Repeated intermittent amphetamine enhances efflux of dopamine through the dopamine transporter in rat basal ganglia and through the norepinephrine transporter in rat pheochromocytoma PC12 cells. Extracellular Ca\(^{2+}\) is required for the detection of this enhancement in the rat. In this study, we examined the role of Ca\(^{2+}\) and Ca\(^{2+}\) channels in the enhanced amphetamine-induced dopamine efflux that develops in PC12 cells following repeated intermittent amphetamine. Repeated pretreatment of PC12 cells with 1 μM amphetamine followed by a drug-free period increased amphetamine-induced efflux of dopamine compared with controls. The enhancement in amphetamine-induced dopamine efflux depended upon the presence of extracellular Ca\(^{2+}\) and was inhibited by the blockade of N-type and L-type Ca\(^{2+}\) channels. The enhanced dopamine efflux was not altered by tetanus toxin or reserpine, treatments that abrogate synaptic vesicle-mediated, exocytotic dopamine efflux. Measurement of intracellular Ca\(^{2+}\) concentrations using fura-2/acetoxymethyl ester revealed that amphetamine increased intracellular Ca\(^{2+}\) by a transporter-dependent mechanism. In amphetamine-pretreated cells, amphetamine elicited a greater increase in intracellular Ca\(^{2+}\); this increase depended upon the presence of extracellular Ca\(^{2+}\) and N- and L-type Ca\(^{2+}\) channel activity. The enhanced amphetamine-induced dopamine efflux requires Ca\(^{2+}\)/calmodulin kinase activity. In vehicle-treated cells, 1 μM amphetamine inhibited the calmodulin kinase activity although it did not in amphetamine-pretreated cells. This study suggests that repeated intermittent amphetamine couples norepinephrine transporter activity and Ca\(^{2+}\) signaling.

The locomotor, reinforcing, and salient incentive effects of the psychostimulant amphetamine are attributed, in part, to the ability of amphetamine to induce outward transport of dopamine through the dopamine transporter, DAT (Wise and Bozarth, 1987). Repeated intermittent treatment with amphetamine leads to a long-lasting behavioral sensitization and persistent neuroadaptations (Robinson and Becker, 1986; White and Kalivas, 1998; Wolf, 1998). Behavioral sensitization is induced by a direct action of amphetamine in the dopamine cell body area, the ventral tegmentum area (VTA) (Vezina, 1993), but is expressed in the terminal areas (Robinson and Becker, 1986) such as the striatum and nucleus accumbens. Although the behavioral sensitization is elicited by the repeated amphetamine, other neurotransmitters and even growth factors influence its development and expression (Wolf, 1998; Flores and Stewart, 2000; Vanderschuren and Kalivas, 2000).

One long-lasting neuroadaptation resulting from repeated intermittent amphetamine treatment in the rat is enhanced efflux of dopamine in response to a subsequent amphetamine challenge (Robinson and Becker, 1986). This neuroadaptation develops later than the behavioral sensitization but is extremely persistent and thus could contribute to the endurance of sensitization. An interesting feature of this neuroadaptation is its dependence upon extracellular Ca\(^{2+}\) (Pierce and Kalivas, 1997; Kantor et al., 1999). Although amphetamine-induced outward transport does not normally require extracellular Ca\(^{2+}\), the enhancement in amphetamine-mediated dopamine efflux following repeated amphetamine requires extracellular Ca\(^{2+}\) and is blocked by inhibitors of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II) (Kantor et al., 1999).

In the rat, this neuroadaptation is expressed in the terminal areas of striatum and nucleus accumbens but is initiated by amphetamine in the VTA (Vezina, 1993). We have shown, however, that this neuroadaptation can be developed in cell
lines containing monoamine transporters for which amphetamine is a substrate (Kantor et al., 2002). Repeated intermittent treatment of rat pheochromocytoma PC12 cells or human neuroblastoma SH-SY5Y cells resulted in an enhancement in amphetamine-induced efflux of dopamine (Kantor et al., 2002). The development of the neuroadaptation in the PC12 cells did not alter the number of monoamine transporters, the amount of dopamine in the cell, or the uptake of dopamine. There was a requirement for intermittent but not continuous dosage of amphetamine with several drug-free days following the repeated treatment. These experiments demonstrated that the inception of this neuroadaptation did not require a complex intact neuroanatomy. Furthermore, we have demonstrated that the induction and expression of the neuroadaptation could be developed in the same cell.

It remains to be determined, however, whether the characteristics of the expression of the neuroadaptation in the cells has the same Ca\(^{2+}\) dependence as in the rat, that is, whether the enhanced amphetamine-induced efflux of dopamine is dependent upon extracellular Ca\(^{2+}\) and CaM kinase II activity. The use of the PC12 cells gives us the advantage of measuring biochemical responses to amphetamine challenge in one cell type rather than the mixture of terminals and cell types in dopamine terminal areas of the rat. The PC12 cell has proven an ideal single-cell model in which to investigate molecular mechanisms of acute and repeated amphetamine.

Measurement of release of endogenous dopamine, rather than norepinephrine, is suitable in the PC12 cells because they contain more dopamine than norepinephrine (Greene and Tischler, 1976); both dopamine and amphetamine are excellent substrates for the norepinephrine transporter (Wall et al., 1995), and dopamine is readily released in PC12 cells in response to depolarization (Kittner et al., 1987) and amphetamine (Kantor et al., 2001).

In this study, we examined whether the Ca\(^{2+}\) requirement for the expression of the neuroadaptation to repeated amphetamine, enhanced amphetamine-induced dopamine efflux, is the same in PC12 cells as in the rat. Using the PC12 cells, we further explored the ability of amphetamine to alter intracellular Ca\(^{2+}\) by affecting Ca\(^{2+}\) channel activity.

**Materials and Methods**

**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 Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 5% heat-inactivated horse serum, 2 mM L-glutamine, 100 \(\mu\)g/ml streptomycin, and 100 units/ml penicillin (Invitrogen, Carlsbad, CA) and were incubated at 10% CO\(_2\). Cells (5 \(\times\) 10\(^4\) cells/ml) were plated in a 75-cm\(^2\) tissue culture flask at 10% CO\(_2\) in 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. Dopamine efflux was measured in the superfused cells by high-pressure liquid chromatography with electrochemical detection in response to 1 \(\mu\)M amphetamine as described previously (Kantor et al., 2002). All chambers were perfused with KRB buffer or drug for 30 min followed by a 2.5-min bolus KRB buffer or 1 \(\mu\)M amphetamine with or without the drug. For some experiments, cells were perfused for 90 min with 50 nM reserpine before introduction of 1 \(\mu\)M amphetamine.

**[\(^3\)H]DA Uptake.** PC12 cells were grown and harvested as discussed above. Cells (200 \(\mu\)l at 1.2 mg/ml protein) were placed in tubes and equilibrated to 37°C. Uptake of either 50 nM or 1 \(\mu\)M [\(^3\)H]dopamine into PC12 cells was measured as described previously (Kantor et al., 2001).

**Calcium Measurement.** Intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was measured in fura-2-loaded PC12 cells using dual-wavelength spectrofluorometry according to Fisher et al. (1989). PC12 cells were harvested and resuspended in KRB buffer with and without 1.2 mM CaCl\(_2\) to a protein concentration of 3 mg/ml. The pellet was resuspended in KRB buffer with and without added Ca\(^{2+}\). The cells were then incubated with 50 \(\mu\)M fura-2/AM buffer for 15 min at 37°C, washed twice, and resuspended in KRB buffer. Fluorescence measurements were made on 1-nl aliquots of cells maintained at 37°C and constantly stirred. Changes in [Ca\(^{2+}\)] were monitored as variations in the fluorescence ratio of the 340- to 380-nm excitation wavelength in a Shimadzu RF-5000 spectrofluorimeter. Calcium concentrations were calculated by the method of Grynkiewicz et al. (1985). In some experiments, PC12 cells were preincubated for 10 min with 5 \(\mu\)M nitrendipine or 1 \(\mu\)M omega-conotoxin GVIA following loading of fura-2/AM.

**CaM Kinase II Activity.** Cells were homogenized in a buffer containing 50 mM PIPES pH 7.0, 1 mM EGTA, 10% glycerol, 2 mM dithiothreitol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and protease inhibitor cocktail. Homogenates were sonicated in a bath sonicator for 5 min at 4°C and then centrifuged for 20 min at 10,000g. Supernatants were assayed for CaM kinase II in a reaction mixture (50 \(\mu\)l) containing 50 mM PIPES, pH 7.0, 15 mM MgCl\(_2\), 1 mM CaCl\(_2\) or 5 mM EGTA, 0.5 \(\mu\)g of calmodulin, 50 \(\mu\)g of bovine serum albumin, and 10 \(\mu\)M autacamide as substrate. Reactions were initiated by addition of 50 \(\mu\)M [\(\gamma\)-\(^3\)P]ATP (1 Ci/mmol). Samples were incubated at 30°C for 1 min, and the reaction was stopped by adding 50 \(\mu\)l of 10% trichloracetic acid. The samples were centrifuged at 10,000g; 25 \(\mu\)l of supernatant was spotted on P81 paper, washed with four changes of water, dried, and counted in a Beckman scintillation counter LS 5801 using Scintiverse. Specific activity is expressed as nanomoles of \(^{32}\)P transferred to the substrate per minute per milligram of protein.

**Statistics.** Statistical significance was determined in most experiments using one-way ANOVA. Post hoc comparisons of data were made using the Tukey-Kramer multiple comparisons test. All calculations were performed using GraphPad 3.0.

**Results**

**Ca\(^{2+}\) Requirement for Enhanced Amphetamine-Induced DA Efflux.** As we reported previously (Kantor et al., 2002), repeated intermittent treatment of the PC12 cells with 1 \(\mu\)M amphetamine resulted in an enhanced efflux of dopamine to a subsequent challenge with 1 \(\mu\)M amphetamine in the perfusion apparatus. Detection of the enhanced dopamine efflux, however, was contingent upon the presence of Ca\(^{2+}\) in the KRB perfusion buffer (Fig. 1). When KRB buffer not containing CaCl\(_2\) was perfused through amphetamine pretreated cells, the amount of dopamine efflux was the same as that of vehicle-treated cells. The requirement of extracellular Ca\(^{2+}\) for the expression of the enhanced amphetamine-induced dopamine efflux mimics the finding in the rat (Kantor et al., 1999). As shown in Table 1, uptake of [\(^3\)H]dopamine
at either 50 nM or the higher concentration of 1 μM was not different whether or not CaCl₂ was present in the KRB buffer. Furthermore, there was no difference in uptake of [³H]dopamine at either concentration in vehicle- versus amphetamine-pretreated PC12 cells, as also reported by Kantor et al. (2002).

**Effect of Ca²⁺ Channel Blockers on the Expression of Enhanced Amphetamine-Induced DA Efflux after Repeated Intermittent Amphetamine.** The requirement for extracellular Ca²⁺ for detection of the enhanced amphetamine-induced dopamine efflux following repeated amphetamine in PC12 cells. PC12 cells were treated with repeated vehicle (Veh) or 1 μM amphetamine (AMPH) for 5 min/day for 5 days followed by 10 drug-free days, harvested, and perfused as described under Materials and Methods. Cells were washed with KRB buffer prepared with (+Ca²⁺) or without (-Ca²⁺) 1.2 mM CaCl₂ for 1 h, and fractions containing basal dopamine were collected. Amphetamine (1 μM) was administered for 2.5 min and reached the cells at fraction 9 (shown by arrow). Dopamine in the samples was analyzed by high-pressure liquid chromatography with electrochemical detection. Results are given in picomoles of dopamine per milligram of protein in each fraction (500-μl eluent ± S.E.M., n = 4).

### TABLE 1

<table>
<thead>
<tr>
<th>Repeated Intermittent Treatment</th>
<th>Uptake Assay Conditions</th>
<th>[³H]Dopamine Uptake</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>pmol/min/mg protein</td>
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<td></td>
<td></td>
<td>± S.E.M.</td>
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<tr>
<td>Vehicle</td>
<td>-Ca²⁺</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>+Ca²⁺</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>-Ca²⁺</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>+Ca²⁺</td>
<td>0.21 ± 0.03</td>
</tr>
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</table>

**Repeated Intermittent Amphetamine.** Entrace of Ca²⁺ through both L- and N-type Ca²⁺ channels leads to exocytotic neurotransmitter release in PC12 cells (Taylor and Peers, 1999). Ca²⁺ entry through the Ca²⁺ channels could elicit release of dopamine from vesicular stores, which would account for the enhancement in dopamine efflux in response to amphetamine. To assess this possibility, we pretreated the cells with two agents known to block vesicular neurotransmitter release, tetanus toxin and reserpine. Tetanus toxin inhibits vesicular release by the cleavage of synaptobrevin (Link et al., 1992). Reserpine blocks uptake of dopamine and noradrenaline into the vesicles leading to a profound depletion of catecholamines. Although reserpine blocks vesicular monoamine release it does not affect DAT-mediated dopamine efflux (Chiueh and Moore, 1975). Reserpine (50 nM) was perfused through vehicle- or amphetamine-pretreated PC12 cells for 90 min before the challenge with 1 μM amphetamine. This treatment reduced the dopamine content of the vehicle-treated cells by 88 ± 4% (n = 6) and the dopamine content of amphetamine-pretreated cells by 93 ± 2% (n = 6). Vehicle- or amphetamine-pretreated cells were incubated with 10 nM tetanus toxin for 18 h on the 9th drug-free day (day 14 of total treatment). The data in Fig. 3 demonstrate that neither the depletion of vesicular dopamine stores by reserpine nor the blockade of exocytosis by tetanus toxin altered the expression of the enhanced dopamine efflux in response to amphetamine. The effectiveness of the tetanus toxin treatment in blocking exocytotic dopamine release was assessed by measuring the dopamine release in response to 50 mM KCl. There was no dopamine efflux in response to 50 mM KCl in the tetanus toxin-treated cells. In the amphet-
The requirement of extracellular Ca2+ for the expression of the enhanced dopamine efflux in the presence of amphetamine suggests that amphetamine stimulated the entrance of Ca2+ into the cell. To determine whether the amphetamine challenge increased intracellular Ca2+, PC12 cells were pretreated with repeated intermittent vehicle or amphetamine, followed by 10 drug-free days, and then loaded with fura-2/AM to measure intracellular Ca2+. Cells were suspended in KRB buffer made with and without CaCl2. The data in Fig. 4 demonstrate that a challenge dose of 1 μM amphetamine increased intracellular Ca2+ in vehicle-pretreated PC12 cells, and that increase was not dependent upon the presence of extracellular Ca2+. The response to 1 μM bradykinin is also shown. Amphetamine, at 1 μM, elicited a significantly greater increase in intracellular Ca2+ in the PC12 cells that had been pretreated with repeated amphetamine compared with those pretreated with vehicle (Fig. 4). The intracellular Ca2+ rose rather rapidly following administration of amphetamine (Fig. 4, inset), then maintained a plateau. It did not return to baseline values within 5 min. As shown in Fig. 4, the enhanced ability of amphetamine to increase intracellular Ca2+ required the presence of extracellular Ca2+. The increase in intracellular Ca2+ in response to bradykinin, however, was unaffected by pretreatment of PC12 cells with repeated intermittent amphetamine. Thus, the enhancement in response was specific for amphetamine. The ability of amphetamine to elicit the increase in intracellular Ca2+ was blocked by desipramine, demonstrating the involvement of NET. In the presence of 10 μM desipramine, with or without Ca2+ in the KRB buffer, intracellular Ca2+ measurements after administration of 1 μM amphetamine were 36.1 ± 4.8 nM and 25.5 ± 2.3 nM in vehicle- and amphetamine-pretreated cells, respectively (n = 6). These values were not different from baseline values (Fig. 4).

Fig. 3. Tetanus toxin and reserpine do not alter the enhanced amphetamine-induced dopamine efflux in amphetamine-pretreated cells. PC12 cells were treated with repeated vehicle (VEH) or amphetamine (AMPH). Cells were treated with vehicle or 10 nM tetanus toxin on day 14 or were perfused with vehicle or 50 nM reserpine for 90 min on day 15 (the 10th drug-free day) as described under Materials and Methods. Following perfusion with either KRB buffer or reserpine, 1 μM amphetamine was given for 2.5 min followed by a return to KRB buffer. Results are given in fold baseline ± S.E.M. Baseline values for VEH, VEH-tetanus toxin, and VEH-reserpine in picomoles per milligram of protein were: 2.8 ± 0.9, 3.9 ± 1.0, and 4.2 ± 1.9, respectively; baseline values for AMPH, AMPH-tetanus toxin, and AMPH-reserpine were: 3.0 ± 0.8, 1.7 ± 0.5, and 1.8 ± 0.6, respectively; n = 6 to 8. ANOVA was calculated by Kruskal-Wallis nonparametric test, p < 0.002. In post hoc Dunn’s multiple comparison testing, *, p < 0.05 compared with each corresponding VEH value.

Fig. 4. Amphetamine-induced increases in intracellular Ca2+ in PC12 cells pretreated with repeated intermittent vehicle or amphetamine. PC12 cells were treated with repeated vehicle (Veh) or amphetamine (AMPH), harvested, and resuspended in KRB buffer containing (+Ca) or not containing (−Ca) Ca2+. Cells were preincubated with 50 μM fura-2/AM for 20 min and Ca2+ was measured in a spectrofluorometer as described under Materials and Methods. Responses were measured to 1 μM amphetamine or 1 μM bradykinin. Results are given as the Ca2+ concentration (nM ± S.E.M.). Baseline values are given in the open bars and peak Ca2+ values in the shaded bars. ANOVA for all peak Ca2+ values, p < 0.001. In post hoc Bonferroni testing, *, p < 0.01 compared with Veh A + Ca, Veh A − Ca, and AMPH A − Ca, and *p < 0.05 compared with Veh-BK. Inset, representative trace of response to 1 μM amphetamine in amphetamine-pretreated cells. The response to 100 nM ionomycin is also shown.
To determine whether amphetamine was activating L- or N-type Ca\(^{2+}\) channels to increase the intracellular Ca\(^{2+}\), we incubated the cells with either 5 μM nifedipine or 1 μM ω-conotoxin GVIA before amphetamine was added. These treatments did not change baseline Ca\(^{2+}\) measurements. As shown in Fig. 5, both ω-conotoxin GVIA and nifedipine inhibited the enhanced portion of the amphetamine-induced increase in intracellular Ca\(^{2+}\) in cells that had been pretreated with amphetamine. On the contrary, neither Ca\(^{2+}\) channel blocker affected the ability of amphetamine to increase intracellular Ca\(^{2+}\) in vehicle-pretreated cells. Nifedipine and ω-conotoxin GVIA reduced the amphetamine-mediated influx of Ca\(^{2+}\) in amphetamine-pretreated PC12 cells only to the level of vehicle-pretreated cells.

**CaM Kinase Activity Is Required for the Enhanced Amphetamine-Induced Efflux following Repeated Intermittent Amphetamine.** The role of a Ca\(^{2+}\)/CaM-dependent protein kinase was examined by perfusing the cells for 30 min with 10 μM KN-93, a selective inhibitor of CaM kinase II. The data of Fig. 6 demonstrate that KN-93 reduced the ability of amphetamine to release dopamine, but only to the level of efflux exhibited in the vehicle-treated cells. Thus, Ca\(^{2+}\) and CaM played a role only in the enhanced portion of the amphetamine-induced dopamine efflux following repeated intermittent amphetamine treatment. To further explore whether the Ca\(^{2+}\)/CaM-sensitive component of amphetamine-induced dopamine efflux could be attributed to vesicular storage, we examined whether KN-93 could inhibit the enhanced amphetamine-stimulated efflux following reserpine treatment in amphetamine-pretreated cells. In this set of experiments, dopamine efflux in response to 3 μM amphetamine was 1.5-fold baseline ± 0.1 in vehicle-pretreated cells and 2.3 ± 0.2 in amphetamine-pretreated cells (n = 4, p < 0.05). Following 90 min of 50 nM reserpine, amphetamine-stimulated dopamine efflux was unchanged (1.5 ± 0.2 vehicle pretreated and 2.5 ± 0.3 amphetamine pretreated, n = 4, p < 0.05). Following the reserpine pre-treatment, KN-93 was still able to inhibit the amphetamine-stimulated dopamine efflux in the amphetamine-pretreated PC12 cells (1.39 ± 0.04, p < 0.05 compared with amphetamine and reserpine pretreatment). KN-93 had no effect on amphetamine-stimulated dopamine efflux in vehicle- or vehicle- and reserpine-pretreated PC12 cells. Therefore it is unlikely that the CaM kinase II-sensitive portion of the amphetamine-stimulated dopamine efflux following repeated amphetamine depends upon synaptic vesicular storage. This result is reminiscent of that found in the rat. Reserpine pretreatment did not alter the ability of KN-93 to inhibit amphetamine-stimulated dopamine release in the striatum from amphetamine-pretreated rats (Kantor et al., 1999).

Dopamine efflux in response to 1 μM amphetamine was abolished by perfusion with a protein kinase C inhibitor, chelerythrine, in both vehicle- and amphetamine-pretreated cells (Fig. 6). We have shown previously that preincubation with protein kinase C inhibitors block amphetamine-induced dopamine efflux through NET in PC12 cells (Kantor et al., 2001).

**CaM Kinase Activity following Repeated Intermittent Amphetamine.** We examined whether the greater flux of Ca\(^{2+}\) into the cell in response to an amphetamine challenge in the amphetamine-pretreated cells would result in an enhancement of CaM kinase activity. Following the repeated intermittent vehicle or amphetamine pretreatment and the drug-free period, cells were incubated for 2.5 min with vehicle or 1 μM amphetamine in KRB buffer. As shown in Table 2, the challenge with 1 μM amphetamine significantly decreased CaM kinase activity in the PC12 cells treated with repeated intermittent amphetamine. There was no difference in activity between the cells pretreated with vehicle or amphetamine in the absence of amphetamine challenge. In contrast to the vehicle-pretreated cells, however, there was no change in CaM kinase activity following amphetamine chal-

![Graph](image)

**Fig. 5.** The enhanced amphetamine (AMPH)-induced increases in intracellular Ca\(^{2+}\) in amphetamine-pretreated PC12 cells requires N- and L-type Ca\(^{2+}\) channel activity. PC12 cells were treated with repeated vehicle (VEH) or AMPH, harvested, and preincubated with 50 μM fura-2/AM as described under Materials and Methods. Some cells were preincubated for 10 min with 1 μM ω-conotoxin after fura-2/AM loading. Nifedipine (5 μM) was added during the fluorescent measurements prior to the addition of amphetamine. Results are given as the increase in Ca\(^{2+}\) induced by 1 μM amphetamine ± S.E.M., n = 5 to 7. Baseline Ca\(^{2+}\) values for VEH-pretreated cells in the presence of KRB buffer, ω-conotoxin, and nifedipine were: 70 ± 1, 66 ± 4, and 68 ± 5 nM, respectively. Baseline Ca\(^{2+}\) values for amphetamine-pretreated cells in the presence of KRB buffer, ω-conotoxin, and nifedipine were: 73 ± 1, 86 ± 8, and 69 ± 8 nM, respectively. ANOVA, p < 0.02. In post hoc Bonferroni tests, *, p < 0.05 compared with VEH-KRB; **, p < 0.01 compared with AMPH-ω-conotoxin. Statistical significance at the 0.05 level was not achieved in comparing AMPH-KRB and AMPH-nifedipine in a post hoc Bonferroni test, although nifedipine clearly had a diminishing effect in the amphetamine-pretreated cells.
perfused with 1 M amphetamine (AMPH), harvested, and perfused as described in the legend to Fig. 1 and under Materials and Methods. Cells were perfused for 30 min with KRB buffer alone or KRB buffer containing 10 μM KN-93, a CaM kinase II inhibitor, or 1 μM chelerythrine, a PKC inhibitor. All cells were then perfused with 1 μM amphetamine for 2.5 min followed by a return to KRB buffer with or without drugs. Results are given as fold baseline dopamine efflux, n = 4. KN-93 or chelerythrine alone did not alter basal dopamine efflux. Baseline dopamine values for VEH-treated cells with KRB buffer, KN-93, or chelerythrine (in pmol of dopamine/mg of protein ± S.E.M.) were: 0.61 ± 0.07, 0.52 ± 0.2, and 0.37 ± 0.1, respectively (n = 4). Baseline values for amphetamine-pretreated cells with KRB buffer, KN-93, or chelerythrine (in pmol of dopamine/mg of protein ± S.E.M.) were: 0.58 ± 0.07, 0.54 ± 0.07, and 0.57 ± 0.2, respectively, n = 4. ANOVA, p < 0.0001. In Bonferroni post hoc testing, *p < 0.05 compared with VEH-KRB (pretreatment-perfusion), VEH-KN-93, and AMPH-KN-93; **p < 0.05 compared with AMPH-chelerythrine. There were no significant differences between VEH-KN-93 and AMPH-KN-93 or VEH-chelerythrine and AMPH-chelerythrine.

In this study, we have demonstrated that the enhanced amphetamine-induced outward transport of dopamine following repeated intermittent treatment of PC12 cells is dependent on extracellular Ca2+ and CaM kinase II. These characteristics are evocative of those found in rat striatum and nucleus accumbens following repeated intermittent stimulants (Pierce and Kalivas, 1997; Kantor et al., 1999). Moreover, using the advantage of PC12 cells, we have demonstrated that an amphetamine challenge promotes the influx of extracellular Ca2+ through N- and L-type Ca2+ channels into cells that received repeated intermittent amphetamine.

By using the PC12 cell, we have demonstrated both induction and expression of a neuroadaptation resulting from repeated intermittent amphetamine in the same cells. In the

TABLE 2
CaM kinase II activity following amphetamine pretreatment and amphetamine challenge

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Challenge Treatment</th>
<th>CaM Kinase II Activity (nmol/min/mg protein) ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
<td>4.79 ± 0.4</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Amphetamine</td>
<td>2.99 ± 0.2*</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Vehicle</td>
<td>4.11 ± 0.4</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Amphetamine</td>
<td>4.09 ± 0.4</td>
</tr>
</tbody>
</table>

*p < 0.05.

Discussion

In this study, we have demonstrated that the enhanced amphetamine-induced outward transport of dopamine following repeated intermittent treatment of PC12 cells is dependent on extracellular Ca2+ and CaM kinase II. These characteristics are evocative of those found in rat striatum and nucleus accumbens following repeated intermittent stimulants (Pierce and Kalivas, 1997; Kantor et al., 1999). Moreover, using the advantage of PC12 cells, we have demonstrated that an amphetamine challenge promotes the influx of extracellular Ca2+ through N- and L-type Ca2+ channels into cells that received repeated intermittent amphetamine.

By using the PC12 cell, we have demonstrated both induction and expression of a neuroadaptation resulting from repeated intermittent amphetamine in the same cells. In the

![Fig. 6](image-url) Effect of inhibition of CaM kinase and PKC on amphetamine-induced dopamine efflux in vehicle- and amphetamine-pretreated PC12 cells. PC12 cells were treated with repeated vehicle (VEH) or amphetamine (AMPH), harvested, and perfused as described in the legend to Fig. 1 and under Materials and Methods. Cells were perfused for 30 min with KRB buffer alone or KRB buffer containing 10 μM KN-93, a CaM kinase II inhibitor, or 1 μM chelerythrine, a PKC inhibitor. All cells were then perfused with 1 μM amphetamine for 2.5 min followed by a return to KRB buffer with or without drugs. Results are given as fold baseline dopamine efflux, n = 4. KN-93 or chelerythrine alone did not alter basal dopamine efflux. Baseline dopamine values for VEH-treated cells with KRB buffer, KN-93, or chelerythrine (in pmol of dopamine/mg of protein ± S.E.M.) were: 0.61 ± 0.07, 0.52 ± 0.2, and 0.37 ± 0.1, respectively (n = 4). Baseline values for amphetamine-pretreated cells with KRB buffer, KN-93, or chelerythrine (in pmol of dopamine/mg of protein ± S.E.M.) were: 0.58 ± 0.07, 0.54 ± 0.07, and 0.57 ± 0.2, respectively, n = 4. ANOVA, p < 0.0001. In Bonferroni post hoc testing, *p < 0.05 compared with VEH-KRB (pretreatment-perfusion), VEH-KN-93, and AMPH-KN-93; **p < 0.05 compared with AMPH-chelerythrine. There were no significant differences between VEH-KN-93 and AMPH-KN-93 or VEH-chelerythrine and AMPH-chelerythrine.

The reason for the Ca2+ sensitivity of the enhanced amphetamine-mediated outward transport of dopamine is unknown. It could be due to a modification of transporter function or possibly an increase in supply of dopamine to the transporter. If it is a modification of transporter function, it is not one that is reflected in a change in uptake. There was neither a change in [3H]dopamine uptake between vehicle- and amphetamine-pretreated cells nor was there a Ca2+ sensitivity to the uptake process. The lack of change in [3H]dopamine uptake and in whole cell [3H]nisoxetine binding (Kantor et al., 2002) suggests that there is not an increase in surface NET. Although one would logically expect an alteration in efflux to correspond with a change in uptake, the transporter can be asymmetrically regulated (Sitte et al., 1998; Khoshbouei et al., 2004). We recently demonstrated that uptake and efflux through the dopamine transporter

rat, behavioral sensitization and concomitant neuroadaptations are induced by an action of amphetamine at the dopamine cell body area and expressed in the terminals (Vezina, 1993), which are anatomically distanced from the cell body. PC12 cells, however, although not neurons, contain elements present in catecholaminergic cells that could be important for the development of this neuroadaptation: enzymes for catecholamine synthesis and metabolism, including tyrosine hydroxylase, elements for storage of catecholamines, the dense core granules with a vesicular monoamine transporter (Greene and Tischler, 1976), and a plasmalemmal catecholamine transporter (NET) for inward and outward catecholamine transport (Bruss et al., 1997). In addition, the cells contain a CaM kinase activity that is required for the manifestation of the particular neuroadaptation being investigated in this study (Enslen and Soderling, 1994). Although amphetamine acts at the dopamine transporter DAT, in rat VTA, NET shares many important characteristics with DAT. The two proteins are Na+/Cl−-dependent transporters that share over 70% homology (Giros and Caron, 1993). They also share many of the same substrates, including dopamine and amphetamine (Wall et al., 1995) and blockers, such as cocaine (Buck and Amara, 1995). Basic aspects of their regulation, such as sensitivity to regulation by protein kinase C, are the same with the two transporters (Kantor et al., 2001). Therefore it is not unreasonable that repeated intermittent amphetamine would similarly affect the functions of DAT and NET.
have different structural determinants and can be independently regulated (Khoshbouei et al., 2004). Furthermore, there are a number of proteins that bind to the catecholamine transporters (Zahniser and Dolen, 2001). For instance, phosphorylation of a protein associated with the transporter could alter its function by altering a cytoskeletal interaction. A change in binding proteins could also alter the conformation of the transporter (Zahniser and Dolen, 2001).

An increased supply of dopamine to the transporter appears unlikely. The lack of effect of reserpine pretreatment suggests that compromised vesicular function resulting in increased cytosolic dopamine is not an explanation for the enhanced NET-mediated efflux. Similarly, reserpine treatment did not diminish the enhancement in amphetamine-induced dopamine efflux in rat striatum following repeated treatment of rats with amphetamine (Kantor et al., 1999). The repeated amphetamine treatment did not increase the total dopamine in the cell (Kantor et al., 2002), the tyrosine hydroxylase content, or phosphorylation at sites known to increase the enzyme activity in the PC12 cell (Y. H. Park and J. A. Haycock, unpublished data). Although the Ca\(^{2+}\) requirement for the enhanced amphetamine-induced efflux suggests that vesicular dopamine release could be triggered, this possibility is unlikely because neither tetanus toxin pretreatment nor reserpine altered the enhanced response to amphetamine. Therefore, despite the apparent involvement of extracellular Ca\(^{2+}\) as well as L- and N-type calcium channels, vesicular release of dopamine or another vesicular-bound neurotransmitter does not appear to be involved in the effect. Amphetamine, however, can depolarize the membrane (Kahlig et al., 2004). Repeated treatment with amphetamine could alter the membrane potential to make the transporter more responsive to amphetamine. Additionally, increased Ca\(^{2+}\), due to a depolarizing pulse, has been demonstrated to increase trafficking of GABA transporters to the cell surface (Deken et al., 2003). We are presently examining whether a Ca\(^{2+}\)-dependent increased rate of transporter trafficking can explain our results.

Our results suggest that following amphetamine pretreatment, amphetamine acts to enhance L- or N-type Ca\(^{2+}\) channel activity to promote flux of Ca\(^{2+}\) into the cell. Thus, the consequence of the repeated intermittent amphetamine pretreatment is an involvement of Ca\(^{2+}\) channels in the enhancement of two NET-mediated activities, dopamine efflux and Ca\(^{2+}\) influx. Amphetamine elicited an increase in intracellular Ca\(^{2+}\) even in vehicle-pretreated PC12 cells that was independent of Ca\(^{2+}\) channels or extracellular Ca\(^{2+}\). We have demonstrated that amphetamine elicits a cocaine- and thapsigargin-sensitive influx of Ca\(^{2+}\) in HEK 293 cells stably transfected with hDAT (Gnegy et al., 2004). Another mechanism by which amphetamine could increase intracellular Ca\(^{2+}\) is through Na\(^+\)/Ca\(^{2+}\) exchange. Giambalvo (2004) presented evidence suggesting that amphetamine, through an increase in intracellular pH, could increase a Na\(^+\)/Ca\(^{2+}\) exchange activity in rat striatum, which is required for amphetamine to increase PKC activity. An activation of PKC by amphetamine could alter Ca\(^{2+}\) channel activity. N- and L-type Ca\(^{2+}\) channels are substrates for PKC (Catterall, 2000). PKC activation has been shown to enhance Ca\(^{2+}\) currents through both L- and N-type Ca\(^{2+}\) channels (Barrett and Rittenhouse, 2000). We, and others, have shown that amphetamine can activate PKC in rat striatum and PC12 cells (Iwata et al., 1997b; Giambalvo, 2003; Park et al., 2003) and that phosphorylation of some PKC substrates can increase following repeated intermittent amphetamine (Iwata et al., 1997a). Specific forms of N-type Ca\(^{2+}\) channel subunits are also substrates for CaM kinase II (Catterall, 2000). This involvement of L- and N-type Ca\(^{2+}\) channels in a stimulant-induced neuroadaptation is reminiscent of the results of Pierce and Kalivas (1997) who found that behavioral sensitization to repeated cocaine was blocked by both N- and L-type Ca\(^{2+}\) channel blockers.

We were not able to demonstrate a direct activation of CaM kinase II by amphetamine in either vehicle- or amphetamine-pretreated PC12 cells. In fact, incubation of vehicle-pretreated PC12 cells with 1 μM amphetamine for only 2.5 min inhibited CaM kinase II activity. This inhibition mirrors the attenuating effect of a peripheral injection of amphetamine on CaM kinase II activity in several rat brain areas including nucleus accumbens and striatum (Suemaru et al., 2000). CaM kinase II activity in amphetamine-pretreated cells following the 10 drug-free days was no different than the activity from the vehicle-pretreated cells. Moreover, an amphetamine challenge no longer inhibited the CaM kinase II activity. The lack of acute inhibition of CaM kinase II by amphetamine in the cells that had been pretreated with amphetamine suggests that the inhibitory process is no longer operable or that some portion of the enzyme activity is increasing to counterbalance the decrease. We reported an increase in CaM kinase II activity in striatal synaptosomes following treatment of rats with repeated intermittent amphetamine (Iwata et al., 1997a). Our present data may resemble those of Suemaru et al. (2000) more than the synaptosomal data because we are using cells containing a wider variety of regulatory elements than striatal dopaminergic synaptosomes used in our previous study, which are primarily presynaptic structures. Suemaru et al. (2000) used an extract that would contain presynaptic and postsynaptic elements and found that the amphetamine-mediated inhibition of CaM kinase II was mediated by an activation of D1 dopamine receptors. It is possible that amphetamine is altering the activation of a receptor in PC12 cells to inhibit CaM kinase II, although our results suggest that it would not be the dopamine D2 receptor. Similarly, amphetamine could alter the activity of a protein phosphatase, which rapidly alters CaM kinase II activity.

In summary, we have documented that integral characteristics of one neuroadaptation that occurs in brain, enhanced amphetamine-induced dopamine efflux through DAT, can be reproduced in a PC12 cell containing NET. The integral characteristics are the dependence upon extracellular Ca\(^{2+}\) and involvement of CaM kinase II and Ca\(^{2+}\) channels. Moreover, we have made the original finding that repeated treatment with amphetamine results in an activation of N- and L-type Ca\(^{2+}\) channels by amphetamine, which mediates the entry of extracellular Ca\(^{2+}\) into the cell. The increased cellular Ca\(^{2+}\) appears to activate CaM kinase II, which mediates the enhanced amphetamine-induced efflux through the norepinephrine transporter.

Acknowledgments

We thank Dr. Stephen Fisher for the use of the spectrophotometer and invaluable help and advice in discussions concerning intracellular calcium.
References