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H^+ Permeation and pH Regulation at a Mammalian Serotonin Transporter

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The rat serotonin transporter expressed in *Xenopus* oocytes displays an inward current in the absence of 5-HT when external pH is lowered to 6.5 or below. The new current differs from the leakage current described previously in two ways. (1) It is ~10-fold larger at pH 5 than the leakage current at pH 7.5 and reaches 1000 H^+/sec per transporter at extremes of voltage and pH with no signs of saturation. (2) It is selective for H^+ by reversal potential measurements. Similar H^+-induced currents are also observed in several other ion-coupled transporters, including the GABA transporter, the dopamine transporter, and the Na^+/glucose transporter. The high conductance and high selectivity of the H^+-induced current suggest that protons may be conducted via a hydrogen-bonded chain (a "proton-wire mechanism") formed at least partially by side chains within the transporter. In addition, pH affects other conducting states of rat serotonin transporter. Acidic pH potentiates the 5-HT-induced, transport-associated current and inhibits the hyperpolarization-activated transient current. The dose–response relationships for these two effects suggest that two H^+ binding sites, with pK_a values close to 5.1 and close to 6.3, govern the potentiation of the 5-HT-induced current and the inhibition of the transient current, respectively. These results are important for developing structure-function models that explain permeation properties of neurotransmitter transporters.

Key words: protons; sodium; 5-HT; serotonin; SERT; Xenopus oocyte

Neurotransmitter transporters play a key role in synaptic transmission mediated by monoamines and amino acids. After neurotransmitter is released and activates receptors, the transporters help to terminate synaptic transmission by transporting neurotransmitters across the plasma membrane of neurons and, in some cases, of nearby glia (Lester et al., 1994). Recent electrophysiological studies have shown that many neurotransmitter transporters give rise to electrical currents that are comparable, both in magnitude and in unitary properties, to ion-channel currents (Lester et al., 1996; Sonders and Amara, 1996). Thus, neurotransmitter transporters may also participate in intercellular signaling processes in the neuron system.

The serotonin transporter (SERT) is an important target for antidepressants, appetite suppressants, and drugs that decrease obsessive–compulsive behavior. As a result, considerable research has been focused on biochemical and pharmacological properties of the transporter. Most of the earlier work has relied on measurements of radiolabeled substrate flux, on the binding of radiolabeled antagonists and, to some extent, on equilibrium substrate ratios. These studies have led to a classical model in which one Na^+ and one Cl^- are transported with each positively charged 5-HT molecule, and in which one K^+ is countertransported (Rudnick and Clark, 1993). K^+ may play a role in facilitating the return of the unloaded transporter. However, K^+ is not absolutely necessary for 5-HT influx; protons can compete with K^+ and, to some extent, fulfill the requirement for a countertransported cation (Keyes and Rudnick, 1982). Furthermore, an artificially imposed pH gradient (acidic inside) can serve as the sole driving force for 5-HT transport when no other driving forces are present (Keyes and Rudnick, 1982). At the Na^+/glucose transporter (SGLT) protons can substitute for external Na^+ in driving inward transport of glucose (Hirayama et al., 1994).

The classical model also predicts zero net charge movement across the membrane during a transport cycle. However, recent data suggest that other permeation pathways exist at SERT that bypass the substrate-coupled steps. Voltage-clamp recordings (Mager et al., 1994) have revealed three unexpected, but mechanistically important, currents associated with the rat SERT (rSERT) expressed in *Xenopus* oocytes: (1) the 5-HT- and Na^+-dependent transport-associated current, (2) the substrate-independent leakage current, and (3) the substrate-independent, hyperpolarization-activated transient current. The existence of these currents contradicts the stoichiometry prediction and suggests the existence of additional charge movement pathways within the SERT. In searching for these pathways, Lin et al. (1996) found elementary currents that resemble single-channel events of ion channels in oocytes expressing the SERT. The existence of these elementary currents provided a semiquantitative explanation for the observed macroscopic transport-associated and leakage currents. These studies have provided some insights into molecular mechanisms of the transport process.

In the present study, we report another unexpected current associated with the rSERT and several other ion-coupled transporters. This current in rSERT–cRNA-injected oocytes is carried exclusively by H^+, independent of substrate, and separate from the leakage current described previously (Mager et al., 1994). In addition, we report that pH differentially affects other currents associated with the SERT. Acidic pH dramatically potentiates the 5-HT-induced, transport-associated current, but inhibits the 5-HT-independent transient current. We believe that these results

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will provide additional tools to dissect different aspects of neurotransmitter transporter function.

MATERIALS AND METHODS

cRNA and Xenopus *oocytes.* cRNAs were transcribed *in vitro* from linearized plasmids that carry cDNA clones for the rSERT (Hoffman et al., 1991), GABA (GAT1) (Guastella et al., 1990), glycine (GLYT1) (Guastella et al., 1992), bovine dopamine (DAT) (Usdin et al., 1991), or rabbit Na^+/glucose (SGLT) (Hediger et al., 1987) transporters. The rSERT and GAT1 cDNAs were subcloned into a modified pBluescript–SK vector that contains an alfalfa mosaic virus 5′-untranslated region upstream of the cloning site and a poly$(A)_{50}$ sequence downstream of the cloning site (Lin et al., 1996; Mager et al., 1996).

Stage V and VI oocytes were isolated as described (Quick and Lester, 1994) and injected with ~20 ng of mRNA in 50 nl of water. The injected oocytes were then incubated 3–7 d at 19°C for translation.

Electrophysiology. Voltage-clamp experiments were performed using the two-electrode voltage-clamp technique (Mager et al., 1994). Normal Na^+ Ringer's solution contains (in mM): 100 NaCl, 5 KCl, 1 $MgCl_2$, 5 HEPES, and 5 MES. Solution pH was adjusted with NaOH or HCl to values indicated in the text. For *N*-methyl-D-glucamine (NMDG) Ringer's solutions, the NaCl was replaced with NMDG-Cl. None of the solutions contained added Ca^{2+}. Solution changes were made with electrically operated valves (Auto-Mate Scientific, San Francisco, CA). All recordings were performed at room temperature (21°–22°C).

5-HT uptake. 5-HT uptake was measured by a 3 min incubation in 150 μl of Na^+ Ringer's solutions with various pH values (see above for solution composition). Longer incubation times up to 30 min were also tried and gave comparable results. Oocytes were washed once with the uptake solution before uptake began and 3 times after uptake ended. Oocytes were then solubilized in 2% SDS. The [^{3}H]5-HT uptake was determined by liquid scintillation counting.

RESULTS

H^+-induced current in the absence of 5-HT

When the pH of external Na^+ Ringer's solution was lowered to 6.5 or below for oocytes expressing rSERT and the membrane potential was held at −60 mV, we observed a reversible increased inward current of ~40 nA at pH 5.5 or ~150 nA at pH 4.5 (Fig. 1*A*). In some batches of oocytes with greater expression, the inward current exceeded 1 μA at pH 3.5 (data not shown). In uninjected oocytes, acidic external pH induced a much smaller inward deflection (usually <20 nA at pH 4.5) under the same recording conditions (Fig. 1*B*). This small inward deflection actually reflects the inhibition of the background K^+ conductance by H^+ (Woodward and Miledi, 1992) and can be reduced by holding membrane potentials closer to the K^+ equilibrium potential (−80 mV) (data not shown). Another endogenous H^+-induced current that is oscillatory and occurs via a Ca^{2+}-activated Cl^- channel (Woodward and Miledi, 1992) was minimized in this and all subsequent experiments by removal of external Ca^{2+}. Nevertheless, we have verified that the H^+-induced current in rSERT-injected oocytes was also observed in the presence of 1 mM Ca^{2+} (data not shown).

The dose–response relationship for the H^+-induced current in rSERT-injected oocytes showed a steep rising slope when external $[H^+]$ is <30 μM, pH >4.5, and a less steep rising slope when external $[H^+]$ is >30 μM, pH <4.5, (Fig. 1*C*). This dose–response relationship cannot be fit by a rectangular hyperbola. The H^+-induced current did not seem to saturate even at pH 3.0, the lowest pH value tested (data not shown).

The H^+-induced current was blocked at least 70% by the SERT inhibitors desipramine (10 μM) and fluoxetine (10 μM) (Fig. 2*A*). The blockade was reversible, although the recovery from the fluoxetine blockade was much slower (time constant ~1 min) than that from desipramine blockade (time constant <10 sec). A similar pattern of slower reversibility from fluoxetine blockade than for desipramine blockade was observed in our previous experiments (Mager et al., 1994) and presumably arises from the much higher affinity of fluoxetine for rSERT. The two inhibitors had no effect on uninjected oocytes (data not shown).

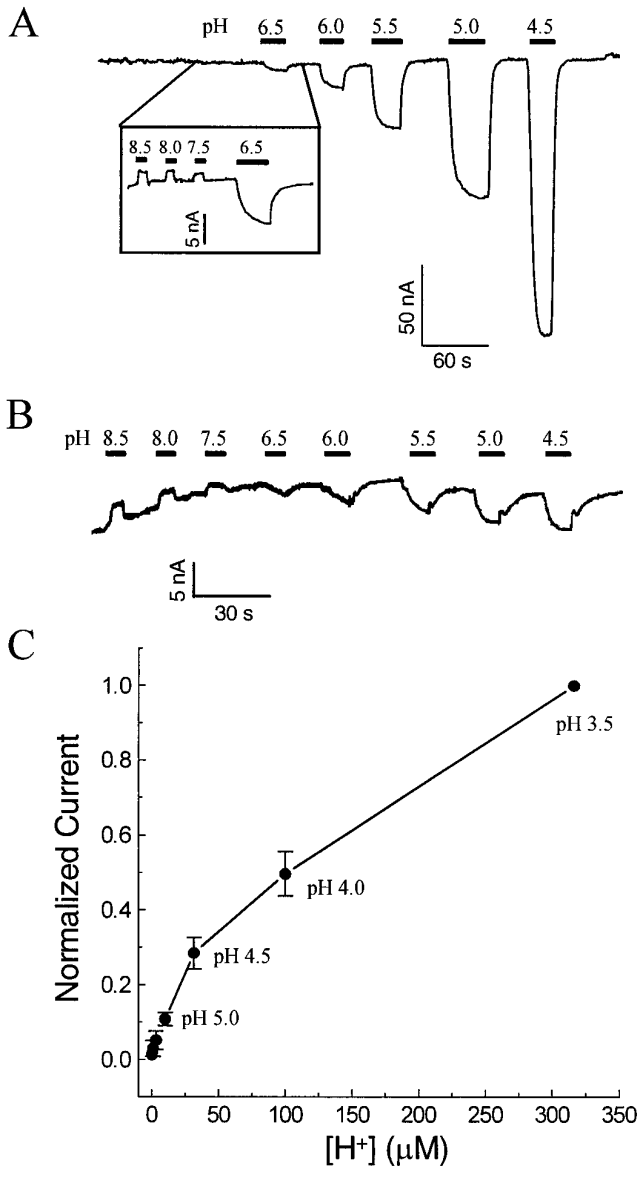

Figure 1. Acidic pH induces current in rSERT-injected and uninjected oocytes. *A*, *B*, Acidic pH induced current in an rSERT-injected and an uninjected oocyte, respectively. Holding potential, −60 mV. Base solution, Na^+ Ringer's solution, pH 7.0. Application of Na^+ Ringer's solutions with pH values other than 7.0 is indicated by *bars* above the current traces. *C*, Dose–response relationship for the pH-induced current in rSERT-injected oocytes. Current was normalized to the value recorded at pH 3.5 (632 ± 136 nA, mean ± SD, $n = 4$ oocytes). *Vertical bars* indicate the SD.

The H^+-induced current was present even in the absence of Na^+ (NMDG substitution) (Fig. 2*B*). Actually, the current amplitude in Na^+ Ringer's solution was only 30–50% of that in NMDG Ringer's solution from the same oocyte, indicating that Na^+ may partially inhibit the H^+-induced current. This result also suggests that Na^+ is not the carrier of the H^+-induced current.

To determine whether H^+ carries the current, reversal potentials were measured at three different external pH values (5.5, 6.0, and 6.5) (Fig. 3). It was first necessary to decrease the internal

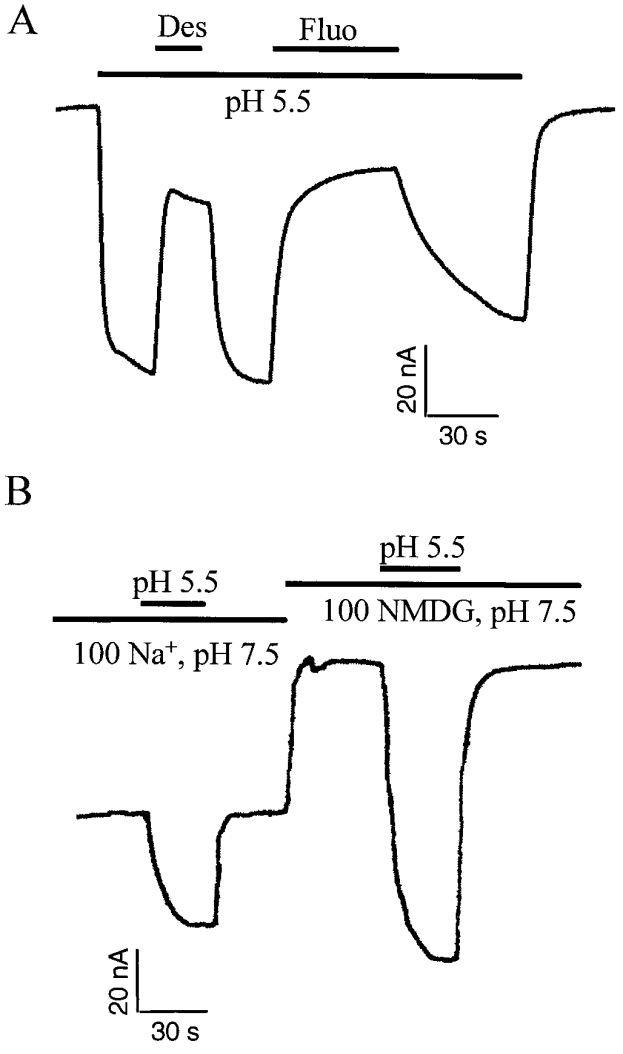

Figure 2. Inhibition of the H^+-induced current. *A*, The H^+-induced current in an rSERT-injected oocyte was inhibited by the SERT inhibitors desipramine (10 μM) and fluoxetine (10 μM). Holding potential, −40 mV. Base solution, Na^+ Ringer's solution, pH 7.5. *B*, The H^+-induced current was partially inhibited in Na^+ Ringer's solution compared with the current in NMDG Ringer's solution. Holding potential, −40 mV.

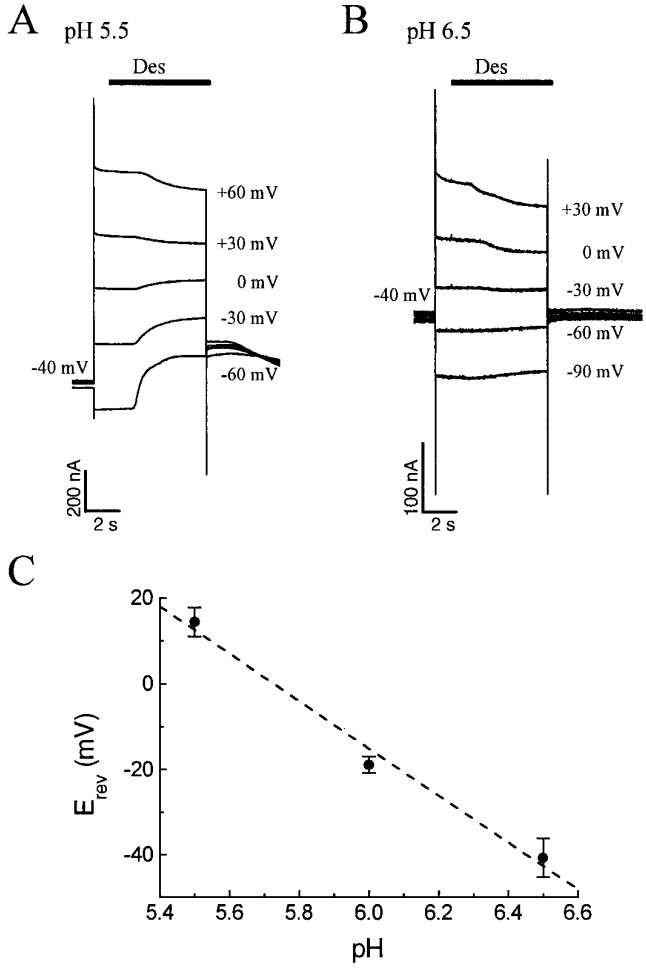

Figure 3. Reversal potential of the H^+-induced current. *A*, *B*, Reversal potential measurements in NMDG Ringer's solutions at pH 5.5 and 6.5, respectively. The membrane potential was held at −40 mV and jumped for 6 sec to the test potentials noted adjacent to each current trace. The *bars* above current traces indicate the period when desipramine (10 μM) was applied. *C*, Reversal potentials measured by experiments shown above were plotted as a function of pH. *Vertical lines* indicate the SD (n = 3). *Dashed line* is the least-square fit, with a slope of −55 mV/pH U.

[H^+] to a value giving an experimentally accessible proton reversal potential. We therefore incubated oocytes in an acidic (pH 5.5) Na^+-free (NMDG substitution) Ringer's solution for 30–60 min to prevent endogenous H^+/Na^+ exchange. This method has been shown to effectively lower the oocyte internal pH (Sasaki et al., 1992). We then transferred oocytes into a recording chamber, held the oocyte membrane at −40 mV, and then applied a series of 6 sec steps to various test potentials. During each voltage step, desipramine (10 μM) was added between the second and sixth second. Examples of current traces recorded at pH 5.5 and 6.5 are shown in Figure 3, *A* and *B*, respectively. The recordings were performed in Na^+-free solutions and within a short time (<5 min for each oocyte) to minimize the internal pH change during an experiment. Desipramine inhibits the H^+-induced current and thus causes a deflection of current traces. At external pH 5.5, the deflection reversed direction at +14 ± 3 mV, whereas at pH 6.5, the reversal potential was −41 ± 5 mV (mean ± SD, n = 3). The desipramine-sensitive reversal potential versus pH relationship is shown in Figure 3*C*. There was a 55 mV shift in reversal potential per pH unit change. This value is close to the expected value (58 mV) for a pure H^+-selective current, indicating that the H^+-induced current is indeed carried by H^+. Thus, we called this current H^+-leakage current.

To study additional details of the voltage dependence and time course of the H^+-leakage current, we conducted voltage-jump relaxation experiments on oocytes with normal internal pH (~7.6) (Sasaki et al., 1992) (Fig. 4). We isolated the pure desipramine-sensitive H^+-leakage current by subtracting the current recorded at pH 5.0 and in the presence of 10 μM desipramine (Fig. 4*A*) from the total current recorded at pH 5.0 with no desipramine (Fig. 4*B*). The subtracted current traces (shown in Fig. 4*C*) showed that the H^+-leakage currents reached a new steady state more rapidly than the settling time of our voltage-clamp circuit (~3 msec) and were then maintained for the 600 msec duration of the test pulses. (The inactivation at −140 mV is an artifact of instability in the control trace and was not reproduced in other experiments.) Under conditions that produced large H^+-leakage current (e.g., cells with greatest expression or very low external pH), we have noted modest time-dependent decreases in H^+-

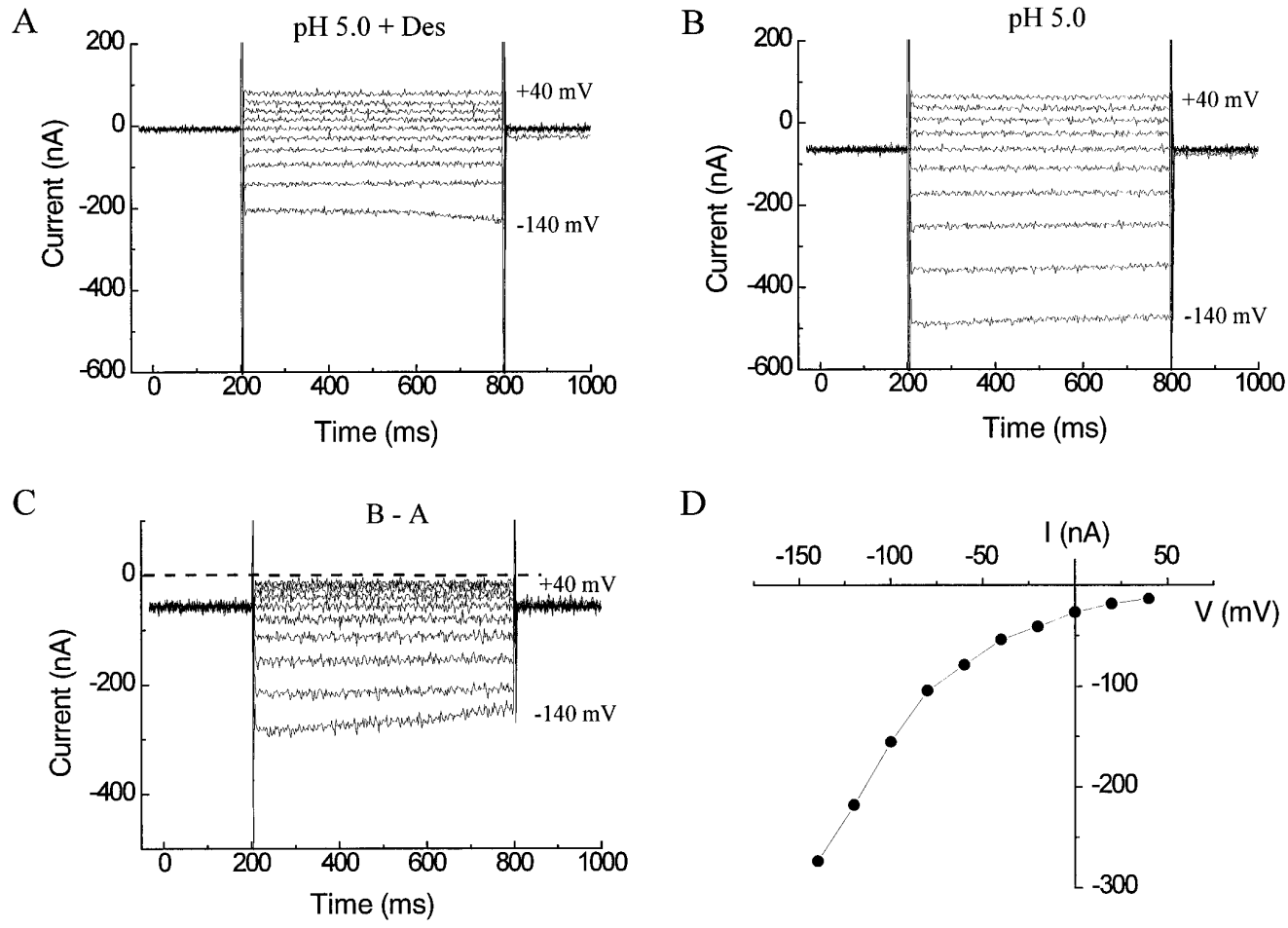

Figure 4. Current–voltage relationship and voltage-jump relaxation kinetics for the H^+-induced current. *A*, *B*, Voltage-clamp recordings from an rSERT-injected oocyte perfused with NMDG Ringer's solution, pH 5.0, in the presence (*A*) or absence (*B*) of 10 μM desipramine. The membrane potential was held at −40 mV and then shifted for 600 msec to a series of test potentials ranging from −140 mV to +40 mV in 20 mV increments. *C*, Pure H^+ leakage current obtained by subtracting *A* from *B*. *Dashed line* is at zero subtracted current. *D*, Steady-state currents obtained from *C* were plotted as a function of membrane potential. Currents were averaged from the first 50 msec at the test potential.

leakage current, but we have not studied such decreases systematically.

Under the conditions of Figure 4 ($pH_i \approx 7.6$, $pH_o = 5.0$), reversals at experimentally accessible membrane potentials were neither expected nor observed. In the current–voltage relationship averaged over the first 50 msec at the test potential (Fig. 4*D*), the inward H^+-leakage current increases more than linearly with the driving force. This nonlinear current–voltage relationship differentiates the H^+-leakage current from most other voltage-activated H^+ channel currents (DeCoursey and Cherny, 1994).

H^+ increases the 5-HT-induced, transport-associated current

Next, we examined the effect of H^+ on the 5-HT-induced (transport-associated) current. Oocytes were perfused sequentially with Na^+ Ringer's solutions with pH values ranging from 8.5 to 4.5. During each solution perfusion, 5-HT (5 μM) was added (10–30 sec, shown as *short bars* in Fig. 5*A*) to induce the transport-associated current. When pH was >6.5, the amplitude of the 5-HT-induced current was almost independent of external pH. However, when pH was ≤6.5, we observed not only the H^+-leakage current as described above, but also an increase (by up to 20-fold at pH 4.5) in the 5-HT-induced current (Fig. 5*A*). This increased current is not caused by changes in the protonation state of 5-HT itself; the pK_a for the amino group is 9.8, and the molecule is therefore fully protonated at all pH values studied in our experiments. As an additional argument that pH changes affect the transporter rather than the substrate, we note that this increased 5-HT-induced current was not observed in the closely related human SERT (hSERT) (data not shown).

The amplitude of the low-pH-potentiated, 5-HT-induced current was not always proportional to that of the leakage current. Occasionally, for some unknown reason, the H^+-leakage current was so large that adding 5-HT actually decreased the total inward current (data not shown). This result indicates that 5-HT may inhibit the H^+-leakage current while inducing the transport-associated current. In fact, 5-HT did inhibit the H^+-leakage current when external Na^+ was replaced by NMDG (Fig. 5*B*). Thus, we take the combination of the H^+-leakage current and the low-pH-potentiated component of the 5-HT-induced current as the total transport-associated current. In fact, the amplitude of this combined current was less variable among oocytes than that of each of the two currents alone. The dose–response relationship for this total transport-associated current is shown in Figure 5*C*. Unlike the H^+-leakage current, the total transport-associated current tends to saturate, displaying a typical Michaelis–Menten relationship with an EC_{50} of 7.8 ± 1.4 μM H^+, pH 5.1 ± 0.1, and

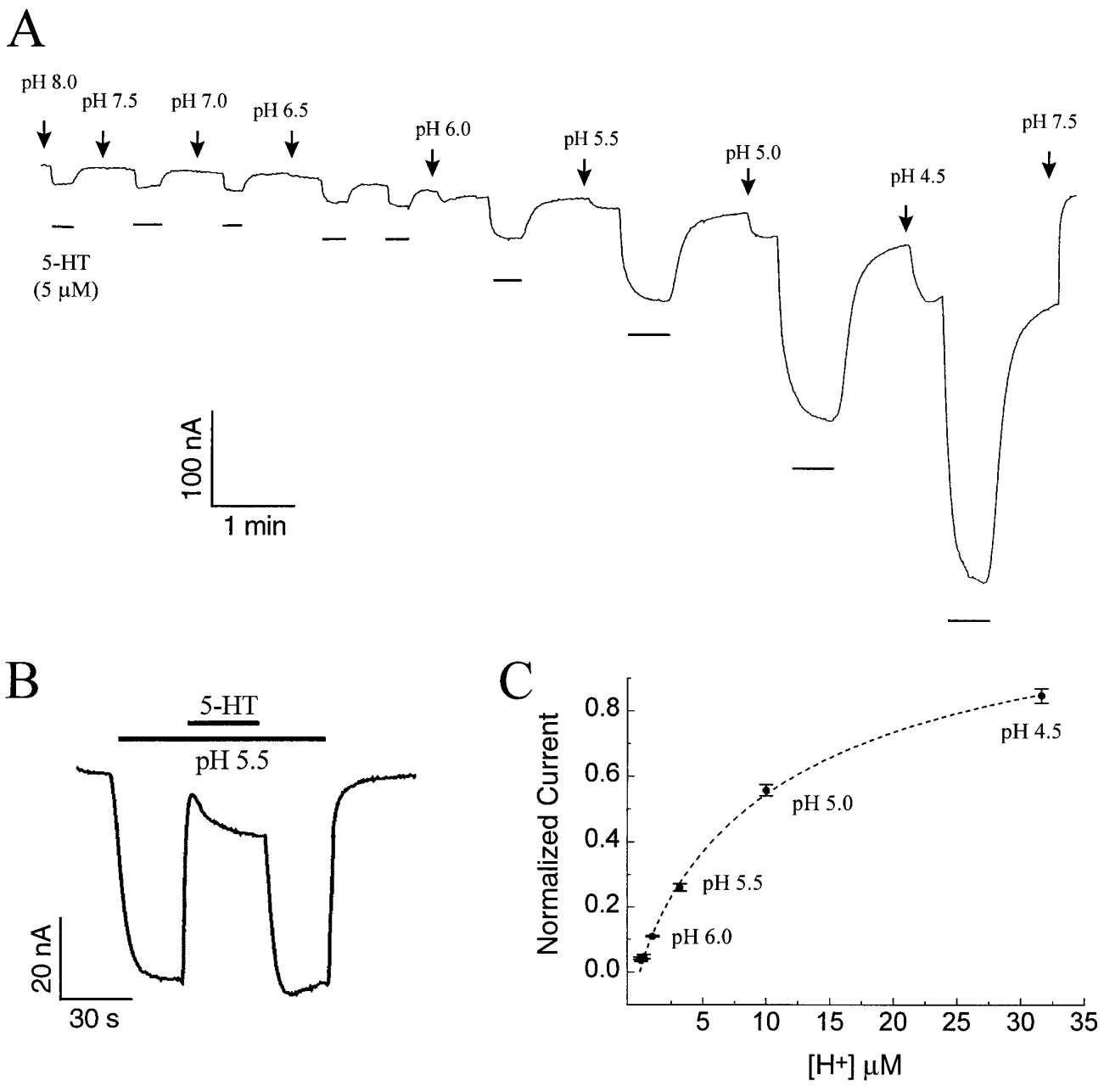

Figure 5. Acidic pH potentiates the 5-HT-induced, transport-associated current. *A*, 5-HT-induced current recorded in Na^+ Ringer's solutions with various pH values. Solution changes are indicated by *arrows* above the current trace. 5-HT (5 μM) applications are indicated by *bars* under the current trace. Holding potential, −40 mV. *B*, 5-HT (5 μM) does not induce the transport-associated current, but rather inhibits the H^+ leakage current in the absence of Na^+ (NMDG substitution). Holding potential, −40 mV. Base solution, NMDG Ringer's, pH 7.5. *C*, Dose–response relationship for the total transport-associated current. The H^+leakage current and the 5-HT-induced current at each pH value were combined. This combined current was normalized to the maximal current obtained after a nonlinear regression fitting to the Hill equation (*dashed line*). EC_{50} = 7.8 ± 1.4 μM H^+, pH 5.1 ± 0.1, Hill coefficient n = 1.1 ± 0.1 (mean ± SD). *Vertical lines* indicate the SD (n = 4 oocytes).

a Hill coefficient of 1.1 ± 0.1 (mean ± SD, n = 4). This indicates that there may be a single H^+ binding site responsible for potentiating the transport-associated current.

To determine the ionic basis of the H^+-potentiated, transport-associated current, we sought to measure the reversal potential. Interestingly, the 5-HT-induced current was inward over the entire range of membrane potentials and pH values tested (−160 to +40 mV, pH 5.5–7.5), even when external [Na^+] was reduced to 50 mM, so that E_{Na} is approximately +20 mV. In our previous macroscopic and single-channel measurements at pH 7.5, in which the 5-HT-induced current is carried mostly by Na^+, we also observed no reversal (Mager et al., 1994; Lin et al., 1996). We have not systematically investigated the mechanism for this inward rectification of the transport-associated current; but at present, we can make no firm conclusions about the current carrier(s) (Na^+ and/or H^+) for the component of the transport-associated current that is potentiated by H^+.

We also tested pH effects on [^{3}H]5-HT uptake. At pH 5.5, the [^{3}H]5-HT uptake was undetectably different from the value at pH 7.5 (Fig. 6), despite the fact that the transport-associated current

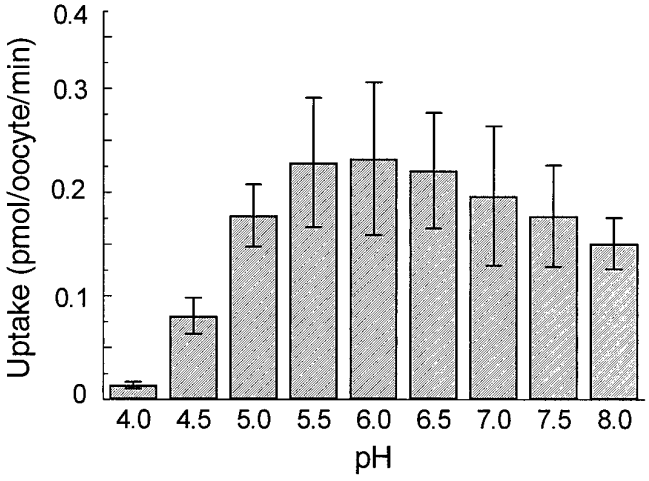

Figure 6. Effect of pH on [^{3}H]5-HT uptake. Final [^{3}H]5-HT concentration, 1 μM. *Vertical lines* show SD in measurements from 6 oocytes. Similar results were obtained in at least three separate batches of oocytes.

was increased by more than fivefold compared with that at pH 7.5 (Fig. 5*A*). Neither the EC_{50} nor the V_{max} for uptake were affected (data not shown). One possible explanation for the different effects of pH on the 5-HT-induced current and the 5-HT uptake could be that the current was measured under voltage-clamp conditions, whereas the uptake was not. This is unlikely to account for the more than fivefold differences, however, because our previous data show that 5-HT uptake is independent of membrane potentials in the range of −30 to −80 mV (Mager et al., 1994). Our results indicate that 5-HT uptake does not vary directly with the size of the transport-associated current, consistent with the notion that the single-channel openings, which underlie the transport-associated current, do not represent an obligatory step in the normal transport cycle (Lin et al., 1996). At pH <5.0, the 5-HT uptake decreased dramatically (Fig. 6). This may arise from intracellular accumulation of Na^+/H^+ or from other sequelae of the large leakage and transport-associated currents.

H^+ inhibits the transient current

In the absence of 5-HT, voltage jumps to negative potentials in oocytes expressing rSERT induce transient inward current. This transient current is greatly enhanced if a depolarizing prepulse is applied before the test pulse (Mager et al., 1994). To test whether H^+ affects this transient current, we performed voltage-jump experiments in Na^+ Ringer's solutions with various pH values ranging from 8.0 to 4.5. In the experiment shown in Figure 7*A*, the membrane potential was held at −40 mV. The voltage was first jumped to +60 mV, then to −140 mV, and then to +60 mV again. Current traces from the same oocyte, but at various pH values, were superimposed. The data show that lowering external pH gradually inhibited the transient current, with nearly complete inhibition at pH 5.0. At pH 4.5, much of the transient current remained inhibited, but because the H^+-leakage current became obvious at this pH, we actually observed an increased steady-state inward current. The inhibition of the transient current was reversible; returning the pH to 7.5 at the end of the experiment recovered all the transient current (Fig. 7*A*, compare the two pH 7.5 traces). Figure 7*B* shows traces in which the remaining current at pH 5.0 was subtracted from all other traces recorded at pH >5.0. The dose–response relationship for the inhibition of the peak transient current is shown in Figure 7*C*. These data were fitted to

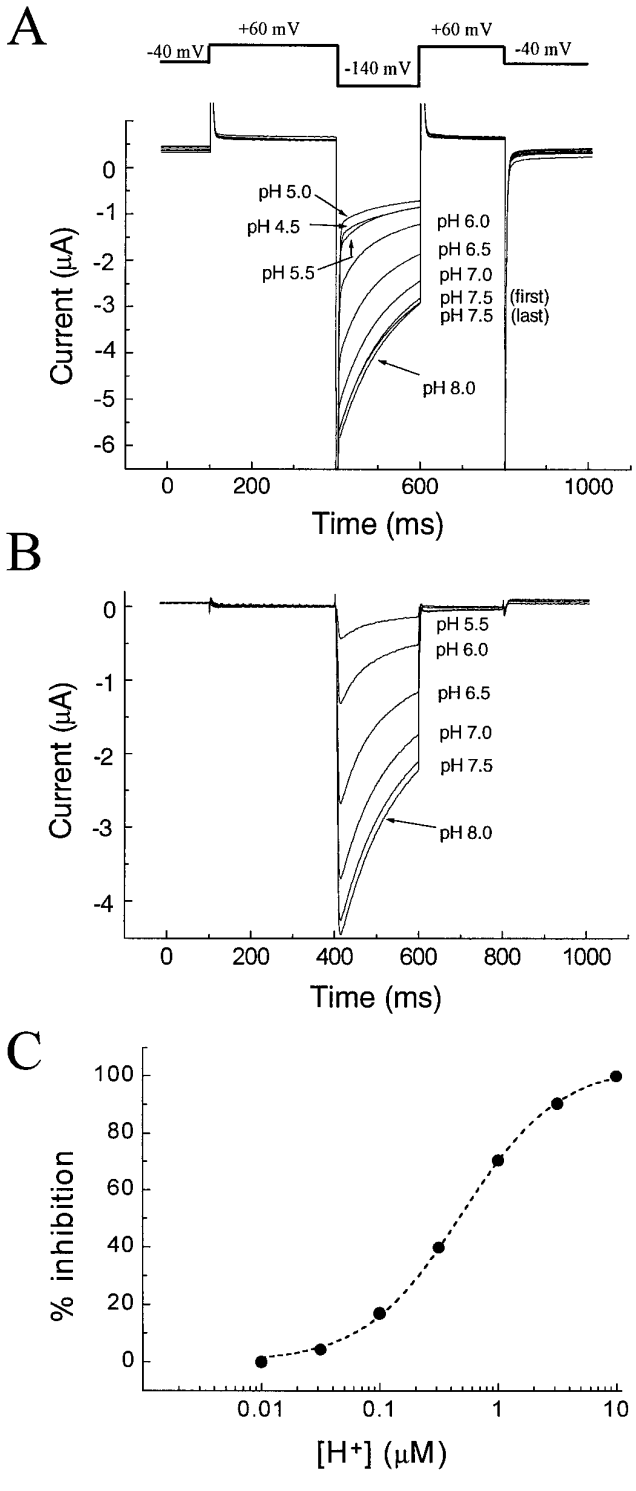

Figure 7. Protons inhibit the transient current. *A*, Superimposed current traces recorded in Na^+ Ringer's solutions with various pH values. Holding potential, −40 mV. During each trial, the oocyte membrane potential was jumped to +60 mV, −140 mV, and +60 mV (protocol is at *top*). The pH 7.5 solution was tested at both the beginning and the end of the experiment. *B*, Traces after subtracting the current remaining at pH 5.0 from all other currents recorded at pH >5.0. *C*, The peak of transient current in *B* was plotted as a function of $[H^+]$. Data were fitted by nonlinear regression to the Hill equation (*dashed line*). EC_{50} = 0.49 ± 0.02 μM H^+, pH 6.31 ± 0.02, Hill coefficient n = 1.06 ± 0.04 (mean ± SD, n = 4 oocytes).

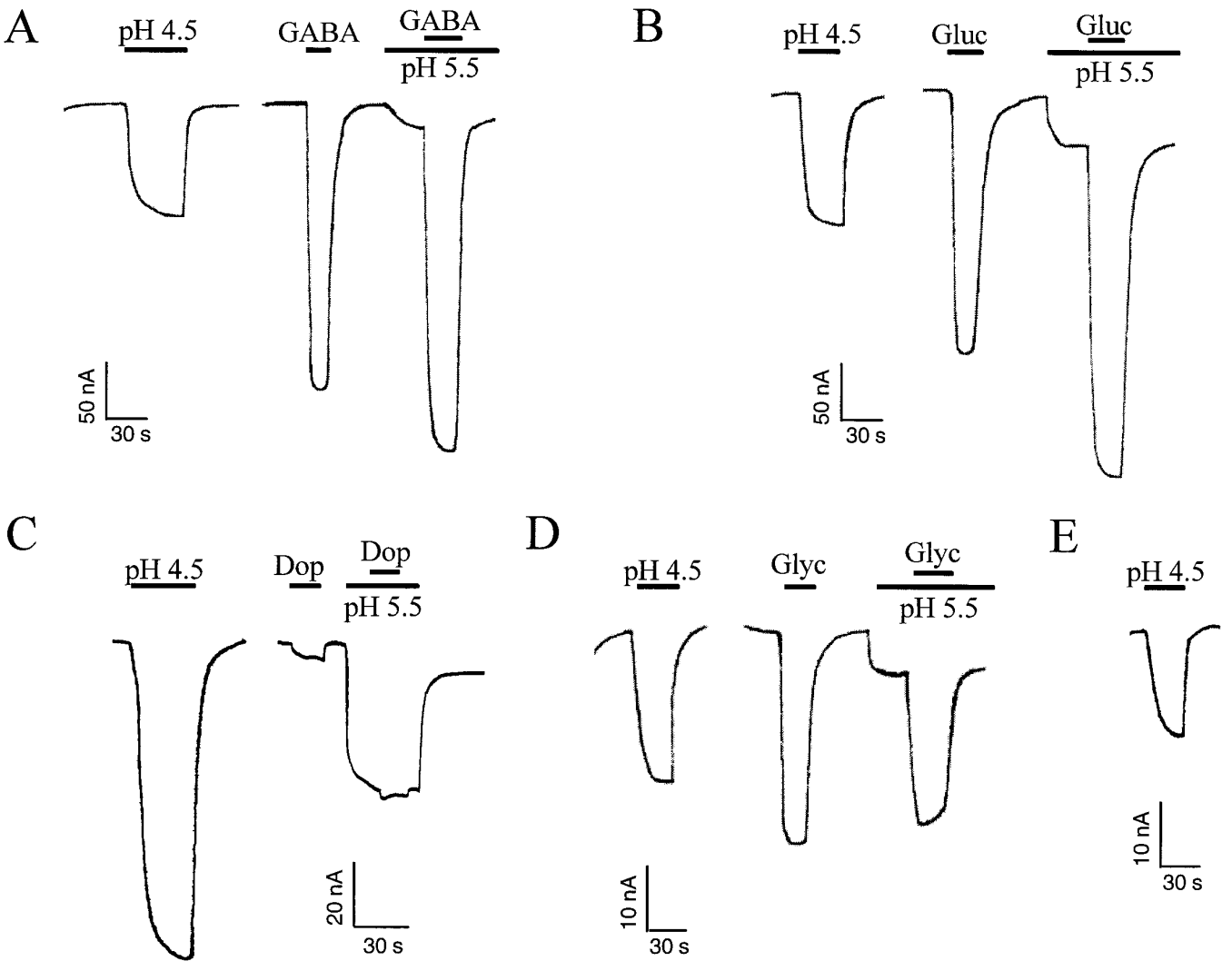

Figure 8. Effect of pH on other transporters. Current traces were recorded from oocytes injected with cRNA for GABA (GAT1) (*A*), Na^+/glucose (SGLT) (*B*), dopamine (DAT) (*C*), or glycine (GLYT1) (*D*) transporters, or from an uninjected oocyte from the same batch (*E*). Holding potential, −40 mV. Base solution, NMDG Ringer's solution, pH 7.5 (*left panels* in *A–D*, and *E*), or Na^+ Ringer's solution, pH 7.5 (*right panels* in *A–D*). Concentrations of organic substrates were *GABA*, 100 μM; glucose (*Gluc*), 1 mM; dopamine (*Dop*), 10 μM; glycine (*Glyc*), 100 μM.

the Hill equation (*dashed line*) with an EC_{50} of 0.49 ± 0.02 μM H^+, pH 6.31 ± 0.02, and a Hill coefficient (n) of 1.06 ± 0.04 (mean ± SD, $n = 4$ oocytes), suggesting that a single H^+ binding site governs inhibition of the transient current and that this binding site differs from that governing potentiation of the transport-associated current (EC_{50} at pH 5.1).

Effect of H^+ on other transporters

We surveyed H^+ effects on several other transporters; the rat GABA (GAT1) (Guastella et al., 1990) and glycine (GLYT1) (Guastella et al., 1992), the bovine dopamine (DAT) (Usdin et al., 1991), and the rabbit Na^+/glucose (SGLT) (Hediger et al., 1987) transporters (Fig. 8). All except the glycine transporter displayed a significantly increased inward current at pH 4.5 in the absence of Na^+ and organic substrates (Fig. 8*A–D*, *left panels*) (for comparison, the same pH 4.5-induced current in an uninjected oocyte from the same batch is shown in Fig. 8*E*; notice the scale changes in Fig. 8*C–E*). The exceptionally large proton permeability in DAT has also been reported (Sonders et al., 1997). Thus, proton currents are a common, but not universal, feature of Na^+-coupled transporters.

However, unlike the case for rSERT-injected oocytes, protons produced little or no potentiation of the transport-associated current in GAT1- and SGLT-injected oocytes (Fig. 8*A*,*B*, *right panels*). This lack of potentiation at SGLT occurred despite the fact that protons support glucose transport by SGLT (Hirayama et al., 1994). Protons partially inhibited the transport-associated current in GLYT1-injected oocytes (Fig. 8*D*, *right panel*). The dopamine transport-associated current was too small for systematic measurements, but there was no marked potentiation at low pH (Fig. 8*C*, *right panel*). Thus, robust H^+ enhancement of the transport-associated current is unique to rSERT among the transporters surveyed.

DISCUSSION

Précis of proton effects on the SERT

Our data show several distinct effects of protons at rSERT expressed in *Xenopus* oocytes. (1) There is a bona fide substrate-independent proton leakage current. This result is rather general, in the sense that we observe it also for the GABA, dopamine (independently reported by Sonders et al., 1997), and Na^+/glu-

cose transporters. Because 5-HT and uptake blockers inhibit this current, we favor the hypothesis that the proton permeation pathway is at least partially congruent with the 5-HT pathway. (2) Low pH potentiates the 5-HT-induced, transport-associated current by up to 20-fold. The current carrier (Na^+ vs H^+) is uncertain, because no reversal was observed. This potentiation does not appear to generalize to hSERT and other transporters we have surveyed. (3) Low pH also inhibits the transient voltage-dependent current. Because the transient current has been reported only for SERT, we cannot comment on the generality of this effect.

Possible mechanisms of H^+ permeation in the absence of 5-HT

Our data indicate the existence of a proton leakage current expressing oocytes of ~40 nA at pH 5.5 and 600 nA at pH 3.5 at −60 mV. Combined with an expression level of 76 fmol per oocyte (measured previously from oocytes with transport-associated currents comparable with those of the present experiments) (see Mager et al., 1994), we estimated that ~20 protons/sec flow through each transporter at pH 5.5 at −60 mV and ~300 protons/sec at pH 3.5, where the current still shows no signs of saturation with $[H^+]$ (Fig. 1). The current is three times higher at −140 mV (Fig. 4) and again shows no saturation with membrane potential, so that maximum H^+ flux is at least 1000/sec. It is not surprising to observe proton permeation through SERT, because it has already been shown that H^+ can replace K^+ in countertransporting 5-HT and that a H^+ gradient alone can serve as the driving force for serotonin accumulation (Keyes and Rudnick, 1982). What is surprising is the amplitude of the H^+-leakage current, the extremely high selectivity for H^+, and the existence of this current even in transporters that do not normally require K^+/H^+ for transport.

Macroscopic proton currents present a general challenge for modern biophysics, because the current carriers are present at such low concentrations (DeCoursey and Cherny, 1994). What are the expected molecular components of such currents at SERT? Recent electrophysiological studies have shown that many Na^+-dependent transporters display a leakage current in the absence of substrates at normal pH (Umbach et al., 1990; Mager et al., 1994, 1996; Galli et al., 1995; Kanai et al., 1995; Vandenberg et al., 1995; Sonders et al., 1997); however, those Na^+ leakage currents have many qualitative differences from the H^+ leakage current studied here. The existence of a leakage current in rSERT at normal pH has been attributed to the spontaneous opening (~2 msec duration) of single channels at a very low rate (one per ~700 sec) (see Lin et al., 1996). We have sought, and failed to find, single-channel recordings in oocyte membrane patches exposed to low pH, Na^+-free solutions ($n = 5$, from two batches of oocytes) (F. Lin and Y. Cao, unpublished observations). This is hardly surprising, because unitary proton currents in biological membranes have thus far escaped direct detection but are thought to have amplitudes on the order of ~10 fA at pH 5, corresponding to diffusion-limited access to a pore of diameter ~5 Å (DeCoursey and Cherny, 1994). If channel-like currents of this magnitude do underlie the proton leakage conductance, then the open probability P_o at pH 5.5 would equal $\sim 10^{-3}$, a factor of some 10^3 greater than P_o for the single-channel events recorded by Lin et al. (1996). Furthermore, we have to assume that protons also alter the ionic pathway so that the new pathway is almost exclusively permeable to H^+.

If, on the other hand, the protons permeate singly and constantly at each transporter, the measured rates can be explained by a scheme that does not necessarily rely on protein conformational changes, but relies on intrinsic physical properties of protons and water. It has been well documented that the mobility of H^+ in bulk solution is five- to sevenfold higher than that of other biologically relevant cations because of its ability to hop along transient hydrogen-bonded clusters of water molecules (Robinson and Stokes, 1965). This type of H^+ conduction mechanism has been proposed for H^+-conducting channels that have a water-filled pore such as the gramicidin A channel (Myers and Haydon, 1972; Deamer and Nichols, 1989; DeCoursey and Cherny, 1994; Pomès and Roux, 1996). Thus, H^+ mobility within the pore could be substantially greater than that of other cations. Furthermore, a variety of amino acid side chains in membrane proteins could form networks of hydrogen bonds that would efficiently transport H^+ (Nagle and Morowitz, 1978). In such a scenario, only protons but not other ions could jump across the membrane. This explains how the current is extremely selective for H^+. This model evidently bears a resemblance to recent formulations of ion-coupled transporters as pores that allow single-file diffusion of substrates with minimal conformational changes (Su et al., 1996).

Nature of the H^+-potentiated transport-associated current

At neutral pH and in the presence of Na^+, 5-HT induces an inward current that is not part of a transport cycle (Mager et al., 1994). This current is carried mostly by Na^+ and has been attributed to the spontaneous opening of a second conducting state at the single-channel level (Lin et al., 1996). At acidic pH, we observed an increased 5-HT-induced current. The enhancement, by up to 20-fold, is not accompanied by increased [^{3}H]5-HT flux, further increasing the quantitative mismatch between charge entry and substrate flux (Mager et al., 1994) and providing yet more evidence for the inadequacy of the classic stoichiometric model.

The increased transport-associated current is different from the H^+-leakage current, because the former (1) adds to the H^+-leakage current and (2) depends on Na^+. At present, we cannot formally exclude the possibility that H^+ at least partially carries the increased transport-associated current, because the reversal potential measurements were unsuccessful. However, in the most straightforward explanation, H^+ binding at a certain amino acid side chain changes the property of the transport-associated pathway and perhaps increases the channel open probability and thus increases the transport-associated current. The side chain involved can be characterized partially by the fact that the dose–response curve for the H^+-potentiated transport-associated current resembles a saturable Michaelis–Menten-type curve with an EC_{50} at pH 5.1. The increased transport-associated current seems a likely candidate for additional single-channel studies.

Inhibition of the transient current

Voltage jumps to high negative potentials induce a transient current carried by Na^+. Because low pH inhibits this current but potentiates the transport-associated current, we have another reason to believe that these two currents represent distinct states of rSERT (Mager et al., 1994). The inhibition by pH may become a diagnostic tool in future single-channel studies on the nature of the transient current.

Significance of H^+ permeation and pH regulation

Several homologous Na^+-dependent transporters display the H^+-leakage current in the absence of their own substrates (Fig. 8). Such a conserved feature may suggest some important functions. However, in rSERT, the H^+-leakage current becomes obvious only when the external pH is <6.5. This pH value is not normally seen in brain tissues under physiological conditions. Under certain pathophysiological conditions such as ischemia, brain tissue pH could fall below 6.5 (Csiba et al., 1983). Billups and Attwell (1996) showed that a 1 U acid shift of external pH inhibited transporter-mediated release of glutamate. This inhibition plays an important role in preventing the neuronal damage during transient ischemia. Whether acidic pH also inhibits reversed uptake of other neurotransmitters is not known, but at least the forward uptake of 5-HT is not inhibited at pH values between 5.5 and 7.5. The H^+-leakage current and/or the H^+-potentiated transport-associated current may also add to detrimental effects of acidosis such as disturbed ionic fluxes and cell swelling that many cells experienced after traumatic brain injury (Hovda et al., 1992).

A number of channels that conduct monovalent cations also conduct H^+ at low pH. These include the gramicidin A channel (Hladky and Haydon, 1972), voltage-gated Na^+ channels (Mozhayeva and Naumov, 1983), and amiloride-sensitive Na^+ channels (Gilbertson et al., 1992) (for review, see DeCoursey and Cherny, 1994). On the other hand, several ion-coupled transporters that conduct H^+ as shown in this study also display channel-like conductance for monovalent cations (Cammack et al., 1994; Mager et al., 1994; Cammack and Schwartz, 1996; Galli et al., 1996; Mager et al., 1996). These data raise the interesting question of whether H^+ permeation is an inherent feature of nonselective channels. Perhaps these channels and transporters have water-filled pores in which protons permeate by a water-wire mechanism. However, the aquaporin CHIP28 water channel, which is obviously a water-filled pore, was not detectably permeable to H^+ and other cations (Zeidel et al., 1994). Thus, it is still too early to draw a conclusion on the mechanism of H^+ conduction. However, this study will provide some tools to address these questions for transporters.

Although proton permeation may be a widespread feature of Na^+-coupled transporters, other effects of low pH–potentiation of the transport-associated current and inhibition of the transient current–seem specific to the SERT and, therefore, may each be governed by just one or a few residues. It is now a reasonable goal to locate these residues. Sequence alignment among various ion-coupled transporters and site-directed mutagenesis will be appropriate for this purpose. We believe that the results of such studies will help us develop a more detailed structure-function picture that describes the permeation pathway of ion-coupled transporters.

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