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PKC β co-localizes with the dopamine transporter in mesencephalic neurons

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Abstract

The dopamine transporter (DAT) is a critical regulator of dopaminergic neurotransmission. Research in both rat striatum and heterologous cells suggests that protein kinase C beta (PKC β) is important for proper trafficking of DAT. However, a critical gap that is missing from the literature is the localization of PKC β to mesencephalic dopaminergic neurons. In this study we examined the co-localization of DAT, which serves to identify dopaminergic neurons, and PKC β in mesencephalic dopaminergic cells. Using immunofluorescence and confocal microscopy, we demonstrated co-localization of DAT and PKC β in primary cultures of mesencephalic neurons and in dopamine neurons in rat substantia nigra and ventral tegmental area. PKC β was not specific for dopamine neurons in the two brain regions. This is the first demonstration of co-localization of PKC β and DAT in mesencephalic neurons. The co-localization of PKC β with DAT in mesencephalic neurons corroborates our previous studies demonstrating a role for PKC β in DAT function.

Keywords

protein kinase C; substantia nigra; ventral tegmental area; primary cultures; dopamine

Introduction

In the basal ganglia, the dopamine transporter (DAT), the site of reuptake of dopamine on dopaminergic cells, is a crucial determinant of the duration of the dopamine signal in the synaptic cleft [28]. DAT is also the principal site of action for the rewarding properties of the psychostimulants amphetamine and cocaine [3,10]. As a substrate, amphetamine binds to DAT and is transported into the nerve terminal, whereupon dopamine binds to the inward-facing transporter and is subsequently pumped into the synapse [7]. Monoamine transporters, including DAT, are regulated by protein kinases, especially protein kinase C (PKC) [8]. We and others found that PKC inhibitors block the ability of amphetamine to stimulate dopamine efflux and to elicit locomotor activity [11,17]. Our recent studies suggest that PKC activity is important for the ultra-rapid trafficking of DAT to the plasmalemmal membrane upon amphetamine or dopamine stimulation [2,9,14]. Persistent

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activation with phorbol esters, however, reduces DAT function by desensitizing and internalizing DAT [6,22,27].

PKC isozymes are a family of serine/threonine protein kinases that are divided into three subfamilies based on structural differences in their amino-terminal regulatory domains [23]. The conventional, or cPKC, isoforms are sensitive to activation by diacylglycerol, phorbol esters and calcium and consist of the isoforms α , β I, β II and γ . PKC β II is an alternatively spliced isoform of PKC β I which contains an additional 43 residues at the amino terminus. Evidence from our laboratory, using both heterologous expression systems [9,15] and PKC β knockout mice [2], suggests that PKC β regulates rapid substrate-stimulated DAT trafficking to the surface and affects dopamine uptake and efflux [2,9,15]. Overexpression of PKC β II, in particular, enhanced amphetamine-stimulated dopamine efflux in hDAT-HEK293 cells [15]. PKC β has been detected in the mesencephalic dopamine cell body areas, substantia nigra and the ventral tegmental area in both rats [24] and humans [13]. However, PKC β has not been localized to dopaminergic cells in those areas and is reported not to be located in nigrostriatal neurons [26]. In this report we use immunocytochemistry in primary cultured mesencephalic neurons and rat brain to demonstrate that PKC β is co-localized with DAT in mesencephalic dopamine neurons.

Materials and Methods

Generation of primary neuronal cultures

Rat midbrain mesencephalic neurons from 1 to 3-day-old pups were isolated and grown on a monolayer of glial cells based on a modified version of the protocol of Rayport et al. [21]. Poly-D-lysine-coated glass-bottomed culture dishes (MatTek, Ashland, MA) were coated with 10 μ g/ml laminin. A monolayer of rat C6 glial cells was plated 2 to 3 days before culturing neurons and maintained in Neurobasal-A media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 30 U/ml penicillin, 30 mg/ml streptomycin, and 0.6 mM L-glutamine. The neurons were used for immunostaining 7 days after preparation. Neurons were fixed in 4% formaldehyde, washed with PBS, permeabilized with methanol and blocked with 4% goat serum and 1% gelatin. The neurons were incubated with the following primary antibodies: rat monoclonal anti-DAT prepared against residues 180-218 in the second extracellular loop as described [12] (dilution 1:100, a generous gift of Dr. Allan Levey) and anti-PKC β II (dilution 1:50, (C-18) rabbit, Santa Cruz, catalog no. sc-210). Both antibodies have been used successfully for immunocytochemistry [4,12]. Secondary antibodies were goat anti-rabbit or chicken anti-rat (as appropriate) conjugated to either Alexa 488 (green) or Alexa 594 (red) (Molecular Probes, Carlsbad, CA).

Rat brain cryosections

Female Holtzman rats, aged 8-10 weeks, were deeply anaesthetized with sodium pentobarbital and sacrificed by decapitation. All animal procedures were approved by the University of Michigan Committee on the Use and Care of Animals and rats were housed in the University of Michigan Unit for Laboratory Animal Medicine. Brains were removed and fixed in 4% paraformaldehyde, followed by sequential cryoprotection steps in 10% and 30% sucrose. Brains were then snap-frozen in 2-methylbutane and stored at -80°C until time of sectioning. Coronal cryosections were generated at 10 μ m and fixed with 4% paraformaldehyde for 10 minutes. Sections were washed 3 times in 0.05M phosphate buffer and then blocked for a minimum of 1 hour in PBTGS (0.1M PB, 0.3% Triton X-100 and 10% normal goat serum). Sections were then incubated overnight in primary antibody diluted in PBTGS. Primary antibodies for brain immunocytochemistry were as follows: mouse monoclonal anti-PKC β (dilution 1:250, Zymed, Carlsbad, CA, catalog no. 13-3700) and rat anti-DAT (dilution 1:200, see above). The following day, sections were washed 3

times with 0.1M PB before incubation with secondary antibody diluted in PBTGS. Secondary antibodies were goat anti-mouse or anti-rat (as appropriate) conjugated to either Alexa 488 (green) or Alexa 594 (red) (Molecular Probes, Carlsbad, CA). Sections were washed 3 times with 0.1M phosphate buffer before being air-dried and placed on coverslips using GelMount anti-fade mounting medium (Biomedica, Foster City, CA).

Microscopy

Digital images of cultured neurons and cryosections were collected using an Olympus FluoView 500 confocal microscope located in the Department of Pharmacology, University of Michigan. Images were collected using either a 60 x 1.4 numerical aperture (NA) oil objective or a 100 x 1.35 NA oil objective. Composite images were assembled using Adobe Photoshop CS4.

Results

We first examined the expression of DAT and PKC β in primary cultured neurons. Immunofluorescence for DAT (green, Fig. 1A) and PKC β II (red, Fig. 1B) in a primary mesencephalic cultured neuron is presented in Fig. 1. As shown in Fig. 1C (merge), PKC β II is expressed in DAT-positive primary mesencephalic cultured neurons. The inset in Fig. 1C shows a region of the neuron displaying a high degree of overlap between the DAT and PKC β II fluorescent signals. Controls with secondary antibody only (data not shown) showed no labeling. There was some level of labeling in the nucleus with both antibodies that was likely nonspecific.

Since primary neonatal mesencephalic cultures may not accurately represent protein expression patterns in adult neurons, we performed immunocytochemistry for DAT and PKC β in regions of adult rat brain containing mesencephalic cell bodies. Both PKC β II and PKC β I may affect DAT trafficking and function [9,15], and both isozymes co-immunoprecipitated with DAT from rat striatum [15], so we consequently used an antibody with demonstrated specificity for immunofluorescence that would detect both isoforms [20]. The data in Fig. 2 demonstrate that DAT and PKC β co-localize in both the substantia nigra pars compacta (SNc) and in the ventral tegmental area (VTA). Dopaminergic neurons in the SNc were identified by labeling with anti-DAT (Figure 2A, red). Double-labeling with anti-PKC β antibody demonstrated that these DAT-positive neurons were also immunoreactive for PKC β (Figure 2B, green, and merged image in Figure 2C). The inset in Fig. 2C presents a zoomed-in view of the colocalization of the DAT and PKC β fluorescent signals in an individual mesencephalic neuron. Control experiments lacking primary antibodies as well as experiments labeling with only one secondary antibody at a time were also performed in order to confirm the specificity of primary antibodies used and to ensure that anti-mouse and anti-rat secondary antibodies did not cross-react (data not shown). The merged image shown in Fig. 2C demonstrates that most of the neurons in the rat SNc expressed PKC β but only some expressed DAT. Thus, PKC β is expressed in a number of neuronal cell types. Confocal images of the VTA were also obtained from the same coronal sections in which PKC β -DAT colocalization in the SNc was observed. Co-localization of PKC β and DAT was again observed in the VTA (Figure 2D, red anti-DAT; Figure 2E, green anti-PKC β , and Figure 2F, merge). The VTA can be seen to contain a great many DAT-expressing cell bodies and fibers. Again, most cells expressed PKC β but not all cells expressed DAT. However, all of the dopaminergic, DAT-expressing cells in the SNc or the VTA were also positive for PKC β . Interestingly, the neuropil was densely labeled by anti-DAT but not by anti-PKC β . The inset in Fig. 2F presents a zoomed-in view of two neurons positive for both DAT and PKC β . Although the images shown are from female rats, parallel experiments performed using male rats demonstrated no female-male differences (data not shown).

Discussion

This is the first demonstration of co-localization of PKC β and DAT in mesencephalic neurons. PKC β is a prominent PKC isozyme in striatal medium spiny neurons [26] and we have now identified its existence in mesencephalic dopamine neurons in both the SNc and the VTA. Our finding that a majority of the cells contained PKC β confirms studies reporting robust amounts of PKC β in rat SNc and VTA [24]. PKC β has been localized to GABAergic cells in the SNc [26] and we have now demonstrated that PKC β is also in dopaminergic cells. Yoshihara et al [26] reported that PKC α was localized in nigrostriatal dopaminergic neurons but co-localization of PKC β I and tyrosine hydroxylase was not detected. PKC β I immunoreactivity was detected in GABAergic cell bodies in the SNc, while PKC β II immunoreactivity was detected primarily in the neuropil. The difference in our results is unclear but could be attributable to different methods and perhaps different antibodies. Steketee et al. [24] reported much higher levels of PKC β 1 than PKC β II in both the SNc and the VTA, but the co-localization of DAT and PKC β II shown in the primary cultured neurons in Fig. 1 demonstrates that some dopaminergic neurons contain PKC β II.

Activation of PKC has a biphasic effect on DAT function most likely by altering trafficking of the transporter. Rapid activation of PKC increases DAT-mediated dopamine (DA) efflux in rat striatal slices [5,15]. Similarly, amphetamine stimulation of dopamine efflux is blocked by PKC antagonists [16], particularly by select PKC β antagonists [15]. Stimulation of DAT-containing cells or synaptosomes with amphetamine elicits a rapid trafficking of DAT to the surface, which is dependent upon PKC β [2,9,14]. However, longer-term, persistent activation of heterologous cells or striatal synaptosomes with phorbol esters, reduces DAT function by desensitizing and internalizing DAT [6,22,27]. The down-regulation of DAT activity by persistent phorbol ester activation is also mediated by a conventional, Ca²⁺-dependent isoform of PKC [6]. One explanation for the biphasic effect of PKC activation on DAT trafficking is that one cPKC isozyme, PKC β , is important for recycling of DAT to the surface, especially rapid substrate-stimulated recycling, and another cPKC isozyme, perhaps PKC α which is also located in nigrostriatal cells [26], mediates PKC-dependent internalization. Although amphetamine itself elicits desensitization and down-regulation of DAT, this effect is not dependent on PKC [1].

PKC β is widely located throughout the brain [19,25], including mesencephalic dopaminergic cells as we have shown in this report. Conventional forms of PKC are important for a variety of events in DA neurons, including long-term potentiation in the VTA [18]. This study is the first to report localization of PKC β to mesencephalic dopamine neurons and corroborates our earlier studies demonstrating both coimmunoprecipitation of PKC β and DAT and the effects of PKC β on DAT function.

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Figure 1.

Primary cultured mesencephalic neurons express both DAT and PKC β II. (A): Anti-DAT, (B): Anti-PKC β II, (C): merged image. The inset in (C), zoomed in from the smaller boxed regions shown in A-C, displays a region of strong overlap between DAT and PKC β II fluorescent signal. Scale bar: 20 μ m.

Figure 2.

Mesencephalic neurons in the SNc and the VTA express both DAT and PKC β . Insets in (C) and (F) show zoomed-in views of individual neurons positive for both DAT and PKC β in the SNc (C) and VTA (F). (A-C): DAT-positive neurons in the SNc also express PKC β . (A): Anti-DAT, (B): Anti-PKC β , (C): merged image. (D-F): DAT-positive neurons in the VTA are also positive for PKC β . (D): Anti-DAT, (E): Anti-PKC β , (F): merged image. Scale bar: 50 μ m.