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Characterization of the Translocation of Protein Kinase C (PKC) by 3,4-methylenedioxymethamphetamine (MDMA/Ecstasy) in Synaptosomes:

Evidence for a Presynaptic Localization Involving the Serotonin Transporter (SERT)

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3,4-methylenedioxymethamphetamine (MDMA or Ecstasy) is a substituted amphetamine whose acute and long-term effects on the serotonin system are dependent on an interaction with the 5-HT uptake transporter (SERT). Although much of the work dedicated to the study of this compound has focused on its ability to release monoamines, this drug has many important metabolic consequences on neurons and glial cells. The identification of these physiological responses will help to bridge the gap that exists in the information between the acute and neurotoxic effects of amphetamines. Substituted amphetamines have the ability to produce a long-term translocation of protein kinase C (PKC) in vivo, and this action may be crucial to the development of serotonergic neurotoxicity. Our earlier results suggested that PKC activation occurred through pre- and postsynaptic mechanisms. Because the primary site of action of these drugs is the 5-HT transporter, we now expand on our previous results and attempt to characterize MDMA's ability to translocate PKC within cortical 5-HT nerve terminals. In synaptosomes, MDMA produced a concentration-dependent increase in membrane-bound PKC (as measured by ^{3}H-phorbol 12, 13 dibutyrate, ^{3}H-PDBu) bindings sites. This response was abolished by cotreatment with the specific serotonin reuptake inhibitor (SSRI), fluoxetine, but not by the 5-HT$_{2A/2C}$ antagonist, ketanserin. In contrast, full agonists to 5-HT$_{1A}$ and 5-HT$_2$ receptors did not produce significant PKC translocation. MDMA-mediated PKC translocation also requires the presence of extracellular calcium ions. Using assay conditions where extracellular calcium was absent prevented the in vitro activation of PKC by MDMA. Prolonged PKC translocation has been hypothesized to contribute to the calcium-dependent neurotoxicity produced by substituted amphetamines. In addition, many physiological processes within 5-HT nerve terminals, including 5-HT reuptake and vesicular serotonin release, are susceptible to modification by PKC-dependent protein phosphorylation. Our results suggest that prolonged activation of PKC within the 5-HT nerve terminal may contribute to lasting changes in the homeostatic function of 5-HT neurons, leading to the degeneration of specific cellular elements after repeated MDMA exposure. ***[Neuropsychopharmacology 19:265–277, 1998]*** *© 1998 American College of Neuropsychopharmacology. Published by Elsevier Science Inc.*

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The increased use and abuse of psychoactive stimulants such as amphetamine (AMPH), methamphetamine (METH), and their structural analog, 3,4-methylenedioxymethamphetamine (MDMA or Ecstasy), has encouraged research on their addictive potential and putative neurotoxicity. These drugs all produce acute psychoactive and long-term neurotoxic effects, which seem to be dependent on their ability to promote a calcium-independent release of monoamines from nerve terminals (Sanders-Bush and Strenaka 1978; Nichols et al. 1982; Schmidt 1987; O'Hearn et al. 1988; Appel et al. 1989; Schmidt et al. 1990; Berger et al. 1992a,b). MDMA shows a unique selectivity for the serotonergic system through its efficacy for releasing 5-HT, inhibiting its reuptake, and producing the degeneration of presynaptic serotonergic elements (Johnson et al. 1986; Commins et al. 1987; Schmidt 1987; Battaglia et al. 1988b; O'Hearn et al. 1988; Ricaurte et al. 1988; Insel et al. 1989; Fischer et al. 1995).

Several laboratories, including our own, have reported on the intermediate metabolic consequences of repeated exposure to substituted amphetamines (SA). Some of these effects include: an increase in striatal dopamine release, chronic inhibition of tryptophan hydroxylase, hyperthermia, increased Ca^{2+} uptake, sustained glycogenolysis in astrocytes, and the prolonged translocation and activation of the Ca^{2+} and phospholipid-dependent kinase, protein kinase C (PKC) (Park and Azmitia 1991; Malberg et al. 1994; Poblete and Azmitia 1995; Pu and Vorhees 1995; Kramer et al. 1997). Many of these processes seem to be directly involved in the neurotoxicity produced by MDMA, because the inhibition of several of these responses prevents the loss of monoamine nerve terminals (Schmidt 1987; Farfel et al. 1992; Gu and Azmitia 1993).

Because the prolonged translocation of PKC has been implicated in several calcium-dependent neurodengenerative processes, its putative role in MDMA-induced neurotoxicity is of interest. Our previous studies have shown that MDMA stimulates a delayed but prolonged translocation of PKC to cortical and hippocampal membranes (Kramer and Azmitia 1994; Kramer et al. 1995; Kramer et al. 1997). However, the localization (either pre- or postsynaptic) of the MDMA-sensitive PKC pool is not yet defined. One hypothesized location of action for MDMA has been the 5-$HT_{2A/2C}$ receptor, a site for which this drug shows high affinity (Battaglia et al. 1988a; Pierce and Peroutka 1988; Poblete and Azmitia 1995). The 5-$HT_{2A/2C}$ receptor subtype is a G-protein coupled receptor, which stimulates PKC activity through the classical hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP_2) (Conn and Sanders-Bush 1985, 1986; Wang and Friedman 1990). Consequently, MDMA may produce PKC translocation through stimulation of the 5-$HT_{2A/2C}$ receptor. Numerous experiments have shown that several of MDMA's acute and neurotoxic effects arise through interactions with the central 5-$HT_{2A/2C}$ receptor (Schmidt et al. 1991; Malberg et al. 1994; Poblete and Azmitia 1995). These findings are consistent with MDMA's agonist-like properties at the 5-$HT_{2A/2C}$ receptor (Nash 1990; Poblete and Azmitia 1995). In culture, pretreatment of fetal serotonergic neurons with the 5-$HT_{2A/2C}$ antagonist, ketanserin, reduces their susceptibility to toxic concentrations of MDMA (Azmitia et al. 1990). In vivo, Schmidt and co-workers (1990) showed that hyperthermia and long-term reductions in cortical, hippocampal, and striatal 5-HT content are competitively reversed by pretreatment with the 5-$HT_{2A/2C}$ antagonist, MDL 11,939. Because MDMA produces a 5-$HT_{2A/2C}$ receptor and Ca^{2+}-dependent form of neurotoxicity, the postsynaptic activation of PKC may be required for its development.

Alternatively, PKC translocation could be occurring presynaptically within the 5-HT nerve terminal. This type of activation may occur in response to MDMA binding to the SERT, which is known to result in increased calcium influx into 5-HT nerve terminal synaptosomes (Azmitia et al. 1993). MDMA increases $^{45}Ca^{2+}$ uptake into cortical synaptosomes at drug concentrations known to release ^{3}H-5-HT (Azmitia et al. 1993). Increases in intracellular calcium can promote PKC translocation to the plasma membrane in the absence of receptor-stimulated phospholipid hydrolysis (Melloni et al. 1985). In vivo, fluoxetine is capable of totally inhibiting MDMA's effect on PKC translocation; presumably by preventing MDMA from binding to the SERT (Kramer et al. 1997). This inhibitory action of fluoxetine will prevent 5-HT release and any subsequent stimulation of postsynaptic 5-HT receptors, which may contribute to further, postsynaptic PKC activation (Schmidt et al. 1987; Berger et al. 1992a,b).

This series of experiments expands on the role of the SERT in MDMA-mediated PKC translocation. It is evident that substituted amphetamines are capable of translocating PKC in vivo, but our previous experiments raised two important questions about their mechanisms of action. First, ketanserin produced a partial, but significant, attenuation of PKC translocation in the cortex of MDMA-treated animals (Kramer et al. 1997). However, membrane PKC density remained significantly above that recorded in saline-treated rats. This evidence suggests that stimulation of postsynaptic receptors (including the 5-HT_2 and 5-HT_3 subtypes) is only partly responsible for SA-mediated PKC translocation. Second, both pCA and MDMA are unable to activate PKC when animals are pretreated with fluoxetine 30 minutes prior to receiving MDMA. Fluoxetine may inhibit PKC translocation by competing with MDMA for its preferential binding site on the SERT. Despite the

actual inhibitory mechanism of fluoxetine, it is apparent that MDMA's partial agonist activity at the 5-$HT_{2A/2C}$ receptor is not, by itself, sufficient to promote a long-term translocation of PKC and that some activity at the SERT is required.

For the present studies, the effect of MDMA and several other serotonergic compounds were tested for their ability to translocation PKC in rat cortical synaptosomes. Synaptosomes are capable of binding and internalizing many classes of drugs and neurotransmitters, and they have been extensively used to characterize the pharmacology of compounds like MDMA and pCA (Johnson et al. 1991; Berger et al. 1992b; Rudnick and Wall 1992b). Furthermore, 5-HT nerve terminals and the surrounding glial cells are abundant in several PKC isoforms and many of their substrate proteins (Conn and Sanders-Bush 1985; Conn and Sanders-Bush 1986; Kagaya et al. 1990; Wang and Friedman 1990; Masliah et al. 1991; Gott et al. 1994). Our results demonstrate that MDMA produces a Ca^{2+}-dependent, 5-HT receptor-independent form of PKC translocation, within 5-HT nerve terminals, though its interaction with the serotonin uptake transporter.

METHODS

Animal Care and Handling

Female Sprague–Dawley rats (weighing between 150–200 g; Taconic Farms, Germantown, NY) were used for all experiments. All animals were maintained on a 12-hour light/dark cycle and had unlimited access to food and water. All animal protocols were reviewed and approved by the New York University animal welfare committee.

Tissue Preparation for PKC Translocation in Rat Cortical Synaptosomes

For the preparation of corticol synaptosomes, rats were placed under deep anesthesia (CO_2), decapitated, and their brains rapidly removed followed by careful dissection of the cerebral cortex on ice. Fresh brain regions were homogenized at 10 × volume/wet brain weight (v/ww) in an ice-cold (+4°C) buffer (homogenization buffer) that limited active proteolysis and contained (mM): NaCl 134; KCl 3.0; $MgCl_2$ 1.2; EGTA 0.1; 4-(2-hydroxyethyl)-1-piperazine ethane sulfonate (HEPES) 10; pH 7.4 (buffer A). These brain regions were homogenized using a Cole–Palmer glass/Teflon homogenizer and centrifuged at 1,000 *g* for 10 min using a Sorvall SS-34 fixed-angle rotor. The supernatant (S1) was placed on ice, and the pellet (P1) was resuspended in 3.0 ml of homogenization buffer and recentrifuged as indicated above. The two supernatants were combined and centrifuged at 6,000 *g* for 5 min. The pellet, representing the crude synaptosomal fraction (P2), was resuspended at 10 × v/ww in synaptosomal assay buffer containing (mM): NaCl 134; KCl 3.6; $MgCl_2$ 1.2; $CaCl_2$ 1.2; HEPES 10; pH 7.4 (buffer B). Synaptosomes were incubated at 30°C in the presence of buffer or the test compounds for 60 min to allow for the maximum translocation of PKC. At the end of the incubation period, the synaptosomes were washed by centrifugation at 6,000 *g* for 10 min, and the pellet was sonicated in 10 volumes of a buffer containing (mM): Tris-HCl 20; ethylenediaminetetraammonium (EDTA) 2.0; phenylmethylsolfonyl fluoride 0.2; ethyleneglycoltetraacetate (EGTA) 0.5; pH 7.4 (buffer C). The resuspension was centrifuged at 45,000 *g* for 10 min to separate the particulate (membrane) from the cytosolic fraction. The final pellet was resuspended in 10 volumes of radioligand assay buffer (buffer B) and prepared for plating. These cortical membrane fractions were then used to assay the translocation of PKC binding sites as explained in the following subsection.

^{3}H-phorbol 12, 13 dibutyrate (^{3}H-PDBu) Binding to Treated and Untreated Tissue as a Measure of Membrane-Bound PKC Binding Sites

For radioligand binding, 40 μl (0.1 mg of protein as assessed by the method of Lowry et al., 1951) of membranes were plated onto 96-well plates (Nunc, Denmark) and allowed to equilibrate with 20 μl of either assay buffer or 3.0 μM phorobol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) to determine nonspecific binding. Scatchard analyses were performed to determine total binding and the initial binding parameters (K_D and B_{max}) using ^{3}H-phorbol dibutyrate (^{3}H-PDBu) (1–40 nM) (New England Nuclear, Boston, MA, specific activity 18.6–20.0 Ci/mmol) in a total well volume of 200 μl for 45 minutes at room temperature. One-point determinations were used to assay the density of membrane PKC binding sites (7 nM ^{3}H-PDBu) in some experiments. The tissue was harvested onto glass-fiber filtermats (Titertek; coated with 0.1% polyethylimine (PEI) to reduce nonspecific binding) using a Titertek cell harvester, and the filters were placed in scintillation vials containing 3.0 ml Liquiscint (National Diagnostics). Radioactive samples were counted for 5.0 min in a Beckman liquid scintillation counter (efficiency: 40%). CPM data were converted to pmol of ^{3}H-PDBu bound/mg protein.

Data Analysis

^{3}H-PDBu binding curves were analyzed using an iterative curve-fitting program (Ligand) (Munson and Rodbard 1980). All graphs were produced using Sigmaplot for Windows (version 4.0), and all regression analyses were done using the resident curve-fitting program.

Statistics

One-way and two-way analyses of variance (ANOVA) and the post-hoc Tukey test were used for multiple comparisons at a minimum significance level of $p \leq .05$. Student's *t*-test was used when applicable for simple two-sample tests at the same minimum significance level. Statistical data were expressed as mean (±SD or SEM) of the indicated number of observations. In some figures, a representative graph is used to express the results of a particular experiment that was repeated at least three times.

RESULTS

Effect of MDMA, pCA, and 5-HT Receptor Agonists on PKC Translocation in Synaptosomes

The binding characteristics for ^{3}H-PDBu in cortical synaptosomes were not significantly different from those obtained in membrane preparations from the same region with respect to ligand affinity (K_D = 8.21 ± 0.56 nM in synaptosomes versus 9.01 ± 0.21 nM in membranes) (Figure 1). However, the B_{max} of phorbol ester binding to synaptosomal membranes was approximately 50% lower than that measured in membranes prepared from whole brain regions. Synaptosomes prepared from rat corticies were incubated with drugs for 60 min, and all compounds were tested over a wide range of concentrations that bordered their reported K_D for their respective receptors. The 60-min interval was chosen, because our pilot studies showed that MDMA exhibited is most robust effect at this time point (data not shown). MDMA produced a dose-dependent increase in particulate PKC binding sites beginning at a concentration of 1 μM (control: 5.25 ± 0.34 pmol/mg protein v. 6.99 ± 0.07 pmol/mg; $p \leq 0.05$), and this effect reached a plateau at 100 μM (9.89 ± 0.33 pmol/mg; $p \leq 0.01$). MDMA's EC_{50} value for PKC translocation was approximately 2.52 μM. At 100 μM, MDMA's effect seemed to be somewhat desensitized from its peak levels at 1 μM (7.99 ± 0.05 pmol/mg at 1.00 μM v. 9.89 ± 0.33 pmol/mg at 100 μM); however, this response remained significantly above control. In comparison, neither the 5-HT_{1A}-receptor agonist, ipsapirone (IPS), nor the 5-$HT_{2A/2C}$-receptor agonist, (±)-2,5-dimethoxy-4-bromoaminopropane (DOB), was able to induce a significant translocation of ^{3}H-PDBu sites to the plasma membrane using this synaptosomal preparation (Table 1).

Synaptosomes were then pretreated with fluoxetine (100 nM) 30 min before the addition of MDMA, IPS, or DOB. Fluoxetine had no significant effect on the density of PKC binding sites by itself or when co-administered with IPS or DOB. However, fluoxetine was capable of totally abolishing PKC translocation by MDMA in synaptosmal fractions across all concentrations tested (0.1–100 μM, Table 2). pCA, which is a potent activator of PKC in vivo, was similarly effective in synaptosomal

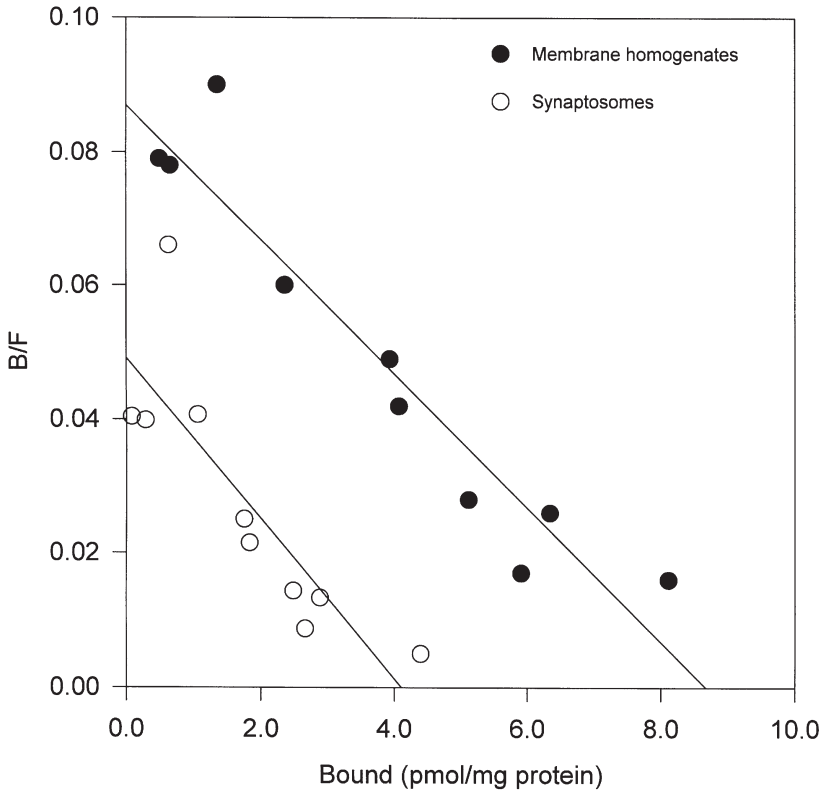

Figure 1. Scatchard transformation of ^{3}H-PDBu binding to cortical membranes and membranes of homogenates derived from cortical synaptosomes. ^{3}H-PDBu (1–40 nM) was incubated either in the presence of Locke's buffer (total binding) of 3 μM PMA (nonspecific) for 45 min at room temperature. The data points represent specific binding of ^{3}H-PDBu to rat brain cortical homogenates (closed circles) or cortical synaptosomal membranes (open circles). This graph represents an experiment that was performed three times. The data were fit by a one-site model (Hill coefficient: 0.957). Results are represented as the K_D (9.01 ± 0.21 nM) and B_{max} (8.88 ± 0.84 pmol of ^{3}H-PDBu bound/mg protein) in cortical membranes (closed circles) and K_D (8.21 ± 0.56 nM) and B_{max} (4.25 ± 0.65 pmol of ^{3}H-PDBu bound/mg protein) in synaptosomes (open circles).

Table 1. Synaptosomal Activation of PKC by Serotonergic Drugs

Compound	Specific ^{3}H-PDBu Binding (pmol/mg Prot.)
Control	5.25 ± 0.34
DOB (nM)	
0.1	5.12 ± 0.14
0.5	5.17 ± 0.47
1	5.25 ± 0.09
5	5.35 ± 0.32
10	5.18 ± 0.44
50	5.23 ± 0.03
100	5.20 ± 0.11
Ipsapirone (nM)	
0.1	5.34 ± 0.55
0.5	5.40 ± 0.23
1	5.00 ± 0.62
5	4.78 ± 0.34
10	5.11 ± 0.47
50	5.42 ± 0.05
100	5.40 ± 0.21
MDMA (μM)	
0.1	5.27 ± 0.08
0.5	5.32 ± 0.04
1	6.99 ± 0.07[a]
5	8.37 ± 0.13[a]
10	8.50 ± 0.11[a]
50	9.89 ± 0.33[b]
100	7.99 ± 0.05[a]

Synaptosomes were prepared as indicated (from rat cortex) in the methods section and incubated with the indicated drugs at the concentrations listed for 60 min. The drugs were washed out by centrifugation and membranes prepared for ^{3}H-PDBu (7 nM) binding to assess any PKC translocation.

[a] $p \leq .05$ by ANOVA and the post-hoc Tukey test versus buffer-treated synaptosomes. Each point is the average ± SD of four determinations and each experiment was repeated at least three times.

[b] $p \leq .01$ by ANOVA and the post-hoc Tukey test versus buffer-treated synaptosomes.

preparations. pCA (5 μM) was able to increase the particulate density of PKC by 39.5% over control after a 60 min incubation (Figure 2). By comparison, MDMA (10 μM) was almost equally effective and increased the density of phorbol ester binding sites by 30.2%. Conversely, neither DOB (100 nM) nor ketanserin had any effect on PKC translocation in synaptosomes (control: 9.53 ± 0.36 pmol/mg, DOB: 8.76 ± 0.31 pmol/mg, and ketanserin: 9.42 ± 0.32 pmol/mg protein). Furthermore, co-incubation of synaptosomes with pCA or MDMA and ketanserin had no effect on amphetamine-mediated PKC translocation in our assay system (Figure 2).

Lesion Studies

Subsets of animals were pretreated with pCA (2 × 10 mg/kg) to lesion cortical 5-HT nerve terminals. This treatment reduces the density of 5-HT nerve terminals labeled by ^{3}H-paroxetine by ≥90% in rats (see Kramer et al. 1995). Synaptosomes prepared from the brains of saline- and pCA-treated rats were then exposed to DOB (100 nM), MDMA (10 μM), ketanserin (100 nM), and pCA (5 μM) in order to assess PKC translocation after the destruction of cortical 5-HT terminals (Figures 2 and 3). pCA pretreatment totally abolished the ability of in vitro MDMA or pCA to induce PKC translocation in synaptosomes. This treatment produced no change in either DOB's or ketanserin's inability to modify particulate PKC density.

Role of Calcium in SA-Mediated PKC Translocation in vitro

The next set of experiments investigated the role of calcium on the in vitro translocation of PKC by MDMA and pCA. These experiments were designed similarly to those in subsection "Effect of MDMA, pCA, and 5-HT receptor, agonists . . ." with the following changes. MDMA (10 μM) and pCA (10 μM) were incubated with cortical synaptosomes either in the presence of absence of fluoxetine (100 nM) or ketanserin (100 nM) in buffers containing physiological levels of calcium (1.2 mM) or 1 mM EGTA. In calcium-containing Locke's buffer, both pCA and MDMA elicited a potent translocation of PKC to the particulate fraction (Figures 4 and 5). PKC activation was again completely inhibited by the presence of 100 nM fluoxetine, but unaffected by ketanserin. However, when the drug incubations were carried out in a Ca^{2+}-free buffer, there were two noticeable effects. First, when synaptosomes are incubated in a calcium-free media, the density of PKC binding sites in the membrane fraction is reduced by approximately 50%, as compared with basal conditions (calcium-containing buffer (control binding): 5.01 ± 0.73 pmol/mg protein versus EGTA-containing buffer (control): 2.40 ± 0.40 pmol/mg protein, Figures 4 and 5). In the EGTA-containing buffer, neither MDMA nor pCA were able to promote any degree of PKC translocation. Consequently, all treatment groups were statistically similar with respect to the density of ^{3}H-PDBu binding sites when the tissue was incubated in a calcium-free buffer.

DISCUSSION

Many of the original studies that demonstrated MDMA and pCA's effects on uptake inhibition and neurotransmitter release took advantage of the synaptosomal (P2) preparation (Johnson et al. 1991; Berger et al. 1992a,b). The primary target of such drugs as amphetamine, methamphetamine, p-chloroamphetamine, and MDMA is the presynaptic nerve terminal, specifically the high-affinity uptake transporter (Rudnick and Wall 1992b). At low concentrations (≤500 nM), SAs inhibit re-uptake by competing with dopamine (DA) and 5-HT for the monoamine binding site (Steele et al. 1987; Johnson et al. 1991; Rudnick and Wall 1992a,b). At increasingly

Table 2. Fluoxetine Inhibition of MDMA-Mediated PKC Translocation in Synaptosomes

Compound	Specific ^{3}H-PDBu Binding (pmol/mg Prot.) Locke's Buffer	Specific ^{3}H-PDBu Binding (pmol/mg Prot.) w/Fluoxetine 100 nM
Control	5.25 ± 0.34	5.17 ± 0.33
DOB (nM)		
0.1	5.12 ± 0.14	5.42 ± 0.36
0.5	5.17 ± 0.47	5.56 ± 0.42
1	5.55 ± 0.09	5.72 ± 0.45
5	5.35 ± 0.32	5.12 ± 0.36
10	5.18 ± 0.44	5.18 ± 0.71
50	5.23 ± 0.03	5.52 ± 0.52
100	5.20 ± 0.11	4.99 ± 0.23
Ipsapirone (nM)		
0.1	5.54 ± 0.55	5.88 ± 0.37
0.5	5.60 ± 0.23	5.10 ± 0.66
1	5.00 ± 0.62	4.74 ± 0.31
5	4.78 ± 0.34	4.88 ± 0.09
10	5.11 ± 0.47	5.18 ± 0.72
50	5.42 ± 0.05	5.65 ± 0.36
100	5.40 ± 0.21	4.87 ± 0.29
MDMA (μM)		
0.1	5.27 ± 0.08	4.73 ± 0.48
0.5	5.32 ± 0.04	5.17 ± 0.44
1	6.99 ± 0.07[a]	5.16 ± 0.14[c]
5	8.37 ± 0.13[b]	5.42 ± 0.55[c]
10	8.50 ± 0.11[b]	4.99 ± 0.35[c]
50	9.89 ± 0.33[b]	5.00 ± 0.28[c]
100	7.99 ± 0.05[a]	5.04 ± 0.74[c]

Synaptosomes were prepared as indicated (from rat cortex) in the methods section and incubated with the indicated drugs at the concentrations listed for 60 min. Fluoxetine (100 nM) was added to some synaptosomes 30 min prior to receiving the experimental drugs as part of a competition study. ^{3}H-PDBu (7 nM) binding was performed as explained in the methods section.

[a] $p \leq .05$ by 01 by ANOVA and the post-hoc Tukey test versus buffer-treated synaptosomes. Each point is the average ± SD of four determinations, and each experiment was repeated at least three times.

[b] $p \leq .01$ by ANOVA and the post-hoc Tukey test versus buffer-treated synaptosomes.

[c] $p \leq .05$ by ANOVA and the post-hoc Tukey test versus MDMA-alone treated synaptosomes.

higher concentrations (≥1 μM), uptake inhibition becomes a neurotransmitter release because of a reversal of the transporter protein or "reverse-flux" (Hekmatpanah and Peroutka 1990). In short, the combination of MDMA's actions on 5-HT re-uptake, release, and metabolism produces an increase in synaptic 5-HT levels, which is believed to lead to the degeneration of fine 5-HT axons (Schmidt 1987; Kokotos Leonardi and Azmitia 1994). From this evidence—and the unique relationship between SAs and the presynaptic nerve terminal—the use of synaptosomes has been a valuable tool in the study of drug action.

The the present report, MDMA induced a significant translocation of PKC to the membrane fraction (Table 1 and Figure 2). MDMA's EC_{50} for PKC translocation in synaptosomes was estimated by a nonlinear, curve-fitting program (Jandel Scientific) to be approximately 2.5 μM. This concentration is within MDMA's optimal concentration range for stimulating ^{3}H-5-HT release from preloaded synaptosomes ($EC_{50\text{-release}}$ = 300 nM-7.96 μM) (Johnson et al. 1991; Berger et al. 1992b; Rudnick and Wall 1992b). This suggests that the PKC-activating properties of MDMA and pCA are linked to their NT release response, which occurs be means of a drug-SERT interaction. In vivo, the stimulation of PKC translocation by MDMA occurs with doses known to induce acute and long-term 5-HT depletion and terminal degeneration (Schmidt 1987; Rudnick and Wall 1992a; Kramer et al. 1997, Table 1).

Two of our experiments support the hypothesis that an interaction of SAs with the SERT is required for PKC translocation in synaptosomes. First, fluoxetine (100 nM) abolished with PKC-activating effects of MDMA in vitro, although fluoxetine, itself, was devoid of any PKC-activating properties when investigated in vivo or in vitro (Kramer et al. 1997, Table 2). Fluoxetine was a higher affinity for the SERT (IC_{50} against ^{3}H-paroxetine = 15 nM) than MDMA, and inhibits MDMA-induced ^{3}H-5-HT release at very low concentrations (40 nM) (Hekmatpanah and Peroutka 1990). Studies by Berger et al. (1992a) showed that such nonamphetamine, 5-HT uptake inhibitors as fluoxetine bind to regionally dis-

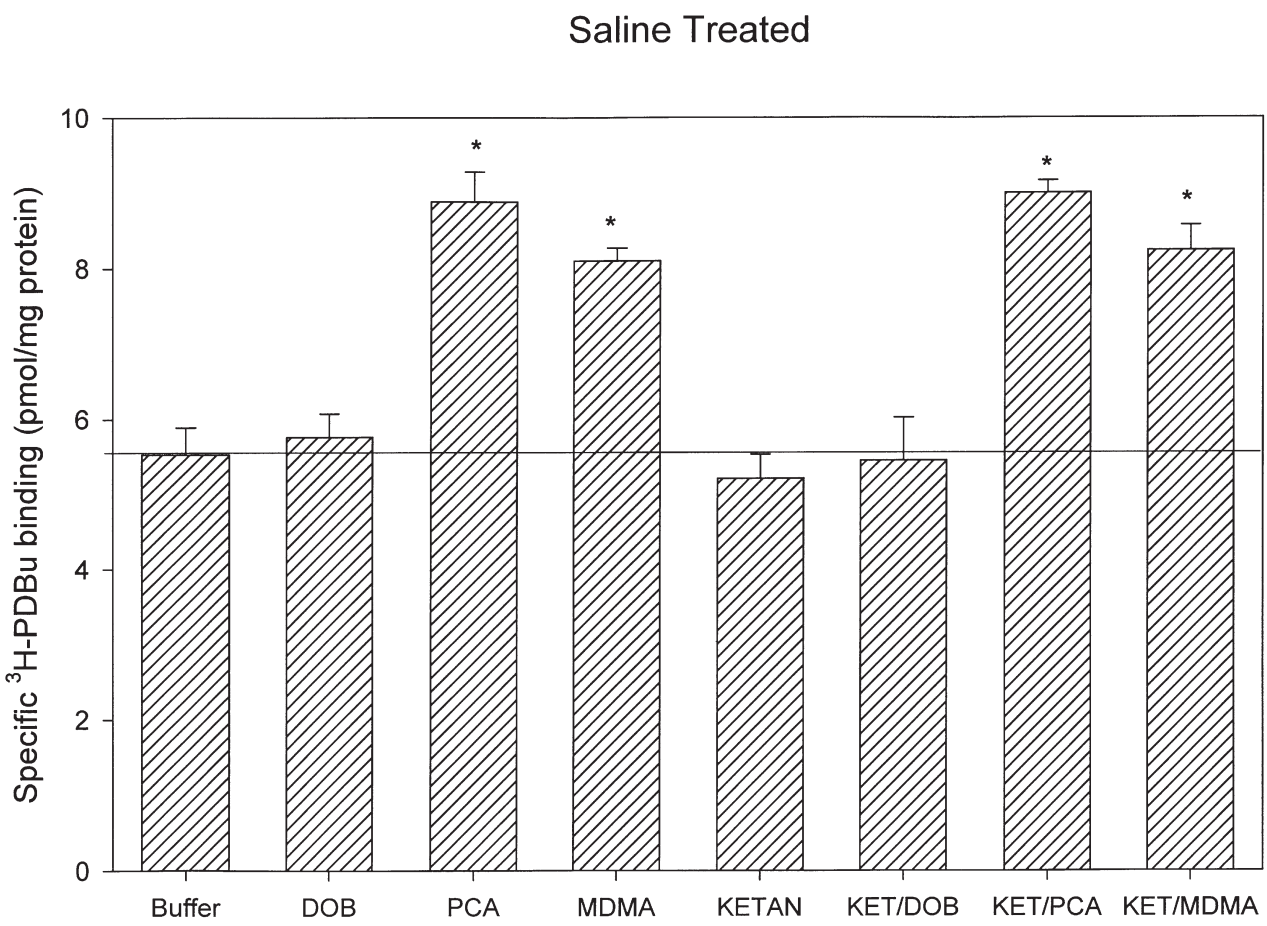

Figure 2. Efficacy of DOB, MDMA, pCA, and ketanserin at mediating PKC translocation in prepared cortical synaptosomes. Synaptosomes were prepared fresh from the corticies of naive rats. These synaptosomes were incubated alone or in combination with DOB (100 nM), pCA (5 μM), MDMA (10 μM), or ketanserin (100 nM) for 60 min. After the drugs were removed, membranes were prepared for ^{3}H-PDBu binding to assess PKC translocation as described in the methods section. Each bar is the average ± SD of four determinations, and each experiment was repeated at least three times. *$p \leq .05$ from control treated synaptosomes by ANOVA and the post-hoc Tukey test.

tinct sites on the SERT from substituted amphetamines. Fluoxetine has been shown to be effective for prevention of the acute and neurotoxic effects of MDMA, and its inhibitory effects against PKC translocation are probably attributable to its ability to block MDMA from gaining access to its binding site on the 5-HT transporter (Schmidt 1987; Hekmatpanah and Peroutka 1990; Berger et al. 1992a).

Second, viable 5-HT nerve terminals seem to be required for such drugs as MDMA and pCA to induce PKC translocation in vitro (Figures 2 and 3). In this experiment, cortical synaptosomes were prepared from rats that received a neurotoxic dose of pCA (2 × 10 mg/kg), followed by a 2-week washout period. PCA selectively destroys 5-HT nerve terminals and reduces the number of cortical ^{3}H-paroxetine-labeled sites by 90%, as compared to saline treated controls (Mamounas and Molliver 1988; Kramer et al. 1995). The loss of ^{3}H-paroxetine binding sites is correlated with the morphological fragmentation of 5-HT-IR axons in the cortex, and these changes are indicative of nerve terminal degeneration (Commins et al. 1987; Battaglia et al. 1988b). A 14-day recovery period is required for development of nerve terminal degeneration, elimination of any residual drug, and cessation of any stress-induced physiological changes. This in vitro work extends and supports the findings of our previous in vivo studies, where a similar dose of pCA prior to an in vivo challenge with MDMA also prevented PKC translocation (Kramer et al. 1995). These data indicate that the prior destruction of 5-HT fibers removes a crucial MDMA bindings site required for PKC translocation. By eliminating MDMA's preferential site of action (the SERT), there is also an attenuation of MDMA's effect on synaptosomal PKC translocation.

Activation of the 5-HT$_2$ receptor family induces the translocation/activation of PKC in brain slices (Kagaya et al. 1990; Wang and Friedman 1990). In synaptosomes, the 5-HT$_{2A/2C}$-receptor agonist (DOB), was unable to increase the particulate density of ^{3}H-PDBu binding sites.

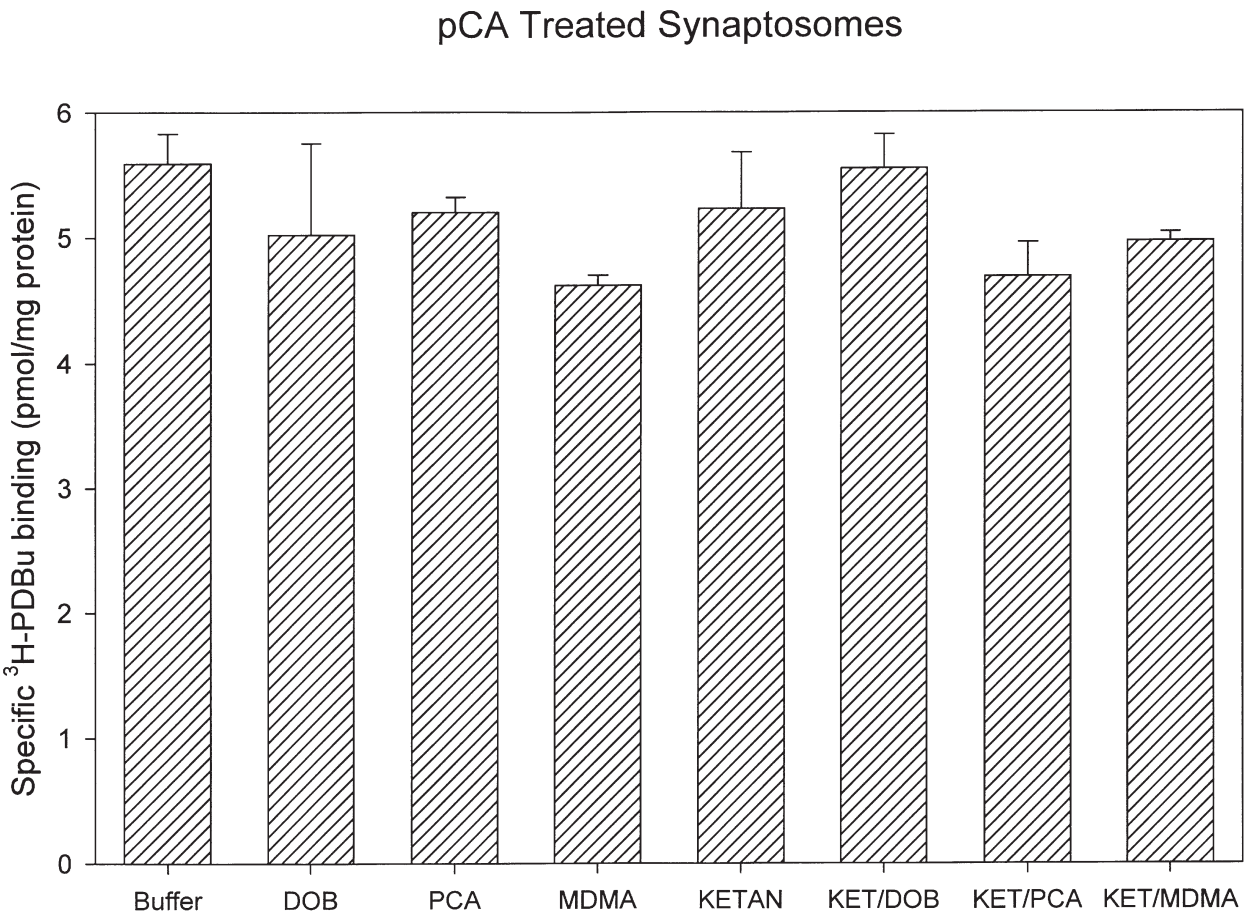

Figure 3. MDMA and pCA require viable 5-HT nerve terminals in order to translocate PKC in synaptosomes. Rats received either two injections of saline or pCA (10 mg/kg) over 2 days. The animals were allowed to sit undisturbed for 2 weeks until sacrifice. Synaptosomes were prepared fresh from the corticies of naive rats. These synaptosomes were incubated alone or in combination with DOB (100 nM), pCA (5 μM), MDMA (10 μM), or ketanserin (100 nM) for 60 min. After the drugs were removed, membranes were prepared for ^{3}H-PDBu binding to assess PKC translocation as described in the methods section. Each bar is the average ± SD of four determinations, and each experiment was repeated at least three times.

Therefore, synaptosomes seem to be insensitive to the PKC-activating properties of postsynaptic 5-$HT_{2A/2C}$ agonists, suggesting that their contribution is minimal in this preparation (Kagaya et al. 1990; Wang and Friedman 1990). This response is not surprising, because 5-$HT_{2A/2C}$ receptors are not indigenous to 5-HT axons or nerve terminals (Quik and Azmitia 1983; Fischette et al. 1987). Similarly, ipsapirone, a 5-HT_{1A}-receptor agonist, was unable to increase the number of PKC binding sites within the particulate fraction. Ipsapirone can be considered a negative control in these experiments, because the 5-HT_{1A} receptor is coupled to the inhibitory G-protein, $G_{i\alpha}$, which is linked to adenylyl cyclase activity (Harrington et al. 1994). Ketanserin, a 5-$HT_{2A/2C}$ antagonist, (100 nM applied 30 min before MDMA) was unable to attenuate the PKC-stimulating effect of pCA and MDMA in synaptosomes. In vivo, ketanserin pretreatment only partially (by 30%) inhibited MDMA-mediated PKC translocation (Kramer et al. 1997). This modulation probably represents the attenuation of PKC activation that is produced by 5-$HT_{2A/2C}$ receptor stimulation in astrocytes and interneurons that express these sites (Conn and Sanders-Bush 1985, 1986; Blue et al. 1988; Hirst et al. 1994; Poblete and Azmitia 1995). Consequently, MDMA may be stimulating PKC translocation, in part, through a non-5-$HT_{2A/2C}$ receptor-mediated mechanism, and this could explain why ketanserin had not effect on MDMA-induced PKC activation in synaptosomes.

MDMA's parent compound, AMPH, has been shown to increase PKC activity both in vivo and in vitro (Giambalvo 1992a,b). AMPH decreases PKC's K_M for cytosolic free calcium, and potentiates its overall catalytic function at resting intracellular Ca^{2+} levels (Giambalvo 1992b). In synaptosomes, PKC translocation produced by AMPH and MDMA does not seem to be affected by DA and 5-HT receptor antagonists, respectively, including those directed against presynaptic autoreceptors. The

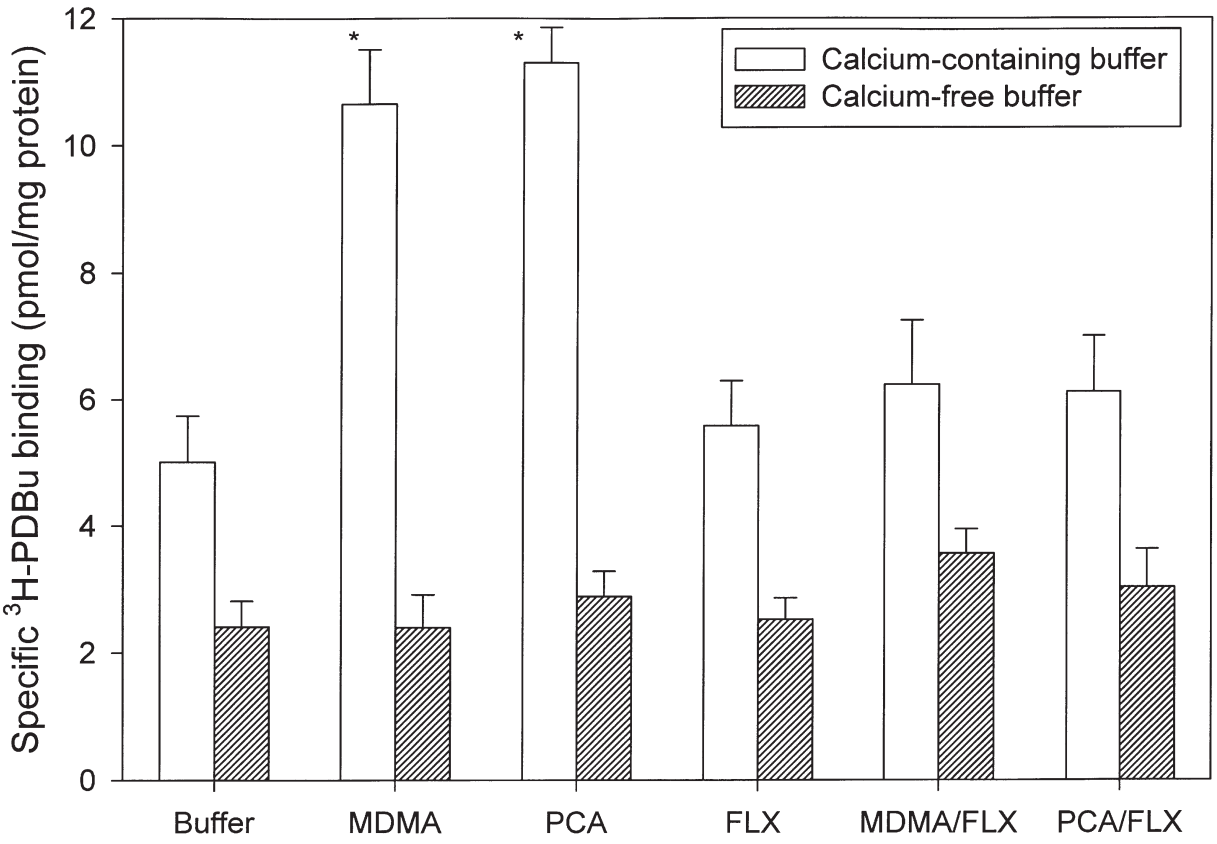

Figure 4. The presence of extracellular calcium is required for substituted amphetamines to translocate PKC: reversal by fluoxetine. Synaptosomes were prepared fresh from the corticies of naive rats. Before the addition of experimental drugs, the final synaptosomal pellet was split and resuspended in Ca^{2+}-containing (1.2 mM) or Ca^{2+}-free (1 mM EGTA) Locke's buffer. These synaptosomes were incubated alone or in combination with pCA (10 μM), MDMA (10 μM), or fluoxetine (100 nM) for 60 min. After the drugs were removed, membranes were prepared for ^{3}H-PDBu binding to assess PKC translocation (in Ca^{2+}-containing buffer) as described in the methods section. Each bar is the average ± SD of four determinations and each experiment was repeated at least three times. *$p \leq .05$ from control-treated cells by ANOVA and the post-hoc Tukey test.

PKC effects of AMPH were also unaffected by pretreatment of rats—24 h before the preparation of synaptosomes—with the nonspecific receptor-coupling agent, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), which irreversibly inactivates 70 to 80% of 5-HT and DA receptors (Meller et al. 1985; Giambalvo 1992b). Overall, the activation of PKC by amphetamine analogs in synaptosomes seems to be a monoamine receptor-independent event mediated through transporter proteins.

An extended period of elevated intracellular calcium concentration is central to the mechanisms that prolong the association of PKC with neuronal membranes (Manev et al. 1988, 1989). Calcium also seems to be an important cofactor for the binding of PKC to biological membranes under basal conditions (Melloni et al. 1985). When synaptosomal ^{3}H-PDBu binding was performed in the absence of extracellular calcium the number of membrane-bound PKC binding sites was reduced by 50% (Figures 4 and 5). In addition, both MDMA and pCA were unable to induce any significant PKC translocation in synaptosomes incubated in this Ca^{2+}-free condition. This suggests that PKC translocation by MDMA and pCA requires elevated concentrations of intracellular calcium within the 5-HT nerve terminal.

In synaptosomes, MDMA has also been shown to stimulate the uptake of extracellular calcium into forebrain synaptosomes (Park and Azmitia 1991; Azmitia et al. 1993). MDMA (10 μM) increases the uptake of $^{45}Ca^{2+}$ by 40% over control in basal (4.5 mM K^+) and stimulating conditions (68.5 mM K^+), indicating that the state of membrane depolarization is not a factor (Park and Azmitia). These results are surprising when analyzed against the calcium-independent nature of MDMA-mediated 5-HT release (Nichols et al. 1982). Although the exact mechanism of calcium entry into synaptosomes by MDMA is not known, work by Azmitia et al. (1993) suggests that the L-type calcium channel may be involved. The presence of nimodipine, a selective L-type calcium channel antagonist, reduces calcium uptake by MDMA and prevents the decrease in intracellular 5-HT that occurs after prolonged MDMA exposure to fetal 5-HT neurons (Azmitia et al. 1990; Azmitia et al. 1993; Gu and

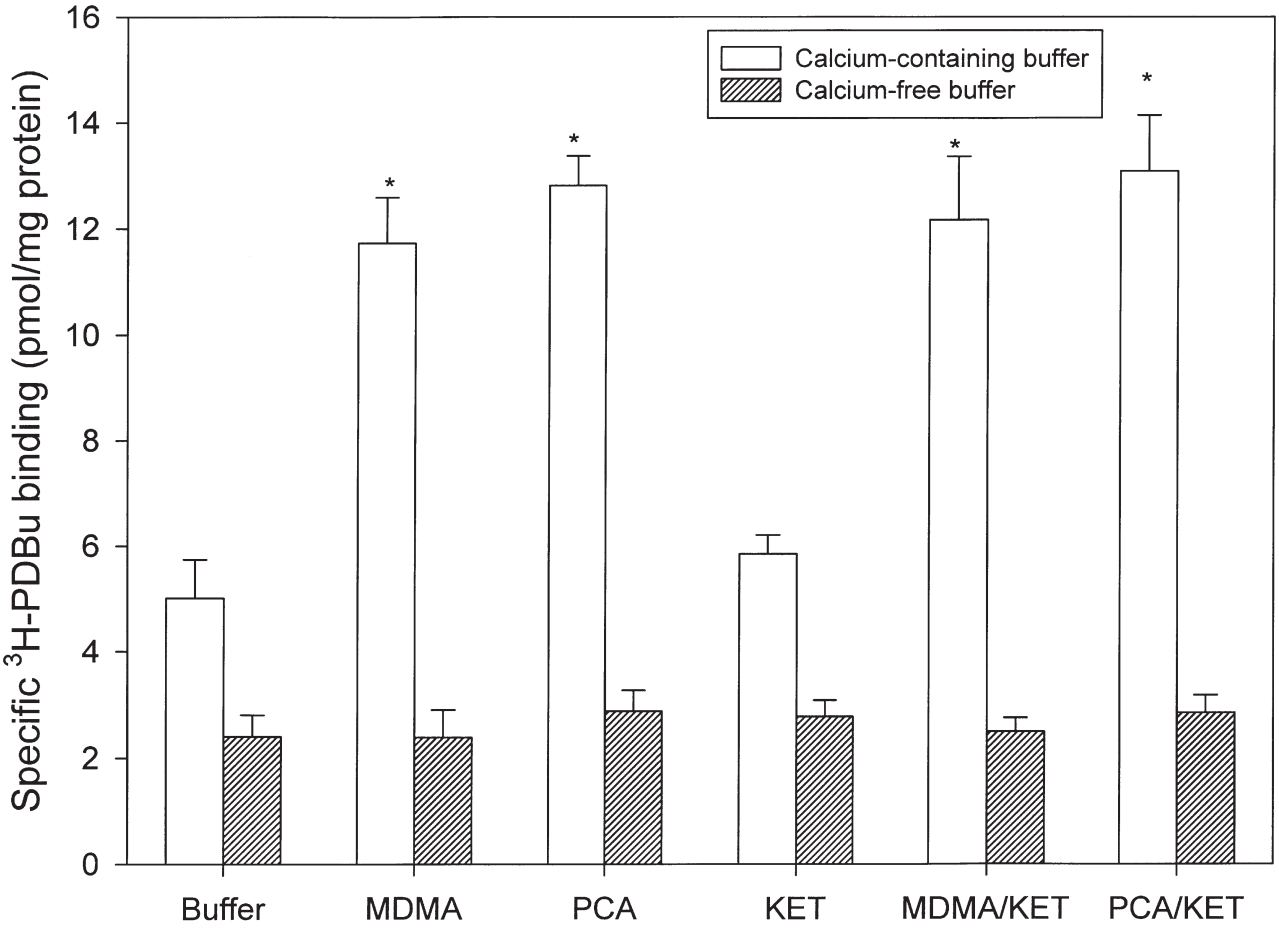

Figure 5. The presence of extracellular calcium is required for substituted amphetamine to translocate PKC: ketanserin is not effective at modulating this response. Synaptosomes were prepared fresh from the corticies of naive rats. Before the addition of experimental drugs, the final synaptosomal pellet was split and resuspended in Ca^{2+}-containing (1.2 mM) or Ca^{2+}-free (1 mM EGTA) Locke's buffer. These synaptosomes were incubated alone or in combination with pCA (10 μM), MDMA (10 μM), or ketanserin (100 nM) for 60 min. After the drugs were removed, membranes were prepared for ^{3}H-PDBu binding to assess PKC translocation (in Ca^{2+}-containing buffer) as described in the methods section. Each bar is the average ± SD of four determinations, and each experiment was repeated at least three times. *$p \leq .05$ from control-treated cells by ANOVA and the post-hoc Tukey test.

Azmitia 1993). The use of different types of calcium channel blockers—some directed against VSCC and others against ligand-gated calcium channels—are effective at reducing the chronic, but not the acute, depletion of 5-HT produced by MDMA in vivo and in vitro (Farfel et al. 1992; Schmidt et al. 1992; Gu and Azmitia 1993). In addition, calcium entry through L-type calcium channels has been associated with the release of intracellular calcium from ryanodine-sensitive stores in cardiac and other peripheral tissues (Suda et al. 1997; Takagishi et al. 1997). This type of extracellular–calcium-stimulated intracellular calcium release may provide a lasting source of cations of prolong PKC translocation. Thus, an interesting relationship between the action of SAs on neurotransmitter uptake inhibition and calcium uptake seems to exist, and this response could be crucial to the mechanism that prolongs PKC translocation within the presynaptic nerve terminal.

As stated earlier, PKC activation is usually associated with stimulation of receptors linked to PIP_2 hydrolysis (Conn and Sanders-Bush 1985, 1986). PKC translocation to the plasma membrane, in the absence of diacylglycerol (DAG) formation and other constituents of PIP_2 hydrolysis, can be achieved by increasing intracellular calcium levels above 1 μM (Melloni et al. 1985). When intracellular Ca^{2+} levels remain elevated, PKC can exist in an "membrane-associated" form, one with a significant catalytic activity, for several hours in the absence of diacyglycerol formation (Mahoney and Huang 1995). The presence of DAG within the inner portion of the lipid bilayer stabilizes the interaction between PKC and the membrane and potentiates its catalytic activity (Mahoney and Huang 1995). Therefore, even in the absence of PIP_2-linked receptor stimulation, amphetamine derivatives may be capable of activating PKC by increasing $[Ca^{2+}]_I$. Furthermore, receptor-medi-

ated PKC translocation is usually a transient event, lasting on the order of only a few seconds before regulatory systems ensure its deactivation (Manev et al. 1990). Therefore, for extended PKC activation to occur within the nerve terminal, a sustained increase in intracellular Ca^{2+} ionic needs to be provided, and this may be satisfied by increases in extracellular calcium uptake or release from internal sequestration sites (Manev et al. 1990). Prolonged PKC translocation may require the contribution of such other mechanisms as increased intracellular calcium concentration to occur. Although it is known that PKC has a strong presence within the 5-HT nerve terminal, all the mechanism by which it can be activated remains to be fully elucidated.

Protein kinase C has been shown to contribute to diverse biochemical processes within the 5-HT nerve terminal. These mechanisms include the acceleration of neutrite extension during development, the enhancement of vesicular neurotransmitter release, and the modulation of 5-HT transporter activity (Benowitz et al. 1987; Gandhi and Jones 1991; Qian et al. 1997). The rapid modulation of these activities suggests that a pool of this enzyme is readily available within the 5-HT nerve terminal and is sensitive to pharmacological manipulation. Qian et al., (1997) has reported that the PKC activation in cells transfected with the human SERT (hSERT) decreases the V_{max} of 5-HT uptake, and this change is attributable to the physical loss of uptake proteins from the cell surface. PKC-mediated phosphorylation also modulates the activity of several other reuptake proteins including those for dopamine, GABA, and glutamate (Miller and Hoffman 1994; Zhang et al. 1997). It is possible that neurotransmitter uptake inhibition, caused by the action of antidepressants and amphetamine derivatives (MDMA), involves the direct phosphorylation of monoamine transporters.

Two preliminary conclusions can be drawn from these experiments: (1) substituted amphetamines elicit their stimulatory effects on synaptosomal PKC translocation through their association with the SERT; and (2) PKC localized to the 5-HT nerve terminals is sensitive to activation by substituted amphetamines. Therefore, two pools of substituted amphetamine-sensitive PKC seem to be present in the rat cortex: one located in SERT expressing neurons and/or glia, while the second may exist in cells that express postsynaptic $5\text{-}HT_{2A/2C}$ receptors. MDMA activates PKC in $5\text{-}HT_2$-expressing cells in vivo, but this requires several steps, including the release of 5-HT, a prolonged increase in extracellular neurotransmitter levels, followed by the stimulation of postsynaptic $5\text{-}HT_2$ receptors. Subsequent studies investigating the involvement of the PKC pool in glial cells and interneurons should be performed to clarify this interesting response further.

In conclusion, substituted amphetamine-mediated PKC translocation occurs within serotonergic nerve terminals, and this seems to occur in the absence of 5-HT receptor stimulation. This response is dependent upon the presence of viable 5-HT axons and requires extracellular calcium and possibly Ca^{2+} uptake into the nerve terminal. Once activated, PKC may modulate local mechanisms within the nerve terminal, which increases a cell's susceptibility to damage by MDMA. Three interesting physiological responses in 5-HT nerve terminals are known to be influenced by changes in PKC activity, including vesicular neurotransmitter release, neurotransmitter re-uptake, and the normalization of intracellular calcium levels. Each of these processes has been shown to contribute to MDMA-mediated neurotoxicity. We now have evidence that MDMA produces PKC translocation through two separate, but interrelated mechanisms, through an interaction with the SERT, and after the stimulation of postsynaptic $5\text{-}HT_{2A/2C}$ receptors. Thus, prolonged PKC activation may begin a specific series of metabolic changes within several types of susceptible cells (neurons and glial cells), which contribute to the neurotoxic potential of MDMA.

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