Early Fluorescence Signals Detect Transitions at Mammalian Serotonin Transporters

Ming Li and Henry A. Lester
Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125 USA

ABSTRACT The mammalian serotonin transporters rSERT or hSERT were expressed in oocytes and labeled with sulforhodamine-MTS. The endogenous Cys-109 residue contributes most of the signal, and the labeled transporter shows normal function. The SERT fluorescence decreases in the presence of 5-HT and also depends on the inorganic substrates of SERT. The fluorescence also increases with membrane depolarization. During voltage-jump experiments, fluorescence relaxations show little inactivation or history dependence. The fluorescence signal has a voltage dependence similar to that of the prepriming step of the previously described voltage-dependent transient current. However, the fluorescence relaxations are the fastest voltage-dependent events yet studied at SERT; their time constants of ~8–30 ms are severalfold faster than the prepriming or inactivation phases of the transient currents. These fluorescence signals are interpreted within the framework of the gate-lumen-gate model. The signals may monitor initial events at the outer gate.

INTRODUCTION

Despite recent strides in understanding the relation between structure and function at neurotransmitter transporters, relatively little is known about the conformational changes that accompany ion-coupled transport. Several tools are available to extend this knowledge. For the mammalian serotonin transporter SERT, previous studies have used ligand binding, radiotracer flux, substituted cysteine accessibility, and electrophysiology, often in conjunction with site-directed mutagenesis. State-dependent fluorescence changes represent another possible source of data. Such measurements have not yet been applied to SERT, but were previously applied to the Na^+-coupled glucose transporter SGLT1 (Loo et al., 1998) and to the GABA transporter GAT1 (Li et al., 2000), which have topological or sequence similarity to SERT. Furthermore, previous fluorescence measurements at GAT1 revealed a signal with time course, voltage dependence, and Na^+ dependence similar to that of the SERT voltage-dependent transient current (Li et al., 2000), but GAT1 does not display SERT-like transient currents. We therefore asked whether fluorescence signals at SERT would resemble those at GAT1 and/or would parallel the SERT voltage-dependent transient current.

In planning site-specific fluorescence experiments for SERT, we noted that several previous studies have utilized methanesulfonate probes at the native Cys-109 group, which probably lies in an extracellular loop between TM1 and TM2 (Chen et al., 1997a; Ni et al., 2001). This residue reports an interaction between Li^+ and SERT (Chen et al., 1997a), is near regions in TM1 thought to participate in 5-HT binding (Adkins et al., 2001), and even appears to report conformation changes in regions of the protein (especially TM7) that are distant from Cys-109 in sequence space (Kamdar et al., 2001). Cys-109 therefore appeared to be a good candidate for fluorescence studies. Alkylation at Cys-109 with small residues such as MTSET inactivates the transporter (Cao et al., 1998; Chen et al., 1997a, 1998); but we hoped that alkylation with a bulkier, zwitterionic fluorescent group would add charge at a lower density, perhaps retaining function.

We report here that this hope is fulfilled and that it is possible to study the fluorescence of a covalently bound sulforhodamine group as the functional SERT molecule undergoes transitions that depend on membrane potential and on substrate concentration. This paper begins to analyze such signals. We first gathered data for fluorescence changes due to bath-applied substrates and for relaxations due to voltage jumps at rSERT and hSERT; we then reexamined current relaxations during voltage jumps, and we then compared electrophysiological and fluorescence data. Voltage-dependent transient currents at rSERT were previously reported (Mager et al., 1994), but this paper reports the first study on the hSERT transient current.

MATERIALS AND METHODS

Reagents and solutions

Two sulfhydryl-reactive MTS reagents, the fluorophore sulforhodamine methanethiosulfonate (sulforhodamine MTS, S699150) and the preblocking reagent MTSET, were purchased from Toronto Research Chemicals Inc. (www.trc-canada.com). The control recording solution, ND96, contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4. The NMDG Cl solution contained 96 mM NMDG instead of Na^+ in the ND96 solution. The sodium gluconate solution contained 96 mM gluconate instead of Cl^- in the ND96 solution. The phosphate-buffered saline (PBS) solution was purchased from Irvine Scientific (Irvine, CA). Other chemicals were purchased from Sigma (St. Louis, MO).

Submitted September 24, 2001, and accepted for publication February 13, 2002.

Address reprint requests to Henry A. Lester, Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125. Tel.: 626-395-4946; Fax: 626-564-8709; E-mail: lester@caltech.edu.

0006-3495/02/07/206/13 $2.00

Expressing hSERT and rSERT in *Xenopus* oocytes

The high-efficiency expression system for human and rat serotonin transporters in *Xenopus* oocytes (Mager et al., 1994; Cao et al., 1998) was used. In brief, 30 ng cRNA of WT rat or human SERT (rSERT, hSERT) or the respective C109A mutant (Mager et. al., 1994; Cao et al., 1998; Y. Tong and M. Li, unpublished results) was injected into stage VI oocytes, and cells were incubated at 18°C for 7–10 days. The incubation solution contained ND96 plus 2% horse serum.

Labeling hSERT and rSERT expressed in oocyte membrane

Seven days after oocytes are injected, expression is optimal for fluorescence labeling. As a result, only exceptionally viable batches of oocytes were eligible for these experiments. All manipulations were performed at 22°C. Oocytes expressing hSERT and rSERT were pretreated with 10 μM MTSET in ND96 solution for 10 min and washed three times. This step, similar to one performed to isolate fluorescence from K^+ channels (Mannuzzu et al., 1996) or GAT1, substantially reduced nonspecific sulforhodamine labeling. Oocytes were then exposed for 15 min to a solution containing 96 mM LiCl instead of NaCl, 100 μM sulforhodamine-MTS, and 1% DMSO (used to solubilize the sulforhodamine-MTS). The fluorescence signal was greater when the oocytes were labeled in a Li^+ solution than in Na^+ (Ni et al., 2001). The labeled oocytes were then washed and incubated in the ND96, ready for recording. The result of labeling was examined by measuring the fluorescence intensity of the oocyte membrane at the animal pole.

Electrophysiology and fluorescence measurement

We used the instruments described in a previous study (Li et al., 2000). In brief, two-electrode voltage clamp procedures were used (Quick and Lester, 1994). The fluorescence of the labeled oocytes is measured with a photomultiplier tube attached to the side port of an inverted fluorescence microscope (Olympus IX-70-FLA) with a stabilized 100 W mercury light source and an objective of 40×, NA1.3. The filter cube is the high-Q TRITC 410012b set (excitation 545 nm, half-width 30 nm; emission 610 nm, half-width 75 nm) from Chroma Technology Corp. (Brattleboro, VT). The oocyte sits on the microscope stage and is visualized for electrophysiology by a separate stereomicroscope. The exciting beam was attenuated by factors approaching 100. Bleaching of the fluorophore amounted to ~0.5% during a typical trial of 100 episodes that lasted a total of 1 min. The emission signal from the oocytes was appropriately amplified and filtered. A HumBug (Qwest Scientific, N. Vancouver, BC) cleaned up the remaining 60 Hz. Signals were acquired and averaged by an Axon Digidata interface and pCLAMP 8 (Axon Instruments, Union City, CA). Traces shown were digitally filtered at 200 Hz except where indicated.

Data analysis

The kinetics of the fluorescence and transient current were fitted to single or two-exponential processes with nonlinear routines in ORIGIN and CLAMPFIT 8.

TABLE 1 Fluorescence intensity of *Xenopus* oocytes labeled with sulforhodamine-MTS

	cRNA Injection		
	Uninjected	rSERT-C109A	WT rSERT
Fluorescence	3.7 ± 0.2 (n = 10)	4.5 ± 0.5 (n = 11)	5.7 ± 0.5 (n = 17)
% Uninjected	100	123 ± 8	153 ± 5

Intensity was measured as the output from the photomultiplier tube at a working voltage of 800 V. Data are given as mean ± SEM.

RESULTS

Fluorescence measurements

The fluorescence signals arise at labeled Cys-109 and depend on 5-HT

We labeled oocytes expressing human or rat SERT (hSERT or rSERT, respectively) cRNA with the sulfhydryl-reactive fluorophore, sulforhodamine-MTS, according to the protocol described in Materials and Methods. Oocytes expressing rSERT exhibit ~53% increase in fluorescence intensity compared to uninjected oocytes (Table 1); a similar percentage increase was observed for hSERT (data not shown). The rSERT-C109A mutant is fully functional and displays transient currents similar to those of wild-type rSERT (Fig. 2 *B* below and Cao et al., 1998), yet oocytes injected with rSERT-C109A cRNA exhibit only ~23% increase in fluorescence intensity compared to uninjected oocytes (Table 1). From this result we conclude that more than half the fluorescence of the SERT-specific fluorescence arises from labeling of Cys-109.

Fig. 1 compares measurements of voltage-clamp current and of fluorescence in a sulforhodamine-labeled rSERT-expressing oocyte exposed repeatedly to 10 μM 5-HT. The 5-HT induced an inward current (Fig. 1 *A*), termed the "transport-associated current" in previous studies (Cao et al., 1997, 1998; Li et al., 2000; Lin et al., 1996; Mager et al., 1994). This indicates that sulforhodamine-labeled rSERT is functional by electrophysiological criteria. The amplitude of the transport-associated current is ~20 nA at a holding potential of −60 mV. The application of 5-HT also reproducibly decreased steady-state fluorescence (Fig. 1 *B*); in the experiment of Fig. 1, this decrease amounted to ~8% within ~50 s at a holding potential of −60 mV. 5-HT-induced changes in current and fluorescence of oocytes expressing SERT have a different time course: when 5-HT is perfused into the recording chamber, the fluorescence changes more slowly than the current. In five oocytes studied systematically 100–120 s after application of 20 μM 5-HT, the fluorescence decrease was 13 ± 2% (mean ± SEM). In the Discussion below, we comment on these differences in time course between the optical and electrophysiological signals. We verified that uninjected oocytes displayed no change in fluorescence when exposed to 5-HT.

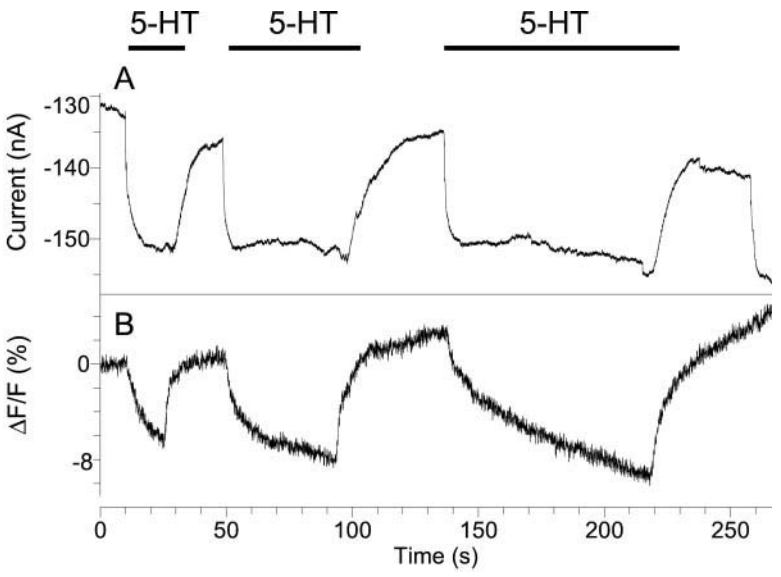

FIGURE 1 5-HT-induced change in current (*A*) and fluorescence (*B*) of an oocyte expressing rSERT and labeled with sulforhodamine-MTS. The holding potential is −60 mV. The signals are filtered at 50 Hz.

Kinetics of the fluorescence relaxations

We measured simultaneous current and fluorescence relaxations in oocytes injected with rSERT, rSERT-C109A, or hSERT cRNA and subjected to voltage jumps (Fig. 2). The electrophysiological responses have been presented in previous publications (Cao et al., 1997, 1998; Lin et al., 1996; Mager et al., 1994). At this point, we note only that hyperpolarizing jumps lead to transient inward currents (denoted by arrows in Fig. 2) in sulforhodamine-labeled rSERT and hSERT-injected oocytes, again showing that the labeled oocytes are electrophysiologically functional. In a later section we describe additional electrophysiological measurements designed for direct comparison with the fluorescence studies.

The fluorescence signals are novel and are therefore presented first. Upon a voltage jump from a holding potential of −40 mV to a test potential of +60 mV, the fluorescence increases to a new steady state ~0.2% higher. The amplitude of the fluorescence increase varied among batches of oocytes, between 0.1% and 0.8%. The fluorescence increase is observed neither with the functional mutant C109A (Fig. 2 *B*), nor with uninjected oocytes (Fig. 2 *C*), suggesting that the voltage-dependent fluorescence originates from the fluorophore label on the Cys-109 residue of SERT. Thus, although the fluorescence relaxations are rather small and required us to average 100 or more sweeps for accurate measurement, they can be unambiguously measured and assigned to a site-specifically labeled fluorophore. Incidentally, these fluorescence relaxations are similar in amplitude to those measured for the tetramethylrhodamine-labeled GABA transporter GAT1 (Li et al., 2000), but the kinetics of the relaxations, and even the sign of the change in response to voltage jumps, differ for the two transporters, again suggesting that the signals arise directly from the transporters rather than from endogenous or artifactual sources.

The two transporters, rSERT and hSERT, have similar relaxation amplitudes when studied under similar conditions (Fig. 2, *D* and *E*). However, the kinetics of the fluorescence increase differ between rSERT and hSERT. In qualitative terms, most of the hSERT relaxation is much faster than the rSERT relaxation. When the fluorescence relaxations are fitted to two-exponential processes, the rSERT signal gives time constants of 50 ± 10 ms and 460 ± 30 ms, with amplitude contributions of 30% and 70%, respectively. For hSERT, the two components are 8 ± 5 ms and 570 ± 40 ms, with amplitude contributions of 80% and 20% (Table 2). Several factors may distort these estimates of time constants. First, the slower component rSERT and hSERT may not be accurate because the fluorescence did not reach steady state under our experimental protocol (longer pulses caused damage to the cells when repeated several hundred times). The time constants given may be underestimates. Second, the faster time constant (8 ms) for hSERT may have been distorted by the settling time of our voltage-clamp circuit. Therefore, this time constant may be an overestimate.

Near the completion of our experiments on rSERT and hSERT fluorescence, we found that both the electrophysiological and the fluorescence signals changed when we omitted HEPES from the recording solutions and substituted phosphate as the buffer. The electrophysiological changes will be reported in a separate publication, but the effects are briefly noted here: the transient currents become larger and slower. The effects of omitting HEPES are more dramatic for rSERT than for hSERT. The rSERT fluorescence relaxations acquire a rapid phase and become similar to the hSERT relaxations (Fig. 2 *E*). We believe that the

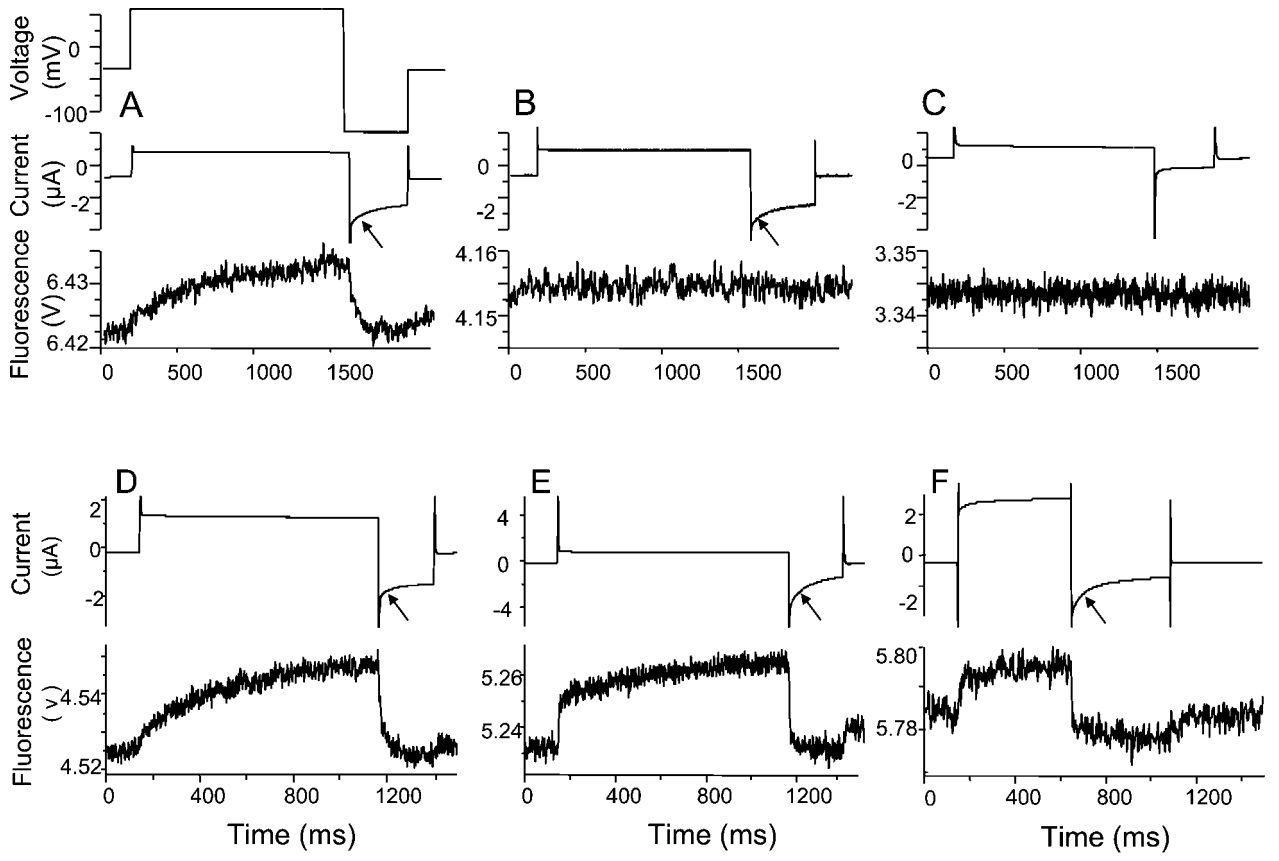

FIGURE 2 Simultaneous measurement of current and fluorescence during voltage jumps in oocytes labeled with sulforhodamine. Each trace is the average of 100–140 sweeps from one cell. The arrows point to the hyperpolarization-activated transient induced currents. For *A*--*C* the membrane potential was held at −40 mV and jumped to +60 mV for 1400 ms, then to −140 mV for 360 ms. (*A*) WT rSERT; (*B*) rSERT-C109A; (*C*) uninjected; (*D*) rSERT. Note that rSERT-C109A exhibits transient currents as large as those for rSERT, but there are no fluorescence relaxations. (*E*) hSERT. For *D* and *E* the membrane potential was held at −40 mV and jumped to +60 mV for 1020 ms, then to −140 for 240 ms. (*F*) rSERT signal in phosphate-buffered saline (PBS) rather than HEPES-buffered saline. For *F* the membrane potential was held at −40 mV and jumped to +60 mV for 500 ms, then to −140 mV for 450 ms. Note that the fluorescence change for the depolarizing step for rSERT consists of one phase occupying several hundred milliseconds, but for hSERT there are two distinct phases, including one phase occupying only tens of milliseconds.

slow phase originates from an interaction between HEPES and rSERT. Thus, omitting HEPES simplifies the description of the relaxations. We have other evidence supporting this hypothesis, as will be discussed later.

Voltage dependence of the rSERT fluorescence relaxations

In addition to changing the steady-state fluorescence of SERT (Fig. 1), 5-HT also changes the voltage depen-

TABLE 2 Kinetic steps of SERT measured by current and fluorescence

	Prepriming Step (−40 to +60 mV)				Conducting Step (+60 to −140 mV)			
	hSERT		rSERT		hSERT		rSERT	
	+ HEPES	− HEPES	+ HEPES	− HEPES	+ HEPES	− HEPES	+ HEPES	− HEPES
Fluorescence	8 ± 5 (80%) 570 ± 40 (20%)	8 ± 3	50 ± 10 (30%) 460 ± 30 (70%)	30 ± 10	<6	<6	33 ± 9	10 ± 5
Current	50 ± 2	94 ± 5	86 ± 12	95 ± 7	63 ± 8 (68%) 218 ± 15 (32%)	46 ± 13 (29%) 216 ± 32 (71%)	45 ± 10 (29%) 325 ± 19 (71%)	53 ± 7 (74%) 493 ± 93 (26%)

Time constants were fitted to the current relaxations, the prepriming envelopes (as in Fig. 8), and the fluorescence relaxations (Figs. 3 and 5). Time constants are given in milliseconds; percent of total amplitude is given in parentheses. Measurements (mean ± SEM) represent 5–10 oocytes in each case.

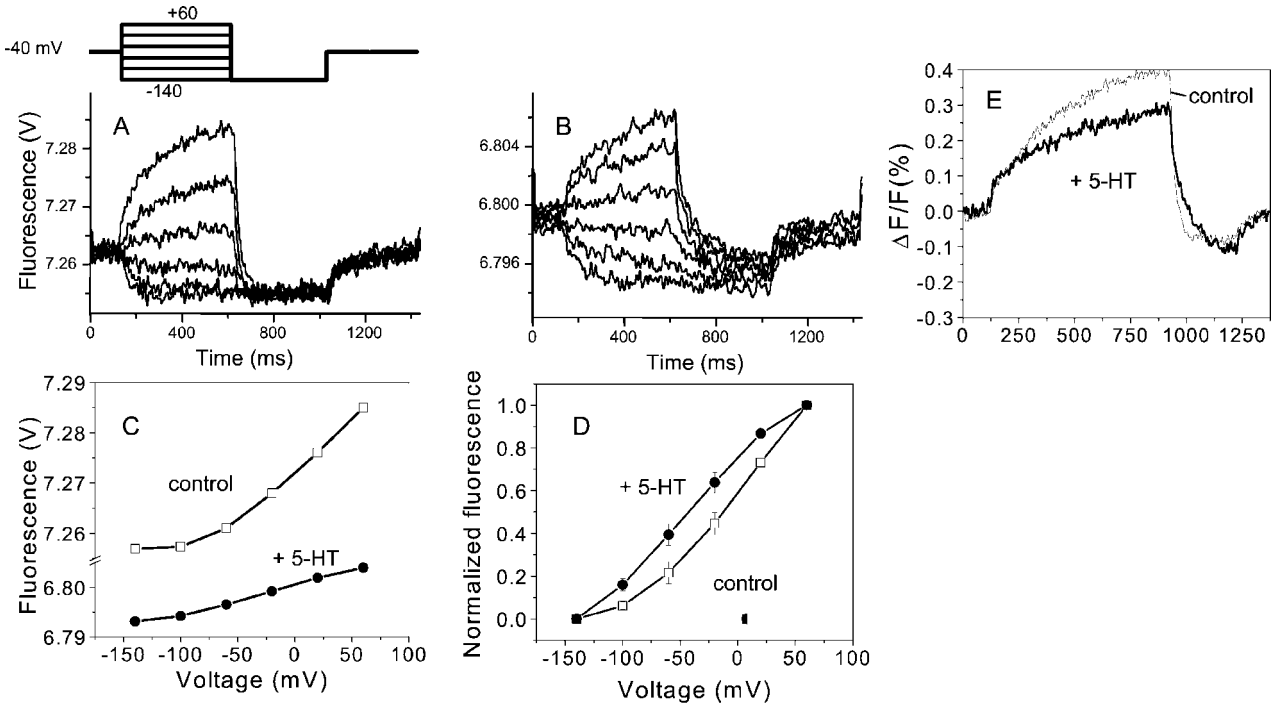

FIGURE 3 Fluorescence of sulforhodamine-labeled rSERT during voltage-jump relaxations in the absence (*A*) and presence (*B*) of 20 μM 5-HT. Each trace is the average of 120 sweeps from a typical cell. (*C*) Value of fluorescence versus voltage at the end of each test pulse in the absence (*squares*) and presence (*circles*) of 5-HT. The data are replotted from *A* and *B*. Note the break in the *y* axis. (*D*) Plots like those in *C* have been normalized to the same vertical range and superimposed. Mean ± SEM, $n = 4$ cells. (*E*) Another typical cell. Superimposed waveforms for jumps from the holding potential of −40 mV to +60 mV, then to −140 mV, then to −40 mV in the absence and presence of 5-HT. ΔF is expressed as a percentage of resting fluorescence.

dence of fluorescence. In the experiment of Fig. 3, fluorescence relaxations of rSERT were measured when the membrane potential was stepped from a holding potential of −40 mV to test potentials between +60 mV and −140 mV at 40-mV decrements. The fluorescence versus voltage (*F-V*) plots are shown in the bottom panels. In the absence of 5-HT, the fluorescence saturated at the most negative potentials studied, −140 to −120 mV (Fig. 3 *A*). This saturation was removed when 10 μM 5-HT was added to the recording chamber (Fig. 3 *B*). The result is best illustrated by normalizing the fluorescence value at +60 mV to 1 and that at −140 mV to 0 (Fig. 3 *D*). If the *F-V* plot in the absence of 5-HT resembles the beginning of a rather shallow Boltzmann relation, the more linear *F-V* in the presence of 5-HT resembles the midregion of a Boltzmann relation.

Fig. 3 *E* presents superimposed traces, in the absence and presence of 5-HT, of the fluorescence relaxations produced by voltage steps from a holding potential of −40 mV to +60 mV, then to −140 mV, and back to −40 mV. 5-HT (10 μM) introduced a faster component in the rise of the fluorescence relaxation produced by a depolarizing jump and a slower component in the falling phase produced by a hyperpolarizing jump.

Ionic dependence of the rSERT relaxations

We also noted the effects of the ionic SERT substrates, Na^+ and Cl^-, on fluorescence. When Na^+ was replaced by NMDG in experiments like those of Fig. 1, rSERT fluorescence increased by 5 ± 3% over a period of several min (mean ± SEM, $n = 5$). There was a slow phase of this increase; but it was too small for systematic measurement. Replacement of Cl^- by gluconate decreased fluorescence by 5 ± 4% (mean ± SEM, $n = 4$); there was no slow phase. Replacement of Na^+ by Li^+ did not change fluorescence detectably (<2%).

We also noted the effects of ionic substitutions on voltage-jump relaxations at rSERT (Fig. 4). When Na^+ was replaced by NMDG, the normalized *F-V* relation was steepest at extreme negative potentials (Fig. 4, *B* and *E*). If the *F-V* plot in the presence of Na^+ resembles the beginning of a rather shallow Boltzmann relation, the more linear *F-V* in the presence of NMDG resembles the positive end of a Boltzmann relation. The *F-V* relations were not changed detectably when Na^+ was replaced by Li^+ (Fig. 4, *C* and *F*) or when Cl^- was replaced by gluconate (Fig. 4, *D* and *E*).

Fig. 4, *H–J* present superimposed traces allowing one to examine the effect of varying SERT substrates on the waveforms of the fluorescence relaxations produced by voltage steps from a holding potential of −40 mV to +60 mV, then

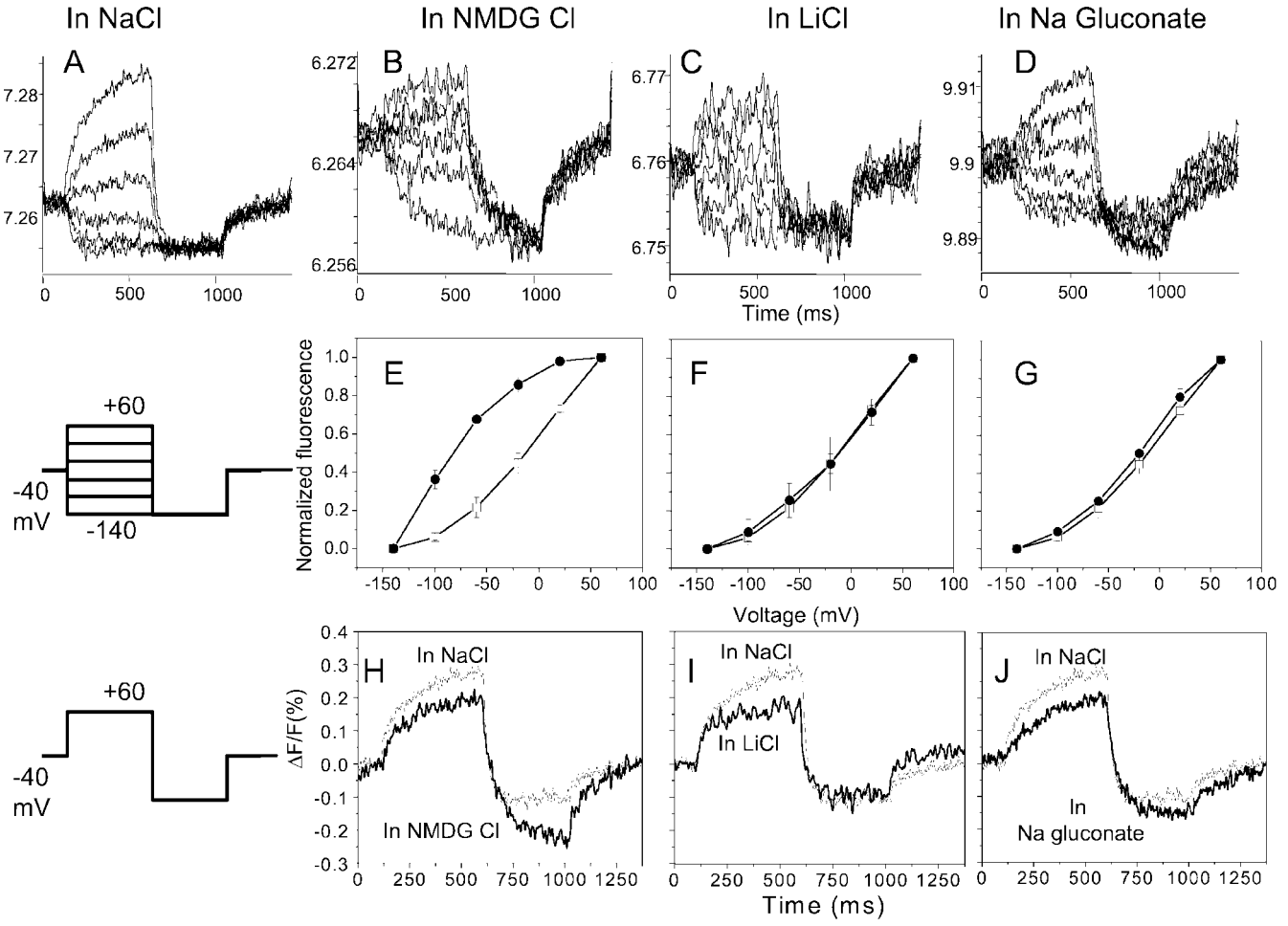

FIGURE 4 rSERT fluorescence relaxations during substitutions of the inorganic substrates. The top panels show fluorescence relaxations for jumps from a holding potential of −40 mV to test potentials between +60 mV and −140 mV, at 40-mV decrements. Each trace is the average of 100–120 sweeps in the same cell. (*A*) Normal saline; (*B*) Na^+ is replaced by NMDG; (*C*) Na^+ is replaced by Li^+; (*D*) Cl^- is replaced by gluconate. Bottom panels show normalized *F-V* plots. Points are mean ± SEM, $n \geq 3$ cells. (*E*) The *F-V* plot in normal Na^+ solution (*squares*) and NMDG solution (*circles*). (*F*) *F-V* plot in normal Na^+ solution (*squares*) and Li^+ solution (*circles*). (*G*) *F-V* plot in normal Cl^- solution (*squares*) and gluconate solution (*circles*). (*H–J*) Waveforms of rSERT fluorescence relaxations during substitutions of the inorganic substrates. Voltage was stepped from a holding potential of −40 mV to +60 mV, then to −140 mV, and back to −40 mV.

to −140 mV, and back to −40 mV. The most significant effect of replacing Na^+ by NMDG is to slow the falling phase of the fluorescence relaxation (Fig. 4 *H*). Replacing Na^+ by Li^+ does not have a significant effect on the kinetics of the fluorescence relaxation (Fig. 4 *I*). The effect of replacing Cl^- by gluconate resembles that of replacing Na^+ by NMDG, but to a lesser extent (Fig. 4 *J*).

Substrate dependence of the hSERT relaxations resembles that of rSERT

Despite the fact that hSERT exhibits faster kinetics in fluorescence relaxations compared to rSERT, the effects of varying substrates on fluorescence relaxations are similar in hSERT and rSERT (Figs. 5 and 6). 5-HT decreased steady-state fluorescence of hSERT (data not shown) and rendered the *F-V* curve more linear (compare Fig. 5 with Fig. 3). Na^+ replacement by NMDG or Li^+, and Cl^- replacement by gluconate, affect the *F-V* relation of hSERT like the analogous replacements at rSERT (compare Fig. 6 with Fig. 4). The effects of manipulating SERT substrates on the kinetics of the fluorescence relaxations were also similar in hSERT and rSERT: 5-HT introduced a slower component in the falling phase of the fluorescence relaxation. Replacement of Na^+ by NMDG or Li^+ and replacing Cl^- with gluconate introduced a slower component in the falling phase of the fluorescence relaxation.

Electrophysiological measurements

We now present a reexamination of current relaxations during voltage jumps at rSERT and hSERT, followed by a

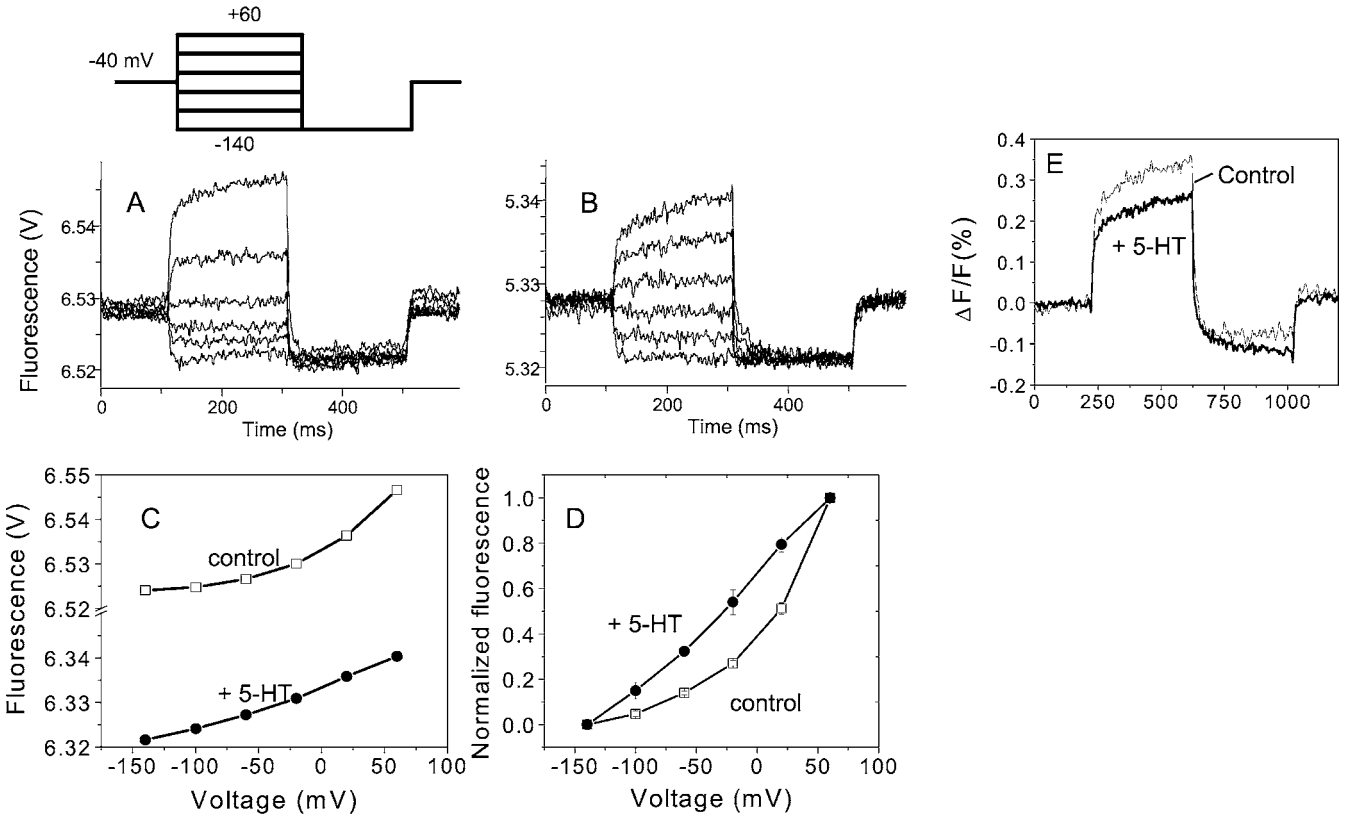

FIGURE 5 Fluorescence of sulforhodamine-labeled hSERT during voltage-jump relaxations in the absence (*A*) and presence (*B*) of 20 μM 5-HT. Each trace is the average of 120 sweeps from a typical cell. (*C*) Value of fluorescence versus voltage at the end of each test pulse in the absence (*squares*) and presence (*circles*) of 5-HT. The data are plotted from *A* and *B*. Note the break in the *y* axis. (*D*) Plots like those in *C* have been normalized to the same vertical range and superimposed. Mean ± SEM, $n = 4$ cells. (*E*) Another typical cell. Superimposed waveforms for jumps from the holding potential of −40 mV to +60 mV, then to −140 mV, then to −40 mV in the absence and presence of 5-HT. ΔF is expressed as a percentage of resting fluorescence.

comparison with the fluorescence relaxations. In the course of this study we also gathered the first data about hyperpolarization-activated transient currents in hSERT-expressing oocytes (Fig. 7). As previously reported for rSERT (Mager et al., 1994), a jump from a positive potential to a large negative potential in the absence of 5-HT produces a distinctive inward transient current. The transient current is eliminated by 5-HT (Mager et al., 1994), and we used this fact to isolate the transient current in the traces of Fig. 7 *A*. The transient currents of hSERT and rSERT have similar dependence on both prepulse and test pulse potentials. However, hSERT seems to display a faster decay in the transient current. Two-exponential fits to the transient currents at −140 mV give time constants of 45 ± 10 and 325 ± 19 ms for rSERT, and 63 ± 8 and 218 ± 15 ms for hSERT (Fig. 7, *B* and *D*; see also Table 2). Because the decay phase of the transient current is dominated (~70%) by the slower component, the hSERT transient currents display a half-decay time (~76 ms at −140 mV) roughly half as large as the rSERT transient currents. The slow component of time constant 325 ms in rSERT may be due to HEPES and other amines binding to SERT, because this component is decreased when HEPES is removed from the recording solution (Table 2).

We detected fluorescence relaxations associated with both hyperpolarizing and depolarizing steps. We therefore designed and conducted additional experiments to probe electrophysiological events during depolarizing pulses. Little or no current actually flows, but previous data show that a sojourn at positive potentials increases the amplitude of a subsequently measured hyperpolarizing transient current (Mager et al., 1994). This phenomenon may be described as an inactivation at negative potentials or as a prepriming at positive potentials. We use the latter term: a depolarizing prepulse preprimes the SERT to a conducting state upon membrane hyperpolarization.

The voltage dependence of the prepriming step in rSERT was previously studied (Mager et al., 1994). In the present experiments we studied the time course of the prepriming process, using prepulses of variable duration (Fig. 8). The single-exponential time constant for the prepriming process at +60 mV is ~86 ms for rSERT (Fig. 8). The same two-pulse protocol was also used to measure the time course of the prepriming process in hSERT (data not shown). In normal solutions the analysis is complicated by the presence of a use-dependent blockade of the transient current in hSERT; this blockade was eliminated by removing HEPES from the recording solution. We found that the time constants of the

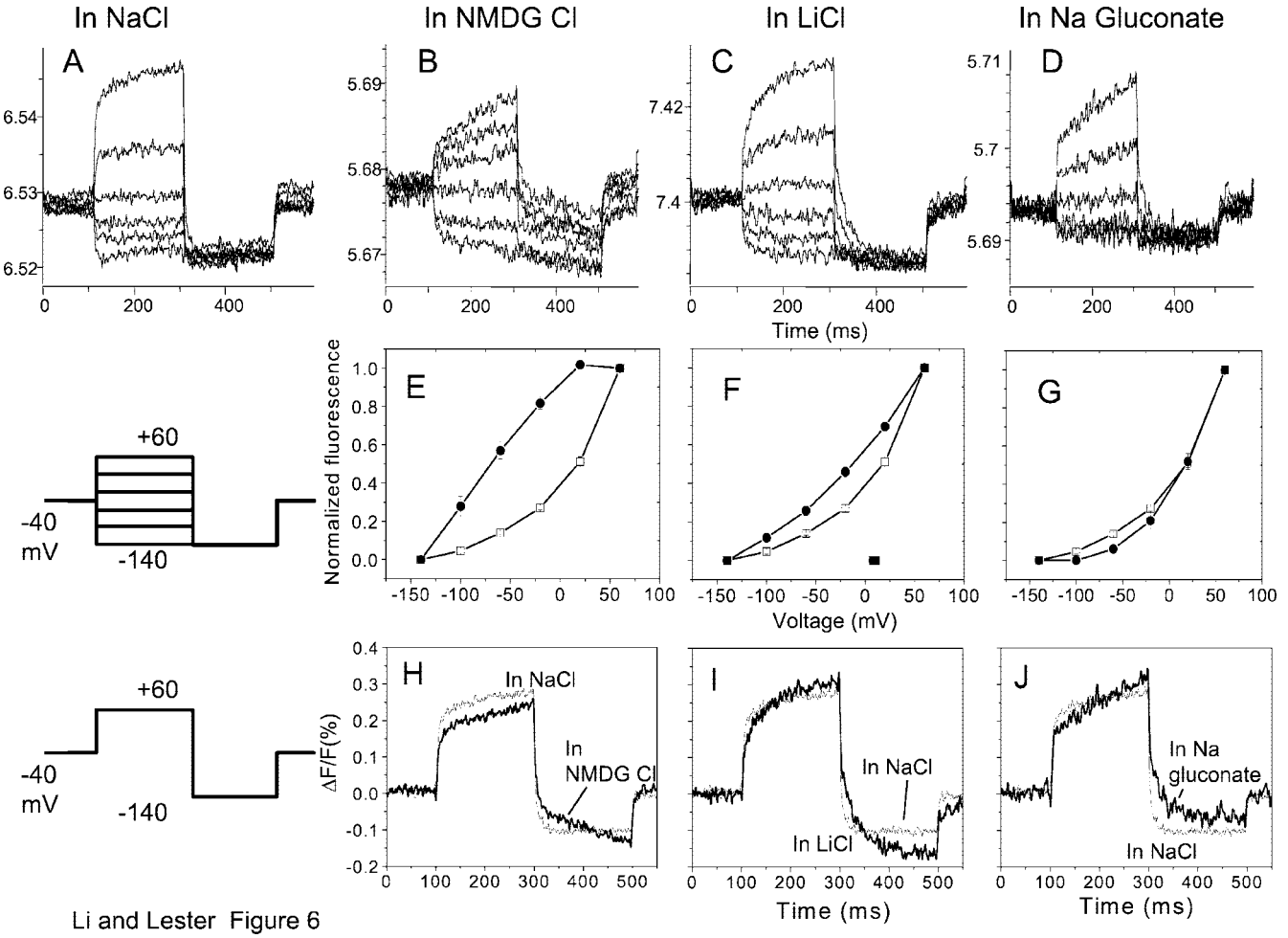

FIGURE 6 hSERT fluorescence relaxations during substitutions of the inorganic substrates. The experiments were conducted and presented similarly to those of Fig. 3. Points are mean ± SEM, $n \geq 3$ cells.

prepriming process in hSERT is near that of rSERT, i.e., ~95 ms at +60 mV membrane potential (Table 2).

Comparing fluorescence and current measurements

The transient current depends on prepulse voltage because of two presumably linked phenomena: inactivation at negative potentials and prepriming at positive potentials. The detailed dependences are presented in Fig. 9 *A* in a format like that of the activation-inactivation plots for ion channels. However, we find that there is little history-dependence or inactivation of the fluorescence signals (Figs. 3–6). Fig. 9 *B* compares *F-V* plots for prepulses to either +60 mV, which fully preprimes the transient current, versus prepulses to −40 mV, which inactivates >80% of the transient current. There is little difference between the *F-V* relations for these two conditions. Fig. 9 *B* also shows that the *F-V* relation is like the "prepriming" relation between prepulse potential and transient current.

Table 2 gathers the information we have obtained about kinetic processes of hSERT and rSERT. Both fluorescence changes and transient currents are summarized. Evidently the situation is much simpler in the absence of HEPES, and these data will be emphasized in the Discussion. The time constants measured by fluorescence signals are all less than those measured by the transient current; e.g., for the prepriming step, the time constants measured by fluorescence are 8 ms for hSERT and 30 ms for rSERT; those measured by transient current are ~95 ms for both hSERT and rSERT. For the conducting step, the time constants measured by fluorescence are <20 ms and those measured by transient current are >50 ms (Table 2).

DISCUSSION

We have detected a component of sulforhodamine fluorescence that occurs when SERT (either rSERT or hSERT) is expressed in oocytes. Specificity is assured by the fact that the C190A transporter shows normal function, but less SERT-dependent fluorescence. The SERT fluorescence also

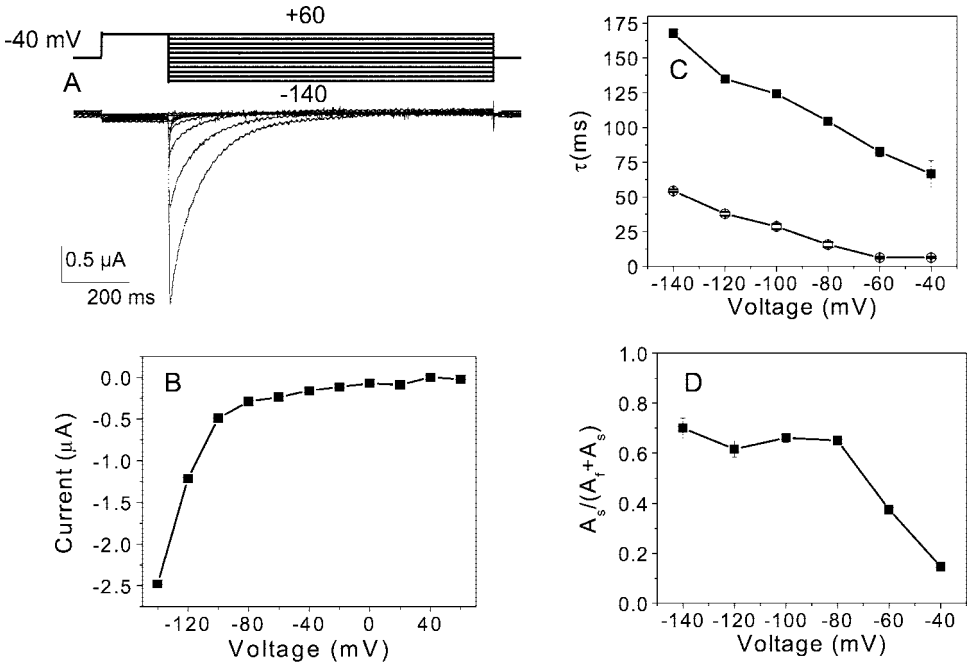

FIGURE 7 Transient currents at hSERT measured in normal saline solution containing 5 mM HEPES. Traces in (*A*) result from the subtraction (traces recorded in the absence of 5-HT) minus (traces recorded in the presence of 20 μM 5-HT). (*B*) Peak transient current versus membrane voltage. (*C* and *D*) Results of two-exponential fit of the transient current to a fast (f) and slow (s) component.

depends on transmembrane potential and on the organic and inorganic substrates of SERT. The fluorescence relaxations are the fastest voltage-dependent events that we have studied at SERT; they are faster than either the prepriming or the inactivation step of the transient current, but they have a voltage dependence similar to that of the prepriming step. Because the fluorescence relaxations amount to $<1\%$ of the background fluorescence even after 7 days of expression, experiments on these relaxations are tedious and limited only to exceptionally viable batches of oocytes. Nonetheless, we can draw some initial conclusions.

Working model

Fig. 10 presents a working model for interpretation of the fluorescence and current measurements within the frame-

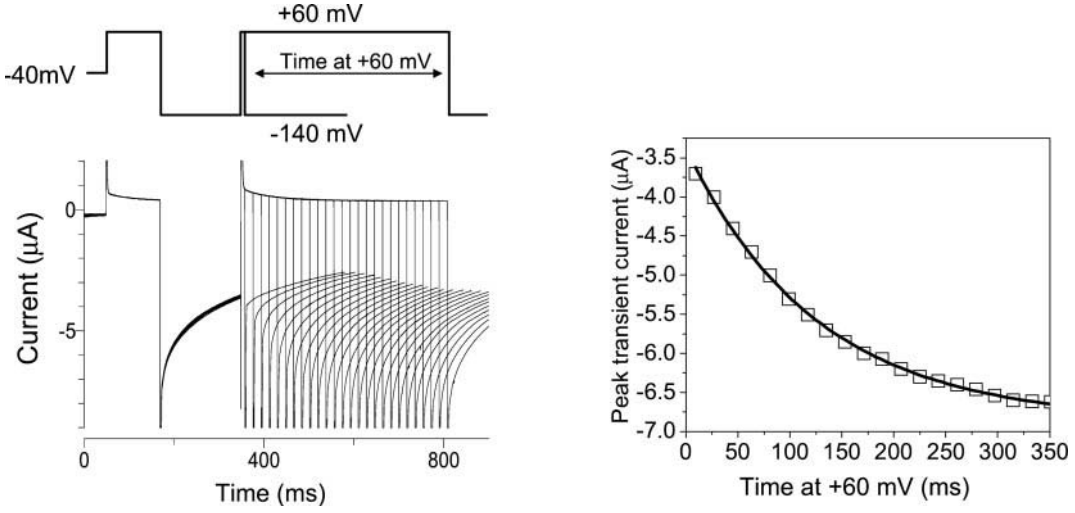

FIGURE 8 Time course of the prepriming process in rSERT. *Left:* the membrane potential was held at −40 mV and jumped to +60 mV for 120 ms, then to −140 ms for 180 ms. Potential was then jumped to +60 mV for a variable duration at 18-ms increments, then back to −140 ms. *Right:* peak transient current versus time at +60 mV. The curve is a single-exponential fit to the data.

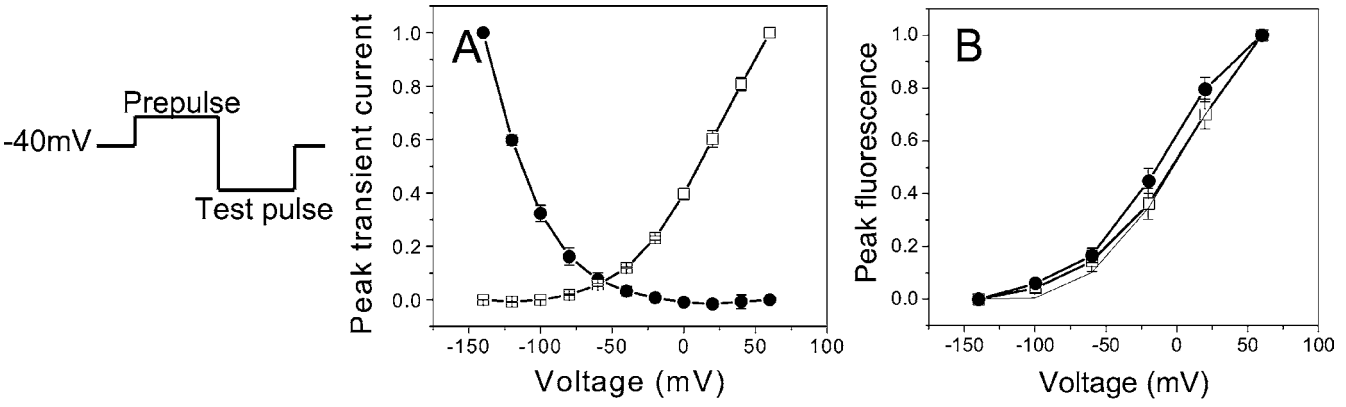

FIGURE 9 Voltage-dependent processes at sulforhodamine-labeled rSERT. (*A*) The transient currents. Circles show peak amplitude of transient current at various test voltages with +60 mV prepulse (the value giving the largest transient current). Squares show the peak amplitude of transient current tested at −140 mV (the value giving largest transient current) with various prepulse voltages. (*B*) The fluorescence relaxations. Circles show the amplitude, at the end of the test pulse, of the fluorescence change from −40 mV holding potential to various test potentials. Squares show the peak amplitude, at the end of the test pulse, of the fluorescence change from a +60 mV prepulse to various test potentials. The dashed line represents the data from *A* for peak transient current at variable prepulse amplitudes.

work of the gate-lumen-gate scheme (Cao et al., 1998). The gate-lumen-gate model basically assumes that the transporter contains a channel-like lumen with gates at each end. Coupled transport involves "alternating access," or cycling of the gates so that the transporter changes the compartmentalization of the substrates. Conducting states, however, involve simultaneous opening of each gate. The voltage-dependent transient current is the conducting state with the largest known currents. We assume that prepriming current is limited by events at the outer gate, i.e., the inner gate

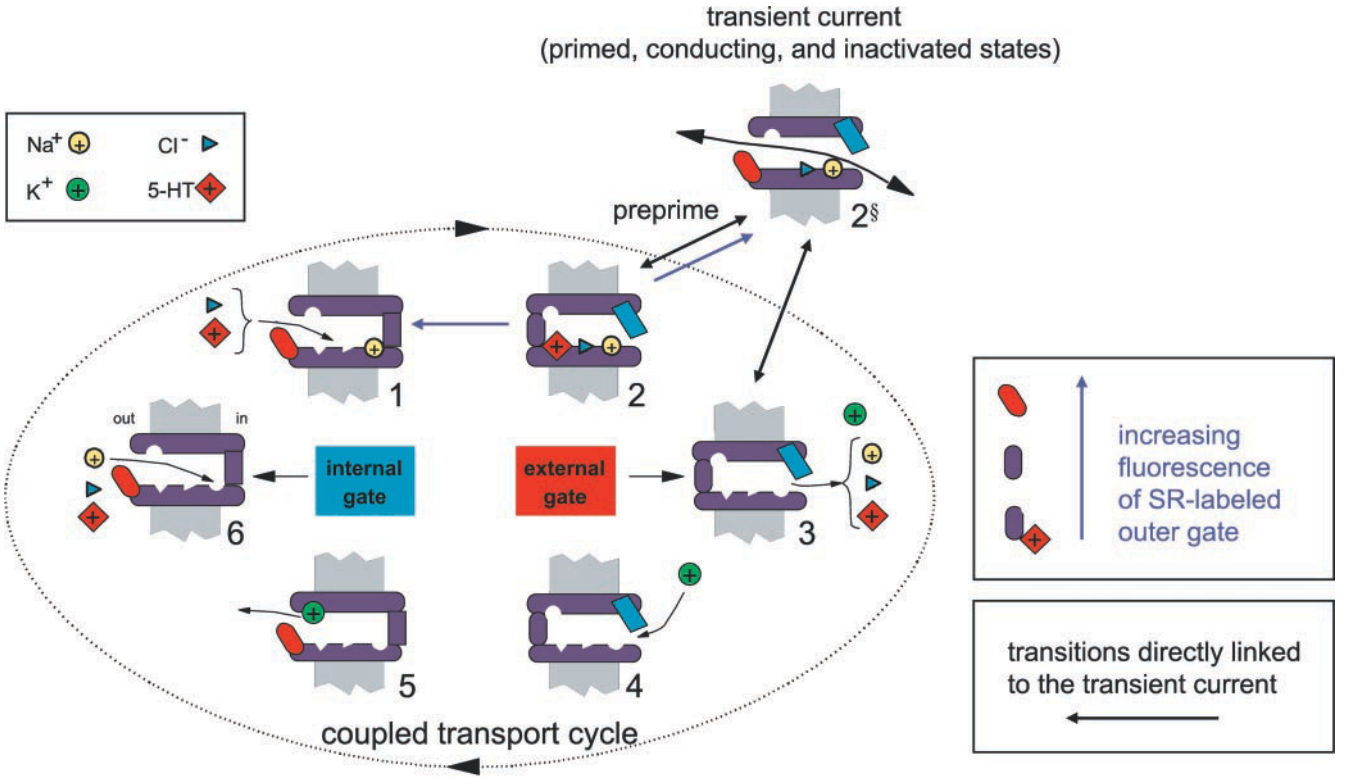

FIGURE 10 Working model for interpretation of the fluorescence and current measurements, within the framework of the gate-lumen-gate scheme (Cao et al., 1998). The fluorescence changes during the transitions are represented by blue arrows, and these transitions involve changes at the outer gate. See text for a full explanation.

would remain continually open during this process. We make this assumption partially because externally applied drugs, such as HEPES, cocaine, and some local anesthetics, can block the transient current and/or modify its waveform. Some of these pharmacological data have been published (Mager et al., 1994); other data will be reported separately. Unfortunately, we have no information about the mechanism(s) of inactivation of the transient current at negative potentials. Our fluorescence signals have no component that matches this inactivation.

We also believe that the outer gate is labeled by MTS-sulforhodamine (SR), because the Cys-109 residue is externally accessible (see Introduction). On this assumption we have assigned fluorescence changes to transitions—either binding or conformational change—at the outer gate; these fluorescence-detected transitions are represented by blue arrows, which point to increased fluorescence. The largest decrease in fluorescence (~13%) occurs when 5-HT binds at the outer gate. The slow fluorescence changes in the presence of external 5-HT (Fig. 1) could arise because this binding site is also somewhat accessible to intracellular 5-HT, which accumulates during prolonged 5-HT application. Because we have not studied whether the slow fluorescence changes occur in the C109A mutant, it is formally possible that the slow fluorescence changes occur at sulforhodamine-labeled residues other than Cys-109; such non-Cys-109 labeling appears to account for nearly half the MTSR signal (Table 1).

We suggest that the fluorescence also increases when the outer gate is primed to open, especially in the absence of 5-HT. That is, the transporter would enter the set of states named $2^{\S}$. This suggestion arises because depolarization is associated both with increased fluorescence and with prepriming of the transient currents. The major components of the fluorescence relaxations are the fastest voltage-dependent events that we have studied at SERT; they are faster than either the prepriming or the activation step of the transient current, but they have a voltage dependence similar to the prepriming step. A hyperpolarizing step activates the transient current at a rate that probably exceeds the resolution of our voltage-clamp circuit. We believe this activation is probably an ohmic phenomenon like an instantaneous *I-V* relation; that is, the hyperpolarizing step generates current through states that are conducting but blocked, or just about to open. In short, we suggest that the fluorescence monitors early, local events near Cys-109 in the prepriming of the outer gate. Analogies to gating currents at ion channels suggest themselves. The state named $2^{\S}$ is presumably a set of interconverting states, including primed, conducting, and inactivated states. Because voltage influences the equilibria among these states, the slower components of the fluorescence relaxations would arise from these changes.

The specific change in the environment of sulforhodamine that produces the signals is unknown. The two major candidates are changes in polarity and changes in quenching. The latter mechanism appears to account for voltage-dependent changes in tetramethylrhodamine fluorescence at K^+ channels (Cha and Bezanilla, 1997, 1998). Thus, environmental changes around the chromophore would result from conformational changes induced by voltage changes or by 5-HT binding. The 5-HT-evoked fluorescence decrease is the largest fluorescence signal yet observed with ion-coupled transporters. Some (Adkins et al., 2001; Barker et al., 1999) but not all (Barker et al., 1994; Buck and Amara, 1994; Chen et al., 1997b; Giros et al., 1994; Kitayama et al., 1992; Lee et al., 2000; Smicun et al., 1999) data suggest that 5-HT, dopamine, or norepinephrine bind at TM1 in their respective transporters. However, 5-HT itself does not absorb detectably at wavelengths >320 nm, and it seems quite unlikely that binding to SERT would shift the spectrum by >200 nm, to cause direct quenching of sulforhodamine. However, 5-HT might favor the binding of an endogenous quenching fluorophore. Na^+ or Li^+ would be required for such binding; NMDG would remove this quenching by disfavoring the binding.

Although the precision of the present data may not merit an explicit scheme like Fig. 10, we include the scheme in this discussion because it has served well in our previous studies. In general, however, we believe that the data can usefully be evaluated against a large class of alternating-access models, in which ion-coupled transport is accomplished by conformational changes that control the compartmentalization of substrates.

Voltage and ionic dependence of the fluorescence relaxations

The voltage dependence of the fluorescence bears comment, but only in a quantitative sense. We assume that the fluorescence behaves as a sigmoid function of voltage and is characterized by a rather shallow Boltzmann. If so, in the absence of 5-HT the midpoint of this relation is more positive than the voltage range we have investigated (−140 to +60 mV). In the presence of 5-HT, the fluorescence becomes linear with voltage over the entire range of our measurements, suggesting that the midpoint is within this range. When Na^+ is replaced by NMDG, the relation begins to saturate at more positive potentials, as though the midpoint of the Boltzmann relation is in a region more negative than the range we have investigated. Assuming that the midpoint of the Boltzmann relation is also the midpoint of a conformational equilibrium sensed by the fluorescence, this midpoint is shifted in the negative direction by 5-HT and even more in this direction by NMDG. Recent data have anticipated the observation that Li^+ and NMDG, two Na^+ substitutes, have distinguishable effects on the environment sensed by Cys-109 (Ni et al., 2001).

Contrast with GAT1 relaxations

The florescence relations that we have measured at SERT-expressing oocytes differ in several ways from those we have previously recorded with covalently labeled tetramethylrhodamine at GAT1, even though 1) the same apparatus was used (Li et al., 2000), and 2) GAT1 was labeled at Cys-74, which aligns with SERT-Cys-109. The sign of the voltage dependence differs: the fluorescence increases and decreases with depolarization, respectively, at GAT1 and SERT. The kinetics of the major phases of the relaxations are roughly 10-fold slower at GAT1 than at SERT. The organic substrate GABA has little effect on the GAT1 fluorescence relaxations, but 5-HT has clear effects on both steady-state and transient SERT fluorescence. Thus we can clearly reject our previous suggestion (Li et al., 2000) that the state revealed by the GAT1 fluorescence signals represents an occult transient current. The speed and voltage dependence of the SERT fluorescence relaxations actually resemble those of SGLT1 labeled in TM11 (Loo et al., 1998) more than those of GAT1 labeled in TM1 (Li et al., 2000).

This contrast between the fluorescence properties of labeled GAT1 and SERT does, however, serve to emphasize the several differences between the transport mechanisms of GAT1 and SERT (Lester et al., 1996). Partially because of this contrast between the GAT1 and SERT fluorescence signals, we have evaluated the SERT fluorescence signals in terms of a physiological state that occurs in SERT, but not in GAT1, rather than in terms of a mechanism or state thought to be common between the two transporters. This unique state is the voltage-dependent transient current.

GAT1 and SERT share the property that they are inactivated (at GAT1-Cys-74 and SERT-Cys-109) by MTSET (Yu et al., 1998) but not by rhodamine derivatives (Li et al., 2000). A reasonable explanation would be that the bulkier, rhodamine group is added charge at a lower density and is therefore not directly occluding the permeation pathway. In addition, sulforhodamine is zwitterionic, so that no net charge is added to the protein.

CONCLUSIONS

These hypotheses about the significance of the SERT fluorescence signals are reasonable interpretations of our data, but alternative explanations are not yet excluded. Regardless of the detailed interpretations, we believe that our observations do establish the principle that substrate- and voltage-dependent movements of the SERT proteins (both rSERT and hSERT) do occur and that the voltage-dependent movements are faster than previously described events. We believe that more detailed schemes should be evaluated after experiments with additional labels and additional labeled residues.

We thank Yanhe Tong for making the C109A mutant of hSERT.

This research was supported by National Institutes of Health Grant DA-019121 and by a National Research Service Award to Ming Li.

REFERENCES

Adkins, E. M., E. L. Barker, and R. D. Blakely. 2001. Interactions of tryptamine derivatives with serotonin transporter species variants implicate transmembrane domain I in substrate recognition. *Mol. Pharmacol.* 59:514–523.

Barker, E. L., H. L. Kimmel, and R. D. Blakely. 1994. Chimeric human and rat serotonin transporters reveal domains involved in recognition of transporter ligands. *Mol. Pharmacol.* 46:799–807.

Barker, E. L., K. R. Moore, F. Rakhshan, and R. D. Blakely. 1999. Transmembrane domain I contributes to the permeation pathway for serotonin and ions in the serotonin transporter. *J. Neurosci.* 19: 4705–4717.

Buck, K. J., and S. G. Amara. 1994. Chimeric dopamine-norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4-phenylpyridinium. *Proc. Natl. Acad. Sci. U.S.A.* 91:12584–12588.

Cao, Y., M. Li, S. Mager, and H. A. Lester. 1998. Amino acid residues that control pH modulation of transport-associated current in mammalian serotonin transporters. *J. Neurosci.* 18:7739–7749.

Cao, Y., S. Mager, and H. A. Lester. 1997. H^+ permeation and pH regulation at a mammalian serotonin transporter. *J. Neurosci.* 17: 2257–2266.

Cha, A., and F. Bezanilla. 1997. Characterizing voltage-dependent conformational changes in the *Shaker* K^+ channel with fluorescence. *Neuron*. 19:1127–1140.

Cha, A., and F. Bezanilla. 1998. Structural implications of fluorescence quenching in the *Shaker* K^+ channel. *J. Gen. Physiol.* 112:391–408.

Chen, J. G., S. Liu-Chen, and G. Rudnick. 1997a. External cysteine residues in the serotonin transporter. *Biochemistry.* 36:1479–1486.

Chen, J. G., S. Liu-Chen, and G. Rudnick. 1998. Determination of external loop topology in the serotonin transporter by site-directed chemical labeling. *J. Biol. Chem.* 273:12675–12681.

Chen, J. G., A. Sachpatzidis, and G. Rudnick. 1997b. The third transmembrane domain of the serotonin transporter contains residues associated with substrate and cocaine binding. *J. Biol. Chem.* 272:28321–28327.

Giros, B., Y. M. Wang, S. Suter, S. B. McLeskey, C. Pifl, and M. G. Caron. 1994. Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. *J. Biol. Chem.* 269:15985–15988.

Kamdar, G., K. M. Penado, G. Rudnick, and M. M. Stephan. 2001. Functional role of critical stripe residues in transmembrane span 7 of the serotonin transporter. Effects of Na^+, Li^+, and methanethiosulfonate reagents. *J. Biol. Chem.* 276:4038–4045.

Kitayama, S., S. Shimada, H. X. Xu, L. Markham, and D. M. Donovan. 1992. Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. *Proc. Natl. Acad. Sci. U.S.A.* 89:7782–7785.

Lee, S. H., M. Y. Chang, K. H. Lee, B. S. Park, Y. S. Lee, and H. R. Chin. 2000. Importance of valine at position 152 for the substrate transport and 2β-carbomethoxy-3β-(4-fluorophenyl)tropane binding of dopamine transporter. *Mol. Pharmacol.* 57:883–889.

Lester, H. A., Y. Cao, and S. Mager. 1996. Listening to neurotransmitter transporters. *Neuron.* 17:807–810.

Li, M., R. A. Farley, and H. A. Lester. 2000. An intermediate state of the γ-aminobutyric acid transporter GAT1 revealed by simultaneous voltage clamp and fluorescence. *J. Gen. Physiol.* 115:491–508.

Lin, F., H. A. Lester, and S. Mager. 1996. Single-channel currents produced by the serotonin transporter, and analysis of a mutation affecting ion permeation. *Biophys. J.* 71:3126–3135.

Loo, D. D., B. A. Hirayama, E. M. Gallardo, J. T. Lam, E. Turk, and E. M. Wright. 1998. Conformational changes couple Na^+ and glucose transport. *Proc. Natl. Acad. Sci. U.S.A.* 95:7789–7794.

Mager, S., C. Min, D. J. Henry, C. Chavkin, B. J. Hoffman, N. Davidson, and H. A. Lester. 1994. Conducting states of a mammalian serotonin transporter. *Neuron.* 12:845–859.

Mannuzzu, L. M., M. M. Moronne, and E. Y. Isacoff. 1996. Direct physical measure of conformational rearrangement underlying potassium channel gating. *Science.* 271:213–216.

Ni, Y. G., J. G. Chen, A. Androutsellis-Theotokis, C. J. Huang, E. Moczydlowski, and G. Rudnick. 2001. A lithium-induced conformational change in serotonin transporter alters cocaine binding, ion conductance, and reactivity of Cys-109. *J. Biol. Chem.* 276:30942–30947.

Quick, M. W., and H. A. Lester. 1994. Methods for expression of excitability proteins in *Xenopus* oocytes. *In* Ion Channels of Excitable Cells. T. Narahashi, editor. Academic Press, San Diego. 261–279.

Smicun, Y., S. D. Campbell, M. A. Chen, H. Gu, and G. Rudnick. 1999. The role of external loop regions in serotonin transport. Loop scanning mutagenesis of the serotonin transporter external domain. *J. Biol. Chem.* 274:36058–36064.

Yu, N., Y. Cao, S. Mager, and H. A. Lester. 1998. Topological localization of cysteine 74 in the GABA transporter, GAT1, and its importance in ion binding and permeation. *FEBS Lett.* 426:174–178.