Zinc Potentiates an Uncoupled Anion Conductance Associated with the Dopamine Transporter*

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Binding of Zn$^{2+}$ to an endogenous binding site in the dopamine transporter (DAT) leads to inhibition of dopamine (DA) uptake and enhancement of carrier-mediated substrate efflux. To elucidate the molecular mechanism for this dual effect, we expressed the DAT and selected mutants in *Xenopus laevis* oocytes and applied the two-electrode voltage clamp technique together with substrate flux studies employing radiolabeled tracers. Under voltage clamp conditions we found that Zn$^{2+}$ (10 μM) enhanced the current induced by both DA and amphetamine. This was not accompanied by a change in the uptake rate but by a marked increase in the charge/DA flux coupling ratio as assessed from concomitant measurements of $[^3]$H]DA uptake and currents in voltage-clamped oocytes. These data suggest that Zn$^{2+}$ facilitates an uncoupled ion conductance mediated by DAT. Whereas this required substrate in the wild type (WT), we observed that Zn$^{2+}$ by itself activated such a conductance in a previously described mutant (Y335A). This signifies that the conductance is not strictly dependent on an active transport process. Ion substitution experiments in Y335A, as well as in WT, indicated that the uncoupled conductance activated by Zn$^{2+}$ was mainly carried by Cl$^-$ . Experiments in oocytes under non-voltage-clamped conditions revealed furthermore that Zn$^{2+}$ could enhance the depolarizing effect of substrates in oocytes expressing WT. The data suggest that by potentiating an uncoupled Cl$^-$ conductance, Zn$^{2+}$ is capable of modulating the membrane potential of cells expressing DAT and as a result cause simultaneous inhibition of uptake and enhancement of efflux.

The dopamine transporter (DAT)\(^1\) belongs to the family of Na$^+$/Cl$^-$-coupled transporters and plays a key role in termina-

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\(^1\) The abbreviations used are: DAT, human dopamine transporter; WT, wild type; DA, dopamine; AMPH, amphetamine; TM, transmembrane segment(s); MES, 4-morpholineethanesulfonic acid.
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nonetheless, the underlying molecular mechanism remained elusive. Pifl et al. (35) have recently corroborated our observations and moreover observed that Zn\(^{2+}\), unexpectedly enhanced the substrate-induced current in X. laevis oocytes. We provide evidence that, in the presence of substrates, Zn\(^{2+}\) potentiates an uncoupled Cl\(^{-}\) conductance mediated by DAT that enhances substrate-dependent membrane depolarization. We propose that potentiation of the uncoupled Cl\(^{-}\)conductance is critical for Zn\(^{2+}\)-induced inhibition of DA uptake and Zn\(^{2+}\)-induced enhancement of carrier-mediated substrate efflux. Altogether, the data support a new principle for modulation of a Na\(^{+}\)/Cl\(^{-}\)-coupled transporter; hence, Zn\(^{2+}\) is the first example of an allosteric modulator that can regulate transporter function by potentiating a specific uncoupled ion conductance.

MATERIALS AND METHODS

Molecular Biology and Expression in Xenopus laevis Oocytes—The cDNA of the human DAT (WT) and the mutants H193A (28), Y335A, and H193A/Y335A (36) were cloned into the oocyte expression vector pMB1, a vector optimized for oocyte expression (37). The resulting plasmids were linearized downstream of the poly(A) segment and in vitro transcribed with the T7 RNA polymerase using the mCAP mRNA capping kit (Stratagene, La Jolla, CA) (37). The resulting cRNA (50 ng/oocyte) was injected into defolliculated stage VI X. laevis oocytes. After incubation in Kuriol medium (90 mM NaCl, 1 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM HEPES, pH 7.4) for 6–9 days at 21°C, experiments were performed (38).

Electrophysiology—The two-microelectrode voltage clamp method was used for recording of whole cell currents in oocytes essentially as described (38). The CA-1B high performance oocyte clamp (Dagan Corporation) interfaced to an IBM-compatible PC via a DigiData 1200 A/D converter and pCLAMP 8.0/9.0 (Axon Instruments) were used to control the membrane potential and other parameters. Electrodes were pulled from borosilicate glass capillaries to a resistance of 0.5–2 M\(\Omega\) and were filled with 2 M KCl. For the experiments, the oocyte was impaled with the two electrodes, the membrane potential (\(V_{\text{m}}\)) was clamped to −40 mV, and the experimental chamber was continuously perfused with the NaCl buffer (see below) until a stable base-line current was obtained. For continuous holding current measurements, the membrane potential was held at −60 mV or as otherwise stated, and currents were low-pass-filtered at 1 Hz and sampled at 10 Hz. Steady-state current/voltage (I/V) relations were obtained by a pulse protocol of 100-ms voltage jumps from the holding potential (\(V_{\text{m}}\)), of −40 mV to a series of test potentials from −60 to −140 mV in steps of 20 mV. The currents were low-pass-filtered at 500 Hz and sampled at 10 kHz. The steady-state current was the average of 3 runs from 50 to 100 ms. The steady-state substrate-induced current was defined as the current measured in the presence of substrate minus the current measured in the corresponding control buffer (\(I_{\text{substrate}} - I_{\text{control}}\)). The NaCl buffer contained 100 mM NaCl, 2 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, pH 7.4. In experiments with low [Cl\(^{-}\] NaCl was exchanged by NaMES or sodium glucuronate, and the reference electrode was connected to the experimental chamber through an agar bridge (3% of agar in 3 M KCl). In the nitrate exchange experiments, 50 mM of the NaCl was exchanged with 50 mM of NaNO\(_3\). In Na\(^{-}\)-free buffer, NaCl was exchanged by 100 mM choline-Cl or by Na\(^{-}\)-free buffer, NaCl was exchanged by NaMES or sodium glucuronate, and the reference electrode was connected to the experimental chamber through an agar bridge (3% of agar in 3 M KCl). In the nitrate exchange experiments, 50 mM of the NaCl was exchanged with 50 mM of NaNO\(_3\). In Na\(^{-}\)-free buffer, NaCl was exchanged by 100 mM choline-Cl or by Na\(^{-}\)-free buffer, NaCl was exchanged by NaMES or sodium glucuronate, and the reference electrode was connected to the experimental chamber through an agar bridge (3% of agar in 3 M KCl).

\(^{3}H\)/DA Uptake Experiments—Uptake experiments were performed in 24-well plates using a concentration of 1.25 \(\mu\)M DA (\(^{3}H\)/DA, 16.8 \(\mu\)Ci/well; specific activity, 5.8 \(\mu\)Ci/mmol Amersham) added to a total of 400 \(\mu\)l NaCl buffer and various concentrations of Zn\(^{2+}\). The oocytes were incubated for 30 min at room temperature, washed three times in 1 ml of Choc buffer, and dissolved in 200 \(\mu\)l of 10% SDS. The content of DA in each oocyte was obtained by standard scintillation counting. For DA uptake under voltage-clamped conditions, the membrane potential was held at −60 mV (or what otherwise stated), and the holding current was continuously monitored. The oocytes were initially perfused in the NaCl (or NaMES) buffer, until a stable base line was established. DA (10 \(\mu\)M) and \(^{3}H\)/DA (67 \(\text{nM}\)) were added to the NaCl (or NaMES) buffer for 10 min. DA was removed from the perfusion solution (holding current returning to base-line level), and the oocytes were removed from the experimental chamber. Determination of DA content was carried out as described above. The net inward charge was obtained from the time integral of the DA-evoked net inward current and correlated with the \(^{3}H\)/DA uptake in the same cell.

Calculations—The data were analyzed by nonlinear regression analysis using Prism 4.0 (GraphPad Software, San Diego, CA) or Clampfit 8.0/9.0 (Axon Instruments Inc., Union City, CA). All of the numbers are given as means ± S.E., and \(n\) equals the number of oocytes tested unless otherwise stated. The unpaired \(t\) test was used for statistical calculations.

RESULTS

Zn\(^{2+}\) Enhances Substrate-induced Currents in the DAT—Fig. 1A shows a representative current trace from an oocyte expressing the wild type DAT (WT). Application of DA or AMPH (10 \(\mu\)M) induced inwardly directed currents in agreement with published results (6, 14). In further agreement with described data, cocaine (100 \(\mu\)M) induced an apparent outwardly directed current most likely as the consequence of inhibition of an inwardly directed tonic leak (14). Co-application of 10 \(\mu\)M Zn\(^{2+}\) with either DA or AMPH led to an enhancement of the inwardly directed currents. Application of Zn\(^{2+}\) alone did not, however, induce any current (Fig. 1A and B). Substrates, cocaine, or Zn\(^{2+}\) did not evoke any response in noninjected oocytes (data not shown).

The substrate-induced currents mediated by the WT were voltage-dependent both in the absence and in the presence of Zn\(^{2+}\) (Fig. 1B). At potentials more negative than −20 mV, the presence of Zn\(^{2+}\) significantly enhanced the inwardly directed current (Fig. 1B). For example, at −140 mV \(I_{\text{AMPH}}\) was 161 ± 7% of that observed in the absence of Zn\(^{2+}\) and, at −40 mV it was 144 ± 5% (means ± S.E., \(n = 9, p < 0.001\). The same effect of Zn\(^{2+}\) was observed with respect to DA-induced currents (data not shown). At potentials more positive than −20 mV, the negative slope of the I-V curve observed in the absence of Zn\(^{2+}\) represented the substrate-mediated inhibition of the tonic leak reported by Sonders et al. (14). Zn\(^{2+}\) shifted this part of the I-V curve upwards, consistent with stimulation by Zn\(^{2+}\) of an outwardly directed current. To visualize the Zn\(^{2+}\)-specific current, we subtracted \(I_{\text{AMPH}}\) from \(I_{\text{AMPH}}\)-Zn\(^{2+}\). This showed a voltage-dependent current that reversed at −10 mV (Fig. 1C).

To verify the specificity of the Zn\(^{2+}\) effect in DAT, we expressed a mutant in which one of the Zn\(^{2+}\) coordinating residues was mutated to lysine (H193K) to eliminate high affinity Zn\(^{2+}\) binding (28). As evident from Fig. 1D, AMPH (as well as DA; data not shown) induced currents in oocytes expressing this mutant that were comparable with those observed in oocytes expressing the WT; however, the ability of Zn\(^{2+}\) to enhance the substrate-induced current was abolished (Fig. 1D).

Rather, we observed a slight, but insignificant, reduction in current at the more hyperpolarizing potentials (\(V_{\text{hold}}\) < −80 mV). Overall, these results corroborate the recent finding by Pifl et al. (35), showing that Zn\(^{2+}\) under voltage-clamped conditions is capable of altering the electrogenic properties of the DAT by interacting with the previously described endogenous Zn\(^{2+}\)-binding site (28).

\(^{3}H\)/DA Uptake Experiments—To further explore the unexpected finding that Zn\(^{2+}\) enhanced the substrate-induced currents, we performed \(^{3}H\)/DA uptake experiments in voltage-clamped oocytes to correlate the net influx of charge to transported DA molecules in the absence and presence of Zn\(^{2+}\) (representative current traces are shown in Fig. 2A). Upon application of substrate, the number of charges translocated across the membrane was calculated from the time integral of the holding current. In parallel, we determined the number of transported radiolabeled DA molecules. The uptake rate of \(^{3}H\)/DA under voltage clamp was not affected by Zn\(^{2+}\) (Fig. 2B), and accordingly we observed a clear increase of the net charge-flux ratio upon co-application of Zn\(^{2+}\) (Fig. 2C). Thus, the net
We also tested the voltage dependence of the coupling and uptake. In agreement with Sonders et al. (14), we found a considerable increase in the coupling ratio at the more hyperpolarizing potentials (i.e., −80 and 0 mV) (Fig. 2D). This dependence of the coupling ratio on the membrane potential supports the existence of ion fluxes, stoichiometrically uncoupled from DA uptake (39). In the presence of Zn$^{2+}$, the amount of charges/DA was not increased by a fixed number of charges but rather elevated in a voltage-dependent manner with an approximate doubling of the charge/DA ratio at the given potentials (Fig. 2D). This suggests that Zn$^{2+}$ affects uncoupled ion fluxes through the DAT and not the magnitude of the stoichiometrically coupled current.

**Effects of Zn$^{2+}$ on the Membrane Potential**—Because we previously observed that Zn$^{2+}$ inhibits the net DA uptake in non-voltage-clamped mammalian cell lines, such as COS-7 cells or HEK-293 cells heterologously expressing the DAT (28, 34), we also measured $[^{3}H]$DA accumulation in non-voltage-clamped oocytes expressing the DAT. In full agreement with our observations in mammalian cell lines, Zn$^{2+}$ inhibited $[^{3}H]$DA uptake in the oocytes in a biphasic, concentration-dependent manner (Fig. 3A). The IC$_{50}$ value was 1.5 ± 0.7 μM for the high affinity phase and 1.1 mM ± 0.2 for the low affinity phase (n = 3) and thus essentially identical to that observed in mammalian cells (28, 34). Based on this and on the above findings, it is tempting to suggest that the functional effects of Zn$^{2+}$ at DAT are related to the ability of Zn$^{2+}$ to potentiate an inwardly directed current associated with DA uptake. Under voltage-clamped conditions this might result in an increase in charge/DA ratio with no change in uptake rate, whereas under non-voltage-clamped conditions it might result in enhanced depolarization of the cell membrane and consequently in attenuation of DA uptake. To test this hypothesis we assessed the effect of substrate on the membrane potential in the absence and presence of Zn$^{2+}$ under non-voltage-clamped conditions. The application of DA (1 μM) caused a marked depolarization of the oocyte membrane consistent with an active transport process (Fig. 3B). Importantly, co-application of Zn$^{2+}$ (10 μM) increased this depolarization as would be predicted from the proposed hypothesis (Fig. 3B). In four separate experiments the depolarization over 1 min in the presence of Zn$^{2+}$ and 1 μM DA was on average 30 ± 10% (p < 0.03) larger than in the absence of Zn$^{2+}$.

**Analysis of the Y335A Mutant**—Recently, we reported that mutation of Tyr$^{335}$ in the third intracellular loop to alanine (Y335A) switched the effect of Zn$^{2+}$ in transfected COS-7 cells from inhibition to activation of transport (36). This was likely the result of an altered equilibrium between distinct conformational states in the transport cycle (36, 40). We decided to test this mutant as a putative tool to further address the effect of Zn$^{2+}$ on DAT currents. Application under voltage clamp conditions of AMPH (Fig. 4A) or DA (not shown) alone to oocytes expressing Y335A failed to induce detectable currents. Application of Zn$^{2+}$ alone, however, induced a voltage-dependent current that reversed at approximately −20 mV (Fig. 4A) and was sensitive to 1 mM cocaine (data not shown). Co-application of AMPH or DA together with Zn$^{2+}$ did not further enhance the Zn$^{2+}$-induced current (Fig. 4A). The effect of Zn$^{2+}$ was concentration-dependent and resulted in a bell-shaped relationship with the most effective concentrations at 10–30 μM (Fig. 4B). The effect of Zn$^{2+}$ was also dependent on the holding potential; at a $V_{\text{hold}}$ of −20 mV we did not detect a net current, whereas at more hyperpolarizing potentials Zn$^{2+}$ activated a net current, which was largest at the extremes (Fig. 4B). Thus, whereas Zn$^{2+}$ enhances substrate-associated currents in the WT, Zn$^{2+}$ by itself induces a current in Y335A.
Ion Substitution Experiments in Y335A—Assessment of the 
Zn$^{2+}$/H$^{+}$-induced current with respect to ion selectivity was diffi-
cult in the WT because of the concomitant Na$^{+}$/H$^{+}$/Cl$^{-}$/H$^{+}$-dependent 
substrate-induced current. Therefore, we took advantage of the 
properties of DAT-Y335A, in which Zn$^{2+}$/H$^{+}$ by itself elicits a 
current and performed ion substitution experiments in oocytes 
expressing this mutant (Fig. 4C). Replacement of Na$^{+}$ with 
either choline or N-methyl-glucosamine (not shown) did not 
change the amplitude of the Zn$^{2+}$-induced current (Fig. 4C). In 
contrast, reduction of Cl$^{-}$ from 104 to 4 mM resulted in a 
substantial inhibition of the Zn$^{2+}$-induced current at depolar-
izing potentials ($V_{\text{hold}} > -20$ mV) (Fig. 4C). Furthermore, the 
reversal potential for the current did not change upon removal 
of Na$^{+}$ but shifted from −20 to +10 mV upon Cl$^{-}$ substitution 
with MES (Fig. 4C) or glucuronate (not shown). The shift 
toward more positive potential from a reversal potential of −20 
mV is consistent with Cl$^{-}$ carrying the ion flux induced by Zn$^{2+}$ 
in DAT-Y335A. Note that we verified a reversal potential of 
−20 mV for Cl$^{-}$ in our oocyte set-up by expressing cystic 
fibrosis transmembrane regulator (41) and analyzing the Cl$^{-}$
conductance mediated by this protein (data not shown).

We further characterized the Zn$^{2+}$-activated current in DAT-
Y335A by substituting extracellular Cl$^{-}$ with the more perme-
able anion, nitrate. Substituting Cl$^{-}$ (104 mM) with 50 mM 
nitrate/54 mM Cl$^{-}$ shifted the reversal potential for the Zn$^{2+}$-
induced current toward more negative potentials (from −20 to 
−50 mV) and caused a marked increase in the amplitude of the 
current (Fig. 4D). These observations are expected for a more 
permeable anion and thus further support that Zn$^{2+}$ promotes 
an anion conductance in DAT-Y335A.
Ion Substitution Experiments and the Effect of Zn^{2+} in the WT—A series of ion substitution experiments were also carried out in the WT (Fig. 5, A–C). At low Cl\(^{-}\) concentration (4 mM; substitution with MES) AMPH was still able to induce an inwardly directed current (Fig. 5B), although it was markedly smaller as compared with that seen in NaCl buffer (Fig. 5A). Zn\(^{2+}\) did not, however, change the net inward current under reduced Cl\(^{-}\) conditions, consistent with Zn\(^{2+}\) promoting a current mainly carried by Cl\(^{-}\) also in the WT (Fig. 5B). Upon substitution of Na\(^{+}\) with choline, AMPH was still able to block the tonic leak conductance (14) as reflected by the apparent outward current at hyperpolarizing potential and the apparent inward current at depolarizing potentials (Fig. 5C). The addition of Zn\(^{2+}\) led to a decrease in both the outward and inward currents (Fig. 5C). At −140 mV, the effect of Zn\(^{2+}\) was statistically significant with a current of 29 ± 4 nA in the absence of Zn\(^{2+}\) and 18 ± 3 nA in the presence of Zn\(^{2+}\) (means ± S.E., n = 7, p < 0.05). Subtraction of \(I_{AMPH}\) from \(I_{AMPH} + Zn^{2+}\) revealed a current with a reversal potential of −20 mV as would be
expected if the current was carried by Cl\(^{-}\) (Fig. 5D).

To further address the issue of ion selectivity we concomitantly measured \(^{3}H\)DA uptake and DA-induced currents (\(V_{\text{hold}} = -60 \text{ mV}\)) in extracellular solutions with minimal [Cl\(^{-}\)] (4 mM). The reduction in [Cl\(^{-}\)] caused a substantial decrease in uptake (0.19 ± 0.1 pmol DA/min/oocyte, \(n = 3\) versus 1.3 ± 0.2 pmol DA/min/oocyte, \(n = 6\) in standard buffer). As in the standard NaCl buffer, uptake of \(^{3}H\)DA was not significantly affected by the presence of Zn\(^{2+}\) under voltage-clamped conditions (DA, 0.19 ± 0.1 pmol DA/min, \(n = 3\); DA + 10 \(\mu M\) Zn\(^{2+}\), 0.10 ± 0.1 pmol DA/min, \(n = 4\), \(p > 0.5\)); however, Zn\(^{2+}\) modulated the coupling ratio even more than in the standard NaCl buffer as reflected by a charge/DA coupling ratio of -30 (Fig. 5E). This also argues for an enhancement of a Cl\(^{-}\) conductance by Zn\(^{2+}\), i.e. at low Cl\(^{-}\) concentrations the outward driving force for Cl\(^{-}\) is enhanced considerably at hyperpolarizing potentials, and thus an enhanced net inward current might be expected.

The Phenotype of Y335A—A remaining question is whether the Zn\(^{2+}\)-promoted anion conductance can explain the phenotype of Y335A, i.e. why is the effect of Zn\(^{2+}\) switched from inhibition to activation of transport? Similar to our observations in COS-7 cells (36), Zn\(^{2+}\) caused under non-voltage-clamped conditions a marked enhancement of basal \(^{3}H\)DA uptake activity (Fig. 6A). This was observed only in oocytes expressing Y335A and not in oocytes expressing the control mutant (DAT-H193K-Y335A) (data not shown). Under voltage-clamped conditions, however, Zn\(^{2+}\) did not affect \(^{3}H\)DA uptake in Y335A (Fig. 6B).

We further explored the electrogenic properties of Y335A by comparing the effect of Na\(^{+}\) substitution with choline in oocytes expressing this mutant with WT-expressing oocytes. As illustrated by the \(I_{\text{Na}} - I_{\text{Cho}}\) subtraction in Fig. 6C, we found a remarkable difference between Y335A and the WT. In oocytes expressing Y335A, we consistently observed a large constitutive inward current at all negative potentials that was not seen in oocytes expressing WT (Fig. 6C). The leak was at -60 mV, approximately five times greater in Y335A than in WT (compare \(53 ± 10\) nA in Y335A to \(-9 ± 2\) nA in WT). This suggests that Y335A possesses a tonic inward leak conductance that is Na\(^{+}\)-dependent. Interestingly, the leak could only be marginally inhibited by even high concentrations of cocaine (data not shown) and must therefore be distinct from the previously described tonic leak in the WT (14). The molecular basis for this apparent large tonic leak remains to be determined; however, it is tempting to speculate that it is strong enough to cause constitutive depolarization of the cell membrane and as a result reduce the driving force for DA uptake. If the cell is depolarized beyond the reversal potential for Cl\(^{-}\) (-20 mV), then application of Zn\(^{2+}\) and stimulation of the Cl\(^{-}\) flux would result in an inwardly directed Cl\(^{-}\) flux (and not an outward directed flux as seen at hyperpolarizing potential) that might in part restore the membrane potential and thus the driving force for DA uptake. Consistent with this idea, we did observe that oocyte expressing Y335A generally had more positive membrane potentials than oocytes expressing WT (Fig. 6D). As predicted we also observed in these oocytes under non-voltage-clamped condition that Zn\(^{2+}\) was capable of causing a hyperpolarization of the oocyte membrane (Fig. 6D). Note that the

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Effect of Zn$^{2+}$ on DAT-mediated Currents

The DAT contains an endogenous Zn$^{2+}$-binding site with a tridentate coordination sphere involving His$^{193}$ in the large second extracellular loop, His$^{375}$ at the external end of transmembrane segment (TM) 7, and Glu$^{396}$ at the external end of TM 8 (28, 29). The present data challenge the paradigm that Zn$^{2+}$-mediated inhibition of DA uptake at this site is caused by simple inhibition of conformational changes critical for the transport process (28, 29). First, in agreement with recent observations (35), we observed that Zn$^{2+}$ did not inhibit the substrate-induced current but instead enhanced it (Fig. 1). Second, we found that Zn$^{2+}$ is able to inhibit DA uptake only in non-voltage-clamped oocytes (Fig. 3A) and not in voltage-clamped oocytes (Fig. 2B). If Zn$^{2+}$-inhibited uptake by constraining critical conformational changes, inhibition would also be expected under voltage clamp conditions. Instead, we observed a substantial increase in the charge/DA coupling ratio under voltage-clamped conditions, suggesting that Zn$^{2+}$ facilitates an ion flux through the transporter (Fig. 2C). We hypothesized that by promoting depolarization of the membrane, this current could explain inhibition of DA uptake in the WT DAT. Similarly, it might explain the enhancement of carrier-mediated efflux by Zn$^{2+}$ under non-voltage clamp conditions. In agreement with this, we found that Zn$^{2+}$ enhanced the depolarizing effect on the membrane potential elicited by DA in oocytes expressing DAT (Fig. 3B). This will decrease the driving force for active transport as well as enhance carrier-mediated efflux, which is stimulated by depolarization (7, 8).

We have previously investigated the effect of Zn$^{2+}$ on transporter-mediated currents in the homologous γ-aminobutyric acid transporter-1 containing Zn$^{2+}$-binding sites engineered between the TM 7 and 8. In contrast to the present observation for the endogenous Zn$^{2+}$-binding site in DAT, we found that binding of Zn$^{2+}$ reduced both $[^{3}H]$ γ-aminobutyric acid uptake and the substrate-induced current in these γ-aminobutyric acid transporter-1 mutants (37). Similarly, it was recently shown that the glycine transporter-1 (GlyT1), like the DAT, contains an endogenous Zn$^{2+}$-binding site but that binding of Zn$^{2+}$ to this site resulted in both uptake inhibition and inhibition of the substrate-induced current (42). This suggests that the mechanism for Zn$^{2+}$-mediated inhibition of uptake in the γ-aminobutyric acid transporter-1 mutants as well as in GlyT1 differs from that responsible for Zn$^{2+}$ mediated inhibition in DAT. Interestingly, we have evidence that engineered DAT sites that solely involve coordinates in TM 7 and 8 also differ from the endogenous DAT site by displaying a monophasic Zn$^{2+}$ inhibition curve and no stimulatory effect on efflux (29, 34, 43). It is possible that Zn$^{2+}$ exerts its inhibitory effect at the engineered site between TM 7 and 8 by constraining conformational changes critical for the transport process. At the endogenous DAT site, however, we believe that the primary effect of Zn$^{2+}$ is potentiation of an uncoupled conductance. Apparently, this depends on coordination by Zn$^{2+}$ of His$^{193}$ in the second extracellular loop and thus suggests a role of this loop in regulating the distribution between different functional states of the transporter.

To explore the nature of the Zn$^{2+}$-promoted current in DAT, we performed a series of ion substitutions both in the WT and in a previously described DAT mutant (Y335A). We took advantage of the fact that in this mutant Zn$^{2+}$ by itself elicited a substantial current independent of the presence of substrates. In Y335A, substitution of Cl$^{-}$ with MES decreased substantially the Zn$^{2+}$-promoted current at depolarizing potentials (Fig. 4C). Additionally, the reversal potential of the current shifted from $-20$ mV to $+10$ mV. Because the reversal potential for Cl$^{-}$ was $-20$ mV in our oocyte setup (data not shown),

tonic Na$^{+}$-dependent leak is only slightly voltage-dependent in the range affected by Zn$^{2+}$, i.e. a decrease in the membrane potential from $-9$ to $-30$ mV as induced by Zn$^{2+}$ (Fig. 6D) will only cause a very minor increase in the tonic leak (Fig. 6C).

Thus, although there conceivably is Na$^{+}$ entry through the tonic leak, the hyperpolarization promoted by Zn$^{2+}$ is unlikely to cause an increase in the internal Na$^{+}$ concentration that could counteract the stimulatory effect of hyperpolarization on the transport rate.

![Graph](image-url)
this supports the possibility that Zn$^{2+}$ promotes an uncoupled conductance in Y335A that is mainly carried by Cl$^-$. In agreement, we observed also that the more permeable anion, nitrate, shifted the reversal potential toward more negative potential and increased substantially the magnitude of the Zn$^{2+}$-induced current (Fig. 4D). The ion substitution experiment furthermore supported that Zn$^{2+}$ mainly facilitates a Cl$^-$ conductance in the WT (Fig. 5, A–D). However, when $I_{\text{AMPH}}$ was subtracted from $I_{\text{AMPHe}-\text{Zn}^{2+}}$ in the WT in NaCl, we observed a voltage-dependent current that reversed close to $-10$ mV and not at $-20$ mV as would be expected for a pure Cl$^-$ conductance (Fig. 1C). In contrast, the corresponding current reversed at $-20$ mV when NaCl was substituted with ChoCl (Fig. 5D). It is possible that the more positive reversal potential is because additional ions contribute to the current when the transporter mediates active transport in NaCl. Any conclusion about contributing ions is nonetheless difficult given the obvious complexity of the measured current in the presence of substrate and Zn$^{2+}$ together.

According to the classical alternating access model the transporter operates in modes of selective accessibility: The transporter binds ions as co-substrates for transport (i.e. Na$^+$ and Cl$^-$) and substrates (i.e. DA or AMPH) in an outward facing conformation and releases them in an inward facing conformation (44). The identification of uncoupled ion conductances in the DAT, as well as in many other Na$^+$-coupled transporters, has suggested that although the transport process in itself might follow an alternating access scheme, the model cannot explain all of the functional states of the transporter. It has therefore been suggested that secondary active Na$^+$-coupled transporters structurally and functionally show resemblance to ion channels (27). Channel behavior or channel-like properties have been directly attributed to currents mediated by Na$^+$-coupled neurotransmitter transporter such as the NET (23), the Drosophila SERT (19, 20), and the sodium-glucose transporters (45, 46). A ligand-gated chloride channel activity has also been associated with the Na$^+$-glutamate transporters (e.g. EAAT1) (39). Moreover, it is noteworthy that a bacterial homolog of mammalian CIC Cl$^-$ channels was found to work as an ion exchanger, suggesting a functional and evolutionary similarity between channels and transporters (47).

These considerations are interesting in context of the present data showing that Zn$^{2+}$ can modulate an uncoupled anion conductance in the DAT. Particularly, it is intriguing that Zn$^{2+}$ by itself can activate an uncoupled anion conductance in the Y335A mutant; hence, it could be argued that the transporter has been converted into a “Zn$^{2+}$-gated anion channel.” From a mechanistic perspective the most straightforward explanation for our observations is that the transporter can exist in ion-conducting conformational intermediates. The effect of an allosteric modulator (e.g. Zn$^{2+}$) might then be to increase the probability that the transporter enters such states. In the WT, this is reflected by the increase in the magnitude of the uncoupled conductance during the transport process. Similarly, mutations, which are characterized by altered conformational equilibria, such as for example Y335A (40), could increase the chances that the transporter assume an uncoupled ion-conducting state. It is conceivable that application of Zn$^{2+}$ to such a mutant could further increase the likelihood that the transporter assumes an ion conducting state and accordingly be sufficient to activate an uncoupled current even when substrate is absent.

The present study may provide at least a partial explanation for the peculiar phenotype of the Y335A mutant. We observed not only that Zn$^{2+}$ by itself can promote a Cl$^-$ conductance but also that it possesses a large tonic and Na$^+$-dependent leak conductance (Fig. 6B). This conductance was not sensitive to high concentrations of cocaine and must therefore be distinct from the previously described cocaine-sensitive tonic cation leak (14). In light of the magnitude of this cocaine-insensitive leak, we speculated that overexpression of this mutant in either mammalian cells or oocytes could cause constitutive depolarization of the cell membrane. This will result in a reduced driving force for DA uptake. If the depolarizing effect of the tonic leak conductance caused the membrane potential to increase above the reversal potential for Cl$^-$, opening of a Cl$^-$ flux by Zn$^{2+}$ will lead to a hyperpolarization of the membrane, i.e. at potentials more positive than the reversal potential for Cl$^-$ (~20 mV in our oocytes) will enter the cell. Thus, Zn$^{2+}$ could under these circumstances increase the driving force for DA uptake and cause or contribute to the observed partial restoration of uptake under non-voltage-clamped conditions (Fig. 6A). The stimulatory effect of Zn$^{2+}$ on Y335A-mediated DA uptake under non-voltage-clamped conditions but not under voltage-clamped conditions further supports this notion. Moreover, we obtained more direct evidence by observing that Zn$^{2+}$ can elicit a hyperpolarization of the membrane potential in a non-voltage-clamped oocyte expressing Y335A DAT (Fig. 6D). We can, however, not exclude that other mechanisms contribute to the reversal of transport in the presence of Zn$^{2+}$ such as, for example, simple restoration of a distorted conformational equilibrium. It should also be kept in mind that even under voltage clamp conditions the $[^{3}H]$DA uptake mediated by Y335A was still markedly lower than that observed in the WT (Y335A, $-0.05$ pmol/min/oocyte; WT, $-1.3$ pmol DA/min/oocyte; see “Results” and the legend to Fig. 6). Nevertheless, it must still be emphasized that the electrogenic properties of Y335A strongly underline the importance of being very cautious when interpreting data obtained with DAT mutants. For example, dominant-negative effects of selected mutants, like Y335A, on co-expressed WT (48) may be the simple result of unusual electrogenic properties of the mutant, such as e.g. the tonic depolarizing leak conductance, rather than showing a direct effect that relies on interactions between subunits in an oligomeric complex.

Recently, Ingram et al. (18) described a DAT-mediated uncoupled anion current in midbrain DA neurons stimulated by DA and AMPH. The current promoted an excitatory response, and thus it was inferred that DAT associated currents might modulate their excitability in vivo. It is interesting to reconcile this with the observations here that Zn$^{2+}$ activates a similar anion conductance in DAT. The ability of Zn$^{2+}$ to activate this conductance also in a natural environment of the transporter was supported by our previous observation that Zn$^{2+}$ enhances AMPH-induced efflux of substrate in striatal brain slices (34). Additionally, during neuronal stimulation the synaptic Zn$^{2+}$ concentrations fully sufficient to saturate the endogenous Zn$^{2+}$-binding site of the DAT (30–32). This suggests the possibility that in vivo Zn$^{2+}$ might modulate, in conjunction with substrates, the excitability of midbrain DA neurons and accordingly excitocytotic neurotransmitter release. In this context, it is also important to note that Zn$^{2+}$ levels are significantly elevated in distinct subcortical regions in patients with Parkinson’s disease, i.e. substantia nigra, caudate nucleus, and lateral putamen (49). Furthermore, there is a large body of evidence that Zn$^{2+}$ may also exert detrimental effects in these areas of the brain that have DA neurons. For example, Zn$^{2+}$ induces apoptosis of DA neurons and may be important in the pathophysiology of Parkinson’s disease (50). Nevertheless, to assess an actual physiological and/or pathophysiological role of Zn$^{2+}$, additional studies must be per-
formed in the future such as, for example, generation of a knock-in mouse expressing a DAT mutant insensitive to modulation by Zn2+.

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