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Elizabeth M. Klamo

Mark E. Drew

Scott M. Landfear

Michael Kavanaugh
University of Montana - Missoula

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Kinetics and Stoichiometry of a Proton/*myo*-Inositol Cotransporter*

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Elizabeth M. Klamo‡, Mark E. Drew§, Scott M. Landfear§, and Michael P. Kavanaugh‡¶

From the ‡Vollum Institute and §Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201

Voltage clamp recording was used to measure steady-state and presteady-state currents mediated by a *myo*-inositol transporter cloned from *Leishmania donovani* and expressed in *Xenopus* oocytes. Application of *myo*-inositol resulted in inward currents, which did not require external sodium and which were increased by increasing the extracellular proton concentration and by membrane hyperpolarization. Alkalinization of the extracellular space occurred concomitantly with *myo*-inositol influx. Correlation of membrane currents with radiolabeled *myo*-inositol flux revealed that one positive charge is translocated with each molecule of *myo*-inositol, consistent with cotransport of one proton. The transport concentration dependence on both species suggested ordered binding of a proton followed by a molecule of *myo*-inositol. In the absence of *myo*-inositol, a voltage-dependent capacitance was observed that correlated with the transporter expression level. This charge movement obeyed a Boltzmann function, which was used to estimate a turnover of 0.70 ± 0.06 s^{-1} at −60 mV. The pH and voltage dependence of the charge movements were simulated with a model involving alternating access of internal and external protons to sites within an occluded pore.

Secondary transporters drive concentrative solute flux by utilizing the energy stored in the electrochemical gradients of cotransported ions (Crane, 1977). A central question in transport biophysics concerns the mechanism by which cotransported ions and substrate interact with the transporter to achieve this coupling. While secondary transporters of lower organisms most commonly utilize a transmembrane proton gradient, the evolution and diversification of transport systems in higher organisms has led to utilization of additional ion gradients including those for Na^+, K^+, and Cl^-. Some transporters are capable of utilizing multiple ion gradients, suggesting the possibility of common functional mechanisms that may be conserved among different transporter types. For example, nominally Na^+-dependent glucose transport can be energized by a proton gradient in the absence of external Na^+ (Hoshi *et al.*, 1986; Hirayama *et al.*, 1994). Similarly, the melibiose transporter from *Escherichia coli* is capable of utilizing Na^+ or H^+ (Niiya *et al.*, 1982). In addition, the amino acid transporter, CAT-1, is capable of either Na^+-dependent or Na^+-independent facilitated uptake, depending on the charge on the amino acid transported (Christensen *et al.*, 1969; Wang *et al.*, 1991).

Statistical analysis of predicted amino acid sequences has been used to identify five major gene families within an ancient superfamily of proton-coupled and facilitated transporters, which possess a structural motif predicted to contain 12 transmembrane spanning regions (Marger and Saier, 1993). Recently a genomic DNA clone (D1) related to a family of genes encoding facilitated and proton-coupled hexose transporters was cloned from the parasitic protozoan *Leishmania donovani* and demonstrated to encode a transporter for *myo*-inositol (Drew *et al.*, 1995). The present work uses a combination of isotope flux, voltage-clamp current recording, and pH measurements in order to characterize the kinetics and stoichiometry of *myo*-inositol transport. Protons are shown to be selectively co-transported with *myo*-inositol with a 1:1 stoichiometry. Evidence is presented for ordered binding of a proton followed by *myo*-inositol binding and translocation, and charge movements are observed in the absence of *myo*-inositol that are suggested to reflect this proton binding.

MATERIALS AND METHODS

Transporter Expression and Electrophysiology—Capped cRNA was transcribed from cDNA encoding the *L. donovani myo*-inositol transporter, MIT[1] (Drew *et al.*, 1995). 50 ng was then microinjected into stage V-VI *Xenopus* oocytes, and transport assays were performed 2–5 days later. The frog Ringer's solution used for recording contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and was buffered with 5 mM Na-HEPES (pH 7.5), MES-HEPES (pH 6.0–7.0), or HEPES-Tris (pH 8.0–8.5). In sodium substitution experiments, equimolar choline was substituted for sodium. Recording microelectrodes contained 3 M KCl and had resistances between 0.2 and 1.0 megohm. Two-electrode voltage clamp recordings were performed at 20 °C using a GeneClamp 500 amplifier interfaced to a computer by a Digidata 1200 A/D controlled by pCLAMP software 6.0 (Axon Instruments). Current signals were digitized at 2–5 kHz and low pass-filtered at 1 kHz. Unless indicated, steady-state currents were normalized to the current elicited by application of 1 mM *myo*-inositol at pH 7.5 in the same cells. The kinetic parameters K^s_m and I^s_{max} for either protons or for *myo*-inositol were obtained by least squares fitting the substrate induced current, I, to the Michaelis-Menten equation: $I = I_{max}[S]/(K_m+[S])$, where S represents either *myo*-inositol or protons. Charge movements associated with the transient currents were determined from the product of a fitted exponential relaxation time constant and the corresponding peak current amplitude. The charge was plotted as a function of voltage and fitted by least squares to a Boltzmann equation: $Q = Q_{tot}/1 + \exp(e_o z\delta(V_m - V_{0.5})/kT) + Q_{offset}$, where Q_{tot} is the total charge movement, V_m is the membrane potential, $V_{0.5}$ is the potential at which half the charge has moved, Q_{offset} is the offset that depended on the holding potential, $z\delta$ is the product of the valence of the charge and the fraction of the field through which it moves, e_o is the elementary charge, k is the Boltzmann constant, and T is the absolute temperature. Numerical simulations of kinetic models were generated using SCoP software (Simulation Resources Inc.).

myo-[^{3}H]Inositol Flux—Oocytes expressing MIT were voltage-clamped at −80 mV, and currents were recorded during a 200-s superfusion with 100 μM *myo*-[^{3}H]inositol (1 mCi/ml, DuPont NEN). After a

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¶ To whom correspondence should be addressed: L-474, Vollum Institute, Oregon Health Sciences University, Portland, OR 97201. Tel.: 503-494-4601; Fax: 503-494-2285; E-mail: kavanaug@ohsu.edu.

[1] The abbreviations used are: MIT, *myo*-inositol transporter; MES, 2-(*N*-Morpholino)ethanesulfonic acid.

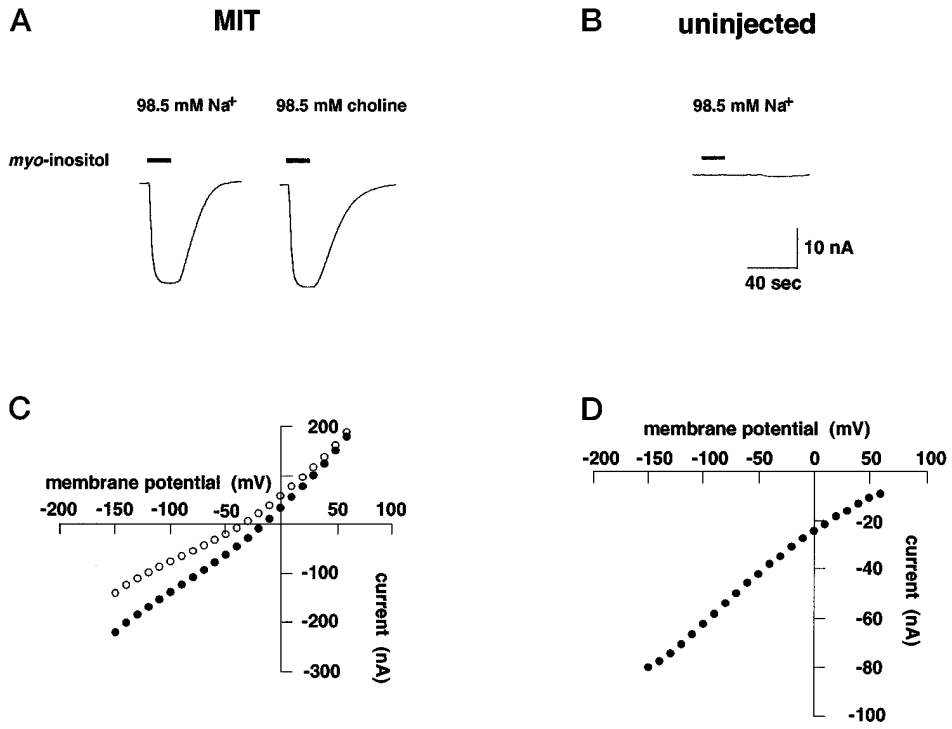

FIG. 1. *A*, membrane currents associated with *myo*-inositol uptake are shown for a representative oocyte expressing MIT voltage-clamped at −60 mV and superfused with 1 mM *myo*-inositol for the duration indicated by the *bar*. The magnitude of the *myo*-inositol induced inward current was unchanged in the absence of Na^+. *B*, no currents were induced by applying 1 mM *myo*-inositol to uninjected oocytes. *C*, current-voltage relationships for an oocyte expressing MIT in Ringer's solution with (*filled symbols*) and without (*open symbols*) 1 mM *myo*-inositol. Subtraction of the latter current from the former reveals the voltage dependence of the steady-state *myo*-inositol transport current (*D*).

20-s wash, oocytes were transferred to scintillation vials, solubilized with 1% SDS, and counted to determine the influx of radiolabeled *myo*-inositol. The total charge translocated during the *myo*-[^{3}H]inositol superfusion was calculated from the current-time integral and correlated with the radiolabel flux measured for each oocyte.

pH Measurements—Measurements of extracellular proton concentrations were made with a pH-sensitive electrode (NMPH3, World Precision Instruments) interfaced to a MacLab A/D converter (AD Instruments). The electrode response was calibrated by superfusion with standard solutions of known pH. Oocyte responses were recorded in sodium-free Ringer's with 1 μM HEPES-Tris, pH 7.5. The pH electrode was positioned immediately adjacent to the oocyte membrane, the bath flow was stopped, and transport was activated in the presence of *myo*-inositol by a voltage jump from +20 to −80 mV while simultaneously recording pH.

RESULTS

Ionic Dependence and Transport Stoichiometry—Inward currents were elicited upon application of *myo*-inositol to voltage-clamped oocytes 2–5 days after injection of the MIT cRNA. These inward currents were independent of external Na^+, as substitution of equimolar choline had no effect on the current amplitudes (n = 3; Fig. 1). The effect of membrane potential on the steady-state currents induced by *myo*-inositol was examined by comparing the currents recorded at the end of a 500-ms command pulse to potentials between −150 and +60 mV in the presence and absence of *myo*-inositol (Fig. 1*C*). The difference between these currents revealed that the inward current induced by *myo*-inositol was increased at more negative potentials (Fig. 1*D*). The *myo*-inositol-induced current approached zero but did not reverse polarity at potentials up to +60 mV (Fig. 1*D*).

Since *myo*-inositol is uncharged at physiological pH, this result suggested that the transporter may utilize the electrochemical gradient of an ionic species other than sodium to drive *myo*-inositol transport. Compounds that dissipate the proton gradient such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone inhibit MIT-mediated uptake of *myo*-inositol, suggesting that protons may be a co-substrate of the transporter (Drew *et al.*, 1995). To directly test this hypothesis, a pH-sensitive electrode positioned next to the oocyte membrane was used to monitor changes in the extracellular proton concentration that accompanied *myo*-inositol influx under voltage-clamp conditions. Measurements were made during voltage jumps from +20 mV to −80 mV, a membrane potential change expected to increase transport approximately 3-fold (Fig. 1). Consistent with the hypothesis of proton symport, hyperpolarizing voltage jumps in the presence of *myo*-inositol resulted in an alkalinization of the extracellular space (Fig. 2*A*). This alkalinization did not occur when the oocyte membrane was stepped from +20 to −80 mV in solution without *myo*-inositol (Fig. 2*A*; n = 4) or in uninjected oocytes (n = 4) under either of the conditions (Fig. 2*B*). In 4 oocytes expressing MIT, the mean pH change caused by activation of *myo*-inositol influx was 0.045 ± 0.001.

To determine the stoichiometry of proton/*myo*-inositol symport, oocytes were voltage-clamped at −80 mV and the current induced during a 200-s superfusion of 100 μM *myo*-[^{3}H]inositol was recorded. After a brief washout, oocytes were removed and radioactivity counted to measure the amount of *myo*-inositol taken up. The total charge translocated during the *myo*-[^{3}H]inositol application was calculated from the current-time integral, and the quantity of charge transferred was correlated with the radiolabel flux in six oocytes (Fig. 3). Linear regression analysis yielded a slope of 96,648 coulomb/mol of *myo*-inositol, indicating that 1.002 elementary charges are translocated per molecule of *myo*-inositol (96,648/Faraday's constant; r = 0.94). Because *myo*-inositol is uncharged at the pH used in these measurements, the results indicate a transport stoichiometry of 1 proton:1 molecule of *myo*-inositol.

Steady-state Kinetics—The influences of *myo*-inositol concentration, pH, and membrane potential on the steady-state transport rate were studied by systematically varying each param-

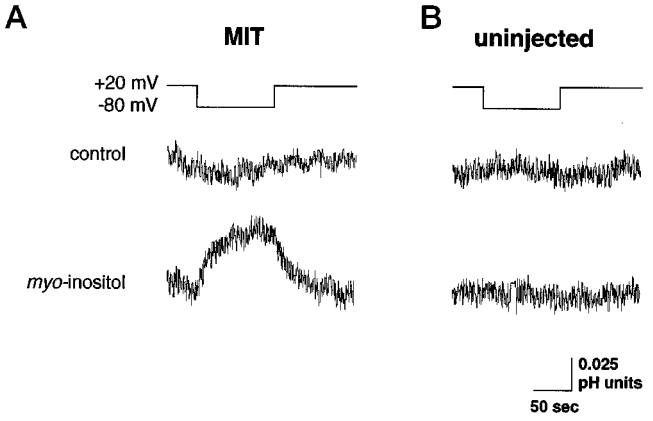

FIG. 2. ***myo*-Inositol influx is associated with alkalinization of the extracellular space.** *Traces* show the response measured by a pH electrode placed adjacent to a representative oocyte expressing MIT (*A*) or an uninjected oocyte (*B*) when the membrane potential is stepped from +20 mV to −80mV in control solution or in the presence of 1 mM *myo*-inositol as indicated. Similar results were seen in 4 oocytes.

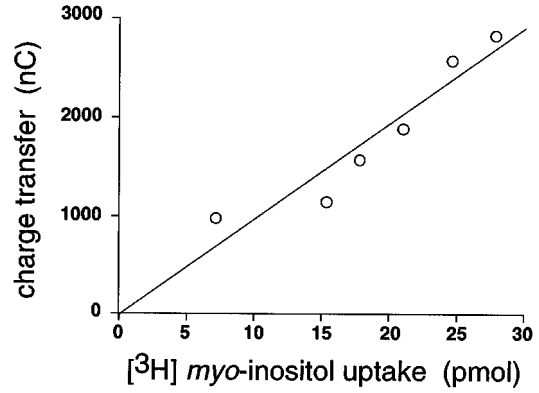

FIG. 3. **Charge coupling stoichiometry determined by correlation of current-time integrals and [^{3}H]-*myo*-inositol uptake in six oocytes expressing different quantities of MIT.** The *line* represents least squares linear regression fit of the data points. The slope of the fit is 96,648 C/mol (1.002 *F*), suggesting that 1 elementary charge is translocated per *myo*-inositol transport cycle.

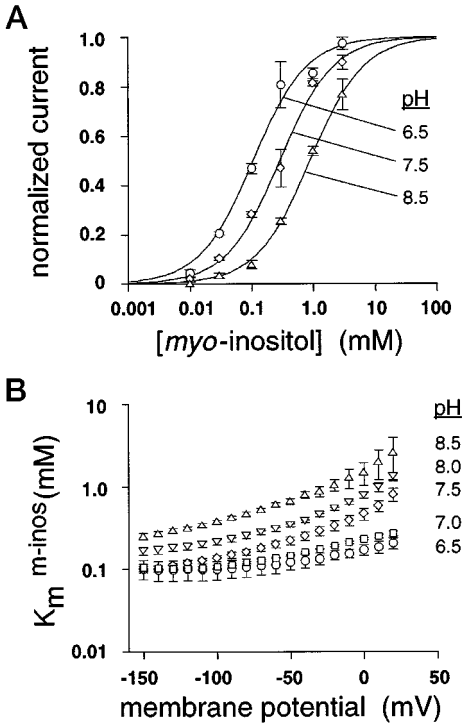

FIG. 4. **Influence of pH on the kinetic parameters $K_m^{\text{m-inos}}$ and $I_{\text{max}}^{\text{m-inos}}$.** *A*, *myo*-inositol concentration-response curves at fixed pH values between 6.5 and 8.5; *curves* shown are least squares fit of the data (mean ± S.E. n = 3–4) to the Michaelis-Menten equation with K_m values 95 μM (pH 6.5), 334 μM (pH 7.5), and 1.4 mM (pH 8.5). Currents were normalized to the $I_{\text{max}}^{\text{m-inos}}$ at pH 7.5 in the same cells (membrane potential = −30 mV). Varying the pH did not result in a statistically significant change in I_{max}. *B*, effect of membrane potential on the K_m for *myo*-inositol at different proton concentrations.

eter. At any given pH between 6.5 and 8.5, application of varying concentrations of *myo*-inositol resulted in current responses that were well fitted by the Michaelis-Menten equation (Fig. 4). The apparent affinity for *myo*-inositol increased with increasing $[H^+]_{out}$; the $K_m^{\text{m-inos}}$ ranged from 95 μM at pH = 6.5 to 1.4 mM at pH = 8.5 (V_m = −30 mV). There was no significant change in the $I_{\text{max}}^{\text{m-inos}}$ as a function of pH over this range (Fig. 4*A*). At a given pH, the transporter displayed an increased apparent affinity for *myo*-inositol at more negative potentials (Fig. 4*B*). The $K_m^{\text{m-inos}}$ appeared to approach a limiting value with membrane hyperpolarization, and this saturation occurred at less negative potentials as the proton concentration was raised (Fig. 4*B*), suggesting the possibility of a voltage-dependent transporter interaction with protons (see below).

The proton concentration dependence of the steady-state transport current was also well fitted by the Michaelis-Menten equation (Fig. 5*A*). Analogous to the effect of increasing $[H^+]_{out}$ on the apparent *myo*-inositol affinity, increasing $[myo\text{-inositol}]_{out}$ increased the apparent affinity for protons. The $K_m^{H^+}$ ranged from 490 nM at 10 μM *myo*-inositol to 4 nM at 1 mM *myo*-inositol (V_m = −30 mV). In contrast to the lack of effect of $[H^+]_{out}$ on $I_{\text{max}}^{\text{m-inos}}$, $I_{\text{max}}^{H^+}$ was strongly dependent on $[myo\text{-inositol}]_{out}$ (Fig. 5*C*). The $I_{\text{max}}^{H^+}$ increased at negative membrane potentials, but it became markedly less voltage-dependent with decreasing *myo*-inositol concentration, suggesting that with limiting concentrations of *myo*-inositol, the rate-limiting step in transport becomes voltage-independent (Fig. 5*C*).

Presteady-state Currents—In the absence of *myo*-inositol, oocytes injected with MIT cRNA displayed current relaxations following voltage jumps that persisted for tens of milliseconds after the time required to charge the membrane capacitance (Figs. 6*A* and 7*A*). These relaxations were not seen in uninjected oocytes (Fig. 6*A*), and they were unaffected by removal of Na^+ from the bathing solution (data not shown). These transient currents decayed completely by the end of a 500-ms voltage pulse, and the mean currents at the end of the pulse (corresponding to the steady-state leak) were not significantly different in oocytes expressing the transporter and in uninjected oocytes. In addition, no changes in pH were detected following voltage jumps in the absence of *myo*-inositol (Fig. 2), suggesting that the transporter does not mediate a significant uncoupled proton flux. The transient currents decayed exponentially with two time constants over a range of voltages from −180 to +90 mV. The faster component represented 45–75% of the charge movement and decayed with time constants ranging from 5 to 10 ms, while the slower component decayed with time constants between 40 and 80 ms. During voltage jumps in the presence of *myo*-inositol, the slow component of the transient current was selectively attenuated (Fig. 6*C*). The total charge movement during a voltage jump in the absence of *myo*-inositol was calculated for a range of test potentials. The charge movement at the onset of the voltage pulse (Q_{on}) was found to be equal and opposite to the charge movement at the return to the prepulse potential (Q_{off}), suggesting that the transient currents were capacitive in nature (Fig. 7, *inset*). The increased capacitance correlated with transporter expression levels as measured by *myo*-inositol-induced currents (r = 0.91; Fig. 7*B*). The

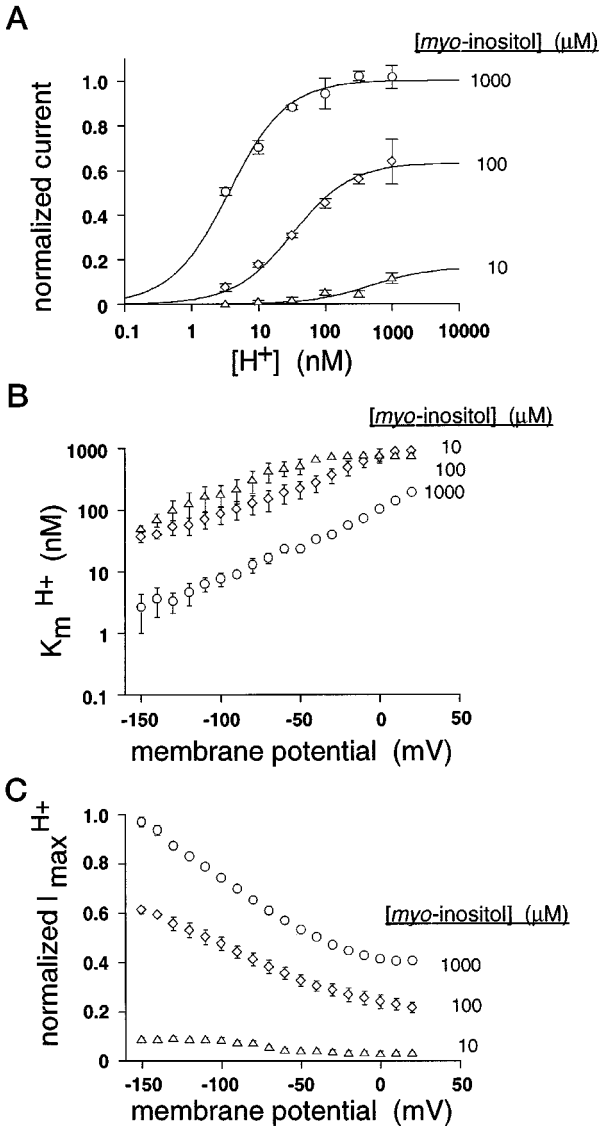

FIG. 5. ***myo*-Inositol influence on the kinetic parameters $K_m^{H^+}$ and $I_{max}^{H^+}$.** *A*, proton concentration dependence of currents at *myo*-inositol concentrations between 10 μM and 1000 μM obtained at −30 mV; *curves* shown are least squares fit of the data (mean ± S.E.; n = 3–4) to the Michaelis-Menten equation with K_m values of 490 nM at 10 μM, 32 nM at 100 μM, and 4 nM at 1000 μM *myo*-inositol. Currents were normalized to the maximal response obtained in the presence of 1 mM *myo*-inositol. *B*, effect of membrane potential on the apparent affinity for protons ($K_m^{H^+}$) at indicated concentrations of *myo*-inositol. *C*, effect of membrane potential on $I_{max}^{H^+}$. Data were normalized as in *A*. Data points represent the mean ± S.E. of the $I_{max}^{H^+}$ values obtained from Michaelis-Menten fits of concentration-response data in individual oocytes (n = 3–4).

charge movements depended on the membrane potential in a manner well described by a Boltzmann function. The total charge movement (Q_{tot}) in 5 oocytes expressing the transporter was 20.6 ± 1.2 nC. The voltage midpoint of the charge movement ($V_{0.5}$) was −110.0 ± 3.4 mV, and the slope factor of the function was 61.5 ± 3.7 mV, corresponding to an effective valence for the charge movement of 0.41.

Analysis of the charge movements in the absence of *myo*-inositol allows an estimation of the number of transporters, N, from the total charge movment (Q_{tot}) since $Q_{tot} = Ne_o z\delta$, where e_o is the elementary charge and z is the valence of the charge moving through a fraction δ of the electric field (Mager *et al.*, 1993; Wadiche *et al.*, 1995). The average number of transporters per oocyte was 3.20 ± 0.36 × 10^{11} (n = 5). Assuming a membrane surface area of 2.85 × 10^7 μm^{-2} (Wadiche *et al.*, 1995), this corresponds to a transporter density of 11,228 μm^{-2}. The transporter turnover rate can be directly calculated from the transporter density and the steady-state current measurements, since one elementary charge is translocated per molecule of *myo*-inositol (Fig. 3). The turnover rate in e_o s^{-1} is given by $I_{ss}/(Q_{tot}/z\delta)$, where I_{ss} is the steady-state current resulting from the application of 1 mM *myo*-inositol. This turnover rate was voltage-dependent, ranging from 0.49 ± 0.04 s^{-1} at −30 mV to 1.37 ± 0.13 s^{-1} at −150 mV.

Origin of Charge Movements—The transient currents could reflect ion binding to the transporter at sites within the electric field and/or charge movements associated with conformational transitions of the protein. The effect of membrane potential on the apparent affinity for protons suggests that the binding of the proton to a site on the transporter may be voltage-dependent (Figs. 4*B* and 5*B*). This voltage dependence might arise in several ways. First, an "ion well" effect could occur due to the proton traversing a fraction δ of the membrane field to reach its binding site (Läuger, 1991). In this case, changes in membrane potential would result in a change in the equilibrium dissociation constant (Woodhull, 1973). If this occurred, then the voltage midpoint ($V_{0.5}$) of the Boltzmann distribution should shift as a function of external proton concentration according to the equation: $[H^+]_1/[H^+]_2 = \exp[\delta F(V_1 - V_2)/RT]$, where $[H^+]_1$ and $[H^+]_2$ are the proton concentrations that give the corresponding $V_{0.5}$ values V_1 and V_2 (Wadiche *et al.*, 1995). Accordingly, using a value for δ = 0.41 (obtained from the slope of the Boltzmann fit of the charge movements at pH 7.5), a 10-fold decrease in $[H^+]_{out}$ should result in a −140 mV shift in $V_{0.5}$ of the charge movement (Fig. 8). To test this prediction, charge movements were measured at pH 8.5 and compared to the charge movements measured at pH 7.5 in the same oocytes. The observed shift in the $V_{0.5}$ was only −16 mV (Fig. 8), suggesting that the charge movements cannot be solely accounted for by external proton binding to a site in the electric field. Because poor control of intracellular pH precluded a direct test of varying $[H^+]_i$, the effect on the external pH dependence of the charge movements of ion binding at a cytoplasmic site were tested using a computer simulation. With a conventional ordered kinetic scheme, a cytoplasmic proton binding site within the electrical field was required to adequately describe the shift of the voltage midpoint of the charge movements with changing external pH (Fig. 8).

An alternative explanation for the origin of the transient currents in the absence of *myo*-inositol is a voltage- and pH-dependent "empty state" transition of the protein. Voltage-dependent state transitions have been postulated to occur in alternating access schemes for glucose (Parent *et al.*, 1992) and arginine transporters (Kavanaugh, 1993). Because global conformational transitions of proteins are likely to be more temperature-dependent than diffusion processes, the temperature dependence of the transient current kinetics was examined. Reducing the temperature from 21 °C to 10 °C resulted in greater than 85% reduction in the steady-state transport rate (Fig. 9). In contrast, the charge movement kinetics were significantly less affected; the relative amplitude of the slow component of the transient current decay was increased without slowing the time constants (τ_{fast} = 8.0 ± 0.3 ms and 8.3 ± 0.3 ms and τ_{slow} = 53.7 ± 6.0 ms and 31.4 ± 5.1 ms for a step from −30 to −150 mV at 21 °C and 10 °C, respectively; Fig. 9). These results imply that the rate-limiting step in the transport cycle is highly temperature-dependent ($q_{10} > 5$), and that the charge movements are not likely to reflect this slow (~1 s^{-1}) rate-limiting step because of their faster kinetics and lesser temperature dependence.

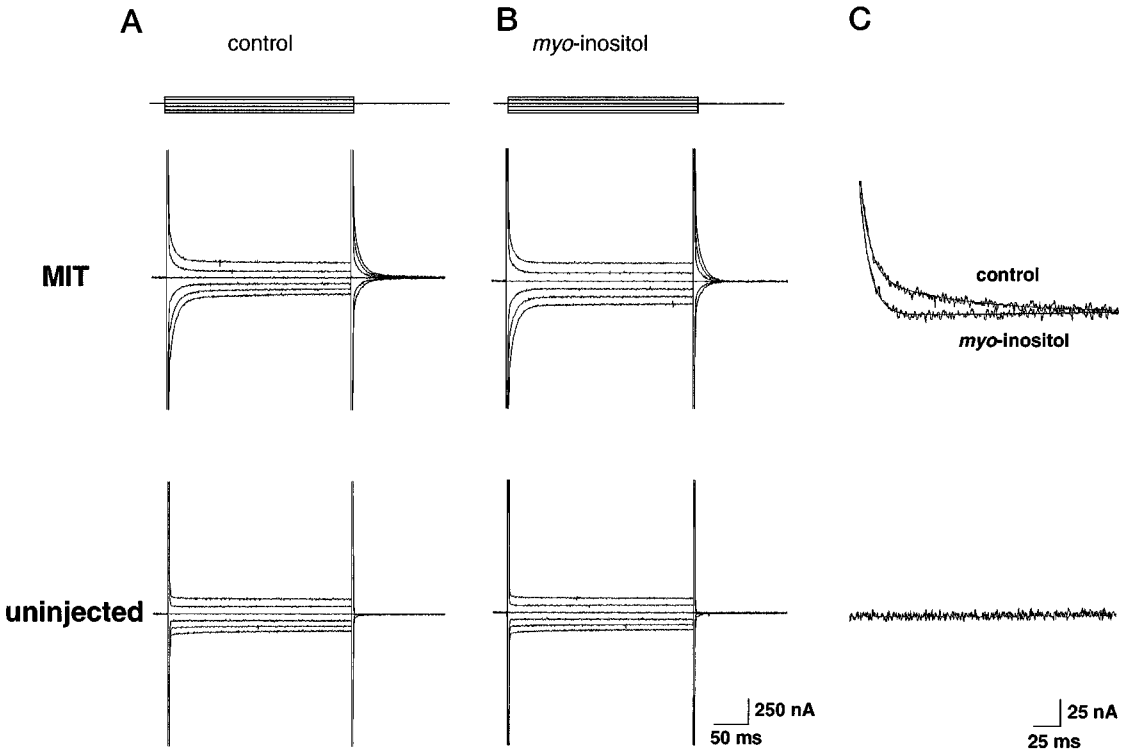

FIG. 6. **Currents elicited by voltage jumps in the presence and absence of *myo*-inositol.** *A*, an MIT-expressing oocyte displays slow current relaxations not seen in uninjected or water-injected control oocytes in response to voltage jumps performed in Ringer's solution (*A*) or 1 mM *myo*-inositol (*B*). Membrane voltage was stepped from a holding potential of −30 mV to a range of potentials between −150 mV and +50 mV in 40-mV increments. The pH of both solutions was 7.5. *C*, in the absence of *myo*-inositol, the current decayed exponentially with two time constants (fits shown represent τ_{fast} = 7.3 ms and τ_{slow} = 57.6 ms). In the presence of 1 mM *myo*-inositol, the relaxation decayed with a single time constant (τ_{fast} = 7.6 ms). Currents associated with the linear membrane capacitance during the first 3 ms following the voltage jump were deleted for clarity.

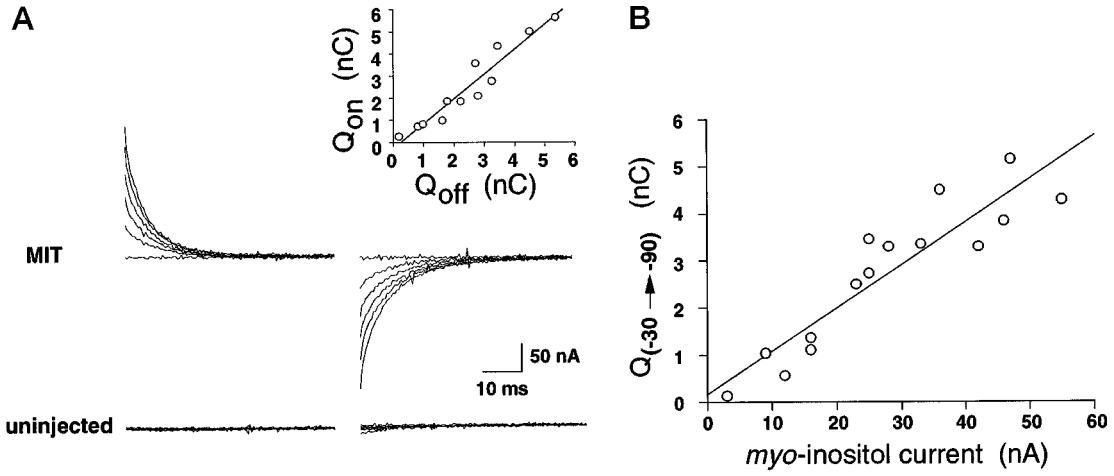

FIG. 7. *A*, current relaxations in a representative oocyte expressing MIT following voltage jumps to potentials between −10 mV and 70 mV in 20-mV increments from a holding potential of −30 mV. Steady-state leak current has been subtracted. *Inset* shows the correlation of charge movement obtained from time integration of transient currents following command pulses to a range of potentials between −150 mV and 70 mV with charge movement following return to the holding potential (*line* shows least-squares linear regression; slope = 1.1). *B*, charge movements during a voltage jump from −30 mV to −90 mV were correlated (r = 0.91) with transporter expression level as determined by steady-state currents induced by 1 mM *myo*-inositol superfusion (n = 15).

DISCUSSION

Measurement of flux mediated by a cloned *L. donovani* *myo*-inositol transporter revealed that a steady-state flow of electrical current occurs during transport such that one charge is translocated with each molecule of *myo*-inositol. Transport of *myo*-inositol was sodium-independent and was accompanied by an alkalinization of the extracellular space, consistent with coupled proton/*myo*-inositol symport. The transport kinetic parameters K_m and I_{max} revealed an interaction between *myo*-inositol and protons, which was in turn influenced by the membrane potential. The apparent affinity for *myo*-inositol increased with increasing $[H^+]_{out}$, and the apparent affinity for protons increased with increasing $[myo\text{-inositol}]_{out}$. Membrane hyperpolarization caused an increased apparent affinity for *myo*-inositol, an effect that saturated at very negative potentials (Fig. 4*B*). The voltage dependence of $K_m^{m\text{-}inos}$ appears to reflect the voltage dependence of proton binding since this saturation occurred at less negative potentials as the external proton concentration was raised. Consistent with this possibility, the apparent affinity

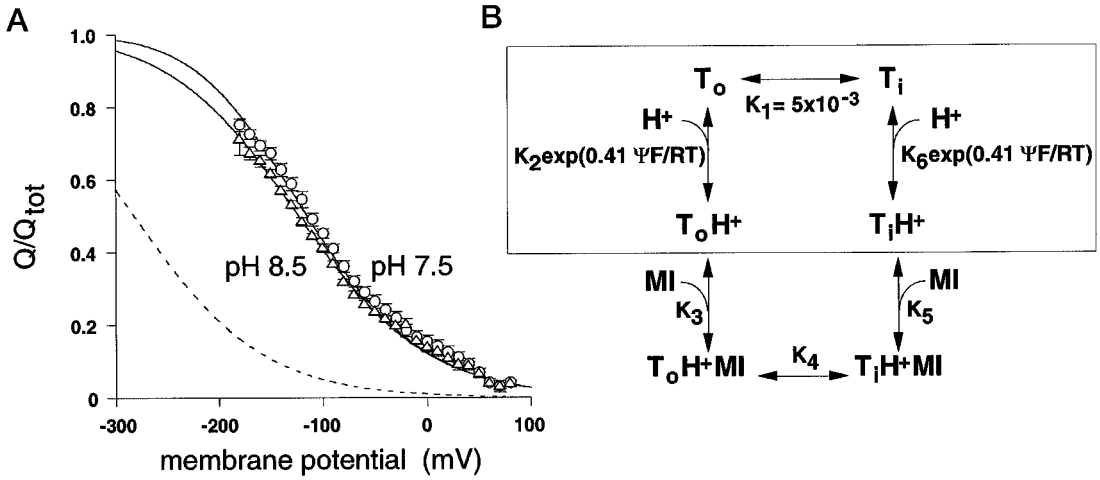

FIG. 8. **Effect of external pH on voltage dependence of charge movements.** *A*, data points (mean ± S.E., $n = 5$) represent charge movements measured at pH 7.5 (*circles*) and pH 8.5 (*triangles*). Smooth curves are simulations from kinetic scheme shown in the *boxed region* of *B*, with proton binding 0.41 of the electrical distance through the membrane ($K_6 = 4.2 \times 10^{-9}$ M exp[0.41Ψ/*RT*] and $K_2 = 6.6 \times 10^{-9}$ M exp[0.41Ψ/*RT*]), with a voltage-independent equilibrium constant $K_1 = 5 \times 10^{-3}$. A similar fit could be obtained assuming voltage independence for external proton binding ($K_2 = 6.2 \times 10^{-10}$ M). *Dashed line* shows −140 mV shift in the voltage midpoint at pH 8.5 predicted when K_2 is the only charge moving transition (see text).

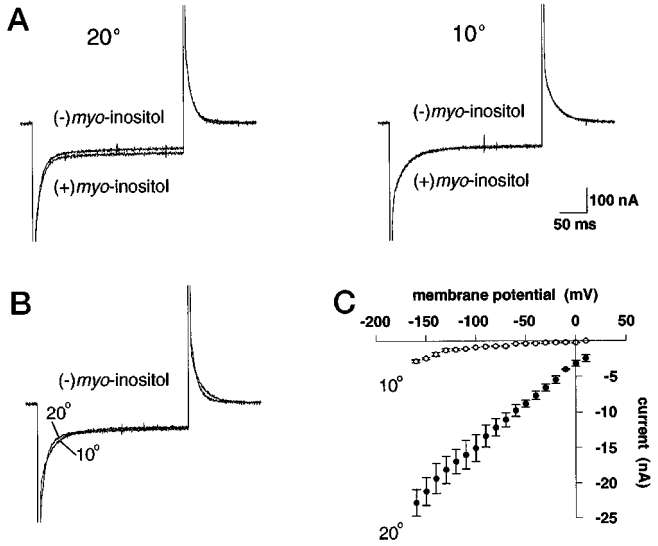

FIG. 9. **Temperature dependence of current relaxations and steady-state current.** *A*, currents recorded from an oocyte expressing MIT during a voltage step from −30 mV to −150 mV in the presence and absence of 1 mM *myo*-inositol at 20 °C and 10 °C. *B*, superimposed traces recorded in the absence of *myo*-inositol at 20 °C and 10 °C. *C*, subtracted *myo*-inositol-induced steady-state current at 20 °C (*filled circles*) and 10 °C (*open circles*). Data points represent the mean ± S.E., $n = 3$.

for protons was increased by hyperpolarization, and this effect did not saturate at negative membrane potentials even with high concentrations of *myo*-inositol (Fig. 5). The $I_{max}^{H^+}$ increased at negative membrane potentials, but it became markedly less voltage-dependent with decreasing *myo*-inositol concentration. This result suggests that with very low concentrations of *myo*-inositol, the rate-limiting step in transport becomes voltage-independent. This voltage-independent step may be the binding of *myo*-inositol to the transporter.

Information about binding order of substrates for well defined cotransport processes can be obtained from analysis of steady-state kinetic parameters (see Stein (1990)). As shown in Fig. 4, $K_m^{m\text{-inos}}$ is increased by reducing $[H^+]_{out}$, while $I_{max}^{m\text{-inos}}$ is unaffected. Therefore, limiting $[H^+]$ can be overcome by increasing [*myo*-inositol] to reach the same maximal rate. This is in contrast to the effect of limiting [*myo*-inositol], in which $K_m^{H^+}$ is increased while $I_{max}^{H^+}$ is reduced (Fig. 5). These data are consistent with a sequential ordered binding mechanism (Jauch and Läuger, 1986) with one proton binding to the transporter first, followed by a molecule of *myo*-inositol. This behavior differs from that of a H^+/hexose transporter, STP1, cloned from *Arabidopsis thaliana* (Sauer *et al.*, 1990), which is reported to mediate H^+/hexose flux via a random binding consecutive transport mechanism (Boorer *et al.*, 1994).

Following voltage jumps, presteady-state charge movements were evident, which were well resolved from membrane capacitance currents and which correlated in magnitude with transporter expression levels (Fig. 7*B*). The charge movements associated with the *myo*-inositol transporter depended on the membrane potential in a manner that could be described by a Boltzmann function. Qualitatively similar currents reflecting reversible charge movements have been observed in a number of transporters (Parent *et al.*, 1992; Mager *et al.*, 1993; Boorer *et al.*, 1994; Wadiche *et al.*, 1995). Estimation of transporter numbers can be obtained from the Boltzmann parameters of the charge movements (Mager *et al.* 1993). Using this approach, we obtained estimates of *myo*-inositol transporter densities in the oocyte membrane of approximately 11,000 μm^{-2}. The turnover rate inferred from these estimates is on the order of 1 s^{-1}. The temperature sensitivity of the transport rate was much greater than were the transient current relaxation time constants (Fig. 9), suggesting that the charge movements are not likely to reflect protein transitions associated with a rate-limiting step. The pH and voltage dependence of the charge movements could, however, be reasonably well described by a standard alternating access kinetic scheme with proton binding to sites in an occluded pore (Fig. 8). In this scheme, binding of a proton is followed by a molecule of *myo*-inositol, with coupled translocation through a transporter lumen alternately accessed from the cytoplasmic and external sides of the membrane. This scheme shares features with ion channel-like models of transport in which permeant ions interact with sites in an occluded or partially occluded pore (Läuger, 1980; Gadsby *et al.* 1993). The identification and further study of such charge movements should help to resolve these processes in greater detail.

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Kinetics and Stoichiometry of a Proton/*myo*-Inositol Cotransporter

Elizabeth M. Klamo, Mark E. Drew, Scott M. Landfear and Michael P. Kavanaugh

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