

Zinc regulates the dopamine transporter in a membrane potential and chloride dependent manner

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ABSTRACT

The dopamine transporter (DAT), a membrane protein specifically expressed by dopaminergic neurons and mediating the action of psychostimulants and dopaminergic neurotoxins, is regulated by Zn^{2+} which directly interacts with the protein. Herein, we report a host-cell-specific direction of the Zn^{2+} effect on wild type DAT. Whereas low μ molar Zn^{2+} decreased dopamine uptake by DAT expressing HEK293 cells, it stimulated uptake by DAT expressing SK-N-MC cells. Inhibition or stimulation was lost in a DAT construct without the binding site for Zn^{2+} . Also reverse transport was differentially affected by Zn^{2+} , dependent on whether the DAT was expressed in HEK293 or SK-N-MC cells. Pre-treatment of DAT expressing cells with phorbol-12-myristate-13-acetate, an activator of protein kinase C, attenuated the inhibitory effect of Zn^{2+} on uptake in HEK293 cells and increased the stimulatory effect in SK-N-MC cells. Patch-clamp experiments under non-voltage-clamped conditions revealed a significantly higher membrane potential of HEK293 than SK-N-MC cells and a reduced membrane potential after phorbol ester treatment. Lowering chloride in the uptake buffer switched the stimulatory effect of Zn^{2+} in SK-N-MC cells to an inhibitory, whereas high potassium depolarization of HEK293 cells switched the inhibitory effect of Zn^{2+} to a stimulatory one. This study represents the first evidence that DAT regulation by Zn^{2+} is profoundly modulated by the membrane potential and chloride.

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1. Introduction

The dopamine transporter (DAT) is a specific membrane protein in dopaminergic neurons and decisively involved in the function of neurons which use dopamine (DA) as neurotransmitter. Reuptake of DA by DAT regulates the extracellular DA concentration in brain regions with dopaminergic innervation. The important behavioural role of the DAT is demonstrated by the profound neurochemical and behavioural effects of cocaine which blocks the transporter, of amphetamine-like drugs which reverse its action, and of genetic deletion by targeted recombination (Piffl and Caron, 2002). The DAT may also play a role in the neurodegeneration of dopaminergic neurons, since various substrates of the DAT, including the natural substrate DA, have been shown to be neurotoxic (Uhl and Kitayama, 1993; Edwards, 1993; Miller et al., 1999; Kawai et al., 1998; Nass and Blakely, 2003). Regulation of the DAT might therefore have a broad impact on the physiology and pathophysiology of brain function.

The DAT is regulated by low μ molar concentrations of zinc ions (Richfield, 1993; Bonnet et al., 1994). Although basal concentrations of free extracellular Zn^{2+} are low in the brain, activity-dependent release of Zn^{2+} at specific glutamatergic synapses might lead to concentrations of more than 20 μ M (Assaf and Chung, 1984; Vogt

et al., 2000). For the striatum, the brain area with the highest density of DAT, an innervation by zinc-containing neuronal fibers has been demonstrated (Howell et al., 1989; Frederickson et al., 2000). The regulation of DAT by Zn^{2+} ions is based on a direct molecular interaction with the transporter protein (Norregaard et al., 1998). Zn^{2+} ions promptly decrease DA uptake by interaction with defined histidins in two extracellular loops of the DAT protein (H193, H375). However, various substitutions of amino acid residues in the DAT (Y335A, K264A, D345A/N, D436A) have been shown to switch the inhibitory effect of Zn^{2+} to an activating one (Loland et al., 2002, 2004; Chen et al., 2004; Meinild et al., 2004). These mutations are located on intracellular loops and lead to lower uptake rates explained by disruption of intramolecular interactions which normally stabilize the transporter in the outward facing conformation; it was argued that Zn^{2+} would re-establish a conformational equilibrium similar to wild type by stabilizing the outward facing conformation and thereby restore substrate binding and translocation. However, in a study on DAT expressing *Xenopus laevis* oocytes, the modulation of transport by Zn^{2+} was explained by potentiation of an uncoupled chloride conductance which modulates the membrane potential of the cells (Meinild et al., 2004). It was suggested that, depending on the membrane potential being above or below the reversal potential of chloride, Zn^{2+} may stimulate or inhibit transport by increasing or inhibiting the driving force for uptake.

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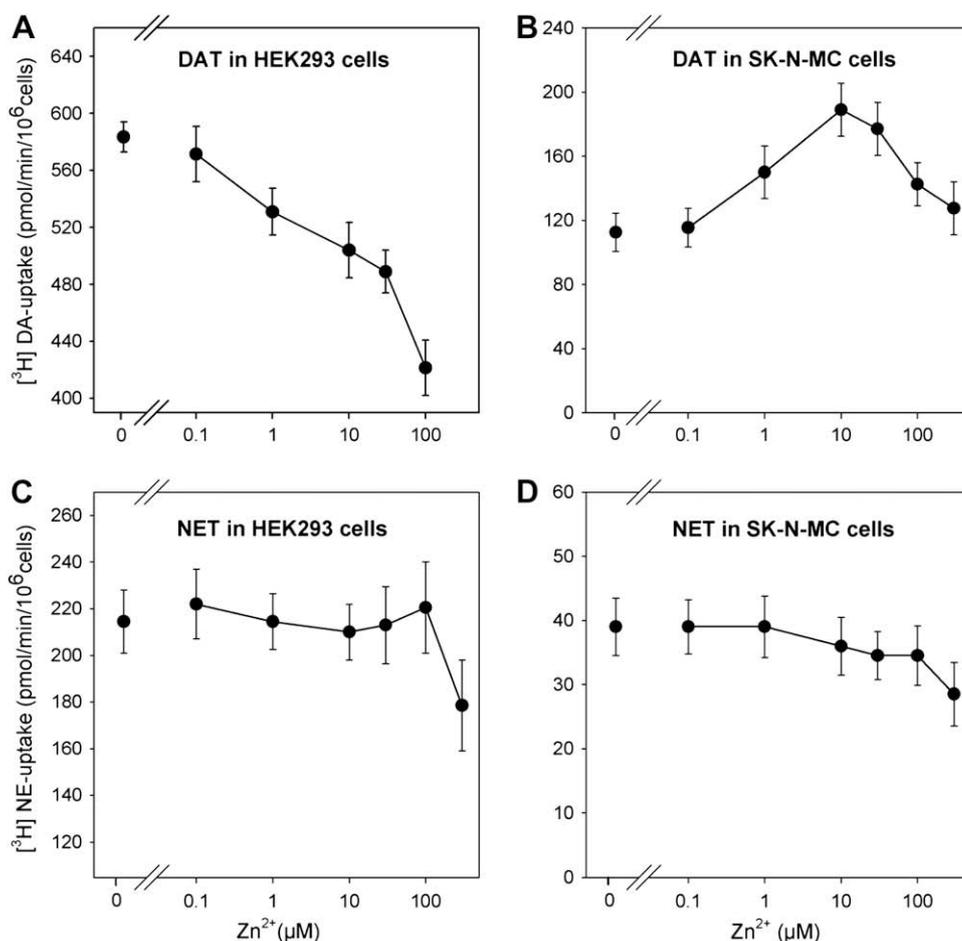


Fig. 1. Concentration-dependence of the Zn^{2+} effect on transport by the DAT or NET in different cellular hosts. HEK293 cells (A,C) or SK-N-MC cells (B,D) stably expressing the human DAT (A,B) or NET (C,D) were seeded in 24-well plates and incubated, in the presence of the concentrations of $ZnSO_4$ indicated, with $10 \mu M$ [3H]DA (A,B) or $10 \mu M$ [3H]NE (C,D) in uptake buffer for 2.5 min at $25^\circ C$, and incorporation of tritium was determined as described under "Section 2.2". Nonspecific uptake was estimated in the presence of $10 \mu M$ mazindol. Symbols represent means \pm standard error of four to six independent experiments, each in duplicates.

Here, we show an unexpected host-cell-specific direction of the Zn^{2+} effect on DA uptake by the human DAT wild type protein in mammalian cells. We stably expressed the DAT either in human embryonic kidney 293 cells (HEK293) or in human neuroblastoma SK-N-MC cells and surprisingly found opposite actions of Zn^{2+} on DA uptake that is an inhibitory effect in HEK293 and a stimulatory action in SK-N-MC cells. A thorough investigation of this novel finding reveals that the direction of the Zn^{2+} effect depends on the membrane potential and chloride distribution of the cells.

2. Methods

2.1. Cell culture

HEK293 and SK-N-MC cells were grown in minimum essential medium with Earle's salts and L-glutamine, 10% heat-inactivated fetal bovine serum and 50 mg/l gentamicin on 100 mm tissue culture dishes (Falcon) at $37^\circ C$ and 5% $CO_2/95\%$ air. The human DAT, human norepinephrine transporter (NET) or human DAT-H193K cDNA was stably expressed using methods as described (Piffl et al., 2004). Cells were selected with 0.8 g/l (HEK293 cells) or 1 g/l (SK-N-MC cells) geneticin in the medium. More than one cell clone of each, HEK293 and SK-N-MC cells, were used in the experiments.

2.2. Uptake of monoamines

Cells were seeded in poly-D-lysine-coated 24-well plates (1×10^5 HEK293 cells/well and 1.5×10^5 SK-N-MC cells/well) and, 1 day later, uptake was performed at $25^\circ C$ for 2.5 min as described previously (Piffl et al., 2004). 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 $MgSO_4$; 5 D-glucose; 0.5 ascorbic acid; pH 7.1. In experiments with various concentrations of potassium the buffer

composition was the same except (mmol/l): 0 KCl and 34 D-glucose; 5 KCl and 20 D-glucose; 20 KCl and 5 D-glucose; 50 KCl and 5 D-glucose. In ion dependence experiments, chloride was replaced with isethionate. After incubation with buffer containing various concentrations of $ZnSO_4$ for 5 min, uptake was started by addition of tritium labelled DA, norepinephrine (NE) or 1-methyl-4-phenyl-pyridinium (MPP^+) at a final concentration of $10 \mu M$. In experiments with phorbol esters, cells were pretreated with $0.5 \mu M$ of the active phorbol ester phorbol-12-myristate-13-acetate (PMA) or, as a negative control, with $0.5 \mu M$ of the inactive ester 4 α -phorbol-12,13-didecanoate (4 α PDD) in uptake buffer at $37^\circ C$ for 20 min. Nonspecific uptake was estimated in the presence of $10 \mu M$ mazindol. For uptake inhibition experiments data of each separate experiment were fitted to the equation $f = \min + (\max - \min) / (1 + x^{Hillslope} / IC_{50}^{Hillslope})$, "min" being nonspecific uptake, "max" is the uptake in the absence of inhibiting drug, x is the molar concentration of the inhibiting drug, and IC_{50} is the drug concentration that inhibits 50% of specific uptake.

2.3. Superfusion

Cells were seeded onto poly-D-lysine-coated 5-mm-diameter glass coverslips in 96-well tissue culture plates and loaded with $6 \mu M$ [3H]MPP $^+$ (0.1 Ci/mmol) at $37^\circ C$ for 20 min. Coverslips were transferred to small chambers and superfused with the same buffer as used in uptake experiments ($25^\circ C$, 1.0 ml/min) as described (Piffl et al., 2004; Piffl and Singer, 1999). The radioactivity released during consecutive 4 min fractions was expressed as percentage of the total radioactivity present in the cells at the beginning of each fraction. Experiments with and without Zn^{2+} were run in parallel in each superfusion session and therefore statistical differences between these conditions were analysed by paired Student's t -test.

2.4. Binding

Binding on whole cells, seeded as described for uptake experiments, was performed in 24-well plates as described previously (Piffl et al., 1996). Cells were incubated on ice for 2 h in the same buffer as used in uptake experiments containing 2 nM [3H]- β -carbomethoxy-3 β -(4-fluorophenyl)tropane ([3H]CFT). For binding to

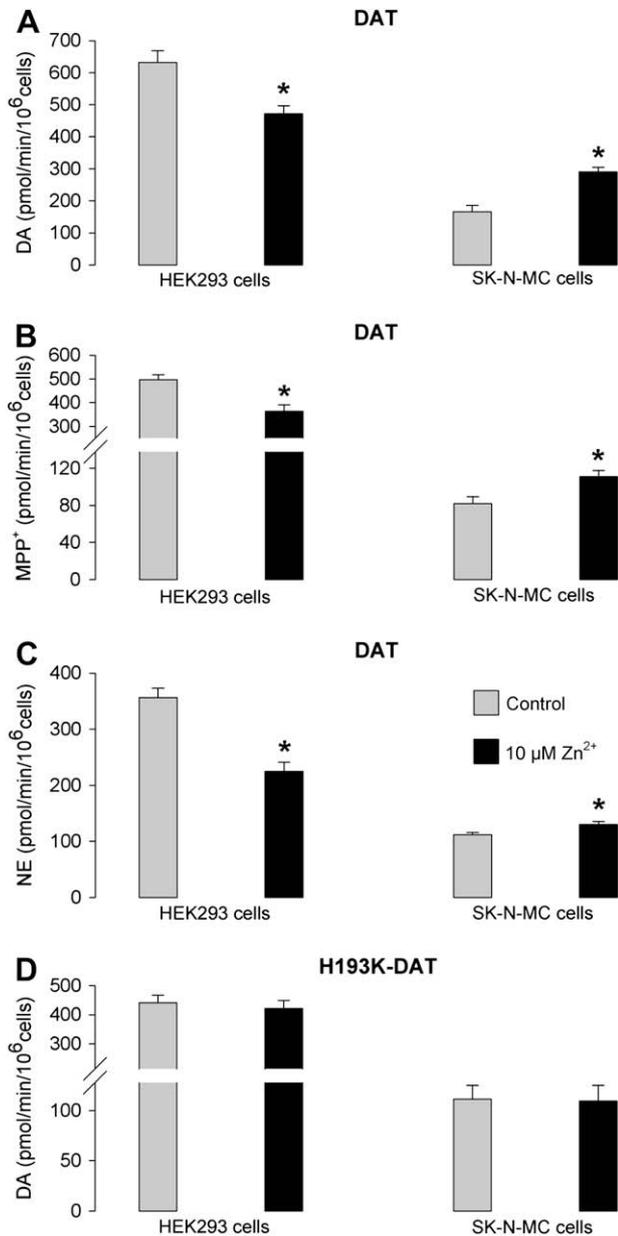


Fig. 2. Host-cell-specific direction of the Zn²⁺ effect on the transport of various substrates by the DAT. HEK293 cells (left) or SK-N-MC cells (right) stably expressing the human DAT (A–C) or H193K-DAT (D) were seeded in 24-well plates and incubated, in the absence or presence of 10 μM ZnSO₄, with 10 μM [³H]DA (A,D), 10 μM [³H]MPP⁺ (B) or 10 μM [³H]NE (C) in uptake buffer for 2.5 min at 25 °C, and incorporation of tritium was determined as described under “Section 2.2”. Nonspecific uptake was estimated in the presence of 10 μM mazindol. Columns represent means of specific uptake ± standard error of three independent experiments, each in duplicates. **p* < 0.05 vs. control by Student’s *t*-test.

membranes, membranes were prepared by lysis of cells with 2 mM HEPES, 1 mM EDTA, pH 7.6, centrifugation of the lysate at 31,000 × *g* for 20 min, and homogenisation of the pellet with a polytron in uptake buffer. 12–30 μg of membrane protein (Bio-Rad assay) were incubated in 125 μl of uptake buffer for 2 h on ice and incubation was stopped by the addition of 4 ml of ice-cold buffer and rapid filtration through Whatman (Hassel, Munich, Germany) GF/B filters presoaked for 1 h in polyethyleneimine (0.3% in H₂O), using a 48-place Brandel (Gaithersburg, MD) harvester. Filters were washed twice with 4 ml buffer and transferred into counting vials. After addition of 2.5 ml scintillation standard cocktail (Ultima Gold, Packard Instruments), vials were warmed to 40 °C, agitated for 1 h, and counted in a liquid scintillation counter.

2.5. Uptake into striatal synaptosomes

Male Sprague–Dawley rats were killed by decapitation. The brains were rapidly removed and immediately chilled in ice-cold 0.9% NaCl. Striata were dissected from

the unfrozen brains on a cold-plate (+1 °C) and fresh crude synaptosomes were prepared and uptake performed as described previously (Piffl et al., 1993) with minor modifications. Tissue was initially homogenized in 10 volumes (g tissue/ml) and the final pellet resuspended in 7 volumes of ice-cold 0.3 M sucrose. Twenty-five μl of the synaptosomal suspension were added to 600 μl of the uptake buffer as described above (without MgSO₄) and incubated with 1 μM [³H]DA at 25 °C for 2.5 min. Stop of the incubation by filtration and liquid scintillation counting was as described above for binding experiments. Nonspecific uptake was estimated in the presence of 10 μM mazindol.

2.6. Patch-clamp experiments

About 6 × 10⁴ HEK293 and 1.2 × 10⁵ SK-N-MC cells were split into 35 mm tissue culture dishes two days before. The external (bathing) solution for recordings was essentially the uptake buffer with minor modifications (130 mM NaCl and 34 mM D-glucose) for obtaining the final osmolality of 300 mOsm/l. Patch pipettes were filled with (mmol/l): 20 KCl and 125 K-gluconate (130 KCl in current-clamp experiments), 0.1 CaCl₂, 1.1 EGTA, 2 MgCl₂, 4 Tris–HCl; 6.25 HEPES, pH 7.2, with an osmolality of 270 mOsm/l and patch electrodes pulled from borosilicate glass capillaries (GB150F-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. 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) and clamping the cells to zero current or to the holding potential indicated. In voltage-clamp experiments test currents 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 were continuously superfused with bathing solution.

2.7. Uptake of ³⁶chloride

Cells were seeded in poly-D-lysine-coated 12-well plates (1.9 × 10⁵ HEK293 cells/well and 3.5 × 10⁵ SK-N-MC cells/well, respectively) and, 2 days later, cells were once washed and incubated for 10 min at 30 °C with 200 μl uptake buffer (see above). Uptake was started with addition of 0.12 μCi ³⁶chloride (sodium salt) in 100 μl buffer in the absence or presence of DA and/or Zn²⁺ at a final concentration of 10 μM. After incubation for 5 min at 30 °C, uptake was stopped by aspirating the incubation mixture and washing each well twice with 3 ml ice-cold buffer. The radioactivity remaining in each well was determined by incubating with 0.8 ml 1% sodium dodecyl sulphate and transferring this solution into scintillation vials containing 4 ml scintillation cocktail.

2.8. Materials

Media, sera and other tissue culture reagents were obtained from Life Technologies (Vienna, Austria). [³H]dopamine (22 Ci/mmol), (–)[³H]norepinephrine (12 Ci/mmol) and [³H]MPP⁺ (79.9 Ci/mmol) were obtained from New England Nuclear GmbH (Vienna, Austria), ³⁶chloride (14 mCi/g) from GE Healthcare (Buckinghamshire, UK), D-amphetamine-sulphate was kindly donated by SmithKline & French (Welwyn Garden City, Herts, UK), mazindol from Sandoz GmbH (Vienna, Austria). PMA and 4αPDD were purchased from Calbiochem, the other chemicals were from Sigma–Aldrich or Merck.

3. Results

Zn²⁺ inhibits DA uptake by the DAT expressed in HEK293 cells (Fig. 1A), but stimulates uptake by the DAT expressed in SK-N-MC cells in concentrations up to 30 μM (Fig. 1B). This is observed on at least two clones, each of the cell lines stably expressing the human DAT. Up to 100 μM Zn²⁺ do not affect NE uptake by the closely related norepinephrine transporter (NET), neither in HEK293 (Fig. 1C) nor in SK-N-MC cells (Fig. 1D).

Zn²⁺ inhibits uptake by the DAT in HEK293 cells and stimulates uptake by the DAT in SK-N-MC cells, irrespective of whether dopamine (Fig. 2A), MPP⁺ (Fig. 2B) or NE (Fig. 2C) are used as substrates. Uptake is neither stimulated nor inhibited by Zn²⁺ in cells expressing a DAT construct in which the binding of Zn²⁺ is impeded by substitution of histidine 193 with a lysine (Fig. 2D).

Zn²⁺ inhibits DA-induced carrier-mediated release of tritium from DAT expressing HEK293 cells preloaded with [³H]MPP⁺ (Fig. 3A), but stimulates it slightly in SK-N-MC cells (Fig. 3B). In contrast to DA uptake, amphetamine-induced release from DAT expressing HEK293 cells is stimulated and not inhibited by

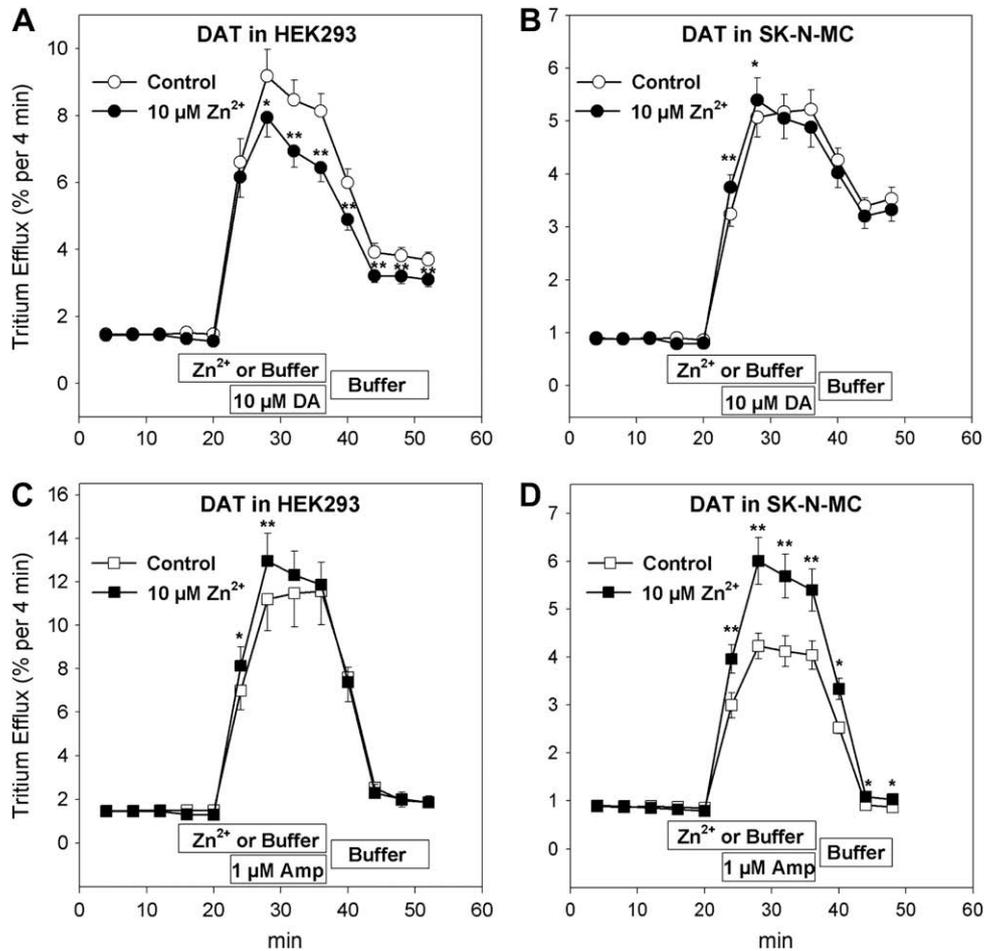


Fig. 3. Effect of Zn^{2+} on DA- or amphetamine-induced release of $[^3H]MPP^+$ from different cellular hosts stably expressing the DAT. HEK293 cells (A,C) or SK-N-MC cells (B,D) stably expressing the human DAT were grown on 5-mm-coverslips, preloaded with $6 \mu M [^3H]MPP^+$, superfused at $25^\circ C$, and 4-min fractions were collected. Open frames indicate fractions collected after exposure to Zn^{2+} , DA or D-amphetamine (Amp) in the superfusion buffer, respectively. (A,B) $10 \mu M$ DA (circles) and (C,D) $1 \mu M$ D-amphetamine (squares) in the absence (open symbols) or presence (closed symbols) of $10 \mu M ZnSO_4$. Efflux of tritium was determined and presented as described under "Section 2.3". Symbols represent means \pm standard error of four to six independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control by paired Student's *t*-test.

Zn^{2+} (Piffl et al., 2004; Scholze et al., 2002); here we show that this stimulation is much more pronounced in DAT expressing SK-N-MC cells (compare D to C in Fig. 3).

Differences in the action of proteins with identical amino acid sequence in different cellular hosts might be due to differences in posttranslational modifications in these cells. An established posttranslational modification of the DAT is phosphorylation (Vaughan et al., 1997; Huff et al., 1997). Therefore, we investigated if stimulation of protein kinase C with phorbol-12-myristate-13-acetate (PMA) might modify the regulatory effect of Zn^{2+} in our cells. In fact, pre-treatment of the cells with $0.5 \mu M$ PMA as compared to pre-treatment with the inactive phorbol ester $4\alpha PDD$, attenuated the inhibitory effect of Zn^{2+} in HEK293 cells and potentiated the stimulatory effect in SK-N-MC cells (Fig. 4).

In addition to translocating substrates, the DAT also binds cocaine and cocaine-derivatives. In binding experiments at $4^\circ C$ on whole cells expressing the DAT, Zn^{2+} stimulated binding of the cocaine analogue $[^3H]CFT$ in both cell types, which is in agreement with the literature (Bonnet et al., 1994; Norregaard et al., 1998). By contrast, binding of $[^3H]CFT$ to membranes prepared from these cells was not affected by Zn^{2+} at all (Fig. 5).

The main difference between the DAT in whole cells and in a membrane preparation is the membrane potential, to which the DAT is exposed only in intact cells. Therefore, we measured the membrane potential of DAT expressing SK-N-MC and HEK293 cells

by patch clamping the cells to zero current. Under this condition the membrane potential of HEK293 cells (mean value \pm SE, 12 cells: -61.8 ± 2.6 mV) was significantly more negative than that of SK-N-MC cells (-44.7 ± 3.6 mV, 20 cells; $p < 0.005$).

The modulation of DA uptake by Zn^{2+} was explained by the potentiation of an uncoupled chloride conductance (Meinild et al., 2004). We therefore studied the accumulation of $^{36}Cl^-$ (Fig. 6). Whereas in cells without DAT $^{36}Cl^-$ incorporation was not affected, in DAT expressing HEK293 cells $^{36}Cl^-$ uptake was significantly increased in the presence of DA, but was at control levels in the presence of both, Zn^{2+} and DA. By contrast, in DAT expressing SK-N-MC cells $^{36}Cl^-$ incorporation was only increased significantly by the combination of Zn^{2+} and dopamine. After growing the cells in the presence of $^{36}Cl^-$ for three days, $^{36}Cl^-$ levels were lower in SK-N-MC than HEK293 cells (0.097 ± 0.028 vs. 0.22 ± 0.02 pmol/cell; $p < 0.05$).

Ion substitution experiments revealed a strong chloride dependence of the modulation of DA uptake by Zn^{2+} . Lowering the concentration of Cl^- by substitution with isethionate significantly increased the inhibitory action of Zn^{2+} on DA uptake in HEK293 cells (Fig. 7, left panels) and switched the stimulatory effect of Zn^{2+} to an inhibitory effect in SK-N-MC cells (Fig. 7, right panels).

We now reasoned that the differing membrane potential of HEK293 and SK-N-MC cells might be the cause of the different direction of the Zn^{2+} effect, and investigated, how membrane

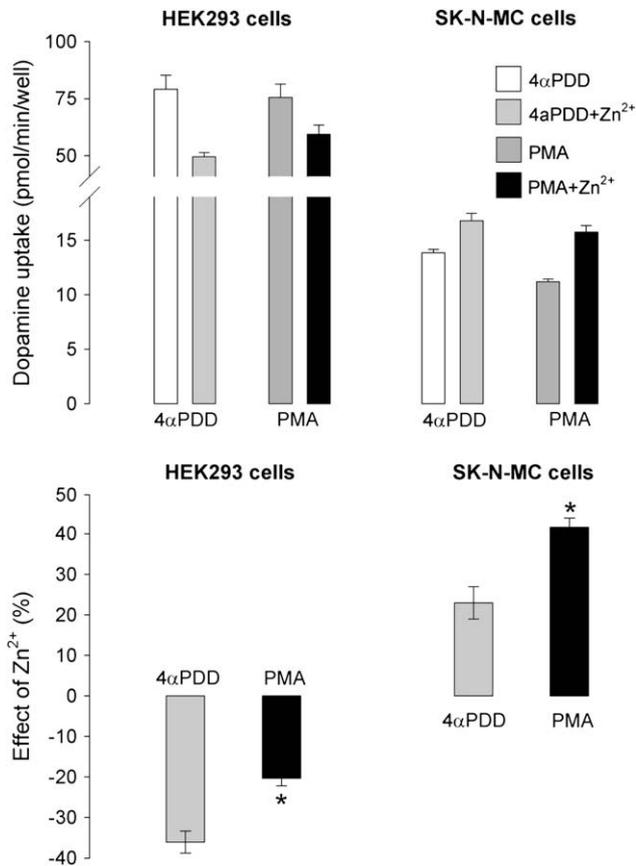


Fig. 4. Effect of protein kinase C stimulation on the regulation of DA uptake by Zn^{2+} . HEK293 cells (left) or SK-N-MC cells (right) stably expressing the human DAT were seeded in 24-well plates and pretreated with 0.5 μ M of the active phorbol ester phorbol-12-myristate-13-acetate (PMA) or with 0.5 μ M of the inactive ester 4 α -phorbol-12,13-didecanoate (4 α PDD) in uptake buffer at 37° for 20 min. Then, in the absence or presence of 10 μ M $ZnSO_4$, DA uptake was determined by incubation with 10 μ M [3 H]DA for 2.5 min at 25 °C, and incorporation of tritium was determined as described under "Section 2.2". Nonspecific uptake was estimated in the presence of 10 μ M mazindol. Columns represent means of specific uptake \pm standard error of six to ten independent experiments, each in duplicates. * $p < 0.05$ vs. 4 α PDD by Student's *t*-test.

potential changes induced by altering the concentration of K^+ in the assay buffer would influence the modulation of DA uptake by Zn^{2+} . In fact, the Zn^{2+} effect on HEK293 cells could be switched from an inhibitory one at zero K^+ to a stimulatory one at a concentration of 20 or 50 mM K^+ , and in SK-N-MC cells the depolarization of the cellular membrane by high K^+ increased further the stimulatory effect of Zn^{2+} (Fig. 8).

Membrane depolarization by 50 mM K^+ also made disappear differences between the two DAT expressing cell lines in the sensitivity of [3 H]DA uptake to inhibition by mazindol: In SK-N-MC cells, mazindol blocks with lower potency (IC_{50} 0.235 μ M) than in HEK293 cells (IC_{50} 0.101 μ M; see Fig. 9A,B). In both cell lines, addition of 10 μ M Zn^{2+} increased this potency to about the same value (0.055/0.052 μ M). In HEK293 cells, simple depolarization by increasing [K^+] from 5 to 50 mM leads to a similar uptake inhibition by mazindol as in SK-N-MC cells (Fig. 9C).

Transporter substrates induce inward currents in patch-clamp measurements on DAT expressing HEK293 cells (Sitte et al., 1998; Piffl et al., 2004). Zn^{2+} increased this inward current by about 67 ± 21 pA in 7 cells clamped to -30 mV ($p < 0.02$ by paired Student's *t*-test, Fig. 10), but only insignificantly by 14 ± 11 pA if the same cells were clamped to -80 mV (difference between -30 mV and -80 mV condition: $p < 0.03$ by paired Student's *t*-test). We could not reliably measure substrate-induced currents in DAT expressing

SK-N-MC cells, presumably due to the lower expression of DAT per cell in these lines.

In order to examine the possibility that the modulation of the Zn^{2+} effect by the phorbol ester might be indirect *via* modulation of the membrane potential, we measured it under non-voltage-clamped conditions: pre-treatment of HEK293 cells expressing the DAT with 1 μ M PMA depolarized 24 cells to a mean value \pm SE of -50.1 ± 2.6 mV as compared to the -57.4 ± 1.8 mV of 20 cells pre-treated with 1 μ M of the inactive ester 4 α PDD (significance by *t*-test $p < 0.05$; pre-treatment time did not differ significantly between groups: 19.5 ± 2.7 min vs. 21.2 ± 3.9 min). This membrane depolarization was smaller than that induced by 50 mM K^+ : -47.7 ± 1.6 mV as compared to -58.9 ± 2.0 mV at 5 mM K^+ (11 cells; $p < 0.001$). Potential mechanisms of membrane depolarization by phorbol esters might be inhibition of potassium channels (Boland and Jackson, 1999) which were found in HEK293 and SK-N-MC cells (Yu and Kerchner, 1998; Lee et al., 1993) or a decrease of the activity of Na^+ , K^+ -ATPase by protein kinase C (McDonough and Farley, 1993).

Could these findings of a membrane potential dependent modulation of the Zn^{2+} effect on dopamine transport also have relevance *in vivo*? There is evidence for presynaptic glutamate receptors on dopaminergic nerve terminals in the striatum (Krebs et al., 1991; Mercuri et al., 1992; Paquet and Smith, 2003). A depolarizing effect upon activation of these receptors by glutamate might modulate the Zn^{2+} effect on DA uptake. In fact, in *in vitro* experiments on striatal synaptosomes 1 mM glutamate attenuated the inhibitory effect of Zn^{2+} on DA uptake (Fig. 11, left panels). This attenuation of the Zn^{2+} effect by glutamate was not due to an unspecific interaction of the amino acid with the zinc ions since in DAT-transfected HEK293 cells, which lack glutamate receptors and are therefore unlikely to be depolarized by glutamate, 10 μ M Zn^{2+} blocked DA uptake in presence of 1 mM glutamate to the same extent as in the absence of glutamate (Fig. 11, right panels).

4. Discussion

Our findings demonstrate that the polarization of the cell membrane and the cellular chloride distribution into which the DAT is embedded, profoundly affect the regulation of the DAT by Zn^{2+} . Whereas at the more negative membrane potential of the DAT expressing HEK293 cells Zn^{2+} inhibited uptake, attenuation of the membrane potential in DAT expressing HEK293 cells by high extracellular potassium resulted in a stimulatory effect of Zn^{2+} on DA uptake, and this stimulatory effect was even stronger at the still less polarized membrane of DAT expressing SK-N-MC cells. Lowering the extracellular chloride concentration increased the inhibitory effect of Zn^{2+} in HEK293 cells and shifted the stimulatory effect in SK-N-MC to an inhibitory one. Also the reduced inhibitory potency of Zn^{2+} after pre-treatment with the phorbol ester PMA might not be due to posttranslational modification of the DAT protein by phosphorylation, as initially hypothesized, but caused by a weakening effect of PMA on the membrane potential independent of the DAT. The opposite Zn^{2+} effect in HEK293 and SK-N-MC cells was obviously induced by interaction of Zn^{2+} with the well characterized histidins in the DAT (Norregaard et al., 1998), since both, the inhibitory effect in HEK293 and the stimulatory effect in SK-N-MC cells were abolished in cells expressing the NET which has no matching histidins as well as in cells expressing a mutant DAT with one of the critical histidins removed.

Since the translocation process of the DAT is electrogenic, DA uptake depends on the membrane potential. In DAT expressing oocytes held under voltage clamp, hyperpolarization increased the rate of DA accumulation (Sonders et al., 1997). That Zn^{2+} changes DA uptake by changing the membrane potential was recently suggested in another study on DAT expressing oocytes:

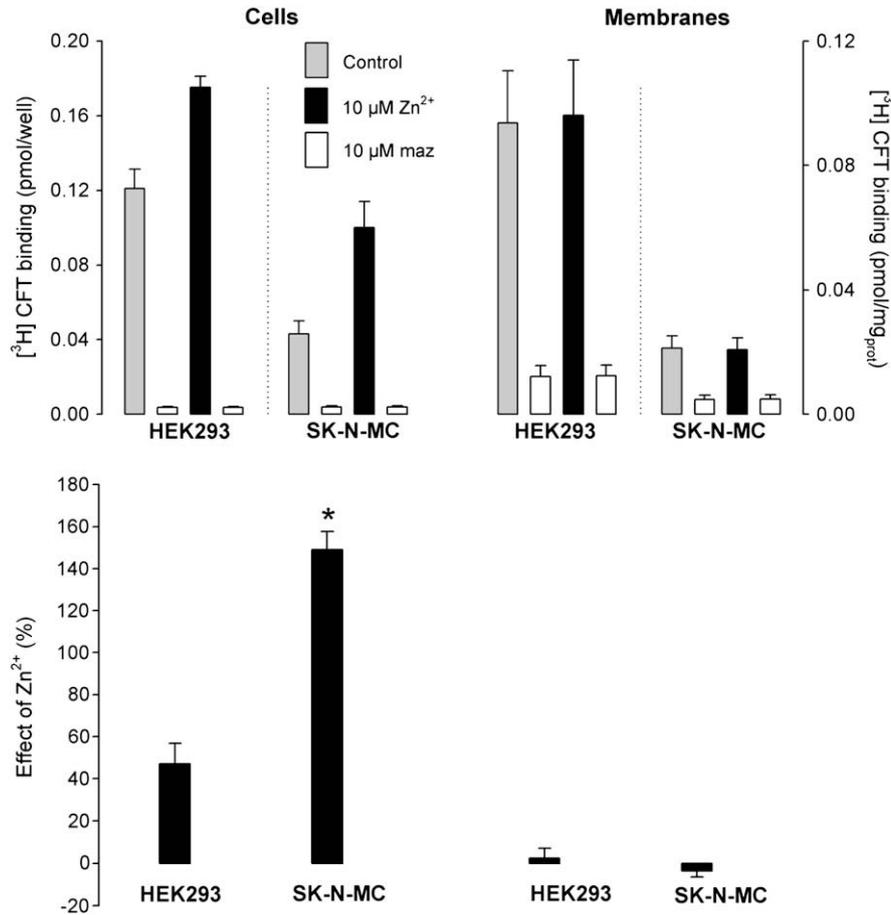


Fig. 5. Effect of Zn^{2+} on binding of $[^3\text{H}]\text{-2}\beta\text{-carbomethoxy-3}\beta\text{-(4-fluorophenyl)tropane}$ ($[^3\text{H}]\text{CFT}$) in different cellular hosts stably expressing the DAT. Cells stably expressing the DAT (left) and seeded in 24-well plates or membranes prepared from these cells (right) were incubated in uptake buffer containing 2 nM $[^3\text{H}]\text{CFT}$ for 2 h in the absence or presence of 10 $\mu\text{M ZnSO}_4$ at 4 $^\circ\text{C}$, and binding of tritium was determined as described under "Section 2.4". Columns represent means of binding \pm standard error of three independent experiments, each in duplicates. $*p < 0.05$ vs. control by Student's *t*-test.

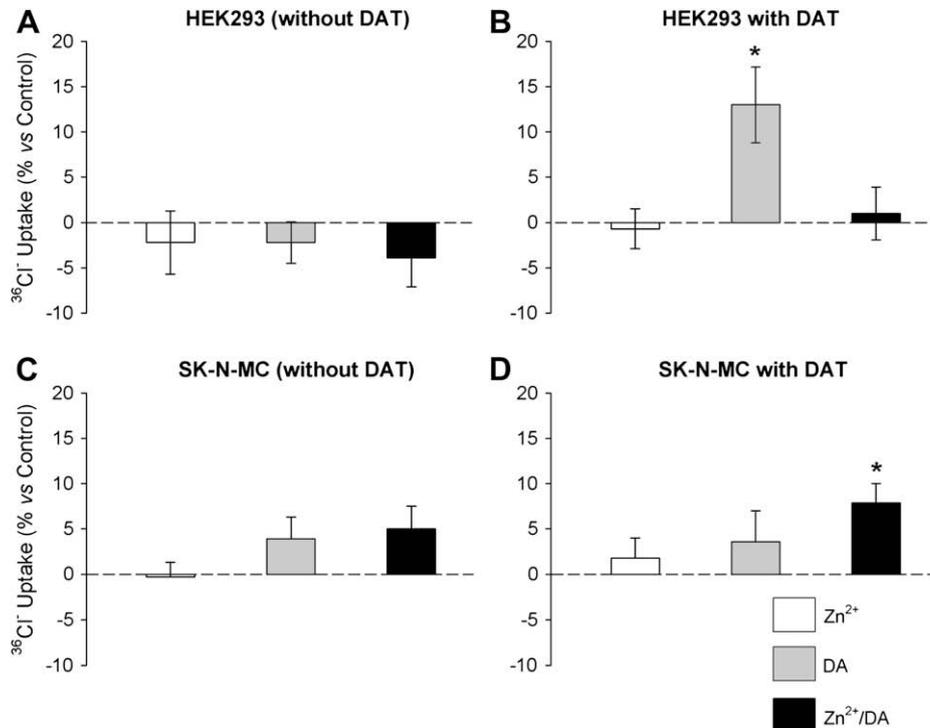


Fig. 6. Effect of Zn^{2+} and DA on the uptake of chloride. HEK293 cells (A,B) or SK-N-MC cells (C,B) in the absence (A,C) or the presence of the stably expressed human DAT (B,D) were seeded in 12-well plates and incubated in the absence or presence of 10 $\mu\text{M ZnSO}_4$ and/or DA in uptake buffer with 0.12 $\mu\text{Ci } ^{36}\text{chloride}$ for 5 min at 30 $^\circ\text{C}$, and incorporation of $^{36}\text{chloride}$ was determined as described under "Section 2.7". Columns represent means \pm standard error of six to seven independent experiments, each in triplicates. Control uptake was 12170 ± 970 , 8105 ± 525 , 4967 ± 666 , and 6492 ± 392 pmol/min/ 10^6 cells for A-D respectively. $*p < 0.05$ vs. control by paired Student's *t*-test.

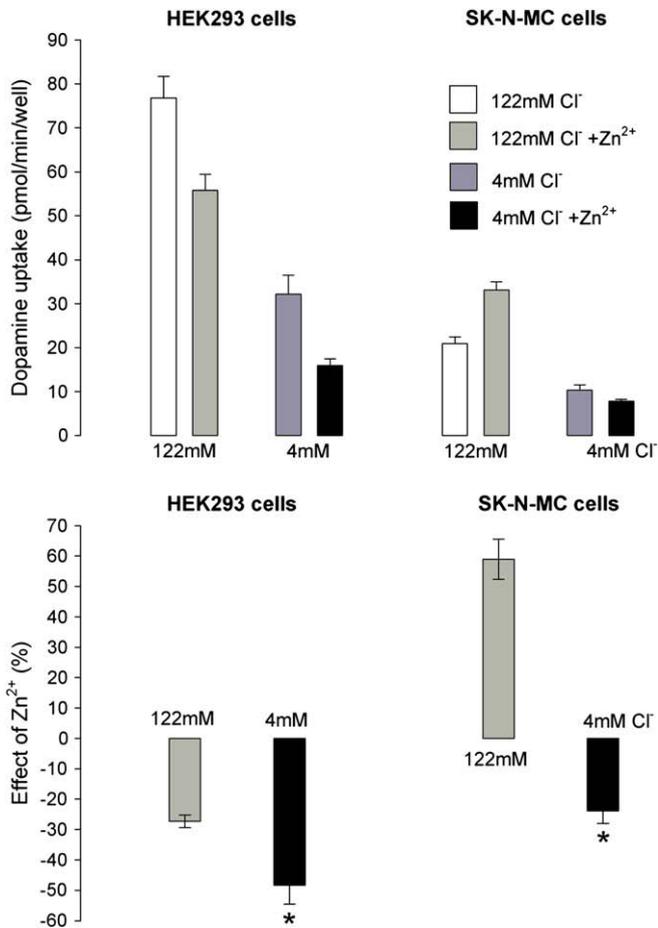


Fig. 7. Effect of low chloride on the regulation of DA uptake by Zn²⁺. HEK293 cells (left) or SK-N-MC cells (right) stably expressing the human DAT were seeded in 24-well plates and incubated in normal uptake buffer with 122 mM Cl⁻ or in uptake buffer with 4 mM Cl⁻, Cl⁻ iso-osmotically replaced by isethionate, with 10 μM [³H]DA for 2.5 min at 25 °C, and incorporation of tritium was determined as described under "Section 2.2". Nonspecific uptake was estimated in the presence of 10 μM mazindol. Columns represent means of specific uptake ± standard error of three to four independent experiments, each in duplicates.

Zn²⁺ inhibited uptake in non-voltage-clamped oocytes, but did not affect uptake under voltage-clamped conditions (Meinild et al., 2004). The authors explained both, inhibition by Zn²⁺ of wild type, but stimulation by Zn²⁺ of a mutated DAT (Y335A) with a tonic leak current constitutively depolarizing the membrane, by facilitation of a DA-induced chloride conductance (Loland et al., 2002; Meinild et al., 2004). However, for artificial mutants of the DAT other mechanisms such as a distorted conformation cannot be excluded as a cause for the peculiar uptake stimulation by Zn²⁺. By contrast, our findings of a stimulatory effect of Zn²⁺ on uptake by wild type DAT in cells with a constitutively low membrane potential (DAT/SK-N-MC cells) and presumably low intracellular chloride concentration or with a depolarized membrane (50 mM K⁺ treated DAT/HEK293 cells) demonstrate that membrane potential and chloride distribution are in fact the key determinants for Zn²⁺ modulation of the DAT wild type protein as well.

Our ³⁶chloride experiments support the contention that Zn²⁺-induced chloride flux modulates DA transport (Meinild et al., 2004). In addition to differences in membrane potential, differences in intracellular Cl⁻ concentration between HEK293 and neuronal SK-N-MC cells due to for example differences in Na⁺-K⁺-2Cl⁻ and neuronal specific K⁺-Cl⁻ co-transporters (Stein et al., 2004), might contribute to differential effects of a Cl⁻ conductance on Cl⁻ flux between the cellular hosts of the DAT. Increased ³⁶Cl⁻ uptake in the

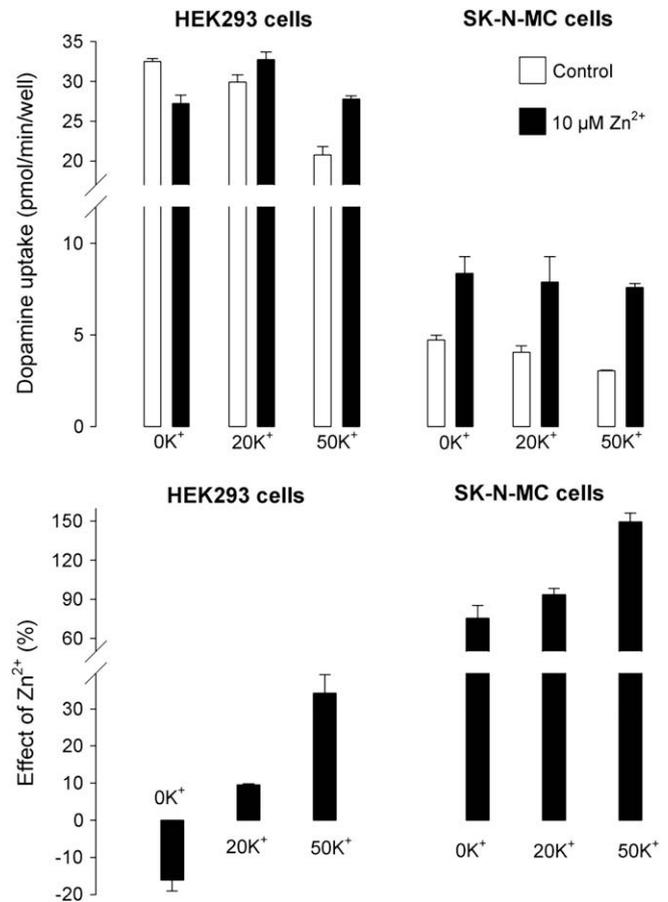


Fig. 8. Effect of membrane depolarization by high K⁺ on the regulation of DA uptake by Zn²⁺. HEK293 cells (left) or SK-N-MC cells (right) stably expressing the human DAT were seeded in 24-well plates and incubated in the absence of potassium ions (0K⁺) or in the presence of 20 mM (20K⁺) or 50 mM potassium chloride (50K⁺) with 10 μM [³H]DA in uptake buffer for 2.5 min at 25 °C, and incorporation of tritium was determined as described under "Section 2.2". Nonspecific uptake was estimated in the presence of 10 μM mazindol. Columns represent means of specific uptake ± standard error of three independent experiments, each in duplicates.

presence of DA in DAT expressing HEK293 cells might be due to stoichiometric co-transport of Cl⁻. This DA-induced influx was no longer detectable in the presence of Zn²⁺. At the applied concentration (10 μM) Zn²⁺ blocks only about 20% of DA transport, the major part of co-transported Cl⁻ should still flow into the cells. If we assume that Zn²⁺, in addition to its effect on DA transport, leads to the opening of a Cl⁻ conductance associated with the DAT, we can expect Cl⁻ efflux at the negative membrane potential and presumably higher intracellular Cl⁻ of HEK293 cells and consequently a lower Cl uptake in the presence of DA and Zn as compared to DA alone. A depolarizing effect of this Cl⁻ efflux could explain the lower DA uptake in the presence of Zn²⁺. In the less polarized SK-N-MC cells with likely lower intracellular Cl⁻, opening of the Cl⁻ conductance by Zn²⁺ in the presence of DA might lead to Cl⁻ influx and this influx was detected in our experiments with ³⁶Cl⁻ (Fig. 6D). Influx of Cl⁻ would hyperpolarize the cells and stimulate DA uptake in SK-N-MC cells. This Cl⁻ influx would be shifted to a Cl⁻ efflux by lowering Cl⁻ concentration in the uptake buffer and in fact, substitution of Cl⁻ by isethionate switched the stimulatory effect of Zn²⁺ on DA uptake in SK-N-MC cells to an inhibitory one. The fact that we could not detect significant effects of DA on (stoichiometric) Cl⁻ uptake in SK-N-MC cells could be due to the more than fourfold lower expression level of the DAT in these cells.

Our voltage-clamp experiments parallel our uptake experiments in as far as Zn²⁺ clearly stimulated the transporter current

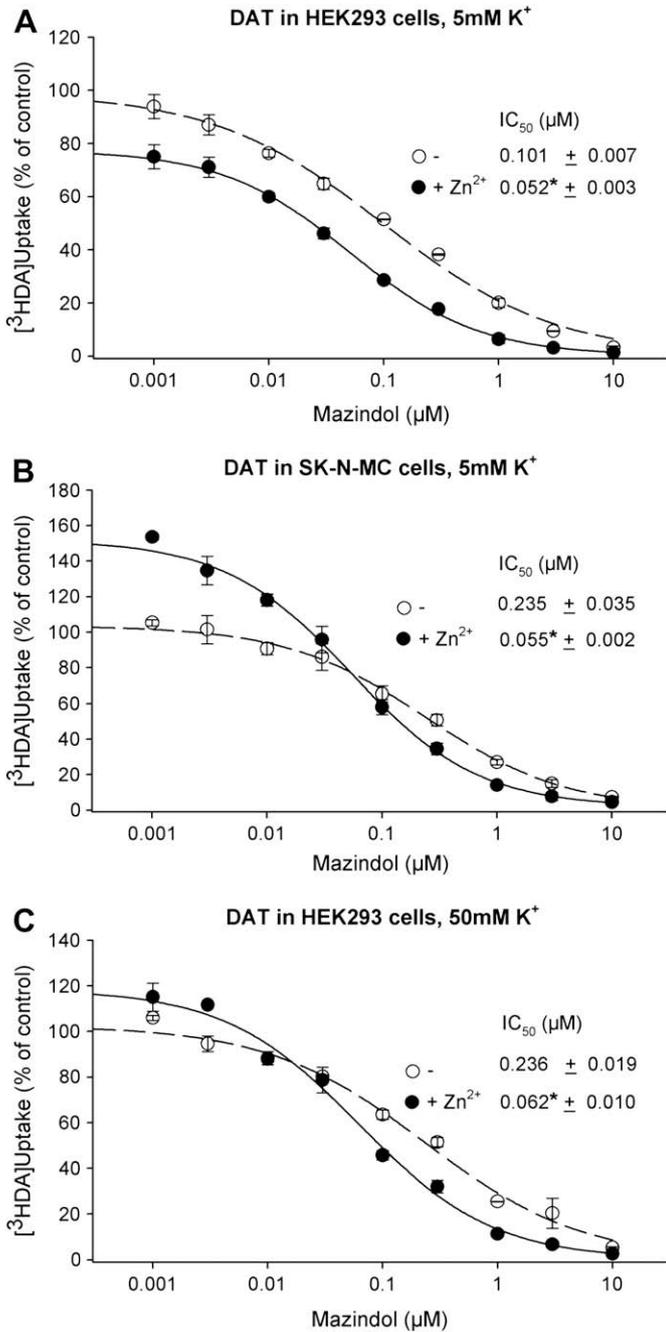


Fig. 9. Effect of Zn²⁺ on the inhibition of DA uptake by mazindol in different cellular hosts stably expressing the DAT. HEK293 cells (A,C) or SK-N-MC cells (B) stably expressing the human DAT were seeded in 24-well plates and incubated in an uptake buffer containing 5 mM (A,B) or 50 mM potassium chloride (C) and the concentrations of mazindol indicated, in the absence or presence of 10 μM ZnSO₄, with 10 μM [³H]DA in uptake buffer for 2.5 min at 25 °C, and incorporation of tritium was determined as described under “Section 2.2”. Symbols represent means of specific uptake normalized to the 0 Zn²⁺/0 mazindol condition ± standard error of three independent experiments, each in duplicates. The data of each experiment were fitted by nonlinear regression, and the means of the IC₅₀ values ± standard error are inserted into the panels. **p* < 0.01 vs. 0 Zn²⁺ by Student’s *t*-test.

induced by DA at –30 mV and increased uptake in cells with low membrane potential, whereas Zn²⁺ affected the current at –80 mV only insignificantly and inhibited uptake in cells with high membrane potential. That Zn²⁺ did not decrease the DA-induced inward current at –80 mV might be due to the additional inward current by Cl[–] efflux at –80 mV.

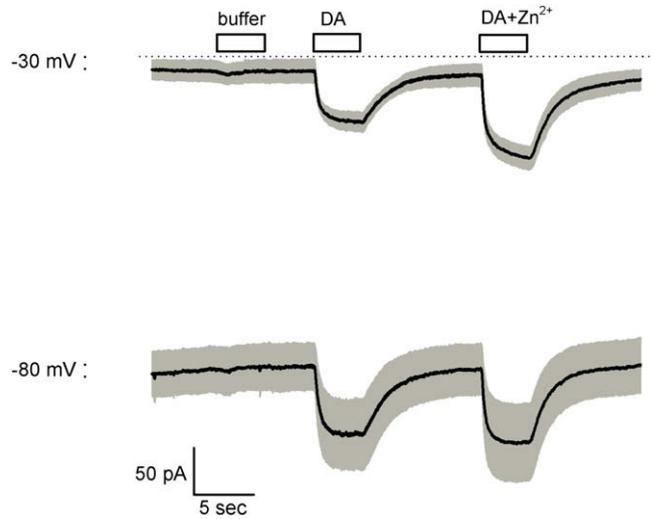


Fig. 10. Effect of Zn²⁺ on whole-cell patch-clamp recordings of HEK293 cells stably expressing the DAT. The cells were voltage-clamped first at a holding potential of –80 mV and superfused at the times marked by the bars for 4 s with 10 μM DA in the absence or presence of 10 μM ZnSO₄ and then on the same cells the protocol was run at –30 mV. Shown are mean values of current traces ± standard error (grey area) of 7 cells. The dotted line denotes zero current.

Differential effects of Zn²⁺ on carrier-mediated release in the two DAT expressing cell lines can be explained as follows. DA-induced release follows the concept of facilitated exchange-diffusion (Fischer and Cho, 1979): uptake of DA provides inward-facing

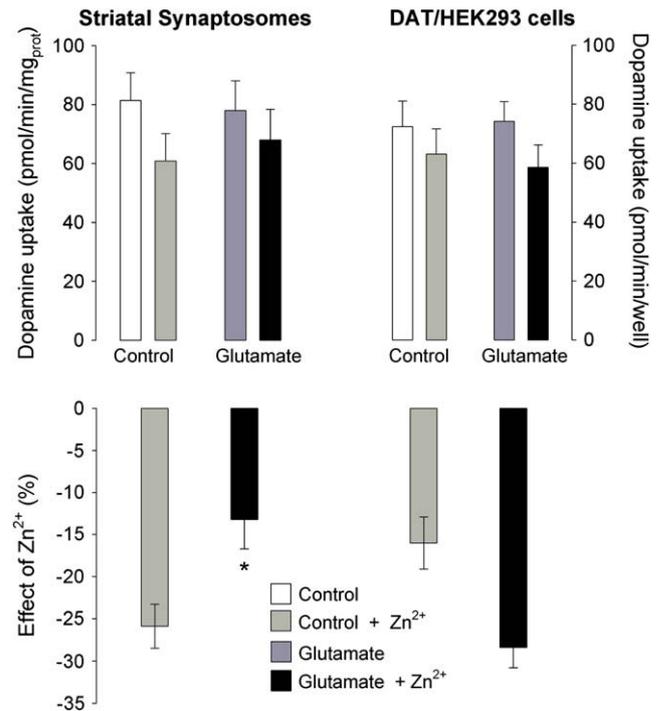


Fig. 11. Effect of glutamate on the regulation of DA uptake by Zn²⁺. Rat striatal synaptosomes (left panels) or HEK293 cells stably expressing the human DAT (right panels) were incubated in the absence or presence of 10 μM ZnSO₄ with 1 or 10 μM [³H]DA, respectively, for 2.5 min at 25 °C in uptake buffer (without MgSO₄), and incorporation of tritium was determined as described under “Section 2.2”. In experiments with glutamate, incubation was performed in the presence of 1 mM glutamate and 1 μM glycine which was reported to lower the free Zn²⁺ concentration by chelation to 4.1 μM (Spiridon et al., 1998). Nonspecific uptake was estimated in the presence of 10 μM mazindol. Columns represent means of specific uptake ± standard error of three independent experiments, each in duplicates. **p* < 0.05 vs. control by Student’s *t*-test.

transporter sites for reverse transport of preloaded substrate; consequently, the inhibition of uptake of DA by Zn^{2+} in HEK293 cells results in inhibition of DA-induced release whereas in SK-N-MC cells, we observe parallel stimulation of both processes. By contrast, amphetamine-induced release via the DAT has a different mechanism (Piffl and Singer, 1999): amphetamine induces release mainly by inducing an inward current of sodium which increases sodium at the inner face of the transporter and thereby reverses transport (Sitte et al., 1998; Khoshbouei et al., 2003). The additional release induced by concomitant action of Zn^{2+} fits with the additional inward current during amphetamine plus Zn^{2+} as measured in patch-clamp experiments (Piffl et al., 2004; Meinild et al., 2004). The even stronger stimulation of amphetamine-induced release by Zn^{2+} in SK-N-MC cells may be due to the suggested inward flux of chloride, which would reduce the chloride gradient. A reduced chloride gradient was shown previously to cause carrier-mediated release in DAT expressing cells (Piffl and Singer, 1999; Piffl et al., 1997).

Binding of uptake inhibitors to the DAT was found to be stimulated by low μ Molar concentrations of Zn^{2+} in several studies (Richfield, 1993; Bonnet et al., 1994; Norregaard et al., 1998). We observed the stimulatory effect of Zn^{2+} only in intact DAT expressing cells, not in membrane preparations of lysed cells. It seems that under our assay condition, the concentration gradient of ions of intact cells and/or their membrane potential is essential for the conformational change of the DAT by Zn^{2+} which leads to a more favourable configuration for binding of the cocaine analogue. Furthermore, we found that also in uptake inhibition experiments the interaction of mazindol with the DAT and its modulation by Zn^{2+} was regulated by the membrane potential. Mazindol had a two-fold lower potency as uptake-blocker in SK-N-MC cells than in HEK293 cells. The potency was switched to the same low level in HEK293 cells by high potassium depolarization. The observation that 10μ M Zn^{2+} increased the potency of mazindol under all 3 conditions to the same high level suggests, that, under the influence of Zn^{2+} , the DA uptake site exhibited similar conformation, independent of cell type or potassium concentration. Interestingly, also cells expressing a Y335A DAT mutant exhibited an attenuated membrane potential (Meinild et al., 2004), and also in these the potency of mazindol as a blocker of DA uptake was reduced, but restored, at least partially, by addition of Zn^{2+} (Loland et al., 2002).

In conclusion, we have shown that the influence of Zn^{2+} on the DAT critically depends on the membrane potential and chloride distribution. The DAT decisively regulates the sphere of influence and lifetime of released DA beyond the synapse (Cragg and Rice, 2004). Released DA enacts inhibitory feedback mechanisms by inhibiting DA synthesis and release and by stimulating DA reuptake, all these effects mediated by stimulation of presynaptic D_2 receptors (Elsworth and Roth, 1995; Cass and Gerhardt, 1994; Meiergerd et al., 1993). The voltage-dependence of the Zn^{2+} effect on DA reuptake would provide another negative feedback for dopaminergic neurotransmission: DA exocytotically released from depolarized nerve endings, depolarized either by propagation of action potentials down the axon or by excitatory neurotransmitters, will be taken up more efficiently by the DAT stimulated by Zn^{2+} under depolarizing conditions. On the other hand, if Zn^{2+} stimulated the DAT under the conditions of lowered membrane potential, such as in pathologically impaired DA neurons, it might aggravate neurodegeneration by increasing the uptake of potentially neurotoxic substrates (including DA). This mechanism may be a factor in Parkinson's disease where Zn^{2+} levels have indeed been found to be increased in those brain areas (substantia nigra, striatum; Dexter et al., 1991) bearing the brunt of the neurodegenerative process.

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