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Protein Kinase C Beta Regulates the D₂-Like Dopamine Autoreceptor

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Abstract

The focus of this study was the regulation of the D₂-like dopamine autoreceptor (D₂ autoreceptor) by protein kinase C β , a member of the protein kinase C (PKC) family. Together with the dopamine transporter, the D₂ autoreceptor regulates the level of extracellular dopamine and thus dopaminergic signaling. PKC regulates neuronal signaling via several mechanisms, including desensitizing autoreceptors to increase the release of several different neurotransmitters. Here, using both PKC $\beta^{-/-}$ mice and specific PKC β inhibitors, we demonstrated that a lack of PKC β activity enhanced the D₂ autoreceptor-stimulated decrease in dopamine release following both chemical and electrical stimulations. Inhibition of PKC β increased surface localization of D₂R in mouse striatal synaptosomes, which could underlie the greater sensitivity to quinpirole following inhibition of PKC β . PKC $\beta^{-/-}$ mice displayed greater sensitivity to the quinpirole-induced suppression of locomotor activity, demonstrating that the regulation of the D₂ autoreceptor by PKC β is physiologically significant. Overall, we have found that PKC β downregulates the D₂ autoreceptor, providing an additional layer of regulation for dopaminergic signaling. We propose that in the absence of PKC β activity, surface D₂ autoreceptor localization and thus D₂ autoreceptor signaling is increased, leading to less dopamine in the extracellular space and attenuated dopaminergic signaling.

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Keywords

Dopamine; autoreceptor; exocytosis; quinpirole; synaptosomes

1. Introduction

Tight regulation of extracellular dopamine is crucial for normal dopaminergic signaling and is primarily achieved at the presynaptic terminal by activation of the dopamine transporter (DAT) and the D₂-like dopamine autoreceptor (D₂ autoreceptor). The primary function of the DAT is to take up dopamine from the extracellular space, terminating dopaminergic signaling (Giros et al., 1996). The D₂ autoreceptor regulates extracellular dopamine levels through a negative feedback mechanism (L'Hirondel et al., 1998). Both the D₂-like dopamine receptor (D₂R) and DAT are substrates for the widely expressed serine/threonine kinase protein kinase C (PKC) (Foster et al., 2002; Namkung and Sibley, 2004).

In addition to its regulation of D₂R and DAT, PKC impacts neurotransmitter exocytosis. Activation of PKC by phorbol esters increases the release of various neurotransmitters, including dopamine, following a depolarizing stimulus (Cubeddu et al., 1989; Huang et al., 1989; Barrie et al., 1991). PKC affects exocytosis through several different mechanisms, including inhibiting potassium channels, blocking calcium channel inactivation through G-proteins, increasing the size and replenishing rates of vesicle pools, and increasing the availability of the Soluble NSF Attachment Protein Receptor (SNARE) complex proteins involved in vesicle fusion (Barrett and Rittenhouse, 2000; Leenders and Sheng, 2005; Majewski and Iannazzo, 1998; Tanaka and Nishizuka, 1994). Cubeddu and colleagues (1989) demonstrated that PKC activation with a phorbol ester reduced the ability of the D₂ autoreceptor to inhibit evoked dopamine release. Additionally, inhibition of D₂Rs by phospholipase C and calcium-dependent PKCs has been shown in ventral tegmental dopaminergic neurons (Nimitvilai et al., 2013). PKC phosphorylates D₂R, resulting in internalization and desensitization of the receptor (Namkung and Sibley, 2004; Morris et al., 2007). While there is evidence showing that PKC affects the regulation of both extracellular dopamine and D₂R, it has yet to be determined which of the ten mammalian PKC isoforms interacts with D₂R to cause these changes.

We previously reported that the PKC β isoform regulates DAT trafficking and activity in response to amphetamine (Johnson et al., 2005; Furman et al., 2009; Chen et al., 2009). Additionally, we have shown that PKC β is present in dopaminergic neurons and co-localizes with DAT (O'Malley et al., 2010). More recently, we determined that PKC β is crucial for coordinating the interaction between the D₂ autoreceptor and DAT (Chen et al., 2013). Because both D₂ autoreceptors and PKC β are expressed in the dopaminergic neurons and D₂ autoreceptors are a known substrate of PKC, we hypothesized that PKC β regulates the D₂ autoreceptor. In the present study we used mice genetically lacking PKC β along with specific PKC β inhibitors to determine the impact of this kinase on D₂ autoreceptor activity, which was assessed by measuring dopamine exocytosis following chemical stimulation of synaptosomes or electrical stimulation of brain slices, as well as measuring D₂R-mediated changes in locomotor activity in the presence of the D₂R/D₃R agonist quinpirole. We

determined that PKC β influences the D₂ autoreceptor to regulate its surface localization and activity. Coupled with our findings regarding the regulation of DAT by PKC β , this work identifies a role for PKC β as a key regulator of extracellular dopamine levels and thus dopaminergic signaling.

2. Materials and Methods

2.1 Animals

All animal use and procedures were approved by the Institutional Animal Care and Use Committee at the University of Michigan and were in accordance with the National Institutes of Health guidelines. We made every effort to reduce the number of animals used and minimize animal suffering. The generation of PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice was previously described (Leitges et al., 1996). These mice were backcrossed with C57BL/6J mice more than ten times. Mice had free access to water and standard laboratory chow. Experimental mice were gender- and age-matched and were used between two and four months of age.

2.2 Chemicals

LY379196 was a generous gift from Eli Lilly (Indianapolis, Indiana). Enzastaurin was purchased from LC Labs (Woburn, MA). [³H]-sulpiride was from PerkinElmer (Waltham, MA). Complete Mini protease inhibitor was purchased from Roche Diagnostics (Indianapolis, IN). All other chemicals, including 4-aminopyridine, quinpirole, sulpiride, and butaclamol, were purchased from Sigma Aldrich (St. Louis, MO).

2.3 Striatal dopamine release via superfusion

Synaptosomes from whole striata were prepared as described previously (Chen et al, 2009). Briefly, mice were sacrificed by cervical dislocation. Striata were dissected on ice and homogenized in 0.32 M sucrose containing Complete Mini protease inhibitor cocktail. Homogenates were centrifuged at 4°C (800 × g, 10 minutes) to remove cellular debris. The supernatant was centrifuged again (12,000 × g, 15 minutes, 4°C).

The pellet containing crude synaptosomes was resuspended in oxygenated Krebs-Ringer's Buffer (KRB) (145 mM NaCl, 2.7 mM KCl, 1.2 mM KH₂PO₄, 1.0 mM MgCl₂, 10 mM glucose, 24.9 mM NaHCO₃, 0.05 mM ascorbic acid, 0.05 mM pargyline, pH 7.4). Synaptosomes were loaded into the chambers of a Brandel superfusion apparatus (Brandel Inc., Gaithersburg, MD). The samples were perfused with oxygenated KRB at approximately 800 μ l/min. Following a 60-minute wash to achieve a steady dopamine baseline, 14 fractions were collected at one-minute intervals. Dopamine release was stimulated at fractions seven and eight with 50 μ M 4-aminopyridine (4AP) in the presence or absence of quinpirole or sulpiride. The PKC β inhibitor LY379196 was applied during the 60-minute wash period and continued throughout fraction collection. An internal standard solution composed of 50 mM perchloric acid, 25 μ M EDTA, and 10 nM 2-aminophenol was added to each collected fraction. Dopamine content in each fraction was measured using high-performance liquid chromatography (HPLC) with electrochemical detection (Thermo Scientific/ESA, Sunnyvale, CA).

2.4 Striatal dopamine release via electrical stimulation

Brain slices were prepared as described previously (Mateo et al., 2005). Briefly, mice were decapitated, brains rapidly removed, and the coronal brain slices (400 μm thick) containing the nucleus accumbens were prepared using a vibrating tissue slicer. Slices were maintained at 32°C in oxygen-perfused (95% O_2 –5% CO_2) modified Krebs buffer, which consisted of (in mM): NaCl, 126; NaHCO_3 , 25; D-glucose, 11; KCl, 2.5; CaCl_2 , 2.4; MgCl_2 , 1.2; NaH_2PO_4 , 1.2; L-ascorbic acid, 0.4; pH 7.4. A capillary glass-based carbon-fiber electrode (active area $\sim 100 \mu\text{m}$ long, 7 μm wide) was positioned approximately 75 μm below the surface of the slice in the nucleus accumbens core. Dopamine release was evoked every 5 min by a 4-ms, one-pulse stimulation (monophasic, 300 μA) from a bipolar stimulating electrode (Plastics One, Roanoke, VA, USA) placed 100–200 μm from the carbon-fiber electrode.

Fast-scan cyclic voltammetry recordings were performed and analyzed using locally written software (Demon Voltammetry and Analysis; Yorgason et al., 2011). The electrode potential was linearly scanned as a triangular waveform from -0.4 to 1.2 V and back to -0.4 V (Ag vs. AgCl) using a scan rate of 400 V/s. Cyclic voltammograms were recorded at the carbon-fiber electrode every 100 ms by means of a potentiostat (Dagan, Minneapolis, MN, USA). Once the stimulated dopamine response was stable for at least three successive collections, baseline measurements were taken. Evoked extracellular concentrations of dopamine were assessed by comparing the current at the peak oxidation potential for dopamine with electrode calibrations of known concentrations of dopamine (1–3 μM). Data were modeled using Michaelis-Menten kinetics to determine dopamine released and V_{max} (Yorgason et al., 2011). The selective D_2R agonist (–)-quinpirole hydrochloride was used to induce autoreceptor activation. Quinpirole-induced decreases in electrically stimulated dopamine release were compared with pre-drug values (each animal served as its own control) to obtain a percent change in stimulated dopamine release. The $\text{PKC}\beta$ inhibitor enzastaurin (200 nM) was applied after stable baselines were obtained, 60 minutes before quinpirole was added, and continued throughout the experiment. Quinpirole dose–response curves were plotted as log concentration (M) of quinpirole vs. percent of control dopamine response.

2.5 D_2R Binding

The D_2R binding protocol was modified from previously published methods (Namkung and Sibley, 2004). Briefly, striatal synaptosomes were prepared as described above and were resuspended in KRB. To measure surface D_2R binding, synaptosomes were treated for 5 minutes at 37°C with vehicle or the $\text{PKC}\beta$ inhibitor enzastaurin. Then the synaptosomes were incubated with 10 nM [^3H]-sulpiride, a hydrophilic D_2 receptor antagonist, for 3.5 hours on ice. Non-specific binding was determined in the presence of 10 μM (–)-butaclamol. Overall D_2R expression was determined using a membrane preparation from the striatal synaptosomes prepared above (Rubinstein et al., 1990). These synaptosomes were resuspended in 50 mM Tris-HCl (pH 7.4) and centrifuged at $40,000 \times g$ for 15 minutes. The resulting membrane fraction was resuspended in KRB and then treated with vehicle or enzastaurin for 5 minutes at 37°C. Membranes were incubated with 10 nM [^3H]-sulpiride $\pm$ 10 μM (–)-butaclamol for 90 minutes at room temperature (Namkung and Sibley, 2004). The reaction was terminated by filtering over GF/B Whatman filters and washing 3X with

ice cold KRB. The radioactivity bound was quantified by scintillation counting. Data are expressed as the amount of specific [^3H]-sulpiride bound, normalized to the vehicle treated control.

2.6 Locomotor suppression by acute quinpirole treatment

Locomotor suppression following quinpirole treatment in a novel environment was measured using radiotrigger implantation (Mini Mitter Co., Bend, OR) as previously described (Chen et. al, 2007). Briefly, a radiotrigger was implanted into the peritoneal cavity of each mouse. Following recovery, PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice were injected with saline and quinpirole (0.03, 0.1, or 0.3 mg/kg i.p.). Locomotor activity (gross activity count) was recorded immediately after the injection for 15 minutes and the data are expressed as the sum of the recorded locomotor activity as a percent of the saline control treatment.

2.7 Statistical Analysis

Results were analyzed using GraphPad Prism 6 software (San Diego, CA) and are plotted as mean $\pm$ SEM. Statistical significance was set at $p < 0.05$. Comparisons between multiple groups or treatments were made using one-, two- or three-way ANOVA with Bonferroni post-test. Three-way ANOVA was performed using Systat (Chicago, IL). When only two groups were compared, a paired, two-tailed Student's t -test was used.

3. Results

3.1 Suppression of PKC β activity increases the control of dopamine release by D $_2$ autoreceptors

The primary function of the D $_2$ autoreceptor is to control the amount of dopamine in the extracellular space through a negative feedback mechanism. Activation of the D $_2$ autoreceptor reduces dopamine exocytosis. We optimized a superfusion assay to measure D $_2$ autoreceptor control of dopamine exocytosis. In this assay, striatal synaptosomes are prepared from mice and perfused with KRB to achieve steady basal release of endogenous dopamine at a speed that minimizes dopamine reuptake ($\sim 800 \mu\text{l}/\text{min}$). The amount of dopamine in each collected fraction is determined using HPLC coupled with electrochemical detection. Dopamine exocytosis is stimulated using the potassium channel blocker 4-aminopyridine (4AP, 50 μM) to depolarize the synaptosomes (L'Hirondel et al., 1998). This stimulation increases dopamine release 2-3 fold over basal (Figure 1). D $_2$ autoreceptor control of dopamine exocytosis is determined by adding the D $_2$ R agonist quinpirole simultaneously with 4AP. Activation of D $_2$ R by agonist reduces 4-AP-stimulated dopamine release. In Figure 1, treatment with 3 μM quinpirole, a maximally effective concentration, inhibits 4AP-stimulated dopamine release. A one-way ANOVA revealed a significant effect of quinpirole treatment ($N = 3$, $F(5, 12) = 46.28$, $p < 0.0001$). To demonstrate D $_2$ R specificity for the quinpirole suppression of dopamine release, we included the D $_2$ R antagonist sulpiride, which had no effect on either basal release or 4AP-stimulated dopamine release. However, sulpiride treatment blocked the quinpirole suppression of dopamine exocytosis, demonstrating 4AP-stimulated dopamine release is indeed D $_2$ -autoreceptor dependent.

To determine if PKC β influences the D₂ autoreceptor activity, we measured the 4AP-stimulated dopamine exocytosis in the presence and absence of quinpirole in striatal synaptosomes prepared from PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice (Figure 2). Addition of 100 nM quinpirole decreased 4AP-stimulated dopamine release from PKC $\beta^{+/+}$ mice, as expected. 4AP-stimulated dopamine release was not statistically different in PKC $\beta^{-/-}$ mice as compared to PKC $\beta^{+/+}$ controls (N = 4). There was, however, an enhanced suppression of dopamine release in response to quinpirole. A three-way ANOVA with repeated measures yielded a significant main effect of genotype, $F(1,12) = 8.998$, $p < 0.05$, and drug, $F(1,12) = 7.23$, $p < 0.05$, and a significant interaction between time and genotype, $F(12,144) = 2.44$, $p < 0.05$.

To ensure any differences observed between PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ were not due to compensatory changes resulting from life-long deficiency of PKC β , we inhibited PKC β activity in wild type mice using specific inhibitors. We repeated the dopamine exocytosis experiment using the PKC β -specific inhibitor LY379196 (IC₅₀ = 30 nM, Jirousek et. al., 1996). Striatal synaptosomes from wild type mice were pretreated with vehicle or 100 nM LY379196 for 60 minutes prior to addition of 50 μ M 4AP and 30 nM quinpirole. A lower concentration of quinpirole was used to better detect potential increases in sensitivity due to PKC β inhibition. 4AP-stimulated dopamine release following quinpirole treatment in the presence and absence of LY379196 is shown in Figure 3, corrected for baseline release.

In the vehicle-treated control samples, 30 nM quinpirole did not significantly decrease stimulated dopamine release. Acute inhibition of PKC β by LY379196 potentiated the ability of the D₂ autoreceptor to suppress 4AP-stimulated dopamine release (one-way ANOVA, $F = 4.681$, $p = 0.0119$; N = 4). The absolute decrease (delta) in 4AP-stimulated dopamine release between quinpirole and 4AP in the absence and presence of 100 nM LY479196 (in pmol/mg protein/min) is -0.13 ± 0.05 and -0.32 ± 0.06 , respectively ($p = 0.0068$ in two-tailed paired t-test, $n=4$). The increased sensitivity to quinpirole following acute PKC β inhibition mimics the increased quinpirole reactivity measured in the PKC $\beta^{-/-}$ mice. Acute PKC β inhibition had no effect on 4AP-stimulated dopamine release in the absence of quinpirole, again replicating the results obtained with the PKC $\beta^{-/-}$ mice.

3.2 Lack of PKC β activity increases D₂ autoreceptor-mediated control of dopamine release in the nucleus accumbens

The superfusion experiments described above used a depolarizing chemical stimulus to trigger dopamine release. We then applied fast-scan cyclic voltammetry to determine if PKC β inhibition would increase D₂ autoreceptor-mediated inhibition of electrically evoked dopamine release. Dopamine release was evoked from mouse nucleus accumbens core in brain slices with single 300 μ A stimulations. The slices were pretreated for 60 minutes with either vehicle or the PKC β inhibitor enzastaurin (200 nM, IC₅₀ = 6 nM, Graff, 2005) and a concentration-response curve was generated for dopamine release suppression in response to quinpirole. Enzastaurin treatment on its own had no effect on stimulated dopamine release (Figure 4A; vehicle treatment: 1067.24 ± 80.23 nM, N = 10; enzastaurin treatment: 1058.55 ± 88.11 nM, N = 8). Similar to the superfusion results described above, inhibition of PKC β using enzastaurin increased the effectiveness of the D₂R agonist quinpirole, leading to

increased suppression of dopamine release (Figure 4B). A two-way ANOVA revealed a significant effect of pretreatment group as well as quinpirole, but no significant interaction (interaction $F(5, 82) = 1.00$, $p = 0.4231$; quinpirole $F(5, 82) = 109.4$, $p < 0.0001$; treatment group $F(1, 82) = 16.00$, $p = 0.0001$; $N = 8-10$). PKC β inhibition had no effect on the reuptake of dopamine via DAT (Vmax, vehicle treatment: 2335 ± 308.3 nM/sec, $N = 11$; enzastaurin treatment: 2291 ± 260.9 nM/sec, $N = 8$).

3.3 PKC β inhibition increases surface localization of the D₂R

We then investigated the mechanism by which PKC β inhibition would increase the activity of the D₂ autoreceptor. Previous reports found that PKC phosphorylation elicits internalization and desensitization of D₂R in a heterologous cell system (Namkung and Sibley, 2004; Morris et al., 2007). We therefore hypothesized that inhibition of PKC β would increase D₂R surface localization. For this experiment, striatal synaptosomes from wild type mice were treated with vehicle or 200 nM enzastaurin for 5 minutes prior to using the hydrophilic antagonist [³H]-sulpiride to bind the D₂R located on the surface of the synaptosomes. To ensure PKC β inhibition was not altering overall D₂R expression or binding, receptor binding was repeated on crude membranes prepared from striatal synaptosomes. PKC β inhibition significantly increased D₂R surface binding in intact synaptosomes but did not affect D₂R binding in crude membranes (Figure 5, $N = 3$ with 4-5 replicates per N , paired t-test, $p < 0.01$; specific [³H]-sulpiride binding in vehicle treated samples: Intact synaptosomes: 79.08 ± 0.01 fmol/mg protein, Lysed membranes: 82.6 ± 0.01 fmol/mg protein). Thus an increase in D₂R surface localization following inhibition of PKC β may underlie the increased D₂ autoreceptor activity.

3.4 PKC $\beta^{-/-}$ mice show increased quinpirole-induced locomotor suppression

To determine the physiological relevance of PKC β inhibition on D₂ autoreceptor activity, we measured suppression of locomotor activity in response to the D₂R agonist quinpirole in both PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice. Studies using mice with a deletion of the D₂ autoreceptor (*Drd2^{loxP/loxP}*; *Dat^{+IRES-cre}*) in midbrain dopamine neurons have concluded that the suppression of locomotor activity by low doses of quinpirole is primarily mediated by the D₂ autoreceptor (Bello et al., 2011). Here, the locomotor activity following an injection of either saline or an increasing dose of quinpirole in PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice was measured and the results are plotted as the total locomotor activity in fifteen minutes, normalized to the saline control (Figure 6). Quinpirole dose-dependently suppressed locomotor activity in both genotypes ($N = 6-15$). The PKC $\beta^{-/-}$ mice showed significantly greater locomotor suppression to quinpirole than did the PKC $\beta^{+/+}$ controls. A two-way ANOVA indicated a significant main effect of quinpirole dose, $F(4, 77) = 81.73$, $p < 0.0001$, and genotype, $F(1, 77) = 12.02$, $p < 0.001$, as well as a significant interaction, $F(4, 77) = 2.73$, $p < 0.05$. The results of this experiment demonstrate that the PKC β regulation of the D₂ autoreceptor measured in our suprafusion assays is physiologically relevant.

4. Discussion

To our knowledge, we are the first to determine that PKC β regulates presynaptic D₂ autoreceptor surface location and activity, expanding on previous reports demonstrating that

PKC activation regulates D₂R (Cubeddu et al., 1989; Namkung and Sibley, 2004; Morris, 2007). Using both PKC $\beta^{-/-}$ mice and specific PKC β inhibitors, we found that a reduction in PKC β activity resulted in increased D₂ autoreceptor function, likely by increasing receptor surface localization. We are the first to demonstrate the physiological relevance of the PKC regulation by demonstrating greater suppression of locomotor behavior by quinpirole in PKC $\beta^{-/-}$ as compared to PKC $\beta^{+/+}$ mice. Our model for this finding supports the hypothesis that under normal conditions, PKC β activity enhances phosphorylation of the D₂ autoreceptor, causing internalization and desensitization of the receptor, leading to a blunting of D₂ autoreceptor-mediated control of extracellular dopamine. However, when PKC β is inhibited, more D₂ autoreceptor is on the neuronal surface, leading to increased signaling and a greater D₂ autoreceptor-mediated inhibition of dopamine exocytosis. The greater quinpirole-mediated reduction in dopamine exocytosis would result in lower extracellular dopamine and less postsynaptic dopaminergic signaling, causing increased suppression of locomotor activity, as we observed in PKC $\beta^{-/-}$ mice.

PKC increases the release of neurotransmitters through many different mechanisms. By either inhibiting potassium channels (Colby and Blaustein, 1988) or preventing G protein blockade of calcium channels (Barrett and Rittenhouse, 2000), PKC can cause cell depolarization and an increase in neurotransmitter release. PKC increases vesicle fusion and neurotransmitter release by interacting with release machinery (de Vries et al., 2000). PKC also interacts with autoreceptors to alter neurotransmitter release, which is most pertinent to this study. Activation of PKC by phorbol esters increased electrical stimulation of [³H]-dopamine from rabbit striatal and prefrontal cortex slices (Cubeddu et al., 1989, but see (Iannazzo et al., 1997)). PKC activation antagonized the activity of several D₂R agonists, including quinpirole, leading the authors to hypothesize that PKC activation could decrease the surface localization of D₂ autoreceptors. PKC-dependent desensitization of D₂R was also reported by Nimitvilai et al. (2013). In that study, a reduced sensitivity of the D₂Rs in the ventral tegmental area was mediated by a Gq-coupled receptor signaling cascade, including calcium-dependent PKC. Taken together, these reports support our hypothesis that PKC activity decreases autoreceptor control of neurotransmitter exocytosis and in reverse, inhibition of PKC, especially PKC β , increases autoreceptor control. A recent study found that the Ca²⁺-regulated isozyme PKC γ , increases striatal dopamine release by phosphorylating a Cdc42/Rac1 guanine nucleotide exchange factor substrate (Shirafuji et al., 2014). That study and our current study are both identifying how specific PKC isoforms are regulating dopamine release, either through signaling cascades, or as in our study, through inhibition of the D₂ autoreceptor.

Extracellular dopamine levels, and thus dopaminergic signaling, are tightly controlled by both DAT and the D₂ autoreceptor. Our lab and others demonstrated that PKC regulates both of these proteins through phosphorylation. PKC phosphorylates the D₂R receptor to change surface localization and receptor activity. Several PKC phosphorylation sites have been identified on the receptor using mutagenesis studies, particularly on the third intracellular loop. Phosphorylation at these sites causes internalization and desensitization of the receptor (Namkung & Sibley, 2004; Morris et al., 2007; Thibault et al., 2011). Overexpression of PKC β in HEK cells suggests that this isoform specifically phosphorylates D₂R (Namkung & Sibley, 2004). In addition to inhibiting dopamine exocytosis, the D₂

autoreceptor can increase the surface localization and uptake of dopamine through DAT to control extracellular dopamine levels (Bolan et al., 2007).

This coordination between DAT and the D₂ autoreceptor allows for more precise control of dopaminergic signaling and may occur through a separate population of D₂ autoreceptors that is associated with DAT (Wu et al., 2002). Using PKC β ^{-/-} mice and PKC β -specific inhibitors we found that PKC β signaling is required for this D₂ autoreceptor-DAT coordination (Chen et al., 2013). Additionally, the D₂ autoreceptor and DAT are reported to physically interact between the third intracellular loop of D₂R and the N-terminus of DAT (Lee et al., 2007). Both the D₂R third intracellular loop (Morris et al., 2007; Namkung and Sibley, 2004) and the DAT N-terminus (Foster et al., 2002) contain PKC phosphorylation sites, suggesting that PKC phosphorylation may be involved in this D₂ autoreceptor-DAT interaction and thus the regulation of extracellular dopamine. Studies are currently underway to further understand how PKC regulates this D₂ autoreceptor-DAT interaction.

In the present study, we used the potassium channel blocker 4-aminopyridine (4AP) to stimulate dopamine exocytosis in our suprafusion assay. Some studies have reported that 4AP or other potassium channel blockers interfere with autoreceptor regulation of neurotransmitter release (norepinephrine: Hu & Fredholm, 1989; acetylcholine: Drukarch et al., 1989; dopamine: Cass & Zahniser, 1991 and Fulton et al., 2011). Unlike our assay, these studies did not use 4AP stimulation alone, but combined potassium channel blockade with electrical stimulation. This combined stimulation appears to cross an upper threshold of neurotransmitter release above which autoreceptors are no longer able to regulate neurotransmitter release. This threshold is crossed following prolonged depolarization, such as that seen using high concentrations of KCl which is not subject to autoreceptor regulation (Tibbs et al., 1989; L'Hirondel et al., 1998). Additionally, the similarity of our findings using 4AP as a stimulus to those with electrical stimulation in the fast-scan cyclic voltammetry experiments reinforces that our findings are physiologically relevant.

Our results demonstrate that PKC β regulates the D₂ autoreceptor *in vivo*. Further work is needed to understand the conditions under which PKC β is activated, leading to the modulation of the D₂ autoreceptor. It does not appear that agonist-induced phosphorylation of D₂R occurs through PKC (Namkung & Sibley, 2004). PKC is canonically activated by diacylglycerol and IP₃ generated by phospholipase through the G_q or G_i-protein signaling cascades. Activation of G_q-coupled GPCRs such as the neurotensin receptor causes internalization and desensitization of D₂R through a PKC-dependent mechanism in cultured cells (Thibault et al., 2011). However, the specific mechanisms for the *in vivo* activation of PKC β leading to the phosphorylation and subsequent regulation of the D₂ autoreceptor remain unknown.

This study focused on the interaction of PKC β with presynaptic D₂ autoreceptors; however, the majority of D₂Rs are located postsynaptically. In mice lacking the D₂ autoreceptor, quinpirole treatment no longer suppresses locomotor activity, indicating that the locomotor suppression is controlled by the presynaptic D₂R population (Bello et al., 2011). Our results showing that PKC β ^{-/-} mice have increased suppression of locomotor activity following quinpirole treatment strongly suggest that we are measuring regulation of the D₂

autoreceptor population by PKC β . Otherwise, we are unable to definitively differentiate experimentally between postsynaptic and presynaptic receptor populations. The D₂R is also expressed as two splice variants, short and long, with the long D₂R having an additional 29 amino acids in the third intracellular loop (Uziel et al., 2000). Conflicting reports indicate that PKC may or may not differentially regulate the long variant of D₂R compared to the short (Namkung & Sibley, 2004; Morris et al., 2007). Future work is needed to understand if PKC β regulation is the same for both populations of D₂R and if this regulation is the same for other D₂-like family members, such as the closely related D₃R receptor.

5. Conclusion

In conclusion, we have found that PKC β regulates the D₂ autoreceptor *in vivo*, adding an additional layer of regulation to the control of dopamine signaling. We demonstrated that loss or inhibition of PKC β activity increases the sensitivity of the D₂ autoreceptor to the agonist quinpirole, as measured by increased suppression of dopamine release. Mechanistically, PKC β inhibition increased the surface localization of the D₂R, which, for D₂ autoreceptors, would increase receptor signaling. Finally, we demonstrated that this PKC β regulation of the D₂ autoreceptor is physiologically relevant, as loss of PKC β activity increased the sensitivity of mice to quinpirole suppression of locomotor activity. These findings increase our understanding of how the D₂ autoreceptor is regulated and may aid in the development of therapeutics targeting disorders and disease states associated with dopamine signaling.

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Abbreviations

DAT	dopamine transporter
D₂R	D ₂ -like dopamine receptor
D₂ autoreceptor	D ₂ -like dopamine autoreceptor
SNARE	Soluble NSF Attachment Protein Receptor
PKC	protein kinase C
KRB	Krebs-Ringer buffer
4AP	4-aminopyridine
QP	quinpirole
HPLC	high-performance liquid chromatography

References

- Barrett CF, Rittenhouse AR. Modulation of N-type calcium channel activity by G-proteins and protein kinase C. *J Gen Physiol.* 2000; 115:277–286. [PubMed: 10694257]
- Barrie AP, Nicholls DG, Sanchez-Prieto J, Sihra TS. An ion channel locus for the protein kinase C potentiation of transmitter glutamate release from guinea pig cerebrocortical synaptosomes. *J Neurochem.* 1991; 57:1398–1404. [PubMed: 1716664]
- Bello EP, Mateo Y, Gelman DM, Noain D, Shin JH, Low MJ, Alvarez VA, Lovinger DM, Rubinstein M. Cocaine supersensitivity and enhanced motivation for reward in mice lacking dopamine D2 autoreceptors. *Nat Neurosci.* 2011; 14:1033–1038. [PubMed: 21743470]
- Bolan EA, Kivell B, Jaligam V, Oz M, Jayanthi LD, Han Y, Sen N, Urizar E, Gomes I, Devi LA, Ramamoorthy S, Javitch JA, Zapata A, Shippenberg TS. D2 receptors regulate dopamine transporter function via an extracellular signal-regulated kinases 1 and 2-dependent and phosphoinositide 3 kinase-independent mechanism. *Mol Pharmacol.* 2007; 71:1222–1232. [PubMed: 17267664]
- Cass WA, Zahniser NR. Potassium channel blockers inhibit D2 dopamine, but not A1 adenosine, receptor-mediated inhibition of striatal dopamine release. *J Neurochem.* 1991; 57:147–152. [PubMed: 1828829]
- Chen R, Daining CP, Sun H, Fraser R, Stokes SL, Leitges M, Gnegy ME. Protein kinase C β is a modulator of the dopamine D2 autoreceptor-activated trafficking of the dopamine transporter. *J Neurochem.* 2013; 125:663–672. [PubMed: 23458603]
- Chen R, Furman CA, Zhang M, Kim MN, Gereau R. W. t. Leitges M, Gnegy ME. Protein kinase C β is a critical regulator of dopamine transporter trafficking and regulates the behavioral response to amphetamine in mice. *J Pharmacol Exp Ther.* 2009; 328:912–920. [PubMed: 19098163]
- Chen R, Zhang M, Park S, Gnegy ME. C57BL/6J mice show greater amphetamine-induced locomotor activation and dopamine efflux in the striatum than 129S2/SvHsd mice. *Pharmacol Biochem Behav.* 2007; 87:158–163. [PubMed: 17524461]
- Colby KA, Blaustein MP. Inhibition of voltage-gated K channels in synaptosomes by sn-1,2-dioctanoylglycerol, an activator of protein kinase C. *J Neurosci.* 1988; 8:4685–4692. [PubMed: 3199201]
- Cubeddu LX, Lovenberg TW, Hoffman IS, Talmaciu RK. Phorbol esters and D2-dopamine receptors. *J Pharmacol Exp Ther.* 1989; 251:687–693. [PubMed: 2530340]
- de Vries KJ, Geijtenbeek A, Brian EC, de Graan PN, Ghijsen WE, Verhage M. Dynamics of munc18-1 phosphorylation/dephosphorylation in rat brain nerve terminals. *Eur J Neurosci.* 2000; 12:385–390. [PubMed: 10651895]
- Drukarch B, Kits KS, Leysen JE, Schepens E, Stoof JC. Restricted usefulness of tetraethylammonium and 4-aminopyridine for the characterization of receptor-operated K⁺-channels. *Br J Pharmacol.* 1989; 98:113–118. [PubMed: 2553183]
- Foster JD, Pananusorn B, Vaughan RA. Dopamine transporters are phosphorylated on N-terminal serines in rat striatum. *J Biol Chem.* 2002; 277:25178–25186. [PubMed: 11994276]
- Fulton S, Thibault D, Mendez JA, Lahaie N, Tirota E, Borrelli E, Bouvier M, Tempel BL, Trudeau LE. Contribution of Kv1.2 voltage-gated potassium channel to D2 autoreceptor regulation of axonal dopamine overflow. *J Biol Chem.* 2011; 286:9360–9372. [PubMed: 21233214]
- Furman CA, Chen R, Guptaroy B, Zhang M, Holz RW, Gnegy M. Dopamine and amphetamine rapidly increase dopamine transporter trafficking to the surface: live-cell imaging using total internal reflection fluorescence microscopy. *J Neurosci.* 2009; 29:3328–3336. [PubMed: 19279270]
- Giros B, Jaber M, Jones SR, Wightman RM, Caron MG. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature.* 1996; 379:606–612. [PubMed: 8628395]
- Graff JR, McNulty AM, Hanna KR, Konicek BW, Lynch RL, Bailey SN, Banks C, Capen A, Goode R, Lewis JE, Sams L, Huss KL, Campbell RM, Iversen PW, Neubauer BL, Brown TJ, Musib L, Geeganage S, Thornton D. The protein kinase C β -selective inhibitor, Enzastaurin (LY317615.HCl), suppresses signaling through the AKT pathway, induces apoptosis, and

- suppresses growth of human colon cancer and glioblastoma xenografts. *Cancer Res.* 2005; 65:7462–7469. [PubMed: 16103100]
- Hu PS, Fredholm BB. Alpha 2-adrenoceptor agonist-mediated inhibition of [3H]noradrenaline release from rat hippocampus is reduced by 4-aminopyridine, but that caused by an adenosine analogue or omega-conotoxin is not. *Acta Physiol Scand.* 1989; 136:347–353. [PubMed: 2568729]
- Huang HY, Hertting G, Allgaier C, Jackisch R. 3,4-Diaminopyridine-induced noradrenaline release from CNS tissue as a model for action potential-evoked transmitter release: effects of phorbol ester. *Eur J Pharmacol.* 1989; 169:115–123. [PubMed: 2574686]
- Iannazzo L, Sathananthan S, Majewski H. Modulation of dopamine release from rat striatum by protein kinase C: interaction with presynaptic D2-dopamine-autoreceptors. *Br J Pharmacol.* 1997; 122:1561–1566. [PubMed: 9422799]
- Jirousek MR, Gillig JR, Gonzalez CM, Heath WF, McDonald JH 3rd, Neel DA, Rito CJ, Singh U, Stramm LE, Melikian-Badalian A, Baevsky M, Ballas LM, Hall SE, Winneroski LL, Faul MM. (S)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16, 21-dimetheno-1H, 13H-dibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecene-1,3(2H)-dione (LY333531) and related analogues: isozyme selective inhibitors of protein kinase C beta. *J Med Chem.* 1996; 39:2664–2671. [PubMed: 8709095]
- Johnson LA, Guptaroy B, Lund D, Shamban S, Gnegy ME. Regulation of amphetamine-stimulated dopamine efflux by protein kinase C beta. *J Biol Chem.* 2005; 280:10914–10919. [PubMed: 15647254]
- L'Hirondel M, Cheramy A, Godeheu G, Artaud F, Saiardi A, Borrelli E, Glowinski J. Lack of autoreceptor-mediated inhibitory control of dopamine release in striatal synaptosomes of D2 receptor-deficient mice. *Brain Res.* 1998; 792:253–262. [PubMed: 9593923]
- Lee FJ, Pei L, Moszczynska A, Vukusic B, Fletcher PJ, Liu F. Dopamine transporter cell surface localization facilitated by a direct interaction with the dopamine D2 receptor. *Embo j.* 2007; 26:2127–2136. [PubMed: 17380124]
- Leenders AG, Sheng ZH. Modulation of neurotransmitter release by the second messenger-activated protein kinases: implications for presynaptic plasticity. *Pharmacol Ther.* 2005; 105:69–84. [PubMed: 15626456]
- Leitges M, Schmedt C, Guinamard R, Davoust J, Schaal S, Stabel S, Tarakhovsky A. Immunodeficiency in protein kinase cbeta-deficient mice. *Science.* 1996; 273:788–791. [PubMed: 8670417]
- Majewski H, Iannazzo L. Protein kinase C: a physiological mediator of enhanced transmitter output. *Prog Neurobiol.* 1998; 55:463–475. [PubMed: 9670214]
- Mateo Y, Lack CM, Morgan D, Roberts DC, Jones SR. Reduced dopamine terminal function and insensitivity to cocaine following cocaine binge self-administration and deprivation. *Neuropsychopharmacology.* 2005; 30:1455–1463. [PubMed: 15702135]
- Morris SJ, Van H II, Daigle M, Robillard L, Sajedi N, Albert PR. Differential desensitization of dopamine D2 receptor isoforms by protein kinase C: the importance of receptor phosphorylation and pseudosubstrate sites. *Eur J Pharmacol.* 2007; 577:44–53. [PubMed: 17869243]
- Namkung Y, Sibley DR. Protein kinase C mediates phosphorylation, desensitization, and trafficking of the D2 dopamine receptor. *J Biol Chem.* 2004; 279:49533–49541. [PubMed: 15347675]
- Nimitvilai S, McElvain MA, Brodie MS. Reversal of dopamine D2 agonist-induced inhibition of ventral tegmental area neurons by Gq-linked neurotransmitters is dependent on protein kinase C, G protein-coupled receptor kinase, and dynamin. *J Pharmacol Exp Ther.* 2013; 344:253–263. [PubMed: 23019137]
- O'Malley HA, Park Y, Isom LL, Gnegy ME. PKCbeta co-localizes with the dopamine transporter in mesencephalic neurons. *Neurosci Lett.* 2010
- Rubinstein M, Muschietti JP, Gershanik O, Flawia MM, Stefano FJ. Adaptive mechanisms of striatal D1 and D2 dopamine receptors in response to a prolonged reserpine treatment in mice. *J Pharmacol Exp Ther.* 1990; 252:810–816. [PubMed: 2138223]
- Shirafuji T, Ueyama T, Yoshino K, Takahashi H, Adachi N, Ago Y, Koda K, Nashida T, Hiramatsu N, Matsuda T, Toda T, Sakai N, Saito N. The Role of Pak-Interacting Exchange Factor-beta

- Phosphorylation at Serines 340 and 583 by PKC γ in Dopamine Release. 2014; 34:9268–9280.
- Tanaka C, Nishizuka Y. The protein kinase C family for neuronal signaling. *Annu Rev Neurosci*. 1994; 17:551–567. [PubMed: 8210187]
- Thibault D, Albert PR, Pineyro G, Trudeau LE. Neurotensin triggers dopamine D2 receptor desensitization through a protein kinase C and beta-arrestin1-dependent mechanism. *J Biol Chem*. 2011; 286:9174–9184. [PubMed: 21233215]
- Tibbs GR, Barrie AP, Van Mieghem FJ, McMahon HT, Nicholls DG. Repetitive action potentials in isolated nerve terminals in the presence of 4-aminopyridine: effects on cytosolic free Ca²⁺ and glutamate release. *J Neurochem*. 1989; 53:1693–1699. [PubMed: 2553862]
- Usiello A, Baik JH, Rouge-Pont F, Picetti R, Dierich A, LeMeur M, Piazza PV, Borrelli E. Distinct functions of the two isoforms of dopamine D2 receptors. *Nature*. 2000; 408:199–203. [PubMed: 11089973]
- Wang Y, Xu R, Sasaoka T, Tonegawa S, Kung MP, Sankoorikal EB. Dopamine D2 long receptor-deficient mice display alterations in striatum-dependent functions. *J Neurosci*. 2000; 20:8305–8314. [PubMed: 11069937]
- Wu-Zhang AX, Newton AC. Protein kinase C pharmacology: refining the toolbox. *Biochem J*. 2013; 452:195–209. [PubMed: 23662807]
- Wu Q, Reith ME, Walker QD, Kuhn CM, Carroll FI, Garriss PA. Concurrent autoreceptor-mediated control of dopamine release and uptake during neurotransmission: an in vivo voltammetric study. *J Neurosci*. 2002; 22:6272–6281. [PubMed: 12122086]
- Yorgason JT, Espana RA, Jones SR. Demon voltammetry and analysis software: analysis of cocaine-induced alterations in dopamine signaling using multiple kinetic measures. *J Neurosci Methods*. 2011; 202:158–164. [PubMed: 21392532]

Highlights

- We studied the regulation of the D₂-like dopamine autoreceptor by protein kinase C β
- Lack of PKC β activity increased quinpirole-stimulated decrease in dopamine release
- PKC β inhibition increased surface D₂-like receptor localization
- PKC $\beta^{-/-}$ mice display increased quinpirole-induced locomotor suppression
- PKC β regulates dopaminergic signaling through the D₂ autoreceptor

Figure 1.

Stimulation of the D₂ autoreceptor inhibits dopamine exocytosis. Striatal synaptosomes from PKC $\beta^{+/+}$ mice were perfused with KRB and one minute fractions were collected for 14 minutes. Dopamine release was stimulated with 50 μ M 4AP at fractions seven and eight + 3 μ M quinpirole (QP) + 10 μ M sulpiride. The amount of dopamine in each fraction collected was determined using HPLC-EC and normalized to protein concentration. N = 3, *** p < 0.0001 vs. 4AP control via one-way ANOVA with *post-hoc* Bonferroni analysis.

Figure 2.

Quinpirole (QP)-induced suppression of 4AP-stimulated dopamine release is enhanced in PKC $\beta^{-/-}$ mice. Striatal synaptosomes from PKC $\beta^{+/+}$ (A) and PKC $\beta^{-/-}$ mice (B) were perfused with KRB and one minute fractions were collected for 14 minutes. Dopamine release was stimulated with 50 μ M 4AP at fractions seven and eight $\pm$ 100 nM QP, as indicated by horizontal bar. The amount of dopamine in each fraction collected was determined using HPLC-EC and normalized to protein concentration. N = 4, * p < 0.05, ** p < 0.01, # p < 0.0001 vs. each 4AP control via three-way ANOVA with *post-hoc* Bonferroni analysis.

Figure 3.

Acute PKC β inhibition increases dopamine release suppression in response to quinpirole (QP). Striatal synaptosomes from wild type mice were perfused with vehicle control or 100 nM LY379196 for 60 minutes; one minute fractions were collected for 14 minutes. Dopamine release was stimulated using 50 μ M 4AP $\pm$ 30 nM QP at fractions seven and eight. A lower concentration of QP was used here to better detect potential increases in sensitivity due to PKC β inhibition. Dopamine content was determined via HPLC-EC and was normalized to protein concentration and is shown here as peak stimulated dopamine release subtracted from baseline release, which did not differ among groups. N = 4, * $p < 0.05$ via one-way ANOVA with *post-hoc* Bonferroni analysis. Peak dopamine release from PKC $\beta^{-/-}$ mouse synaptosomes treated with 4AP + 100 nM QP is included for comparison.

Figure 4.

Acute inhibition of PKC β increases the effectiveness D₂R agonist quinpirole (QP). (A) Representative traces of electrically stimulated dopamine release in nucleus accumbens core in brain slices was inhibited by QP in a dose-responsive manner. Pretreatment (60 min) of slices with 200 nM enzastaurin significantly decreased the QP dose-response curve (B), normalized to % pre-QP baseline values to the left, indicating supersensitivity of the D₂ autoreceptor (two-way ANOVA $F(1, 82) = 16.00, p = 0.0001$). $N = 8-10$, * $p < 0.05$ via two-way ANOVA with *post-hoc* Bonferroni analysis.

Figure 5.

Inhibition of PKC β increases surface localization of D₂R. Striatal synaptosomes from wild type mice were prepared. Half of the synaptosomes were lysed and membranes were isolated. The tissue preparations were incubated with vehicle or 200 nM enzastaurin for 5 minutes at 37° C. Following treatment, intact synaptosomes were incubated with [³H]-sulpiride on ice for 3.5 hours to measure surface D₂ autoreceptor binding. Lysed membranes were incubated with [³H]-sulpiride for 1.5 hours at room temperature to measure total D₂R binding. Binding was terminated by filtering samples and radioligand binding was determined via scintillation counting. N = 3, with 3-5 replicates per N, ** p < 0.01 via paired t-test.

Figure 6.

Quinpirole (QP) suppression of locomotor activity is increased in PKC $\beta^{-/-}$ mice. PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice were implanted with Mini-mitter tracking devices and placed in a novel environment. Mice were injected IP with saline or increasing doses of QP (0.032 – 1 mg/kg). Locomotor activity was measured for 15 minutes following injection and total activity normalized to saline control is shown. N = 5-15, * p < 0.05, ** p < 0.01 via two-way ANOVA with *post-hoc* Bonferroni analysis.