A Lithium-induced Conformational Change in Serotonin Transporter Alters Cocaine Binding, Ion Conductance, and Reactivity of Cys-109*

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Inactivation of serotonin transporter (SERT) expressed in HeLa cells by [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) occurred much more readily when Na\(^+\) in the reaction medium was replaced with Li\(^+\). This did not result from a protective effect of Na\(^+\) but rather from a Li\(^+\)-specific increase in the reactivity of Cys-109 in the first external loop of the transporter. Li\(^+\) alone of the alkali cations caused this increase in reactivity. Replacing Na\(^+\) with N-methyl-D-glucamine (NMDG\(^+\)) did not reduce the affinity of cocaine for SERT, as measured by displacement of a high-affinity cocaine analog, but replacement of Na\(^+\) with Li\(^+\) led to a 2-fold increase in the \(K_D\) for cocaine. The addition of either cocaine or serotonin (5-HT) protected SERT against MTSET inactivation. When SERT was expressed in Xenopus oocytes, inward currents were elicited by superfusing the cell with 5-HT (in the presence of Na\(^+\)) or by replacing Na\(^+\) with Li\(^+\) but not NMDG\(^+\). MTSET treatment of oocytes in Li\(^+\) but not in Na\(^+\) decreased both 5-HT and Li\(^+\)-induced currents, although 5-HT-induced currents were inhibited to a greater extent. Na\(^+\) antagonized the effects of Li\(^+\) on both inactivation and current. These results are consistent with Li\(^+\) inducing a conformational change that exposes Cys-109, decreases cocaine affinity, and increases the uncoupled inward current.

Serotonin transporter (SERT)\(^3\) regulates the extracellular serotonin (5-HT) concentration by transporting this neurotransmitter into neurons at sites adjacent to areas of transmitter release (1). SERT is similar in sequence and function to transporters for the other biogenic amines dopamine (DAT) and norepinephrine (2). These three biogenic amine transporters are part of a larger family of amine and amino acid transporters that couple substrate transport to the cotransport of ions (3). For SERT, as for most family members, Na\(^+\) is co-transported with 5-HT. Although 5-HT binding was found not to be dependent on Na\(^+\), binding affinities of the tricyclic antidepressant imipramine and a cocaine analog were both weaker when Na\(^+\) was replaced with Li\(^+\) (4). The reactivity of a cysteine at position 179 (in SERT mutant I179C) toward [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) was also increased by the presence of Na\(^+\) (5). This residue may be part of an external gate that must be open for 5-HT to bind from the cell exterior and that closes to prevent dissociation to the outside when bound 5-HT is released to the cytoplasm.

SERT contains eighteen cysteine residues, three of which are predicted to be extracellular. Of these, two (Cys-200 and Cys-209) were proposed to be in a disulfide bond, whereas the third, Cys-109, reacted with externally added (2-aminoethyl)methanethiosulfonate (MTSEA) and MTSET (6). Inactivation at Cys-109 was extremely slow in normal Na\(^+\)-containing medium but was much faster when Li\(^+\) replaced Na\(^+\) (6). The corresponding residue in DAT, Cys-90, also reacts with MTSEA (7); however, the consequence of modifying this residue in SERT and DAT were quite different. In SERT, MTSEA modification of Cys-90 inactivated transport (6), but in DAT, MTSEA modification of Cys-90 did not inactivate but rather stimulated binding of a cocaine analog. Moreover, cocaine actually potentiated the effects of MTSEA and MTSET, as if ligand binding increased the accessibility or reactivity of Cys-90 (7).

In addition to Na\(^+\)-coupled substrate transport, SERT, norepinephrine transporter, and DAT were all found to catalyze uncoupled ion fluxes in the presence and the absence of substrate (8–10). In the case of SERT, these currents were induced by both 5-HT in the presence of Na\(^+\) or by replacing Na\(^+\) with Li\(^+\) (8, 11). The effect of Li\(^+\) seemed to result from an increase in the frequency with which the transporter opens as an ion channel and not from changes in single channel conductance or open time (11). This observation suggests that rather than permeating faster than Na\(^+\), Li\(^+\) changed the properties of SERT to increase the probability of channel opening. The Li\(^+\) current was also studied in SERT in Drosophila melanogaster (dSERT) and found to be antagonized by Na\(^+\) relative to an inert cation such as N-methyl-D-glucamine (NMDG\(^+\)) (12). This study also demonstrated that 5-HT at high concentrations could inhibit the current in a manner consistent with its permeation through the SERT ion channel. Given the apparently direct effects of Li\(^+\), it is reasonable to question whether the effects of replacing Na\(^+\) with Li\(^+\) (4, 13, 14) were due to decreased Na\(^+\) or increased Li\(^+\).

We considered the possibility that Li\(^+\) caused a conforma-
tional change in SERT that affected multiple functions of the transporter. In this study we examined the effect of Li⁺ on exposure of Cys-109, cocaine binding, and SERT-mediated currents. The results are consistent with a conformational change that affects all of these properties.

**EXPERIMENTAL PROCEDURES**

**Expression of rSERT in HeLa Cells**—Rat SERT was expressed in HeLa cells as described previously (15). In this method, HeLa cells were infected with recombinant vTF-7 vaccinia virus and transfected with plasmid DNA encoding rSERT under control of the T7 promoter. The cells were assayed 16–24 h post-transfection.

**Transport and Binding Measurements**—Transport was assayed in intact cells in 48-well culture plates. 5-HT uptake was initiated by the addition of 23 mM [³²⁵]H-5-HT (21.8 Ci/mmol, PerkinElmer Life Sciences) in 200 μl of phosphate-buffered saline (PBS) containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS/CM). The reactions were terminated after 10 min by aspiration of the substrate followed by three rapid washes with ice-cold PBS/CM. The cells were lysed with 200 μl of 1% SDS and transferred to scintillation vials for counting.

Binding of the high affinity cocaine analog, 2α-carboxymethoxy-3β-(4'-[¹²⁵]iodophenyl)tropane (β-CIT) was measured as described previously (16, 17). Kᵦ values for cocaine were determined by displacement of [¹²⁵]β-CIT.

**Treatment with MTSET, Reactivation, and Protection**—Transfected cells in 48-well plates were washed once with PBS/CM and once with incubation buffer, and then 200 μl of incubation buffer was added as specified in text. Freshly made MTSET or MTSEA stock solutions in fresh medium also contained the indicated concentrations of Na⁺, Li⁺, and NMDG, respectively. Cells as above were washed into PBS/CM in which Na⁺ was replaced to the indicated extent with Li⁺ (squares) or with various mixtures of NMDG and Li⁺ replacing all Na⁺ (circles). After a 10-min incubation with 0.1 mM MTSET in the indicated medium, the cells were washed with PBS/CM and assayed for 5-HT influx.

**Results**

As previously reported (6), SERT is sensitive to inactivation by methanethiosulfonate reagents in lithium medium but not in normal sodium medium. Fig. 1A demonstrates that this inactivation results from the absence of lithium rather than the absence of sodium. The cells expressing SERT retain the ability to concentrate 5-HT after treatment with MTSET in PBS or in medium in which Na⁺ was replaced by NMDG⁺. However, when Na⁺ was replaced with Li⁺, MTSET inactivated SERT. In each of these experiments, Na⁺ was replaced with Li⁺ or NMDG⁺ only during the preincubation with MT-
SET. In the absence of MTSET, precubation in Li<sup>+</sup> or NMDG<sup>+</sup> medium had no effect on activity. As described previously (6), inactivation of SERT by external methanethiosulfonate reagents did not occur, even in Li<sup>+</sup>, in a mutant containing alanine in place of cysteine at position 109. Similar results were obtained when MTSEA or (2-sulfonatoethyl)methanethiosulfonate was used instead of MTSET (data not shown). MTSET reaction with a cysteine sulfhydryl yields a mixed disulfide as its product (19). As previously demonstrated (5), the inactivation of SERT was readily reversed by reducing MTSET-treated cells with thiols. Under conditions where 83 ± 10% of the transport activity in the cells was inactivated by MTSET, 5 min of treatment with 10 mM free cysteine restored ~85 ± 10% of the original activity. In contrast to inactivation, however, reactivation with cysteine was equally effective in Na<sup>+</sup> and Li<sup>+</sup> media (data not shown).

To further investigate the nature of the inactivation, we examined the effect of Cys-109 modification by MTSET on binding of the cocaine analog β-CIT. Under conditions where ~65% of the transport activity was inactivated, less than 25% of β-CIT binding activity was affected by MTSET treatment (Table I). Furthermore, both cocaine and 5-HT were able to displace β-CIT after MTSET modification. These results suggest that modification of Cys-109 does not affect substrate binding but rather a subsequent step in the catalytic cycle of SERT. To investigate the selectivity of the Li<sup>+</sup> effect, we tested the ability of other alkali cations to increase the reactivity of Cys-109. Table II shows that, relative to Na<sup>+</sup>, MTSET induced inactivation was enhanced only by Li<sup>+</sup> and not by K<sup>+</sup>, Rb<sup>+</sup>, or Cs<sup>+</sup>.

The ability of Li<sup>+</sup> to stimulate Cys-109 modification depended on the presence of Na<sup>+</sup> in the medium. In the absence of Na<sup>+</sup> (replaced by NMDG) the amount of inactivation increased almost linearly with [Li<sup>+</sup>] up to 150 mM, the highest concentration tested. However, in the presence of Na<sup>+</sup>, Li<sup>+</sup> replacement increased the rate of inactivation less at low [Li<sup>+</sup>] and more at higher concentrations (Fig. 1B). This behavior might be expected if multiple Li<sup>+</sup> ions interacted with SERT in a cooperative manner to expose Cys-109 to reaction with MTSET. However, the same cooperativity was not observed when Li<sup>+</sup> replaced NMDG. Other possibilities are that Li<sup>+</sup> and Na<sup>+</sup> competed for binding at the same site or that Li<sup>+</sup> and Na<sup>+</sup> binding at separate sites induced mutually exclusive conformational changes.

**Table I**

**Effect of Cys-109 modification on transport and binding**

| Ion | Remaining activity (percentage of rate in PBS/CM) |
|-----|-----------------------------------------------|
| Na<sup>+</sup> | 100 ± 10 |
| Li<sup>+</sup> | 56 ± 5 |
| K<sup>+</sup> | 111 ± 9 |
| Rb<sup>+</sup> | 99 ± 12 |
| Cs<sup>+</sup> | 96 ± 16 |

Although Cys-109 was predicted to lie in an extracellular loop (18, 20), the variable reactivity of this residue to modification with MTSET suggested that its accessibility might be influenced by conformational changes, such as those induced by substrate or inhibitor binding. Fig. 2A shows that the amount of inactivation by MTSET was decreased in the presence of either cocaine or 5-HT. In the experiment shown in Fig. 2A, SERT-expressing cells were treated with 0.1 mM MTSET for 10 min in PBS/CM with Li<sup>+</sup> replacing Na<sup>+</sup> in the presence of the indicated concentrations of either 5-HT or cocaine. The cells were then washed extensively to remove unreacted MTSET and then assayed for transport activity in PBS. As shown in Fig. 2A, MTSET in the absence of 5-HT or cocaine inactivated transport to ~40% of the original level, but the presence of a sufficient concentration of either ligand completely blocked SERT inactivation. The concentration required for half-maximal protection was 0.76 ± 0.12 µM for 5-HT and 0.89 ± 0.21 µM for cocaine. It is unlikely that this protection represents direct occlusion of Cys-109, because cocaine and 5-HT bound to the transporter after modification (Table I).

We previously demonstrated that binding of β-CIT to platelet plasma membrane vesicles containing SERT was weaker when Li<sup>+</sup> replaced Na<sup>+</sup> (4). Fig. 2B shows that the same is true for cocaine itself and that the decrease in affinity results not from a lack of Na<sup>+</sup> but from the presence of Li<sup>+</sup>. In this experiment, the membranes from cells expressing SERT were incubated with a low, subsaturating amount of labeled β-CIT in the presence of the indicated concentrations of cocaine. When Na<sup>+</sup> was substituted with NMDG<sup>+</sup>, cocaine binding was actually slightly enhanced, as demonstrated by its ability to displace β-CIT from the membranes. Replacement of Na<sup>+</sup> with Li<sup>+</sup>, however, increased by almost 2-fold the concentration of cocaine required to displace half of the bound β-CIT from 1.10 ± 0.19 µM (0.70 ± 0.08 µM in NMDG<sup>+</sup>) to 1.92 ± 0.10 µM in Li<sup>+</sup>. Although the shifts in K<sub>B</sub> for cocaine are not large, they were very reproducible. The same order was observed in three separate experiments, and the experiment shown was closest to the average values for the three conditions. An unexpected difference was observed between β-CIT and cocaine in that cocaine affinity was highest in NMDG<sup>+</sup> medium (Fig. 2), but β-CIT affinity was highest in Na<sup>+</sup> (Fig. 2 legend). Thus, both the increased susceptibility of Cys-109 and the decrease in cocaine affinity were due to the presence of Li<sup>+</sup> and not the absence of Na<sup>+</sup>.

Lithium is known to increase uncoupled ionic currents mediated by SERT (8, 11, 12). Petersen and DeFelice (12) showed that dSERT conducted inward currents in Li<sup>+</sup> Ringer and that these currents were inhibited in mixtures of Li<sup>+</sup> and Na<sup>+</sup>. Because this behavior was mirrored by the inactivation of SERT by MTSET (Fig. 1B), we verified that the currents conducted by rSERT, like dSERT, were affected similarly by Na<sup>+</sup> and Li<sup>+</sup>. Fig. 3 shows currents elicited by 5-HT and Li<sup>+</sup> in Xenopus oocytes injected with cRNA encoding rat SERT. The
In the absence of cocaine was 0.374 ± 0.037, 0.163 ± 0.009, and 0.268 ± 0.029 pmol/mg for Na\(^+\), Li\(^+\), and NMDG media, respectively. The relatively higher amount of control binding in Na\(^+\) was a consequence of higher Kd effect on cocaine binding and protection by ligands (data not shown). In contrast to inactivation, this reactivation, which also restored transport activity (5), was the same whether the reaction mixture contained Na\(^+\) or Li\(^+\) (data not shown).

**DISCUSSION**

The results presented above suggest that Li\(^+\) has a direct effect on SERT structure that increases reactivity of Cys-109, decreases cocaine affinity, and increases an inward current carried by the transporter. It had not previously been appreciated that these disparate effects (6, 8, 21) were all due to Li\(^+\) rather than the absence of Na\(^+\). We also show here that the effect of Li\(^+\) on both SERT current and reactivity of Cys-109 is antagonized in the same way by Na\(^+\), lending support to the possibility that a single Li\(^+\)-induced conformational change is responsible for each of these effects.

Evidence from external loop chimeras suggests that these loops are important for the conformational changes that follow binding (22). It is conceivable that the structural basis for our observations lies in the conformational changes in the first external loop, which contains Cys-109, that accompany ligand and ion binding to SERT. Changes in the conformation of external loops under the influence of Li\(^+\) may favor an alternate conformation with greater accessibility of Cys-109 and lower affinity for cocaine and β-CIT. In contrast, the conformational changes induced by Na\(^+\) (5), which are essential for completion of the reaction cycle, may favor a more compact conformation of this loop in which Cys-109 is not exposed.

Modification of Cys-109 with MTSET places a highly charged and bulky substituent on the loop, possibly restricting it from to inactivation by MTSET than were Li\(^+\) elicited currents. The expected product of MTSET reaction with Cys-109 is a disulfide (19), and consequently treatment with 10 mM free cysteine reduced the disulfide and regenerated native SERT with normal response to 5-HT and Li\(^+\) (data not shown). In contrast to inactivation, this reactivation, which also restored transport activity (5), was the same whether the reaction mixture contained Na\(^+\) or Li\(^+\) (data not shown).

The SERT currents elicited by 5-HT and Li\(^+\) were sensitive to inhibition by MTSET (Fig. 4). As with inhibition of transport, the inactivation was not observed in Na\(^+\) Ringer (Fig. 4B) but only when Na\(^+\) was replaced by Li\(^+\) (Fig. 4C). The presence of 5-HT during the incubation with MTSET in Li\(^+\) Ringer protected against inactivation (data not shown) just as it did for inactivation of transport (Fig. 2A). The extent to which MTSET decreased the 5-HT elicited current varied. Inhibition always occurred, and in some cases the current was completely inactivated. By comparison, inactivation of the Li\(^+\) elicited current was never complete and was always less than inactivation of the 5-HT elicited current in the same experiment. The combined results from eight oocytes are summarized in Fig. 4D. These results demonstrate that inactivation of the currents occurred only when MTSET was incubated with the oocytes in Li\(^+\) Ringer and that 5-HT elicited currents were more sensitive to inactivation by MTSET than were Li\(^+\) elicited currents. The expected product of MTSET reaction with Cys-109 is a disulfide (19), and consequently treatment with 10 mM free cysteine reduced the disulfide and regenerated native SERT with normal response to 5-HT and Li\(^+\) (data not shown). In contrast to inactivation, this reactivation, which also restored transport activity (5), was the same whether the reaction mixture contained Na\(^+\) or Li\(^+\) (data not shown).

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adopting the conformation in which the cysteine sulfhydryl is sequestered from solution. Such a restriction would prevent the conformational transitions required for the transport cycle.

The accessibility of Cys-109 in unmodified SERT depended on the presence of Li⁺ (Fig. 1), but after modification with MTSET, Li⁺ did not change the reactivity of this residue. We previously showed that reactivation of MTSET-inactivated SERT cysteine mutants could be used as a measure of the conformation around the modified residue (5). For example, the reactivation of MTSET-inactivated SERT I179C was increased by Na⁺ and 5-HT. The observation that SERT modified with MTSET at Cys-109 was reactivated by free cysteine equally well in Na⁺ and Li⁺ medium suggests that this residue, once modified, does not change its accessibility in response to Li⁺ like unmodified SERT. This change in the response to Li⁺ suggests that modification with MTSET restricted the conformational mobility of the first external loop, consistent with inhibition of transport.

The altered loop conformation in Li⁺ containing solutions may be responsible also for the increased likelihood of SERT conducting as an ion channel. However, it is unlikely that the conformation that exposes Cys-109 and binds cocaine with lower affinity is identical to the conducting state of SERT responsible for increased current in Li⁺ Ringer. Lin et al. (11) showed that single channel conductances underlie the currents carried by SERT and the N177G mutant of SERT. Li⁺ increased the open probability of the channel but did not change the single channel conductance, consistent with a Li⁺ induced conformational change rather than an increased permeation of Li⁺ relative to Na⁺ (11). From their work (11), the transporter, even in Li⁺, spends only a small fraction of its time in a conductive state, too small to effect a 2-fold change in cocaine affinity. It is more likely that the conformational disruption induced by Li⁺ increases the probability for entering a short-lived conductive state.

The effects of Li⁺ on inactivation and current were antagonized by Na⁺ as shown in Figs. 1B and 3. Such antagonism could result from a direct competition between Li⁺ and Na⁺ at the Na⁺ binding site. However, in other experiments, Li⁺ does not act like a competitive antagonist of Na⁺ in 5-HT transport (23). We consider it more likely that Na⁺ and Li⁺, acting at different sites, both induce conformational changes in the transporter and that these changes are mutually exclusive. Recent results with the F380Y mutant of SERT indicates the opposing effects of Na⁺ and Li⁺ on the reactivity of Cys-109 (24). Another indication of this phenomenon is that Na⁺ and Li⁺ had opposite effects on β-CIT binding to SERT (Fig. 2 legend). That only Li⁺ of all the alkali cations has this effect (Table I) raises the possibility that the conformational change induced by Li⁺ represents a “salting in” phenomenon by agents that cause protein denaturation at much higher concentrations (25).

The ability of Na⁺ to induce conformational changes has been documented in many transporters (5, 26–28). Together with data from the F380Y mutant of SERT (24), the present data suggest that for SERT also, Na⁺ induces a conformational change or stabilizes the native conformation of SERT to oppose the conformational change induced by Li⁺. In previous studies Li⁺ has been used as an inert replacement for Na⁺ (4, 13, 14). Determinations of the effect of Na⁺ on ligand association and dissociation rates and affinity assumed that Li⁺ had no effect of its own on SERT (4). These earlier studies obviously need to be re-evaluated in light of the current findings. The requirement for Na⁺ in 5-HT transport, however, has firmer support, because other ions have been shown not to replace Na⁺ (13).

Inactivation of SERT transport activity by MTSET modification of Cys-109 was paralleled by inhibition of 5-HT- and Li⁺-induced currents (Fig. 4). We repeatedly observed greater inhibition of 5-HT-induced current than Li⁺-induced current, suggesting that these two conductance levels may result from different states of the transporter. This conclusion is consistent with data from single channel recordings showing that the unitary conductance in 5-HT and Na⁺ was smaller than the conductance in Li⁺ (11). MTSET blockade of the 5-HT-induced current is consistent with the inactivation of transport under the same conditions. However, this block did not result from a blockade of 5-HT binding, because binding was substantially intact after MTSET treatment (Table I).

There are important ways in which these results with SERT contrast with similar experiments performed with DAT. DAT Cys-90 corresponds to SERT Cys-109, and it also reacts with methanethiosulfonate reagents. Modification of DAT Cys-90 by MTSEA increased the binding of a high affinity cocaine analog (7) and did not inhibit transport (29), but modification of the corresponding Cys-109 in SERT by MTSEA or MTSET inactivated transport activity (6) (Fig. 1A). In membranes from cells expressing DAT, modification of Cys-90 was potentiated by binding of cocaine, but in cells expressing SERT, cocaine protected against the inhibition of transport (Fig. 2A). Despite these seeming differences, there are similarities between the two proteins. In cells expressing SERT, Li⁺ potentiated the modification of Cys-109, presumably by an increase in the rate of reaction (Fig. 1A), and Li⁺ potentiated the modification also of Cys-90 in cells expressing DAT. Furthermore, both in DAT and SERT, cocaine binding caused a change in accessibility of Cys-109. Apparently that change decreased the cysteine accessibility in SERT and increased it in membranes from cells.

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2 M. M. Stephan, K. M. Y. Penado, and G. Rudnick, unpublished results.

3 J. Javitch, personal communication.
expressing DAT. Because of the potentiation in DAT, it is unlikely that Cys-90 is in the cocaine binding site. This is consistent with the present results showing that SERT Cys-109 still bound 5-HT and cocaine after MTSET modification (Table I). Rather, it is likely that DAT Cys-90 and SERT Cys-109 are in a conformationally flexible region that is affected allosterically by ligand binding.

In summary, Cys-109 in SERT seems to be part of a conformationally flexible first external loop that is influenced by ligand binding and Li+. Binding of cocaine, 5-HT, or Na⁺ favors a conformation where Cys-109 is less accessible to modification by MTSET. Li⁺ causes a different conformation change that increases both the accessibility of Cys-109 and also the open probability of the SERT ion channel. Modification of Cys-109 in SERT inactivates transport, presumably by blocking conformational flexibility in that region. The modification blocks the 5-HT-induced current much more than it does the Li⁺-elicited current, consistent with the proposal (11) that the conducting states of SERT in 5-HT and Li⁺ are not identical.

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