed protective effect of the PKCδ inhibitor rottlerin in an MPTP model of PD strongly supports our previous mechanistic studies demonstrating proapoptotic function of PKCδ in dopaminergic neuronal cell death (Kaul et al. 2003; 2005; Yang et al., 2005).

Rottlerin is also shown to be able to inhibit CaM kinase III and MAPKAP kinase 2. MAPKAP kinase 2 is an essential component in the inflammatory response which regulates biosynthesis of TNF-alpha at a post-transcriptional level. Inhibition of MAPKAP kinase 2 might help to against MPTP toxicity (Kotlyarov et al., 1999).

A number of researchers have attempted to develop neuroprotective agents targeting cell death signaling molecules. Inhibitors targeted against mixed lineage kinase (MLK) and poly-ADP-ribose polymerase (PARP) have been shown to protect nigral dopaminergic neurons in animal models (Cosi et al., 1996), and some of these agents are currently being evaluated in human clinical trials (Parashar et al., 2005). Previous studies have shown PKCδ can indirectly regulate PARP and MLK (Merritt et al., 1999; Yoshida et al., 2002; Kitazawa et al., 2004), suggesting that PKCδ may be an attractive upstream neuroprotective therapeutic target. Saporito et al. showed systemic injection of the c-Jun N-terminal kinase (JNK) inhibitor CEP-1347/KT-7515 protected against MPTP toxicity in the C57 black mouse model (Saporito et al., 1999). However, CEP-1347 was effective only in the pretreatment regimen, and not in the post-exposure period to MPTP. The outcome of recent clinical trials with the CEP-1347 compound was not encouraging due to the lack of clinical improvement.
in PD patients, which may be related to the failure of the compound in the preclinical model
during post-treatment to prevent the progression of the disease (Burke, 2007). In the
present study, rottlerin post-treatment protects against degenerative processes even 3 days
after MPTP exposure, demonstrating its neurorescue properties. Normally, PD patients lose
around 70% of dopaminergic innervation before they develop signs of PD and therefore, the
effective rottlerin post-treatment regimen shows real promise for therapeutic development.
Advantageously, rottlerin is orally active.

Maruyama et al. developed a class of compounds with multiple putative properties,
including iron chelation, MAO inhibition, and antioxidant effects (Maruyama et al., 2002),
but the preclinical and clinical evaluations of these compounds have not yet been evaluated.
Recent Phase II clinical trials with 12 compounds have demonstrated creatine and
minocycline as effective; these compounds have been recommended to proceed to Phase III
trials to determine if they alter the long term progression of PD (NINDS, 2006). Minocycline,
a tetracycline derivative with anti-inflammatory properties, prevented
dopaminergic neurodegeneration in MPTP models of PD (Du et al., 2001). Minocycline
also inhibits microglial-associated inflammation and apoptosis (Ravina et al., 2003; Kelly et
al., 2004). Oral administration of the nutritional supplement creatine attenuated MPTP
toxicity, which is similar to our findings with rottlerin (Matthews et al., 1999). Creatine
exhibits neuroprotective properties by inhibiting mitochondrial permeability, as well as
indirectly acting as an antioxidant (Tarnopolsky and Beal, 2001). Very recently, the National
Institute of Neurological Disorders and Stroke (NINDS) launched a multicenter large scale 7
year phase III clinical trial, at the cost of about 60 million dollars, to evaluate the
neuroprotective effect of a purified form of creatine (Couzin, 2007). In addition to creatine,
the antioxidant Coenzyme-Q is being evaluated in PD clinical trials (Shults and Haas, 2005).
Release of cytochrome C resulting from increased mitochondrial permeability during
oxidative stress has been shown to initiate PKCδ activation (Anantharam et al., 2002), and
antioxidant treatment attenuates proapoptotic action of the kinase (Kaul et al., 2003),
indicating that PKCδ is closely associated with two leading PD pathological mechanisms, i.e., oxidative insult and mitochondrial dysfunction.

In conclusion, we demonstrate that inhibition of a PKCδ isoform with rottlerin can offer protection against behavioral deficits, neurochemical depletion, and nigral dopaminergic neuronal damage in animal models of PD. Our results provide evidence that PKCδ may serve as a novel therapeutic target for development of neuroprotective agents and suggest that development of selective and systemically-active small molecule PKCδ isoform inhibitors may translate to an effective neuroprotective agent for treatment of PD.

References


FIGURE 1.

Fig. 1. Chemical structure of rottlerin.
Fig. 2. Effect of rottlerin on the number of TH-positive cells and their neurite length in MPP⁺-treated mesencephalic primary culture. Primary neurons were cultured and grown on laminin-coated cover slips. The cultures were then exposed to 10 µM MPP⁺ for 48 hr in the presence or absence of 0.3 µM or 1 µM rottlerin. After treatment, primary neurons were fixed and immunostained for TH and viewed under a Nikon TE2000 fluorescence microscope, as described in the methods section. TH cell count and neuronal process length were quantified using Metamorph image analysis software, as described in the methods section. Asterisks (*p<0.05 and **p<0.001; N=4) indicate significant difference compared with MPP⁺-treated neurons.
Fig. 3. Effect of rottlerin on PKCδ kinase activity in mouse substantia nigra. C57 black mice were treated with rottlerin (3-20 mg/kg) for 5 days. Substantia nigra tissue was homogenized and used in the immunoprecipitation kinase assay as described in the methods. The bands were quantified by a PhosphoImager after scanning the dried gel, and are expressed as a percentage of vehicle-treated bands. The data represent mean ± SEM from 4-6 animals per group. Asterisks (**) indicate significant differences compared with control.
Fig. 4. Effects of rottlerin on MPTP-induced depletions of dopamine, DOPAC, and HVA. C57 black mice were pretreated with different doses of rottlerin (3-20 mg/kg, i.p.
or p.o.) 24 hr before MPTP treatment and then co-treated with MPTP (30 mg/kg, i.p.) once a day for 5 days. 1% DMSO served as vehicle control. Animals were sacrificed 7 days following the treatment, and neurochemical analysis was performed by HPLC in striatal tissues. The data represent mean ± SEM from 6-8 animals per group. Asterisks (**p<0.01 and ***p<0.001) indicate significant difference compared with the MPTP group.
FIGURE 5.

Fig. 5. Effect of rottlerin on MPTP-induced PKCδ kinase activity. C57 black mice were pretreated with different doses of rottlerin (20 mg/kg, p.o.) 24 hr before MPTP treatment and then co-treated with MPTP (30 mg/kg, i.p.) once a day for 5 days. 1% DMSO served as vehicle control. Substantia nigra tissue was homogenized and used in the immunoprecipitation kinase assay. Recombinant PKCδ protein was used as a positive control. PKCδ kinase activity was measured in the absence of lipid activators. The bands were quantified by a Phospholmager after scanning the dried gel, and are expressed as a percentage of control. The data represent mean ± SEM from 4-6 animals per group. Asterisks (**p<0.01; N=4) indicate significant differences compared with control.
Stereological evaluation of neuroprotective effect of rottlerin on number and morphology of SNpc neurons in brains of MPTP-treated mice. C57 black mice were pretreated with different doses of rottlerin (20 mg/kg, p.o.) 24 hr before MPTP treatment and then co-treated with MPTP (30 mg/kg, i.p.) once a day for 5 days. 1% DMSO served as vehicle control. Total numbers of TH+ neurons in the substantia nigra pars compacta in groups exposed to vehicle (1% DMSO), 30 mg/kg MPTP, or the combination with rottlerin (20 mg/kg) were counted and are shown in the figure. TH+ neurons were measured using unbiased stereology two days after the last treatment. The data represent mean ± SEM from 3 animals per group. Asterisks (**p<0.01) indicate significant difference compared with MPTP group, n=3 per group.
Fig. 7. Effect of different doses of rottlerin on TH expression level in SN of MPTP-treated mice. C57 black mice were pretreated with different doses of rottlerin (3-20 mg/kg, i.p. or p.o.) 24 hr before MPTP treatment and then co-treated with MPTP (30 mg/kg, i.p.) once a day for 5 days. 1% DMSO served as vehicle control. Substantia nigra tissue was homogenized and used for Western blotting, as described. Tyrosine hydroxylase expression was detected using monoclonal antibody raised against TH. The membrane was reprobed with β-actin antibody to confirm equal protein loading in each lane. The data represent mean ± SEM from 3 animals per group. Asterisks (**p<0.01) indicate significant difference compared with MPTP group, n=3 per group.
Fig. 8. Effects of rottlerin on MPTP-induced locomotor deficits. C57 black mice were pretreated with different doses of rottlerin (20 mg/kg, p.o.) 24 hr before MPTP treatment and then co-treated with MPTP (30 mg/kg, i.p.) once a day for 5 days. 1% DMSO served as vehicle control. Locomotor activity was measured using VersaMax Analyzer 1-2 days before sacrificing the animals. A, Moving track of mice. B, Total horizontal and vertical movement. The vehicle-treated group served as control and was set at 100%. The data represent mean ± SEM from 6 animals per group. Asterisks (*p<0.05) indicate significant difference compared with MPTP group.
Fig. 9. Effects of rottlerin post-treatment on MPTP-induced depletions of dopamine, DOPAC, and HVA. C57 black mice were treated with MPTP (20 mg/kg, i.p.) once a day for 3 days and then rottlerin was administered (20 mg/kg, p.o.) once a day for an additional 10 days. 1% DMSO served as vehicle control. Animals were sacrificed following the treatment and neurochemical analysis was performed by HPLC in striatal tissues. The data represent mean ± SEM from 6 animals per group. Asterisks (*p<0.05) indicate significant difference compared with MPTP group.
CHAPTER IV: INHIBITION OF PKC-DELTA ISOFORM IN NIGROSTRIATAL DOPAMINERGIC SYSTEM ENHANCES DOPAMINE SYNTHESIS AND NEUROBEHAVIORAL ALTERATIONS IN MICE: DUAL NEUROPROTECTIVE STRATEGIES IN PARKINSON’S DISEASE

Danhui Zhang, Arthi Kanthasamy, Vellareddy Anantharam and Anumantha Kanthasamy

Abstract

Recently, we reported a novel finding that PKCδ, a novel PKC isoform, negatively regulates tyrosine hydroxylase (TH) and curtails dopamine synthesis through activation of protein phosphatase PP2A. In the present study, we examined whether inhibition of PKCδ by the kinase inhibitor rottlerin enhances dopaminergic neurotransmission and neurobehavioral characteristics in animal models. Treatment with the PKCδ inhibitor rottlerin in C57 black mice effectively increased a number of key neurochemical events in the dopamine pathway, including TH phosphorylation, TH enzymatic activity, and striatal dopamine and DOPAC levels. Time course studies revealed that TH-ser31 and -ser40 phosphorylation and dopamine synthesis were increased within 1hr of rottlerin treatment. Increased dopamine synthesis was accompanied by stimulation of locomotor activity and stereotypic behavior. To further explore the neurochemical mechanisms responsible for the behavioral super-sensitivity observed during rottlerin treatment, neuropharmacological approaches were used. Administration of a nonselective DA receptor agonist, apomorphine (10 mg/kg), significantly augmented locomotor activity in rottlerin-treated mice, suggesting that postsynaptic dopaminergic function is altered during rottlerin treatment. Additionally, pretreatment with the D1-like antagonist SCH 23390 or D2-like antagonist spiperone effectively suppressed rottlerin-induced hyperlocomotion and stereotypy, indicating the possible involvement of both D1 and D2 receptors in the behavioral changes. Consistent
with the pharmacological effects of rottlerin, naïve PKCδ-knockout mice showed enhanced striatal dopamine levels and behavioral function as compared to wild-type mice. Taken together with our recent studies showing the neuroprotective effect of rottlerin in MPTP models of PD, these results suggest that inhibition of PKCδ in the nigrostriatal dopaminergic system can offer dual benefits of neuroprotection and enhanced dopaminergic function in the development of therapeutic agents for treatment of Parkinson’s disease.

Introduction

Parkinson’s disease (PD) is a major neurodegenerative disorder characterized by progressive and substantial loss of dopaminergic neurons in the substantia nigra compacta (SNc), resulting in debilitating motor signs including tremors, bradykinesia, and rigidity. The PKC family consists of more than 12 isoforms and is subdivided into three major subfamilies, which include conventional PKC (α, βI, βII, γ), novel PKC (δ, ε, μ, η, θ), and atypical PKC (τ, λ, ζ) (Gschwendt 1999; Dempsey, Newton et al. 2000; Maher 2001; Kanthasamy, Kitazawa et al. 2003). PKCδ, a key member of the novel PKC family, plays a role in a variety of cell functions including cell differentiation, proliferation and secretion. Our recent studies demonstrate that PKCδ is an oxidative stress-sensitive kinase, and activation of this kinase via caspase-3 dependent proteolysis induces apoptotic cell death in cell culture models of Parkinson’s disease (Kanthasamy, Kitazawa et al. 2003; Kaul, Kanthasamy et al. 2003; Yang, Kaul et al. 2004; Latchoumycandane, Anantharam et al. 2005). Blockage of proteolytic activation of PKCδ by overexpression of the kinase-dominant negative PKCδ mutant, cleavage-resistant PKCδ mutant, or siRNA directed against PKCδ almost completely prevented the dopaminergic cell death (Kaul, Kanthasamy et al. 2003; Kitazawa, Anantharam et al. 2003; Anantharam, Kitazawa et al. 2004; Yang, Kaul et al. 2004; Latchoumycandane, Anantharam et al. 2005). Furthermore, we demonstrated that inhibition of PKCδ activation by the pharmacological inhibitor rottlerin offers neuroprotection in the MPTP-induced animal model of PD (Zhang, Kanthasamy et al. 2007). We also observed a high level of
PKCδ expression in the nigral dopaminergic neurons and a physical association of PKCδ with tyrosine hydroxylase (TH). TH is a rate-limiting enzyme in the biosynthesis of catecholamines and catalyzes the first step of a biochemical synthetic pathway in which L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-dopa). Phosphorylation and dephosphorylation of TH represent important post-translational regulatory mechanisms of the enzymatic activity that mainly determine the amount of catecholamine synthesis. A number of phosphorylation sites have been identified in TH, and phosphorylation of TH greatly influences the enzyme activity (Lee, Lew et al. 1989).

General PKCs can phosphorylate TH-ser40 and TH-ser31 (Albert, Wu et al. 1984; McTigue, Cremins et al. 1985; Tachikawa, Tank et al. 1987; Cahill, Horwitz et al. 1989; Haycock, Ahn et al. 1992; Bunn and Saunders 1995; Bobrovskaya, Cheah et al. 1998), however, direct phosphorylation of TH by PKA and not by PKC results in activation of the enzymatic activity (Funakoshi, Okuno et al. 1991). The role of PKC isoforms in the regulation of TH activity is not well studied. Recently, we showed that PKCδ can negatively regulate TH activity and dopamine synthesis by enhancing protein phosphatase-2A activity in dopaminergic neurons (Zhang, Anantharam et al. 2007). In the present study, we characterized the dopamine synthesis and neurobehavioral alterations in PKCδ-knockout mice, as well as PKCδ inhibition in treated mice.

Materials and Methods

Chemicals and Biological Reagents. Rottlerin, Apomorphine, SCH 23390, spiperone, protease cocktail, 3-hydroxybenzylhydrazine (NSD-1015), ATP, Protein A-Sepharose®, and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO); mouse tyrosine hydroxylase (TH) antibody, PhosphoTH-ser40 and ser31 antibodies were purchased from Chemicon (Temecula, CA); rabbit PKCδ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-rabbit and anti-mouse secondary antibodies and the ECL chemiluminescence kit were purchased from Amersham Biosciences (Piscataway,
NJ). [γ-32P]ATP was purchased from Perkin Elmer Life Science (Boston, MA). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

**Animal studies.** Six to 8-week-old C57/bL mice weighing approximately 30g were housed in standard conditions. Rottlerin was dissolved in 1% DMSO and administered intraperitoneally or orally at various doses (3-20 mg/kg). An equal volume of 1% DMSO was given to the vehicle control group. Mice were sacrificed after the last dose of rottlerin, and the striata were dissected for catecholamine analysis. To characterize dopaminergic behavioral changes, mice were given 10mg/kg apomorphine or 0.1mg/kg D1 receptor antagonist SCH-23390 (s.c.) or 0.1mg/kg D2 receptor antagonist spiperone (s.c.) 30min before rottlerin treatment.

**PKCδ kinase assay.** PKCδ enzymatic activity was assayed using an immunoprecipitation kinase assay as described in our previous publications (Kaul, Kanthasamy et al. 2003; Zhang, Kanthasamy et al. 2007). Briefly, substantia nigra brain tissue from rottlerin treated mice and control mice was washed once with PBS and resuspended in 1 ml of PKCδ lysis buffer (25 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 10 mM NaF, and 4 µg/ml each aprotonin and leupeptin). The lysates were cooled on ice for 30 min, and then centrifuged at 13,000 × g for 5 min, and the supernatants were collected as cytosolic fraction. Protein concentration was determined using a Bradford assay. Cytosolic protein (0.25-0.5 mg) was immunoprecipitated overnight at 4°C using 2 µg of anti-PKCδ antibody. The immunoprecipitates were then incubated with Protein A-Sepharose® (Sigma) for 1 hr at 4°C. The protein A-bound antigen-antibody complexes were then washed three times with 2× kinase buffer (40 mM Tris, pH 7.4, 20 mM MgCl2, 20 µM ATP, and 2.5 mM CaCl2), and resuspended in 20 µl of 2× kinase buffer. The reaction was started by adding 20 µl of reaction buffer containing 0.4 mg histone H1, 50µg/ml phosphatidylserine, 4.1 µM dioleoylglycerol,
and 5 µCi of [γ-32P] ATP (3000 Ci/mM) to the immunoprecipitated samples and incubated for 10 min at 30°C. SDS gel-loading buffer (2×) was added to terminate the reaction, the samples were boiled for 5 min, and the products were separated on a 12.5% SDS-PAGE gel. The H1 phosphorylated bands were detected using a Personal Molecular Imager (FX model; Bio-Rad), and quantified using Quantity One 4.2.0 software.

**Tyrosine hydroxylase activity.** Tyrosine hydroxylase activity was measured via L-DOPA levels in striatum after blocking DOPA decarboxylase activity using NSD-1015 (Nir, Haque et al. 2000). The drug was dissolved in phosphate-buffered saline (pH 7.0) and injected intraperitoneally (150 mg/kg body weight) 30min before the mice were sacrificed.

**HPLC assay for catecholamines.** DA, DOPAC, HVA and L-DOPA levels in brain striatal tissues were determined by high-performance liquid chromatography with electrochemical detection (HPLC-EC); samples were prepared as described previously (Zhang, Anantharam et al. 2007; Zhang, Kanthasamy et al. 2007). Briefly, neurotransmitters were extracted from samples using 0.1 M perchloric acid containing 0.05% Na2EDTA and 0.1% Na2S2O5. The extracts were filtered in 0.22 µm spin tubes, and 20 µl of the samples was loaded for analysis. DA, DOPAC and HVA were separated isocratically by a reversed-phase column with a flow rate of 0.7 ml/min. An HPLC system (ESA Inc., Bedford, MA) with an automatic sampler (ESA model 542) was used for these experiments. The electrochemical detection (EC) system consisted of an ESA Coulochem model 5100A with a microanalysis cell (model 5014A) and a guard cell (model 5020) (ESA Inc., Bedford, MA). Peaks representing DA, DOPAC and HVA in the samples were identified on the basis of their retention times and co-chromatography of the respective authentic standards in several different chromatographic conditions. The DA, DOPAC and HVA levels were measured as ng /mg protein.

**Locomotor and stereotypy activity.** Behavioral data were collected using VersaMax Animal
Activity Monitors (AccuScan Model RXYZCM-16, Columbus, OH). Each chamber was 40×40×30.5 cm, made of clear Plexiglas and covered with a Plexiglas lid with holes for ventilation. Infrared monitoring sensors were located every 2.54 cm along the perimeter (16 infrared beams along each side) and 2.5 cm above the floor. Two additional sets of 16 sensors were located 8.0 cm above the floor on opposite sides. Data were collected and analyzed by a VersaMax Analyzer (AccuScan Model CDA-8, Columbus, OH) which in turn sent information to a computer where it was stored for future analyses. All data are expressed as percent of the vehicle-treated control group (mean ± SEM, n=6) and were obtained in a 2hr test session.

**Western blot.** Brain lysates containing equal amounts of protein were loaded in each lane and separated on a 10-12% SDS-PAGE gel as described previously (Kaul, Kanthasamy et al. 2003). After the separation, proteins were transferred to a nitrocellulose membrane, and nonspecific binding sites were blocked by treating with 5% nonfat dry milk powder. The membranes were then treated with primary antibodies directed against TH (mouse monoclonal, 1:1000) or PhosphoTH-ser40 (rabbit, 1:1000) or phosphoTH-ser31 (rabbit, 1:1000). The primary antibody treatments were followed by treatment with secondary HRP-conjugated anti-mouse IgG (1:2000) or anti-rabbit IgG (1:1000) for 1 hr at room temperature. Secondary antibody-bound proteins were detected using an ECL chemiluminescence kit (Amersham Biosciences). To confirm equal protein loading, blots were reprobed with a β-actin antibody (1:5000 dilution). Western blot images were captured with a Kodak 2000 MM imaging system and data were analyzed using 1D Kodak Imaging Analysis software (Zhang, Anantharam et al. 2007).

**Data analysis.** Data analysis was performed using Prism 4.0 software (GraphPad Software, San Diego, CA). Data were first analyzed as raw by using one-way ANOVA and then Bonferroni’s post-test was performed to compare all treatment groups, and differences with p
< 0.05 were considered significant.

Results

Rottlerin inhibits PKCδ activity in the mouse substantia nigra. In this experiment, we examined whether rottlerin effectively inhibits PKCδ kinase activity in mouse substantia nigra. In order to determine the optimal route of rottlerin treatment, we chose the intraperitoneal route for low doses of rottlerin (3 and 7 mg/kg) and the oral route for high doses (10 and 20 mg/kg). Vehicle-injected animals were used as controls. As shown in Fig. 1, PKCδ kinase activity was significantly suppressed in the substantia nigra of rottlerin-treated animals in a dose-dependent manner compared to vehicle-treated control animals. Densitometric analysis of phosphorylated histone H1 bands revealed 48%, 37%, and 29% decreases in protein kinase activity in SN of mice treated with 20, 10, and 7 mg rottlerin, respectively. These results indicate that rottlerin significantly inhibits PKCδ activity, as observed in our previous cell culture experiments (Kaul, Kanthasamy et al. 2003).

Rottlerin increases dopamine and its metabolites in the striatum in mice. We previously showed that rottlerin significantly enhanced dopamine synthesis in dopaminergic cells (Zhang, Kanthasamy et al. 2007). So after verifying rottlerin’s ability to suppress PKCδ activity in the brain, we examined the effect of rottlerin on the dopamine synthesis level in the mouse brain. Mice were treated with 20mg/kg rottlerin for 5 days, and DA, DOPAC and HVA levels between rottlerin-treated animals and vehicle-treated animals were compared. HPLC analysis revealed that striatal DA and DOPAC levels were significantly higher in the striatum of rottlerin-treated animals compared to vehicle-treated animals (Fig. 2). DA levels were determined to be 235.7±41.03 ng/mg protein in rottlerin-treated mice compared to 190.6±19.25 ng/mg protein in vehicle-treated animals, an increase of 20%. Similarly, striatal DOPAC levels were estimated to be 47.29±5.99 ng/mg protein in rottlerin-treated mice compared to 31.77±3.21 ng/mg protein in PKC vehicle-treated animals,
an increase of 40%. Together, these in vivo data further demonstrate that rottlerin enhances dopamine synthesis in the mouse brain.

**Rottlerin increases TH activity and TH phosphorylation level in the substantia nigra.** Tyrosine hydroxylase (TH) is a rate-limiting enzyme in the biosynthesis of catecholamines and catalyzes the first step of a biochemical synthetic pathway in which L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-dopa). Since rottlerin can increase dopamine synthesis, we next determined whether the effect of rottlerin occurs by altering TH activity in dopaminergic neurons in the mouse brain. To check TH activity, mice were injected intraperitoneally with AAAD inhibitor NSD-1015 (150mg/kg) 30 min before sacrifice (Nir, Haque et al. 2000). Inhibition of AAAD results in accumulation of DOPA, which is used as an estimation of TH activity and DA synthesis. The level of DOPA in rottlerin-treated (20mg/kg, 5 days) mice was 149.8±11.41 ng/mg protein, while DOPA levels in vehicle-treated animals was 110.2±10.04 ng/mg protein (Fig. 3A), indicating increased TH activity in mouse brain induced by rottlerin treatment.

Since rottlerin treatment resulted in enhanced TH activity, we examined whether rottlerin has any effect on the phosphorylation status of TH. It is well established that TH activity can be regulated by phosphorylation of multiple serine residues (Campbell, Hardie et al. 1986; Mitchell, Hardie et al. 1990), and serine phosphorylation at positions 31 and 40 has been suggested to play a key role in TH activation and increased dopamine biosynthesis (Haycock 1990). We measured the extent of TH-ser40 phosphorylation in immunoblots using phosphospecific antibodies directed against TH-ser40. As shown in Fig. 3B, the level of TH-ser40 phosphorylation was significantly enhanced in substantia nigra of rottlerin-treated animals. But the level of total TH was unaltered in rottlerin-treated animals as compared to vehicle-treated animals, indicating that rottlerin treatment does not alter the expression of TH in mouse brain. Nitrocellulose membranes were reprobed with β-actin antibody and the density of the 43 kDa β-actin band was identical in all lanes, confirming
equal protein loading (Fig. 3B).

**Rottlerin increases striatal dopamine and its metabolites in a time dependent manner.** Continuous treatment with rottlerin for 5 days increased dopamine synthesis in mouse brain (Fig. 2). To further determine whether acute treatment with rottlerin also promotes dopamine synthesis in mouse brain, we treated mice with 20mg/kg rottlerin for different time points, from 1-7 hr. Dopamine levels in striatum were determined by similar methods previously shown. Interestingly, dopamine levels significantly increased first at 1hr of rottlerin treatment and continued increasing to the 7hr time point. However, the trend of increase is not time dependent. Dopamine metabolites DOPAC and HVA increased in the same pattern as dopamine (Fig. 4).

**Rottlerin treatment promotes TH activity and phosphorylation in mice.** Since dopamine synthesis was enhanced in acute rottlerin treated mice, we further examined whether rottlerin increases the activity and phosphorylation status of TH in acute rottlerin treated mouse brain. Tyrosine hydroxylase activity was measured via L-DOPA levels in striatum after blocking DOPA decarboxylase activity using NSD-1015 (Nir, Haque et al. 2000). The drug was injected intraperitoneally (150 mg/kg body weight) 30min before the mice were sacrificed. As shown in Fig. 5A, L-DOPA levels significantly increased at 1hr, 3hr, 5hr and 7hr rottlerin treatment. Increase in TH activity should result in increase in dopamine synthesis, which is consistent with the increased dopamine level shown in Fig 4. Substantia nigra tissue was used to check TH phosphorylation level after different time points of rottlerin treatment. We measured the extent of TH-ser31 and TH-ser40 phosphorylation in immunoblots using phosphospecific antibodies directed against TH-ser31 and TH-ser40. As shown in Fig. 5B, the level of TH-ser40 and TH ser31 phosphorylation was significantly enhanced in substantia nigra of rottlerin-treated animals. But the level of total TH was unaltered in rottlerin-treated animals as compared to vehicle-treated animals, indicating that
rottlerin treatment does not alter the expression of TH in mouse brain. Nitrocellulose membranes were reprobed with β-actin antibody and the density of the 43 kDa β-actin band was identical in all lanes, confirming equal protein loading (Fig. 5B).

**Rottlerin stimulates locomotor activity and stereotypy in mice.** Increased brain dopamine content should be accompanied by behavior changes in animals; after 20mg/kg rottlerin treatment, the spontaneous locomotor activity was measured during the dark phase of the cycle for 2hr (Figure 6). Rottlerin treated mice exhibited increased locomotion as expected: 40% increased locomotor activity compared to vehicle treated mice. Stereotypic movement was also examined at the same time and was increased: 37% increased movement in rottlerin treated mice compared with vehicle treated mice. These results indicate that the rottlerin-treated mice were hyperlocomotive.

**Apomorphine enhances hyperlocomotor activity and stereotypy induced by rottlerin.** To further explore the mechanism responsible for the behavioral supersensitivity to rottlerin observed in the mice, we tested the locomotor response to the nonselective DA receptor agonist apomorphine. A high dose (10 mg/kg) was used in order to probe postsynaptic DA receptor function. Data shown in Figure 7 revealed that the rottlerin treated mice exhibited significantly higher (two-fold) locomotor activity as compared to vehicle treated mice in both locomotor activity and stereotypy behavior. This result indicates that PKCδ inhibition by acute rottlerin treatment is associated with postsynaptic DA receptor supersensitivity.

**D₁ and D₂-like antagonist SCH 23390 and spiperone alter rottlerin-induced locomotor and stereotypy stimulation.** Since rottlerin induces locomotor stimulation in mice, we next investigated the role of dopamine receptors and presynaptic dopamine neurons in the locomotor effects of rottlerin. The selective D₂ antagonist spiperone (0.1 mg/kg, s.c.)
significantly diminished the stimulant actions of rottlerin (Fig. 8), whereas D₁ antagonist R(±)-SCH 23390 (0.1 mg/kg, s.c.) did not, although both antagonists reduced the locomotor and stereotypy activity of mice when administered alone (Fig. 8). However, the locomotor stimulant effect of rottlerin can be expressed in the presence of D₁ dopamine receptor blockade. These results support a role for dopamine receptor in the stimulation of normally coordinated locomotion by rottlerin.

Enhanced striatal dopamine levels and behavioral function in PKCδ knockout mice. Further validation of the role of PKCδ in the regulation of DA synthesis was sought by extending these studies to PKCδ (-/-) knockout animals. We compared DA and DOPAC levels between PKCδ (-/-) animals and PKCδ (+/+) animals. HPLC analysis revealed that striatal DA and DOPAC levels were significantly higher in the striatum of PKCδ (-/-) animals compared to PKCδ (+/+) animals (Fig. 9A). DA levels were determined to be 311.7±24.88 ng/mg protein in PKCδ (-/-) compared to 190±14.24 ng/mg protein in PKCδ (+/+) animals, an increase of 60%. Similarly, striatal DOPAC levels were estimated to be 83.64±12.02 ng/mg protein in PKCδ (-/-) compared to 59.41±8.697 ng/mg protein in PKCδ (+/+) animals, an increase of 40%.

Spontaneous locomotor activity measured during the dark phase of PKCδ (+/+) and PKCδ (-/-) mice is shown in Fig. 9B. PKCδ (-/-) mice displayed increased locomotion in comparison with PKCδ (+/+) mice. Stereotypy activity of PKCδ (-/-) mice was slightly higher than PKCδ (+/+) mice, but not significantly. These results indicate that the KO mice were hypolocomotive.

Discussion

In the present study, we characterized the in vivo regulation of TH and dopamine synthesis by a member of the novel class of the protein kinase C family, PKCδ, and demonstrated that (i) inhibition of PKCδ enhances TH activity and TH phosphorylation in an
animal model, (ii) inhibition of PKCδ increases striatal DA levels, (iii) locomotor activity and stereotypy are significantly elevated during rottlerin treatment or in PKCδ knockout in mice, and (iv) PKCδ inhibition-induced hyperlocomotor and stereotypy activity are significantly enhanced by DA receptor agonists and inhibited by DA receptor antagonists.

The PKC family of kinases consists of 13 isoforms classified into three distinct sub-families based on their activation profiles. Conventional isoforms of PKCs (cPKCs), PKCα, βI, βII, and γ, require both intracellular calcium and diacylglycerol (DAG) for their activation, while PKCδ, ε, η, θ, and μ require only DAG. The atypical PKCs (aPKCs), PKCζ and λ(ι), require neither calcium nor phospholipids for activation. The expression patterns and biological functions of these isoforms in CNS are only beginning to be recognized. For example, PKCγ is expressed predominantly in the brain, and has been implicated in neural plasticity, including long term potentiation (Saito and Shirai 2002) and ischemic injury (Aronowski and Labiche 2003). The levels of another novel PKC isoform, PKCε, are greatly reduced in Alzheimer’s brains, and are linked to increased production of amyloid β protein (Kinouchi, Sorimachi et al. 1995; Matsushima, Shimohama et al. 1996). In the CNS, PKCε is localized mainly in the presynaptic region and induces neurite outgrowth during differentiation and mediates apoptosis during embryonic development, among other functions (Matsushima, Shimohama et al. 1996; Akita 2002).

Recent studies from our laboratory have demonstrated PKCδ is highly expressed in nigral dopaminergic neurons, and that suppression of PKCδ activity by kinase inhibitors, dominant negative mutants or RNAi-mediated knockdown increases TH-ser40 phosphorylation, TH activity, and dopamine levels. PKCδ phosphorylates PP2A to promote dephosphorylation of TH-ser40, and inhibition of PKCδ attenuates PP2A activity, resulting in elevated TH-ser40 phosphorylation, TH activity, and dopamine levels in a cell culture model (Zhang, Kanthasamy et al. 2007). In this study, we showed that PKCδ inhibition by its pharmacological inhibitor of PKCδ, rottlerin, and PKCδ knockout enhances TH activity and dopamine synthesis in mouse brain. Excessive DA production may not only alter
neurotransmission, but may also contribute to neuronal cell death through increased oxidative stress (Hoyt, Reynolds et al. 1997; Luo, Umegaki et al. 1998). In this regard, we wish to point out that PKCδ can be activated by at least two independent mechanisms in neuronal cells: membrane translocation and caspase-3-dependent proteolytic cleavage (Kikkawa, Matsuzaki et al. 2002; Brodie and Blumberg 2003; Kanthasamy, Kitazawa et al. 2003). Of the two activation mechanisms, PKCδ activated by membrane translocation following tonic stimulation by lipid activators contributes to cell survival and proliferation (Kanthasamy, Kitazawa et al. 2003; Jackson and Foster 2004). As demonstrated in our recent studies, another form of activation is caspase-3-dependent proteolytic cleavage of native PKCδ into regulatory and catalytic fragments resulting in persistent activation during exposure to neurotoxic agents such as MPP⁺, MMT, manganese or dieldrin (Anantharam, Kitazawa et al. 2002; Kaul, Kanthasamy et al. 2003; Latchoumycandane, Anantharam et al. 2005). This form of proteolytic activation contributes to apoptotic cell death of dopaminergic neurons (Kanthasamy, Kitazawa et al. 2003). In the case of a lipid activator, TPA induced membrane translocation of native PKCδ, but did not induce apoptotic cell death in dopaminergic cell lines (Anantharam, Kitazawa et al. 2002). Furthermore, recent studies from our lab and others have shown that unlike the native 74 kDa PKCδ, the 41 kDa catalytically active PKCδ fragment, upon proteolytic cleavage, is targeted to various sub-cellular organelles including the mitochondria (Majumder, Pandey et al. 2000) and nucleus (DeVries, Neville et al. 2002) to activate apoptotic cell death signaling molecules. These results suggest that native and cleaved PKCδ have different substrate profiles. Small molecule inhibitors of protein kinases are being increasingly evaluated for therapeutic use for various human diseases. Rottlerin is isolated from the seeds of *Mallotus phillippinensis* and has been shown to be a potent inhibitor of PKCδ, with an IC₅₀ of 3-6 μM, whereas the Kis for other PKC isoforms (α, β, γ, ε and λ) are at least 5-10 times higher (Gschwendt, Muller et al. 1994; Samokhin, Jirousek et al. 1999; Davies, Reddy et al. 2000; Soltoff 2001). Toxicity data for rottlerin indicate that the compound has a low toxicity profile (LD₅₀ 750 mg/kg, rat
oral); 120 mg/kg (oral 6-day rat study) is the lowest toxic dose (Varma, Sareen et al. 1959). Previous studies have shown that the PKCδ inhibitor rottlerin protects against MPTP-induced motor deficits, striatal dopamine depletion, and nigral dopaminergic neuronal loss (Zhang, Anantharam et al. 2007); in addition, rottlerin inhibits PKCδ enzyme activity in vitro (Anantharam, Kitazawa et al. 2002). In the present study, we provide evidence that rottlerin treatment can increase brain dopamine synthesis by enhancing TH activity and phosphorylation in an animal model. PKCδ knockout mice also have higher brain dopamine levels than wild-type mice. This negative regulation may be important for maintaining optimal dopamine levels. In addition to the function of regulating dopamine synthesis, under the condition of enhanced oxidative stress induced by neurotoxic insults, PKCδ serves as a key downstream proapoptotic effector of caspase-3, resulting in proteolytic activation of the kinase which contributes to cell death (Anantharam, Kitazawa et al. 2002; Kaul, Kanthasamy et al. 2003; Kitazawa, Anantharam et al. 2003; Yang, Kaul et al. 2004). Rottlerin is also shown to inhibit CaM kinase III and MAPKAP kinase 2 (Kotlyarov, Neininger et al. 1999), but these kinases are not related to dopamine synthesis in animal studies.

Regulation of TH activity and DA levels is critical for normal dopaminergic neurotransmission in the CNS. Excessive DA production may alter neurotransmission (Hoyt, Reynolds et al. 1997; Luo, Umegaki et al. 1998). In the present study, PKCδ knockout induced mouse hyperlocomotor activity and stereotypy activity following acute rottlerin administration demonstrate an important role of PKCδ in modulating basal locomotor activity. A relatively high dose of apomorphine (10 mg/kg) resulted in enhanced locomotion in the rottlerin-treated animals. Selective activation of D1- or D2-like receptors produces different locomotor activity in mice. As a nonselective DA receptor agonist, apomorphine induced a modest increase in locomotor activity in the rottlerin treated mice but did not change control animals. This may well reflect a combined effect of apomorphine on both D1- and D2-like receptors. Only the D2 receptor antagonist spiperone attenuated rottlerin-induced locomotor stimulation, while the D1 receptor antagonist SCH did not, indicating that these
hyperlocomotor responses caused by PKCδ inhibition might be mediated via D2-like receptors. Previous studies suggested that PKC-mediated phosphorylations of D1 and D2 receptors were involved in receptor internalization (Namkung and Sibley 2004), and this rapid receptor sequestration is involved in agonist induced receptor desensitization. Thus, PKCδ inhibition may affect the postsynaptic sensitivity by altering the receptor phosphorylation state and/or interfering with receptor desensitization. Mice treated acutely with rottlerin showed good response to dopamine receptor agonist and antagonist but PKCδ knockout mice did not (data not shown here). Permanent knockout of PKCδ protein might change the dopamine receptor sensitivity or cause desensitization, but this possibility needs to be further tested.

Taken together with our recent studies, these results demonstrate that PKCδ negatively regulates TH activity and that inhibition of the kinase enhances dopaminergic function.

References


Fig. 1. Effect of rottlerin on PKCδ activity in mouse substantia nigra. C57 black mice were treated with rottlerin (3-20 mg/kg) for 5 days. Substantia nigra tissue was homogenized and used in the immunoprecipitation kinase assay as described in the methods. The bands were quantified by a PhosphoImager after scanning the dried gel, and are expressed as a percentage of vehicle-treated bands. The data represent mean ± SEM from 4-6 animals per group. Asterisks (**p<0.01) indicate significant differences compared with control.
Fig. 2. Effect of rottlerin on dopamine content in mouse striatum. C57 black mice were treated with rottlerin (20 mg/kg, p.o.) once a day for 5 days. 1% DMSO served as vehicle control. Animals were sacrificed 1 day after the last dose of rottlerin, and neurochemical analysis was performed by HPLC in striatal tissues. The data represent mean ± SEM from 6-8 animals per group. Asterisks (*p<0.05) indicate significant difference compared with the vehicle treated group.
Fig. 3. Effects of rottlerin on TH activity and TH phosphorylation level in the substantia nigra. A, TH activity: Tyrosine hydroxylase activity was measured via L-DOPA levels in striatum after blocking DOPA decarboxylase activity using NSD-1015. The drug was dissolved in phosphate-buffered saline (pH 7.0) and injected intraperitoneally (150 mg/kg body weight) 30 min before the mice were sacrificed. L-DOPA level was determined by HPLC. Inhibition of AAAD results in accumulation of DOPA, which is used as an estimation of TH activity. The data represent a mean ± SEM from 6-10 animals. Asterisks (*p<0.05) indicate significant difference compared with vehicle-treated animals. B, Western blot analysis of P-TH-ser40. Substantia nigral lysates from vehicle treated mice and rottlerin treated mice were subjected to immunoblotting of P-TH-ser40, and total TH. To confirm equal protein loading in each lane, the membranes were reprobed with β-actin antibody. The immunoblots were visualized using the Amersham’s ECL detection agents.
Fig. 4. Effects of rottlerin on striatal dopamine and its metabolites levels at different treatment time points. Mice were treated with 20mg/kg rottlerin (p.o) for different time points from 0-7 hr. Striatum tissue was collected at 0hr, 1hr, 3hr, 5hr and 7hr time points. Striatal dopamine, DOPAC and HVA levels were determined by HPLC. The data represent mean ± SEM from 6-8 animals per group. Asterisks (*p<0.05 and **p<0.01) indicate significant differences compared with the 0hr time point group.
FIGURE 5.

**Fig. 5. Effects of acute rottlerin treatment on TH activity and phosphorylation in mice.**

**A, TH activity:** Mice were treated with 20mg/kg rottlerin (p.o) for different time points ranging from 0-7 hr. 150 mg/kg NSD-1015 was injected intraperitoneally 30min before the mice were sacrificed. Striatal tissue was collected at 0hr, 1hr, 3hr, 5hr and 7hr time points. Striatal L-DOPA level was determined by HPLC. The data represent a mean ± SEM from 6-10 animals. Asterisks (*p<0.05) indicate significant difference compared with the 0hr time point rottlerin-treated animals.

**B, Western blot analysis of P-TH-ser40.** Substantia nigral tissue was collected at 0hr, 1hr, 3hr, 5hr and 7hr time points from 20mg/kg...
rottlerin-treated mice. SN lysates from all these groups were subjected to immunoblotting of P-TH-ser40, and total TH. To confirm equal protein loading in each lane, the membranes were reprobed with β-actin antibody. The immunoblots were visualized using Amersham’s ECL detection agents
Fig. 6. Effects of rottlerin on mouse locomotor activity and stereotypy. C57 black mice were treated with a single dose of rottlerin (20 mg/kg, p.o.), and locomotor and stereotypy activity of mice was measured using a VersaMax Analyzer right after rottlerin treatment. Control mice were treated with 1% DMSO. The vehicle-treated group served as control and was set at 100%. The data represent mean ± SEM from 6 animals per group. Asterisks (*p<0.05) indicate significant difference compared with vehicle-treated group.
Fig. 7. Effects of apomorphine on mouse hyperlocomotor activity and stereotypy induced by rottlerin. Mice were treated with 10mg/kg apomorphine (i.p.) 10min before a single dose of rottlerin (20 mg/kg, p.o.). Immediately after rottlerin treatment, mouse locomotor and stereotypy activity was measured using a VersaMax Analyzer. The apomorphine alone group was treated with 10mg/kg apomorphine (i.p.), and the rottlerin alone group was treated with a single dose of 20mg/kg rottlerin (p.o.). Control mice were treated with 1% DMSO. The vehicle-treated group served as control, set at 100%. The data represent mean ± SEM from 6 animals per group. Asterisks (*p<0.05, **P<0.01) indicate significant difference compared with vehicle-treated group.
Fig. 8. Effects of SCH 23390 and spiperone on rottlerin-induced locomotor and stereotypy stimulation. Mice were treated with selective D₁ antagonist R(±)-SCH 23390 (0.1 mg/kg, s.c.) or selective D₂ antagonist spiperone (0.1 mg/kg, s.c.) 10 min before a single dose of rottlerin (20 mg/kg, p.o.). Immediately after rottlerin treatment, mice A, locomotor activity and B, stereotypy activity were measured using a VersaMax Analyzer. The SCH alone group was treated with 0.1 mg/kg R(±)-SCH 23390 (s.c.), and the spiperone alone group was treated with 0.1 mg/kg spiperone (s.c.). Control mice were treated with 1% DMSO. The vehicle-treated group served as control, set at 100%. The data represent mean ± SEM from 6 animals per group. Asterisks (**(p<0.01, ***(p<0.001) indicate significant difference compared with vehicle-treated group.
Fig. 9. Striatal dopamine levels and behavioral changes of PKCδ knockout mice.

A, Neurotransmitter levels: DA and DOPAC levels were determined in the striatal extracts of PKCδ (-/-) and PKCδ (+/+) animals by HPLC. The data represent a mean ± SEM from 6-10 animals. Asterisks (*p<0.05) indicate significant differences between PKCδ (-/-) and PKCδ (+/+) animals.

B, Locomotor and stereotypy activity. Locomotor and stereotypy activity of PKCδ (+/+) and PKCδ (-/-) mice was measured using a VersaMax Analyzer. The PKCδ (+/+) group served as control, set at 100%. The data represent mean ± SEM from 6 animals per group.
CHAPTER V: EFFECTS OF MANGANESE ON TYROSINE HYDROXYLASE ACTIVITY AND PHOSPHORYLATION IN DOPAMINERGIC NEURONAL CELLS

Abstract

Manganese (Mn) exposure causes manganism, a neurological disorder similar to Parkinson's disease. However, the cellular mechanism by which Mn induces dopaminergic neuronal cell death remains unclear. We found that caspase-3-dependent proteolytic activation of protein kinase Cδ (PKCδ) plays a key role in Mn-induced apoptotic cell death. Tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, can be regulated by phosphorylation at multiple serine residues, including serine-31 and serine-40. In the present study, we report that acute Mn treatment promotes TH activity by increasing TH phosphorylation, which can’t be reduced by PKCδ inhibition; chronic Mn treatment suppresses TH activity, which is accompanied by increased PKCδ cleavage in the dopaminergic cells. The reduced TH activity induced by chronic Mn treatment can be attenuated by the PKCδ inhibitor rottlerin. Both the acute and chronic Mn treatment concentrations were not cytotoxic to dopaminergic cells, so the effects of Mn on TH activity and phosphorylation in this study occurred under physiological conditions. Previously, we reported an interaction between PKCδ and TH, in which the kinase modulates dopamine synthesis by negatively regulating TH activity via protein phosphatase 2A (PP2A). Here we also showed that Mn treatment can increase PP2A activity, which may contribute to the decreased TH activity induced by MnCl2. The increased PP2A activity induced by chronic MnCl2 treatment is suppressed by PKCδ inhibitor rottlerin, which may suggest that the activation of PKCδ induced by Mn increased PP2A activity, which might decrease the TH activity induced by chronic Mn treatment.

Introduction

Exposure to high levels of manganese (Mn) has been shown to cause a
Parkinson's-like syndrome known as manganism. Increased incidences of manganism have been observed among miners and industrial welders as well as farmers exposed to Mn-based pesticides such as fungicides Maneb (Mn ethylene-bis-dithiocarbamate) and Mancozeb (Mn Cu Zn ethylene-bis-dithiocarbamate) (Roth and Garrick, 2003; Dobson et al., 2004; Olanow, 2004). Adverse neurological effects of Mn also occurred in people who drank water containing high levels of Mn in Japan, Greece, and Australia and in abusers who used the Mn-containing compound Bazooka, a cocaine-based drug (Ensing, 1985). Several lines of evidence suggest that exposure to Mn or Mn-containing compounds induces a variety of cellular changes, including glutathione and dopamine depletion, increased oxidative stress, and impairment of energy metabolism and antioxidant systems (Hirata, 2002; Kitazawa et al., 2002; Roth and Garrick, 2003; Dobson et al., 2004; Olanow, 2004). Recently, several studies have demonstrated effects of Mn on neuronal cells, but the mechanism of Mn-induced dopaminergic neurodegeneration remains unclear. Recently, we developed an in vitro model of dopaminergic neurotoxicity, namely, N27 cells derived from the mesencephalon, a brain region directly affected by PD. N27 cells also represent a homogenous population of dopaminergic tyrosine hydroxylase-positive cells with functional characteristics, including cellular signaling, similar to dopaminergic neurons (Anantharam et al., 2002; Kaul et al., 2003).

The PKC family consists of more than 12 isoforms and is subdivided into three major subfamilies, which include conventional PKC (α, βI, βII, γ), novel PKC (δ, ε, μ, η, θ), and atypical PKC (τ, λ, ζ) (Gschwendt, 1999; Dempsey et al., 2000; Maher, 2001; Kanthasamy et al., 2003). PKCδ, a key member of the novel PKC family, plays a role in a variety of cell functions including cell differentiation, proliferation, and secretion. Our recent studies demonstrate that PKCδ is an oxidative stress-sensitive kinase, and activation of this kinase via caspase-3 dependent proteolysis induces apoptotic cell death in cell culture models of Parkinson’s disease (Kanthasamy et al., 2003; Kaul et al., 2003; Yang et al., 2004; Latchoumycandane et al., 2005). Tyrosine hydroxylase (TH), a rate-limiting enzyme in the
biosynthesis of catecholamines, catalyzes the first step of a biochemical synthetic pathway in which L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-dopa). Phosphorylation and dephosphorylation of TH represent important post-translational regulatory mechanisms of the enzymatic activity that mainly determines the amount of catecholamine synthesis. A number of phosphorylation sites have been identified in TH, and phosphorylation of TH greatly influences the enzyme activity (Lee et al., 1989). Phosphorylation of TH at amino-terminal serine (Ser) amino sites at Ser8, Ser19, Ser31, and Ser40 leads to activation of TH. A number of protein kinases have been shown to phosphorylate these serine residues to varying degrees. For example, THser19 is phosphorylated by CaMKII, TH-ser40 by Protein kinase A (PKA), Protein kinase G by MSK, and Protein kinase C (PKC) and TH-ser31 by ERK1/ERK2 kinases and indirectly by PKC (Haycock, 1990). Among these serine phosphorylation sites, TH-ser40 is a major residue that positively regulates the TH activity in vivo (Campbell et al., 1986; Wu et al., 1992). The phosphorylation state of TH can also be regulated by dephosphorylation reactions mediated by phosphatases. Haavik et al. demonstrated that phosphatase 2A (PP2A) is the major serine/threonine phosphatase that dephosphorylates TH, resulting in reduced TH activity (Haavik et al., 1989). General PKCs can phosphorylate TH-ser40 and TH-ser31 (Albert et al., 1984; McTigue et al., 1985; Tachikawa et al., 1987; Cahill et al., 1989; Haycock et al., 1992; Bunn and Saunders, 1995; Bobrovskaya et al., 1998); however, direct phosphorylation of TH by PKA and not by PKC results in activation of the enzymatic activity (Funakoshi et al., 1991). We reported a novel functional interaction between PKCδ and TH in which PKCδ negatively regulates TH activity and dopamine synthesis by enhancing PP2A activity in dopaminergic neurons (Zhang et al., 2007). We demonstrated that caspase-3-mediated proteolytic activation of PKCδ plays a pivotal role in Mn-induced apoptosis in N27 mesencephalic dopaminergic cells (Latchoumycandane C et al., 2005). We further examined in the present study whether Mn has any physiological role in regulating TH activity or phosphorylation, and if PKCδ has a functional role during this process. Herein, we report that activation of PKCδ induced by
chronic Mn treatment can increase PP2A activity and lead to a suppression of TH activity in dopaminergic cells.

**Materials and Methods**

**Chemicals.** Rottlerin, manganese chloride (MnCl₂, 99%), NSD-1015, dibutyryl cAMP, protease cocktail, and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO); Z-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-DEVD) was obtained from Alexis Biochemicals (San Diego, CA); phosphoTH-ser40 antibody was purchased from Chemicon (Temecula, CA); rabbit PKCδ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and the Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Anti-rabbit and anti-mouse secondary antibodies and the ECL chemiluminescence kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). RPMI, fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Invitrogen (Gaithersburg, MD).

**Cell culture models.** Differentiated N27 cells were cultured as described earlier (Adams et al., 1996; Anantharam et al., 2002; Kaul et al., 2003). Briefly, cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units of penicillin, and 50 µg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. N27 cells were differentiated with 2 mM dibutyryl cAMP for 3-5 days and then used for experiments described below.

**Treatment paradigm.** Differentiated N27 dopaminergic cells were exposed to 0.1-10 µM MnCl₂ for the duration of the experiment. 3µM Rottlerin and 30µM Z-DEVD were used for PKCδ inhibition during chronic MnCl₂ treatment. For measurement of TH activity, cells were exposed to 2 mM NSD-1015 for 1 hr prior to MnCl₂ treatment. Untreated cells were used as control samples. We derived the concentrations of rottlerin used in this study based on
previously published literature. Rottlerin inhibits PKCδ kinase activity with a Ki of 3-6 μM, while PKCα, β, γ, ε and λ Ki values are at least 5-10 times higher (Gschwendt, 1999; Davies et al., 2000; Way et al., 2000; Soltoff, 2001). In our previous study, we showed 3-10 μM rottlerin dose-dependently attenuated kinase activity to a greater extent (Anantharam et al., 2002). We used 3 μM rottlerin in the study, which was lower than Ki values of other PKC isoforms.

**Tyrosine hydroxylase activity.** TH enzyme activity was measured by the modified method of Hayashi et al. (Hayashi et al., 1988) in which DOPA levels are quantified as an index of TH activity after inhibition of DOPA decarboxylase with the decarboxylase inhibitor NSD-1015 (Hayashi et al., 1988). Briefly, cells were incubated with Krebs-HEPES buffer (pH 7.4) containing 2 mM NSD-1015 at 37°C for 1 hr prior to subjection to the treatment paradigm as described earlier. After treatment, cells were collected and resuspended in antioxidant solution, sonicated, centrifuged, and DOPA levels in the supernatants were measured by high-performance liquid chromatography with electrochemical detection (HPLC-EC); samples were prepared as described previously (Kitazawa et al., 2001; Sun et al., 2006). Briefly, neurotransmitters were extracted from samples using 0.1 M perchloric acid containing 0.05% Na2EDTA and 0.1% Na2S2O5. The extracts were filtered in 0.22 micron spin tubes and 20 μl of the samples was loaded for analysis. DOPA was separated isocratically by a reversed-phase column with a flow rate of 0.7 ml/min. An HPLC system (ESA Inc., Bedford, MA) with an ESA automatic sampler (model 542) was used for these experiments. The electrochemical detection (EC) system consisted of an ESA coulochem model 5100A with a microanalysis cell model 5014A and a guard cell model 5020 (ESA Inc., Bedford, MA). The peak areas of standard DOPA were compared with peak areas of samples. The DOPA level in the samples was measured and expressed as pg/μg protein, with a retention time for DOPA of 2.5-4 min.
**PP2A assay.** To determine PP2A phosphatase activity, we used the Serine/Threonine phosphatase assay kit from Promega. For in vivo PP2A activity measurement, N27 cells were homogenized in lysis buffer (25 mM Tris-HCl, 10 mM β-mercaptoethanol, 2 mM EDTA, protease inhibitor) supplied with the kit. After centrifugation, the supernatants were used for the assay. PP2A activity was determined by measuring the amount of free phosphate generated in a reaction by measuring the absorbance of a molybdate: malachite green: phosphate complex at 600 nm using a Spectramax plate reader (Molecular Devices). The effective range for the detection of phosphate released in this assay is 100–4,000 pmol of phosphate.

**Western blotting.** Cell lysates containing equal amounts of protein were loaded in each lane and separated on a 10-12% SDS-PAGE gel as described previously (Kaul et al., 2003). After the separation, proteins were transferred to nitrocellulose membrane, and nonspecific binding sites were blocked by treating with 5% nonfat dry milk powder. The membranes were then treated with primary antibodies directed against PKCδ (rabbit polyclonal or mouse monoclonal, 1:2000 dilution) and phospho TH-ser40 (rabbit polyclonal, 1:1000). The primary antibody treatments were followed by treatment with secondary HRP-conjugated anti-rabbit IgG (1:2000) for 1 hr at RT. Secondary antibody-bound proteins were detected using Amersham's ECL chemiluminescence kit. To confirm equal protein loading, blots were reprobed with a β-actin antibody (1:5000 dilution). Western blot images were captured with a Kodak 2000 MM imaging system and data were analyzed using 1D Kodak imaging analysis software.

**Data analysis.** Data analysis was performed using Prism 4.0 software (GraphPad Software, San Diego, CA). Data were first analyzed using one-way ANOVA and then Bonferroni's post-test was performed to compare all treatment groups, and differences with p<0.05 were considered significant.
Results

Acute Mn exposure induces increases in TH activity in differentiated dopaminergic cells. We recently demonstrated that protein kinase C delta negatively regulates tyrosine hydroxylase activity and dopamine synthesis by enhancing protein phosphatase-2A activity in dopaminergic neurons both in cell culture and in animal brain (Zhang et al., 2007). We have also shown that PKCδ mediates Mn-induced cytotoxic and apoptotic cell death in undifferentiated N27 dopaminergic neuronal cells (Latchoumycandane et al., 2005). In this study we examined whether Mn at non-toxic doses has any effect on TH activity. Differentiated N27 cells were exposed to 3 µM and 10 µM Mn; after 3 hr, cells were harvested, lysed, and TH activity was measured. As shown in Fig. 1A, the both 3 µM and 10 µM Mn increased TH activity by 77% compared to untreated control cells. To determine if PKCδ mediates Mn-induced increases in TH activity, N27 cells were pretreated with 3 µM rottlerin for 30 min prior to Mn treatment. The results show that Mn-induced increases in TH-activity were unaffected by rottlerin treatment (Fig. 1B).

Acute Mn exposure increases TH phosphorylation in dopaminergic cells. We previously demonstrated that PKCδ colocalizes with TH and also negatively regulates TH-ser40 phosphorylation (Zhang et al., 2007). Since acute Mn exposure resulted in enhanced TH activity, we examined whether acute Mn treatment has any effect on the TH-ser40 phosphorylation status. Differentiated N27 cells were exposed to 3 µM and 10 µM Mn for 3 hr; the cell lysates were subjected to Western blot analysis and the membranes were probed with phospho-specific-antibody directed against P-TH-ser40. As shown in Fig. 2, Mn treatment dose-dependently increased the levels of P-TH-ser40. Densitometry analysis of the 60 kDa P-TH-ser40 band in Fig. 2 revealed a 2-fold increase in phosphorylation in 10 µM Mn treated cells compared to untreated control cells. Nitrocellulose membranes were reprobed with β-actin antibody and the density of the 43 kDa β-actin band was identical in all
lanes, confirming equal protein loading. Densitometric analysis of the 60 kDa P-TH-ser40 band in Fig. 2 revealed a 2-fold increase in phosphorylation as compared to control cells. These results demonstrate that acute MnCl₂ exposure results in enhanced TH phosphorylation.

Acute Mn exposure is not toxic to differentiated N27 cells. In this experiment we determined if 3 hr exposure to 3-10 µM Mn induces cytotoxicity in different N27 cells. Cytotoxicity was measured using the Sytox Green assay. An increase in the number of Sytox-positive green cells indicates an increase in cell death, because the Sytox green dye permeates compromised cell membranes to stain nuclear chromatin. Fig. 3 shows the phase-contrast and fluorescent images of Sytox Green treated cells. No Sytox positive cells were observed in N27 cells exposed to either 3 or 10 µM Mn for 3 hr (Fig. 3). Quantitative analysis of Sytox fluorescence using a fluorescence plate reader also revealed that different N27 cells were unaffected by acute Mn treatment (Fig. 3). These results suggest that the Mn concentration used in acute experiments is not toxic to the cells.

PKCδ regulates TH activity in N27 cells chronically exposed to Mn. In this experiment we examined whether chronic Mn treatment also increases TH activity. N27 cells were exposed to 0.1-1 µM Mn, and after 24 hr TH activity was measured in the cell lysates. As shown in Fig. 4A, Mn dose-dependently decreased TH activity. Densitometric analysis revealed that TH activity was decreased to 70%, 61%, and 59% in 0.1, 0.3, and 1 µM Mn-treated cells, respectively, compared to untreated control cells (Fig. 4B). Therefore, unlike acute exposure, chronic Mn treatment decreases TH activity in differentiated N27 cells. To determine whether PKCδ mediates Mn-induced decreases in TH activity, differentiated N27 cells were cotreated with 3 µM rottlerin. As shown in Fig. 4C, TH activity levels in Mn and rottlerin-treated cells were restored to levels comparable to untreated control cells. These data strongly suggest that PKCδ mediates Mn-induced decreases in TH activity levels.
Chronic Mn exposure is not toxic to differentiated N27 cells. In this experiment we determined if 24 hr exposure to 0.1-1µM Mn induces cytotoxicity in different N27 cells. Cytotoxicity was measured using the Sytox Green assay. Fig. 5A shows the phase-contrast and fluorescent images of Sytox Green treated cells. No Sytox positive cells were observed in N27 cells exposed to 0.1, 0.3, and 1µM Mn for 24 hr (Fig. 5). Quantitative analysis of Sytox fluorescence using a fluorescence plate reader also revealed that different N27 cells were unaffected by chronic Mn treatment (Fig. 5). These results suggest that the Mn concentration used in chronic experiments is not toxic to the cells.

Chronic Mn exposure induces increased PKCδ kinase activity. Since rottlerin treatment prevented Mn-induced decreases in TH activity, we examined if chronic Mn treatment increased PKCδ kinase activity. Immunoprecipitation kinase assays were performed by measuring PKCδ phosphorylation of histone H1 using [32P]-ATP, as described previously (Kaul et al., 2005). Differentiated N27 cells were exposed to 1 µM Mn in the presence or absence of 3 µM rottlerin, and the cell lysates were subjected to kinase assay. As shown in Fig. 6, 24 hr exposure to 1µM Mn resulted in a 48% increase in histone phosphorylation compared to untreated control cells. Rottlerin cotreatment completely suppressed the 1 µM Mn-induced increase in kinase activity. Together, these data imply that chronic Mn exposure induces increases in PKCδ kinase activity, which can be prevented by cotreatment with 3 µM rottlerin. Previously, we determined that caspase-mediated PKCδ cleavage and activation occur with 300 µM Mn treatment for 24 hr. We believe that increased PKCδ kinase activity induced by 0.1-1µM Mn may be due to activation of native PKCδ.

PKCδ mediates Mn-induced increases in PP2A activity in dopaminergic cells. We previously demonstrated that PKCδ negatively regulates TH activity by enhancing protein phosphatase-2A activity in dopaminergic neurons (Zhang et al., 2007). In that study we
showed that PKCδ can directly phosphorylate PP2A and increase its phosphatase activity. Activated PP2A would then dephosphorylate P-Ser40 and inactivate TH. Since chronic Mn treatment induced increases in PKCδ kinase activity and decreased TH activity, we examined whether PP2A activity is increased in Mn-treated cells. As shown in Fig. 7, PP2A enzyme activity was increased by almost 50% in differentiated N27 cells exposed to 1 µM Mn for 24 hr. Further, 3 µM rottlerin significantly blocked Mn-induced increases in PP2A activity, suggesting that PKCδ may be activating PP2A by direct phosphorylation, consistent with our previous study findings (Zhang et al., 2007).

**Discussion**

In the present study, we demonstrate that an inorganic form of Mn changes tyrosine hydroxylase activity following different treatment methods in mesencephalic dopamine-producing N27 cells. We demonstrated that (i) acute manganese exposure activates tyrosine hydroxylase; (ii) chronic manganese exposure suppresses tyrosine hydroxylase activity; (iii) chronic manganese exposure causes PKCδ activation and increases PP2A activity; and (iv) the reduced TH activity induced by chronic Mn exposure might occur through the PKCδ-induced activation of PP2A. Importantly, to our knowledge, this is the first report showing that manganese modifies tyrosine hydroxylase in dopaminergic neuronal cells and that PKCδ is a key biochemical target in this process.

Mn has been shown to primarily target the nigrostriatal system, including the globus pallidus and substantia nigra (Baek et al., 2003). Mn exposure varies from moderate, as occurs via drinking water and food sources, to high, occurring via occupational and industrial exposure through mining, welding, and steel manufacturing (Woolf et al., 2002; Roth and Garrick, 2003; Dobson et al., 2004; Olanow, 2004). Mn can cross the blood-brain barrier via specific carriers such as transferrin and divalent metal transporter 1 and also by diffusion (Yokel and Crossgrove, 2004). The normal concentration of manganese in human adult
tissues ranges from 3 to 20 µM (Roth and Garrick, 2003), with an average human blood manganese level of 7.2 µg/l (Hauser et al., 1996). Markesbery et al. (1984) reported that the mean brain manganese level is 0.261 µg/g, and Zecca et al. (1994) reported Mn levels in putamen, substantia nigra, and neuromelanin of 6.31, 0.34, and 58.5 ng/mg wet weight, respectively. Depending on the level of exposure, blood manganese concentrations can increase from 10- to 200-fold (Hauser et al., 1996; Lucchini et al., 1999; Mergler et al., 1999; Woolf et al., 2002; McKinney et al., 2004).

Tyrosine hydroxylase is the enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to dihydroxyphenylalanine (DOPA). DOPA is a precursor for dopamine, which in turn is a precursor for norepinephrine (noradrenaline) and epinephrine (adrenaline). The enzyme, an oxygenase, is found in the cytosol of all cells containing catecholamines. This initial reaction is the rate limiting step in the production of catecholamines. Tyrosine hydroxylase is regularly used as a marker for dopaminergic neurons, which is particularly relevant for Parkinson's disease research. Since Mn primarily targets the nigrostriatal system, we sought to evaluate the effect of Mn on the activity of tyrosine hydroxylase, an enzyme with a key role in the physiology of adrenergic neurons, in dopaminergic neurons. We chose lower, nontoxic concentrations of MnCl₂ for our studies. We also found that acute Mn exposure leads to increased TH activity and phosphorylation, while chronic Mn exposure causes decreased TH activity. Neither kinds of exposure are lethal to dopaminergic neuronal cells.

Phosphorylation is a key post-translational mechanism to regulate TH activity. Phosphorylation of serine residues at 8, 19, 31 and 40 can activate TH, resulting in enhanced dopamine synthesis (Campbell et al., 1986; Haycock, 1990; Mitchell et al., 1990; Lindgren et al., 2001; McCulloch et al., 2001; Dunkley et al., 2004). A number of kinases, including PKC, PKA, CaMPK-II, and MAPkinase, have been shown to phosphorylate one or more of these sites to increase TH activity, depending on the cell type. General PKC increases ser40 phosphorylation and TH activity (Cahill et al., 1989; Haycock, 1990; Haycock and Haycock,
1991; Waymire et al., 1991; Haycock, 1993; Bobrovskaya et al., 1998); however, the effect of PKC subtypes has never been explored. At the present time, 12 different isoforms of PKC have been identified and grouped into three major classes (Gschwendt, 1999; Dempsey et al., 2000; Maher, 2001; Kanthasamy et al., 2003). The conventional class of PKC isoforms, PKCα, βI, βII, and γ, require DAG and Ca$^{2+}$ for activation, the novel PKC isoforms, PKCδ, ε, μ, η, and θ, require only DAG but not Ca$^{2+}$ for activation, and the atypical PKCs, PKCτ, λ, and ζ, require neither Ca$^{2+}$ nor DAG for activation. The isoform-specific physiological functions of each subtype of PKCs are yet to be characterized in CNS. The recently available, more specific pharmacological inhibitors, genetic mutants, and siRNAs specific for subtypes of isoforms are extremely useful in characterizing the functional significance of PKC isoforms. The enhanced TH activity we observed induced by pharmacological inhibitors and siRNA in three different cell culture models clearly demonstrated that PKCδ can negatively regulate TH activity (Zhang et al., 2007). In Fig. 6 we showed that chronic Mn exposure increased cleavage of PKCδ, which was attenuated by the PKCδ cleavage inhibitor Z-DEVD. PKCδ can be proteolytically cleaved by caspase-3 at the 324DIPD327 residue, resulting in 41-kDa catalytic and 38-kDa regulatory subunits, leading to a persistent activation of the kinase (Kaul et al., 2003; Yang et al., 2004; Anantharam et al., 2004). While TH was inactivated by chronic Mn exposure, PKCδ was activated in dopaminergic cells. To determine if TH inactivation by Mn treatment is PKCδ dependent, we cotreated Mn with the PKCδ inhibitor rottlerin. We found that the PKCδ inhibitor can successfully recover from the suppressed TH activity induced by Mn.

An active PP2A enzyme consists of a heterotrimer of the structural A subunit, a catalytic C subunit, and a regulatory B subunit (Dobrowsky and Hannun, 1993; Sontag et al., 1995; McCright et al., 1996; Ruvolo et al., 2002). The exact nature of the physical association and dynamic regulation of TH, PKCδ, and PP2A are yet to be characterized; however, recent literature provides some information regarding this interaction. PKCδ, TH, or PP2A have recently been shown to physically associate with each other, as well as other
putative chaperone proteins such as α-synuclein and 14-3-3 (Ostrerova et al., 1999; Kleppe et al., 2001; Srivastava et al., 2002; Kjarland et al., 2006). Recently, Peng et al. (Peng et al., 2005) demonstrated that a functional interaction between α-synuclein and PP2A can regulate TH phosphorylation and TH activity. Srivastava et al. reported a physical interaction between PKCδ and PP2A in NIH3T3 cells, and that dephosphorylation of PKCδ by PP2A results in its inactivation (Srivastava et al., 2002). In our recent study, we showed that α-synuclein interacts with PKCδ and regulates its activity following neurotoxic insults (Kaul et al., 2005). Therefore, in the dopaminergic system, the physical and functional association between PKCδ, TH, and PP2A could be facilitated and/or regulated by chaperone proteins such as α-synuclein (Kaul et al., 2005; Peng et al., 2005) and 14-3-3 (Ostrerova et al., 1999; Kleppe et al., 2001; Kjarland et al., 2006). We previously provided novel evidence that PKCδ negatively regulates TH activity and DA synthesis via activation of PP2A (Zhang et al., 2007). According to our hypothesis, after activation of PKCδ by chronic Mn treatment, the PP2A phosphorylation level will increase and activated PP2A will cause more dephosphorylation of TH, which will lead to the inactivation of TH enzyme. To check our hypothesis, we measured PP2A activity after chronic Mn treatment with or without rottlerin, and the results showed that chronic Mn treatment significantly promotes PP2A activity, which is attenuated by the PKCδ inhibitor rottlerin. Collectively, our data on Mn, TH, PKCδ, and PP2A suggest that chronic Mn induces activation of PP2A through activation of PKCδ, which leads to the decreased TH activity in the dopaminergic neuronal cells. Following acute Mn exposure, the PKCδ inhibitor does not attenuate the increased TH activity induced by Mn exposure, suggesting that PKCδ might not be involved in this process.

Regulation of TH activity and DA levels is critical for normal dopaminergic neurotransmission in the CNS. Excessive DA production may not only alter neurotransmission, but may also contribute to neuronal cell death through increased oxidative stress (Hoyt et al., 1997; Luo et al., 1998). In this regard, we wish to point out that PKCδ can be activated by at least two independent mechanisms in neuronal cells. These include
membrane translocation and caspase-3-dependent proteolytic cleavage (Kikkawa et al., 2002; Brodie and Blumberg, 2003; Kanthasamy et al., 2003). Of the two activation mechanisms, PKCδ activated by membrane translocation following tonic stimulation by lipid activators contributes to cell survival and proliferation (Kanthasamy et al., 2003; Jackson and Foster, 2004). As demonstrated in our recent studies, another form of activation is caspase-3-dependent proteolytic cleavage of native PKCδ into regulatory and catalytic fragments resulting in persistent activation during exposure to neurotoxic agents such as MPP+, MMT, manganese, or dieldrin (Anantharam et al., 2002; Kaul et al., 2003; Latchoumycandane et al., 2005). This form of proteolytic activation contributes to apoptotic cell death of dopaminergic neurons (Kanthasamy et al., 2003). In the case of lipid activator, TPA induced membrane translocation of native PKCδ, but did not induce apoptotic cell death (Anantharam et al., 2002). Furthermore, recent studies from our lab and others have shown that unlike the native 74 kDa PKCδ, the 41 kDa catalytically active PKCδ fragment, upon proteolytic cleavage, is targeted to various sub-cellular organelles including the mitochondria (Majumder et al., 2000) and nucleus (DeVries et al., 2002) to activate apoptotic cell death signaling molecules. These results suggest that native and cleaved PKCδ have different substrate profiles.

In conclusion, we provide novel evidence that Mn exposure leads to changes in TH enzyme activity, while chronic Mn exposure induces activation of PP2A through activation of PKCδ, which leads to the decreased TH activity in the dopaminergic neuronal cells. We suggest that Mn-mediated regulation of TH through PKCδ may have important implications in neurological dopaminergic system disorders, such as Parkinson’s disease.

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FIGURE 1.

Figure 1. Effect of acute Mn treatment on TH activity in differentiated N27 cells. N27 cells were differentiated by using 2 mM dbcAMP for 3 days; differentiated N27 cells were incubated with 3 or 10 μM MnCl$_2$ for 3 hr with or without 3 μM rottlerin. For measurement of TH activity, cells were exposed to 2 mM NSD-1015 for 1 hr prior to MnCl$_2$ treatment.
Cells were lysed after treatment, and extracts were used for determining L-DOPA levels by HPLC. \textbf{A}, Data were presented as pg DOPA/μg protein; \textbf{B}, data were presented as percentage of control. The data represent a mean ± SEM of six to eight individual measurements. Asterisks (*p<0.05) indicate significant differences between MnCl₂-treated cells and control cells.
Figure 2. Effect of acute Mn treatment on TH phosphorylation level in differentiated N27 cells. Differentiated N27 cells were exposed to 3 or 10 μM MnCl₂ for 3 hr. Cell extracts were prepared and separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. TH antibody (mouse, 1:1000) and phospho-specific antibodies directed against P-TH-ser40 (rabbit, 1:1000) were used for immunoblotting. To confirm equal protein loading in each lane, the membranes were reprobed with β-actin antibody. The immunoblots were visualized using the Amersham’s ECL detection agents. Densitometric analysis of 60 kDa P-TH-ser40 bands represents the mean ± SEM from three separate experiments (*p < 0.05, **p < 0.01).
Figure 3. Cytotoxicity of acute MnCl₂ treatment in differentiated N27 cells. Differentiated N27 cells were treated with 3 μM or 10 μM MnCl₂ for 3 hr. The effect of acute manganese treatment on cell death was quantified by Sytox Green fluorescence assay (green dots represent the dead cells). Then the density of fluorescence was measured using a fluorescence plate reader and was presented as % of control. 100 μM H₂O₂ treated cells were used as positive control. The data represent a mean ± SEM of four individual measurements. Asterisks (***p<0.001) indicate significant differences between H₂O₂-treated cells and control cells.
FIGURE 4.

Figure 4. Effect of chronic MnCl₂ on TH activity and phosphorylation level in
**differentiated N27 cells.** Differentiated N27 cells were incubated with 0.1 μM, 0.3 μM, or 1 μM MnCl₂ for 24 hr. For measurement of TH activity, cells were exposed to 2 mM NSD-1015 for 1 hr prior to MnCl₂ treatment. Cells were lysed after treatment, and extracts were used for determining L-DOPA levels by HPLC. **A,** Data were presented as pg DOPA/μg protein; **B,** data were presented as percentage of control. The data represent a mean ± SEM of six to eight individual measurements. Asterisks (*p<0.05) indicate significant differences between MnCl₂-treated cells and control cells. **C,** Differentiated N27 cells were treated with 1 μM MnCl₂ or cotreated with 3 μM rottlerin for 24 hr. Cells were pretreated with 2 mM NSD-1015 for 1 hr before MnCl₂ treatment, were lysed and extracts were used for determining L-DOPA levels by HPLC. The data represent a mean ± SEM of six to eight individual measurements. Asterisks (**p<0.01) indicate significant differences between MnCl₂-treated cells and control cells.
Figure 5. Cytotoxicity of chronic MnCl₂ treatment in differentiated N27 cells. Differentiated N27 cells were treated with 0.1 μM, 0.3 μM, or 1 μM MnCl₂ for 24 hr. The effect of chronic manganese treatment on cell death was quantified by Sytox Green fluorescence assay (green dots represent the dead cells). Then the density of fluorescence was measured using a fluorescence plate reader and was presented as % of control. 100 μM H₂O₂ treated cells were used as positive control. The data represent a mean ± SEM of four individual measurements. Asterisks (***p<0.001) indicate significant differences between H₂O₂-treated cells and control cells.
Figure 6. Effect of MnCl₂ on PKCδ activity in differentiated N27 cells. Differentiated N27 cells were treated with 1 μM MnCl₂ or cotreated with 3 μM rottlerin for 24 hr. Cell extracts from the above treatment were used in the immunoprecipitation kinase assay. Recombinant PKCδ protein was used as a positive control. The bands were quantified by a PhosphoImager after scanning the dried gel, and are expressed as a percentage of control. The data represent a mean ± SEM of three individual measurements. Asterisks (**p<0.01) indicate significant differences between MnCl₂ treated and control cells.
Figure 7. Effect of Mn on PP2A activity in dopaminergic cells. Differentiated N27 cells were treated with 1 μM MnCl₂ or cotreated with 3 μM rottlerin for 24 hr, then cells were lysed and PP2A enzyme activity was measured using a serine/threonine phosphatase assay kit from Promega. The data represent a mean ± SEM of four to six individual measurements. Asterisks (*p<0.05) indicate significant differences between MnCl₂ treated and control cells.
Chapter VI: GENERAL CONCLUSION

The major findings of each research chapter included in this thesis have been described, and the implications for dopaminergic function have been interpreted in the discussion section of each individual chapter. This section presents an overview of the results and findings of the thesis, with special emphasis on the contributory role of PKCδ protein in the dopaminergic system, and its relevance to the pathogenesis of Parkinson’s disease.

Interaction of PKCδ-PP2A-TH regulates dopamine synthesis in both dopaminergic cells and animal brain. Tyrosine hydroxylase (TH) is a rate-limiting enzyme in the biosynthesis of dopamine and catalyzes the first step of a biochemical synthetic pathway in which L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-dopa). Phosphorylation and dephosphorylation of TH represent important post-translational regulatory mechanisms of the enzymatic activity that largely determine the amount of dopamine synthesis (Lee et al., 1989). Regulation of TH activity and DA levels is critical for normal dopaminergic neurotransmission in the CNS. The results from our studies show that PKCδ phosphorylates PP2Ac to enhance its activity and thereby reduces TH-ser40 phosphorylation and TH activity and ultimately dopamine synthesis in dopaminergic neurons. PKCδ, TH, or PP2A sometimes physically associate together, or with other putative chaperone proteins such as α-synuclein and 14-3-3 (Ostrerova et al., 1999; Kleppe et al., 2001; Srivastava et al., 2002; Kjarland et al., 2006). Recently, Peng et al. (Peng et al., 2005) demonstrated that a functional interaction between α-synuclein and PP2A can regulate TH phosphorylation and TH activity. Srivastava et al. reported a physical interaction between PKCδ and PP2A in NIH3T3 cells, and that dephosphorylation of PKCδ by PP2A results in its inactivation (Srivastava et al., 2002). The novel finding of our study demonstrates that physical association of PKCδ and PP2A was accompanied by PP2Ac phosphorylation by PKCδ, resulting in PP2A activation. We found that PP2A activity was significantly
decreased, without altering the PP2A protein levels, by the PKC\(\delta\) inhibitor rottlerin. Basal PP2A enzymatic activity was also significantly reduced in dopaminergic cells stably expressing loss of function kinase inactive PKC\(\delta\)-DN mutant compared to LacZ cells. Furthermore, substantia nigra of PKC\(\delta\) \((-/-)\) mice showed significantly lower basal PP2A activity compared to naïve animals, indicating that PKC\(\delta\) can augment PP2A activity. Additionally, in vitro kinase assays also revealed that PKC\(\delta\) can phosphorylate PP2Ac. Treatment with the PP2A inhibitor okadaic acid increased the TH-ser40 level and enhanced TH activity, suggesting that PP2A effectively regulates TH activity and ser40 phosphorylation. Our results agree with a recent study showing attenuation of TH activity and TH-ser40 phosphorylation by PP2A (Peng et al., 2005). Collectively, our data on PKC\(\delta\) and PP2A suggest that PKC\(\delta\) negatively regulates TH-ser40 phosphorylation and activity and dopamine synthesis via increased PP2A activity by direct phosphorylation of PP2A. Future studies may need to focus on finding the phosphorylation sites of PP2A by PKC\(\delta\) in dopaminergic neurons.

**PKC\(\delta\) inhibitor rottlerin rescued MPTP-induced dopaminergic cell death and dopamine loss in mice.** Although the existing approaches to PD treatment alleviate the symptoms, they fail to prevent the progression of the neurodegenerative process. Therapeutics that prevent the progressive loss of nigral dopaminergic neurons are not currently available. The mechanisms underlying the dopaminergic degenerative process observed in PD are not well understood, which has hampered development of successful neuroprotective drugs. Recent studies from our laboratory demonstrated that the PKC\(\delta\) isoform is an oxidative stress-sensitive kinase and a key mediator of apoptotic cell death in PD models (Kaul et al., 2003; Yang et al., 2004). We showed that native PKC\(\delta\) is proteolytically activated by caspase-3 and that suppression of PKC\(\delta\) by dominant negative mutant or siRNA against the kinase can effectively block apoptotic cell death in cellular models of PD. In an attempt to translate the mechanistic studies to a neuroprotective strategy targeting PKC\(\delta\), we systematically characterized the neuroprotective effect of a PKC\(\delta\)
inhibitor, rottlerin, in MPP⁺-treated primary mesencephalic neuronal cultures, as well as in an MPTP animal model of PD. Administration of rottlerin, either intraperitoneally or orally, to C57 black mice significantly protected against MPTP-induced locomotor deficits and striatal depletion of dopamine and its metabolite DOPAC. Importantly, stereological analysis of nigral neurons revealed rottlerin treatment significantly protected against MPTP-induced TH-positive neuronal loss in the substantia nigra compacta. Collectively, our findings demonstrate the neuroprotective effect of rottlerin in both cell culture and preclinical animal models of PD, and suggest that pharmacological modulation of PKCδ may offer a novel therapeutic strategy for treatment of PD. Small molecule inhibitors of protein kinases are being increasingly evaluated for therapeutic use for various human diseases. A number of researchers have attempted to develop neuroprotective agents targeting cell death signaling molecules. Inhibitors targeted against mixed lineage kinase (MLK) and poly-ADP-ribose polymerase (PARP) have been shown to protect nigral dopaminergic neurons in animal models (Cosi et al., 1996), and some of these agents are currently being evaluated in human clinical trials (Parashar et al., 2005). Previous studies have shown PKCδ can indirectly regulate PARP and MLK (Merritt et al., 1999; Yoshida et al., 2002; Kitazawa et al., 2004), suggesting that PKCδ may be an attractive upstream neuroprotective therapeutic target. Saporito et al. showed systemic injection of the c-Jun N-terminal kinase (JNK) inhibitor CEP-1347/KT-7515 protected against MPTP toxicity in the C57 black mouse model (Saporito et al., 1999). Maruyama et al. developed a class of compounds with multiple putative properties, including iron chelation, MAO inhibition, and antioxidant effects (Maruyama et al., 2002). Minocycline, a tetracycline derivative with anti-inflammatory properties, prevented dopaminergic neurodegeneration in MPTP models of PD (Du et al., 2001). Minocycline also inhibits microglia-associated inflammation and apoptosis (Ravina et al., 2003; Kelly et al., 2004). Oral administration of the nutritional supplement creatine attenuated MPTP toxicity, which is similar to our findings with rottlerin (Matthews et al., 1999). Creatine exhibits neuroprotective properties by inhibiting mitochondrial permeability,
as well as indirectly acting as an antioxidant (Tarnopolsky and Beal, 2001). In addition to creatine, the antioxidant Coenzyme-Q is being evaluated in PD clinical trials (Shults and Haas, 2005). Release of cytochrome C resulting from increased mitochondrial permeability during oxidative stress has been shown to initiate PKCδ activation (Anantharam et al., 2002), and antioxidant treatment attenuates proapoptotic action of the kinase (Kaul et al., 2003), indicating that PKCδ is closely associated with two leading PD pathological mechanisms, i.e., oxidative insult and mitochondrial dysfunction. Our results provide evidence that PKCδ may serve as a novel therapeutic target for development of neuroprotective agents and suggest that development of selective and systemically-active small molecule PKCδ isoform inhibitors may translate to an effective neuroprotective agent for treatment of PD.

**PKCδ inhibition causes hyperactivity in mice.** Regulation of TH activity and DA levels is critical for normal dopaminergic neurotransmission in the CNS. Excessive DA production may alter neurotransmission (Hoyt, Reynolds et al. 1997; Luo, Umegaki et al. 1998). In the present study, acute rottlerin administration and PKCδ knockout induced hyperlocomotor activity as well as stereotypy in mice, demonstrating an important role of PKCδ in modulating basal locomotor activity. A relatively high dose of apomorphine (10 mg/kg) resulted in enhanced locomotion in the rottlerin-treated animals. Selective activation of D1- or D2-like receptors produces different locomotor activity in mice. The nonselective DA receptor agonist apomorphine induced a modest increase in locomotor activity in the rottlerin-treated mice, but did not change activity in control animals. This may well reflect a combined effect of apomorphine on both D1- and D2-like receptors. Previous studies suggested that PKC-mediated phosphorylations of D1 and D2 receptors were involved in
receptor internalization (Namkung and Sibley 2004), and this rapid receptor sequestration is involved in agonist-induced receptor desensitization. Thus, PKCδ inhibition may affect the postsynaptic sensitivity by altering the receptor phosphorylation state and/or interfering with receptor desensitization. Mice administered an acute rottlerin treatment responded well to the dopamine receptor agonist and antagonist, while the PKCδ knockout mice did not (data not shown here). Permanent knockout of the PKCδ protein might change the dopamine receptor sensitivity or desensitization, but this possibility needs to be further tested.

Manganese exposure changes tyrosine hydroxylase activity, which might be PKCδ involved. Exposure to high levels of manganese (Mn) causes a Parkinson's-like syndrome known as manganism. Increased incidences of manganism have been observed among miners and industrial welders as well as farmers exposed to Mn-based pesticides such as fungicides Maneb (Mn ethylene-bis-dithiocarbamate) and Mancozeb (Mn Cu Zn ethylene-bis-dithiocarbamate) (Roth and Garrick, 2003; Dobson et al., 2004; Olanow, 2004). Several studies have recently demonstrated effects of Mn on neuronal cells, but the mechanism of Mn-induced dopaminergic neurodegeneration remains unclear. In the present study, we report that chronic Mn treatment suppresses TH activity, which is accompanied by increased PKCδ cleavage in the dopaminergic cells. The reduced TH activity induced by chronic Mn treatment can be attenuated by the PKCδ inhibitor rottlerin. And such levels of chronic Mn treatment are not cytotoxic to dopaminergic cells, indicating physiological concentrations of Mn influenced TH activity and phosphorylation in this study. Previously,
we reported an interaction between PKCδ and TH, in which the kinase modulates dopamine synthesis by negatively regulating TH activity via protein phosphatase 2A (PP2A). Here we also showed that Mn treatment can increase PP2A activity, possibly due to decreased TH activity induced by MnCl₂. The increased PP2A activity induced by chronic MnCl₂ treatment is suppressed by the PKCδ inhibitor rottlerin, which may suggest that the activation of PKCδ induced by Mn caused increased PP2A activity, which may decrease TH activity induced by Mn treatment. Mn primarily targets the nigrostriatal system, including the globus pallidus and substantia nigra (Baek et al., 2003). Our novel finding is that Mn exposure leads to changes in TH enzyme activity. Specifically, chronic Mn induces activation of PP2A through activation of PKCδ, which leads to decreased TH activity in dopaminergic neuronal cells. We suggest that Mn-mediated regulation of TH through PKCδ may have important implications in neurological dopaminergic system disorders, such as Parkinson’s disease. Identifying pharmaceutical or genetic methods which inhibit PKCδ to prevent Mn-induced Parkinson's-like syndrome will be a promising future research endeavor.

In summary, PKCδ enhances phosphatase 2A (PP2A) activity by phosphorylation of PP2Ac. Increased PP2A activity decreases TH phosphorylation at site ser40, which leads to inactivation of TH and reduction of its activity and an ultimate decrease in dopamine synthesis. Inhibition of PKCδ by its inhibitor rottlerin, PKCδ-DN mutant, PKCδ KO, or PKCδ siRNA, suppresses PP2A activity due to a decreased phosphorylation of PP2A. Our data suggest that PKCδ negatively regulates TH and dopamine synthesis via PP2A
Inhibition of PKCδ by the kinase inhibitor rottlerin enhances dopaminergic neurotransmission and neurobehavioral characteristics in animal models. The neuroprotective effect of rottlerin in both cell culture and preclinical animal models of PD suggests that pharmacological modulation of PKCδ may offer a novel therapeutic strategy for treatment of PD. PKCδ inhibition can also attenuate reduction of TH activity induced by manganese exposure in dopaminergic cells. These findings suggest that inhibition of PKCδ in the nigrostriatal dopaminergic system can offer dual benefits of neuroprotection and enhanced dopaminergic function in the development of therapeutic agents for treatment of Parkinson’s disease.

**Scheme-1**
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