Amphetamine-induced Dopamine Efflux

A VOLTAGE-SENSITIVE AND INTRACELLULAR Na\(^+\)-DEPENDENT MECHANISM*

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Amphetamine (AMPH) elicits its behavioral effects by acting on the dopamine (DA) transporter (DAT) to induce DA overflow into the synaptic cleft. Facilitated exchange diffusion is the classical model used to describe AMPH-induced DA efflux. This model hypothesizes that AMPH-induced DA efflux is mediated by DAT and results from the transport of AMPH into the cell followed by a counter movement of DA out to the extracellular compartment. To further characterize the action of AMPH, we used the patch clamp technique in the whole-cell configuration combined with amperometry on human embryonic kidney HEK-293 cells stably transfected with the human DAT (DAT cells). In DAT cells, AMPH-induced DAT-mediated currents were blocked by cocaine. We demonstrate that DA efflux mediated by DAT is voltage-dependent, electrogenic, and dependent on intracellular Na\(^+\) concentration in the recording electrode. Intracellular Na\(^+\) fluorescence, as measured by confocal microscopy using a Na\(^+\)-sensitive dye, was enhanced by AMPH application. Furthermore, the ability of AMPH to induce DA efflux was regulated by intracellular Na\(^+\) concentration and correlated with the size of the DAT-mediated, AMPH-induced ion flux across the plasma membrane. In the absence of intracellular Na\(^+\), but not the presence of high intracellular Cl\(^-\), AMPH-induced inward currents elicited DA efflux proportionally to their dimension and duration. Thus, we propose that AMPH-induced DA efflux depends on two correlated transporter processes. First, AMPH binds to the DAT and is transported, thereby causing an inward current. Second, because of this AMPH-induced inward current, Na\(^+\) becomes more available intracellularly to the DAT, thereby enhancing DAT-mediated reverse transport of DA.

The dopamine transporter (DAT)\(^1\) is thought to control the temporal and spatial action of released dopamine (DA) by rapid reuptake of the neurotransmitter into presynaptic terminals. The interaction of amphetamine (AMPH) with DAT, which induces DA overflow into the synaptic cleft, is thought to mediate the acute behavioral and reinforcing effects of this psychostimulant (1). Although some of the initial steps of AMPH action have been clarified, the mechanism driving the AMPH-induced reverse transport of DA at the plasma membrane remains controversial. The reverse transport of DA occurs by a carrier-mediated release, which is not dependent on action potential depolarization and is only slightly calcium-dependent (2). One of the models used to explain this AMPH effect is the facilitated exchange diffusion model (3, 4). This model proposes that AMPH-induced DA release is mediated by DAT and results from translocation of AMPH into the cell followed by a counter movement of DA out to the extracellular compartment. By acting as a substrate, AMPH increases the number of inward-facing transporter binding sites and thus increases the rate of reverse transport. In contrast, the weak base or vesicle depletion model (5, 6) proposes that the elevated cytoplasmic DA concentration and its altered gradient across the plasma membrane caused by the AMPH-mediated depletion of synaptic vesicles induce a reversed transport of DA. This process could be independent of AMPH interacting with DAT (7). Based mainly on the observation that DA displacement from vesicles by Ro4–1248 and reserpine-like compounds does not cause DA efflux, Jones et al. inferred that, although vesicular depletion is rate-limiting, the facilitated exchange diffusion mechanism is critical for AMPH-induced DA release (8). Therefore, simply increasing the intracellular DA concentration was not enough to cause DA efflux, and an interaction of AMPH with DAT was essential as well.

However, other intriguing experiments have challenged a simple model of facilitated exchange diffusion (9–13). For example, it has been shown that PKC activation leads to an immediate increase in outward transport of DA from the homologous norepinephrine transporter (13) and that protein kinase C (PKC) inhibitors block AMPH-induced DA release via DAT in the striatum in a synaptic vesicle-independent process (14). In addition to signaling pathways, intracellular ions such as Ca\(^{2+}\) (13), Na\(^+\), and Cl\(^-\) (9, 10, 15, 16) have been implicated in AMPH-induced DA efflux. In particular, a relationship between intracellular Na\(^+\) and the mechanism of substrate-induced monoamine efflux has been suggested (9, 10, 16–18), and an increased intracellular Na\(^+\) concentration has been proposed to be an important step for reversing the action of monoamine transporters (9, 18, 19). The ability of AMPH to stimulate reverse transport of DA was further proposed to result from its ability to stimulate inward ion fluxes through DAT (9, 10).

In such a model, the inward current generated by DAT substrates such as AMPH, which is most likely produced by the flow of Na\(^+\) into the cell, is sufficient to stimulate the efflux of...
intracellular substrate because of an elevation of intracellular Na⁺. Indeed, results from Sitte et al. support the hypothesis that the releasing properties of DAT substrates are not proportionally related to their ability to be taken up by DAT but instead to their ability to elicit DAT-mediated inward currents (see above) (9). To date, it has not been possible to directly test the role of intracellular Na⁺ on AMPH-induced DA efflux. Here, we demonstrate that the following events occur in DAT-expressing cells: 1) AMPH increases intracellular Na⁺. 2) Intracellular Na⁺ regulates AMPH-induced DA efflux. 3) AMPH-induced DA efflux is voltage-dependent and electrogenic. 4) AMPH-induced inward currents elicit DA efflux proportionally to their dimension and duration.

MATERIALS AND METHODS

Plasmid Construction, Transfection, and Cell Culture—The synthetic human DAT gene, modified to express a FLAG epitope fused to the N terminus of DAT, was subcloned into a bicistronic expression vector (20) modified to express the synthetic DAT from a cytomegalovirus promoter and the hygromycin resistance gene from an internal ribosomal entry site as described previously (pciHyp) (21). EM4 cells, which are from a human embryonic kidney HEK-293 cell line stably transfect with macrophage scavenger (R. Horlick, Pharmacoepia, Cranberry, NJ), were transfected with the FLAG-DAT using LipofectAMINE (Invitrogen), and a stably transfected pool was selected in 250 μg/ml hygromycin as described (22). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. 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 (21). A fluorescently tagged DAT was constructed by fusing the C terminus of the coding region of enhanced yellow fluorescent protein (YFP) from pEYFP-N1 (Clontech) to the N terminus of the human synthetic DAT cDNA, thereby creating the fusion construct YFP-DAT. This construct was subcloned into pciHyp, and stable pools of EM4 cells expressing YFP-DAT (YFP-DAT cells) were obtained as described above.

Electrophysiology—Before recording from parental or stably transfected cells, cells were plated at 10⁵ 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 CaCl₂, 0.5 mM MgSO₄, and 1.5 mM KH₂PO₄ adjusted to pH 7.35. The pipette solution for the whole-cell recording contained 120 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 1.1 mM EGTA, 10 mM Hepes, and 30 mM dextrose plus DA (2 mM or 100 μM, as specified in the text) adjusted to pH 7.35. Free Ca²⁺ was 0.1 mM. In ion replacement experiments, the intracellular Na⁺ concentration was adjusted by iso-osmotically changing the concentration of KCl in the pipette solution. 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 Besse filter set at 1,000 Hz. Current-voltage relations were generated using a voltage step (1 s) protocol ranging from −160 to 100 mV separated by 20 mV from a given holding potential. Current and oxidative (amperometric) signals were measured simultaneously. Data were recorded and analyzed off-line using the software pCLAMP 8 from Axon Instruments.

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. At voltages more negative than −60 mV the amperometric signal approaches zero, making −80 mV a convenient membrane potential to hold the YFP-DAT cells. Unlike the usual amperometric calibration, which requires conversion to concentration, we recorded DA independently without converting the amperometric signal. Thus, our requirements are a defined baseline, and our data represent a lower limit to the DA efflux because some transporter 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 msec after the step. Confocal Microscopy—EM4 cells expressing FLAG-DAT or YFP-DAT were cultured on round glass coverslips in 35-mm dishes ~48–72 h prior to the experiments and grown to 50–70% confluence. The cells were incubated with 5 μM Sodium Green³⁺-AM (Molecular Probes) in Me₂SO (Sigma) at room temperature for 30 min under light-protected conditions. The coverslips containing the cells were washed twice in bath solution and placed into the confocal chamber (Biotecs). Real time confocal imaging was performed on an Olympus IX-70 inverted microscope using an Olympus Fluoview 500 confocal microscope equipped with an argon laser. Images were acquired every 15 s using a 40× objective excitation wavelength with a 505 long pass filter. After acquiring two basal images, seven images were recorded during continuous perfusion of 10 μM AMPH or vehicle. To determine the concentration of free intracellular Na⁺, the following equation was used,

\[ [Na⁺]_{\text{free}} = \frac{K_c (F - F_{\text{max}}) (F_{\text{max}} - F)}{F} \]  

\[ \text{(Eq. 1)} \]

where F is the value of the average fluorescence intensity within the cytoplasm of a cell, \( K_c \) is the dissociation constant of the fluorescent probe for sodium, and \( F_{\text{max}} \) is the maximum fluorescence intensity, calculated as described above, after permeabilizing the cell with digitonin (15 μM) in a bath solution containing 300 mM NaCl, 10 mM HEPES, 34 mM dextrose, 1.5 mM CaCl₂, 0.5 mM MgSO₄, and 1.3 mM KH₂PO₄ (n = 12 and 10 for FLAG and YFP-DAT cells, respectively). \( F_{\text{max}} \) is the minimum fluorescence intensity calculated from FLAG and YFP-DAT cells after incubating the cells for 6 h, in a Na⁺-free medium solution. Confocal imaging was conducted at 25 °C. Image analysis was performed using the public domain ImageJ imaging program (rsb.info.nih.gov/ij/).

RESULTS

DA uptake was not observed in EM4 cells not transfected with the YFP-DAT (data not shown). Furthermore, in untransfected cells neither 10 μM AMPH nor 10 μM AMPH together with 10 μM cocaine produced either whole-cell currents or DA efflux, as measured by amperometry with DA in the whole-cell pipette (see below). Thus, the EM4 cells provided a suitable null background in which to study DA-efflux mediated by DAT and stimulated by AMPH.

To facilitate the selection of cells expressing adequate DAT for electrophysiological and amperometric studies, we created a pool of cells stably expressing a YFP-DAT fusion construct and selected for analysis cells with easily visualized plasma membrane fluorescence (Fig. 1A, inset). Addition of the N-terminal YFP tag to DAT did not significantly alter [³H]DA uptake (data not shown) and did not alter the ability of the transporter to produce substrate-induced currents (Fig. 1A). YFP-DAT-mediated currents were recorded in the whole-cell configuration, and the membrane voltage was stepped from a holding potential of −20 to −120 mV for 500 msec while acquiring a control current (Fig. 1A, CO). Perfusion of the cell with 10 μM AMPH caused an increase of the steady-state inward current (Fig. 1A, AMPH), which was blocked in the presence of 10 μM cocaine (COC) with AMPH still present (Fig. 1A, AMPH + COC). Cocaine also reduced the control current because of its block of a DAT-mediated leak current, which has been described both for DAT and other neurotransmitter transporters (9, 23–25). Therefore, the AMPH-induced current was defined as the current recorded in the presence of AMPH minus the current recorded after the addition of cocaine to the bath with AMPH still present. Fig. 1B, shows a current voltage relationship for the AMPH-induced current. The membrane potential was held at −20 mV, and then the voltage was stepped to a new potential between −160 and 100 mV in 20 mV increments. Outward currents were recorded at membrane voltages more positive than 20 mV.

The effects of AMPH were further investigated by following real time changes in intracellular Na⁺ concentration by fluoro
deterministic with a cell-permeant Na⁺ green tetraacetate. We collected confocal microscopy images every 15 s, and for each time point we subtracted the background (fluorescence measured from a confocal plane in the control condition (rest)) from the fluorescence recorded in the same z section upon the addition of AMPH. An increase in intracellular fluorescence
was detected within 15 s of AMPH application (Fig. 2A). In both YFP-DAT and FLAG-DAT cells, AMPH increased intracellular Na\(^+\) with similar kinetics (Fig. 2B). No significant changes in intracellular fluorescence were detected either in EM4 cells treated with AMPH or in untreated YFP-DAT cells (Fig. 2C). A 60 s application of 10 \(\mu\)M AMPH increased intracellular free Na\(^+\) to 25.9 ± 3.3 mM (n = 10) and 46 ± 12 mM (n = 12) in YFP-DAT and FLAG-DAT, respectively. These data suggest that AMPH is able to modify ionic gradients across the plasma membrane in cells expressing DAT. To test whether such AMPH-induced changes in intracellular Na\(^+\) concentration regulate reverse transport of DA by DAT, we combined patch clamp recording with amperometry. YFP-DAT cells were voltage clamped with a whole-cell patch pipette while an amperometric electrode was placed onto the plasma membrane (Fig. 3A). The whole-cell electrode was filled with a solution containing 2 mM DA and different concentrations of NaCl substituted with KCl to maintain a constant osmolarity of 270 mOsm. The amperometric electrode was held at 700 mV, a potential greater than the redox potential of DA. We recorded DAT-mediated currents with the whole-cell pipette by stepping the membrane voltage from a holding potential of −80 mV to potentials between −100 and 100 mV. To isolate the DAT current, we subtracted the current recorded in the presence of external COC from the current recorded in the control condition. Fig. 3B shows an example of transporter currents recorded between −100 and 100 mV with a whole-cell pipette filled with an internal solution containing DA and 90 mM NaCl. DAT-mediated outward currents were voltage-dependent and increased at positive voltages in an exponential fashion. For a fixed voltage, the DAT-mediated outward current was increased by increasing the intracellular Na\(^+\) concentration. Thus, at an intracellular Na\(^+\) concentration equal to 90 mM, the whole-cell current recorded at 100 mV was 12.4 ± 4.2-fold greater than the current recorded with no Na\(^+\) in the pipette solution. At the same time, we monitored DA efflux with the amperometric electrode (Fig. 3C). We isolated the DAT-mediated DA efflux by subtracting the background currents (traces recorded in the presence of 10 \(\mu\)M cocaine) from amperometric traces recorded under control conditions (DA and high intracellular Na\(^+\)) for each potential tested. An upward deflection in the amperometric current corresponds to an outward flux of DA. At the "on" of the voltage step, for voltages more positive than −60 mV the amperometric electrode recorded an oxidation current (positive), which is indicative of DA efflux. At the termination "off" of the voltage step, the amperometric current relaxed to baseline. The on and off of the voltage step are defined by vertical arrows in Fig. 3C. Moving the carbon fiber away from the patch caused the oxidative response to become smaller and slower. Furthermore, in the absence of intracellular Na\(^+\) in the whole-cell pipette, a barely detectable signal was recorded with the amperometric electrode. Finally, as expected for DA oxidation, the oxidative response diminished upon reduction of the carbon fiber voltage to 300 mV and disappeared completely with further reduction of the voltage. In Fig. 3D, we plot oxidation currents obtained using different concentrations of Na\(^+\) (between 0 and 90 mM) in the whole-cell pipette against the test voltage applied to the cell. For each single concentration of intracellular Na\(^+\) used, DA efflux increased at positive voltages without reaching saturation in the range of voltages studied. Similarly, at a defined membrane voltage, increasing intracellular Na\(^+\) concentration increased DA efflux. The dependence of DA efflux on internal Na\(^+\) was determined by fitting the values of the steady-state amperometric currents recorded at different intracellular Na\(^+\) concentrations by nonlinear regression to a Hill equation. The \(K_m\)s obtained were 54 ± 8 mM at 100 mV, 46 ± 6 mM at 80 mV, and 50 ± 12 mM at 60 mV, with a Hill coefficients of 2 ± 0.2, 3 ± 0.7, and 3.3 ± 0.7, respectively. At voltages more negative than 60 mV, the reduced values of the amperometric currents, obtained at low Na\(^+\) concentrations, impaired our ability to estimate the affinity of DAT for intracellular Na\(^+\). Finally, we were also able to record amperometric currents with 100 \(\mu\)M DA (data not shown), and these, like those measured with higher DA, were voltage dependent and sensitive to intracellular Na\(^+\) concentration as well.

To determine whether AMPH-induced DA efflux was also regulated by intracellular Na\(^+\), we recorded DA efflux using the experimental configuration described in the legend for Fig. 3A in the presence of 10 \(\mu\)M AMPH in the bath solution. At a DAT saturating concentration of intracellular DA (2 mM), the steady-state amperometric currents recorded in the presence of 10 \(\mu\)M AMPH were plotted against different voltages (Fig. 4). DA efflux increased exponentially at voltages more positive than −60 mV in a manner dependent on the intracellular Na\(^+\) concentration. Although DAT did not require the presence of AMPH to mediate DA efflux (Fig. 3D), AMPH substantially
enhanced its ability to cause carrier-mediated DA release at low concentrations of intracellular Na\(^+\) (Fig. 4). For example, with 10 mM intracellular Na\(^+\), the amperometric current recorded at 100 mV in the present of AMPH was twice the current magnitude recorded in its absence (0.12 \(\pm\) 0.015 pA versus 0.06 \(\pm\) 0.02 pA, respectively, \(n = 4\), \(p < 0.005\) by paired Student's t test) (compare Figs. 3 and 4, open diamonds). Concomitantly, we recorded DAT-mediated whole-cell currents. At 100 mV in the presence of AMPH, the DAT whole-cell current increased 1.72 \(\pm\) 0.23 times with respect to the current recorded in its absence. In contrast, at an intracellular Na\(^+\) concentration of 90 mM, no significant increase of DA efflux or DAT-mediated outward currents was detected upon AMPH application.

Our data show that DA efflux mediated by DAT is voltage-dependent, electrogenic, and dependent on the intracellular Na\(^+\) concentration (Fig. 3). Moreover, DAT does not require AMPH to generate DA efflux, but AMPH appears to play a major role in stimulating the reverse transport mechanism of DAT at low intracellular Na\(^+\) concentrations. In contrast, at high intracellular Na\(^+\) concentrations the presence of AMPH did not affect DAT-mediated DA efflux, which was already substantial. Therefore, we considered the possibility that substrate-induced DA efflux depends on two independent transporter processes. First, substrate (e.g. AMPH) translocation by DAT produces an inward current (Fig. 1). This inward current, in part carried by Na\(^+\), results in an increase in intracellular Na\(^+\) concentration (Fig. 2), thereby enhancing the ability of DAT to produce DA efflux.

To test this hypothesis, we used a whole-cell patch pipette containing an internal solution with 2 mM DA and no Na\(^+\) while 10 \(\mu\)M AMPH and a physiological concentration of NaCl (130 mM) were present in the bath solution. The concentration of Cl\(^-\) was 124.2 mM intracellularly and 133 mM extracellularly. The membrane potential of the cell was held at \(-20\) mV, a voltage close to the equilibrium potential of the AMPH-stimulated, DAT-mediated outward current recorded under these experimental conditions. In the absence of Na\(^+\) and the presence of intracellular DA, we were able to record only a small AMPH-induced outward current and a small amperometric signal at 100 mV (Fig. 5, left side of panels A and B, respectively). In the same cell, with AMPH still present in the bath solution, we stepped the membrane potential to \(-140\) mV for 3 s (negative prepulse) and then again to 100 mV for 1 s. As expected, AMPH stimulated an inward current at \(-140\) mV (Fig. 5A, center). This is consistent with cations entering the cell via DAT. In fact, we detected an increase of intracellular Na\(^+\) upon AMPH application (Fig. 2). The DAT-mediated current caused by the negative prepulse stimulation substantially enhanced the ability of DAT to carry an outward current at 100 mV (Fig. 5A, right side). Simultaneously, we recorded DA efflux with the amperometric electrode. The negative prepulse also facilitated the reverse transport of DA mediated by DAT (Fig. 5B, right side). At 100 mV, upon negative prepulse stimulation the AMPH-induced whole-cell and amperometric current increased 1.63 \(\pm\) 0.19-fold and 2.08 \(\pm\) 0.14-fold, respectively, with respect to the current obtained in the absence of a negative prepulse.

**Fig. 2.** AMPH induces an increase of intracellular Na\(^+\) that is DAT-mediated. A, intracellular Na\(^+\) fluorescence obtained from a single z section of a patch of FLAG-DAT cells. Sodium Green\textsuperscript{TM} fluorescence was measured from confocal images and used to monitor temporal changes of intracellular Na\(^+\) levels upon AMPH application. Background fluorescence (rest) was subtracted from each single time point. Upon AMPH application (Amph), an increase of intracellular fluorescence was detected. B, the relative changes (\(\Delta F/F\)) in Na\(^+\) sensitive green fluorescence induced by AMPH were evaluated by ImageJ imaging analysis by tracing a region of interest defining the cytoplasmic area of a single z section of the cell. The images were collected every 15 s for 2 min. The ratio \(\Delta F/F\) was measured for YFP-DAT (open circles) and in YFP-DAT cells (open squares) under control conditions in the absence of AMPH. C, changes in the ratio \(\Delta F/F\) over 2 min in EM4 cells after the application of AMPH (open circles) and in YFP-DAT cells (open squares) under control conditions in the absence of AMPH.
In contrast, the negative prepulse stimulation failed to significantly increase either the amperometric or whole-cell currents recorded at 100 mV at concentrations of intracellular Na\(^+\)/H\(^+\) equal to or greater than 30 mM (\(n = 10\)). In Fig. 5C, we show the correlation between the amount of charge crossing the plasma membrane during the negative prepulse stimulation (Q) and the increase (fold) of the amperometric and whole-cell currents recorded at 100 mV. The coefficient of correlation (R) between Q and the amperometric and whole-cell currents was 0.73 and 0.8, respectively (\(n = 8\)), indicating that upon AMPH exposure we were able to increase DAT-mediated currents and DA efflux by increasing the amount of cotransported ions crossing the plasma membrane during the negative prepulse.

To further demonstrate that the ability of AMPH to induce DA efflux also relies on its ability to cause ion fluxes across the plasma membrane, we varied the duration of the negative prepulse and, therefore, the dimension of the AMPH-induced current. Using an experimental setting as in Fig. 5, we increased the duration of the negative prepulse between 0.5 and 3 s and then recorded the amperometric signal at 100 mV (Fig. 6). The amperometric currents were normalized to currents recorded at 100 mV in the absence of the negative prepulse in the same cell (y axis) and plotted against the duration of the prepulse stimulation (x axis). The amperometric signal increased without reaching saturation in the range of prepulse duration studied, confirming that the amount of elementary charges crossing the plasma membrane stimulated by AMPH regulates DAT-mediated DA efflux.

**DISCUSSION**

This study shows for the first time that AMPH increases intracellular Na\(^+\) availability and that the intracellular Na\(^+\) concentration and transmembrane potential regulate AMPH-induced, DAT-mediated DA efflux. Because changes in DA...
neurotransmission are thought to play an important role in the addictive properties of psychostimulants such as AMPH, the AMPH-induced increase of intracellular Na\(^+\) and its regulation of DA efflux may be an important mechanism in the development of its psychostimulant action. Our initial experiments were designed to assess the ability of AMPH to increase intracellular Na\(^+\) concentration. Our finding that incubation of YFP-DAT cells with AMPH evoked a significant increase in intracellular Na\(^+\) concentration is consistent with the hypotheses of previous reports (9, 10). In the present study we have shown that a 60 s of exposure to 10 \(\mu\)M AMPH increases intracellular Na\(^+\) to \(-46\) mM. It is likely that the increase in cytoplasmic Na\(^+\) induced by AMPH is because of the substrate-like properties of AMPH (3, 9, 16, 26). Indeed, extracellular substrates (e.g., AMPH) induce an inward current (Fig. 1), which is most likely partially produced by a flow of Na\(^+\) into the cell generated by DAT (9, 23, 27). In fact, although DAT-mediated currents have been shown to be comprised primarily of anions (25), a contribution of Na\(^+\) ions to the response mediated by DAT substrates has not been excluded. In support of such a role for Na\(^+\) ions, our real time confocal data showed that cocaine prevented the AMPH-induced increase in intracellular Na\(^+\) in DAT cells (data not shown) and that AMPH failed to increase intracellular Na\(^+\) in HEK-293 cells not expressing DAT (Fig. 2C). From our data it may also been inferred that the increase of Na\(^+\) green fluorescence upon AMPH application is greater near the plasma membrane, the location of DAT (Fig. 2A).

Dopamine uptake by DAT has been shown to be electrogenic in a heterologous expression system (24), but a recent study described voltage-independent DA uptake in mesencephalic, dopaminergic neurons (28). The slow turnover rate of DAT relative to neuronal firing and its voltage independence has been suggested as a possible mechanism for increased DA signaling during burst firing. In contrast, reversal of DA transport by depolarization has recently been proposed in experiments conducted in neurons ex vivo (29). Mintz and co-workers suggested that glutamate-gated channels and Na\(^+\) channels could eventually depolarize the membrane potential of the neuron beyond the reversal potential of DAT to induce reversal of DAT transport (29). Here, we showed that DA efflux mediated by DAT is voltage-dependent. The DA transport process reversed at voltages more positive than \(-60\) mV, increasing exponentially without reaching saturation in the range of membrane potentials studied (Fig. 3C). As expected, DAT-mediated whole-cell currents reversed at positive voltages as well (Fig. 1B). Several lines of evidence suggested the possibility that the influx of extracellular Na\(^+\) could be the trigger for the transporter-mediated release of the neurotransmitter (9, 10, 29–31). Substrate-induced release of norepinephrine from the dog saphenous vein was augmented by inhibition of the Na\(^+\),K\(^+\)-ATPase (32). Inhibition of the Na\(^+\),K\(^+\)-ATPase produces increased internal Na\(^+\) concentration as well as membrane depolarization. These two events might contribute to the enhanced amine efflux. Stimulation of DA efflux by ouabain, an

Fig. 5. DAT-mediated inward currents stimulate AMPH-induced DA efflux. The whole-cell patch pipette internal solution contained 2 \(\mu\)M DA and no Na\(^+\). The bath solution was an external solution plus 10 \(\mu\)M AMPH. The membrane potential of the cell was held at \(-20\) mV. In the absence of Na\(^+\) and the presence of intracellular DA, a small AMPH-induced DAT outward current was recorded at \(+100\) mV (A, left side). In the same cell, with AMPH still present in the bath solution, the membrane potential was stepped to \(-140\) mV and then rapidly to \(+100\) mV. An AMPH-stimulated inward current was recorded at \(-140\) mV (A, center). The DAT-mediated current caused by the negative prepulse stimulation enhanced the subsequent DAT-mediated outward current recorded at \(+100\) mV (A, right side). Similarly, the cocaine-sensitive amperometric signal also increased at \(+100\) mV upon negative prepulse stimulation (B). The whole-cell current increased on average 1.63 ± 0.19-folds with respect to the outward current obtained in the absence of negative prepulse, whereas the amperometric signal increased on average 2.08 ± 0.14-folds (C). Panel D illustrates the correlation between the amount of charge carried by the AMPH-induced current during the negative prepulse (Q) and either the increase of whole-cell (open circle) or amperometric (open square) current. Q was normalized to the maximum movement of charge obtained during the negative prepulse (\(n = 8\), whereas the amperometric and whole-cell currents were normalized to the respective currents recorded at \(+100\) mV in the absence of the negative prepulse (I).
inhibitor of Na\(^+\),K\(^+\)-ATPase, was also reported in LLC-PK cells stably expressing the human norepinephrine transporter (18). The authors inferred that the ouabain stimulation of efflux was most likely because of an increase in intracellular Na\(^+\), although they were unable to demonstrate a voltage dependence of substrate-mediated DA efflux (18). In addition, a strong correlation has been found between the ability of a substrate to induce DAT-mediated currents, which are Na\(^+\), Cl\(^-\) and substrate-dependent, and its releasing action (9). Our studies demonstrate that increasing the cytoplasmic Na\(^+\) concentration increases the ability of DAT to mediate DA efflux, particularly at voltages more positive than \(-60\) mV (Fig. 3D). At a saturating concentration of DA over the range of voltages studied (between 60 and 100 mV), the \(K_m\) for intracellular Na\(^+\) was \(-50\) m\(\text{s}\) and was voltage-independent (Fig. 3). These results suggest that the voltage dependence of DA efflux does not result from altered DAT affinity for intracellular Na\(^+\) and that intracellular Na\(^+\) regulates DA efflux.

We found that DA efflux was barely detectable, even in the presence of AMPH, at a level of intracellular Na\(^+\) approaching zero (Figs. 3 and 4, open circles). In this experimental configuration with zero Na\(^+\) solution in the whole-cell pipette, AMPH may not increase the intracellular Na\(^+\) sufficiently to drive DA efflux. In contrast, at an intracellular Na\(^+\) concentration of \(-10\) m\(\text{s}\), the amperometric current recorded at 100 mV in the present of AMPH was twice the current magnitude recorded in its absence (compare Figs. 3 and 4, open diamonds). With an intracellular Na\(^+\) concentration equal to or higher than 30 m\(\text{s}\), large amperometric and whole-cell currents were recorded, and no significant increase of DA efflux or DAT-mediated outward currents was detected upon AMPH application (compare Figs. 3 and 4). Thus, we conclude that normal intracellular Na\(^+\) concentration is required for AMPH to reverse the DAT cycle and that high intracellular Na\(^+\) can substitute for AMPH in causing DA efflux.

Sitte et al. demonstrated a correlation between the releasing properties of a substrate and its ability to induce DAT-mediated inward current (9). To test the hypothesis that AMPH-induced Na\(^+\) influx regulates AMPH-induced DA reverse transport, we performed amperometric experiments without Na\(^+\) in the whole-cell patch pipette (Fig. 5). The relatively small DA efflux recorded in the presence of AMPH at 100 m\(\text{V}\) doubled in size when preceded by a negative prepulse that stimulated an AMPH-induced inward current. Because we used a saturating concentration of Cl\(^-\) both intracellularly and extracellularly, these data suggest that the potentiation of the AMPH-induced DA efflux results from an influx of Na\(^+\). Moreover, the negative prepulse failed to potentiate DA efflux when the whole-cell patch pipette contained Na\(^+\) at a concentration equal to or greater than 30 m\(\text{s}\). This was because the signal recorded at 100 m\(\text{V}\) was already reasonably sustained by the high intracellular Na\(^+\) concentration. We also demonstrated that DA efflux increased in proportion to the number of elementary charges crossing the plasma membrane through DAT upon AMPH stimulation (Fig. 5D). Because transport of substrate through DAT is coupled to both Na\(^+\) and Cl\(^-\) ions, these data further support the role of the activity of DAT and the influx of Na\(^+\) in the DAT-mediated release of DA. Increasing the duration of the negative prepulse and, therefore, the amount of cotransported ions crossing the plasma membrane also increased DA efflux (Fig. 6). The negative prepulse failed to increase the amperometric signal in the absence of AMPH, suggesting that substrate-induced DAT activity is essential for the stimulation of DA efflux.

Based on these data, we propose a model for AMPH-induced DA efflux in which the ability of AMPH to increase intracellular Na\(^+\) concentration is essential for its stimulation of DA efflux. Future experiments will be required to define the role of Cl\(^-\) in the regulation of this AMPH action.

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**Fig. 6.** Increasing the duration of the negative prepulse increases AMPH-induced DA efflux. YFP-DAT cells were perfused with a bath solution plus 10 \(\mu\text{M}\) AMPH. The whole-cell patch pipette contained an internal solution with no Na\(^+\) plus 2 mM DA. The membrane potential of the cell was stepped from a holding potential of \(-20\) to \(-140\) mV for different time periods (between 0.5 and 3 s) and then rapidly to 100 mV to record amperometric currents. The amperometric currents recorded at 100 mV after the negative prepulse were normalized to the amperometric currents obtained in the absence of negative prepulse in the same cell. The increase of the amperometric signals, generated by negative prepulses, is represented on the y axis. The x axis represents the duration of the negative prepulses. Asterisk indicates significant changes in DA efflux compared with a prepulse stimulation of 0.5 s (analysis of variance (ANOVA) followed by Tukey’s test; level of significance equal to \(p < 0.01\) for *. )
Amphetamine-induced DA Efflux, a Na\(^+\)-dependent Mechanism

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