Repeated amphetamine treatment induces neurite outgrowth and enhanced amphetamine-stimulated dopamine release in rat pheochromocytoma cells (PC12 cells) via a protein kinase C- and mitogen activated protein kinase-dependent mechanism

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Abstract

Repeated intermittent treatment with amphetamine (AMPH) induces both neurite outgrowth and enhanced AMPH-stimulated dopamine (DA) release in PC12 cells. We investigated the role of protein kinases in the induction of these AMPH-mediated events by using inhibitors of protein kinase C (PKC), mitogen activated protein kinase (MAP kinase) or protein kinase A (PKA). PKC inhibitors chelerythrine (100 nM and 300 nM), Ro31-8220 (300 nM) and the MAP kinase kinase inhibitor, PD98059 (30 μM) inhibited the ability of AMPH to elicit both neurite outgrowth and the enhanced AMPH-stimulated DA release. The direct-acting PKC activator, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA, 250 nM) mimicked the ability of AMPH to elicit neurite outgrowth and enhanced DA release. On the contrary, a selective PKA inhibitor, 100 μM Rp-8-Br-cAMPS, blocked only the development of AMPH-stimulated DA release but not the neurite outgrowth. Treatment of the cells with acute AMPH elicited an increase in the activity of PKC and MAP kinase but not PKA. These results demonstrated that AMPH-induced increases in MAP kinase and PKC are important for induction of both the enhancement in transporter-mediated DA release and neurite outgrowth but PKA was only required for the enhancement in AMPH-stimulated DA release. Therefore the mechanisms by which AMPH induces neurite outgrowth and the enhancement in AMPH-stimulated DA release can be differentiated.

Keywords: cultured cells, extracellular signal-regulated kinase, neurite outgrowth, protein kinases, repeated amphetamine, transporter-mediated dopamine release.

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Amphetamine (AMPH), a synthetic psychomotor stimulant, acts as an indirect agonist of catecholaminergic systems. AMPH exerts its physiological effects by enhancing the release of dopamine (DA) and norepinephrine from nerve terminals into the synapse through a reversal of their respective plasmalemmal transporters, the dopamine transporter (DAT) and norepinephrine transporter (NET) (Seiden *et al*. 1993). Repeated, intermittent treatment of rats with AMPH leads to both a behavioral sensitization and neuroadaptations that include an enhanced release of DA from striatum and nucleus accumbens in response to AMPH (Robinson and Becker 1986; Robinson 1991; Kantor and Gnegy 1998a; Wolf 1998). There is morphological evidence that AMPH can remodel synapses in rat brain by increasing the density of synapses and number of dendritic spines in rat nucleus accumbens and prefrontal cortex (Robinson and Kolb 1997, 1999).

We found that neurite outgrowth and enhanced transporter-mediated DA release occur in rat pheochromocytoma PC12 cells in response to repeated, intermittent AMPH,

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Abbreviations used: AMPH, amphetamine; DA, dopamine; DAT, dopamine transporter; MAP kinase, mitogen activated protein kinase; MEK, MAP kinase kinase; NET, norepinephrine transporter; NGF, nerve growth factor; PKA, protein kinase A; PKC, protein kinase C; TBST, Tris-buffered saline (containing 0.1% Tween 20); TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; VTA, ventral tegmental area.

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reminiscent of those occurring in brain. The PC12 cell can be a useful single-cell model in which to investigate molecular mechanisms of acute and repeated AMPH, although the PC12 cells are not neurons. These cells contain endogenous norepinephrine and DA which can be released in response to AMPH through plasmalemmal NET (Greene and Tischler 1976; Bonisch 1984; Kantor *et al*. 2002). In addition, PC12 cells characteristically differentiate in response to trophic factors such as nerve growth factor (NGF) (Koike 1983; Nakafuku and Kaziro 1993). Repeated, intermittent treatment with AMPH induced neurite outgrowth in PC12 cells in a process that required the plasmalemmal transporter (Park *et al*. 2002). The identical AMPH treatment concurrently induced an enhancement in AMPH-stimulated DA release in PC12 cells that had characteristics analogous to those found in striatum and nucleus accumbens (Kantor *et al*. 2002). The fact that repeated, intermittent AMPH can elicit these neuroadaptations in the PC12 cells demonstrates that these responses can be achieved within the catecholaminergic cell and does not require an intact neuroanatomy. It also suggests that PC12 cells are an appropriate model in which to investigate the mechanisms of induction of the neuroadaptations by repeated AMPH.

Protein kinase-dependent signal transduction is a factor both in the action of stimulants such as AMPH and in neurite outgrowth. Protein kinase C (PKC), a Ca^{2+}/lipid-dependent serine and threonine kinase, plays a pivotal role in the regulation of many cellular processes, including release of catecholamines, catecholamine plasmalemmal transporter trafficking (Blakely *et al*. 1998; Daniels and Amara 1999) and cellular neurite outgrowth (Nishizuka 1988; Ben-Shlomo *et al*. 1991; Hug and Sarre 1993). PKC activation is involved in AMPH-induced outward transport of DA through both DAT, as demonstrated in brain, and through NET, as shown in PC12 cells (Ben-Shlomo *et al*. 1991; Giambalvo 1992a,b; Kantor and Gnegy 1998b; Cowell *et al*. 2000; Kantor *et al*. 2001, 2002). Similarly, PKC plays a part in AMPH-mediated locomotion (Browman *et al*. 1998).

Mitogen activated protein kinase (MAP kinase) is also involved in the behavioral sensitization in animals to psychostimulants and dopamine transporter function (Rothman *et al*. 2002; Licata and Pierce 2003). Intra-ventral tegmental area (VTA) injection of MAP kinase kinase (MEK) inhibitors blocks the initiation of behavioral sensitization to cocaine (Pierce *et al*. 1999). Similarly, repeated treatment with cocaine increases the phosphorylation of MAP kinase in the VTA (Berhow *et al*. 1996). Since protein kinase A (PKA) also contributes to psychostimulant sensitization (Self *et al*. 1998), this kinase could be important in the induction of the neuroadaptations elicited in response to repeated, intermittent AMPH as well. cAMP signal transduction is important for the induction of the persistent behavioral sensitization following repeated administration of AMPH in the DA cell bodies in the VTA (Tolliver *et al*. 1999).

All three of these protein kinases, PKA (Yao *et al.* 1998; Hansen *et al*. 2000; Jessen *et al*. 2001), PKC (Hall *et al*. 1988; Borgatti *et al*. 1996) and MAP kinase (Koike 1983; Nakafuku and Kaziro 1993) play a role in neurite outgrowth in PC12 cells.

Therefore we investigated whether PKC, MAP kinase or PKA is involved in the induction of AMPH-elicited neurite outgrowth and enhanced DA release in PC12 cells after repeated, intermittent treatment with AMPH. In addition, we also determined whether the AMPH-induced neurite outgrowth and enhanced AMPH-stimulated DA release are induced through activation of identical signaling pathways.

Materials and methods

Materials

D-Amphetamine sulfate, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) and forskolin were purchased from Sigma (St. Louis, MO, USA). Chelerythrine, Ro31-8220, Rp-8-Br-cAMPS, PD98059 and H-89 were purchased from Calbiochem (La Jolla, CA, USA).

Cell culture

PC12 cells were maintained in a 75-cm^2 tissue culture flask in growth medium composed of Dulbecco's modified Eagle's medium from BioWhittaker (Walkersville, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 5% heat-inactivated horse serum, 2 mM L-glutamine, 100 μg/mL of streptomycin and 100 U/mL of penicillin (Gibco, Rockville, MD, USA) and were incubated at 10% CO_2. To induce neurite outgrowth, 5×10^4 cells/mL were plated in a 75-cm^2 tissue culture flask at 10% CO_2 in the same growth medium. PC12 cells were treated with 1 μM AMPH (Sigma) for 5 min a day for 5 days. After a 10-day drug-free period (withdrawal), cells were analyzed for neurite outgrowth and AMPH-stimulated DA release. To block the activity of protein kinases, the inhibitors were added to the media for either 30 min (chelerythrine, Ro31-8220, PD98059 or H-89) or 1 h (Rp-8-Br-cAMPS) before the AMPH. This treatment paradigm is outlined in the schema in Fig. 1(a). To activate PKC or PKA, PC12 cells were treated with 250 nM TPA or 10 μM forskolin for 5 min a day for 5 days in the same manner as with AMPH.

Quantification of neurite outgrowth

To analyze neurite outgrowth, pictures of the PC 12 cells were taken with a digital camera (SPOT RT, Diagnostic Instrument Inc., Sterling Heights, MI, USA) at 200× phase contrast using a Leica DMI RB inverted microscope. For each treatment, randomly chosen individual areas (containing ≥ 80 cells) were scored in the digital pictures.

A neurite was counted when a cellular process was longer than the diameter of the cell body. The percentage of neurite outgrowth was calculated as the number of cells with neurites divided by total cell numbers (Park *et al*. 2002). Each experiment was conducted in triplicate.

Superfusion assay

Cells were harvested by washing the flasks with Kreb's Ringer buffer containing 125 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$,

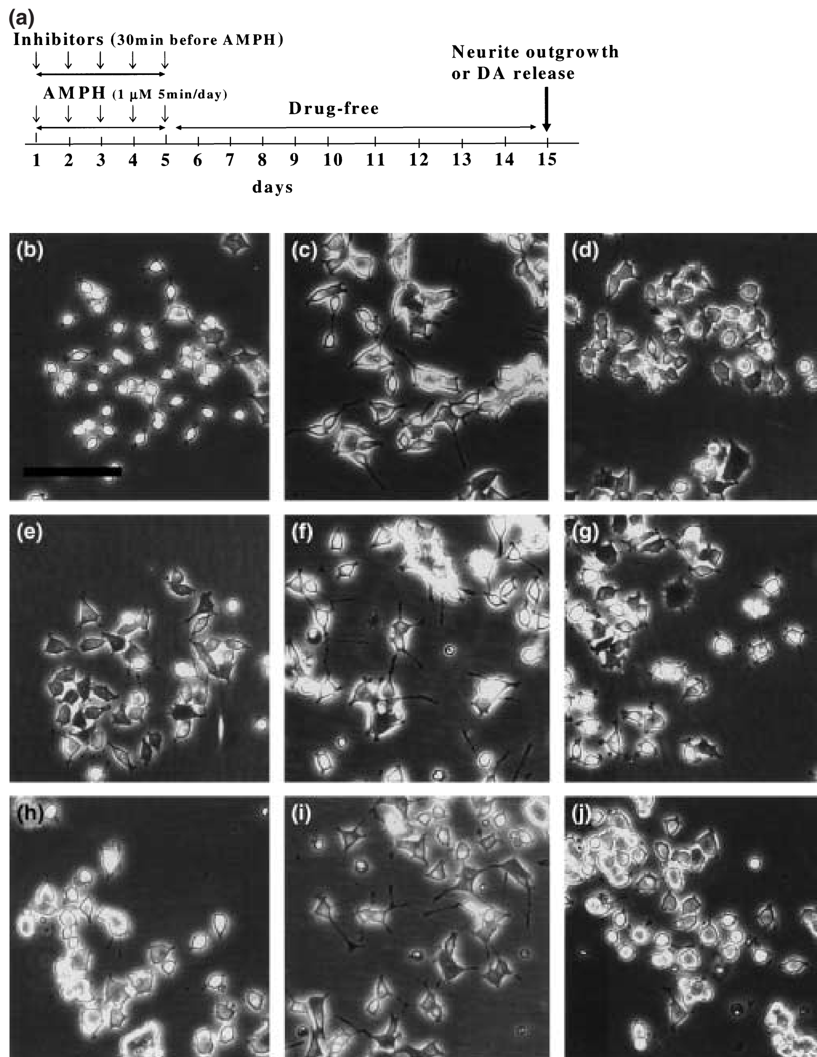

Fig. 1 (a) Schema representing repeated, intermittent treatment of PC12 cells. PC12 cells were treated with vehicle (control) or 1 μM AMPH following a 30 or 60 min pre-treatment with protein kinase inhibitors or vehicle. Following a 10-day drug-free period, neurite outgrowth and DA release in response to a challenge of 1 μM AMPH was measured. In some experiments, 250 nM TPA or 10 μM forskolin is substituted for the 1 μM AMPH in the repeated treatments. (b–j) Involvement of protein kinases in AMPH-induced neurite outgrowth in PC12 cells. PC12 cells were treated with repeated, intermittent vehicle (b), AMPH (c–e, g–i) or protein kinase activators (f, j) for 5 min per day for 5 days as described in Materials and Methods and Fig. 1(a). Protein kinase inhibitors (d, e, g–i) were given before each AMPH treatment as shown in Fig. 1(a). The treatments were: (b) vehicle, (c) 1 μM AMPH, (d) 100 nM chelerythrine + AMPH, (e) 300 nM Ro31-8220 + AMPH, (f) 250 nM TPA, (g) PD98059 + AMPH, (h) 500 nM H89 + AMPH, (i) 100 μM Rp-8-Br-cAMPS + AMPH and (j) forskolin. Ten days following the last AMPH treatment, pictures of PC12 cells were taken by digital camera SPOT RT at 200× phase contrast. Scale bar represents 100 μm.

1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 24.9 mM $NaHCO_3$ and 0.25 mM ascorbic acid and oxygenated by 95% O_2 and 5% CO_2 for 1 h. DA release was measured in the superfused cells by HPLC with electrochemical detection as described previously (Kantor *et al.* 2002).

PKC assay

Cells were washed in serum-free media and resuspended in the same media and incubated at 37°C with or without 3 μM AMPH for 5 min. Cold phosphate-buffered saline was added to the samples and the cells were centrifuged at 800 × *g* for 3 min. Cells were washed twice with cold phosphate-buffered saline and lysed in an extraction buffer containing 25 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 0.5 mM EDTA, 10 mM dithiothreitol and protease inhibitor cocktail, by sonication for 60 s in an ice bath. Lysates were centrifuged at 800 × *g* to remove cell debris and then centrifuged at 100 000 × *g* for 1 h. The supernatant was designated as the cytosolic fraction. The pellet was then extracted with extraction buffer containing 0.25% Triton X-100 at 4°C for 1 h and centrifuged at 100 000 × *g* for 30 min. The solubilized material was designated as the membrane fraction. Both the cytosolic and membrane fractions were loaded on 0.5 mL DE-52 columns pre-equilibrated with their respective extraction buffers. Columns were washed with five column volumes of buffer and then eluted with buffer containing 0.2 M NaCl. Eluates were assayed for PKC activity.

PKC activity was assayed using SignaTECT PKC assay system from Promega (Madison, WI, USA). Briefly, extracts were assayed for PKC activity using a biotinylated substrate peptide, corresponding to the PKC phosphorylation site in neurogranin. The assay (25 μL volume) contained 10 mM $MgCl_2$, 0.4 mM $CaCl_2$, 100 μM substrate, and 100 μM ATP (containing 0.05 μCi of [γ-^{32}P]ATP/assay) in the presence and absence of 0.32 mg/mL phosphatidyl serine and 0.032 mg/mL diacylglycerol. Reactions were started by adding the sample and incubating at 30°C for 5 min. Reactions were terminated by adding 7.5 M guanidine hydrochloride and 10 μL of the reaction was spotted on a special biotin capture membrane. The membrane was washed sequentially four times in 2 M NaCl followed by four times in 2 M NaCl in 1% phosphoric acid and dried and counted by liquid scintillation. Enzyme activity is expressed as pmols of ^{32}P transferred to the substrate per min per mg protein.

PKA assay

PC12 cells were washed with serum-free media and resuspended in the same media. Cells were then incubated at 37°C and treated without or with 3 or 10 μM AMPH for 5 min. At the end of the incubation, 0.5 mL of cold phosphate-buffered saline was added to the cells and they were centrifuged at 800 *g* for 3 min. Cells were washed twice with cold phosphate-buffered saline and lysed in 0.2 mL of lysis buffer (10 mM potassium phosphate, 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol containing protease inhibitor cocktail) by sonication for 5 min in an ice bath. The lysate was centrifuged at 10 000 × *g* for 15 min in a microcentrifuge and the supernatant was used for the assay of PKA activity.

PKA activity was assayed in a volume of 50 μL in a buffer containing 10 mM HEPES, pH 7.4, 10 mM dithiothreitol, 5 mM sodium fluoride, 10 mM magnesium chloride, 0.5 mM IBMX and 0.2 mM ATP using 30 μM kemptide as substrate in the presence and absence of 30 μM cAMP. The reaction was allowed to proceed for 5 min at 30°C and stopped by spotting 25 μL of the reaction mix on a P81 strip. The strips were washed four times in 75 mM phosphoric acid, dried and counted using Scintverse BD in a Beckman LS5800 liquid scintillation counter. The cAMP-dependent activity of PKA is defined as pmols of radioactive phosphate transferred to the substrate per min per mg protein.

Protein extraction and immunoblotting for GAP-43 and MAP kinases

Cells were treated at 37°C without or with 3 μM AMPH for 5 min. For GAP-43 phosphorylation, PC12 cells were extracted with 150 μL of 2.5% perchloric acid, 150 mM NaCl and 1% Triton X-100. Extracts were sonicated for 15 min in a water bath and centrifuged at 15 000 × *g* for 10 min. Percholoric acid-soluble protein was neutralized with 2 N NaOH. For MAP kinase, PC12 cells were lysed in 10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA and 5 mM EDTA with the protease inhibitor cocktail tablets, Complete Mini (1 tablet/10 mL of buffer) (Roche, Mannheim, Germany) and 1 mM sodium orthovanadate. Nuclei and undisrupted cells were removed by spinning at 1000 × *g*. Protein concentration was determined by a modified Lowry method (D-C protein assay kit, Bio-Rad, Hercules, CA, USA).

Samples (20 μg protein each) were resolved by electrophoresis on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated in 2.5% milk in TBST with rabbit anti-phosphoserine^{41}-GAP-43 (Chemichon, Temecula, CA, USA) (1 : 1000 dilution, overnight), rabbit anti-GAP-43 (Chemichon) (1 : 1000, 1 h), mouse anti-phospho-p44/42 MAPK (Cell signaling, San Francisco, CA, USA) (1 : 1000 dilution, overnight) or rabbit anti-p44/42 MAPK (Cell signaling) (1 : 1000 dilution, 1 h). After three washes with TBST, membranes were incubated with a secondary antibody conjugated with horseradish peroxidase. (1 : 10 000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoblots were developed using enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ, USA). To quantify the GAP-43 blots, the films were scanned and the density of each band was measured by using the NIH images program (Scion corporation, Frederick, MD, USA).

Data analysis

Statistical significance between two groups was determined using a two-tailed Student's *t*-test. Analysis among three or more groups was conducted using one-way analysis of variants (ANOVA) with post-test Tukey–Kramer multiple comparison analysis.

Results

AMPH-induced neurite outgrowth and enhanced AMPH-stimulated DA release depended on PKC in PC12 cells

To investigate the role of PKC in AMPH-induced neurite outgrowth, PC12 cells were pre-treated with a PKC inhibitor, either chelerythrine or Ro31-8220, for 30 min before each daily AMPH treatment. These drugs are structurally dissimilar and have been shown to block AMPH-stimulated DA release in rat striatum and PC12 cells (Kantor and Gnegy 1998b; Kantor *et al*. 2001). After 10 drug-free days, neurite outgrowth was measured using digital pictures. As we showed previously (Park *et al*. 2002), AMPH induced neurite outgrowth in PC12 cells (Figs 1b and c). Both chelerythrine (Fig. 1d) and Ro31-8220 (Fig. 1e) significantly blocked the ability of AMPH to induce neurite outgrowth in PC12 cells but did not alter basal neurite outgrowth (Fig. 2a). As shown in Fig. 2(a), both drugs at 300 nM blocked the induction of the neurite outgrowth in response to AMPH. To further demonstrate participation of PKC in the neurite outgrowth, PC12 cells were treated with 250 nM of the PKC activator TPA in a manner identical to that of AMPH. This concentration of TPA was previously shown to induce DA release non-additively with AMPH through NET (Cowell *et al*. 2000). Ten days following the repeated TPA treatment, neurite outgrowth was measured. The repeated, intermittent TPA treatment induced a significant increase in neurite outgrowth in PC12 cells as compared to vehicle (Fig. 1f). There was no significant difference in the percentage of cells exhibiting neurites following the AMPH and TPA treatments (Fig. 2b).

Repeated, intermittent treatment of the PC12 cells with AMPH results in an enhancement of DA release to a subsequent challenge of AMPH in the perfusion (Kantor *et al*. 2002) just as occurs in the rat striatum following repeated treatment of the rat with AMPH (Kantor *et al*. 1999). To investigate whether the induction of the enhanced DA release is also dependent on PKC activity, the PC12 cells were pre-treated with chelerythrine or Ro31-8220 before the daily AMPH treatment as described above. After the 10 drug-free days, AMPH-mediated DA release was measured. Pre-treatment of the PC12 cells with the PKC inhibitors chelerythrine and Ro31-8220 before each AMPH treatment effectively inhibited the induction of enhanced DA release to a challenge dose of AMPH (Figs 3a and b). The drugs had no effect on basal DA release. To confirm that a repeated activation of PKC by AMPH results in the enhancement in

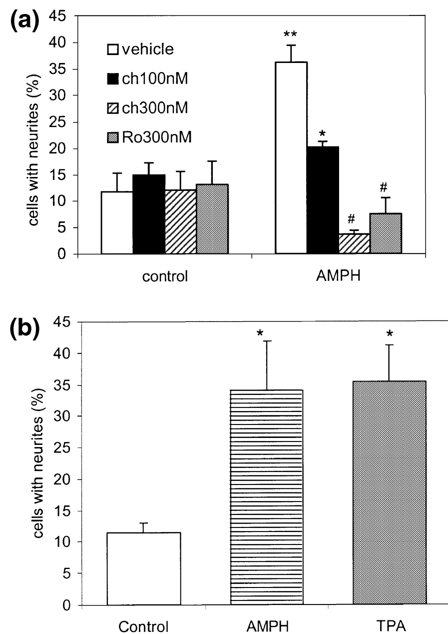

Fig. 2 Effect of PKC inhibition or activation on AMPH-induced neurite outgrowth. (a) PC12 cells were treated with 1 μM AMPH (AMPH, *x*-axis) or vehicle (control, *x*-axis) for 5 min per day for 5 days. Before each vehicle or AMPH treatment, PC12 cells were pre-treated with vehicle, 100 nM or 300 nM chelerythrine or 300 nM Ro 31–8220 for 30 min (pre-treatments are given in the legend in the Figure). After 10 drug-free days, neurite outgrowth was measured as described in Material and Methods. Values represent means ± SEM. $n \geq 3$. ANOVA, $p < 0.0001$. In *post hoc* Tukey–Kramer analysis $^{**}p < 0.001$ vs. control. $^{*}p < 0.05$ and $\#p < 0.001$ vs. vehicle-AMPH. (b) PC12 cells were treated with 1 μM AMPH or 250 nM TPA for 5 min per day for 5 days. After 10 drug-free days, neurite outgrowth was measured. Both repeated AMPH and repeated TPA elicited an increase in neurite outgrowth. Values represent means ± SEM. $n \geq 3$. ANOVA, $p < 0.03$. In *post hoc* Tukey analysis, $^{*}p < 0.05$ vs. control.

transporter-mediated DA release, the PC12 cells were treated with 250 nM TPA instead of 1 μM AMPH for each of 5 days and given 10 drug-free days. As shown in Fig. 3(c), the repeated TPA treatment mimicked the ability of repeated AMPH to induce the enhancement in AMPH-stimulated DA release.

The MEK inhibitor, PD98059 blocked AMPH-induced neurite outgrowth and enhanced AMPH-stimulated DA release in PC12 cells

To investigate whether MAP kinase activity is required for the induction of AMPH-induced neurite outgrowth, PC12 cells were treated with 1 μM AMPH for 5 min a day for 5 days in the absence or presence of 30 μM of the MEK inhibitor, PD98059. After 10 drug-free days, neurite outgrowth was measured. PD98059 pre-treatment inhibited the repeated AMPH-induced neurite outgrowth in the PC12 cells by 70% (Figs 1g and 4a). We investigated whether MAP kinase activation is similarly necessary for the induction of enhanced AMPH-stimulated DA release after repeated, intermittent AMPH. PC12 cells were treated with the same protocol as for neurite outgrowth. After 10 drug-free days, PC12 cells were challenged with AMPH and DA release was measured by HPLC. Pre-treatment with the MEK inhibitor, PD98059, inhibited the induction of AMPH-stimulated enhanced DA release demonstrating that both neuroadaptations require the activation of MAP kinase (Fig. 4b). It has been reported that PD98059 can block DA uptake (Rothman *et al.* 2002). However, 30 μM PD98059 did not inhibit DA release in response to acute AMPH (in the perfusion) in untreated cells.

The PKA inhibitor Rp-8-Br-cAMPS did not block AMPH-induced neurite outgrowth but inhibited enhanced AMPH-stimulated DA release in PC12 cells

To determine whether PKA contributes to AMPH-induced neurite outgrowth, the PKA inhibitors H-89 and Rp-8-Br-cAMPS were used. Pre-treatment with 500 nM H-89, given 30 min before each repeated AMPH treatment, blocked the induction of neurite outgrowth in response to repeated, intermittent AMPH (Figs 1h and 5a). On the contrary, Rp-8-Br-cAMPS, a potent cell-permeable metabolically stable cAMP antagonist that is highly specific for inhibition of PKA, did not block AMPH-induced neurite outgrowth when given at either 30 μM or 100 μM 1 h before each AMPH treatment (Figs 1i and 5a). Rp-8-Br-cAMPS alone did not affect basal neurite outgrowth. To further understand the role of PKA in the neurite outgrowth, PC12 cells were also treated with 10 μM forskolin, an adenylyl cyclase activator, in a manner identical to that of AMPH. Ten days following the repeated forskolin treatment, neurite outgrowth was measured. In contrast to AMPH, the repeated, intermittent forskolin treatment did not induce neurite outgrowth in PC12 cells (Figs 1j and 5b).

To further compare the mechanism of induction of neurite outgrowth and enhanced DA release, the role of PKA in enhanced AMPH-stimulated DA release was investigated. Either Rp-8-Br-cAMPS or H-89 was added to the media before each of the five AMPH treatments. After 10 drug-free days, DA release in response to a challenge of 1 μM AMPH was measured in perfused cells. In contrast to the development of neurite outgrowth, both Rp-8-Br-cAMPS and H-89 pre-treatment significantly blocked the ability of repeated AMPH to induce an enhancement in AMPH-stimulated DA release as compared to vehicle (Fig. 5c). The drugs had no effect on basal DA release. To determine whether direct activation of PKA can mimic AMPH in the induction of enhanced DA release, PC12 cells were treated with 10 μM forskolin for 5 min a day for 5 days. After 10 drug-free days, AMPH-stimulated DA release was measured. In contrast to direct PKC activation, repeated forskolin did not mimic the ability of repeated AMPH to elicit enhanced

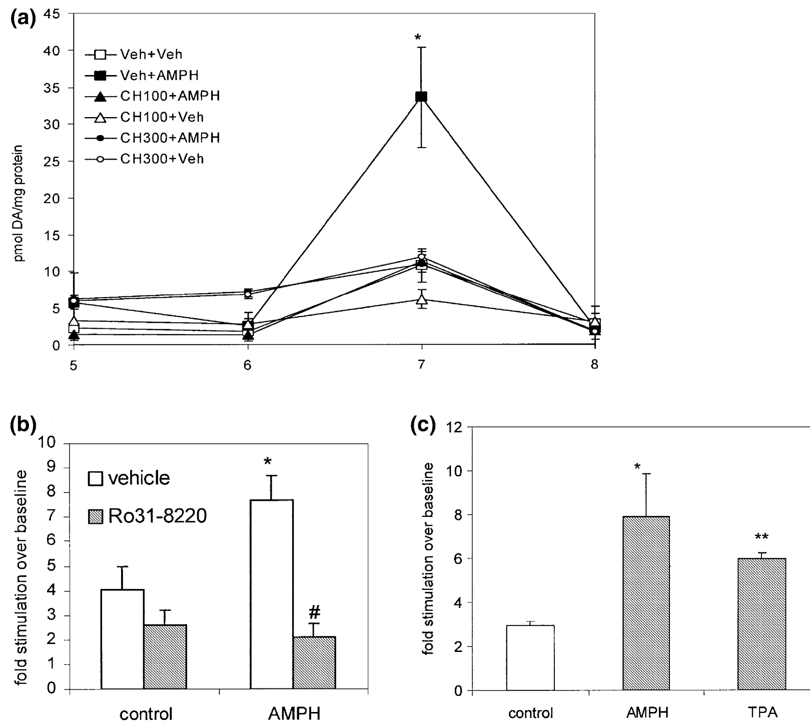

Fig. 3 Effect of PKC inhibitors and TPA on the induction of enhanced AMPH-stimulated DA release. (a) PC12 cells were treated for 5 min a day for 5 days with 1 μM AMPH or vehicle preceded by a 30-min pre-treatment with (a) 100 nM or 300 nM chelerythrine (CH). The legend indicates the pre-treatment + the inducing treatment (vehicle or AMPH, 30 min later). After 10 drug-free days, PC12 cells were harvested and perfused as described in Methods and Materials. All samples received a bolus of 1 μM AMPH for 2.5 min at fraction 4. Due to the length of tubing and rate of perfusion, the DA elutes at fraction 7. Results are given in pmol DA/mg protein ± SEM. $n \geq 3$. For fraction 7, ANOVA $p \leq 0.003$. In *post hoc* Tukey analysis $*p < 0.01$ vs. A + CH100 and A + CH300 (AMPH and chelerythrine 100 or 300 nM treatment). (b) PC12 cells were treated with vehicle or 300 nM Ro31-8220 followed 30 min later by 5 min of 1 μM AMPH (AMPH, *x*-axis) or vehicle (control, *x*-axis) for 5 days (pre-treatments are given in the legend in the Figure). Following 10 drug-free days, AMPH-mediated DA release was measured in the perfusion as described in part (a) and Materials and Methods. Results are expressed as the fold stimulation of the DA in fraction #7 over baseline. $*p < 0.05$ compared to control-vehicle, $\#p < 0.05$ compared to AMPH-vehicle, by two-tailed Student's *t*-test. (c) PC12 cells were treated for 5 min a day for 5 days with vehicle (control), 1 μM AMPH or 250 nM TPA, given 10 drug-free days, harvested and perfused as described in Methods and Materials. Results are given in fold-stimulation of the DA in fraction #7 over baseline. AMPH and TPA significantly differed from vehicle at $*p < 0.05$ or $**p < 0.001$, respectively, by two-tailed Student's *t*-test.

AMPH-stimulated DA release (fold stimulation by 3 μM AMPH after repeated forskolin = 1.84 ± 0.12, $n = 6$, not different from vehicle).

Acute treatment with AMPH increased the activation of PKC and MAP kinase but not PKA in PC12 cells

Our results have demonstrated that PKC inhibitors block and a PKC activator mimics the AMPH-mediated induction of neurite outgrowth and enhancement in transporter-mediated DA release. These results imply that an acute AMPH treatment could increase PKC activity in the PC12 cells. We investigated this possibility in two ways: (i) by determining whether AMPH would elicit the translocation of PKC activity from the cytosol to the membrane, and (ii) by examining whether AMPH would increase the phosphorylation of a protein at a specific PKC substrate site. As shown in Fig. 6(a), acute treatment with AMPH at a concentration and time commensurate to those used in the induction experiments (3 μM AMPH for 5 min) decreased the activity of PKC in the cytosol and increased the activity of PKC in the membrane as compared to vehicle-treated cells. The reduction in cytosol PKC activity and increase in membrane-bound activity of PKC demonstrates translocation and activation of the enzyme (Nishizuka 1988). Since growth associated protein-43 (GAP-43) is specifically phosphorylated by PKC, we used an antibody specific for phosphoserine^{41}-GAP-43 to determine whether AMPH can induce the phosphorylation of GAP-43. Acute treatment with 3 μM AMPH for 5 min showed an increase in the phosphorylation of GAP-43 at serine^{41} as compared to vehicle control. TPA (250 nM), used as a positive control, also showed the increase in the phosphorylation of GAP-43 (Fig. 6b). The amount of total GAP-43 was not changed by the AMPH or TPA. The blot in Fig. 6(b) is representative of four experiments which

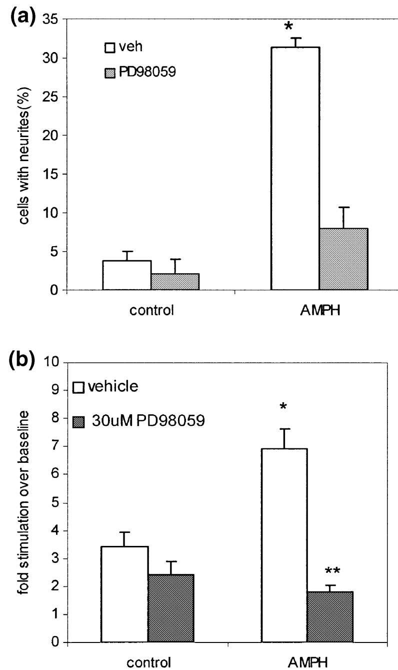

Fig. 4 Effect of the MEK inhibitor, PD98059 (30 μM), on the ability of repeated, intermittent AMPH to induce (a) neurite outgrowth and (b) enhanced AMPH-stimulated DA release. PC12 cells were treated for 5 min a day for 5 days with 1 μM AMPH (*x*-axis) or vehicle (control, *x*-axis) following a 30-min pre-treatment with 30 μM PD98059 or vehicle (legend in Figure). After 10 days of withdrawal (a) neurite outgrowth and (b) DA release in response to 1 μM AMPH were measured as described in Material and Methods. In (b), results are given in fold-stimulation of DA in fraction #7 over baseline. Values represent means ± SEM. $n \geq 3$. (a) ANOVA, $p < 0.0001$. In *post hoc* Tukey–Kramer analysis, $^{*}p < 0.001$ AMPH vs. control-vehicle or AMPH-PD98059. (b) ANOVA, $p < 0.0001$. In *post hoc* Tukey–Kramer analysis, $^{*}p < 0.001$ for vehicle-control vs. vehicle-AMPH; $^{**}p < 0.001$ for PD98059-AMPH vs. vehicle-AMPH.

were quantified by densitometry. The results were calculated as a ratio of the optical density (O.D.) for the phosphoser^{41}-GAP-43 band/O.D. for the total GAP-43. The ratio was 0.04 ± 0.009 for the vehicle treatment and 0.52 ± 0.1 for the AMPH treatment ($p < 0.005$, $n = 4$).

Similarly, the ability of the MEK inhibitor, PD98059, to inhibit the ability of AMPH to induce the enhancement in DA release suggests that acute AMPH would also increase MAP kinase activity. As shown in Fig. 7(a), acute treatment with 3 μM AMPH for 5 min increased the phosphorylation of MAP kinase with respect to vehicle. NGF (50 ng/mL), used as a positive control, also showed an increase in the phosphorylation of MAP kinase. The total amount of MAP kinase was not altered by the AMPH or NGF treatments.

In contrast, when PC12 cells were treated with 3 μM or even 10 μM AMPH for 5 min, no significant difference in the activation of PKA among the treatment groups was detected (Fig. 7b).

Discussion

We have investigated the role of protein kinases in the mechanism of induction of two known neuroadaptations following repeated, intermittent AMPH: neurite outgrowth and enhanced AMPH-stimulated DA release. Although these two neuroadaptations are known to occur in brain and PC12 cells following repeated, intermittent AMPH, our study is the first to demonstrate that induction of these two neuroadaptations result from divergent downstream signaling pathways. PKC is clearly important in the induction of both neuroadaptations. This conclusion is based on the fact that two structurally dissimilar PKC inhibitors, chelerythrine and Ro31-8220, blocked the induction of both neuroadaptations and that direct activation of PKC by TPA induced both neuroadaptions. The demonstration of activation of PKC in response to AMPH further confirms that AMPH elicits a rapid activation of PKC that initiates induction of these events. MAP kinase similarly plays a critical role in the induction of both neurite outgrowth and the enhancement in AMPH-mediated DA release. This was confirmed by the fact that the MEK inhibitor, PD98059, blocked the induction of both neuroadaptations. In addition, acute AMPH increased the phosphorylation of MAP kinase. On the contrary, PKA appears to be required only for the induction of the enhanced AMPH-stimulated DA release, suggesting that PKA activation is an event downstream of PKC activation. This conclusion is further supported by the fact that a short treatment of the cells with AMPH did not activate PKA and that direct activation of PKA did not mimic AMPH-induced neurite outgrowth and DA release.

A role for PKC in the induction of the neuroadaptations in response to repeated, intermittent AMPH might be expected based on previous studies demonstrating the requirement of PKC activity for AMPH-induced reverse transport (Giambalvo 1992a; Kantor and Gnegy 1998b). Inhibitors of PKC block AMPH-stimulated DA release whereas PKC activators increase DA release through the plasmalemmal transporter in both rat brain and PC12 cells (Kantor and Gnegy 1998b; Kantor *et al.* 2001). Similarly, PKC inhibitors block the expression of the enhanced AMPH-stimulated DA release in rat striatum following repeated AMPH (Kantor *et al.* 1999). Although a role for PKC in the induction of behavioral sensitization following repeated AMPH has not been examined, increases in PKC activity have been related to psychostimulant action. Locomotion stimulated by intra-accumbens AMPH in the rat was inhibited by prior intra-accumbal injection of Ro31-8220 (Browman *et al.* 1998). Both acute AMPH and repeated AMPH increase the phosphorylation of neuromodulin (GAP-43, F1) in rat striatum *in vivo* and rat striatal synaptosomes *in vitro* at serine-41, the PKC-substrate site (Gnegy *et al.* 1993; Iwata *et al.* 1996, 1997a,b). Kramer *et al.* (1998) demonstrated that 3,4-methylenedioxymethamphetamine, acting through the serotonin

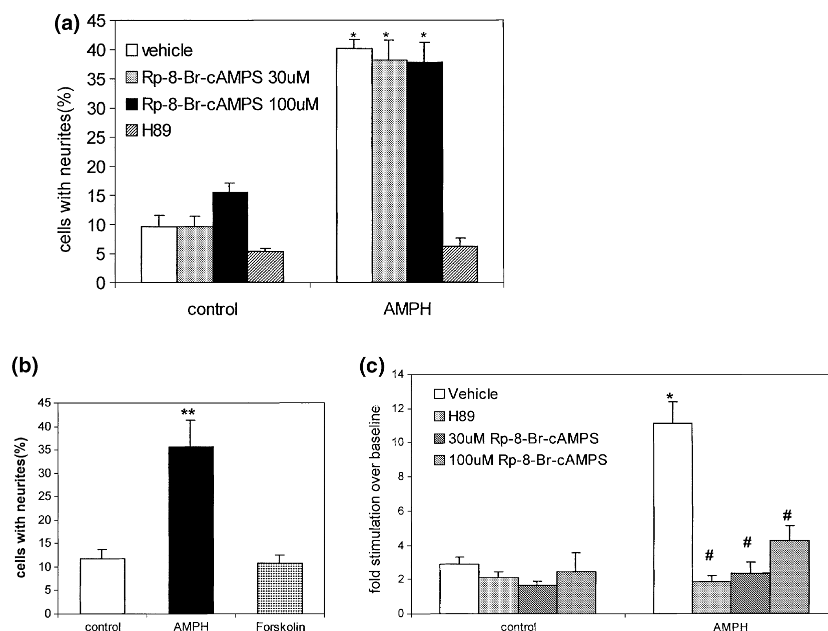

Fig. 5 Effect of PKA on AMPH-induced neurite outgrowth (a, b) or enhanced AMPH-induced DA release (c) in PC12 cells. (a) PC12 cells were treated with 1 μM AMPH (*x*-axis) or vehicle (control, *x*-axis) for 5 min per day for 5 days. Before each vehicle or AMPH treatment, PC 12 cells were pre-treated with vehicle, 30 μM or 100 μM Rp-8-Br-cAMPS (1 h) or 500 nM H-89 (30 min) (legend in Figure) as described in Materials and Methods. Neurite outgrowth was measured after 10 drug-free days. Values represent means ± SEM. $n \geq 3$. ANOVA $p < 0.0001$. In *post hoc* Tukey–Kramer analysis, $*p < 0.001$ vs. all controls and H-89-AMPH. (b) Effect of forskolin on AMPH-induced neurite outgrowth in PC12 cells. PC12 cells were treated with 10 μM forskolin for 5 min a day for 5 days as described in Materials and Methods. Values represent means ± SEM. $n \geq 3$. ANOVA $p \leq 0.004$. In *post hoc* Tukey–Kramer analysis, $*p \leq 0.01$ vs. vehicle, forskolin. (c) Effect of H-89 and Rp-8-Br-cAMPS on the induction of enhanced AMPH-stimulated DA release by repeated, intermittent AMPH. PC12 cells were treated for 5 min a day for 5 days with 1 μM AMPH (*x*-axis) or vehicle (control, *x*-axis) with or without pre-treatment with vehicle, 30 μM or 100 μM Rp-8-Br-cAMPS (1 h) or 500 nM H-89 (30 min). After 10 drug-free days, PC12 cells were harvested and perfused and DA release was measured as described in Methods & Materials. Results are given in fold-stimulation of DA in fraction #7 over baseline ± SEM. $n \geq 3$. ANOVA, $p < 0.0001$. In post hoc Tukey–Kramer analysis, $*p < 0.001$ vs. vehicle-control, $\#p < 0.001$ vs. vehicle-AMPH.

transporter in cerebral cortical synaptosomes, leads to an activation of PKC within the nerve terminal. Finally, increased protein kinase C activity in the VTA may be important in the development of cocaine-induced sensitization (Steketee 1997).

It is not clear, however, how AMPH or AMPH derivatives such as 3,4-methylenedioxymethamphetamine activate PKC. It is unlikely that released DA or norepinephrine is activating PKC through a DA receptor, since neither D1 receptor nor D2 receptor antagonists block the induction of these neuroadaptations by repeated AMPH in PC12 cells (Park *et al.* 2002). PC12 cells do not contain α1-adrenergic or α2-adrenergic receptors (Williams *et al.* 1998; Molderings *et al.* 2002). AMPH is a ligand of a recently identified trace amine receptor, but that receptor is coupled to an inhibition of adenylyl cyclase through a Go/Gi mechanism (Bunzow *et al.* 2001). σ_2 receptor ligands are reported to enhance AMPH-stimulated DA release via activation of PKC activity (Derbez *et al.* 2002). AMPH, however, appears not to be activating the σ_2 receptor itself since a σ_2-specific antagonist had no effect on AMPH-mediated DA release in the absence of a σ_2 receptor agonist (Derbez *et al.* 2002). Since phosphatase enzymes such as PP2A have been demonstrated to be associated with monoamine transporters in the membrane, it is possible that protein kinases could be similarly associated with the transporter (Ramamoorthy *et al.* 1998; Bauman *et al.* 2000). Either binding of AMPH or the conformational change associated with inward transport could activate an associated kinase or recruit a nearby kinase, perhaps by an effect on membrane lipids or production of arachidonic acid. Arachidonic acid alters transporter function and can increase PKC activity (Ingram and Amara 2000). There are reports that AMPH may increase the concentration of intracellular Ca^{2+} (Chen *et al.* 1998; Kantor *et al.* 2001; Derbez *et al.* 2002), which could elicit PKC translocation and activation as shown in 1321 N1 astrocytoma cells (Trilivas and Brown 1989). We have evidence that AMPH can increase intracellular Ca^{2+} in PC12 cells (Kantor and Gnegy, preliminary results). Further, we have demonstrated that intracellular Ca^{2+} is required for AMPH-mediated outward transport of DA in both brain and PC12 cells (Kantor and Gnegy 1998a; Kantor *et al.* 2001).

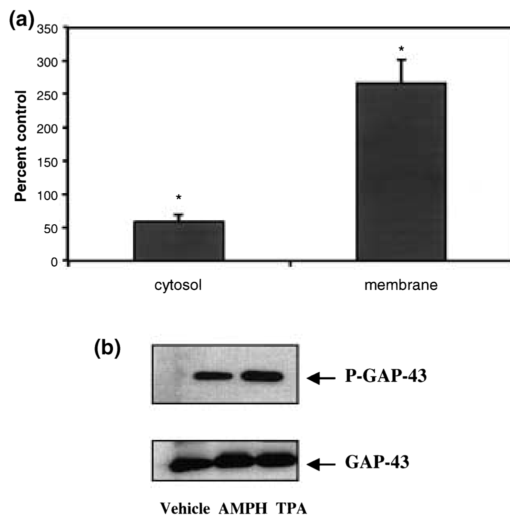

Fig. 6 Effect of acute AMPH treatment on PKC activity. (a) PKC activity measured in cytosol and membrane after acute AMPH treatment in PC12 cells. PC12 cells were incubated for 5 min with or without 3 μM AMPH. Cytosolic and membrane extracts were prepared and PKC activity was determined as described in Methods and Materials. Results are presented as percentage control (vehicle) ± SEM. Control PKC values in the cytosol and membranes were 584 ± 86 pmol/(min mg protein) and 281 ± 56 pmol/(min mg protein), respectively, $n = 6$. *$p < 0.01$ as compared to control (100%) by two-tailed Student's *t*-test. (b) Effect of acute AMPH and TPA on the phosphorylation of GAP-43. PC 12 cells were treated with 3 μM AMPH or 250 nM TPA for 5 min. Western blot analysis was performed as described in Materials and Methods using anti-phosphoser[41]-GAP-43 (top). The blot was stripped and reprobed for total GAP-43 (bottom).

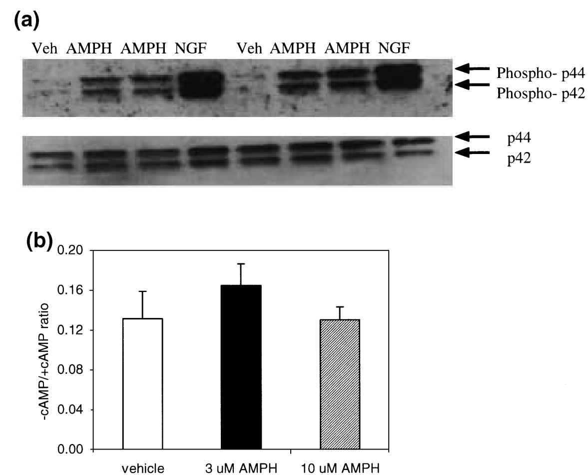

Fig. 7 Effect of acute AMPH on MAP kinase activity (a) or PKA activity (b). (a) PC12 cells were treated with 3 μM AMPH for 5 min and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described in Material and Methods. Western blot analysis was performed to detect phosphorylated MAP kinase (top, two forms: phospho-p44 and phospho-p42). The blot was subsequently stripped and reprobed with an antibody measuring total MAP kinase (bottom, p44 and p42). Cells in lanes 1 and 5 received 5 min of vehicle, lanes 2, 3, 6 and 7 received 3 μM AMPH and lanes 4 and 8 received 50 ng/mL of nerve growth factor as a positive control. (b) PC12 cells were prepared and incubated for 5 min with or without 3 μM AMPH. Whole cell lysates were prepared and PKA activity was determined as described in Materials and Methods. Results represent the ratio of the activity without cAMP (– cAMP) over that with cAMP (+ cAMP) ± SEM. The activity of PKA in the control samples is 844 ± 65 pmols/min/mg/protein. $n = 3$.

MAP kinase plays an important role in the induction of neurite outgrowth in PC12 cells (Vaudry *et al.* 2002). As expected, the pre-treatment of PC12 cells with the MEK inhibitor PD98059 blocked the induction of AMPH-induced neurite outgrowth and it additionally blocked the ability of AMPH to induce enhanced AMPH-mediated DA release. PD98059 did not, however, block acute AMPH-stimulated DA release (data not shown). This result is consistent with the finding that intra-VTA injection of PD98059 did not influence the acute behavioral response to cocaine but it blocked the development of sensitization (Pierce *et al.* 1999). MAP kinase has been demonstrated to play a pronounced role in cocaine-induced behavioral sensitization (Pierce *et al.* 1999; Valjent *et al.* 2000) but its role in the induction of behavioral sensitization and neuroadaptations resulting from repeated AMPH has not been explored. AMPH increases extracellular signal-regulated kinase phosphorylation in rat striatum through activation of metabotropic glutamate receptors (Choe *et al.* 2002). Activation of D1 receptors, D2 receptors, group I mGluRs and group II mGluRs have all been demonstrated to lead to MAP kinase phosphorylation and activation in brain and cultured cells (Otani *et al.* 1999; Yan *et al.* 1999; Narkar *et al.* 2001; Oak *et al.* 2001). However, we found that the D2 dopamine receptor antagonist, sulpiride, and the D1 dopamine receptor antagonist, SCH23390, did not block AMPH-mediated neurite outgrowth (Park *et al.* 2002) or enhanced DA release (Kantor and Gnegy, preliminary results). Another possibility is that AMPH can activate PKC followed by MAP kinase activation. There is evidence that PKC can regulate MAP kinase by activating Raf-1 (Kolch *et al.* 1993). We are presently characterizing the amphetamine-mediated phosphorylation of MAP kinase in PC12 cells.

In contrast to the ability of PKC and MAP kinase inhibitors to block both neuroadaptations, PKA inhibition blocked the induction of enhanced AMPH-stimulated DA release in PC12 cells but not the AMPH-mediated induction of neurite outgrowth. This differentiates the mechanisms by which AMPH induces neurite outgrowth and enhances DA release. The role of PKA in the induction of enhanced AMPH-stimulated DA release is confirmed by the use of two different PKA inhibitors: H-89, a competitive inhibitor at the ATP substrate site, and Rp-8-Br-cAMPS (Gjertsen *et al.* 1995), which binds to the regulatory site of PKA and blocks enzyme activation. Unexpectedly, H89 but not

Rp-8-Br-cAMPS blocked AMPH-induced neurite outgrowth. Rp-8-Br-cAMPS blocked the induction of the enhanced AMPH-stimulated DA release, even at the lower dose of 30 μM, demonstrating that it was active at both the 30 μM and 100 μM concentrations. An action of H-89 other than PKA inhibition could be responsible for the blockade of AMPH-induced neurite outgrowth. H-89 has been shown to inhibit mitogen- and stress-activated protein kinase 1 and S6-activated protein kinase 1 with a potency similar to or greater than that for PKA (Davies *et al*. 2000). Therefore AMPH-mediated neurite outgrowth may be dependent on PKC and a MAP kinase pathway and independent of PKA. PACAP38-induced neurite outgrowth in PC12 cells, for example, is dependent on PKC and MAP kinase but not on PKA (Lazarovici *et al*. 1998), despite the fact that PACAP38 activates adenylyl cyclase activity and increases the formation of cAMP.

cAMP and PKA activation appear to have some role in the induction of behavioral sensitization to AMPH but manipulations of this system neither totally mimic nor block the full range of responses obtained with repeated systemic AMPH. Repeated intra-VTA injections of cholera toxin, which activates adenylyl cyclase, has been reported to induce a short-lasting behavioral sensitization by itself but there was no enhancement of accumbal AMPH-mediated DA release (Tolliver *et al*. 1996). The PKA activator Sp-cAMPS could not elicit sensitization (Tolliver *et al*. 1999). These results would correlate with the inability of forskolin, which increases cAMP production and PKA activation, to induce either neuroadaptation in the present study. Conversely, inhibition of PKA by direct intra-VTA injection of either an adenylyl cyclase inhibitor or Rp-cAMPS blocked the sensitization induced by repeated intra-VTA AMPH but not systemic AMPH (Tolliver *et al*. 1999). AMPH-induced locomotor sensitization is not seen in RII beta PKA knockout mice but it is difficult to know whether this is due to a problem with induction or expression (Brandon *et al*. 1998). Therefore, our results in the PC 12 cell correspond with the results found in rats, that PKA activation is necessary but not sufficient in the induction of select behaviors and neuroadaptations resulting from repeated AMPH.

We were unable to detect a direct effect of AMPH on PKA activity. Since AMPH can release DA within 3 min, an AMPH-mediated release of DA or other activity that would increase cAMP should have been apparent in our 5 min incubation of the cells with AMPH. This suggests that the activation of PKA is a later effect of AMPH action. In addition, as stated above, pre-treatment of the cells with DA D1 or D2 antagonists before each AMPH treatment did not block the induction of the neuroadaptations (Park *et al*. 2002). The more rapid activation of PKC could lead to downstream activation of the PKA system. For instance, PKC activation could alter cAMP phosphodiesterase activity, as shown in PC18 and vascular smooth muscle cells (Yingling *et al*. 1994; Liu *et al*. 2000), or could lead to increased cAMP biosynthesis (Yingling *et al*. 1994; Mons *et al*. 1998).

In summary, induction of neurite outgrowth and DA release after repeated, intermittent AMPH treatment is dependent on PKC and MAP kinase activation in PC12 cells. The cAMP-dependent signaling pathway also contributes to AMPH-induced enhanced DA release in PC12 cells, suggesting that enhanced neurite outgrowth and DA release have different mechanisms of induction by repeated AMPH. Understanding the regulation of the neurite outgrowth and DA release in PC12 cells after repeated, intermittent treatment with AMPH will give greater insights into the neuronal adaptations following withdrawal from repeated AMPH. They could also contribute to the therapeutics of neurodegenerative disease. Repeated, intermittent AMPH has proven useful in ameliorating effects of cerebral ischemia in rats (Stroemer *et al*. 1998) and in treatment of stroke in humans (Crisostomo *et al*. 1988).

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