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Protein kinase C β is a modulator of the dopamine D2 autoreceptor-activated trafficking of the dopamine transporter

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Abstract

The strength and duration of extracellular dopamine concentrations are regulated by the presynaptic dopamine transporter (DAT) and dopamine D2 autoreceptors (D2autoRs). There is a functional interaction between these two proteins. Activation of D2autoRs increases DAT trafficking to the surface whereas disruption of this interaction compromises activities of both proteins and alters dopaminergic transmission. Previously we reported that DAT expression and activity are subject to modulation by protein kinase C β (PKC β). Here, we further demonstrate that PKC β is integral for the interaction between DAT and D2autoR. Inhibition or absence of PKC β abolished the communication between DAT and D2autoR. In mouse striatal synaptosomes and transfected N2A cells, the D2autoR-stimulated membrane insertion of DAT was abolished by PKC β inhibition. Moreover, D2autoR-stimulated DAT trafficking is mediated by a PKC β -ERK (extracellular signal-regulated kinase) signaling cascade where PKC β is upstream of ERK. The increased surface DAT expression upon D2autoR activation resulted from enhanced DAT recycling as opposed to reduced internalization. Further, PKC β promoted accelerated DAT recycling. Our study demonstrates that PKC β critically regulates D2autoR-activated DAT trafficking and dopaminergic signaling. PKC β is a potential drug target for correcting abnormal extracellular dopamine levels in diseases such as drug addiction and schizophrenia.

Keywords

dopamine transporter; dopamine D2 autoreceptor; Protein kinase C β ; trafficking; extracellular signal regulated protein kinase

Introduction

Extracellular dopamine (DA) levels are regulated by presynaptic DA transporter (DAT) and DA D2 autoreceptor (D2autoR). Reuptake of DA through DAT into neurons is the primary

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The authors declare no conflict of interests.

Authorship credit

RC and MEG designed the experiments; RC, CPD, RF and SLS performed the experiments and collected the data; RC and MEG analyzed the data and wrote the manuscript; ML donated the PKC β knockout mice on SV129 background and critically reviewed the manuscript.

mechanism for removing extracellular DA and terminating DA action, whereas D2autoR provides an inhibitory feedback on DA exocytosis, DA synthesis, and dopaminergic neuron firing (Jones *et al.* 1999, L'Hirondel *et al.* 1998, Cubeddu & Hoffmann 1982, Pothos *et al.* 1998, Haubrich & Pflueger 1982). There is an anatomical and a functional interaction between DAT and D2autoR. DAT and D2autoR co-localize in striatal presynaptic terminals (Hersch *et al.* 1997), and demonstrate a physical coupling in heterologous cells (Lee *et al.* 2007). D2autoR agonists increase surface DAT expression and activity (Eriksen *et al.* 2010, Bolan *et al.* 2007). Coexpression of D2autoR facilitates the expression of surface DAT (Lee *et al.* 2007), whereas disruption of the DAT and D2autoR interaction compromises the activity of both proteins (Dickinson *et al.* 1999, L'Hirondel *et al.* 1998, Lee *et al.* 2007, Jones *et al.* 1999). More significantly, the interaction between DAT and D2autoR has clinical relevance. A reduced coupling of striatal DAT and D2autoR has been reported in postmortem brains of schizophrenics (Lee *et al.* 2009). Moreover, a DAT polymorphism in humans with attention deficit/hyperactive disorder (ADHD) disrupts the normal interaction between DAT and D2autoR, leading to an anomalous DA efflux (Bowton *et al.* 2010). These data suggest an important physiological role of DAT and D2autoR interaction in regulation of dopaminergic transmission.

DAT undergoes trafficking upon exposure to DAT substrates or DA D2 receptor agonists. Although DAT substrate-elicited DAT trafficking and its regulation by second-messenger systems have been studied extensively [see review (Chen *et al.* 2010)], knowledge of the molecular mechanisms underlying D2autoR-stimulated DAT trafficking is limited. A potential candidate to govern the interaction between DAT and D2autoR is protein kinase C (PKC), a common modulator for DAT and D2R trafficking and function (Giambalvo & Wagner 1994, Giambalvo 2004, Namkung & Sibley 2004). Recently, we reported that a specific PKC isoform, PKC β , promotes rapid DAT trafficking to the surface in N2A neuroblastoma cells upon short-term exposure to DAT substrate amphetamine (Chen *et al.* 2009, Furman *et al.* 2009a) whereas long-term PKC activation leads to DAT internalization (Chen *et al.* 2010, Schmitt & Reith 2010). Further, there are reports that PKC can regulate D2autoR function. PKC activation reduces the inhibitory effect of D2autoR on electrical stimulation-evoked DA exocytosis (Cubeddu *et al.* 1989) and reduces D2autoR-stimulated immobilization of calcium (Liu *et al.* 1992, Morris *et al.* 2007). D2autoR-stimulated DAT trafficking in heterologous cells is dependent on activation of ERK (extracellular signal-regulated kinase), a downstream signaling molecule (Bolan *et al.* 2007, Lee *et al.* 2007). Here we make a new observation that, in addition to ERK, PKC β is required for D2autoR-elicited trafficking of intracellular DAT to the membrane surface. Moreover, PKC β is upstream of ERK. This study reveals an important contribution of PKC β in maintaining homeostasis of the presynaptic dopaminergic transmission.

Materials and Methods

Dopamine receptor nomenclature

Both DA D2 receptor (D2R) and D3 receptor (D3R) are expressed in the dopaminergic neurons (Diaz *et al.* 2000, Sesack *et al.* 1994), thus we use D2autoR as a general term for presynaptic D2-like autoreceptors for experiments conducted in animal tissues. Although DA D3 receptors exist in dopamine neurons, evidence is strong that the dopamine autoreceptor in mice is the D2 subtype (Mercuri *et al.* 1997). D2R has two splice variants: short and long (D2S and D2L, respectively). Evidence suggests that the short variant D2S functions as a presynaptic autoreceptor (Wang *et al.* 2000, Usiello *et al.* 2000). The short splice variant D2S was transfected into N2A cells. We determined by quantitative PCR that N2A cells do not express D2R or D3R receptors.

Cell culture and transfection

N2A neuroblastoma cell lines were grown in Opti-MEM I media (Life Technology) supplemented with 10% fetal growth serum and 1% penicillin/streptomycin. Cells were transiently transfected with human D2S with FLAG tag (FLAG-hD2S, a gift from Dr. David Sibley) and human DAT with HA tag (HA-hDAT, a gift from Dr. Jonathan Javitch) using the calcium phosphate method. The FLAG tag was fused at the extracellular N terminus of D2S. The HA tag was inserted in the second extracellular loop of DAT as described previously (Furman *et al.* 2009b).

Confocal microscopy of DAT trafficking in N2A cells

Cells were seeded on coverslips 12 hrs after transfection. Coverslips were coated with 0.5 mg/ml poly-D-lysine. Forty-eight hours after the transfection, cells were incubated with vehicle (Veh), a specific PKC β inhibitor LY379196 (LY, 100 nM, 30 min) or the ERK inhibitor PD98059 (PD, 10 μ M, 15 min) at 37°C before treatment with the D2R/D3R agonist quinpirole (Quin, 1 μ M) or Veh for 30 min. The reaction was stopped by washing cells with cold PBS, and blocked with 4% normal goat serum in PBS for 1 hr on ice. To label surface DAT protein, cells were incubated with a mouse anti-HA antibody (Covance, Gaithersburg, MD) followed by secondary goat anti-mouse Alexa Fluor 594 at 4°C. Then cells were fixed with 4% paraformaldehyde in PBS for 15 min on ice and permeabilized by 0.1% Triton X-100 in PBS for 10 min at room temperature for the subsequent cytosolic DAT labeling. Cells were incubated with a rabbit anti-HA antibody (Covance) followed by secondary goat anti-rabbit Alexa Fluor 647. Cells were mounted onto glass slides with ProLong Gold anti-fade reagent (Life Technology) for imaging.

Images of cells displaying fluorescent signals were acquired on an Olympus FluoView 500 confocal microscope with a 60x by 1.35N.A. oil objective. Images were obtained by taking a series of images every 0.2 μ m through the cell and combing the images into a composite stack. Sequential scans were taken to prevent the overlap of laser signal. For the laser configuration, Alexa Fluor 594 was excited by a 543nm laser and passed through a BA560-600nm bandpass filter whereas Alexa Fluor 647 was excited by a 633nm laser and passed through a BA660IF bandpass filter. Z-slices of cells were quantified using Image J software (NIH). The background signal from neighboring untransfected cells was subtracted from the total fluorescence for all quantified signals to determine the specific intensity of fluorescence. The surface DAT content was calculated as the ratio of the specific intensity of surface labeling to that of the intracellular labeling of DAT.

D2S-activated DAT recycling and internalization in N2A cells

N2A cells transfected with HA-DAT and FLAG-D2S were treated with vehicle (V) or quinpirole (Q, 100 nM) in the presence or absence of LY379196 (LY, 100 nM). DAT recycling and internalization were determined according to the published protocol (Loder & Melikian 2003, Boudanova *et al.* 2008b). To determine whether quinpirole or LY379196 had an effect on DAT recycling, cells were biotinylated (1 mg/kg) at 4°C for 60 min. Then cells were warmed up to room temperature and treated with V+V, V+Q, LY+V and LY+Q in the presence of sulfo-NHS-SS-biotin (1.5 mg/ml) for 30 min. Biotinylation was rapidly quenched with 100 mM glycine for 15 min at 4°C. Cells were lysed in RIPA buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton X-100) containing protease inhibitors. Biotinylated and non-biotinylated proteins from equal amounts of cell lysates were separated by streptavidin pull-down. Samples were analyzed by SDS-PAGE and immunoblotted by DAT antibody (MAB369). The biotinylated DAT was normalized by the DAT from the non-biotinylated fraction. The data were calculated as the percent of the vehicle treatment.

To determine whether DAT internalization was affected by quinpirole or LY379196 treatment, cells were biotinylated with 2 mg/ml sulfo-NHS-SS-biotin. Following biotinylation, one set of cells were washed with PBS/Ca/Mg and kept on ice to determine the total initial surface DAT and the stripping efficiency. To initiate endocytosis, the other set of cells were warmed up to room temperature and treated with V+V, V+Q, LY+V and LY+Q for 30 min. The endocytic reaction was stopped by replacing the reaction solution with the cold NT buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.6). The remaining surface biotin was stripped twice by the reducing agent Tris(2-carboxyethyl)phosphine (TCEP) in NT buffer for 20 min at 4°C. The strip efficiency was determined in each experiment and was more than 95%. After stripping, cells were washed with cold PBS and lysed in RIPA buffer. The internalized proteins were pulled down by streptavidin beads. The biotinylated DAT was normalized by the DAT from the non-biotinylated fraction. The data were calculated as the percent of the vehicle treatment.

Animals

Generation of PKC β wild type (PKC $\beta^{+/+}$) and knockout mice (PKC $\beta^{-/-}$) was described previously (Leitges *et al.* 1996). PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mouse breeders had been backcrossed with C57BL/6J mice ten times. Experimental PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice were age- (2–3 months old) and gender-matched. Mice had free access to the standard Purinarodent chow and water and were maintained in a temperature- and humidity-controlled environment on a 12-h dark/light cycle with lights on at 7:00 AM. Animal use and procedures were in accordance with the National Institutes of Health guidelines, and approved by the Institutional Animal Care and Use Committee at University of Michigan.

Quinpirole-stimulated DAT trafficking in mouse striatal synaptosomes

Preparation of mouse striatal synaptosomes and the procedure for surface striatal DAT biotinylation were previously described (Chen et al. 2009). The striatal tissues were dissected by a means of brain matrix, and included both the ventral and the dorsal striatum. To study the time course of quinpirole-induced DAT trafficking, mouse synaptosomes were treated for 1, 5, 15, 30 or 60 min at 37°C with quinpirole (10 μ M) or vehicle in Krebs-Ringer's buffer (KRB) containing 24.9 mM NaHCO₃, pH 7.4, 1.2 mM KH₂PO₄, 146.2 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 10 mM glucose, 0.05 mM ascorbic acid, 50 μ M pargyline. To study the effect of PKC β inhibition on quinpirole-induced DAT trafficking, synaptosomes were pretreated with a specific PKC β inhibitor LY379196 (100 nM) or KRB for 1 hour before the quinpirole treatment (15 min). Then samples were biotinylated by membrane non-permeable sulfo-NHS-SS-biotin. Surface DAT expression was calculated as the ratio of the biotinylated DAT to the total DAT. Data were expressed as the relative surface DAT content upon quinpirole treatment vs. vehicle treatment in the same time frame of drug exposure for comparison of genotype difference in DAT trafficking.

Quinpirole-stimulated DA uptake in mouse striatal synaptosomes

Striatal synaptosomes were pre-incubated with KRB or 10 μ M quinpirole for 10 min at 37°C. DA uptake was measured for 1 min at 37°C in KRB containing 10 nM [³H]DA (specific activity 23.5 Ci/mmol; Perkin Elmer) and 500 nM unlabeled DA. Non-specific [³H]DA uptake was determined in the presence of 30 μ M cocaine. The DA uptake assay was conducted as described (Chen et al. 2009). Data were expressed as percent of the vehicle treatment, and analyzed by paired-Student's t-test for each genotype.

To ensure that potential compensatory changes in PKC $\beta^{-/-}$ mice were not confounding the results, the effect of the PKC β inhibitor LY379196 on [³H]DA uptake was determined. Synaptosomes were incubated with LY379196 (100 nM) for 1 hr, PD98059 (10 μ M) for 15 min or KRB at 37°C before treatment with quinpirole (10 μ M) or vehicle for 5 min prior to

measurement of [³H]DA uptake. DA uptake was performed for 5 min at 37°C in the presence of 500 nM unlabeled DA and 10 nM [³H]DA.

Percoll-purification of striatal synaptosomes

To dissect the presynaptic D2autoR signaling from the postsynaptic D2R/D3R signaling upon agonist quinpirole stimulation, striatal synaptosomes were purified on a percoll gradient as described (Dunkley *et al.* 1988) to eliminate the majority of the postsynaptic components. Briefly, crude striatal synaptosomes pooled from striata of 4 wildtype mice were suspended in KRB and loaded on a percoll gradient consisting of 23%, 15%, 10% and 3% percoll made in 0.32M sucrose containing 1 mM EDTA. The gradient was centrifuged, and layers 3 and 4 were combined, washed and resuspended in KRB as purified striatal synaptosomes for determination of PKC β and ERK phosphorylation.

Phosphorylation of PKC β and ERK in percoll-purified mouse synaptosomes

Phosphorylation of PKC β I and PKC β II were used as a measurement of PKC β activation. Percoll-purified striatal synaptosomes from PKC $\beta^{+/+}$ mice were resuspended in KRB and pretreated with or without the ERK inhibitor PD98059 (10 μ M) for 15 min at 37°C before addition of quinpirole (10 μ M) or KRB for 5 min. The reaction was stopped with cold KRB, and samples were immediately centrifuged. The pellets were lysed in RIPA buffer containing phosphatase and protease cocktail inhibitors (PhosStop and Complete Mini, respectively, Roche, Indianapolis, IN). PKC β activation was determined by detecting phosphorylated PKC β I and PKC β II (pPKC β I and pPKC β II) at threonine 642 and threonine 641, respectively, using phosphospecific antibodies. The total content of PKC β I and PKC β II (tPKC β I and tPKC β II, respectively) was determined using rabbit anti-PKC β I or -PKC β II antibody. PKC β I or PKC β II activity was expressed as a ratio of pPKC β I to tPKC β I or pPKC β II to tPKC β II.

To determine ERK activity, percoll-purified striatal synaptosomes from both PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice were pretreated with or without 100 nM LY379196 for 1 hour at 37°C before treatment with KRB, phorbol 12-myristate 13-acetate (PMA, 1 μ M), or quinpirole (10 μ M) for 5 min. The levels of phospho-ERK (pERK) and total ERK (tERK) were detected using rabbit anti-pERK and mouse anti-ERK antibodies, respectively. ERK activity was expressed as a ratio of pERK to tERK.

Drugs and reagents

Amphetamine, dopamine and quinpirole were purchased from Sigma Aldrich (St. Louis, MO). PD98059 and phorbol 12-myristate 13-acetate were from Calbiochem (San Diego, CA). Sulfo-NHS-SS-biotin, streptavidin and Tris(2-carboxyethyl)phosphine (TCEP) were from Proteochem Inc. (Denver, CO). LY379196 was a generous gift from Eli Lilly Company (Indianapolis, IN). The DAT antibody MAB369 was from Millipore (Billerica, CA), antibodies for ERK and phosphorylated ERK were from Cell Signaling Technology (Danvers, MA), antibodies for PKC β and phosphorylated PKC β were from Santa Cruz Biotechnology (Santa Cruz, CA). All the other chemicals were from Sigma Aldrich (Saint Louis, MO) unless otherwise indicated. Goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 647 were from Life Technology (Grant Island, NY)

Statistical analyses

Results are presented as means \pm standard error of the means (SEM). Comparisons between two groups were made by paired two-tailed Student's t-test. Comparisons among multiple groups were made by one- or two-way ANOVA with *post hoc* Bonferroni test using Systat (Chicago, IL). Statistical significance was set at $P < 0.05$.

Results

Inhibition of PKC β and ERK disrupts D2S-activated DAT trafficking in N2A cells

We initially examined whether D2S-activated DAT trafficking is PKC β and ERK dependent in HA-DAT-FLAG-D2S-N2A cells using a highly specific PKC β inhibitor LY379196 (Jirousek *et al.* 1996). Bolan *et al.* (2007) demonstrated that quinpirole enhanced trafficking of DAT to the surface in heterologous N2A cells. Further, we previously showed that PKC β was instrumental in promoting rapid substrate-induced trafficking of DAT to the surface in N2A cells (Furman *et al.* 2009a). We utilized confocal microscopy to determine that PKC β critically modulated D2S-stimulated DAT trafficking in N2A cells. Representative images for staining of surface and cytosolic DAT upon quinpirole treatment in the presence or absence of LY379196 are shown (Fig. 1A). A one-way ANOVA indicated a significant main effect of quinpirole and LY379196 on trafficking of DAT, $F(3,85)=7.833$, $p<0.01$ ($N=14-42$). Compared to vehicle, quinpirole (1 μ M, 30 min) increased the surface content of DAT (Fig. 1B, $p<0.05$). The PKC β inhibitor, LY379196, alone did not increase surface DAT, but blocked quinpirole-induced DAT trafficking to the surface (Fig. 1B). Next, we examined whether inhibition of ERK would affect DAT trafficking in a similar manner as did inhibition of PKC β . Similarly to LY379196, the ERK inhibitor PD98059 (10 μ M) blocked quinpirole-stimulated DAT trafficking to the surface without significantly affecting the basal DAT surface content (Fig. 1C).

PKC β promotes D2S-stimulated DAT recycling in N2A cells

The increased surface DAT expression upon D2S stimulation could result from an increase in the insertion of DAT into the membrane or a reduction in the internalization of DAT. We interrogated this by examining the effect of quinpirole on both recycling and internalization of DAT in N2A cells co-expressing DAT and D2S. As shown in Fig. 2A, quinpirole (100 nM) significantly increased the recycling rate of DAT over 30 min compared to vehicle (two-tailed paired t-test, $t = 2.788$, $df = 5$, $p<0.05$). Incubation with LY379196 alone did not alter the recycling rate of DAT, but blocked the effect of quinpirole. On the contrary, there was no effect of either quinpirole or LY379196 on the internalization rate of DAT when co-expressed with D2S (Figure 2B, $N=5$). These data suggest that the increased surface DAT expression by quinpirole indeed resulted from accelerated DAT trafficking to the membrane instead of decreased DAT internalization.

Inhibition of PKC β abolishes quinpirole-induced increases in surface DAT content in mouse striatal synaptosomes

In DAT-D2S-N2A cells, we demonstrated that PKC β and ERK were involved in D2S-stimulated DAT trafficking. However, we wished to establish this in a more physiological system and to verify our results without reliance on a small molecule PKC inhibitor. Therefore we continued our interrogation into the role of PKC β in D2autoR-stimulated DAT trafficking by using mice with a deletion of PKC β . To investigate whether PKC β regulates D2autoR-modulated DAT trafficking in striatal synaptosomes, surface DAT content in PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice was determined by biotinylation in the presence or absence of quinpirole. Treatment with quinpirole (10 μ M) significantly increased surface DAT expression in PKC $\beta^{+/+}$ mice (two-tailed paired t-test, $t=5.476$, $df = 3$, $p<0.05$), but not in PKC $\beta^{-/-}$ mice (Fig. 3A). To exclude the possibility that neuronal adaptations in the absence of PKC β might have complicated the response to quinpirole in PKC $\beta^{-/-}$ mice, synaptosomes from PKC $\beta^{+/+}$ mice were treated with the PKC β inhibitor LY379196. Mimicking PKC $\beta^{-/-}$ mice, pretreatment with LY379196 (100 nM) significantly prevented the quinpirole-induced increase in striatal surface DAT content in PKC $\beta^{+/+}$ mice (two-tailed paired t-test, $t=4.480$, $df=3$, $p<0.05$), and had no effect on PKC $\beta^{-/-}$ mice (Fig. 3B). These data are consistent with our findings in N2A cells (Fig. 1).

To further ascertain that PKC $\beta^{-/-}$ mice indeed had a blunted response to quinpirole stimulation in surface DAT expression, a time course of quinpirole treatment on DAT trafficking was conducted. A two-way ANOVA revealed a significant main effect of genotype on the time course of quinpirole-induced DAT trafficking, $F(1, 73) = 38.73$, $p < 0.001$ (Fig. 3C) $F(5,45) = 5.265$, $p < 0.01$, $N = 6-15$. In *post-hoc* analyses, compared with the vehicle treatment, surface DAT expression in PKC $\beta^{+/+}$ mice was significantly increased at 1, 5, 15 and 30 min of quinpirole, but not at 60 min. By contrast, quinpirole did not increase the surface DAT content in PKC $\beta^{-/-}$ mice at any time point. Rather, quinpirole significantly reduced the surface DAT after 5 and 10 min treatment.

To verify that quinpirole-stimulated insertion of DAT into surface membranes had functional significance, DA uptake was measured in striatal synaptosomes from PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice. Quinpirole pretreatment (10 μ M) significantly increased DA uptake in PKC $\beta^{+/+}$ mice (Fig. 3D, two-tailed paired t-test, $t = 4.438$, $df = 6$, $p < 0.005$), but not in PKC $\beta^{-/-}$ mice. Moreover, LY379196 significantly blocked the quinpirole-induced increase in DA uptake in PKC $\beta^{+/+}$ mice (Fig. 3E, two-tailed paired t-test, $t = 5.005$, $df = 3$, $p < 0.05$), and had no effect in PKC $\beta^{-/-}$ mice (Fig. 3E), suggesting that the absence of quinpirole-stimulated DAT trafficking in PKC $\beta^{-/-}$ mice indeed resulted from the absence of PKC β . These data demonstrate that quinpirole-stimulated DAT trafficking to the surface paralleled the increased DAT activity.

Quinpirole activates presynaptic PKC β in purified mouse striatal synaptosomes

If D2autoR-stimulated DAT trafficking is PKC β dependent, then activation of the D2autoRs should activate PKC β . We assessed this by examining the effect of quinpirole on phosphorylation of the individual PKC β isozymes at threonine 642 (in PKC β I) and threonine 641 (in PKC β II). Autophosphorylation of these carboxyl terminal threonines is required for active PKC β (Edwards *et al.* 1999). Since PKC β exists presynaptically in dopaminergic terminals (O'Malley *et al.* 2010) and postsynaptically (Yoshihara *et al.* 1991) in the striatum of animals, quinpirole-stimulated PKC β activity was assayed in percoll-purified striatal synaptosomes. The percoll purification procedure eliminates the majority of postsynaptic membranes (Dunkley *et al.* 1988), and enriches synaptosomal signaling. Purified synaptosomes from PKC $\beta^{+/+}$ mice were treated with vehicle, quinpirole (1 μ M or 10 μ M), or the PKC activator PMA (1 μ M). A one-way ANOVA indicated a significant drug effect on phosphorylation of PKC β II, $F(3,11) = 6.46$, $p < 0.05$. Both 1 μ M and 10 μ M quinpirole stimulated PKC β II activity by increasing phosphorylation of PKC β II compared to vehicle treatment ($N = 4$, Fig. 4, $p < 0.05$). However, neither dose of quinpirole enhanced phosphorylation of PKC β I (the values for pPKC β I/tPKC β I relative to the Veh treatment were 1.03 ± 0.09 , 0.99 ± 0.76 , and 1.55 ± 0.19 for quinpirole 1 and 10 μ M and PMA, respectively). PMA significantly increased both pPKC β I and pPKC β II compared to the vehicle treatment (*post hoc* Bonferroni, $p < 0.05$). These data suggest a specific role of PKC β II in D2autoR signaling.

The PKC β -ERK signaling cascade modulates D2autoR-activated DAT trafficking in mouse striatal synaptosomes

Our experiments in heterologous N2A cells demonstrated a role for both PKC β and ERK in D2S-stimulation of DAT trafficking. Analogous to our studies in heterologous N2A cells, we further explored the effect of ERK in D2autoR modulation of DAT trafficking in the PKC $\beta^{-/-}$ mice and queried whether or not PKC β is a downstream or upstream signaling molecule of ERK. We first verified that ERK was required for D2S-stimulated DAT trafficking in PKC $\beta^{+/+}$ mice and blocked the effect of quinpirole to increase striatal DA uptake in PKC $\beta^{+/+}$ mice (Fig. 5A, $N = 6$). Moreover, quinpirole increased phosphorylation of ERK in synaptosomes from PKC $\beta^{+/+}$ mice (Fig. 5B, lanes 1 and 2, $p < 0.05$, $N = 5$). We then

examined whether PKC β was upstream or downstream of ERK. If PKC β were downstream of ERK, then deletion or inhibition of PKC β should have no effect on the activation of ERK by quinpirole. Quinpirole-induced ERK phosphorylation (pERK) was examined in percoll-purified striatal synaptosomes from PKC $\beta^{-/-}$ mice (N=5). A two-way ANOVA showed a significant effect of genotype on ERK activation, $F(1,12) = 10.21$, $p < 0.01$. Quinpirole increased pERK in PKC $\beta^{+/+}$ mice but not in PKC $\beta^{-/-}$ mice (Fig. 5B), suggesting that PKC β is upstream of ERK following D2autoR activation. However, PKC $\beta^{-/-}$ mice exhibited a significant increase in the basal pERK compared to PKC $\beta^{+/+}$ mice (Fig. 5B, $p < 0.05$). The inability of quinpirole to further stimulate ERK activity in PKC $\beta^{-/-}$ mice could have resulted from the absence of PKC β or from a ceiling effect due to adaptation.

To further analyze the effect of PKC β on quinpirole-stimulated ERK activity, striatal synaptosomes from PKC $\beta^{+/+}$ mice were pretreated with the PKC β inhibitor LY379196 before quinpirole treatment. A one-way ANOVA revealed a significant effect of drug on ERK activity, $F(3,31) = 3.957$, $p < 0.05$ (Fig. 5C, lanes 3 and 4, N=8). LY379196 itself did not increase pERK, suggesting that the increased basal pERK in PKC $\beta^{-/-}$ mice was due to adaptation. These data indicate that PKC β is an upstream signaling molecule of ERK in quinpirole stimulation of D2autoR.

To further clarify that ERK is downstream of PKC β in D2autoR signaling, pPKC β II was determined from percoll-purified striatal synaptosomes from PKC $\beta^{+/+}$ mice treated with quinpirole in the presence or absence of the ERK inhibitor PD98059. A one-way ANOVA revealed a significant effect of drug, $F(3,31) = 3.886$, $p < 0.05$ (N=8). Quinpirole-stimulated phosphorylation of PKC β II was unchanged in the presence of PD98059 (Fig. 5D), verifying that ERK is downstream of PKC β II in quinpirole-induced D2autoR signaling.

Discussion

The present study reveals a significant new role of PKC β in regulation of D2autoR-activated DAT trafficking in heterologous cultured cells and mouse striatal synaptosomes. We have demonstrated that PKC β promotes D2autoR-activated insertion of DAT into the membrane by accelerating DAT recycling. A specific PKC $\beta \rightarrow$ ERK signaling cascade is critical in D2autoR modulation of DAT trafficking.

The novel trafficking role of PKC β in D2autoR-activated DAT translocation

PKC regulation of dynamic trafficking of neurotransmitter transporters is well documented (Chen et al. 2010, Schmitt & Reith 2010), but the importance of the specific PKC isoforms, notably PKC β , in trafficking processes is newly emerging. PKC β promotes trafficking of insulin-stimulated glucose transporter 4 and synaptic vesicles to membranes in adipocytes (Walaas *et al.* 1997, Wright *et al.* 2003) and primary cultured neurons (Lu *et al.* 1998, Yang *et al.* 2002), respectively. In this study we contribute a new example of PKC β -dependent trafficking by delineating the role of PKC β in promoting translocation of DAT to surface membranes upon D2autoR/D2S activation by accelerating the recycling of DAT. We found that quinpirole transiently decreased surface DAT in striatal synaptosomes from PKC $\beta^{-/-}$ mice (Fig. 3C). The reduction in surface DAT may have occurred because normal recycling of DAT is dysregulated in the absence of PKC β (Chen et al. 2009). The requirement of PKC β for quinpirole-stimulated recycling of DAT to the surface could lead to a reduction in surface DAT when PKC β is deleted. However, inhibition of PKC β by LY379196 did not reduce surface DAT below control levels in the heterologous N2A cells (Fig. 1) whether cells were incubated with quinpirole for 30 min as shown in Fig. 1 or even times as short as 5 min (data not shown). Therefore it is possible that the transient decrease in DAT in response to quinpirole could be unique to PKC $\beta^{-/-}$ mice.

The PKC β -ERK signaling cascade modulates DAT trafficking

D₂S-modulated DAT trafficking is sensitive to pertussis toxin and thus mediated by G $\alpha_{i/o}$ (Bolan et al. 2007). Our study suggests the additional requirement of PKC β II stimulation upon activation of D2autoR. Quinpirole stimulation of PKC β II activity promotes DAT trafficking while inhibition of PKC β abolishes DAT trafficking mediated by D2autoR. PKC β I and PKC β II are isozymes encoded by a single gene locus which differ only in their C-terminus 50–52 amino acids (Ono et al. 1987). *In vivo*, they can behave as unique isoenzymes with distinct properties. Although both PKC β I and PKC β II couple to DAT (Johnson et al. 2005b, Hadlock et al. 2011), the PKC β II isozyme enhanced AMPH-stimulated reverse transport of DAT (Johnson et al. 2005b). In purified striatal synaptosomes from PKC $\beta^{+/+}$ mice, the selective activation of PKC β II but not PKC β I by quinpirole may be due to differing cellular localization of the proteins. For instance, activated PKC β II translocates to the membrane and interacts specifically with actin and the actin cytoskeleton (Blobe et al. 1996), which facilitates vesicle trafficking and exocytosis. Substrate-induced DAT trafficking to the surface is an exocytotic event that is regulated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins (Furman et al. 2009a). A specific PKC β II interaction with the cytoskeleton element may facilitate the trafficking and membrane insertion of DAT through activation of D2autoR. PKC β II activation by quinpirole is likely due to activation of PLC β by the G $\beta\gamma$ subunit of G $\alpha_{i/o}$ (Liu et al. 2003). Cocaine has been shown to facilitate PKC maturation to prime PKC for response to the second messenger system (Xue et al. 2012).

Both D2autoR and DAT are subject to PKC regulation and contain identified PKC phosphorylation sites (Morris et al. 2007, Foster et al. 2002). Therefore, a PKC β -dependent phosphorylation of DAT, D2autoR or both may underlie the association between DAT and D2autoR and facilitate DAT recycling. In fact, G protein-coupled receptor kinases (GRKs) regulate recycling of phosphorylated but not unphosphorylated D2autoR in HEK293 cells (Cho et al. 2010), supporting the notion that phosphorylation of D2S by protein kinases may result in altered trafficking of DAT. It warrants further investigation as to whether or not the increased surface DAT expression by quinpirole is due to co-trafficking of DAT and D2autoR. Nonetheless, our study not only adds a new member to the PKC-ERK signaling family, but also delineates PKC β II as the specific PKC form involved in the signaling.

D2autoR-induced DAT trafficking is distinct from DAT substrate-induced DAT trafficking

Although DAT trafficking induced by either D2autoR or DAT substrate involves PKC β , the characteristics are distinct. D2autoR-stimulated DAT trafficking is relatively long-lived (15 min in striatal synaptosomes and 30 min in N2A-hDAT-D₂S cells) as demonstrated in the current study compared to that stimulated by DAT substrate amphetamine (15 min) that we reported previously (Chen et al. 2009, Furman et al. 2009a, Johnson et al. 2005a). DAT substrate-induced DAT trafficking does not require D2autoR (Furman et al. 2009a). Interestingly, the processes may also be related to different conformational states of DAT. The increased surface DAT stimulated by amphetamine parallels increased amphetamine-induced DA efflux through DAT suggesting that it may be in a predominantly inward-facing conformation (Furman et al. 2009a). In contrast, the increased surface DAT stimulated by quinpirole parallels increased DA uptake (Fig. 3) which is reflected by an outward-facing conformation. Since quinpirole is a ligand for both D2R and D3R, the presynaptic D3R could also function as an autoreceptor (Zapata et al. 2001). Further investigation on the regulatory machinery of D3R-DAT trafficking will determine whether PKC β is a common signaling pathway for presynaptic D2S and D3R regulation of DAT trafficking.

Conclusion

We show here that PKC β is an important modulator of D2autoR-activated DAT trafficking in both mouse striatal synaptosomes and cultured cells. PKC β facilitates D2autoR-stimulated DAT trafficking by increasing the recycling rate of DAT without an effect on DAT internalization. The signaling cascade of PKC β -ERK critically modulates D2autoR-activated DAT trafficking. This study provides a new avenue for intervening in the interaction between DAT and D2autoR and controlling synaptic DA levels. This is of clinical significance for diseases such as schizophrenia, ADHD and drug addiction which may involve a dysfunctional dopamine autoreceptor function.

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Abbreviations

DA	dopamine
DAT	dopamine transporter
D2autoR	dopamine D2-like autoreceptor
D2S	dopamine D2 receptor short variant
PKCβ	protein kinase C β
ERK	extracellular signal-regulated kinase

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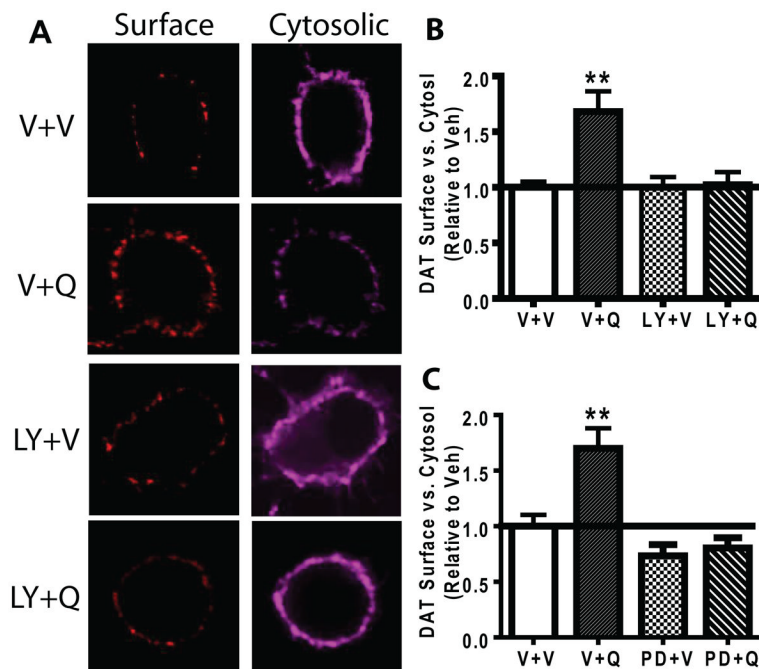


Fig. 1. PKC β and ERK inhibitor blocked quinpirole-stimulated DAT trafficking to the surface in N2A cells. N2A cells were transiently transfected with HA-DAT and FLAG-D2S. Cells were pretreated with LY379196 (LY, 100 nM, 30 min), PD98059 (PD, 10 μ M, 15 min) or vehicle (V) prior to quinpirole (Q, 1 μ M, 30 min) or V treatment. Following the treatment, live cells were labeled for surface DAT, then fixed and permeabilized for intracellular DAT labeling with different fluorophores. The surface DAT content was calculated as the ratio of the fluorescent intensity of surface labeling to that of the intracellular labeling. (A) Representative confocal images of surface and cytosolic DAT treated with quinpirole (Q) or vehicle (V) in the presence or absence of LY379196 (LY). (B) Quinpirole stimulated DAT trafficking to the surface ($p < 0.05$, $N = 19$) whereas LY379196 blocked quinpirole-induced DAT trafficking ($n = 15$). (C) The ERK inhibitor blocked quinpirole-stimulated DAT trafficking to the surface. PD98059 alone did not have an effect on basal DAT trafficking ($N = 14$). Error bars represent SEM. * $p < 0.05$ vs. the vehicle treatment.

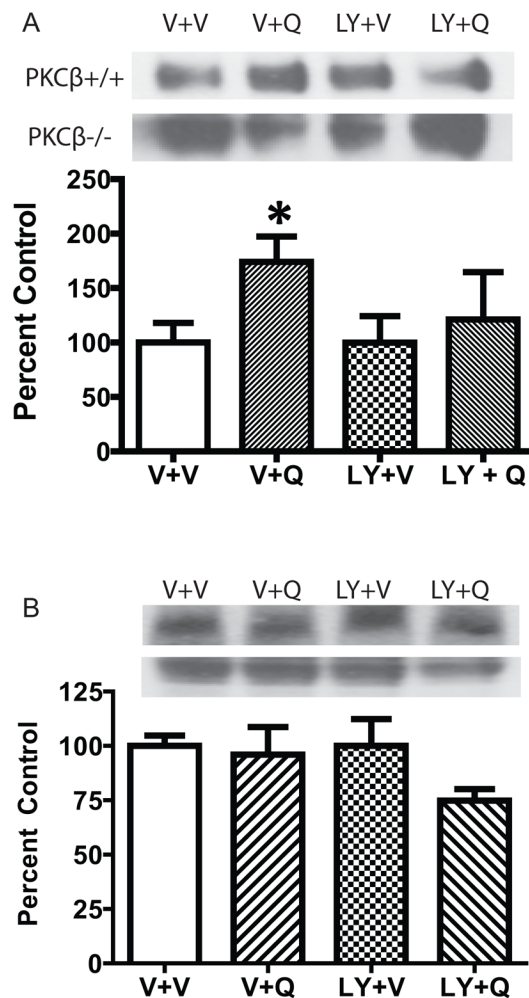


Fig. 2. Quinpirole stimulated DAT recycling without an effect on DAT internalization. N2A cells transiently transfected with HA-DAT and FLAG-D₂S were treated with vehicle (V), quinpirole (100 nM, 30 min), LY379196 (100 nM), or LY+Q. (A) Representative blots and summary of the DAT recycling (N=6). Quinpirole accelerated the DAT recycling rate compared to the vehicle treatment (*p<0.05). LY379196 itself did not have an effect on DAT recycling, but blocked quinpirole-induced increased DAT recycling. (B) Representative blots and summary of DAT internalization (N=5). The DAT internalization rate did not differ between quinpirole and vehicle treatment and LY379196 did not affect DAT internalization either. The data from the LY+Q group did not differ from V+V or LY+V. Error bars represent SEM.

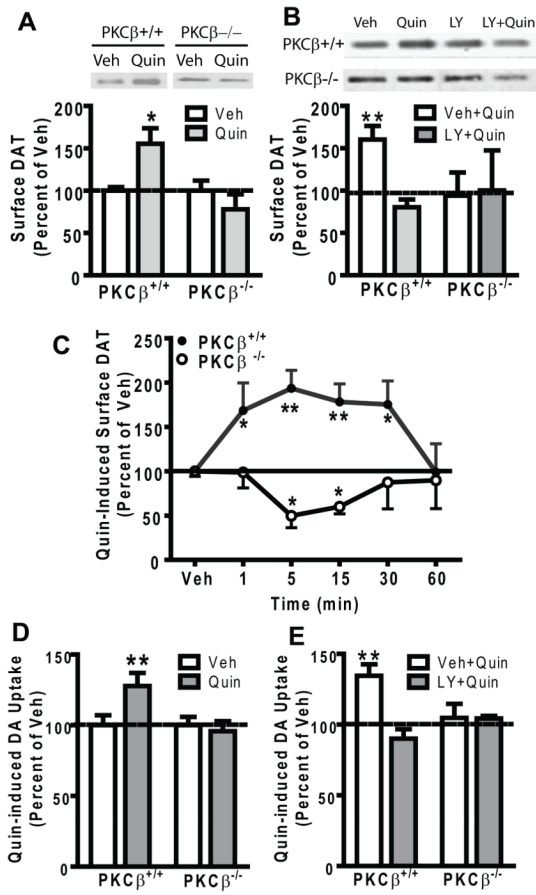


Fig. 3. PKCβ inhibition abolishes quinpirole-induced increase in striatal surface DAT expression and DA uptake in mice. (A) Striatal synaptosomes from the PKCβ^{+/+} and PKCβ^{-/-} mice were incubated for 15 min with vehicle (Veh) or quinpirole (Quin, 10 μM) before biotinylation of surface DAT. Quinpirole increased surface DAT content in PKCβ^{+/+} mice but not in PKCβ^{-/-} mice. Representative blots for surface DAT biotinylation under various conditions are shown. Surface DAT was calculated as a ratio of surface biotinylated DAT to the total DAT from the lysate. Data were expressed as percent of the Veh treatment in each genotype. Veh values for ratios of biotinylated/total DAT for PKCβ^{+/+} and PKCβ^{-/-} mice were 1.1 ± 0.1 and 0.7 ± 0.2, respectively. (B) Pretreatment with the PKCβ inhibitor LY379196 (LY, 100 nM, 1 hr) blocked the quinpirole-induced increase in surface DAT content in PKCβ^{+/+} mice compared to the Veh treatment and had no effect on surface DAT in PKCβ^{-/-} mice. Representative blots for surface DAT biotinylation under various conditions are shown. Data were calculated as percent of the Veh+Veh treatment. (C) Quinpirole induced a significant time-dependent DAT trafficking in PKCβ^{+/+} mice, There was a significant increase in surface DAT expression upon quinpirole treatment at 1, 5, 15 and 30 min compared to the Veh treatment. By contrast, quinpirole did not increase surface DAT expression at any time point in PKCβ^{-/-} mice (N=13, 6, 6, 9, 3 and 5 for Veh, 1, 5, 15, 30 and 60 min, respectively). *p<0.05, **p<0.01 vs. Sal, *post hoc* Bonferroni test. (D) Quinpirole (Quin) increased DA uptake in striatal synaptosomes from PKCβ^{+/+} mice (N=6, Veh vs. Quin, paired t-test, **p<0.01) but not from PKCβ^{-/-} mice. Data were expressed as percent of Veh treatment in each genotype. Veh values for [³H]DA uptake in PKCβ^{-/-} mice were 94% of that in PKCβ^{+/+} mice, N=6. (E) The PKCβ inhibitor LY379196 (LY, 100 nM) inhibited quinpirole-stimulated DA uptake in striatal synaptosomes from PKCβ^{+/+} mice

(** $p < 0.01$). Data were calculated as percent of Veh+Veh treatment. Error bars represent SEM.

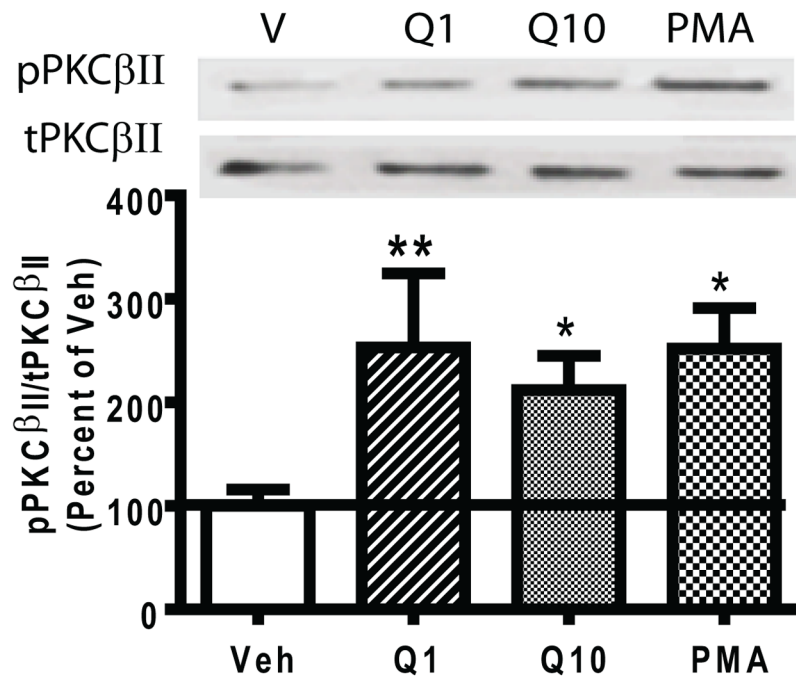


Fig. 4. Quinpirole stimulated PKCβII activity. Percoll-purified striatal synaptosomes prepared from PKCβ^{+/+} mice were incubated with vehicle (V), quinpirole (Q, 1 or 10 μM, 5 min) or PMA (1 μM, 1 min) as a positive control. The activity of PKCβII was determined by the level of phosphorylated PKCβII (pPKCβII) vs. total PKCβII (tPKCβII). Both 1 and 10 μM quinpirole increased PKCβII activity. Error bars represent SEM.

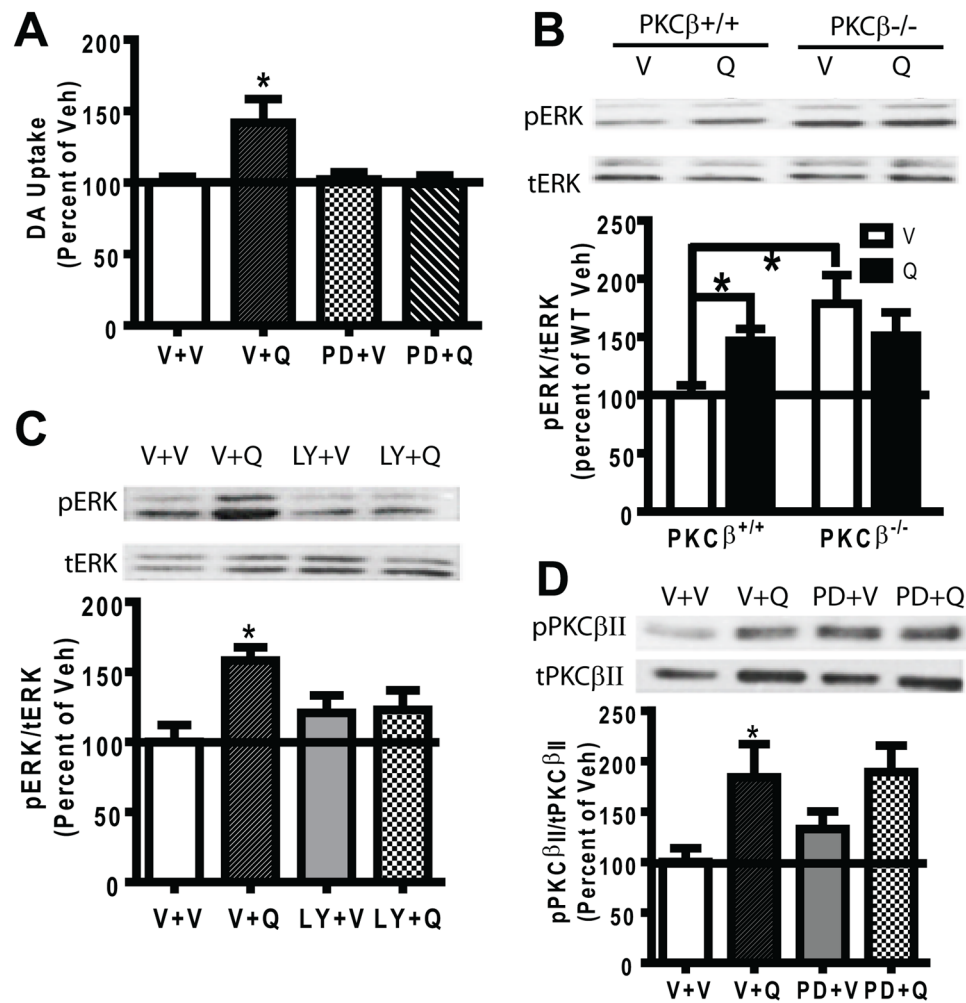


Fig. 5. ERK activation by quinpirole required PKC β . (A) A 15-min pretreatment with PD98059 (PD, 10 μ M) blocked quinpirole (Q, 1 μ M, 5 min)-stimulated DA uptake in striatal synaptosomes from PKC $\beta^{+/+}$ mice compared to vehicle (V) treatment. (B) Percoll-purified striatal synaptosomes from PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mice were treated with V or 10 μ M quinpirole (Q) for 5 min (N=5). The upper panel shows representative blots of pERK (phosphorylated ERK) and tERK (total ERK) from a representative PKC $\beta^{+/+}$ and PKC $\beta^{-/-}$ mouse. Data were converted as a ratio of pERK to tERK as a measurement of ERK activity, and calculated as a percent of the V treatment in PKC $\beta^{+/+}$ mice. Quinpirole significantly increased the pERK level in PKC $\beta^{+/+}$ but not in PKC $\beta^{-/-}$ mice. The basal pERK level was significantly higher in PKC $\beta^{-/-}$ mice as compared to that from PKC $\beta^{+/+}$ mice ($p < 0.05$). (C) Percoll-purified striatal synaptosomes from PKC $\beta^{+/+}$ mice were pretreated with the PKC β inhibitor LY379196 (LY, 100 nM) or vehicle (V) for 1 hr, and the level of pERK in response to quinpirole (Q, 1 μ M, 5 min) was determined (N=8). Representative blots of pERK and tERK were shown. LY379196 did not change the basal pERK, and blocked quinpirole-stimulated pERK. (D) Percoll-purified striatal synaptosomes were pretreated with the ERK inhibitor PD98059 (PD, 10 μ M, 15 min) before vehicle or quinpirole treatment (Q, 1 μ M, 5 min). The activity of PKC β II was determined by pPKC β II (phosphorylated PKC β II) vs. tPKC β II (total PKC β II) (N=8). Representative blots of pPKC β II and tPKC β II

blots are shown in the upper panel. PD98059 itself did not change the basal pPKC β II level and did not block quinpirole-stimulated pPKC β II. Error bars represent SEM.