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Intracellular Ca^{2+} Regulates Amphetamine-Induced Dopamine Efflux and Currents Mediated by the Human Dopamine Transporter

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ABSTRACT

Although it is clear that amphetamine-induced dopamine (DA) release mediated by the dopamine transporter (DAT) is integral to the behavioral actions of this psychostimulant, the mechanism of this release is not clear. In this study, we explored the requirement for intracellular Ca^{2+} in amphetamine-induced DA efflux and currents mediated by the human DAT. The patch-clamp technique in the whole-cell configuration was used on Na^+ and DA-preloaded human embryonic kidney 293 cells stably transfected with the human DAT (hDAT cells). Chelation of intracellular Ca^{2+} by inclusion of 50 μM BAPTA in the whole-cell pipette reduced the voltage-dependent amphetamine-induced hDAT current, with the greatest effect seen at positive voltages. Likewise, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) reduced amphetamine-induced DA efflux as measured by amperometry. Furthermore, preincubation of the cells with 50 μM BAPTA acetoxy methyl ester (AM) or thapsigargin also blocked amphetamine-induced release of preloaded *N*-methyl-4-[^{3}H]phenylpyridinium from superfused hDAT cells. BAPTA-AM also reduced DA release from striatal synaptosomes. Amphetamine also led to an increase in intracellular Ca^{2+} that was blocked by prior treatment with 5 μM thapsigargin or 10 μM cocaine. These studies demonstrate that amphetamine-induced DAT-mediated currents and substrate efflux require internal Ca^{2+} and that amphetamine can stimulate dopamine efflux by regulating cytoplasmic Ca^{2+} levels through its interaction with DAT.

The plasmalemmal dopamine transporter, DAT, is essential for regulation of synaptic levels of dopamine (DA). Although the primary physiological function of DAT is to clear DA from the synapse through reuptake, DAT can also mediate DA efflux. As hypothesized for other gated carriers, the function of DAT seems to depend on conformational changes that alternately expose extracellular and intracellular substrate binding sites (Rudnick and Clark, 1993; Abramson et al., 2003). Thus, after transport and dissociation of substrate, the "inward-facing" conformation of DAT is able to bind cytoplasmic DA and carry it to the outside. DA efflux is most apparent in response to a substrate such as amphetamine (AMPH). Because the locomotor and reinforcing effects of AMPH are a result of its ability to release DA into the synapse (Seiden et al., 1993; Giros et al., 1996; Koob and Nestler, 1997), AMPH-induced DA efflux has been highly investigated. AMPH increases the prevalence of the inward-facing conformation of the plasmalemmal catecholamine transporter (Langeloh et al., 1987), inhibits monoamine oxidase activity, blocks the vesicular transporter (VMAT2), and disrupts the vesicular proton gradient, making more cytoplasmic DA available to the inward-facing DAT (Seiden et al., 1993; Sulzer et al., 1995). Emerging evidence, however, indicates that AMPH-mediated DA efflux does not rely exclusively on the ability of AMPH to increase the availability of inward facing DATs (Kantor and Gnegy, 1998; Pifl and Singer, 1999; Sitte et al., 1998; Chen and Justice, Jr., 2000). The efficacy of AMPH in stimulating DA efflux correlates strongly with its ability to stimulate inward ion fluxes (Sitte

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ABBREVIATIONS: DA, dopamine; DAT, dopamine transporter; hDAT, human dopamine transporter; AMPH, amphetamine; [^{3}H]MPP$^+$, *N*-methyl-4-[^{3}H]phenylpyridinium; AM, acetoxymethyl ester; HEK, human embryonic kidney; KRH, Krebs-Ringers-HEPES; ANOVA, analysis of variance; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; VEH, vehicle; PKC, protein kinase C.

et al., 1998; Pifl and Singer, 1999; Khoshbouei et al., 2003) and DAT-associated transport currents are greater than would be expected from the normal stoichiometric Na^+, Cl^- and substrate transport function (Sonders et al., 1997). Moreover, AMPH-induced DA efflux has been shown to be dependent on the intracellular Na^+ concentration and on the membrane potential (Khoshbouei et al., 2003). Most recently, we have shown that phosphorylation of the N terminus of DAT is essential for AMPH-induced DA efflux (Khoshbouei et al., 2004).

Transporter-mediated efflux of DA into the synapse has been differentiated from vesicular or exocytotic release of DA by their differential requirement for extracellular Ca^{2+}. Exocytotic release of DA requires extracellular Ca^{2+}, whereas AMPH-induced DA efflux is thought to be independent of extracellular Ca^{2+} (Arnold et al., 1977; Ross and Kelder, 1979; Lynch et al., 1985; Carboni et al., 1989; Hurd and Ungerstedt, 1989; Kantor et al., 2001). The effect of intracellular Ca^{2+} on AMPH-induced DA efflux, however, has not been thoroughly investigated. We found previously that chelation of intracellular Ca^{2+} significantly reduced the transporter-mediated DA released in response to AMPH in rat pheochromocytoma (PC-12) cells (Kantor et al., 2001), suggesting a requirement for intracellular Ca^{2+} for AMPH-induced efflux through the homologous plasmalemmal norepinephrine transporter.

To better understand the role of Ca^{2+} in DAT-mediated outward transport and ion fluxes, we characterized the requirement for intracellular Ca^{2+} in AMPH-induced DAT-mediated currents and DA efflux. Moreover, in an initial step toward elucidating the mechanism of action of AMPH, we demonstrated that through its interaction with DAT, AMPH can trigger an increase in the intracellular Ca^{2+} concentration.

Materials and Methods

Drugs and Materials. *d*-Amphetamine sulfate was obtained from Sigma (St. Louis, MO). *N*-methyl-4-[^{3}H]phenylpyridinium ([^{3}H]MPP$^+$) was obtained from PerkinElmer Life and Analytical Sciences. BAPTA acetoxymethyl ester (AM) was purchased from Calbiochem (La Jolla, CA).

Plasmid Construction, Transfection, and Cell Culture. The synthetic hDAT gene tagged at the 5′ end with a FLAG epitope was subcloned into a bicistronic expression vector (Rees et al., 1996) modified to express the synthetic hDAT from a cytomegalovirus promoter and the hygromycin resistance gene from an internal ribosomal entry site as described previously (pciHyg) (Saunders et al., 2000). EM4 cells, an HEK 293 cell line stably transfected with macrophage scavenger (R. Horlick, Pharmacopeia, Cranberry, NJ), were transfected with the FLAG-hDAT using LipofectAMINE (Invitrogen, Carlsbad, CA), and a stably transfected pool (hDAT cells) was selected in 250 μg/ml hygromycin as described previously (Ferrer and Javitch, 1998). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Previous studies have shown that addition of the N-terminal FLAG tag does not alter the ability of the transporter to produce substrate-induced currents (Saunders et al., 2000).

Electrophysiology. Cells were plated at 10^5 per 35 mm culture dish. Attached cells were washed three times with bath solution at room temperature. The bath solution contained 130 mM NaCl, 10 mM HEPES, 34 mM dextrose, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 1.3 mM KH_2PO_4, adjusted to pH 7.35. The pipette solution for the whole-cell recordings contained 30 mM KCl, 90 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.1 mM EGTA, 10 mM HEPES, and 30 mM dextrose plus 2 mM DA, adjusted to pH 7.35. The free Ca^{2+} in this solution was calculated to be 0.1 mM. For the experiments in which we buffered the intracellular Ca^{2+} concentration, 50 μM BAPTA was added to the pipette solution in the absence of $CaCl_2$. Patch electrodes were pulled from quartz pipettes on a P-2000 puller (Sutter Instruments, Novato, CA) and filled with the pipette solution. Whole-cell currents were recorded using an Axopatch 200B with a low-pass Bessel filter set at 1000 Hz. After establishing the whole-cell configuration, we waited 10 min to allow perfusion of the intracellular milieu with the pipette solution. Current-voltage relationships were generated using a voltage step (1 s) protocol ranging from −120 to +100 mV separated by 20 mV from a holding potential of −20 mV. Current and oxidative (amperometric) signals were measured simultaneously. Data were recorded and analyzed off-line using the software pCLAMP 8 from Axon Instruments (Union City, CA). hDAT-mediated whole-cell and amperometric current is defined as the current obtained in the presence of 10 μM AMPH minus that of AMPH plus 10 μM cocaine.

Amperometry. A carbon fiber electrode connected to a second amplifier (Axopatch 200B) was attached to the plasma membrane of the cell held at +700 mV for all experiments unless noted otherwise. The carbon fiber electrodes (ProCFE; fiber diameter is 5 μm) were obtained from Axon Instruments. Oxidative (amperometric) current-voltage relationship was generated as above. As described previously (Khoshbouei et al., 2003), unlike the usual amperometric calibration, which requires conversion to concentration, we report the current

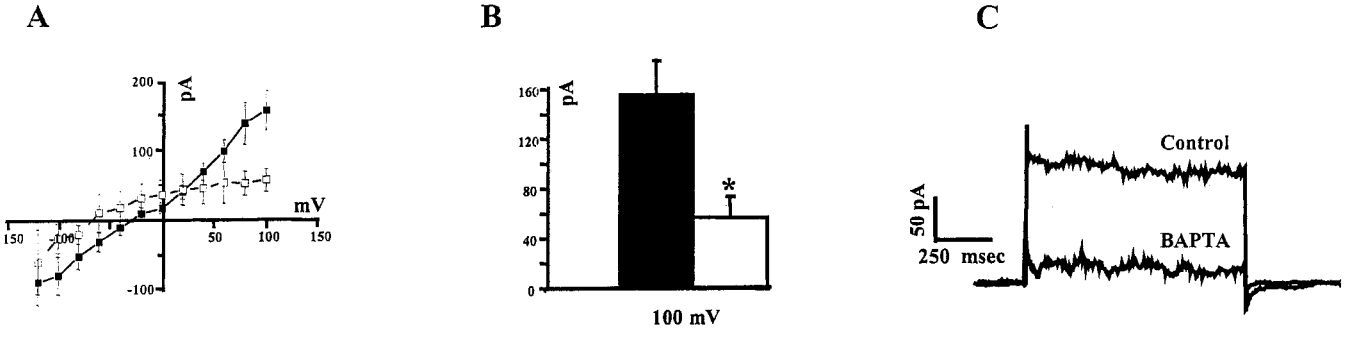

Fig. 1. Removal of internal Ca^{2+} by the membrane-impermeant Ca^{2+}-chelator BAPTA reduces AMPH-induced current in hDAT cells. Cells were voltage-clamped with a whole-cell patch pipette. hDAT-mediated currents were recorded by stepping the membrane voltage from a holding potential of −20 mV to potentials between +100 and −120 mV. The whole-cell pipette solution contained 2 mM DA and 90 mM NaCl in the presence or absence of 50 μM BAPTA; 10 μM AMPH was added in the bath. A, current-voltage relationships of AMPH-induced hDAT-mediated currents in the presence (□) or absence (■) of 50 μM BAPTA in the whole-cell solution. B, bar graph showing the mean hDAT-mediated outward current at +100 mV in the presence (open bar) or absence (filled bar) of 50 μM BAPTA ($n = 5$; *, $p < 0.05$, Student's t test). C, representative traces of AMPH-induced, hDAT-mediated whole-cell currents recorded at +100 mV from two different cells. Addition of 50 μM BAPTA in the recording pipette solution decreased AMPH-induced current.

directly without considering the effective volume. Thus, our requirements are a defined baseline and our data represent a lower limit to the DA efflux because some transmitter is lost to the bulk solution. The amperometric currents were low-pass filtered at 100 Hz. Data were recorded and analyzed off-line using the software pCLAMP 8 from Axon Instruments. Current-voltage relations were generated by plotting against the test voltage the values of the amperometric currents between 800 and 1000 ms after the step.

Intracellular Calcium Measurements. Increases in intracellular calcium levels ($[Ca^{2+}]_i$) were determined essentially as described previously (Berg et al., 1998). Cells in suspension were loaded with fura-2 AM by incubating the cells in Hanks' balanced salt solution containing 0.1% bovine serum albumin and 5 μM fura-2 AM at 37°C for 30 min in the dark followed by a hydrolysis period of 30 min at room temperature in the dark. Cells were washed once, resuspended in Hanks' balanced salt solution/bovine serum albumin, and placed (2×10^6 cells) in a stirred, temperature-controlled (37°C) cuvette in a fluorescence spectrometer (Photon Technology International, Monmouth Junction, NJ) equipped with automatic data collection/analysis software. After a 5-min equilibration period, data were collected using dual-wavelength excitation at 340 and 380 nm and an emission wavelength of 510 nm at a frequency of 1 Hz. Drugs were added to the cuvette after collection of baseline values for 60 s.

[^{3}H]MPP$^+$ Uptake and Superfusion Assay. hDAT cells, grown on 100-cm plates, were incubated for 20 min at 37°C with 10 μM [^{3}H]MPP$^+$ (1.7 μCi/ml) in a Krebs-HEPES buffer (KRH) containing 25 mM HEPES, pH 7.4, 125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 5.6 mM glucose. After the incubation, cells were washed with KRH, harvested, and resuspended in 0.25 ml of KRH. Cells were placed on a Whatman GF/B filter in a chamber of a Brandel superfusion apparatus (SF-12; Brandel, Gaithersburg, MD). The chambers were perfused at room temperature with KRH and 5-min fractions were collected. At fraction number 8, 10 μM AMPH was added and perfused for 2.5 min followed by KRH. When AMPH is introduced at fraction 8, calculating the time of delivery, it reaches the sample at fraction 9 and elutes at fraction 10. Samples were collected into vials and the radioactivity was determined by liquid scintillation counting. In experiments designed to chelate intracellular Ca^{2+}, cells were incubated with 10 μM [^{3}H]MPP$^+$ with or without 50 μM BAPTA-AM. Thapsigargin, when used, was added in fraction 7, one fraction before the AMPH. The radioactivity released during each fraction is calculated as the fractional rate of release, which is the percentage of the total radioactivity present in the cells at the start of each fraction (Pifl and Singer, 1999).

Endogenous DA Efflux in Rat Striatal Slices. Rat striatal slices were prepared and superfused for 30 min with Krebs Ringer's buffer (containing 125 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 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, oxygenated by 95% O_2 and 5% CO_2 for 1 h) in the Brandel superfusion apparatus as described previously (Kantor and Gnegy, 1998). The slices were superfused for 15 min with or without 50 μM BAPTA-AM followed by a 2.5-min bolus of 1 μM AMPH. The DA content in the perfusate was measured by high-performance liquid chromatography with electrochemical detection using dihydroxybenzylamine as an internal standard (Kantor and Gnegy, 1998).

Statistical Analyses. Statistical significance was determined by one-way analysis of variance (ANOVA) with post hoc Tukey-Kramer

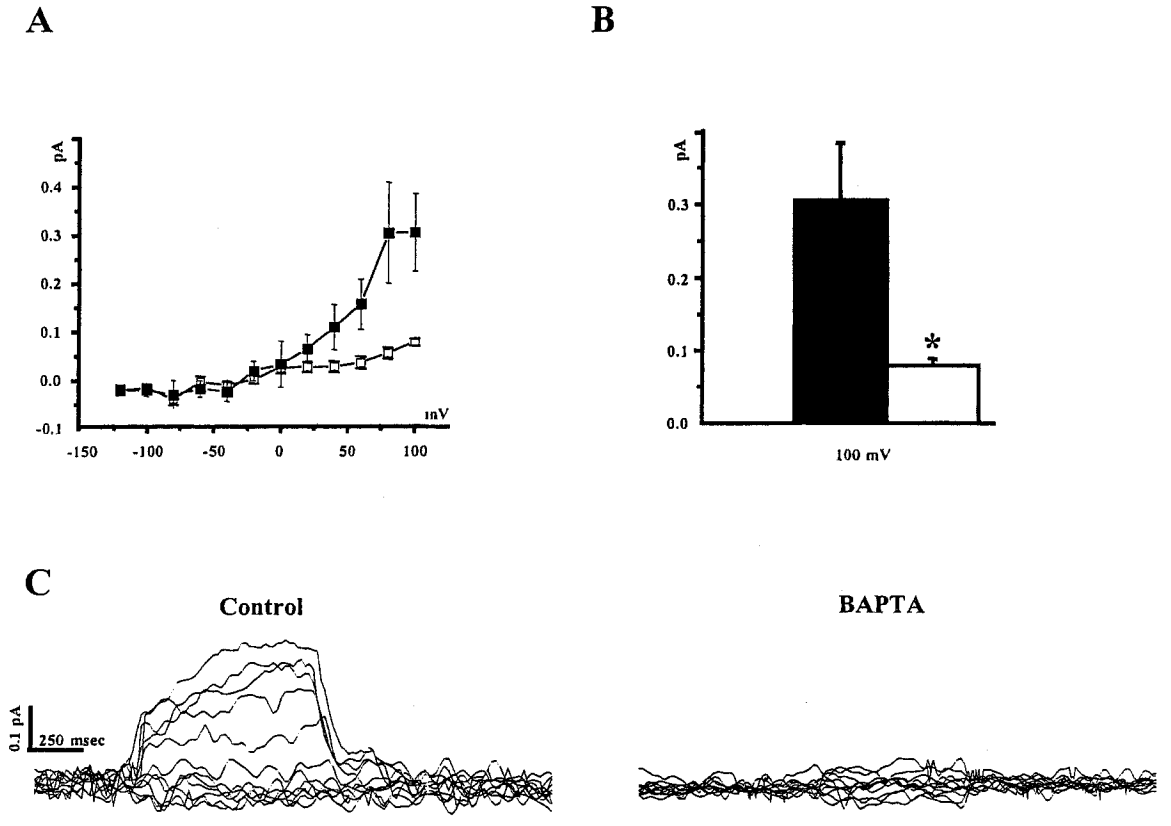

Fig. 2. Removal of internal Ca^{2+} by the membrane-impermeant Ca^{2+} chelator BAPTA reduces AMPH-induced DA efflux from hDAT cells. Cells were voltage-clamped with a whole-cell patch pipette while an amperometric electrode was placed onto the cell membrane. AMPH-induced, hDAT-mediated oxidative currents were acquired concomitantly to the whole-cell currents represented in Fig. 1. AMPH (10 μM) was added in the bath. A, oxidative current-voltage relationships in the presence (□) or the absence (■) of 50 μM BAPTA in the whole-cell patch pipette. B, bar graph showing the mean hDAT-mediated oxidative current at +100 mV in the presence (open bar) or the absence (filled bar) of 50 μM BAPTA in the whole-cell pipette (n = 4; *, $p < 0.03$, Student's t test). C, representative traces of amperometric currents recorded at voltages between −120 mV and +100 mV in the absence (Control) or presence (BAPTA) of BAPTA in the whole-cell pipette solution.

multiple comparisons testing. Comparisons between two groups were made using a two-tailed Student's t test.

Results

Effect of Internal Ca^{2+} on AMPH-Induced Current in hDAT Cells. The effect of internal Ca^{2+} on hDAT-mediated current was examined using the membrane-impermeant Ca^{2+}-chelator BAPTA. HDAT cells were voltage clamped with a whole-cell patch pipette filled with an internal solution containing 90 mM NaCl and 2 mM DA in the presence and absence of 50 μM BAPTA. This intracellular Na^+ concentration facilitated the detection of the AMPH-induced DA efflux obtained by amperometry (Khoshbouei et al., 2003). Figure 1a shows the current-voltage relationship for the AMPH-induced current obtained by bath applying 10 μM AMPH. hDAT-mediated currents were recorded by stepping the membrane voltage from a holding potential of −20 mV to potentials between +100 and −120 mV in 20-mV increments. Outward currents were recorded at voltages more positive than −20 mV. Inclusion of BAPTA in the whole-cell pipette solution significantly reduced the AMPH-induced currents at positive voltages. The mean AMPH-induced, hDAT-mediated outward current obtained at +100 mV was decreased about 60% in the presence of BAPTA (56.4 ± 16 pA) with respect to control conditions (155.4 ± 27 pA) (Fig. 1B). Representative traces of hDAT-mediated whole-cell currents, defined as the current obtained in the presence of 10 μM AMPH minus that of AMPH plus 10 μM cocaine, recorded at +100 mV in the presence or absence of BAPTA, are shown in Fig. 1C.

Effect of Internal Ca^{2+} on AMPH-Induced DA efflux from hDAT Cells. To explore the effect of internal Ca^{2+} on hDAT-mediated DA efflux, amperometry was combined with the patch-clamp recording. The cells were voltage-clamped with a whole-cell patch pipette, whereas an amperometric electrode was placed onto the cell membrane. The AMPH-induced oxidative currents acquired concomitant to the whole-cell currents represented in Fig. 2A were obtained by stepping the membrane voltage of the cell from a holding potential of −20 mV to potentials between +100 and −120 mV with the whole-cell patch pipette. The hDAT-mediated amperometric current is defined as the current obtained in the presence of 10 μM AMPH minus that of AMPH plus 10 μM cocaine. For voltage steps more positive than −50 mV, the amperometric electrode recorded a positive oxidative current that increased at the beginning of the voltage step, reaching a steady-state after several milliseconds. This current was diminished by inclusion of 50 μM BAPTA in the recording pipette (Fig. 2A), demonstrating the importance of intracellular Ca^{2+} for AMPH-induced, hDAT-mediated DA efflux. Fifty micromolar BAPTA reduced the hDAT-mediated oxidative current at +100 mV (0.079 ± 0.007 pA) by approximately 80% with respect to control conditions (0.304 ± 0.07 pA) (Fig. 2B). Amperometric currents recorded at different voltages are shown in Fig. 2C. At the termination of the voltage step, the amperometric current relaxed back to baseline. When 50 μM BAPTA was present in the whole-cell pipette, the signal recorded with the amperometric electrode was barely detectable.

Effect of Chelation of Intracellular Ca^{2+} on AMPH-Mediated [^{3}H]MPP^+ Release in Superfused hDAT Cells. To support the amperometry studies and further demonstrate a role for intracellular Ca^{2+} in DAT-mediated outward transport, the effect of chelation of intracellular Ca^{2+} with BAPTA-AM on AMPH-induced [^{3}H]MPP^+ release was determined in intact hDAT cells. [^{3}H]MPP^+ was used as a

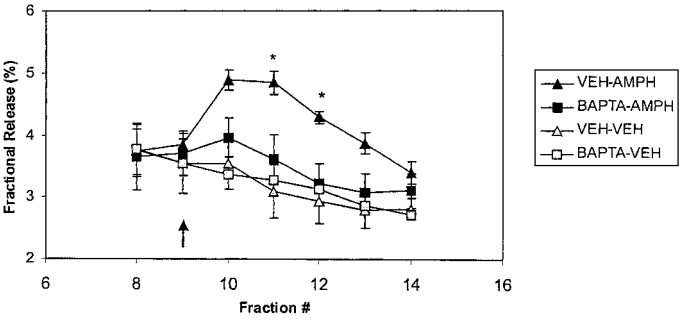

Fig. 3. Effect of the permeant Ca^{2+}-chelator, BAPTA-AM, on AMPH-mediated release of [^{3}H]MPP^+ in hDAT cells. hDAT cells were preincubated with 10 μM [^{3}H]MPP^+ as described under *Materials and Methods* in the absence (VEH) or presence (BAPTA) of 50 μM BAPTA-AM. The cells were then placed in a superfusion apparatus and either KRH (-VEH) or 10 μM AMPH (-AMPH) was added in a 2.5-min bolus, reaching the cells at fraction 9, as shown by the arrow. Results are calculated as fractional rate of release. Note that the y-axis does not begin at zero. In ANOVA of the values for fractions 11 and 12, separately, $p = 0.002$; in post hoc Tukey-Kramer analysis, $p < 0.05$ for VEH-AMPH versus BAPTA-AMPH, and $p < 0.01$ for VEH-AMPH versus VEH-VEH and BAPTA-VEH. $n = 4$; $n = 6$ for VEH-VEH.

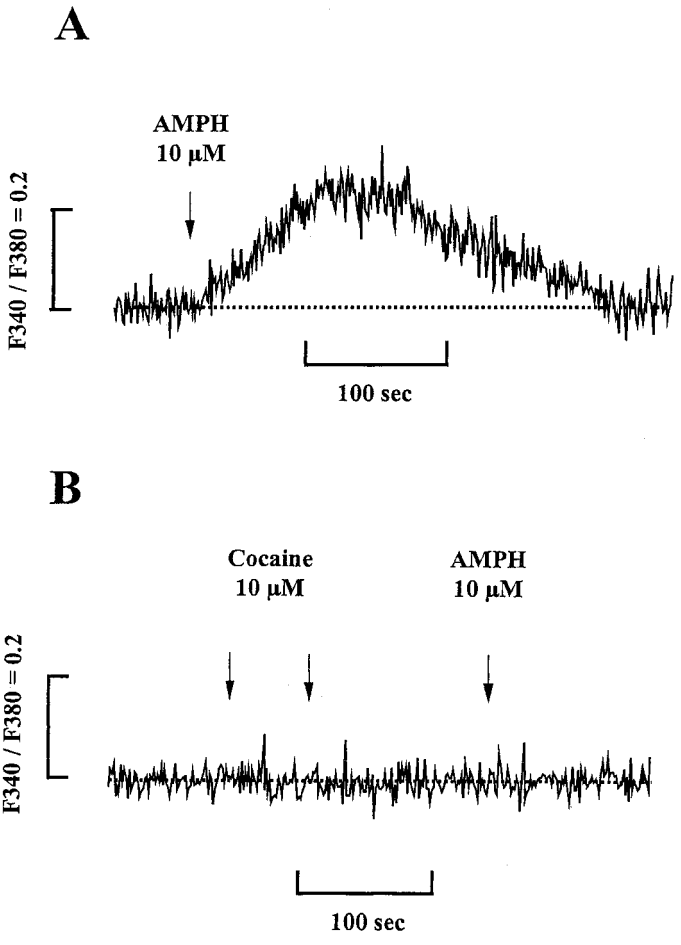

Fig. 4. The intracellular Ca^{2+} concentration is modulated by AMPH. hDAT cells were incubated in buffer containing FURA-2-AM (5 μM) for 30 min before measurements. AMPH (2 μM) application induced an increase in intracellular calcium (A), whereas pretreatment with 10 μM cocaine blocked this effect (B).

DAT substrate because it is not metabolized by catechol-*O*-methyltransferase. hDAT cells were loaded with $[^3H]MPP^+$ in the presence or absence of 50 μM BAPTA-AM (pretreatment). As shown in Fig. 3, superfusion with 10 μM AMPH elicited an overflow of $[^3H]MPP^+$ in vehicle-pretreated cells (VEH-AMPH). No overflow of $[^3H]MPP^+$ was detected in response to AMPH in cells that had been preincubated with 50 μM BAPTA-AM (BAPTA-AMPH). Baseline values of outflow are shown for cells that received only vehicle in the superfusion (VEH-VEH). Preincubation of the cells with 50 μM BAPTA-AM (BAPTA-VEH) did not significantly change the baseline. To further determine the physiological significance of the chelation of intracellular Ca^{2+} for AMPH-induced DA efflux, we determined the effect of BAPTA-AM on the efflux of endogenous DA induced by AMPH in rat striatal slices. Superfusion of rat striatal slices with 50 μM BAPTA-AM for 15 min reduced AMPH-induced release of endogenous DA to essentially baseline values. Values, in picomoles of DA per milligram wet weight, for baseline DA efflux and DA efflux upon perfusion of 1 μM AMPH were 0.06 ± 0.01 and 0.33 ± 0.07, respectively ($p < 0.01$, $n = 3$, two-tailed Student's t test). After a 15-min perfusion of 50 μM BAPTA-AM, values, in picomoles of DA per milligram wet weight, for baseline DA efflux and DA efflux in the presence of 1 μM AMPH were: 0.07 ± 0.01 and 0.10 ± 0.03, respectively (n.s., $n = 3$, two-tailed Student's t test). Therefore, chelation of intracellular Ca^{2+} BAPTA-AM did not alter basal DA efflux but reduced the AMPH-induced efflux almost to baseline values. The peak values of AMPH-induced DA efflux in the presence and absence of 50 μM BAPTA (0.10 ± 0.03 and 0.33 ± 0.07 pmol/mg wet weight, respectively) were significantly different ($p < 0.05$, $n = 3$, two-tailed Student's t test). The reduction in AMPH-induced DA efflux after superfusion of BAPTA-AM in rat striatal slices is similar to the effect of BAPTA-AM on AMPH-induced efflux of endogenous DA in rat pheochromocytoma PC-12 cells (Kantor et al., 2001) and on AMPH-induced efflux of $[^3H]MPP^+$ in HEK-293 cells (this report). Although alternative explanations are possible, these data further support a role for intracellular Ca^{2+} in the AMPH-induced reverse transport of DA.

Modulation of Intracellular Ca^{2+} by Amphetamine. The requirement of intracellular Ca^{2+} for AMPH-induced hDAT-mediated outward transport suggested to us the possibility that AMPH might activate hDAT-mediated reverse transport by increasing intracellular Ca^{2+}. Therefore, we measured real time changes in intracellular Ca^{2+} concentration by fluorometric determination with a cell-permeant Ca^{2+} indicator (fura-2 AM) in response to AMPH. Application of 2 μM AMPH to the cells elicited a rise in intracellular Ca^{2+} as measured by the F_{340}/F_{380} ratio, which reached a maximum within 1 min (Fig. 4A). The rise in internal Ca^{2+} was dependent on hDAT activity because it was blocked by preapplication of 10 μM cocaine to the bath solution (Fig. 4B). The Ca^{2+} response to 2 μM AMPH was prevented by deletion of extracellular Ca^{2+} (Fig. 5A). At high concentrations of AMPH (e.g., 100 μM), the increase of intracellular Ca^{2+} was independent of the presence of extracellular Ca^{2+} and dependent only on intracellular Ca^{2+} (data not shown). The Ca^{2+} response to AMPH was dependent upon intracellular Ca^{2+} stores, because it was also blocked by pretreatment with 5 μM thapsigargin, which inhibits the Ca^{2+}-ATPase pump in the endoplasmic reticulum and thereby depletes intracellular Ca^{2+} (Fig. 5B). Likewise, pretreatment of hDAT cells with 5 μM thapsigargin blocked the ability of AMPH to induce outward transport of $[^3H]MPP^+$ (Fig. 6). Thapsigargin alone had no effect on $[^3H]MPP^+$ release.

Discussion

Our results demonstrate that internal Ca^{2+} regulates both AMPH-induced DAT-mediated currents and outward transport of substrate. In addition, this study is the first to demonstrate an AMPH-induced DAT-mediated increase in intracellular Ca^{2+}.

It has generally been accepted that AMPH-induced DA efflux mediated by DAT does not require extracellular Ca^{2+}

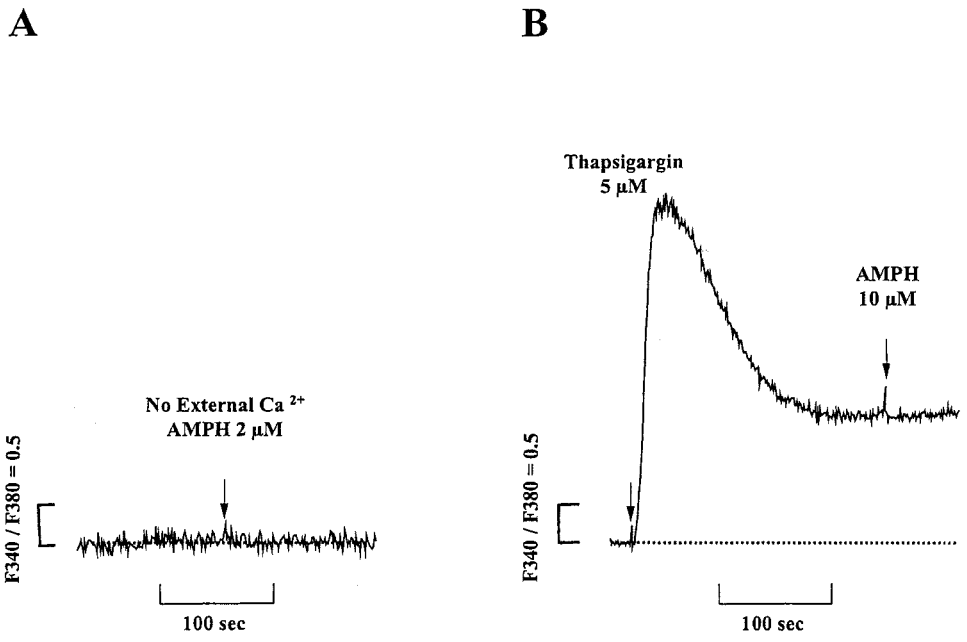

Fig. 5. Extracellular Ca^{2+} and intracellular Ca^{2+} stores are required for the AMPH-induced increase in intracellular Ca^{2+}. The increase in intracellular Ca^{2+} caused by application of 2 μM AMPH was blocked when hDAT cells were perfused with a bath solution not containing extracellular Ca^{2+} (A) and by bath applying 5 μM thapsigargin (arrow) to deplete intracellular Ca^{2+} stores before AMPH application (B).

(Arnold et al., 1977; Ross and Kelder, 1979; Lynch et al., 1985; Carboni et al., 1989; Hurd and Ungerstedt, 1989; Kantor et al., 2001). Indeed, we found that removal of extracellular Ca^{2+} did not affect [^{3}H]MPP^+ efflux. Several reports, however, inferred a requirement of extracellular Ca^{2+} for AMPH-induced DA release. Crespi et al. (1997) found a significant reduction in AMPH-induced release of [^{3}H]DA in rat striatal synaptosomes in a Ca^{2+}-free medium containing 0.03 mM EGTA. However, because the synaptosomes were perfused with the EGTA-containing buffer for 45 min before AMPH addition, it is likely that intracellular Ca^{2+} was depleted as well (Fisher et al., 1989). Mundorf et al. (1999) also reported that the absence of extracellular Ca^{2+} led to a reduction of AMPH-induced catecholamine release in bovine chromaffin cells. This DA release, however, was mediated not by DAT but by exocytotic vesicular catecholamine release that resulted from a local increase in Ca^{2+} concentration.

Although extracellular Ca^{2+} is not required for reverse transport, the ability of 2 μM AMPH to increase intracellular Ca^{2+} was only observed in the presence of extracellular Ca^{2+}. In contrast, the increase of intracellular Ca^{2+} elicited by 100 μM AMPH was detected also in the absence of extracellular Ca^{2+}. The increase in intracellular Ca^{2+} elicited by 2 μM AMPH might be highly localized and too small to be detected in the absence of extracellular Ca^{2+}. Instead, higher concentrations of AMPH might act directly on the intracellular stores to release Ca^{2+} independent of extracellular Ca^{2+} (Mundorf et al., 1999).

AMPH has been shown to induce nonstoichiometrically coupled DAT-mediated currents (Sonders et al., 1997). A strong correlation was found between AMPH-induced DA efflux and these AMPH-induced currents (Sitte et al., 1998; Pifl et al., 1999). These authors suggested that the ability of a releaser (e.g., AMPH) to induce efflux was correlated tightly to its ability to induce transporter-mediated inward currents. Consistent with this, we have shown that AMPH-induced DA efflux is dependent on the intracellular Na^+ concentration, one of the cotransported ions, and is voltage-dependent (Khoshbouei et al., 2003). To facilitate the detection of AMPH-induced DA efflux from a single cell, we loaded the whole cell pipette with a solution containing 90 mM NaCl and 2 mM DA. This experimental configuration allowed us to clamp the membrane potential and the intracellular concentrations of Na^+ and DA, in contrast to studies with cell populations. AMPH is also able to depolarize hDAT cells (Kahlig et al., 2004), which could lead to an increase in intracellular Ca^{2+}. Our results demonstrate that the increase in intracellular Ca^{2+} promoted by AMPH is mediated by DAT and dependent upon thapsigargin-sensitive stores in suspended cells. Similar results were obtained using confocal imaging of single cells expressing the human norepinephrine transporter (DiPace et al., 2003). At low concentrations of AMPH, extracellular Ca^{2+} might enter the HEK cells through voltage-dependent Ca^{2+} channels (Berjukow et al., 1996) or by Na^+/Ca^{2+} exchange (Giambalvo, 2004). The blockade by cocaine of this effect of AMPH delineates this response as DAT-mediated.

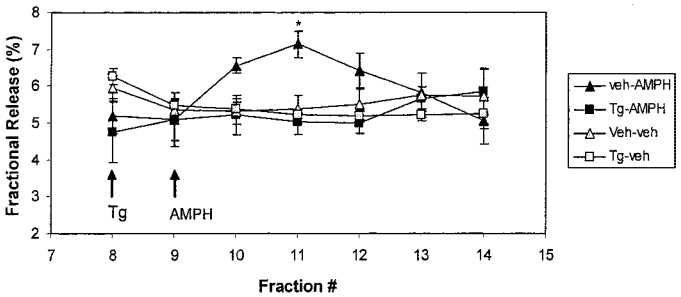

Fig. 6. Effect of thapsigargin on AMPH-mediated release of [^{3}H]MPP^+ in hDAT cells. hDAT cells were preincubated with 10 μM [^{3}H]MPP^+ as described under *Materials and Methods*. The cells were then placed in a superfusion apparatus and 5-min fractions from the KRH perfusate were collected. At fraction 7, either KRH (veh) or 5 μM thapsigargin (Tg) was perfused for 5 min followed by KRH (-veh) or 10 μM AMPH (-AMPH) for 2.5 min, reaching the cells at the fractions shown by the arrow. Results are calculated as fractional rate of release. Note that the *y*-axis does not begin at zero. In ANOVA for fraction 11, $p < 0.05$; in post hoc Tukey-Kramer analysis, $p < 0.05$ for VEH-AMPH versus Tg-AMPH ($n = 3$).

Buffering intracellular Ca^{2+} with BAPTA inhibited AMPH-induced DAT-mediated outward currents and DA efflux. Because intracellular BAPTA did not affect AMPH-stimulated inward currents, it is unlikely that the BAPTA-induced reduction in DA efflux is a result of DAT trafficking. Buffering intracellular Ca^{2+} also decreased the steep voltage dependence of DA efflux (Fig. 2A), suggesting that the voltage sensitivity of reverse transport might rely on the presence of Ca^{2+}. Increases in intracellular Ca^{2+} in response to AMPH have also been reported by Mundorf et al. (1999) in bovine chromaffin cells and Chen et al. (1998) in the African snail *Achatina fulica* Ferussac. In the latter study, however, the concentration of AMPH used was very high (270 μM), and no relationship to transport was demonstrated. In the chromaffin cell study (Mundorf et al., 1999), the source of cytoplasmic Ca^{2+} in the AMPH response was demonstrated to be catecholaminergic vesicles. The mechanism was most probably attributable to a weak base action of AMPH because methylamine showed the same activity. In contrast, the hDAT cells used in the present study do not contain synaptic vesicles.

That thapsigargin reduced both the ability of AMPH to elicit increases in intracellular Ca^{2+} and AMPH-induced DA efflux suggests that the release of Ca^{2+} from the endoplasmic reticulum is essential for DAT-mediated outward transport. Because thapsigargin alone raised intracellular Ca^{2+} (Fig. 5B) but did not cause detectable DA efflux (Fig. 6), intracellular Ca^{2+} by itself is not sufficient to cause DA efflux. The mechanism of internal Ca^{2+} is currently unknown. Increases in internal Ca^{2+} can lead to activation of protein kinase C independent of diacylglycerol production (Trilivas and Brown, 1989), so it is possible that the AMPH-induced increase in internal Ca^{2+} could activate PKC. An increase in PKC activity because of AMPH-induced transporter activation has been demonstrated previously (Iwata et al., 1997; Giambalvo, 1992, 2003). Direct PKC activation by phorbol esters enhances DA efflux, whereas PKC inhibitors block AMPH-induced DA efflux from both rat striatum and PC-12 cells (Giambalvo, 1992; Kantor and Gnegy, 1998; Cowell et al., 2000; Kantor et al., 2001). Moreover, PKC activation leads to phosphorylation of N-terminal serines in DAT (Foster et al., 2002). We have demonstrated recently that N-terminal phosphorylation of DAT is essential for AMPH-induced DA efflux and currents (Khoshbouei et al., 2004), thereby providing a potential mechanistic link between internal Ca^{2+} and efflux.

In summary, our results allow us to propose the following

scenario: AMPH transport through DAT elicits a release of internal Ca^{2+} from the endoplasmic reticulum. This increase in cytoplasmic Ca^{2+} probably activates PKC, which in turn leads to phosphorylation of DAT, which is essential for DAT-mediated outward currents as well as for DAT-mediated DA efflux. Such a mechanism raises new potential targets for therapeutic interference with the actions of psychostimulants such as AMPH.

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