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Amphetamine Increases the Phosphorylation of Neuromodulin and Synapsin I in Rat Striatal Synaptosomes

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ABSTRACT Amphetamine is taken up through the dopamine transporter in nerve terminals and enhances the release of dopamine. We previously found that incubation of rat striatal synaptosomes increases phosphorylation of the presynaptic neural-specific protein, neuromodulin (Gnegy et al., *Mol. Brain Res.* 20:289–293, 1993). Using a state-specific antibody, we now demonstrate that incubation of rat striatal synaptosomes with amphetamine increases levels of neuromodulin phosphorylated at ser^{41}, the protein kinase C substrate site. Phosphorylation was maximal at 5 min at 37°C at concentrations from 100 nM to 10 μM amphetamine. The effect of amphetamine on the phosphorylation of synapsin I at a site specifically phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (site 3), was examined using a state-specific antibody for site 3-phosphosynapsin I. Incubation with concentrations of amphetamine from 1 to 100 nM increased the level of site 3-phospho-synapsin I at times from 30 sec to 2 min. The effect of amphetamine on synapsin I phosphorylation was blocked by nomifensine. The presence of calcium in the incubating buffer was required for amphetamine to increase the level of site 3-phospho-synapsin I. The amphetamine-mediated increase in the content of $phosphoser^{41}$-neuromodulin was less sensitive to extrasynaptosomal calcium. The amphetamine-mediated increase in the content of site 3-phospho-synapsin I persisted in the presence of 10 μM okadaic acid and was not significantly altered by D1 or D2 dopamine receptor antagonists. Preincubation of striatal synaptosomes with 10 μM of the protein kinase C inhibitor, Ro-31-8220, blocked the amphetamine-mediated increases in the levels of both $phosphoser^{41}$-neuromodulin and site 3-phospho-synapsin I. Our results demonstrate that amphetamine can alter phosphorylation-related second messenger activities in the synaptosome. **Synapse 26:281–291, 1997.**

INTRODUCTION

Amphetamine (AMPH) is a powerful psychostimulant whose abuse is escalating in the United States (Miller and Hughes, 1994). In humans and laboratory animals, AMPH induces hyperkinesis and stereotyped behaviors. AMPH increases locomotor and stereotyped behaviors predominantly through enhanced release of dopamine (DA) in the striatum and nucleus accumbens (Segal and Kuczenski, 1994; Seiden et al., 1993). Uptake through the DA transporter is critical for the ability of AMPH to release dopamine and elicit hyperactive behavior (Giros et al., 1996; and see references in Seiden et al., 1993). AMPH-induced DA release is postulated to occur through an exchange-diffusion process (Fischer and Cho, 1979; Liang and Rutledge, 1982). Depending upon the dose, AMPH can release DA from both cytoplasmic and vesicular stores (see references in Seiden et al., 1993). Although AMPH-induced DA release is Ca^{2+}-independent (Raiteri et al., 1979; Rutledge, 1978) there are AMPH-mediated activities that appear to involve Ca^{2+}. Low concentrations of AMPH stimulate DA synthesis in striatal synaptosomes by a Ca^{2+}-dependent mechanism (Fung and

Abbreviations: AMPH,d-amphetamine sulfate; CaM, calmodulin; CaM Kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; DA, dopamine; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; KRB, Krebs ringer buffer; NM, neuromodulin; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-O-tetradecanoylphorbol 13-acetate; SDS, sodium dodecyl sulfate.

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Uretsky, 1982; Roberts and Patrick, 1979; Schwarz et al., 1980; Uretsky and Snodgrass, 1977). In addition, AMPH can increase protein kinase C activity in striatal synaptoneurosomes through a decrease in the Km for Ca^{2+} (Giambalvo, 1992a,b).

We found that AMPH increased phosphorylation of the neural-specific protein neuromodulin (NM; GAP-43, B-50, F1) in rat striatum after acute intraperitoneal injection and in striatal synaptosomes after incubation (Gnegy et al., 1993; Iwata et al., 1996). NM binds calmodulin (CaM), actin and Go and is important for nerve growth, neurotransmitter release, and synaptic plasticity (Coggins and Zwiers, 1991; Skene, 1989). PKC-mediated phosphorylation of NM at ser^{41}, which adjoins the CaM-binding region (Coggins and Zwiers, 1989), results in the dissociation of bound CaM (Alexander et al., 1987). The presence of phospho-ser^{41} on NM appears to be important for the ability of NM to positively modulate neurotransmitter release (Dekker et al., 1989, 1990; Hens et al., 1995). We found an increase in content of phosphoser^{41}-NM in rat striatum upon withdrawal from repeated, intermittent AMPH (Iwata et al., 1996). Upon withdrawal from this regimen of repeated, intermittent AMPH, rats exhibit behavioral sensitization to AMPH, as expressed by a more rapid onset of stereotyped behaviors, more intense stereotyped movements, and a marked increase in AMPH-induced rotation (Robinson, 1991; for review, see Robinson and Becker, 1986). An enhanced stimulus-induced release of dopamine upon withdrawal has been consistently measured in both striatum (Robinson and Becker, 1982) and nucleus accumbens (Robinson et al., 1988) of amphetamine sensitized animals. The phosphorylation state of another CaM-binding protein involved in neurotransmitter release, synapsin I, was also enhanced in striatum of rats treated with repeated, intermittent AMPH (Iwata et al., 1996). Using a state-specific antibody, we found that the phosphorylation state of synapsin I at site 3 was increased after repeated AMPH. Site 3 is phosphorylated by Ca^{2+}/CaM-dependent protein kinase II (CaM Kinase II) and Ca^{2+}/CaM-dependent protein kinase IV. Synapsin I binds to the cytosolic surface of synaptic vesicles and to various cytoskeletal proteins such as F-actin, microtubules, neurofilaments and spectrin (De Camilli et al., 1990; Greengard et al., 1993; Valtorta et al., 1992). Phosphorylation of the nerve terminal-specific synapsin I by CaM Kinase II at sites 2 and 3 decreases its affinity for synaptic vesicles as well as for the cytoskeleton (De Camilli et al., 1990; Greengard et al., 1993; Valtorta et al., 1992) which may affect the number of vesicles available for release (Pieribone et al., 1995; Rosahl et al., 1995). Although acute as well as repeated AMPH increased NM phosphorylation in rat striatum, acute AMPH did not increase the phosphorylation of synapsin I (Iwata et al., 1996). Since AMPH was injected 30 min before striatum removal, a rapid AMPH-induced phosphorylation/dephosphorylation of synapsin I would have been undetected.

The goal of this study was to further investigate the AMPH-mediated phosphorylation of NM in synaptosomes and determine whether AMPH can increase CaM Kinase II activity as assessed by synapsin I phosphorylation. We validated a site of AMPH-mediated phosphorylation of NM as ser^{41} using a state-dependent antibody. In addition, using the state-dependent antibody for synapsin I, we found that incubation of synaptosomes with AMPH increased the content of site 3-phospho-synapsin I. Our results suggest that AMPH can alter phosphorylation-related second messenger activities in the synaptosome.

MATERIALS AND METHODS

Materials

d-AMPH sulfate was purchased from The University of Michigan Laboratory of Animal Medicine. Nomifensine was obtained from Hoechst (Frankfurt, Germany) and dissolved in an aqueous solution at 4 mM with lactic acid to lower the pH. Okadaic acid sodium salt was purchased from LC Laboratory (Woburn, MA). Sulpiride was obtained from the Laboratoire Etudes et Developement Chimiques (Arpajon, France) and first dissolved in dimethylsulfoxide to a concentration of 160 mM. SCH23390 maleate was obtained from Schering, Co. (Bloomfield, NJ) and dissolved in dimethylsulfoxide to a concentration of 40 mM. Ro31-8220 was obtained from Calbiochem-Novabiochemicals (San Diego, CA) and dissolved in dimethylsulfoxide to a concentration of 1.8 mM. ^{125}I-protein A was purchased from Amersham (Arlington Heights, IL). Myelin basic $protein_{4–14}$ and the protein kinase C inhibitor peptide were purchased from Upstate Biochemicals (Lake Placid, NY). [γ-^{32}P]ATP (spec. act. >4,000 Ci/mmol) was from ICN (Irvine, CA). The state-dependent antibody for phosphoser^{41}-NM (2G12/c7) was the generous gift of Dr. Karina Meiri, Department of Pharmacology, SUNY Health Science Center. The state-dependent antibody for site 3-phospho-synapsin I (RU19) was generously donated by Dr. Andrew Czernik, Laboratory of Molecular and Cellular Neuroscience, Rockefeller University.

Preparation of striatal synaptosomes

Synaptosomes were prepared as described by Dunkley et al. (1986). Striata from female Holtzman rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) were dissected using a brain cutting block (Heffner et al., 1980). The striatum was homogenized in a glass-teflon homogenizer in 10 vol of 0.32 M sucrose solution containing 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.25 mM dithiothreitol, 10 µM leupeptin, 10 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4. The homogenate was centrifuged at 1,000*g* for 10 min, the pellet washed, and the combined

supernatants were further centrifuged at 15,000*g* for 30 min. The P2 fraction was resuspended in 13 vol of sucrose solution and layered on Percoll (Pharmacia LKB Biotechnology, Piscataway, NJ) gradients, comprised of 2 ml each of 23, 15, 10, and 3% Percoll (v/v) in sucrose solution. The gradient was centrifuged at 32,500*g* for 5 min without braking. Fraction 4 was collected by aspiration and mixed with either Ca^{2+}-deficient Krebs Ringer Buffer (KRB; 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 24.9 mM $NaHCO_3$, 10 mM glucose, brought to pH 7.4 by oxygenation) for synapsin I measurement or Ca^{2+}-replete KRB containing 1.2 mM $CaCl_2$ for NM measurement. Synaptosomes were centrifuged at 15,000*g* for 15 min. The final pellet was resuspended in 4 vol of either Ca^{2+}-deficient KRB (for synapsin I measurements) or Ca^{2+}-replete KRB (for NM measurements) to give a protein concentration of approximately 1.2 mg/ml. The effect of AMPH on synapsin I phosphorylation was more consistent when synaptosomes were resuspended in Ca^{2+}-deficient KRB.

Synaptosome incubation

Phosphorylation assays were commenced by adding Ca^{2+}-containing KRB (final Ca^{2+} was 1.2 mM) with or without various concentrations of AMPH to synaptosomes for varying lengths of time at 37°C. Final incubation volume was 60 µl. The reaction was terminated with 20 µl SDS-stop solution (final concentration: 52.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue) and boiled at 100°C for 5 min. Nomifensine, okadaic acid, sulpiride, Ro31-8220, and SCH23390 were diluted from stock in Ca^{2+}-free KRB. Synaptosomes were preincubated with the drugs at 4°C for 20–60 min.

Immunoblotting

NM was separated by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto nitrocellulose paper using a Bio-Rad minitransfer apparatus (Bio-Rad Laboratories, Hercules, CA) at 30 V for 14 h. Each lane contained 10–15 µg protein for measurement of phosphoser^{41}-NM or 15 µg of protein for total NM. Blots were blocked in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4) containing 0.1% Tween 20 and 1% bovine serum albumin (blocking buffer), then incubated for 1 h with a monoclonal antibody (2G12/c7) (Meiri et al., 1991) specific for phosphoser^{41}-NM diluted 1:50 in blocking buffer or with antibody for total NM (10E8/E7) (Meiri et al., 1991) diluted 1:100 in blocking buffer. Blots were then incubated for 1 h with anti-mouse IgG coupled to alkaline phosphatase (Calbiochem-Novabiochem Co., San Diego, CA) diluted 1:20,000 in blocking buffer. Immunoreactivity on the blots was also visualized with ECL (enhanced chemiluminescence) purchased from Amersham Co. (Arlington Heights, IL). Results were analyzed by scanning the bands using a Hoefer GS365W scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). The peak areas were quantified by Gaussian integration using Hoefer GS365W electrophoresis data system.

Synapsin I was separated by 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Immobilon P, Millipore Co., Bedford, MA) using a Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) at 100 V for 1 h. For measurement of site 3-phospho-synapsin I, each lane contained 5–10 µg protein. Blots were incubated in blocking buffer for 1 h, then incubated for 2 h with antibody specific for site 3-phosphosynapsin I (RU19) diluted 1:50. The synapsin I antibody was affinity-purified rabbit polyclonal anti-peptide antibody (Czernik et al., 1991, 1995; Yamagata et al., 1995). In vitro, phosphorylation of synapsin I by CaM Kinase II occurs at an equivalent rate and to an equivalent stoichiometry at sites 2 and 3. Using two phosphopeptides corresponding to a distinct sequence surrounding each site, RU19 was determined to be specific for site 3 of synapsin I. Immunoreactivity on the blots was visualized with ^{125}I-protein A (2 mCi). Total radioactivity was quantified by a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA), and densitometry values were obtained. Immunoreactivity was also visualized using an anti-rabbit IgG coupled to alkaline phosphatase (Gibco, Gaithersburg, MD).

Measurement of protein kinase C activity

Synaptosomes were incubated with 1 µM amphetamine for 2 min. Two volumes of cold Ca^{2+}-deficient KRB was added to the incubation; the synaptosomes were pelleted and resuspended in a buffer consisting of 20 mM Tris HCl, pH 7.5, 10 µM leupeptin, and 1 mM PMSF. A set of control and AMPH-incubated synaptosomes were kept intact. After 15 min, the synaptosomes were sonicated for 15 sec and centrifuged at 100,000*g* for 60 min. The pellets were resuspended in a buffer containing 20 mM Tris HCl, pH 7.5 containing 1% Triton X-100, 10 µM leupeptin, and 1 mM PMSF. The membrane protein was diluted over 10-fold into the assay. Protein kinase C activity in non-lysed synaptosomes, supernatants, and pellets was measured in an assay (50 µl) containing 20 mM Tris HCl, pH 7.5, 5 mM magnesium acetate, 50 µM $CaCl_2$, 20 µM ATP (0.5 µC ^{32}P-ATP/assay), 25 µM myelin basic protein$_{4–14}$ with 1–2 µg sample protein. Phosphatidylserine (100 µg) and 10 µg diolein were included in assays of supernatant only. Blanks contained no myelin basic protein$_{4–14}$, which gave values comparable in value to those obtained when 1 µM protein kinase C inhibitor peptide was included in the assay. Samples were incubated for 4 min at 30°C. The reaction was stopped by spotting a 30 µl aliquot of the mixture onto a piece of P-81 paper

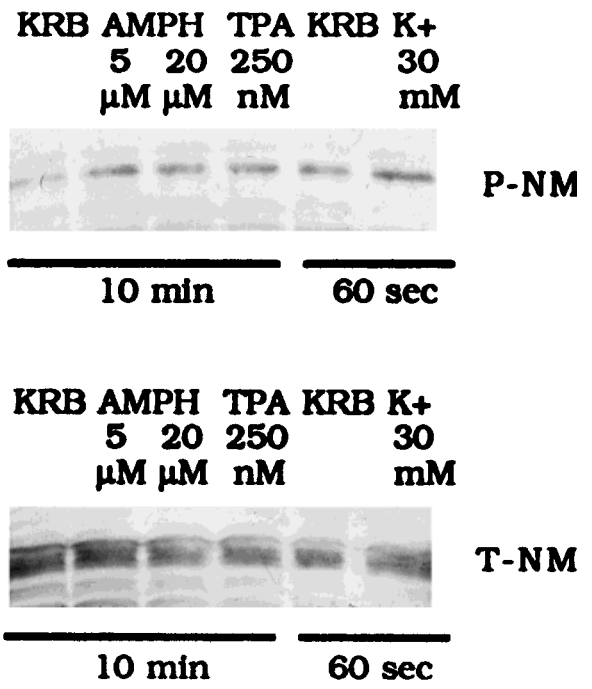


Fig. 1. Effect of AMPH, TPA, and 30 mM K^+ on the level of phosphoser[41]-NM in rat striatal synaptosomes. After preparation, Percoll-purified synaptosomes were resuspended in Ca^{2+}-containing KRB and incubated for 10 min with 5 or 20 μM AMPH, 250 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) or for 60 sec with 30 mM K^+. The NaCl content of the KRB was reduced commensurate with the increase in KCl. Levels of phosphoser[41]-NM and total NM were measured as described in Materials and Methods and detected using alkaline phosphatase-coupled secondary antibody.

(Whatman, Maidstone, UK) which was immediately placed in 75 mM H_2PO_4 and washed four times as described by Yasuda et al. (1990). All radioactivity was determined using β-scintillation counting in a Beckman LS8100. The PKC activity of the membrane fraction was determined to be the activity of the membranes from the lysed synaptosomal fraction minus that of the non-lysed synaptosomes to exclude PKC activity located in adherent postsynaptic densities.

Statistical significance was determined by Student's t-test. BCA (Pierce, Rockford, IL) was used to measure protein.

RESULTS

Phosphorylation of NM in response to AMPH

Experiments investigating the effect of AMPH on NM phosphorylation were conducted in synaptosomes resuspended in Ca^{2+}-replete KRB as described in our previous studies (Gnegy et al., 1993). As shown in Figure 1, incubation of striatal synaptosomes with AMPH elicited an increase in phosphoser[41]-NM with no change in total NM. Phosphorylation of NM at the ser[41] PKC substrate site was also demonstrated in response to the phorbol ester TPA (12-O-tetradecanoylphorbol 13-acetate) and depolarization by 60 mM K^+. Phosphorylation of NM in response to AMPH at ser[41] was rapid, being detected by 30 sec and maximal at 2 min (Fig. 2A,B). After stimulation by AMPH, the phosphate level was stable until at least 10 min. Phosphorylation of NM at ser[41] was maximal at 10 μM AMPH (Table I). The phosphorylation depended upon AMPH uptake since it was blocked by 10 μM nomifensine (data not shown).

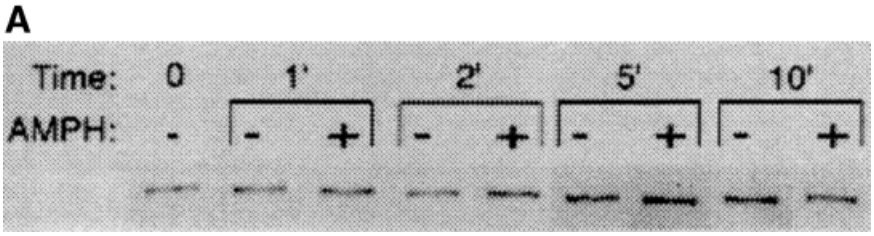
The effect of AMPH on PKC activity was measured in striatal synaptosomes. Striatal synaptosomes were incubated with 1 μM AMPH for times of 45 sec to 2 min. Activation by AMPH was assessed by an increase in PKC activity in the membranes made from lysed synaptosomes and a decrease in activity in the supernatant. AMPH incubation increased PKC activity in the membrane fraction by 133 ± 7 percent (N = 9, $P < 0.005$) over control while the PKC activity in the supernatant was decreased to 85 ± 3 percent of control ($P < 0.005$). Control activities in supernatants and membranes were 2088 ± 422 and 425 ± 43 pmol/min/mg protein, respectively.

Effect of AMPH on phosphorylation of synapsin I at site 3

We investigated whether AMPH would affect the the phosphorylation state of synapsin I in striatal synaptosomes using a state-specific antibody for site 3, the CaM Kinase II substrate site. We found that synapsin I was phosphorylated very rapidly when synaptosomes were resuspended in Ca^{2+}-replete KRB. To minimize this problem, synaptosomes were either resuspended in Ca^{2+}-deficient KRB and Ca^{2+} was added at the start of the assay. Since addition of Ca^{2+} under these conditions can lead to rapid entry of Ca^{2+} into the synaptosome (Bowyer and Weiner, 1990), we performed some experiments where synaptosomes were preincubated in the presence of Ca^{2+} before AMPH was added (as in Figs. 5A, 6B). In these experiments, synaptosomes were warmed to 37°C and then preincubated in Ca^{2+}-replete KRB at 37°C for 1 min before addition of AMPH. Either method gave identical results. As shown in Figure 3A,B, AMPH elicited a time-dependent increase in site 3-phospho-synapsin I in striatal synaptosomes. Phosphorylation of synapsin I at site 3 was maximal at 30 sec and declined hereafter. The stimulatory effect of AMPH was apparent for at least 2 min but there was no stimulation at 10 min. The dose-dependent stimulation of site 3-phospho-synapsin I in response to AMPH was biphasic, being maximal at the low concentration of 1 nM and decreasing at concentrations over 100 nM (Fig. 4). The increase in immunodetectable site 3-phospho-synapsin I in response to 1 nM AMPH was blocked when synaptosomes were preincubated with 10 μM nomifensine (Fig. 5A,B).

Effect of okadaic acid

Since the effect of AMPH on site 3-phospho-synapsin I content was maintained at times beyond 30 sec and



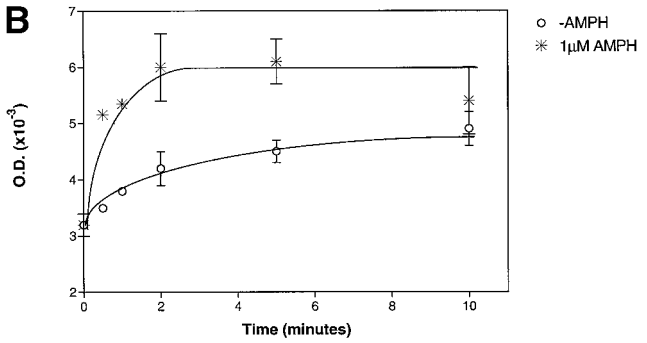


Fig. 2. Time dependent effect of AMPH on the level of phosphoser^{41}-NM in striatal synaptosomes. Percoll-purified synaptosomes were incubated at 37°C for the given times in the absence or presence of 1 μM AMPH. **A:** Immunoblot. Levels of phosphoser^{41}-NM were measured as described in Materials and Methods and detected using ECL. **B:** Synapsomes were incubated in the absence (○) or presence (✳) of 1 μM AMPH. Values from immunoblots were quantified in O.D. units by Gaussian integration using the Hoefer GS365W electrophoresis data system. Values are the average or mean ± S.E.M. of from two to five experiments. For time points of 1, 2, and 5 min, $P < 0.05$ as compared to control with no AMPH.

TABLE I. Dose-dependent effect of amphetamine on phosphoser^{41}-NM content in striatal synaptosomes

Amphetamine concentration	Phosphoser^{41}-NM content (O.D.)
Buffer	3,860 ± 225
0.1 μM	4,812 ± 178*
1.0 μM	5,245 ± 271*
10 μM	5,772 ± 711*

Rat striatal synaptosomes were resuspended in Ca^{2+}-replete KRB and incubated for 5 min at 37°C in the absence or presence of the given concentrations of AMPH. Phosphoser^{41}-NM was determined as described in Materials and Methods. Results from the ECL detection were analyzed by scanning the bands using a Hoefer GS365W densitometer and quantified by Gaussian integration. Results are the mean ± the S.E.M. from four to six experiments. *$P < 0.02$ as compared to buffer control by Student's t-test.

appeared greater at 60 sec than at 30 sec, AMPH treatment could be inhibiting a phosphatase activity rather than a directly activating a kinase activity. To examine this, we determined whether incubation with AMPH would increase the level of site 3-phospho-synapsin I in the presence of okadaic acid. Okadaic acid is an inhibitor of phosphatases 1 and 2A, which can dephosphorylate synapsin I (Sim et al., 1991). At higher concentrations, okadaic acid also inhibits phosphatase 2B. As shown in Table II, okadaic acid at 10 μM elicited a robust increase in content of site 3-phospho-synapsin I, but AMPH was still able to increase the level of site 3-phospho-synapsin I.

Calcium-dependency of the effect of AMPH on phosphoser^{41}-NM and site 3-phospho-synapsin I content

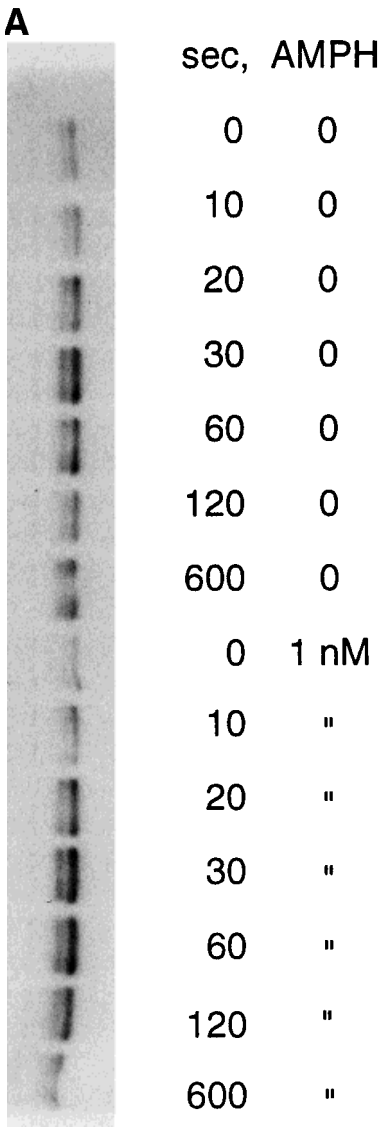
AMPH-mediated increases in the level of phosphoser^{41}-NM were reduced but not abolished when the assay was conducted in KRB containing no added Ca^{2+} (Fig. 6A). In incubations in which Ca^{2+} was simply omitted from the KRB (Fig. 6B), site 3-phospho-synapsin I was detectable, but at lower levels. There was no increase in the level of site 3-phospho-synapsin I by AMPH in the absence of Ca^{2+}, however (Fig. 6B). This was also true at concentrations of AMPH up to 100 nM (data not shown).

Effect of DA receptor blockers on the ability of AMPH to increase the content of site 3-phospho-synapsin I

To determine whether the effects of AMPH on site 3-phospho-synapsin I phosphorylation were due to released DA acting at D1 or D2 dopamine receptors, the effects of the D1 DA receptor antagonist, SCH23390, and the D2 dopamine receptor antagonist, sulpiride, on site 3-phospho-synapsin I phosphorylation were examined. As shown in Table III, there was a partial inhibition of AMPH-mediated increases in site 3-phospho-synapsin I content at all concentrations of SCH23390. Sulpiride had no effect on the stimulatory effect of 1 nM AMPH (Table III). We have found no inhibitory effect of D1 or D2 DA antagonists on phosphoser^{41}-NM phosphorylation.

Effect of the PKC inhibitor, Ro31-8220, on the AMPH-mediated increase in content of phosphoser^{41}-NM and site 3-phospho-synapsin I

If the AMPH-mediated increase in phosphoser^{41}-NM is due to an activation of PKC, the AMPH effect should be blocked by a PKC inhibitor. To test this, and determine whether PKC activation played a role in AMPH-mediated synapsin I phosphorylation, synaptosomes were resuspended in Ca^{2+}-deficient KRB and incubated with the selective PKC inhibitor, Ro31-8220, for 20 min at 4°C prior to addition of 1 nM or 100 nM AMPH.



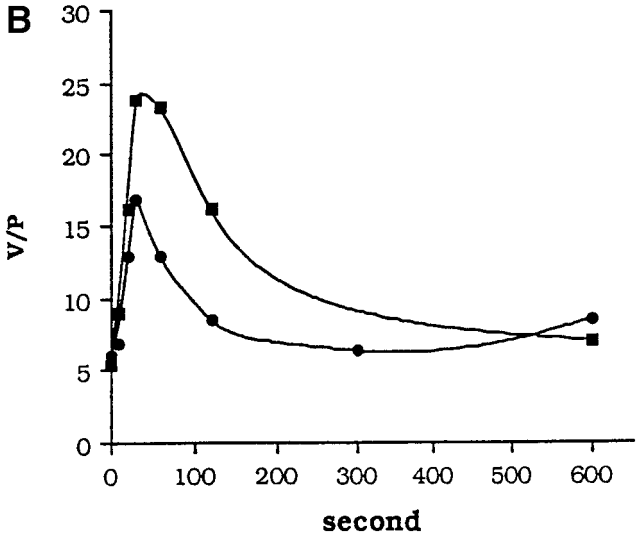


Fig. 3. Time dependent effect of 1 nM AMPH on the level of site 3-phospho-synapsin I in striatal synaptosomes. Percoll-purified striatal synaptosomes were incubated at 37°C in the absence or presence of 1 nM AMPH. **A:** Immunoblot was visualized with ^{125}I-protein A as described in Materials and Methods. **B:** Results from immunoblot were quantified using a Phosphor Imager and densitometry values were obtained as described in Materials and Methods.

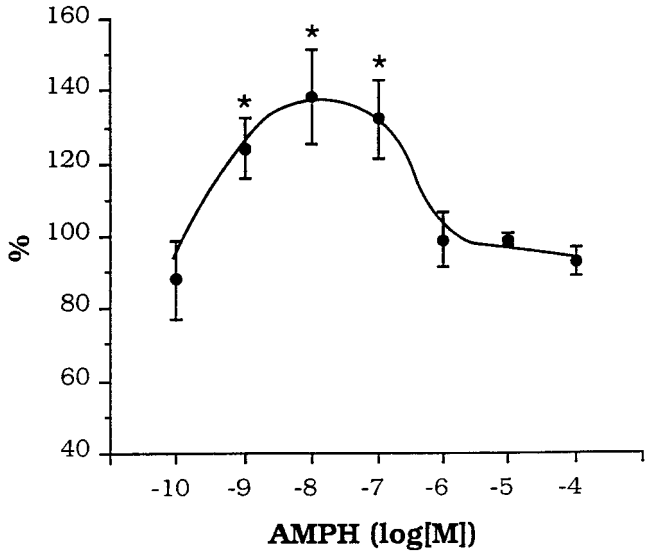


Fig. 4. Dose-dependent effect of AMPH on the level of site 3-phospho-synapsin I in striatal synaptosomes. Percoll-purified synaptosomes were incubated at for 30 sec at 37°C in the presence of various concentrations of AMPH. Results from immunoblots were quantified using a Phosphor Imager and densitometry values were obtained as described in Materials and Methods. Results are the mean ± S.E.M. of five different experiments.

Under the conditions used to measure synapsin I phosphorylation, at 45 sec of incubation, 1 nM AMPH significantly increased the content of phosphoser^{41}-NM by 31 ± 2.7% ($P < 0.007$, N = 3). As shown in Figure 7, the levels of phosphoser^{41}-NM and site 3-phospho-synapsin I were increased by both 1 nM and 100 nM AMPH. Ro31-8220, at 10 μM, had no effect on basal levels of either phosphoser^{41}-NM or site 3-phospho-synapsin I. The AMPH-induced increase in the content of both phosphoser^{41}-NM and site 3-phospho-synapsin I, however, was completely blocked by the PKC inhibitor, Ro31-8220. The AMPH-induced increase in the level of site 3-phospho-synapsin I was also blocked by preincubation with 5 μM of the selective CaM Kinase II inhibitor, KN-62 (data not shown).

DISCUSSION

We have demonstrated that incubation of striatal synaptosomes with low concentrations of AMPH leads to modest but significant increases in phosphoser^{41}-NM and site 3-phospho-synapsin I. The AMPH-mediated increases in phosphorylated synapsin I and NM signifies that AMPH can alter phosphorylation-related sec-

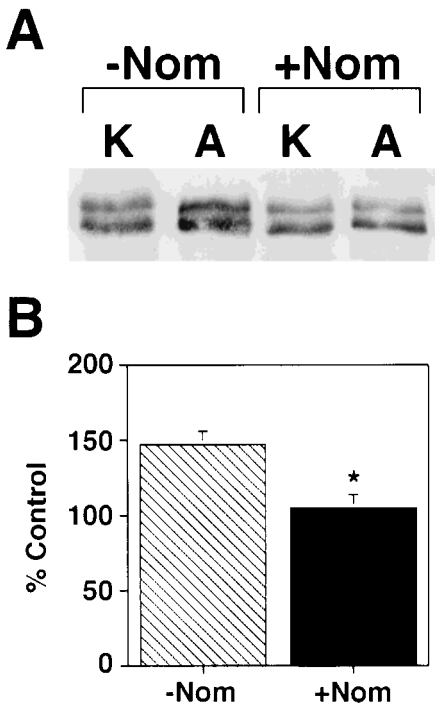


Fig. 5. **A:** Effect of nomifensine on AMPH-mediated increases in the level of site 3-phospho-synapsin I. Percoll-purified synaptosomes were resuspended in Ca^{2+}-deficient KRB and preincubated with buffer or 10 μM nomifensine for 10 min at 25°C. The synaptosomes were warmed to 37°C for 3 min, then preincubated with 1.2 mM $CaCl_2$ for 1 min. Either KRB (K) or AMPH (A, at 1 nM) was added and the incubation was continued for 45 more sec. Levels of phosphoser[41]-NM were measured as described in Materials and Methods and detected using alkaline phosphatase-labeled secondary antibody. **B:** Percentage increase in the level of site 3-phospho-synapsin I by 1 nM AMPH in the absence and presence of 10 μM nomifensine. Results are given as percentage increase over control values in the absence of 1 nM AMPH. Nomifensine alone did not change control values (98 ± 5% of no AMPH control). N = 4. *Value with nomifensine differed from value without at $P < 0.01$ by a Student's paired t-test.

TABLE II. Effect of AMPH and okadaic acid on the level of site 3-phospho-synapsin I in rat striatal synaptosomes

Incubation addition	Site 3-phospho-synapsin I (O.D.)
Buffer	96 ± 6
1 nM AMPH	201 ± 17
10 μM okadaic acid	992 ± 32
Okadaic acid + AMPH	1,271 ± 84*

Synaptosomes were resuspended in Ca^{2+}-deficient KRB and preincubated in the absence or presence of 10 μM okadaic acid at 4°C for 20 min. Following preincubation, Ca^{2+}-replete KRB or 1 nM AMPH with Ca^{2+} was added to synaptosomes for a further 2 min incubation. Site 3-phospho-synapsin I immunoreactivity was determined as described in Materials and Methods. Immunoreactivity visualized with ^{125}I-protein A was quantified by a Phosphor Imager and densitometry values were obtained. *$P < 0.04$ as compared to value for okadaic acid alone, N = 3.

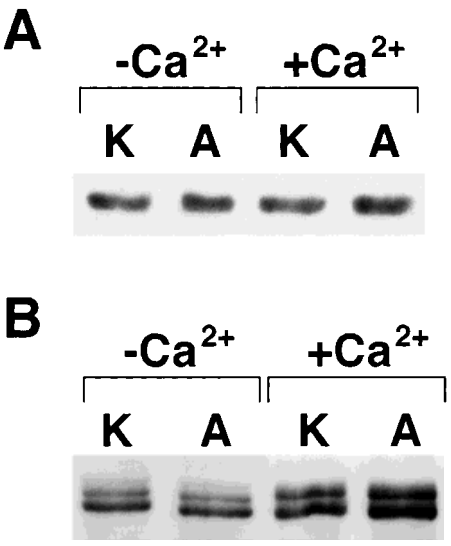


Fig. 6. Calcium dependence of AMPH-mediated increases in levels of phosphoser[41]-NM and site 3-phospho-synapsin I. **A:** Effect of AMPH on levels of phosphoser[41]-NM in synaptosomes incubated in Ca^{2+}-deficient (no added calcium, $-Ca^{2+}$) KRB or Ca^{2+}-replete (1.2 mM $CaCl_2$, $+Ca^{2+}$)-KRB. Synaptosomes were incubated for 5 min at 37°C with 10 μM AMPH (A) or with KRB (K). Levels of phosphoser[41]-NM were measured as described in Materials and Methods and detected using ECL. **B:** Effect of AMPH on levels of site 3-phospho-synapsin I. Synaptosomes were incubated for 3 min at 37°C in Ca^{2+}-deficient (no added calcium, $-Ca^{2+}$) or Ca^{2+}-replete (1.2 mM $CaCl_2$, $+Ca^{2+}$)-KRB. $CaCl_2$ was added to the $+Ca^{2+}$ samples for one min before addition of AMPH (A) or KRB (K) to all samples. Samples were incubated with 1 nM AMPH for 45 sec. The immunoblot was visualized using an alkaline phosphatase-conjugated secondary antibody.

TABLE III. Effect of dopamine antagonists on the AMPH-mediated increases in site 3-phospho-synapsin I

AMPH (nM)	Antagonist concentration (μM)	Percent of control (%) SCH23390	Sulpiride
0	0	100	100
1	0	167 ± 1.1*	137 ± 7**
1	0.1	129 ± 13	127 ± 20
1	1	132 ± 1.7	137 ± 14

Percoll-purified synaptosomes were resuspended in Ca^{2+}-deficient KRB and preincubated with the given doses of SCH23390 or sulpiride for 30–60 min at 4°C. Following preincubation, incubations were conducted with Ca^{2+}-replete KRB in the presence or absence of 1 nM AMPH for 30–60 sec. Site 3-phospho-synapsin I immunoreactivity was determined as described in Materials and Methods. Immunoreactivity visualized with ^{125}I-protein A was quantified by a Phosphor Imager and densitometry values were obtained as described in Materials and Methods. The control value is the value in the absence of AMPH, whether in the presence or absence of drug. Control values did not change in the presence of either SCH23390 or sulpiride. *ANOVA for SCH23390 group, $P < 0.005$, in Tukey-Kramer multiple comparisons post-test, AMPH alone significantly different from any SCH23390-containing sample at $P < 0.01$ and significantly different from 100% at $P < 0.0001$ by Student's t-test. **ANOVA for sulpiride group is n.s. Value for AMPH alone is significantly different from 100% at $P < 0.05$ by Student's t-test.

ond messenger activities in synaptosomes. This work corroborates earlier results in which we found that AMPH increased phosphorylation of NM in rat striatum after acute intraperitoneal injection and in striatal synaptosomes after incubation (Gnegy et al., 1993; Iwata et al., 1996). Acute intraperitoneal injection of either 1 or 2.5 mg/kg AMPH, however, did not increase the level of site 3-phospho-synapsin I in rat striatum although the level was increased after repeated, intermittent AMPH (Iwata et al., 1996). The results of this study show that while AMPH can directly increase the content of site 3-phospho-synapsin I in synaptosomes, the effect is transient and occurs at very low doses of AMPH. Any stimulation would likely have preceeded the 30-min time point chosen for the acute study (Iwata

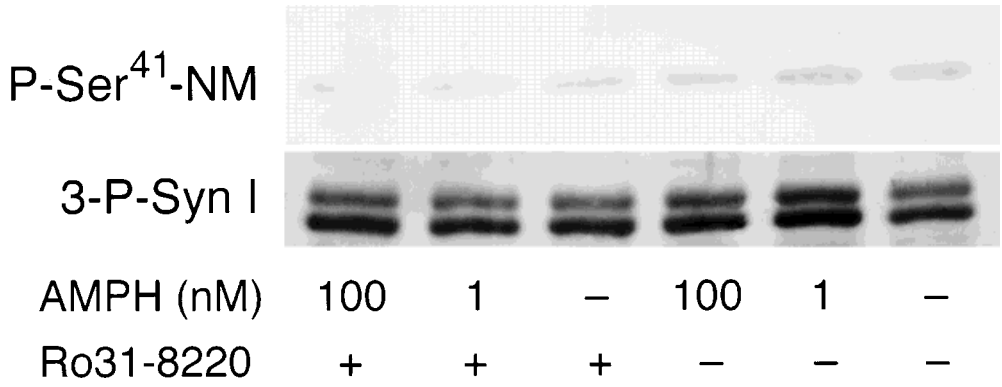


Fig. 7. Effect of the selective protein kinase C inhibitor, Ro31-8220, on the AMPH-mediated increase in the level of phosphoser[41]-NM and site 3-phospho-synapsin I. Percoll-purified rat striatal synaptosomes were preincubated with Ca^{2+}-deficient KRB or 10 μM Ro31-8220 for 20 min at 4°C. Either KRB or the indicated AMPH was then added with Ca^{2+} to give a final [Ca^{2+}] of 1.2 mM and the incubation proceeded for 1 min at 37°C. Immunoreactivity visualized with ^{125}I-protein A.

et al., 1996). In addition, the concentration of AMPH in the striatum 30 min after a 1 mg/kg intravenous injection of AMPH is approximately 10 μM (Melega et al., 1995), suggesting that site 3-phosphosynapsin I levels at that time could be reduced due to the high level of AMPH.

Our results suggest that AMPH can increase PKC activity in synaptosomes as shown by the AMPH-mediated increase in phosphorylation of a specific PKC substrate site in NM and by the direct stimulating effect of AMPH on PKC activation in striatal synaptosomes. Our results support those of Giambalvo (1992b) who found that AMPH increased PKC activity in rat striatal synaptoneurosomes (a 6,000*g* particulate fraction) and in rat striatum when given in vivo by altering the Km for calcium (Giambalvo, 1992a). In addition, drugs that inhibited PKC inhibited AMPH-induced release of DA (Giambalvo, 1992b). Little is known about the action of AMPH in synaptosomes and it is unclear how AMPH would lead to PKC activation. An alteration in levels of calcium or of a lipid, such as diacylglycerol or arachidonic acid, could activate PKC. Although calcium is not required for AMPH uptake through the carrier for DA release, AMPH could have an effect on increasing calcium in the synaptosome. AMPH at concentrations from 1 nM to 100 nM elicited an activation of an inward calcium current in internally perfused neurons of the snail (Vislobokov et al., 1993). Depletion of DA from a specific pool in the synaptosome, elicited by DA release, might have an activating effect on a signal transduction system.

AMPH could be releasing a substance that is increasing PKC activity. Possibilities include dopamine, other monoamines such as norepinephrine and serotonin, glutamate, or even neuropeptides. AMPH-mediated release of some substances in rat striatum, such as glutamate (Mora and Porras, 1993), is mediated by released DA. Our studies with DA antagonists suggest that DA may not mediating the increases in synapsin or NM phosphorylation, although there was some effect of the D1 antagonist SCH23390 on synapsin I phosphorylation. DA has been shown to increase synapsin I phosphorylation in striatal slices but this effect was independent of extracellular Ca^{2+} and cAMP mediated (Walaas et al., 1989) which would likely be reflected at site 1 on synapsin, a substrate site for protein kinase A. It is uncertain whether AMPH releases DA from synaptosomes at concentrations of 1 and 10 nM. Inhibition of [^{3}H]DA release from striatal synaptoneurosomes by 1 and 10 nM AMPH has been reported (Giambalvo, 1992b) although Raiteri (1976) reported measurable [^{3}H]DA release in response to 10 nM AMPH in striatal synaptosomes. An action of released DA at presynaptic autoreceptors, however, could explain the biphasic nature of the dose response curve for phosphorylation of synapsin I. Activation of a presynaptic autoreceptor by DA released at higher doses of AMPH may limit calcium entry into the synaptosome which could account for the decrease in phosphorylation of synapsin I at $\geq$1 μM AMPH. Since AMPH-mediated NM phosphorylation was much sensitive to calcium, it would be less affected by presynaptic DA receptor activation. That could explain the discrepancy between dose response curves for the AMPH effect on NM vs. synapsin I phosphorylation.

The data obtained using the PKC inhibitor Ro31-8220 are consistent with a mechanism whereby stimulation of PKC results in activation of CaM Kinase II which would lead to a phosphorylation of synapsin I at site 3. Similar results have been reported previously (Browning and Dudek, 1992; Ueno and Rosenberg, 1995). Browning and Dudek (1992) found that activation of PKC increased the phosphorylation of synapsin I at CaM Kinase II substrate sites in the rat hippocampal slice. Since purified synapsin I could not be directly phosphorylated by PKC (Browning and Dudek, 1992),

this activation was likely due to cross-talk between PKC and CaM Kinase II. Similarly, Ueno and Rosenberg (1995) reported that phorbol ester stimulation of PKC led to phosphorylation of the myristoylated alanine-rich C-kinase substrate (MARCKS), NM, and synapsin I in rat cerebral cortical synaptosomes. CaM itself may play a role since a PKC-mediated dissociation of CaM from NM or MARCKS would activate CaM Kinase II. The ability of AMPH to extend the time course of phosphorylation of synapsin I at site 3 could be due to a release of CaM providing longer activation of CaM Kinase II. This mechanism has been demonstrated in PC12 cells (MacNicol and Schulman, 1992). Although the dose response curves for AMPH in stimulating synapsin I and NM phosphorylation appeared quite different, low concentrations of AMPH could increase NM phosphorylation when phosphoser^{41}-NM was measured under the conditions used for synapsin I phosphorylation. Possibly the influx of Ca^{2+} upon addition of Ca^{2+} to synaptosomes in Ca^{2+}-deficient KRB (Bowyer and Weiner, 1990) is synergistic with AMPH, providing enhanced activation of PKC and a Ca^{2+}- and PKC-mediated dissociation of CaM from NM which would increase NM phosphorylation (Alexander et al., 1987). Release of CaM from NM or MARCKS could lead to increased activation of the plasma membrane Ca^{2+} pump removing Ca^{2+} from the synaptosome and decreasing CaM Kinase II activation. As mentioned above, an action of released DA in limiting Ca^{2+} influx into the synaptosome could also help explain the difference.

It is unclear whether the phosphorylations or cross-talk occur in the same population of synaptosomes or whether these events even occur in dopaminergic synaptosomes. The mRNA for NM co-localizes with mRNA for tyrosine hydroxylase in rat brainstem and disappears after 6-hydroxydopamine treatment (Bendotti et al., 1991). On the other hand, localization of NM is not selective for nigrostriatal terminals in the striatum since nigral 6-hydroxydopamine lesions decreases the striatal NM content by only 20%, a figure commensurate with the estimated number of dopaminergic terminals in the striatum (S. Iwata, preliminary data). Synapsin I is present in virtually all neurons, primarily in small synaptic vesicles responsible for storage and release of classical transmitters (Greengard et al., 1994). Small, clear vesicles and synapsin I are prominent in glutamatergic synaptosomes (Verhage et al., 1994). There appear to be interactions between dopaminergic and glutamatergic nerve terminals in striatum in that glutamate and DA may alter each other's release (Maura et al., 1988; Mora and Porras, 1993; and see references in Finnegan and Taraska, 1996). Low concentrations of DA suppressed the K^+-induced release of glutamate in a presynaptic-inhibitory manner through dopamine D2 receptors on glutaminergic terminals innervated from cerebral cortex (Maura et al., 1988). If low concentrations of AMPH do decrease DA release (Giambalvo, 1992b), a decrease in DA release could activate the glutamatergic synaptosomes resulting in enhanced phosphorylation of synapsin I at site 3.

The phosphorylation of synapsin I at site 3 by AMPH in synaptosomes is unlikely to be related to the immediate effect of AMPH in releasing DA since synapsin I phosphorylation at site 3 is so strongly calcium-dependent and AMPH-mediated DA release is calcium-independent. On the other hand, there are data suggesting that DA release by AMPH is dependent upon continued synthesis of cytoplasmic pools of DA (Chiueh and Moore, 1975). Low concentrations of AMPH ($\leq$1 µM) increase DA synthesis in striatal synaptosomes (Kuczenski, 1975; Roberts and Patrick, 1979; Uretsky and Snodgrass, 1977). The magnitude of the AMPH effect on DA synthesis is comparable to the magnitude of AMPH-induced increases in levels of phosphorylated proteins in this report. Moreover, the effect of AMPH on DA synthesis is biphasic, with inhibition occurring at concentrations over 1 µM AMPH (Kuczenski, 1975; Roberts and Patrick, 1979; Uretsky and Snodgrass, 1977). Increased DA synthesis by AMPH is calcium-dependent (Schwarz et al., 1980) and occurs at doses of AMPH an order of magnitude less than those eliciting DA release (Kuczenski, 1975). Evidence suggests that depolarization and AMPH stimulate DA synthesis by similar calcium-dependent mechanisms (Roberts and Patrick, 1979; Uretsky and Snodgrass, 1977). Depolarization of synaptosomes leads to a Ca^{2+}-dependent phosphorglation of tyrosine hydroxylase through phosphorylation of a CaM Kinase II-substrate site in the enzyme (Haycock and Haycock, 1991; Waymire and Craviso, 1993). A calcium-dependent increase in DA synthesis in response to AMPH has been reported in vivo (Fung and Uretsky, 1982). Therefore it is possible that AMPH treatment results in an activation of CaM Kinase II which could phosphorylate several substrates, including synapsin I and potentially tyrosine hydroxylase. An AMPH-mediated increase in PKC activity may also contribute to activation of tyrosine hydroxylase since protein kinase C can phosphorylate and activate tyrosine hydroxylase (Albert et al., 1984; Haycock and Haycock, 1991). Calcium-dependent effects of AMPH on behavior have also been demonstrated. Intrastriatal administration of EGTA blocked AMPH-induced circling behavior in unilateral 6-hydroxydopamine lesioned mice and the EGTA effect was reversed with calcium (Fung and Uretsky, 1980). There was no effect on apomorphine-induced circling suggesting a presynaptic mechanism. Therefore, the calcium-dependent effects of AMPH could be important both for DA synthesis, a behavioral component, and other unknown second messenger activities.

In summary, we have demonstrated that incubation of striatal synaptosomes with AMPH can lead to increases in the level of phosphate incorporated into NM at ser^{41}, the PKC substrate site, and of synapsin I at

site 3, the CaM Kinase II substrate site. These results indicate that AMPH can have effects on phosphorylation-related second messenger activity within the synaptosome and that AMPH can affect second messenger function in both a Ca^{2+}-dependent and a Ca^{2+}-independent fashion.

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