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Anomalous Effects of External TEA on Permeation and Gating of the A-Type Potassium Current in *H. aspersa* Neuronal Somata

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Abstract. The model proposed for external TEA block of *Shaker* K^+ channels predicts a proportional relationship between TEA sensitivity and calculated electrical distance derived from measurements of voltage dependence of TEA block. In the present study, we examined this relationship for the A-type K^+ current (I_A) of *Helix aspersa* in neuronal somata using the whole-cell patch-clamp technique. External TEA inhibited I_A with strong voltage dependence, such that the TEA dissociation constant was increased at depolarized test potentials. The half-inhibition constant ($V_{0.5}$) for TEA block was ~21 mM at 0 mV, and $V_{0.5}$ increased to ~67 mM at 50 mV. The calculated electrical distance for TEA block suggested that TEA traversed 65% of the way into the membrane electrical field. TEA also caused significant shifts in the voltage-dependence of A-type K^+ channel gating. For example, at TEA concentrations below that required to fully suppress delayed outward currents, TEA caused depolarizing shifts in the voltage-dependence of A-type channel activation, steady-state inactivation, time for removal of inactivation, and slowed channel activation kinetics. Taken together, these observations suggest that TEA biased the local field potential near voltage-sensing domains of A-type K^+ channels, causing the transmembrane electrical field to be relatively hyperpolarized in the presence of TEA. In summary, the calculated electrical distance of TEA block of A-type K^+ channels in *H. aspersa* neurons is unprecedented among other K^+ channels. This raises concerns about the conventional interpretation of this value. Furthermore, the voltage-dependent properties of I_A are modified by TEA at concentrations previously used to isolate delayed rectifier potassium channels (I_{KDR}) selectively. This lack of specificity has important implications for recent, as well as future studies of I_A in *H. aspersa* and possibly other snail neurons.

Key words: Tetraethylammonium — A-type K^+ current — Electrical distance — Gating — Snail neuron

Introduction

The classical K^+ channel blocker, tetraethylammonium (TEA), is used widely as a molecular probe for the internal and external pore mouth of voltage-dependent K^+ channels. It is well established that TEA, which has the shape of a flattened oblate spheroid, acts by physically plugging the ion permeation pathway, preventing movement of K^+ through the pore (Armstrong, 1969; Armstrong, 1971; MacKinnon & Yellen, 1990; Hartmann et al., 1991; Yellen et al., 1991; Heginbotham & MacKinnon, 1992; Pascual et al., 1995; Crouzy, Bernèche & Roux, 2001). K^+ channels typically fall into one of two categories with respect to external TEA sensitivity: those that are highly sensitive ($K_i < 1$ mM; Hille, 1967) and those that are relatively insensitive ($K_i > 20$ mM; Armstrong & Binstock, 1965). The mechanism of extracellular TEA block of *Shaker* K^+ channels, which have a high affinity for TEA, has been studied extensively. In these channels, the receptor is formed by a bracelet of four aromatic residues positioned at the external mouth of the pore, and TEA interacts simultaneously with these residues to produce high-affinity pore block (Heginbotham & MacKinnon, 1992; Crouzy et al., 2001). When all four residues are present, the dissociation constant for TEA block is only weakly voltage dependent. The degree of voltage dependence of TEA block has been used to calculate

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the site of TEA block within the membrane electrical field, and the weak voltage dependence of TEA block of *Shaker* channels indicates that TEA traverses only 4% of the electrical field within the membrane according to the traditional interpretation. Mutation of two or four of the pore-lining aromatic residues causes a progressive decrease in blocker potency and, interestingly, increases the voltage dependence of TEA block. As a result of mutating the pore-lining residues, TEA appears to enter the pore more deeply and traverses 19% of the electrical field within the cell membrane. TEA does not affect the voltage dependence of channel activation or steady-state inactivation in either wild-type or mutant channels (Heginbotham & MacKinnon, 1992).

Transient A-type K^+ channels, which are critical for regulation of cell excitability in vertebrate and invertebrate neurons (Hille, 1992), generally exhibit a low sensitivity to extracellular TEA. As a consequence, TEA is used routinely to separate A-type K^+ currents (I_A) from TEA-sensitive currents, such as the delayed rectifier K^+ current (I_{KDR}). The relationship between TEA affinity and electrical distance derived from studies of *Shaker* K^+ channels (Heginbotham & MacKinnon, 1992) predicts that TEA will exhibit strong voltage dependence in this class of low-affinity K^+ channels. However, to date, no studies have examined the voltage dependence of TEA block of A-type K^+ channels.

In the present study, we examined the voltage dependence of TEA block of A-type K^+ currents in *H. aspersa* neuronal somata. For reasons described above, we hypothesized that I_A, which has a low affinity for external TEA (Neher & Lux, 1972), would exhibit strong voltage-dependent block by TEA, and we report that the half-inhibition constant for TEA block of I_A is strongly voltage dependent. Indeed, the calculated value for apparent electrical distance suggests that TEA traverses approximately 65% of the membrane electrical field to reach its binding site in the pore, a result that raises concerns about the traditional interpretation of this value. Additionally, during these studies, we observed clear evidence that TEA augments the voltage dependence of channel gating, leading us to explore this systematically. TEA modifies the half-inactivation voltage, time for removal of inactivation, and activation kinetics of I_A at concentrations below that required to fully suppress delayed outward currents. These findings are discussed in reference to recent as well as future studies of I_A in *H. aspersa* neurons.

Materials and Methods

Snails and Dissection

Juvenile *H. aspersa* (1–2 g) were purchased from the Pennsylvania Snail Farm (Meyersdale, PA) and housed for no longer than 4 weeks at room temperature (22–24°C) in a humidified terrarium. Snails were fed lettuce, cucumber, and corn meal 2–3 times per week. Before dissection, snails were anesthetized by injection with 2 ml of a solution with the following composition (in mM): 30 $MgCl_2$, 42 $CaCl_2$, 12 N-[2-Hydroxyethyl]piperazine-N′[2-ethanesulfonic acid] (HEPES) free acid (pH 7.8, 225 ± 5 mOsm). All salts were purchased from Sigma (St. Louis, MO) and were reagent grade. After anesthetizing the snail, the circumesophageal nerve ring was removed and pinned dorsal side up in a 60-mm petri dish half-filled with hardened elastomer (Sylgard, Dow Corning, Midland, MI) and containing normal snail saline of the following composition (in mM): 85 NaCl, 4 KCl, 7 $CaCl_2$, 5 $MgCl_2$, 10 glucose, 10 HEPES (pH 7.8, 225 ± 5 mOsm). The thick outer connective tissue layer covering the subesophageal ganglia was removed with fine-tipped forceps, microscissors, and tungsten microscalpels (Conrad et al., 1993). The thin inner capsule was loosened by proteolytic digestion (2–5 mg/ml protease, type X1V, Sigma) in snail saline for 15–30 min and removed subsequently by manual dissection. To isolate neuronal somata for patch-clamp recordings, unidentified somata (40–75 μm diameter) from the right parietal, left parietal, and visceral ganglia were axotomized 50–200 μm below the cell body with microscissors and transferred to a ~150-μl perfusion chamber (Warner Instruments, Hamden, CT) with a "cell sucker" (Drapeau, Catarsi & Ali, 1999). Cells were allowed to recover for at least 30 min in normal snail saline before initiating patch-clamp experiments. Under Hoffman contrast modulation optics, healthy neurons were easily distinguished from non-viable somata by their smooth, grape-like appearance and absence of membrane blebs and nuclear condensation.

Patch-Clamp Electrophysiology

Whole-cell currents from unidentified somata were recorded in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Patch pipettes were fabricated with a two-stage puller (model BB-CH, Mecanex, Geneva, Switzerland) from borosilicate glass capillaries (Corning #7052 glass, WPI, Sarasota, FL). Patch electrodes had resistances between 1–10 MΩ when filled with intracellular solution of the following composition (in mM): 5 NaCl, 70 K-aspartate, 15 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 ethylene glycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 5 Tris-ATP, HEPES free acid (pH 7.4; 225 ± 5 mOsm). Pipette solutions were sterile-filtered and kept on ice until time of use to minimize ATP degradation. Imposing negative voltage pulses (−5 mV, 5 msec duration) across the pipette tip and holding the tip potential at −50 mV increased the rate and success of gigaseal formation.

After attainment of whole-cell access, current signals were recorded in continuous single-electrode voltage-clamp mode with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Data were filtered with an 8-pole Bessel low-pass filter (Frequency Devices, Haverhill, MA) at a cutoff frequency of 5 KHz and sampled at 10 KHz using a Digidata 1320A A/D interface (Axon Instruments) with pClamp 8.03 software (Axon Instruments). Before initiating voltage-clamp experiments, the voltage-clamp circuitry was tuned by adjusting the clamp multiplier, gain, phase lag, and series resistance compensation (R_s) until V_m changed as a square wave. The performance of the voltage-clamp circuitry was assessed throughout experiments and adjusted when necessary. Typically, loss of clamp performance was accompanied by an increase in R_s, which was presumably due to resealing of the membrane rupture in the patch-pipette tip. The incidence of resealing was significantly reduced by including 5 mM EGTA in the patch-pipette solution. Electrical connections with the amplifier were made with Ag-Ag-Cl wire and 3 M KCl-agar bridges.

Measurement of I_A Activation, Steady-State Inactivation, and Time for Removal of Inactivation

Voltage-clamp routines were generated with Clampex 8.03 software (Axon Instruments). The voltage dependence of I_A activation was measured at 10 mV increments between −70 mV and 50 mV from a 200-msec holding potential of −100 mV. The A-current activated rapidly compared to delayed outward currents (I_{KDR}), such that the peak amplitude of I_A could be easily measured between these test potentials. Moreover, I_{KDR} was not activated below test potentials of 0 mV so that I_A was the only outward current observed at test potentials less than 0 mV. At larger positive test potentials, both the activation kinetics and amplitude of I_{KDR} approximated that of I_A, making it difficult to reliably measure peak I_A above 50 mV without pharmacological suppression of I_{KDR} with high concentrations of external TEA. The A-type K^+ current was easily distinguished from I_{KDR} currents using long (i.e., 1600 msec) test potentials below 50 mV; under these test conditions differences in activation kinetics, current-voltage relationships, and the time course of inactivation between I_A and I_{KDR} were readily discernable.

The steady-state inactivation characteristics of I_A, which reveal voltage dependence of A-type channel availability for activation (i.e., steady-state inactivation), were measured as follows. The peak amplitude of I_A was measured at −10 mV following a 200-msec holding potential that ranged from −120 mV to −20 mV in 10 mV increments. Current amplitude measured at the test potential following each holding potential was normalized to current amplitude recorded from −120 mV. This holding potential removes inactivation from all A-type K^+ channels, rendering 100% of channels available for activation at −10 mV, and −10 mV is below the threshold of activation of I_{KDR}. Normalized current amplitude of I_A was plotted as a function of holding potential, and this relationship was fitted with a Boltzman function of the form: $I/I_{max} = [1 + e(V_{half} - V_{step})/K]^{-1}$, where I_{max} was the maximal current measured from a holding potential of −120 mV, V_{half} was the potential at which half of I_A channels were inactivated, V_{step} was the current measured at a particular step potential, and K was the slope factor, which reflects the voltage dependence of channel inactivation.

The time for removal of inactivation, or deinactivation, was estimated in the following manner. I_A was completely inactivated by clamping V_m to −20 mV for 200 msec. Following this inactivation pulse, V_m was stepped to −60 mV, a voltage that removes inactivation from A-type K^+ channels, for 25–825 msec in 40-msec increments. The current amplitude recorded following each recovery interval was normalized to the maximum current measured after complete removal of I_A inactivation (i.e., current measured following 800-msec recovery interval). The relationship between recovery interval duration and fractional current was plotted and the resulting curves were fitted with the following equation: $y = y_o + a(1 - e^{-\tau/b})$, where τ is the time constant for channel deinactivation.

Electrical-Distance Calculations

To estimate the apparent electrical distance of the TEA binding site within the conduction path, the TEA dissociation constant (K_D) was plotted as a function of test potential. The K_D for TEA block was calculated using the formula $K_D = ([TEA] \times I_{A,TEA})/I_{A,control} - I_{A,TEA})$, where [TEA] is blocker concentration, $I_{A,TEA}$ is peak I_A measured in the presence of blocker, and $I_{A,control}$ is peak I_A recorded in control saline. The slope of the relationship between K_D and test potential is an estimate of the fractional distance within the membrane electrical field of the TEA binding site (Hille, 1992; Woodhull, 1973).

Experimental Solutions

TEA chloride was substituted for NaCl in snail saline on an isosmotic basis to avoid changes in solution ionic strength and osmolarity. The concentrations of TEA used in this study were 0 (control saline), 21.25, 42.5 and 85 mM. Sodium (Na)-free snail saline was made by substituting choline Cl (Sigma) for NaCl. Solution osmolarity was checked routinely with a vapor pressure osmometer (Wescor model 5500, Claremont, Ontario) and adjusted to 225 ± 5 mOsm with dry sucrose.

Experimental Design

Repeated-measures experiments were performed, in which each soma was exposed to the full range of TEA concentrations. To measure the effect of TEA on I_A, control saline was exchanged for TEA-substituted solutions, and peak current amplitude at each TEA concentration was measured at least 30 sec after the solution switch to ensure that the concentration of TEA in the bath had reached a steady state. The order of solution changes was varied to ensure that the effects of TEA-substituted and Na-free saline were independent of the order of solution application. The effects of TEA on permeation and gating of I_A were rapid, occurring within 20 sec of switching the bath solution, and fully reversible in every cell examined. In preliminary experiments, we examined different voltage-clamp protocols and found that the duration of exposure to TEA and the particular voltage increment in the voltage-clamp protocols did not alter the results. Therefore, we used 10-mV test-potential increments and relatively short TEA exposure times (5–10 min) throughout the study.

Statistical Analysis

Data are presented as means ± standard error (SEM). Statistical significance between treatments was assessed using an ANOVA or linear regression as appropriate (Systat 9.0, SPSS, Inc, Evanston, IL). Values of $P < 0.05$ were taken to indicate statistical significance.

Results

Block of I_A by Extracellular TEA Exhibits Strong Voltage Dependence

This study was initiated to examine the voltage dependence of block of A-type K^+ channels by external TEA in *H. aspersa* neuronal somata. From the model proposed for TEA block of *Shaker* K^+ channels, we predicted that block of this low-affinity channel by external TEA would exhibit significant voltage dependence.

The inhibitory action of TEA on I_A and delayed outward currents was first evaluated by measuring whole-cell outward current at various test potentials in the absence (control) and presence of 21.25, 42.5 and 85 mM TEA. The left-hand column of Fig. 1 shows that extracellular TEA suppressed delayed-rectifier currents (I_{KDR}), as well as I_A, in a dose-dependent manner, albeit with different potencies. The right-hand column illustrates the TEA-sensitive current, which represents the current blocked at each TEA

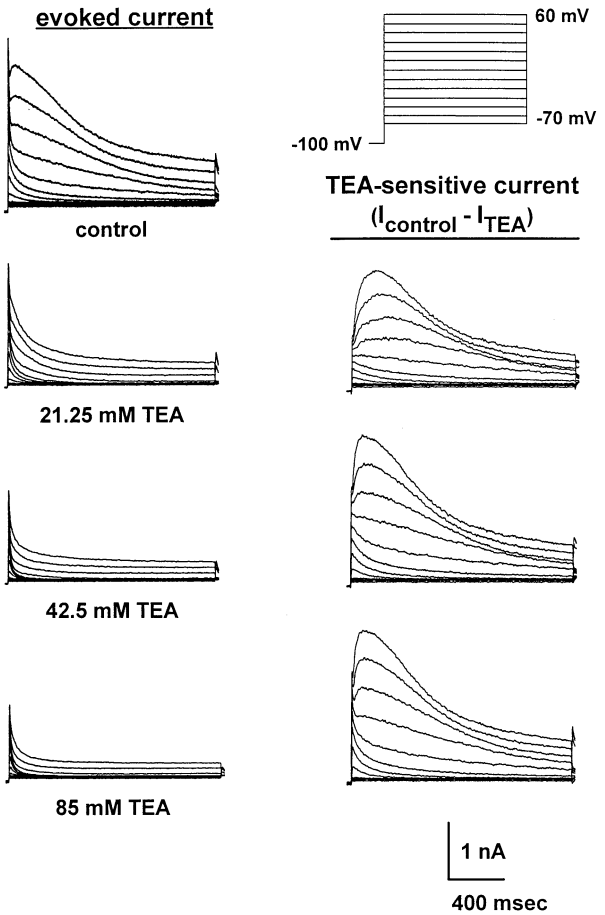

Fig. 1. TEA blocks I_{KDR} and I_A. Currents were evoked at test potentials between −70 and 60 mV in 10-mV increments from a holding potential of −100 mV. Evoked current traces are shown in the left column in control solution and in saline containing 21.25, 42.5, and 85 mM TEA. TEA-sensitive (right column) current represents the current blocked by the indicated concentration of TEA. Note the progressive appearance of I_A in the TEA-sensitive current traces as the concentration of TEA was increased.

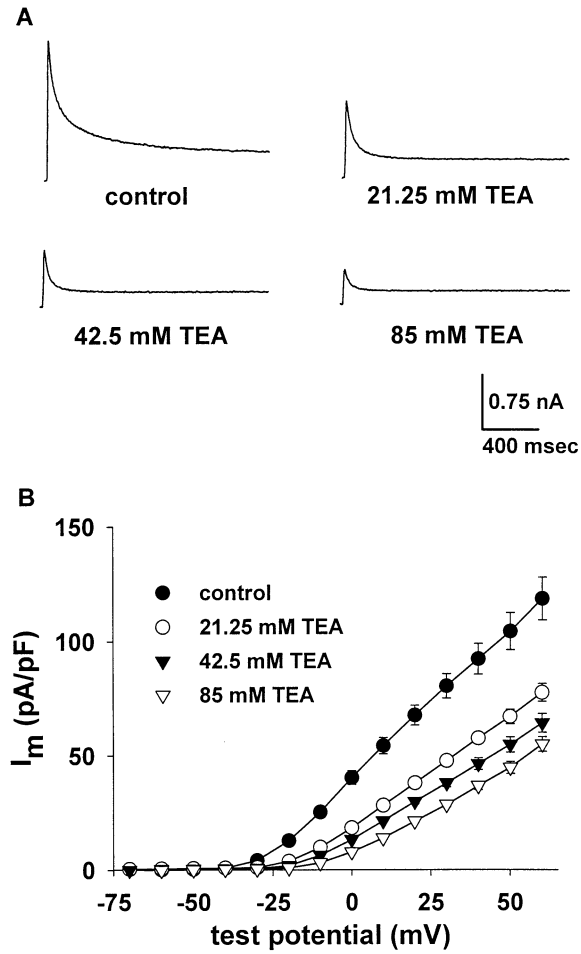

Fig. 2. Dose-dependent TEA block of I_A. In panel *A*, TEA reduces peak I_A in a dose-dependent manner. Panel *B* is a summary graph of the effect of TEA on the peak current-voltage relationship for I_A. A two-way ANOVA (TEA by test potential between −20 and 60 mV) revealed a significant interaction ($P < 0.001$) indicating that the slope of the relationship between test potential and current was altered by the TEA concentration. Linear regression was used to estimate the effect of TEA concentration on the slope conductance, and the regression indicated that the slope conductance decreased on average by 0.007 ± 0.003 nS/pF/mM TEA ($P = 0.02$).

concentration (i.e., $I_{control} - I_{TEA}$). I_{KDR} was almost completely inhibited by 21.25 mM TEA, as evidenced by the disappearance of the delayed outward currents in the evoked current trace (left column) and appearance of I_{KDR} in the TEA-sensitive current trace (right column). Moreover, I_A was evident in the TEA-sensitive trace at this TEA concentration between −10 mV and 10 mV, indicating that it was also weakly inhibited by 21.25 mM TEA. Increasing the concentration of TEA to 42.5 and 85 mM caused progressive inhibition of I_A (left column). This was also evident in the TEA-sensitive current, which showed dose-dependent appearance of I_A as the TEA concentrations increased (right column). This experiment demonstrated that TEA inhibits I_A at concentrations below the concentration of TEA required to fully suppress I_{KDR}.

Figure 2*A* illustrates the dose-dependent, inhibitory effect of extracellular TEA on I_A evoked at −10 mV, a test potential that is more negative than the activation voltage for I_{KDR}. This further supports our conclusion from Fig. 1 that TEA blocks A-type K^+ currents in these neurons. The effect of externally applied TEA on the current-voltage relationship of I_A activation is shown in Fig. 2*B*. In control saline, I_A activated around −40 mV and exhibited a slope conductance of 1.20 ± 0.15 nS/pF between −20 mV and 60 mV (Fig. 2*B*; $n = 4$). Bath application of 21.25, 42.5 and 85 mM TEA decreased the slope conductance to 1.02 ± 0.28, 0.84 ± 0.10, and 0.68 ± 0.07 nS, respectively (Fig. 2*B*; $n = 4$). A two-way ANOVA (TEA at test potential between −20 and 60 mV) revealed a significant interaction ($P < 0.001$), indicating that the slope of the relationship between test potential and current was altered by

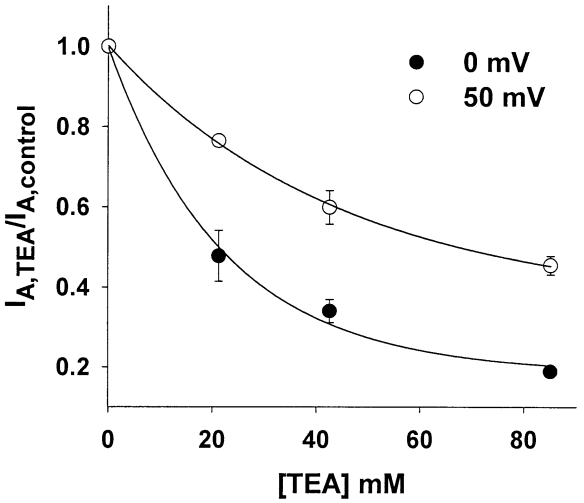

Fig. 3. Voltage dependence of TEA block of I_A. Peak I_A at 0 mV (*filled circles*) and 50 mV (*empty circles*) was recorded in 0, 21.25, 42.5, and 85 mM TEA-containing solution. The amplitude of I_A recorded in the presence of TEA was normalized to that measured in control saline; data are expressed as fractional current (i.e., $I_{A,TEA}/I_{A,control}$). Dose-response curves were fitted with a three-parameter, single-exponential equation of the form: $y = y_o + ae^{-x/b}$, where y_o is the asymptote of residual current at maximal inhibition, a is the maximum current inhibition, and b is the TEA concentration at which 63% of the TEA effect on I_A was reached. $n = 5$ in all cases.

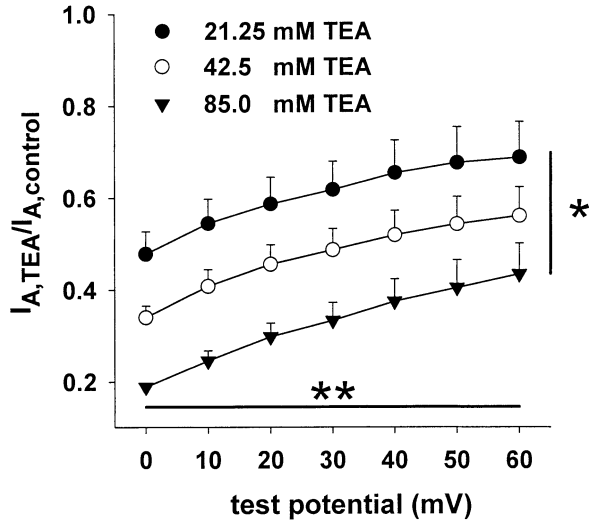

Fig. 4. Relief of TEA block by membrane depolarization. The amplitude of I_A recorded in TEA-containing solution ($I_{A,TEA}$) was normalized to that measured in control saline ($I_{A,control}$). Fractional currents (i.e., $I_{A,TEA}/I_{A,control}$) versus test potential are shown. The TEA block of I_A was voltage dependent at all concentrations of TEA (*, $P = 0.008$) and TEA block was relieved as the membrane depolarization increased at all concentrations of TEA (**, $P < 0.001$). However, there was no significant interaction term. $n = 5$ in all cases, and data are means ± SEM.

the TEA concentration. To estimate the size of this effect, we calculated the slope conductance for each cell at each TEA concentration and used linear regression to estimate the effect of TEA concentration on the slope conductance. The regression indicated that the slope conductance decreased on average by 0.007 ± 0.003 nS/pF/mM TEA ($P = 0.02$).

We next examined the voltage dependence of block of I_A by external TEA. If external TEA traversed the transmembrane field potential to reach its binding site in the pore, we predicted that the half-inhibition constant ($K_{0.5}$) for TEA would increase as function of test potential. Figure 3 shows the dose-response curve for TEA block, from which we estimated $K_{0.5}$ at different test potentials. The zero-voltage $K_{0.5}$ was 21.2 mM; however, $K_{0.5}$ at 50 mV increased to 67.1 mM, indicating that TEA block was indeed voltage dependent. The voltage dependence of TEA block was further characterized across a range of test potentials, and the fraction of I_A remaining in 21.25, 42.5, and 85 mM TEA-containing solution as a function of test potential is shown in Fig. 4. A two-way ANOVA (TEA concentration by test potential) confirmed that block of I_A by TEA was voltage dependent ($P = 0.008$), and block was relieved at more positive test potentials at all concentrations of TEA ($P < 0.001$). For example, 85 mM TEA blocked 81.1 ± 0.6% of I_A at 0 mV, but inhibited only 56.6 ± 6.7% of I_A at 60 mV. However, the slope of the voltage dependence of channel block was not modified by the TEA concentration (there was no significant interaction between TEA and test potential). Taken together, these experiments confirmed that external TEA blocked the A-type K^+ current in *H. aspersa* neuronal somata in a voltage-dependent manner.

The voltage dependence of TEA block of I_A indicated that TEA traversed a fraction of the transmembrane electrical field to bind its receptor in the pore (Woodhull, 1973; Heginbotham & MacKinnon, 1992; Adams et al., 1999). From the model proposed for external TEA block of *Shaker* K^+ channels (*see* Introduction), we predicted that the A-current of *H. aspersa* neurons, which has a relatively low affinity for extracellular TEA, would traverse a large fraction of the field potential to reach its binding site. We calculated the apparent electrical distance of the TEA binding site in the membrane electric field using estimates of the TEA dissociation constant (K_D) plotted as a function of test potential (Woodhull, 1973; Adams et al., 1999). Fig. 5 shows the relationship between K_D for TEA block at different test potentials; the slope of this relationship yields an estimate of the electrical distance of the TEA binding site within the membrane electrical field. Surprisingly, the mean apparent fractional distance for 21.25, 42.5, and 85 mM TEA was 0.65 ± 0.02, suggesting that a single extracellular TEA ion traversed 65% of the membrane electric field.

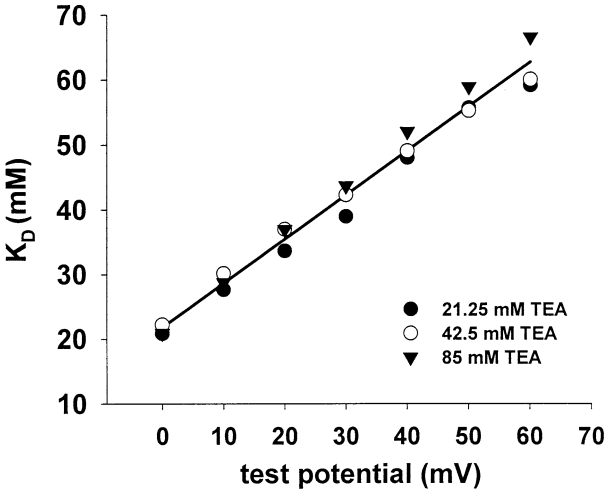

Fig. 5. Voltage dependence of TEA dissociation constant (K_D). The dissociation constant was estimated as follows: $K_D = ([TEA] \times I_{A,TEA})/I_{A,control} - I_{A,TEA})$, where [TEA] is blocker concentration, $I_{A,TEA}$ is peak I_A measured in the presence of blocker, and $I_{A,control}$ is peak I_A recorded in control saline. Linear regression of the K_D on the membrane potential revealed a slope of 0.65 ± 0.02 ($r^2 = 0.99$; $P < 0.01$).

External TEA Modifies A-Type K^+ Channel Gating

During the course of these experiments, we observed that, in the presence of 20–50 mM TEA, an inactivation pulse to −30 mV, which fully inactivates I_A in normal saline, only partially removed I_A from the outward current trace. The outward currents evoked from a holding potential of −30 mV in control saline and 85 mM TEA-containing saline are shown in Fig. 6*A*. In control saline, only the delayed outward current was activated from a holding potential of −30 mV (left panel) due to complete inactivation of I_A. However, in TEA-substituted saline, the same voltage-clamp routine clearly evoked the A-type K^+ current in this representative cell (right panel). This suggested that TEA increased the available fraction of A-type K^+ channels at depolarized holding potentials. We tested this hypothesis by measuring the half-inactivation voltage ($V_{0.5}$) for I_A in control and TEA-substituted saline. Figure 6*B* shows that TEA caused a dose-dependent, depolarizing shift in the steady-state inactivation curve of I_A. For example, the $V_{0.5}$ for I_A in control, 21.25, 42.5, and 85 mM TEA were −54.7 ± 0.37 mV, −49.6 ± 0.4 mV, −47.3 ± 0.69 mV, and −44.8 ± 1.0 mV, respectively ($n = 5$ each). The effect of TEA on the steady-state inactivation characteristics of I_A was reversible, and the TEA-induced shift in $V_{0.5}$ was observed in every cell studied ($n > 30$ cells), implying that this was a general effect of TEA on A-type K^+ channels in *H. aspersa* neurons.

During these experiments, TEA was substituted isosmotically for Na^+. Therefore, it was conceivable

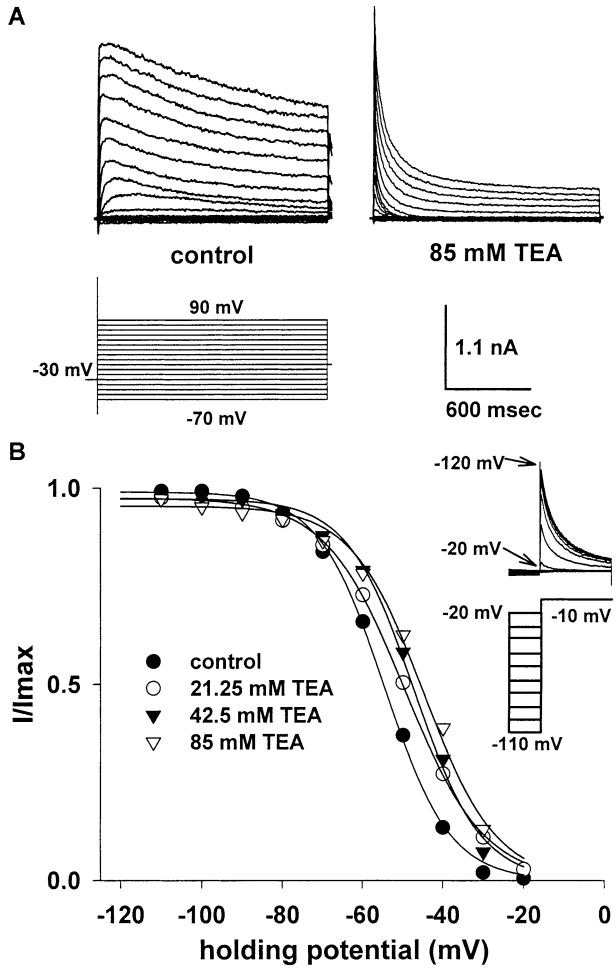

Fig. 6. TEA increased availability of A-type K^+ channels for activation: (*A*) Current families were evoked from a holding potential of −30 mV in control and 85 mM TEA-containing saline. I_A was completely inactivated in control saline (left traces), but was prominent in the presence of 85 mM (right traces). Note that I_A in the right-hand traces had a larger amplitude than the delayed outward current recorded in TEA-free saline. Therefore, TEA did not simply uncover I_A by blocking I_{KDR} and the steady-state current, but promoted the activation of I_A at a holding potential that normally inactivates this current. (*B*) TEA shifted the steady-state inactivation curve of conventional I_A in the depolarizing direction. Peak current amplitude at −10 mV was recorded following 200-msec conditioning pulses between −120 mV and −20 mV and normalized to current evoked from −120 mV. Average V_{half} values were −54.7 ± 0.4 mV, −49.6 ± 0.4 mV, −47.3 ± 0.69 mV, and −44.8 ± 1.0 mV for 0, 21.25, 42.5, and 85 mM TEA-containing saline, respectively ($n = 5$). A one-way ANOVA revealed a significant linear dependence of V_{half} on the TEA concentration ($P = 0.015$), and V_{half} increased approximately 0.13 ± 0.06 mV/mM TEA. Standard error bars were omitted for clarity.

that the effect of TEA on the steady-state inactivation characteristics of I_A was not due to TEA, but resulted from removal of extracellular Na^+ from the bath. To explore this possibility, we measured the effect of complete Na^+ removal on the steady-state inactivation curve for I_A. As shown in Fig. 7, the $V_{0.5}$ for I_A

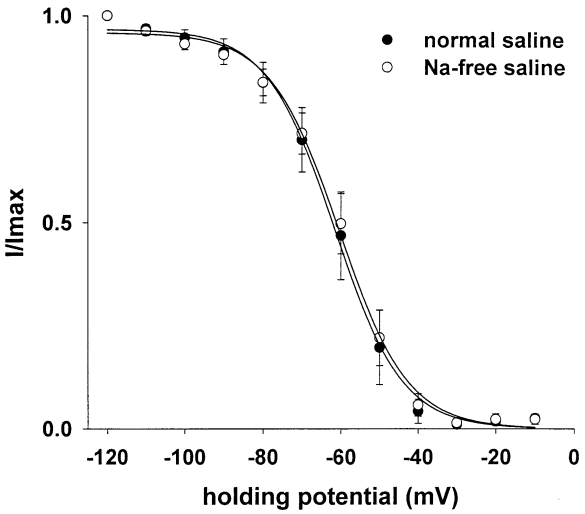

Fig. 7. Sodium-free saline had no effect on availability of A-type K^+ channels. The availability of A-type K^+ channels for activation was evaluated in normal and sodium (Na)-free snail Ringer as described for Fig. 6*B*. Data were fitted with a Boltzman function of the form: $I/I_{max} = [1 + e(V_{half}-V_{step})/K]^{-1}$, where I_{max} was the maximal current measured from a holding potential of −110 mV, V_{half} was the potential at which half of A channels were inactivated, V_{step} was the current measured at a particular step potential, and K was the slope factor. V_{half} values for normal and Na-free saline were −60.4 ± 0.7 mV and −61.6 ± 0.7 mV, respectively, and these values were not significantly different. Likewise, K values between treatments were not significantly different: K for control and Na-free saline were −8.9 ± 0.6 mV and −8.7 ± 0.6 mV, respectively ($n = 6$).

was independent of bath Na^+, indicating that TEA, and not Na^+ removal, was responsible for this effect on gating of A-type K^+ channels.

One possible explanation for the effect of external TEA on the $V_{0.5}$ for I_A was that TEA biased the gating potential detected by A-channel voltage sensors toward a more hyperpolarized potential. To test this hypothesis and to explore the possibility that TEA affected other voltage-dependent processes, we measured the effect of TEA on the time for removal of inactivation (de-inactivation). Figure 8 shows that, in control saline, the removal of inactivation at −60 mV had a time constant of approximately 242 ± 23 msec. In the presence of 85 mM TEA, however, the de-inactivation time constant decreased to 115 ± 10 msec. Since the time for removal of inactivation is voltage-dependent and is faster at hyperpolarized potentials (Neher, 1971), this result was consistent with the idea that TEA hyperpolarized the gating potential sensed by A-type K^+ channels.

These effects of TEA on gating of the A-type channel are similar to those reported for divalent cations, which too are known to also affect the voltage-dependent properties of I_A in *H. aspersa* neurons (Bal et al., 2000). Another common effect of divalent cations is slowing of channel activation kinetics (Hille, 1992; Spires & Begenisich, 1992; Zhang, Kehl & Fedida, 2001a). Therefore we measured the time required to reach peak current amplitude in control and TEA-substituted saline. As shown in Fig. 9, a two-way ANOVA (TEA concentration by test potential) indicated TEA significantly slowed the activation kinetics of I_A at all test potentials between 10 and 40 mV ($P = 0.03$), and as the test potential became more positive, the time to peak was faster at both TEA concentrations ($P = 0.005$). But there was no significant interaction, indicating that the slope of dependence of the time to peak amplitude on test potential was not modified by TEA.

Discussion

In this study, we examined the voltage dependence of external TEA block of A-type K^+ currents in *H. aspersa* neurons, and TEA block of I_A was strongly voltage dependent (Figs. 3–5). Moreover, during the course of these studies, we found evidence that TEA augmented several voltage-dependent properties of I_A at concentrations below that required to fully suppress I_{KDR}. As will be discussed below, we believe these findings have important consequences for recent and future studies of I_A in snail neurons.

Voltage-dependent TEA Block of I_A: Implications for Interpretation of 'Electrical Distance' Values

The most obvious effect of external TEA on I_A was the dose-dependent suppression of peak current amplitude (Fig. 2*A*), which has been reported previously (Neher & Lux, 1972). TEA decreased the slope of the current-voltage relationship at all concentrations tested (Fig. 2*B*). This implies that TEA decreased single-channel conductance and/or open probability of A-type K^+ channels, and the effect of this blocker was not simply due to a depolarizing shift in the voltage dependence of channel activation.

The K_D for block of I_A by external TEA exhibited strong voltage dependence (Fig. 3), and the calculated TEA binding site was 65% of the distance across the membrane potential. To our knowledge, this large electrical distance, a value that normally ranges between 0 and ~30%, is unprecedented for TEA block of a K^+ channel. Traditionally, the calculated electrical distance was interpreted as the physical distance of the TEA receptor within the channel pore (Woodhull, 1973). Thus, in A-type K^+ channels from *H. aspersa* neurons, TEA appeared to traverse 65% of the linear distance of the conduction path to block the channel. However, this interpretation has recently been called into question.

The determination of the three-dimensional structure of the bacterial K^+ channel, KcsA, using X-

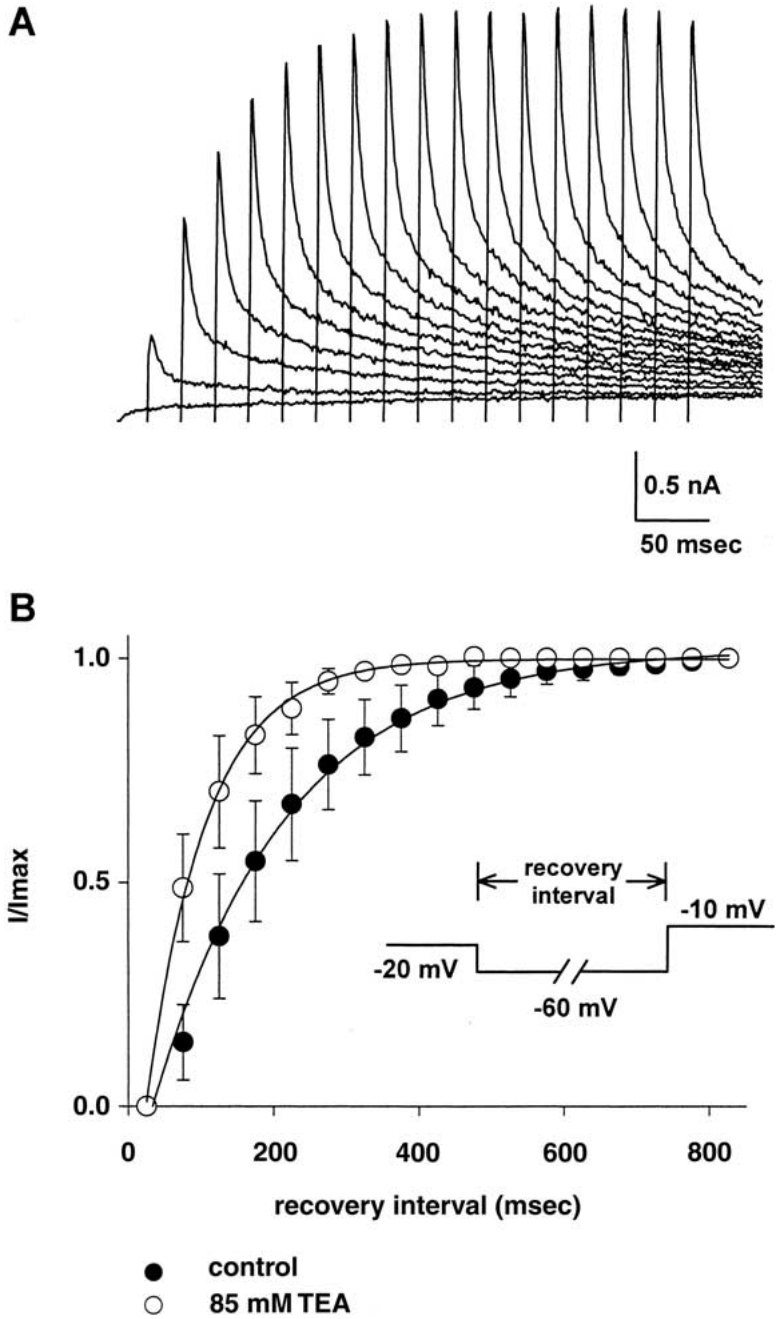

Fig. 8. TEA modulation of time-dependence of I_A de-inactivation: (*A*) Representative current traces showing time-dependent removal of inactivation of I_A. Current was evoked using voltage clamp protocol shown in the inset of panel *B*. I_A was inactivated with a 200-msec conditioning pulse to −20 mV, and peak I_A was recorded at −10 mV following a variable-length recovery interval (RI) at −60 mV. (*B*) Normalized current (I/I_{max}) plotted as a function of recovery-interval duration in control saline and 85 mM TEA-containing saline. The time constants for removal of inactivation in control and TEA-containing saline were 242 ± 22 msec and 115 ± 10 msec, respectively ($n = 5$).

ray crystallography (Doyle et al., 1998) has made it possible to test structural models of TEA-pore interactions deduced from biophysical measurements. Recently, Roux and colleagues used molecular dynamics (MD) simulations of an atomic model of KcsA to reexamine the molecular mechanism of K^+ channel blockade by external TEA (Crouzy et al., 2001). These MD simulations revealed, in remarkable agreement with predictions from electrophysiological data (Heginbotham & MacKinnon, 1992), that TEA interacts simultaneously with four aromatic residues close to the outer pore of the channel to produce high-affinity channel block. Due to its shape and strong coordination by the aromatic residues, TEA behaves as an ideal plug in the wild-type channel. In a mutant channel lacking the pore-lining aromatic residues, however, TEA was off-center and tilted with respect to the axis of the pore. As a consequence, TEA binding was less energetically favorable and occurred with lower affinity.

A surprising result of the MD simulations concerned the location of TEA with respect to the axis of the pore in wild-type and mutant KcsA channels. Due to its shape and strong coordination by aromatic residues, TEA does not physically enter the selectivity filter (Crouzy et al., 2001), where the majority of the

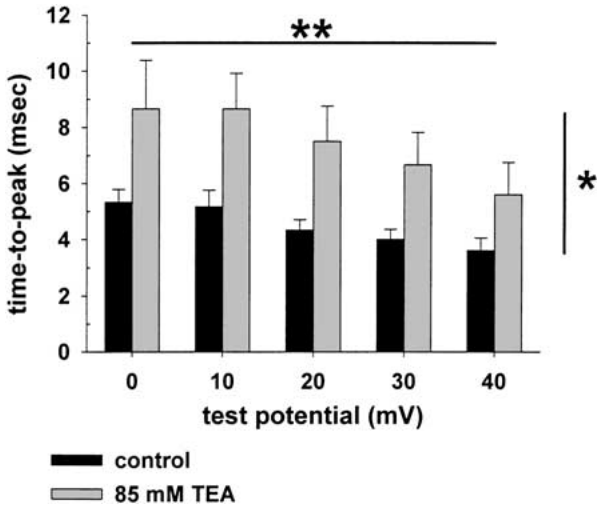

Fig. 9. TEA slows I_A activation kinetics. I_A was evoked at the test potentials shown, and the time required to reach peak amplitude was measured ($n = 5$). TEA slowed the time to reach peak amplitude (*, $P = 0.03$) and as the test potential became more positive, the time to peak was faster (**,$P = 0.005$). There was no significant interaction between the TEA concentration and test potential.

voltage drop across the channel's conduction path is thought to occur (Roux, Berneche & Im, 2000). Furthermore, TEA bound ~1.5 Å deeper along the channel axis in wild-type channels than in mutant channels lacking the aromatic residues. This result was in stark contrast to the interpretation that the increased voltage dependence of channel block of mutant *Shaker* channels resulted from penetration of TEA deeper in the channel pore (Heginbotham & MacKinnon, 1992).

The MD simulations also predicted that TEA and the outermost K^+ ion in the selectivity filter more strongly repelled one another in the mutant channel, because only a single water molecule separated these ions, whereas the wild-type channel accommodated two water molecules between external TEA and K^+. Thus, differences in electrostatic interactions between TEA and permeating K^+ ions may account for differences in potential-dependent block of the K^+ channel pore (Crouzy et al., 2001). We believe that the large electrical-distance value calculated for TEA block of I_A in *H. aspersa* is incompatible with the traditional interpretation that TEA traversed 65% of the voltage drop across the channel pore. We therefore propose that strong electrostatic coupling between TEA and nearby K^+ ions within the water-filled ion conduction pathway may explain the large electrical distance calculated for TEA block of *H. aspersa* A-type K^+ channels.

Effect of TEA on I_A Gating

An unexpected result of this study was that TEA caused substantial depolarizing shifts in the voltage and time dependence of A-type K^+ channel gating below the concentration required to fully suppress I_{KDR}. For example, TEA caused a dose-dependent depolarizing shift in the activation curve for I_A (Fig. 2*B*). TEA also increased the availability of A-type K^+ channels for activation at depolarized holding potentials (Fig. 6), decreased the time for removal of inactivation (Fig. 8), and slowed the activation kinetics of I_A (Fig. 9). Taken together, these observations suggest that TEA biased the transmembrane electrical field potential, and consequently, the voltage sensors of A-type channels were exposed to a relatively hyperpolarized membrane potential.

The effects of TEA on channel gating are similar to those reported for divalent cations, suggesting that these ions may augment gating through a common mechanism. Originally proposed to result from nonspecific screening of anionic surface charges (Frankenhauser & Hodgkin, 1957), a growing literature suggests that divalent cations act by binding to specific charged residues on the channel protein (Spires & Begenisich, 1992; Talkuder & Harrison, 1995; Elinder, Liu & Arhem, 1998; Zhang et al., 2001a). In the present study, TEA, which is a monovalent cation, was substituted on an equimolar basis for Na^+. Thus, net charge and ionic strength were not changed during application of TEA or Na^+-free saline. For this reason, the effects of TEA on I_A gating are unlikely to be explained according to surface charge theory (Hille, 1992) or by confounding effects of bath Na^+ removal on I_A gating (Fig. 7). We propose that TEA interacts with the channel at specific residues or specialized cation-binding structures on the outer portion of the channel protein. Because divalent cations have TEA-like effects on A-type channel gating in *H. aspersa* neurons (Bal et al., 2000), it will be of interest to assess whether TEA competes with divalent cations at a common binding site or if these ions have separate extracellular receptors.

From the available data, we cannot determine whether TEA modifies channel permeation and gating of A-type channels through a single binding site or through multiple TEA receptors. Conceivably, a single TEA molecule binding in the channel pore could induce structural changes that bias the local field potential surrounding voltage-sensing domains and, consequently, modify the voltage dependence of channel gating. Alternatively, TEA could alter channel gating by binding to multiple receptors located inside and outside the pore. Swartz and MacKinnon (1997) provided evidence that hanatoxin modifies K^+ channel gating through binding of four toxin molecules to the same number of receptors outside the pore. In addition, the effects of zinc (Zn^{2+}) on Kv1.5 channel gating support the existence of multiple cation binding sites on the extracellular surface of this channel (Zhang et al., 2001a, b). Future studies will be directed toward understanding the

stoichiometry of TEA binding to *H. aspersa* A-type K^+ channels.

Implications for the Effect of External TEA on Gating of A-type K^+ Channels in Snail Neurons

H. aspersa neurons are used widely as a source of native ion channels for electrophysiological studies of ion channel function (Neher, 1971; Crest & Gola, 1993; Bal et al., 2000) and selectivity (Reuter & Stevens, 1980; Taylor, 1987), and snail neurons are increasingly used in electrophysiological experiments to identify potential ion channel targets of novel pharmacological drugs (Walden et al., 1993; Solntseva et al., 1997; Krishtal et al., 2001; Solntseva, Bukanova & Skrebitsky, 2001) and biologically active peptides (Garateix et al., 2000; Kiss et al., 2000). Such studies often require pharmacological suppression of delayed outward currents to isolate I_A, and TEA is routinely used for this purpose. In the present study, we showed that TEA blocked I_A and modified its gating at concentrations below that required to completely block I_{KDR} (Fig. 1). Thus, in *H. aspersa* neurons, complete suppression of I_{KDR} with TEA necessarily comes at the expense of affecting several gating characteristics of I_A. We believe that this is an underappreciated pharmacological characteristic of I_A in *H. aspersa* neurons that can complicate investigation of I_A when high concentrations of TEA are used to isolate I_A.

For example, Bal et al. (2001) recently reported the identification of a novel A-type K^+ current in *H. aspersa* neurons that differed from conventional I_A in its voltage- and time-dependence of gating (Neher, 1971; Taylor, 1987; Bal et al., 2000). Specifically, this novel A-type current, termed I_{Adepol} (Bal et al., 2001), could be evoked from a holding potential of −40 mV, a potential that completely inactivates conventional I_A. The steady-state inactivation curve of I_{Adepol} was shifted by ~30 mV in the depolarizing direction compared to conventional I_A and, consequently, could be evoked at relatively depolarized holding potentials. The activation curve of I_{Adepol} exhibited a similar rightward shift along the voltage axis, and the activation kinetics of I_{Adepol} were moderately slower than those of conventional I_A. An important detail of this study, however, is that I_{Adepol} was studied after suppression of delayed outward currents with 40–50 mM TEA. Conventional I_A was isolated by standard current subtraction techniques (Hille, 1992) in TEA-free saline.

The results of our study suggest that many of the characteristics attributed to I_{Adepol} may be explained in terms of TEA-induced modification of conventional I_A characteristics. For example, we found that conventional I_A could be evoked even at a holding potential of −30 mV in TEA-containing saline, and that this effect was due to a rightward shift in the steady-state inactivation curve (Fig. 6). We explicitly activated conventional I_A by restricting the test pulses to −10 mV; I_{Adepol} was activated only at voltages more depolarized than −5 mV (Bal et al., 2001). In 42.5 mM TEA, which is close to the concentration of TEA used to isolate $I_{Adelpol}$, the half-inactivation voltage was shifted by ~7 mV (Fig. 6); this is a considerably smaller shift than that reported for I_{Adepol}. However, estimates of the half-inactivation voltage were not derived in the same way in the two studies, and this may explain the discrepancy between these two studies. For instance, in the present study, the steady-state inactivation curves for I_A were constructed by normalizing the current evoked at all test potentials to −120 mV. Bal et al. (2001) normalized currents to the maximum current recorded at −100 mV for conventional I_A, but normalized currents to that recorded at −40 mV when studying I_{Adepol}. This procedure necessarily forced the half-inactivation potential for I_{Adepol} to be more positive than −40 mV. When we duplicated the current normalization protocol used by Bal et al. (2001) with our current measurements recorded in 42.5 mM TEA, the $V_{0.5}$ of conventional I_A was shifted to −30 mV, which represents a 25-mV TEA-induced shift. This value is very close to the ~30-mV shift reported for I_{Adepol}, which again was measured in the presence of 40–50 mM external TEA (Bal et al., 2001). We also found that TEA slowed the activation kinetics of I_A (Fig. 9), a result that may explain the slower activation kinetics reported for I_{Adepol}. Finally, the current amplitude of I_{Adepol} was smaller than that of conventional I_A at equivalent test potentials. From our results, this may be explained by block of conventional I_A by TEA. Hence, we propose that the effect of TEA on a conventional A-type K^+ current, which has been described and studied at the whole-cell and single-channel level by others (Neher, 1971; Taylor, 1987; Bal et al., 2000), accounts for many of the biophysical characteristics attributed to I_{Adepol}.

It is important to note that TEA had a similar effect on the voltage-dependent properties of a conventional A-type K^+ current expressed in *Lymnaea stagnalis* neuronal somata (Alekseev & Ziskin, 1995). To our knowledge, the sensitivity of these molluscan A-currents to external TEA in *Lymnaea* and *Helix* is unique among the considerable number of K^+ currents studied to date. Consequently, important insights into the structural basis of TEA-dependent alteration of K^+ channel gating may be gained from studying cloned genes encoding A-type channels from molluscan neurons.

In summary, extracellular TEA blocked conventional I_A in a voltage-dependent manner. The relationship between external TEA sensitivity and electrical distance agrees with predictions from the model of TEA blockade of low affinity *Shaker* K^+ channels (Heginbotham & MacKinnon, 1992). However, the large electrical distance (65%) that we measured in this study raises concerns about the

traditional interpretation of this value (Hille, 1992). The large electrical distance calculated for TEA block of I_A in this study is unprecedented among other K^+ channels, and we believe that electrical-distance values do not provide useful information about the depth of TEA binding in the external pore. Rather, the strength of electrostatic repulsion between external TEA and K^+ ions exiting the selectivity filter may account for the steep relationship between membrane potential and channel block. Finally, TEA modified gating of conventional I_A at concentrations below that required to fully suppress I_{KDR}. Thus, pharmacological isolation of I_A with high concentrations of TEA necessarily comes at the expense of biasing the voltage-dependent properties of this current. It will be important to consider this caveat when studying A-type currents in *H. aspersa* and, perhaps, other snail A-type K^+ currents (Alekseev & Ziskin, 1995) in the future.

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