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Zn^{2+} modulates currents generated by the dopamine transporter: parallel effects on amphetamine-induced charge transfer and release

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Abstract

The psychostimulant drug amphetamine increases extracellular monamines in the brain acting on neurotransmitter transporters, especially the dopamine transporter. Mediated by this plasmalemmal pump, amphetamine does not only induce release but also charge transfer which might be involved in the release mechanism. To study a potential link between the two phenomena, we used Zn^{2+} as an acute regulatory agent which modulates dopamine uptake by a direct interaction with the transporter protein. Charge transfer was investigated in patch-clamp experiments on HEK 293 cells stably expressing the human dopamine transporter, release was studied in superfusion experiments on cells preloaded with the metabolically inert transporter substrate [^{3}H]1-methyl-4-phenyl-pyridinium. Ten micromoles of Zn^{2+} had only minor effects in the absence of amphetamine but stimulated release and inward currents induced by amphetamine depending on the concentration of the psychostimulant: the effect of 0.2 μM was not significantly modulated, whereas the effect of 1 and 10 μM amphetamine was stimulated, and the stimulation by Zn^{2+} was significantly stronger at 10 μM than at 1 μM amphetamine. The stimulatory action of Zn^{2+} on release and inward current was in contrast to its inhibitory action on dopamine uptake. This supports a release mechanism of amphetamine different from facilitated exchange diffusion but involving ion fluxes through the dopamine transporter.

Keywords: Dopamine transporter; Zinc; Amphetamine; Patch-clamp; Release

1. Introduction

Plasmalemmal monamine transporters are the main molecular sites of action of the psychostimulant amphetamine in the nervous system. In particular, the dopamine transporter (DAT) plays a decisive role in the behavioral effects of amphetamine. This is demonstrated by the lack of locomotor stimulation in knock out mice in which the gene has been inactivated by homologous recombination (Giros et al., 1996). Amphetamine releases dopamine from nerve terminals by reversing the direction of dopamine transport so that dopamine is pumped from the cytosol to the exterior of the cell (Heikkila et al., 1975; Raiteri et al., 1979; Parker and Cubeddu, 1986). How this reverse transport by the DAT is enacted by amphetamine is still a matter of debate. Elucidation of the mechanism is important for the development of remedies against the adverse effects of this widely abused drug. One hypothesis explaining the mechanism is facilitated exchange diffusion, according to which amphetamine is taken up by the transporter and provides intracellular uptake sites for cytosolic dopamine. In exchange, dopamine is transported to the outside of the cell (Fischer and Cho, 1979; Bönisch and Trendelenburg, 1988; Rudnick and Clarke, 1993). This hypothesis has been questioned recently by a detailed analysis of the releasing action of amphetamine on the cloned human DAT; the data on the rates of uptake, release and charge transfer induced by the drug can be better reconciled with a hypothesis according to which influx of extracellular sodium ions through the DAT triggers reverse pumping by the transporter (Sitte et al., 1998, 2001; Pifl and Singer, 1999). Many studies have shown that increase

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of intracellular sodium can make plasmalemmal transporters switch direction of neurotransmitter translocation (Levi et al., 1976; Liang and Rutledge, 1982; Sweadner, 1985; Bönisch, 1986; Yamazaki et al., 1996; Chen et al., 1998). Kinetic studies reveal that the DAT operates by translocating one molecule of dopamine with two Na^{2+} and one Cl^- ions per transport cycle. Amphetamine is translocated by the DAT in a Na^+- and temperature-dependent and cocaine-blockable manner (Zaczek et al., 1991; Sitte et al., 1998) and elicits an inward current in voltage-clamped oocytes or human embryonic kidney (HEK) 293 cells expressing the human DAT (Sonders et al., 1997; Sitte et al., 1998). One would expect the electrogenic cycle to result in an inward current which can be calculated from turnover numbers. However, the charge movement is higher than expected from maximal uptake rates and their stoichiometric coupling with ion translocation (Sitte et al., 1998). This additional amphetamine-induced current and similar extra-currents in other plasmalemmal neurotransmitter transporters have been attributed to a channel mode of the transporters (Galli et al., 1996; Sonders and Amara, 1996). If the inward-current is carried by a flow of sodium uncoupled from the transport cycle, the channel mode of the plasmalemmal neurotransmitter transporters could be considered a cause to locally increase cytoplasmic concentrations of sodium ions which enhances efflux of intracellular substrate.

One possibility to verify the involvement of amphetamine-induced charge transfer in DAT-mediated amphetamine-induced release is to study an acute regulatory effect on both charge transfer and release. For that purpose, we used the regulation of dopamine uptake by Zn^{2+} based on a proven direct molecular interaction with the transport protein (Norregaard et al., 1998). Zinc ions decrease promptly dopamine uptake by interaction with defined histidines in two extracellular loops of the protein. Recently, one investigation on the modulation of reverse transport has been published (Scholze et al., 2002), whereas till now no studies on the effect of Zn^{2+} on DAT-mediated charge transfer have been reported. Therefore, we have undertaken to investigate the effects of Zn^{2+} on amphetamine-induced carrier-mediated release and inward currents by patch-clamp and superfusion experiments on HEK 293 cells stably expressing the human DAT.

2. Methods

2.1. Cell culture and molecular biology

Human embryonic kidney 293 cells were grown in minimum essential medium with Earle's salts 1-glutamine, 10% heat-inactivated fetal bovine serum and 50 mg/l gentamicin on 100 mm tissue culture dishes (Corning) at 37 °C and 5% CO_2/95% air. The cDNA encoding a human DAT with histidine 193 mutated to a lysine (H193K) was constructed by polymerase chain reaction-derived mutagenesis using *Pfu* polymerase according to the manufacturer's instruction (Stratagene, La Jolla, CA) with two complementary primers. The sequences were in the 5′–3′ direction: CTCGGATGC CAAACCCGGGGACTCCAGTGGAGACAGCTCGG GCCT; CACTGGAGTCCCCGGGTTTGGCATC CGA GCAGTTGGGGCTGTT. The mutation and the 604 bp of the construct which were left from *Pfu* synthesis after replacing a *PflM I–BstE II* fragment in the DAT wild-type were confirmed by DNA sequencing. The human DAT, norepinephrine transporter (NET) or DAT-H193K cDNA were stably expressed using the vector pRc/CMV as described recently (Pifl et al., 1996). Cells were selected with 0.8 g/l geneticin in the medium. Two different clonal lines of the DAT were used for superfusion experiments and patch-clamp recordings.

2.2. Uptake experiments

The cells were seeded in poly-D-lysine-coated 24-well plates (1×10^5 cells/well) and, one day later, each well was washed with 0.5 ml uptake buffer and incubated with 0.5 ml buffer containing various concentrations of $ZnSO_4$ for 5 min. Uptake was started by addition of [^{3}H]dopamine or [^{3}H]noradrenaline (0.2 μCi) at a final concentration of 10 μM. After incubation for 2.5 min at 25 °C, it was stopped by aspirating the uptake buffer and washing each well twice with 1 ml icecold buffer. The radioactivity remaining in each well was determined by incubating with 0.4 ml 1% sodium dodecyl sulfate and transferring this solution into scintillation vials containing 3 ml scintillation cocktail (Ultima Gold MV, Packard, Dovners Grove, IL). The uptake buffer consisted of (mmol/l): 4 Tris–HCl; 6.25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 120 NaCl; 5 KCl; 1.2 $CaCl_2$; 1.2 $MgSO_4$; 34 D-glucose; 0.5 ascorbic acid; pH 7.1.

2.3. Superfusion experiments

The cells were seeded onto poly-D-lysine-coated 5-mm-diameter glass coverslips in 96-well tissue culture plates (3×10^4 cells/well). On the following morning, the cells were loaded with 6 μM [^{3}H]1-methyl-4-phenylpyridinium (MPP^+; 0.1 Ci/mmol) at 37 °C for 20 min in uptake buffer. The coverslips were then transferred to small chambers and superfused with the same buffer as used in uptake experiments (25°C, 1.0 ml/min) as described recently (Pifl and Singer, 1999). After a washout period of 45 min to establish a stable efflux of radioactivity, the experiment was started with the collection of 4-min fractions. At the end of the experiment, the cells were lysed by superfusion with 4 ml 1% SDS. The

radioactivity in the superfusates and the SDS-lysates was determined by liquid scintillation counting. The release of tritium was expressed as fractional rate, i.e. the radioactivity released during a fraction was expressed as percentage of the total radioactivity present in the cells at the beginning of that fraction. All the results were expressed as means ± SEM.

2.4. Patch-clamp experiments

Two days before the experiments, about 60,000 cells were split into 35 mm tissue culture dishes. The external (bathing) solution for recordings was essentially the uptake buffer with minor modifications in order to obtain the final osmolality of 300 mosmol/l (mmol/l): 4 Tris–HCl; 6.25 HEPES; 130 NaCl; 5 KCl; 1.2 $CaCl_2$; 1.2 $MgSO_4$; 34 D-glucose; 0.1 ascorbic acid; pH 7.2. Patch pipettes were filled with (mmol/l): 130 KCl; 0.1 $CaCl_2$; 1.1 EGTA; 4 Tris–HCl; 6.25 HEPES; pH 7.2, with an osmolality of 270 mosmol/l. Patch electrodes pulled from borosilicate glass capillaries (GB 150F-8P, Science Products, Hofheim, Germany) with a programmable Brown-Flaming micropipette puller (P-97; Sutter Instruments Co., USA) were heat-polished to a final tip resistance of 3–6 MΩ. The recordings were performed in the whole-cell configuration of the patch-clamp technique using an Axopatch 200B patch-clamp amplifier and the pClamp data acquisition system (Axon Instruments, Foster City, CA, USA) at ambient temperature (25 ± 2°C). Test drugs were applied with a PTR-2000/DAD-12 drug application device (ALA Scientific Instruments Inc., Westbury, NY), which allows a complete exchange of solutions surrounding the cells under investigation within <100 ms. The cells continuously superfused with bathing solution or solutions containing dopamine, amphetamine or $ZnSO_4$ were routinely voltage-clamped at a holding potential of −30 mV. Alternatively, substances were examined in cells at a holding potential of −30 mV with 200 ms test pulses ranging from −100 to +20 mV and data acquisition during the last 50 ms of the pulse. There was a 3 s prerun of superfusion of the various substances. Dopamine was freshly prepared every other hour from a frozen stock solution. All the results were expressed as means ± SEM.

2.5. Materials

Media, sera and other tissue culture reagents were obtained from Life Technologies (Vienna, Austria). [7-^{3}H]Dopamine (22 Ci/mmol), (−)[7-^{3}H]noradrenaline (12 Ci/mmol) and [^{3}H]MPP^+ (79.9 Ci/mmol) were obtained from New England Nuclear GmbH (Vienna, Austria), D-amphetamine-sulfate was kindly donated by SmithKline and French (Welwyn Garden City, Herts, UK), mazindol from Sandoz GmbH (Vienna, Austria). The other chemicals were purchased from Sigma-Aldrich or Merck.

3. Results

3.1. Uptake experiments

In HEK 293 cells stably expressing the human DAT zinc ions concentration-dependently decreased the uptake of 10 μM [^{3}H]dopamine (Fig. 1). Zn^{2+} at concentration of 10–30 μM decreased the uptake rates by about 20%. This effect was non-competitive as shown by a Scatchard analysis of the concentration dependence of dopamine uptake in the presence and absence of 10 μM Zn^{2+} (Fig. 1, inset). It was weaker than that reported by Norregaard et al. (1998) possibly due to experimental differences such as stable vs. transient transfection, COS-7 vs. HEK 293 cells, 25 °C vs. 37°C and 34 mM vs. 5 mM D-glucose. Anyway, the effect of Zn^{2+} was completely absent in cells expressing NET—another member of the transporter family, but without a zinc binding site—where zinc ions did not affect uptake of 10 μM [^{3}H]noradrenaline (Fig. 1). The lower assay temperature and the higher concentration of glucose were necessary for the stability of cells in patch-clamp and superfusion experiments. For further experiments, we

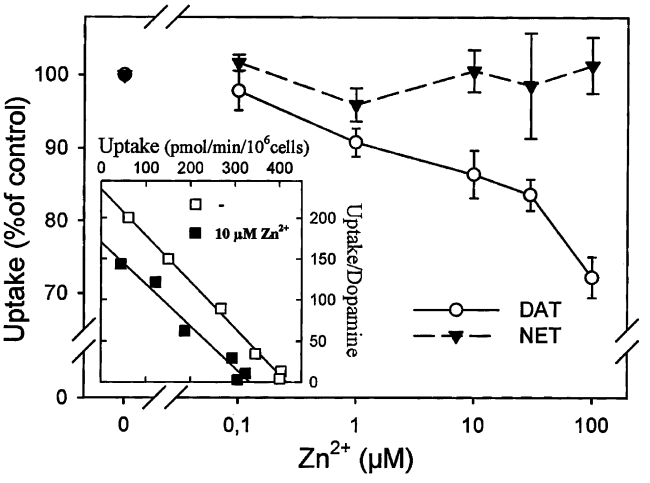

Fig. 1. Effect of Zn^{2+} on uptake by HEK 293 cells stably expressing the human DAT or NET. Cells expressing the DAT (circles) or NET (triangles) were incubated with 10 μM [^{3}H]dopamine or [^{3}H]noradrenaline (0.2 μCi) in 24-well plates at 25 °C for 2.5 min in the presence of the concentrations of $ZnSO_4$ indicated; uptake at 0 $ZnSO_4$ was 389 ± 7 for DAT and 143 ± 9 for NET cells (pmol/min/10^6 cells). *Inset*: Scatchard plot [^{3}H]dopamine uptake by cells expressing the DAT in the absence (open squares) or presence of 10 μM $ZnSO_4$ (closed squares). Uptake was determined by liquid scintillation counting as described in Materials and Methods. Data points are means ± SEM (bars) of 4–5 independent experiments and a representative experiment in the inset.

used 10 μM zinc sulfate in order to avoid unspecific Zn^{2+} effects at higher concentrations (Norregaard et al., 1998).

3.2. Release experiments

Superfusion of DAT-expressing HEK 293 cells preloaded with the metabolically inert DAT substrate [^{3}H]MPP^+ allows a clear distinction between the effect of pure uptake inhibitors such as mazindol and releasing agents such as amphetamine. Mazindol, even at the high concentration of 10 μM, did not induce efflux of radioactivity (Fig. 2A), whereas the strong and reversible releasing action of amphetamine (Fig. 2A) was potently blocked by inhibition of the carrier (Fig. 2B).

Superfusion with 10 μM $ZnSO_4$ did not significantly affect basal efflux or efflux induced by 0.2 μM amphetamine (Fig. 3A). By contrast, it increased the releasing effect of 1 and 10 μM amphetamine potentiating the release by 10 μM amphetamine more strongly than that by 1 μM (at 20 min: increase by 44.0 ± 8.3% vs. 27.4 ± 7.4%, $p < 0.02$ by paired Student's *t*-test on four independent experiments).

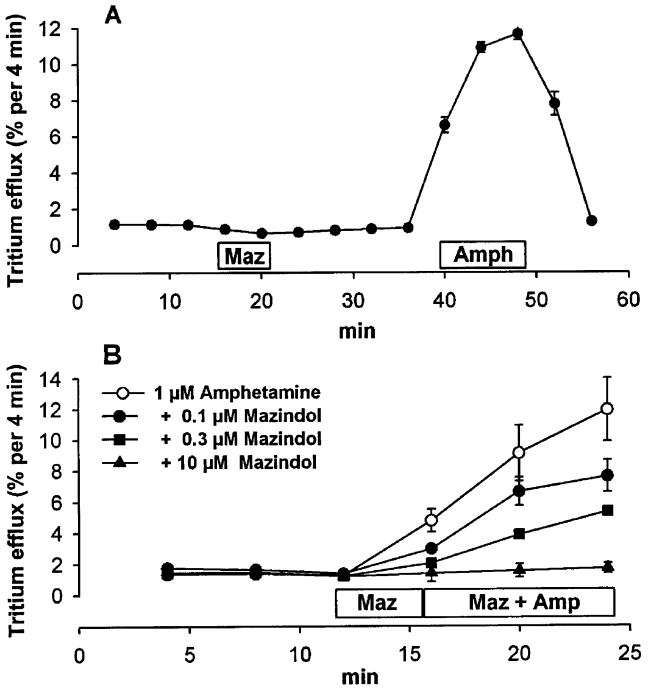

Fig. 2. Effect of mazindol and amphetamine on release of [^{3}H]MPP^+ from HEK 293 cells stably expressing the human DAT. Cells grown on 5-mm-diameter coverslips were preloaded with [^{3}H]MPP^+ and superfused at 25 °C with standard Tris/HEPES buffer, and 4-min fractions were collected. Bars indicate fractions after exposure to mazindol, to amphetamine or to both in the superfusion buffer. (A) 10 μM mazindol or 10 μM amphetamine. (B) 10 μM in the absence (open circles) or presence of 0.1 μM (closed circles), 0.3 μM (squares) or 10 μM mazindol (triangles). Data points are means ± SEM of triplicates of a representative experiment (A) or of three independent experiments (B).

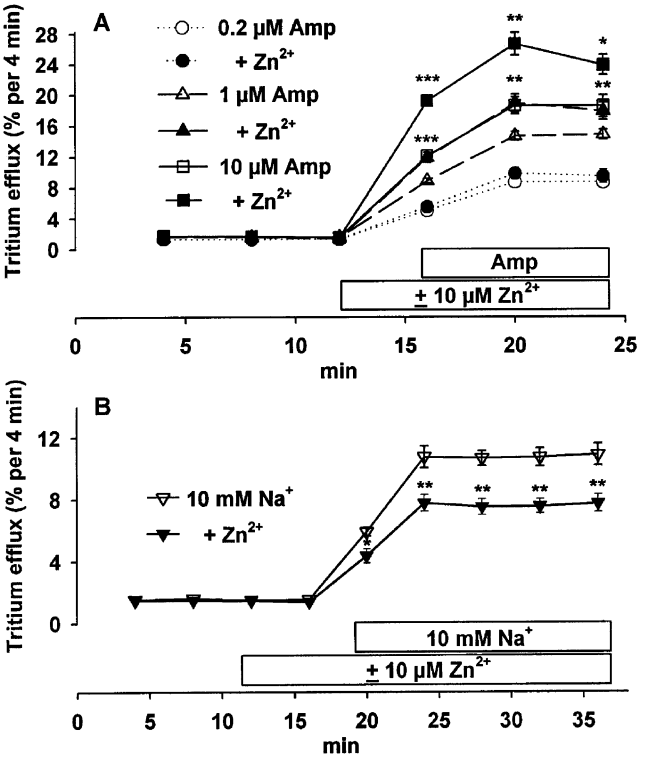

Fig. 3. Effect of Zn^{2+} on amphetamine- or low Na^+-induced release of [^{3}H]MPP^+ from HEK 293 cells stably expressing the human DAT. Cells grown on 5-mm-diameter coverslips were preloaded with [^{3}H]MPP^+ and superfused at 25 °C with standard Tris/HEPES buffer, and 4-min fractions were collected. Bars indicate fractions after exposure to Zn^{2+}, amphetamine, low Na^+ (iso-osmotically replaced by *N*-methyl-glucamine) or combination of them in the superfusion buffer. (A) 0.2 μM (circles), 1 μM (triangles) or 10 μM (squares) amphetamine in the absence (open symbols) or presence (closed symbols) of 10 μM $ZnSO_4$. (B) Na^+ lowered to 10 mM in the absence (open symbols) or presence (closed symbols) of $ZnSO_4$. Data points are means ± SEM of three to five independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. 0 Zn^{2+}.

To find out whether a stimulatory and not an inhibitory action of Zn^{2+} is common to every form of reverse transport, we induced reverse transport by lowering extracellular sodium to 10 mM by iso-osmotic replacement with *N*-methyl-glucamine in the absence or presence of 10 μM $ZnSO_4$. Indeed, Zn^{2+} significantly decreased efflux induced by this ionic manipulation (Fig. 3B).

3.3. Patch-clamp recordings

Patch-clamp experiments in the whole-cell configuration at a holding potential of −30 mV showed that 10 μM zinc ions did not significantly affect the inwardly directed leak currents varying considerably between different cells (compare Fig. 4A–C). Since the leak currents were only partially blocked by mazindol (data not shown), they appear to be mainly caused by a lower elec-

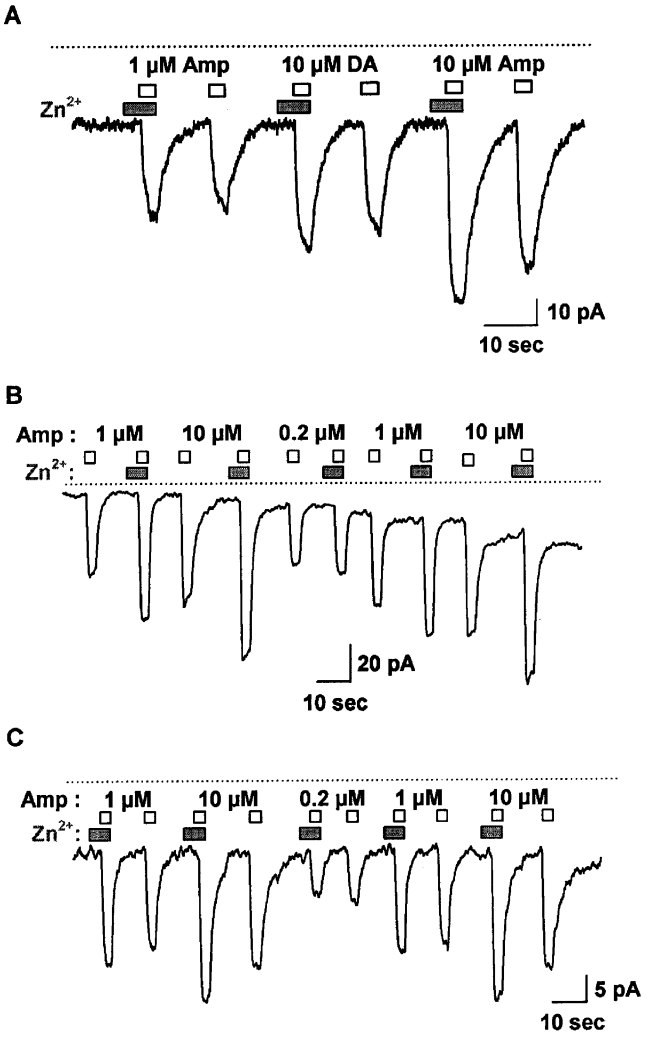

Fig. 4. Effect of Zn^{2+} in whole-cell patch-clamp recordings of HEK 293 cells stably expressing the human DAT. The cells were voltage-clamped at a holding potential of −30 mV and superfused at the times marked by the bars for 2 s with dopamine or amphetamine at the concentrations indicated in the absence or presence of 10 µM $ZnSO_4$ (lower bars, 2 s prerun). Shown are examples of different cells with various leak currents. The dotted lines denote zero current.

trical seal in transfected cells and to be only partially DAT mediated. However, 10 µM Zn^{2+} affected the inward currents that were evoked by 10 µM dopamine or 1 and 10 µM amphetamine (Fig. 4A) The inward currents induced by these drugs were DAT mediated because they were blocked by the transporter inhibitors cocaine or mazindol (data not shown). Zn^{2+} potentiated inward currents induced by 1 or 10 µM amphetamine but not by 0.2 µM regardless of the combination of Zn^{2+} plus amphetamine following (Fig. 4B) or preceding the application of amphetamine-only (Fig. 4C). Although the effect of Zn^{2+} was quite variable, analysis of 28–42 cells in the absence or presence of 10 µM zinc sulfate, applied in random sequence and under random usage of the different drug application syringes, revealed a significant stimulatory effect on currents induced by 10 µM dopamine and 1 or 10 µM amphetamine (Fig. 5A). By contrast, Zn^{2+} was not able to modulate the currents induced by 10 µM amphetamine in cells expressing a DAT with histidine 193 mutated to a lysine which impedes binding of Zn^{2+} (Fig. 5B): In a set of 24 cells expressing DAT-H193K, 10 µM Zn^{2+} did not significantly affect the inward current induced by 10 µM amphetamine ($-4.9 \pm 4.6\%$, $p > 0.05$ by paired Student's *t*-test).

In experiments on 17 cells expressing the wild-type DAT, we investigated the effect of Zn^{2+} on the action of both, 1 or 10 µM amphetamine (inward currents of 73.4 ± 9.5 and 103.6 ± 13 pA, respectively). It revealed

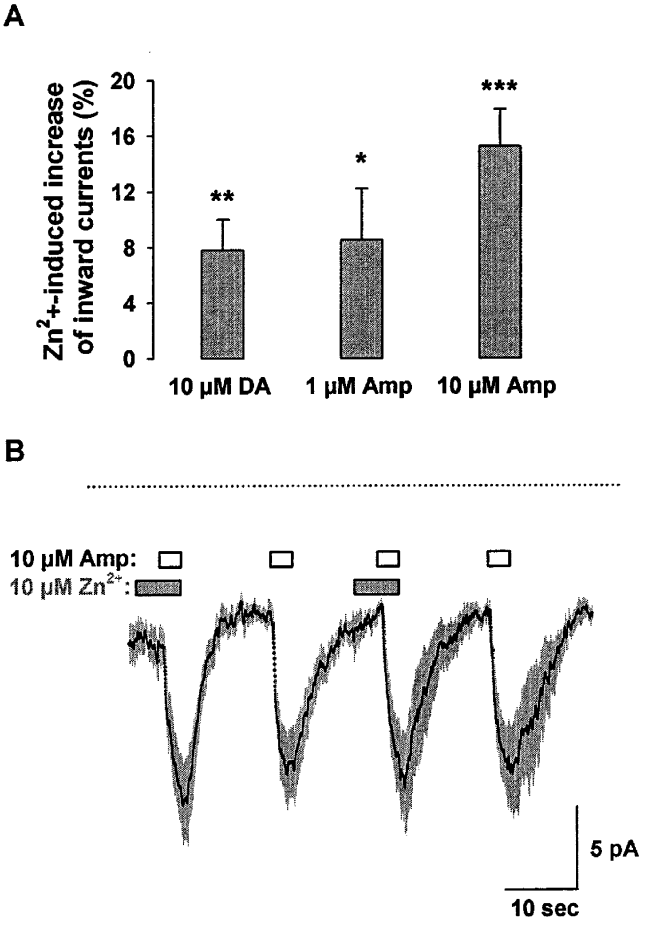

Fig. 5. Effect of Zn^{2+} on inward currents induced by dopamine or amphetamine in HEK 293 cells stably expressing the human DAT. The cells were voltage-clamped at a holding potential of −30 mV and superfused for 2 s with dopamine or amphetamine at the concentrations indicated in the absence or presence of 10 µM $ZnSO_4$ (2 s prerun). (A) Cells expressing the wild-type DAT, stimulation by Zn^{2+} of maxima of inward currents induced by 1 µM dopamine, 1 µM amphetamine and by 10 µM amphetamine determined in the absence or presence of Zn^{2+} in the same cell (38, 42 and 28 cells, respectively). (B): Cells expressing the H193K-DAT, mean values of current traces ± SEM (grey area) of 11 cells. * $p < 0.05$, ** $p < 0.002$, *** $p < 0.0002$ vs. 0 Zn^{2+} by paired Student's *t*-test.

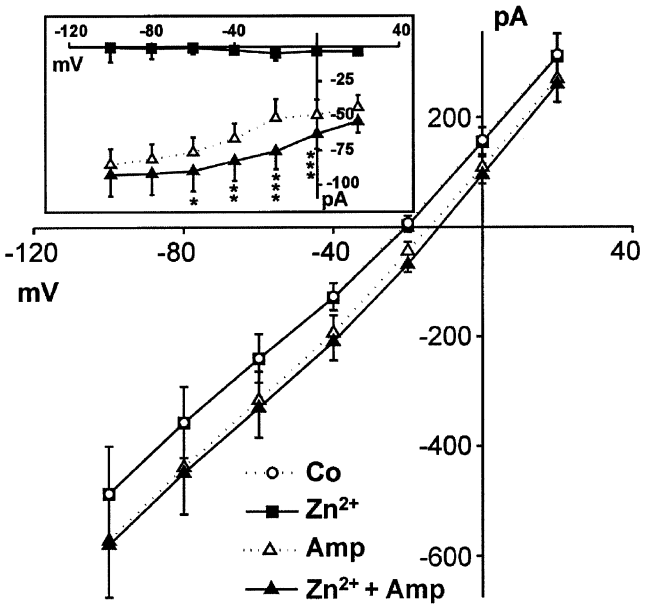

Fig. 6. Effect of Zn^{2+} on the current/voltage relationship in HEK 293 cells stably expressing the human DAT. The cells were voltage-clamped at a holding potential of −30 mV and the indicated levels of test pulses for 200 ms as described in Materials and Methods. Currents in the absence (open symbols) or presence of 10 μM $ZnSO_4$ (closed symbols) in the absence (circles and squares) or presence of 10 μM amphetamine (triangles). *Inset*: Voltage-dependence of zinc-induced currents ($I_{zinc}-I_{leak}$, squares) and amphetamine-induced currents ($I_{amphetamine}-I_{leak}$) in the absence (open triangles) or presence of Zn^{2+} (closed triangles). Data are mean ± SEM (bars) values from nine different cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs. 0 Zn^{2+} by paired Student's *t*-test.

a significantly stronger potentiating effect of Zn^{2+} on the inward current produced by 10 μM amphetamine (increase by 16.7 ± 3.5% vs. 10.0 ± 3.9%, $p < 0.05$ by paired Student's *t*-test). In another set of 14 cells, the effect of Zn^{2+} was investigated on both 0.2 and 1 μM amphetamine (inward currents of 43.1 ± 11.2 and 68.4 ± 17.2 pA, respectively) and Zn^{2+} significantly increased the effect of 1 μM amphetamine (+12.3 ± 3.7%, $p < 0.05$ by paired Student's *t*-test), but not the inward current induced by 0.2 μM amphetamine (−12.1 ± 3.2%, $p > 0.05$ by paired Student's *t*-test). The current traces shown in Fig. 4 also demonstrates the dependence of the Zn^{2+} effect on the concentration of amphetamine.

In current–voltage experiments, there was no significant effect of Zn^{2+} on the voltage-dependent amplitudes of the leak current (Fig. 6). The current–voltage relation measured in the presence of 10 μM amphetamine was distorted to the right by 10 μM Zn^{2+}. This resulted in a significant potentiation of the amphetamine-induced current ($I_{amphetamine}-I_{leak}$) at a membrane potential of 0 to −60 mV (Fig. 6, inset).

4. Discussion

Our results demonstrate that Zn^{2+} modulates two cellular effects in the same direction: amphetamine-induced release and amphetamine-induced inward currents mediated by the DAT. Both effects of μmolar amphetamine - transfer of preloaded substrate and transfer of charges - were stimulated by Zn^{2+} and both were stimulated more strongly by Zn^{2+} if tested at 10 μM amphetamine instead of 1 μM. The action of 0.2 μM amphetamine was not significantly modulated by Zn^{2+}, neither the release nor the current induced by this concentration of the drug. Whereas effects of Zn^{2+} on reverse transport mediated by the DAT were reported recently (Scholze et al., 2002), this is the first publication on the regulation of DAT-associated currents by Zn^{2+}.

In recent reports, Zn^{2+} was found to decrease dopamine uptake but to increase binding of a cocaine analogue in rat striatal synaptosomes as well as COS-7 cells transiently expressing the human DAT (Richfield, 1993; Norregaard et al., 1998). In our study on HEK 293 cells stably expressing the human DAT, Zn^{2+} also decreased dopamine uptake, whereas it increased the inward current induced by dopamine or amphetamine in patch-clamp recordings. The metal ion also increased amphetamine-induced release mediated by the DAT which is in agreement with findings by Scholze et al. (2002). Zn^{2+} was reported to regulate the function of the DAT by binding to one histidine in the large second extracellular loop (His-193) and to another histidine and one glutamate in the third extracellular loop (His-375 and Glu-396; Norregaard et al., 1998; Loland et al., 1999). The binding of Zn^{2+} was suggested to stabilize the transporter in the outward facing conformation which allows binding of dopamine but inhibits its translocation. Switching between outward and inward facing conformation is not only a basic concept for the physiological transport cycle, but also for facilitated exchange diffusion (for review: Rudnick and Clarke, 1993). Whereas the normal transport cycle moves substrate molecules from the extracellular to the intracellular compartment, facilitated exchange diffusion offers an explanation for a carrier-mediated releasing effect of drugs. These drugs must be substrates of the carrier and, being taken up, make the transport protein switch to the inward facing conformation which allows cytoplasmic dopamine to be moved to the outside. We cannot apply this concept on amphetamine-induced release, because stabilisation of the transporter by Zn^{2+} in the outward facing conformation is in contradiction to its amplifying effect on the releasing action of amphetamine as found in our study, since increased release by amphetamine requires facilitated but not restrained cycling between the conformations. Furthermore, the assumption that Zn^{2+} inhibits a conformational change critical for the translocation process is difficult to reconcile with our observation that Zn^{2+}

increases substrate-induced inward currents as long as these currents are attributed to the electrogenicity of the transport cycle. The DAT is a Na^+/Cl^--dependent transporter and the stoichiometry of the ion-dependence of transport can predict inward currents at the DAT produced by the translocation of two sodium and one chloride ion per moved substrate molecule (Sonders et al., 1997; Sitte et al., 1998). Dopamine or amphetamine themselves might contribute one additional positive charge since the cationic form of dopamine is the most likely substrate of the transporter (Berfield et al., 1999). Logically, such a substrate-induced inward current should be blocked by stabilisation of the transporter in the outward conformation rather than potentiated as found in our study.

Therefore, our findings on the zinc effect in release and patch-clamp experiments can be better explained with the following hypothesis: (1) Release is not based on an ongoing transport cycle but on a distinct mode of the transporter, namely amphetamine-induced reverse transport, and this mode is regulated independently of inward transport. (2) Likewise, carrier-mediated inward currents induced by dopamine and amphetamine are not based on the transport cycle but on a channel mode of the DAT which is again regulated independently from inward transport. Reverse transport being a distinct mode of the transporter explains why Zn^{2+} is able to stimulate amphetamine-induced release but slows down the transport cycle as shown in its inhibitory action on dopamine uptake. The stimulatory Zn^{2+} effect on release and current is due to binding to the DAT specific histidines. This was supported (unpublished observation) by the lack of effect on amphetamine-induced release in cells expressing the NET - devoid of these coordinating residues - and proven in cells expressing the DAT-H193K in which a decisive histidine has been turned into a lysine by mutation (see also Scholze et al., 2002 for the effect on release). The higher stimulatory effect of Zn^{2+} at 10 μM amphetamine instead of 1 μM found in our study is also in contrast to the inhibitory Zn^{2+}-effect on dopamine uptake which was strictly non-competitive with uptake rates of low and high dopamine concentrations inhibited to the same extent (Norregaard et al., 1998). There is no obvious explanation for the peculiar dependence of the Zn^{2+}-effect on the amphetamine concentration. Interestingly, in current–voltage experiments, Zn^{2+}, had no significant effect at highly negative holding potentials. This could be due to an inability of the cation to bind to a more positively polarized extracellular part of the protein or to provoke a relevant conformational change at this membrane potential. It can only be speculated whether higher amphetamine concentrations which induce higher inward currents progressively change the DAT structure and lead to more favorable conditions for the Zn^{2+} effect. For the release experiments, it can be argued that high amphetamine concentrations, inducing high inward currents, might depolarize the protein locally, which facilitates the interaction with Zn^{2+}. Such a mechanism is difficult to argue for the electrophysiological experiments where a similar dependence on amphetamine concentrations has been observed under voltage-clamp conditions.

However, the parallel modulation of release and currents by Zn^{2+} suggests a link between these two phenomena on the plasmalemmal transporter. One possible link could be influx of sodium ions via the transporter. The rightward shift of the I–V curve due to amphetamine (Fig. 6) is compatible with an increase of ionic conductance of either Na^+ or Cl^-. When Cl^- was replaced by acetate or isethionate in the patch pipette, amphetamine still induced inward currents (unpublished observation), which suggests that they are carried by Na^+. Increase of cytoplasmic Na^+ by inhibition of the Na^+-K^+-ATPase with ouabain (Levi et al., 1976; Liang and Rutledge, 1982; Sweadner, 1985; Bönisch, 1986; Scholze et al., 2000) or by opening of sodium channels with veratridine or nicotine (Liang and Rutledge, 1983; Bönisch and Trendelenburg, 1987; Gerevich et al., 2001; Drew and Werling, 2001) induced or potentiated reverse transport by plasmalemmal monoamine transporters. Na^+ influx through the DAT induced by amphetamine might increase the concentration of Na^+ just in the vicinity of cytoplasmic parts of the transport protein. This might make the DAT switch its transport direction from influx to efflux more efficiently than voltage-dependent sodium channels opened during membrane depolarization by action potentials. On a molecular level, the opening of sodium channels is more distant and has been shown to have a slower onset in eliciting reverse transport (see Adam-Vizi, 1992; Elverfors et al., 1997). A recent report elegantly demonstrated that DAT-mediated inward currents stimulate amphetamine-induced dopamine efflux (Khoshbouei et al., 2003). The study used in parallel whole-cell patch-clamp recordings and amperometric determination of released dopamine.

Interestingly, only reverse transport induced by amphetamine was stimulated by Zn^{2+}. It seems to be clearly distinct from reverse transport induced by a shift of the sodium gradient. Lowering extracellular sodium to 10 mM induced a DAT-mediated release as shown in previous studies (Pifl et al., 1997; Pifl and Singer, 1999). However, this reverse transport was inhibited and not stimulated by Zn^{2+}. As expected, lowering of extracellular sodium, in contrast to amphetamine, did not induce inward currents in patch-clamp experiments (unpublished observation).

In conclusion, the stimulation of amphetamine-induced inward currents by Zn^{2+} in DAT expressing cells parallels the stimulation of amphetamine-induced DAT-meditated release and contrasts with the inhibitory effect of Zn^{2+} on dopamine uptake. This supports the hypothesis of charge transfer through the DAT rather than

facilitated exchange diffusion being responsible for the releasing action of amphetamine. Furthermore, the present study demonstrates the usefulness of the regulatory effect of Zn^{2+} for elucidating mechanisms related to DAT.

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