

Quaternary Organic Amines Inhibit Na,K Pump Current in a Voltage-dependent Manner: Direct Evidence of an Extracellular Access Channel in the Na,K-ATPase

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ABSTRACT The effects of organic quaternary amines, tetraethylammonium (TEA) chloride and benzyltriethylammonium (BTEA) chloride, on Na,K pump current were examined in rat cardiac myocytes superfused in extracellular Na⁺-free solutions and whole-cell voltage-clamped with patch electrodes containing a high Na⁺-salt solution. Extracellular application of these quaternary amines competitively inhibited extracellular K⁺ (K_o⁺) activation of Na,K pump current; however, the concentration for half maximal inhibition of Na,K pump current at 0 mV (K_Q^0) by BTEA, 4.0 ± 0.3 mM, was much lower than the K_Q^0 for TEA, 26.6 ± 0.7 mM. Even so, the fraction of the membrane electric field dissipated during K_o⁺ activation of Na,K pump current (λ_K), $39 \pm 1\%$, was similar to λ_K determined in the presence of TEA ($37 \pm 2\%$) and BTEA ($35 \pm 2\%$), an indication that the membrane potential (V_M) dependence for K_o⁺ activation of the Na,K pump current was unaffected by TEA and BTEA. TEA was found to inhibit the Na,K pump current in a V_M -independent manner, i.e., inhibition of current dissipated $4 \pm 2\%$ of the membrane electric field. In contrast, BTEA dissipated $40 \pm 5\%$ of the membrane electric field during inhibition of Na,K pump current. Thus, BTEA inhibition of the Na,K-ATPase is V_M -dependent. The competitive nature of inhibition as well as the similar fractions of the membrane electric field dissipated during K_o⁺-dependent activation and BTEA-dependent inhibition of Na,K pump current suggest that BTEA inhibits the Na,K-ATPase at or very near the enzyme's K_o⁺ binding site(s) located in the membrane electric field. Given previous findings that organic quaternary amines are not occluded by the Na,K-ATPase, these data clearly demonstrate that an ion channel-like structure provides access to K_o⁺ binding sites in the enzyme.

KEY WORDS: Na,K-ATPase • K⁺ binding • tetraethylammonium chloride • benzyltriethylammonium chloride

INTRODUCTION

The stoichiometry of ion transport by the Na,K-ATPase under physiologic conditions, 3 Na⁺ extruded from the cell in exchange for the uptake of 2 K⁺ per ATP hydrolyzed (Glynn, 1985; Rakowski et al., 1989), results in one positive charge being moved across the cell membrane per enzyme transport cycle (De Weer et al., 1988; Glynn and Karlsh, 1990; Läuger, 1991; Vasilets and Schwarz, 1993; Rakowski et al., 1997). This movement of positive charge can be measured as a membrane depolarization upon inhibition of the Na,K-ATPase with cardiac glycosides or as cardiac glycoside-inhibitible, extracellular K⁺ (K_o⁺)-dependent steady-state outward current (Na,K pump current) under voltage-clamp conditions where other ionic membrane currents are blocked or inactivated (Cooke et al., 1974; Isenberg and Trautwein, 1974; Eisner and Lederer, 1980; De Weer et al., 1984; Gadsby et al., 1985; Ishizuka et al.,

1996; Berlin and Peluffo, 1997). The net movement of positive charge during the enzyme transport cycle also requires that the rate of at least one reaction step is dependent on the membrane potential (Läuger, 1991). The finding that Na,K pump current is membrane potential (V_M) dependent in extracellular Na⁺ (Na_o⁺) and K_o⁺-containing salt solutions confirmed this theoretical requirement and demonstrated that under some conditions the V_M -dependent reaction(s) can become rate limiting for steady-state ion transport (Hansen et al., 1981; Läuger, 1991). In fact, Na,K pump current has a bell-shaped dependence on V_M in the presence of nonsaturating concentrations of Na_o⁺ and K_o⁺ (Lafaire and Schwarz, 1986; Bielen et al., 1991; Rakowski et al., 1991; Sagar and Rakowski, 1994; Berlin and Peluffo, 1997). Such a biphasic relationship necessitates the presence of at least two V_M -dependent reaction steps in the transport cycle (Läuger, 1991).

One V_M -dependent reaction of the Na,K-ATPase was identified with Na⁺-dependent transport steps by dem-

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Abbreviations used in this paper: BTEA, benzyltriethylammonium ion; NMG, N-methyl-D-glucamine; TMA, tetramethylammonium ion; TPA, tetrapropylammonium ion.

onstrating that the positive slope of the Na,K pump current-voltage relationship, observed at negative V_M in Na^+_{o} - and K^+_{o} -containing solutions, was lost when Na^+ was removed from the extracellular medium (Nakao and Gadsby, 1989; Rakowski et al., 1991; Sagar and Rakowski, 1994). The V_M -dependent step underlying this behavior was characterized by trapping the Na,K-ATPase in Na^+ -transporting conformations (under K^+_{o} -free conditions that promote Na^+ - Na^+ exchange) and studying presteady-state kinetics of Na^+_{o} -dependent charge-moving reaction steps (Nakao and Gadsby, 1986). Such experiments showed that transport of Na^+ to the extracellular medium moves the equivalent of a full charge across two thirds to three quarters of the membrane dielectric (Nakao and Gadsby, 1986; Bühler et al., 1991; Fendler et al., 1993; Rakowski, 1993; Hilgemann, 1994a; Holmgren and Rakowski, 1994; Heyse et al., 1994). Furthermore, the Na^+_{o} concentration dependence of this process provided evidence that the V_M -dependent step occurs during extracellular Na^+ release and binding (Gadsby et al., 1993; Wuddel and Apell, 1995). These results showed that the binding site for at least one Na^+ is within the membrane electric field, i.e., the binding site in the E_2P enzyme conformation is located in an ion well exposed to the extracellular medium.

In Na^+_{o} -free solutions, the Na,K pump current-voltage relationship showed a negative slope in the presence of nonsaturating concentrations of K^+_{o} (Bielen et al., 1991; Rakowski et al., 1991; Sagar and Rakowski, 1994; Berlin and Peluffo, 1997; Peluffo et al., 2000). Because Na^+_{o} -free solutions prevent Na^+_{o} -dependent reactions from influencing the V_M dependence of Na,K pump current, this negative slope demonstrated that a second V_M -dependent reaction was tied to K^+ -dependent transport steps. Measurement of transient charge movements dependent on K^+_{o} or extracellular Tl^+ (Tl^+_{o}), a K^+ congener, under conditions promoting K^+ - K^+ exchange showed that this reaction moved a charge through approximately one third of the membrane dielectric. The Tl^+_{o} concentration dependence of these charge movements also demonstrated that extracellular ion binding was the V_M -dependent reaction (Peluffo and Berlin, 1997). Thus, similar to Na^+_{o} binding, K^+ binds in an extracellularly facing ion well of the Na,K-ATPase.

This extracellularly-facing ion well is most often envisioned conceptually as a narrow access channel in the enzyme, analogous to the pore of an ion channel (De Weer et al., 1988; Rakowski et al., 1997). The V_M dependence of ion binding reactions in this scenario occurs because the membrane electric field strength affects the probability that ions occupy the access channel. However, as first pointed out by Hilgemann (1994b), an equally valid mechanism involves rapid, V_M -depen-

dent microscopic occlusion reactions in which weakly V_M -dependent ion binding/coordination reactions are accompanied by rapid rearrangements of charge in the ion-bound protein with respect to the membrane electric field. Charge rearrangement during microscopic ion occlusion reactions could involve intrinsic protein moieties and/or the bound ion; however, with this mechanism, V_M dependence of ion binding reactions arise because electric field strength affects the probability of charge rearrangements during ion binding and release. The problem is that this alternative mechanism of V_M -dependent ion binding reactions is experimentally indistinguishable from the access channel model as long as the proportion of the ion-bound, but not -occluded enzyme state remains very small. In fact, experimental data suggest that the fraction of Rb^+ (another K^+ congener) -bound, but not -occluded enzyme is indeed very low under conditions that promote K^+ occlusion by the Na,K-ATPase (González-Lebrero et al., 2002). Thus, it could be very difficult to distinguish between the alternate ion binding mechanisms outlined above in the presence of transported cations such as Na^+ and K^+ . However, if ion transport could be blocked in such a manner that a substantial fraction of the enzyme resides in an ion-bound, but not -occluded state, then these alternative scenarios for V_M -dependent ion binding should be experimentally distinguishable.

Organic quaternary amines inhibit ion transport by the Na,K-ATPase in a manner that is competitive with K^+_{o} activation of the enzyme (Kropp and Sachs, 1977) and inhibit Rb^+ occlusion under conditions that would otherwise promote binding of extracellular ions (Forbush, 1988). Of particular note in the context of the present study, quaternary amines do not appear to become occluded by the enzyme (Forbush, 1988). One interpretation of these data is that organic quaternary amines inhibit ion occlusion by the Na,K-ATPase by binding at or near K^+_{o} binding sites in the enzyme. Should this interpretation be correct, these organic amines may allow us to separate binding and occlusion reactions by the enzyme and thereby study the mechanism of V_M -dependent ion binding.

This study reports the concentration and V_M -dependent properties of Na,K pump current inhibition by two such quaternary amines, TEA and its related analogue, benzyltriethylammonium ion (BTEA). The results show that, while these two compounds inhibit K^+_{o} activation of Na,K pump current in a competitive manner, the V_M -dependent properties of enzyme inhibition are quite different. The implications of these results for defining the mechanism of V_M -dependent extracellular ion binding are then discussed.

Preliminary communications for these data have been published previously (Berlin and Peluffo, 1998; Peluffo et al., 2001).

Ventricular myocytes were enzymatically isolated from rat hearts and voltage-clamped with patch electrodes (1.0–1.5 MΩ) as published previously (Ishizuka et al., 1996). After establishing a whole-cell voltage clamp at a holding potential of -40 mV, cells were superfused in a Na⁺- and K⁺-free salt solution (see below) for 5–6 min to allow the contents of the patch electrode solution (see below) to exchange with the cell interior. Na,K pump current was defined as a K⁺_o-activated outward current that was reversibly inhibited in the presence of 1 mM ouabain. During experiments, voltage-clamped cells were exposed to various K⁺-containing superfusion solutions for short periods, <30 s, every 2 min to ensure that bulk cytosolic Na⁺ concentration was maintained at or near patch pipette Na⁺ concentration (115 mM) throughout the experiment. Superfusion solutions containing different K⁺ concentrations were applied in random order. To determine the V_M dependence of Na,K pump current, step changes in V_M between -100 and +40 mV were produced from the holding potential every 500 ms for 100 ms.

The time constant for settling of current after a 5-mV depolarization was typically in the 200–300 μs range without series resistance compensation and cell capacitances were in the range of 120–250 pF.

Solutions

The patch-electrode solution contained (in mM) 85 sodium sulfamate, 20 tetraethylammonium chloride, 10 ATP-Mg²⁺ salt, 5 pyruvic acid, 5 creatine phosphate-Tris salt, 10 EGTA-Tris salt, and 10 HEPES (pH 7.35 with NaOH). The total concentration of Na⁺ in this solution was 115 mM. Na⁺-free superfusion solutions contained the following (in mM): 145 tetramethylammonium (TMA) chloride, 2.3 MgCl₂, 0.2 CdCl₂, 5.5 dextrose, 10 HEPES

(pH 7.4 with Tris). KCl was added to this solution in various concentrations (0.05–5 mM). Our initial experiments were performed with a superfusion solution containing 145 N-methyl-D-glucamine (NMG) instead of TMA; however, TMA blocked contaminating ionic currents more completely than NMG. Furthermore, the K⁺_o dependence of Na,K pump current activation was similar in the presence of NMG and TMA-containing solutions (unpublished data). Tetraethylammonium chloride and benzyltriethylammonium chloride were added to the superfusion solution with equimolar substitution for TMA chloride. All experiments were performed at 37°C. Reagents were obtained from Sigma-Aldrich.

Statistics and Curve Fitting

Data are presented as mean ± SEM for the indicated number of cells. Current density was calculated by dividing K⁺_o-activated currents by cell capacitance measured as the integral of current elicited with 5-mV pulses. Indicated functions were fit to the data using a nonlinear least squares Gauss-Newton algorithm (Rossi and Garrahan, 1989) or with commercial software (SigmaPlot, SPSS).

RESULTS

Na,K pump current has been characterized previously as a K⁺_o-activated, ouabain-inhibitable outward current in single cardiac myocytes (Gadsby et al., 1985; Gadsby and Nakao, 1989; Bielen et al., 1991; Stimers et al., 1991; Ishizuka et al., 1996; Berlin and Peluffo, 1997). Fig. 1 shows this outward current in Na⁺_o-free solutions and briefly summarizes the experimental

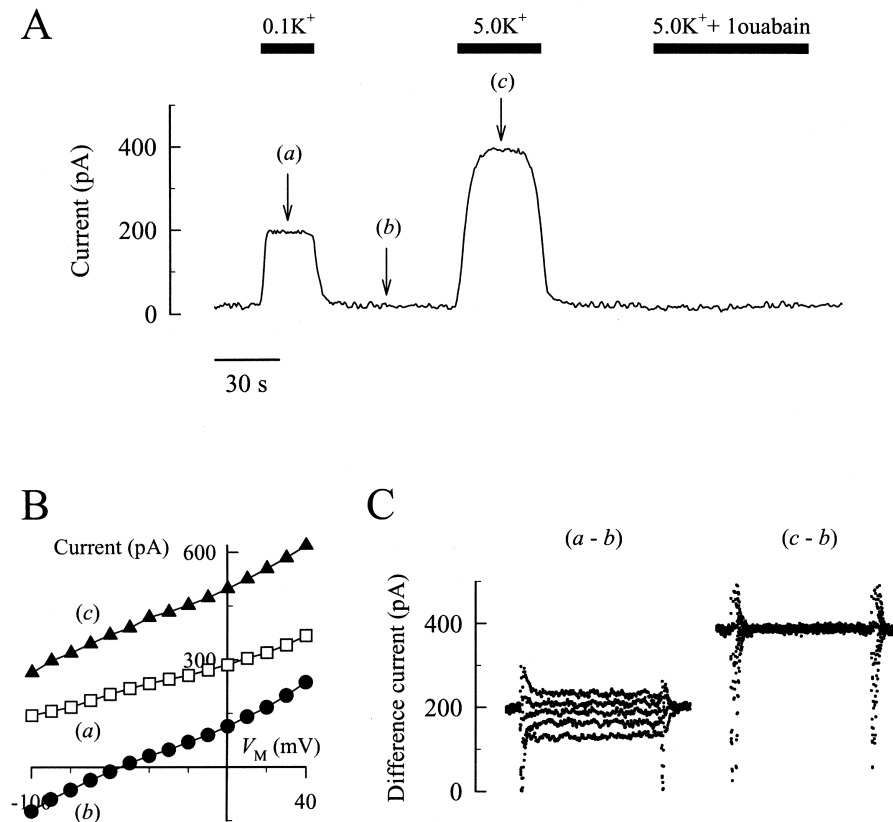


FIGURE 1. Protocol for measurement of Na,K pump current in rat cardiac myocytes. (A) Current record. A cell was held at -40 mV in a Na⁺_o- and K⁺_o-free superfusion solution. K⁺ and ouabain, at the indicated concentrations (in mM), were added to the superfusion solution for the periods indicated by the filled bars. The arrows indicate the time when current-V_M relationships in B were determined. (B) Current-V_M relationships. The displayed data are steady-state current levels averaged over the last 50 ms of each voltage step. (C) K⁺_o-sensitive difference currents. 0.1 mM (a-b) and 5 mM K⁺_o-sensitive currents (c-b) for 100-ms voltage steps are shown. Superimposed tracings (a-b) are currents in response to voltage steps to -80, -50, -20, +10, +40 mV from top to bottom, respectively. Superimposed tracings (c-b) do not show differences at any V_M tested. Na,K pump current levels shown in remaining figures were measured with similar maneuvers.

protocol used throughout this study. After establishing a whole-cell voltage clamp, rat ventricular myocytes were initially superfused with a Na^+ - and K^+ -free salt solution. When the K^+ concentration in the superfusion solution was subsequently increased, an outward current was observed (Fig. 1 A) that was maintained throughout the period of K^+ exposure except when 1 mM ouabain was also present in the superfusion solution. To determine the V_M dependence of Na,K pump current (Fig. 1 B), step changes in V_M from the holding potential of -40 mV were produced at the times indicated in Fig. 1 A and K^+ -sensitive difference currents were calculated at each V_M (Fig. 1 C). When a saturating K^+ concentration (5 mM) was used to activate the Na,K pump, the resulting difference current (c-b), showed very little dependence on V_M in the range of -100 to $+40$ mV; however, when nonsaturating K^+ concentrations (0.1 mM) were used, the Na,K pump current became smaller at more positive V_M (a-b), as reported previously (Rakowski et al., 1991; Sagar and Rakowski, 1994; Berlin and Peluffo, 1997; Peluffo et al., 2000).

The V_M dependence of Na,K pump current was determined over a range of K^+ concentrations between 0.05 and 5 mM (Fig. 2 A). These data make it obvious that, as K^+ is lowered, the current- V_M relationship displays a negative slope and an apparent shift to the left along the V_M axis. To measure the effect of V_M on Na,K pump current, the K^+ dependence of current activation was determined by fitting a Hill equation to the data at each V_M . The calculated maximal current density (I_{max}) at each V_M is shown in Fig. 2 B. As can be seen in this figure, I_{max} appeared to show little change with V_M . This impression was confirmed by regression analysis that showed the slope of a line through the data as a function of V_M was not significantly different than zero. For this reason, I_{max} was averaged over all test V_M to yield a value of 2.40 ± 0.10 pA/pF.

This fitting procedure also yielded the Hill coefficient (γ_K) for K^+ activation of Na,K pump current. Similar to I_{max} , the value of γ_K was found to show no trend with V_M (Fig. 2 B), so γ_K at all 15 V_M tested were averaged to yield a value of 0.58 ± 0.07 . This value is similar to that measured for K^+ activation of Na,K pump current under zero extracellular Na^+ conditions by Sagar and Rakowski (1994) in oocytes and by Peluffo et al. (2000) in HeLa cells expressing heterologous Na,K-ATPase.

By comparison, the concentration of K^+ needed to yield a similar level of current activation increased at more positive V_M , as is reflected by an increase in the K^+ concentration required for half-maximal activation ($K_{0.5}$) from 35 ± 4 to 365 ± 53 μM in going from -100 to $+40$ mV, respectively (Fig. 2 C). Thus, as reported previously (Sagar and Rakowski, 1994; Peluffo

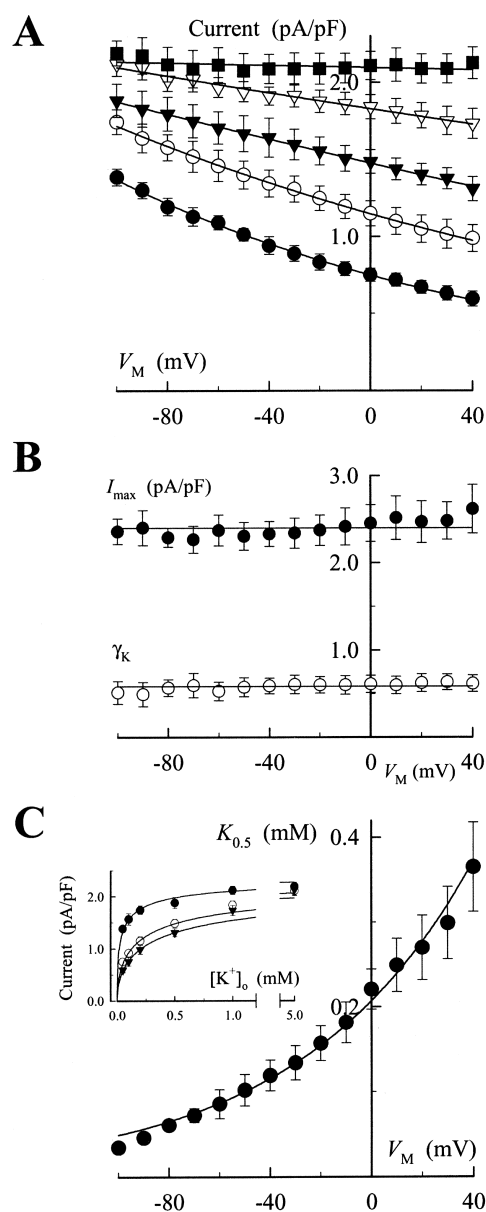


FIGURE 2. V_M and K^+ concentration dependence of Na,K pump current. (A) V_M dependence of Na,K pump current at various K^+ concentrations. Currents were measured as in Fig. 1 and plotted versus V_M for superfusion solutions containing 0.05 (●), 0.2 (○), 0.5 (▼), 1.0 (▽), and 5.0 mM K^+ (■). The curves were fit to the data by eye. (B) The V_M dependence of I_{max} and γ_K . A Hill equation was fit to the data in A at each V_M . The resulting values of I_{max} and γ_K along with their standard errors are shown. The solid lines are the average parameter values calculated over all test V_M . Note: the units of the y-axis differ for I_{max} and γ_K . (C) V_M dependence of the apparent affinity for K^+ activation of Na,K pump current. The $K_{0.5}$ values derived from fitting the data at each V_M with a Hill equation are plotted versus V_M . The solid curve is the best-fit function to Eq. 1 with $K_{0.5}^0 = 204$ μM and $\lambda_K = 0.39$. (Inset) K^+ dependence of Na,K pump current at -100 (●), 0 (○) and $+40$ mV (▼) is shown. The curves were generated by solving Eq. 6 (APPENDIX) at various K^+ concentrations using the parameter values determined from the fitting procedure described in the text.

et al., 2000), the apparent affinity for K^+ activation of the Na,K pump is decreased as the cell interior is made more positive, consistent with the idea that K^+ binding occurs in an extracellularly facing ion well.

Under the conditions of the present experiments, high intracellular and zero extracellular Na^+ , Na,K pump current can be described by a pseudo two-state model that includes one lumped reaction step as well as a second V_M - and K^+ -dependent reaction (Hansen et al., 1981; Sagar and Rakowski, 1994; Peluffo et al., 2000). Using this model, the following equation,

$$K_{0.5} = K_{0.5}^0 \exp(\lambda_K U), \quad (1)$$

can be derived to determine the $K_{0.5}$ at 0 mV ($K_{0.5}^0$) and the portion of the membrane electric field (λ_K) dissipated during K^+ -dependent reactions. The term “ U ” is the unitless form of voltage, zFV_M/RT , where z is equal to 1, the net charge moved during the ion transport cycle. Fitting Eq. 1 to the data in Fig. 2 C yields values of $K_{0.5}^0$ and λ_K of $204 \pm 4 \mu M$ and 0.39 ± 0.01 ($n = 15$), respectively. This value of $K_{0.5}^0$ and the fact that K^+ -dependent reactions dissipate approximately one third of the membrane dielectric are consistent with our previous data (Berlin and Peluffo, 1997; Peluffo et al., 2000).

The two-step fitting procedure described above for the data in Fig. 2 allowed for the determination of four parameters, I_{max} , $K_{0.5}^0$, γ_K , and λ_K , that describe the K^+ concentration and V_M dependence of Na,K pump current under the conditions of these experiments. To determine the utility of this fitting procedure, Eq. 6 in the APPENDIX was solved at each V_M and K^+ concentration tested using these parameter values. The calculated current densities were then compared with the experimental data. Using the entire set of experimentally measured current densities, the root mean square error of the calculated current densities was found to be 0.0057 pA/pF. Similarly, the sum percent error of the calculated current densities, defined as

$$\text{Sum percent error} = \frac{\sum_i \sum_j |\hat{Y}_{ij} - Y_{ij}|}{\sum_i \sum_j Y_{ij}} \times 100,$$

where \hat{Y}_{ij} and Y_{ij} are the calculated and measured current densities, respectively, at the i^{th} K^+ concentration and j^{th} V_M , was determined to be 3.5%. Both error values show that differences between calculated and measured currents are quite small, an indication that our fitting procedure provides an acceptable description of the data. The accuracy of the fitting procedure can also be inferred by comparing the experimental data to curves determined by solving Eq. 6 (APPENDIX) using the fitted parameters values for

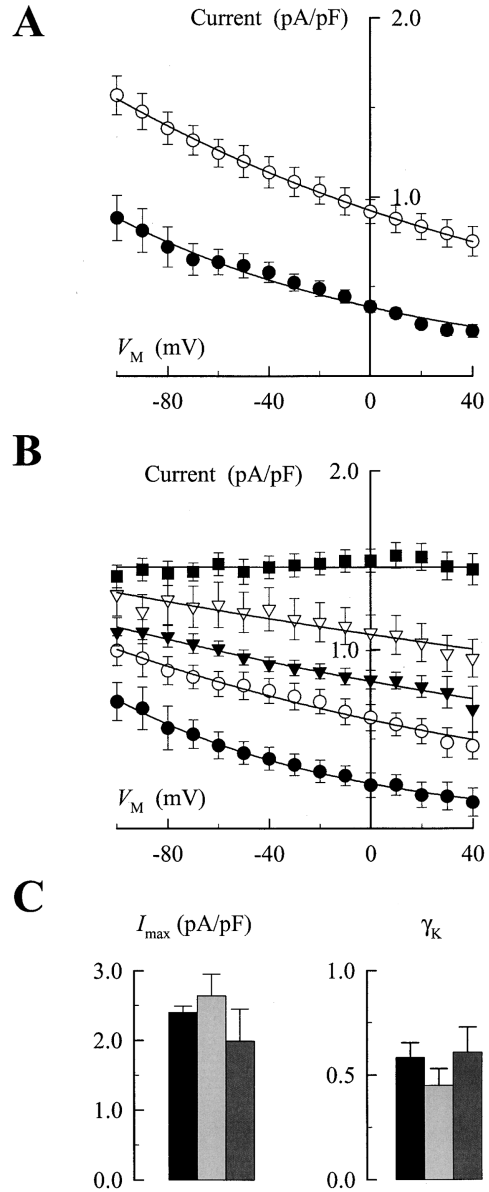


FIGURE 3. Effect of TEA on the V_M and K^+ concentration dependence of Na,K pump current. (A) V_M dependence of 0.1 mM K^+_o -activated Na,K pump current measured in the presence (●) and absence (○) of 25 mM TEA. Curves were fit to the data by eye. (B) V_M dependence of Na,K pump current in the presence of 25 mM TEA. Na,K pump current densities are plotted as in Fig. 2 A for superfusion solutions containing 0.05 (●), 0.2 (○), 0.5 (▼), 1.0 (▽), and 5.0 mM K^+_o (■). Curves were fit to the data by eye. (C) Average I_{max} (left) and γ_K values (right) plus SEM calculated in the presence of 0 (black), 5 (light gray), and 25 mM TEA (dark gray).

I_{max} , $K_{0.5}^0$, γ_K , and λ_K . As can be seen in the inset of Fig. 2 C, the calculated curves closely follow the experimentally measured current densities at -100 , 0 , and $+40$ mV. Given the outcome of these tests, we used a similar two-step fitting procedure when analyzing subsequent data.

Effect of Extracellular Tetraethylammonium ions on Na,K Pump Current

Extracellular TEA has been shown to inhibit activity (Zemková et al., 1988), ion transport (Sachs and Conrad, 1968), and current generation by the Na,K-ATPase (Eckstein-Ludwig et al., 1998). For this reason, our initial characterization of the effects of organic quaternary amines began with TEA. Experiments were performed in the same manner as outlined in Fig. 1 A except that immediately after establishing a whole-cell voltage-clamp, the cells were superfused in the Na⁺_o- and K⁺_o-free salt solution that also contained TEA at the indicated concentrations. TEA was substituted in equimolar fashion for TMA, which itself does not affect the Na,K pump (unpublished data). Fig. 3 A shows that 25 mM TEA decreased the 0.1 mM K⁺_o-activated outward current at all V_M tested. Similar to control conditions (0 TEA), the current-V_M relationship displayed a negative slope.

To describe the inhibitory effects of TEA on Na,K pump current completely, the experiments shown in Fig. 2, measuring K⁺_o and V_M dependence of Na,K pump current, were repeated in the presence of various TEA concentrations. The effect of 25 mM TEA on the K⁺_o and V_M dependence of Na,K pump current was examined in eight cells. The resulting data, shown in Fig. 3 B, appeared to be quite similar to currents recorded under control conditions (Fig. 2 A) in the sense that, with 5 mM K⁺_o, the current displayed little V_M dependence but, at lower K⁺_o concentrations, a negative slope in the current-V_M relationship was still obvious. Just as in Fig. 3 A, however, the current densities at non-saturating K⁺_o concentrations were smaller in the presence of 25 mM TEA.

These data were fit with a Hill equation at each V_M to calculate parameter values for I_{max}, γ_K, and K_{0.5}. As in Fig. 2, the values for I_{max} and γ_K showed no dependence on V_M so they were averaged for all 15 V_M tested to yield values of 1.99 ± 0.46 pA/pF and 0.61 ± 0.12, respectively. Similar experiments and analyses were performed with 13 cells exposed to 5 mM TEA (not depicted) and the calculated average values for I_{max} and γ_K were 2.64 ± 0.31 pA/pF and 0.45 ± 0.08, respectively. These parameter values, along with those determined in the absence of TEA, are summarized in Fig. 3 C.

The calculations above led to two conclusions. First, the values of I_{max} in the presence of 5 and 25 mM TEA are not significantly different than I_{max} in the absence of the quaternary amine. In agreement with previous reports, these results suggest that TEA inhibition of Na,K pump current is competitive with K⁺_o activation of ion transport (Sachs and Conrad, 1968). Second, the values of γ_K were likewise not significantly different in the presence and absence of TEA. Given this lack of effect, the three mean values of γ_K were averaged and the

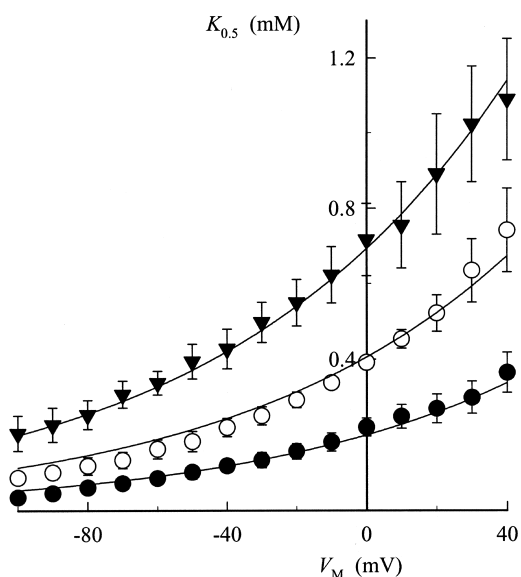


FIGURE 4. Effect of TEA on the apparent K⁺_o affinity for activation of Na,K pump current. Na,K pump current was measured in the presence of 0 (●), 5 (○), and 25 mM TEA (▼) and K_{0.5} values were calculated at each V_M as described in the text. Curves are functions determined by simultaneously fitting all the data to Eq. 2 with γ_K fixed at a value of 0.55. The resulting parameter values for K_{0.5}⁰, K_Q⁰, γ_Q, λ_K, and λ_Q are listed in the text.

result (0.55) was used to fix γ_K during simultaneous fitting of the data in Fig. 4, as discussed below.

Fig. 4 shows the K_{0.5} values calculated at each V_M and then plotted against V_M for each TEA concentration. The K_{0.5} values in Fig. 2 C are plotted again for purposes of comparison. These data show that TEA produces a concentration-dependent increase in K_{0.5} at all V_M. As in control experiments, the apparent affinity for K⁺_o activation of Na,K pump current was decreased at more positive V_M, i.e., K⁺_o activation still appears to be V_M-dependent in the presence of TEA.

To determine how TEA affects the V_M dependence of Na,K pump activation by K⁺_o, the data in Fig. 4 were fit with an equation derived from a pseudo three-state model for the Na,K pump exposed to an extracellular inhibitor that is competitive with K⁺_o for activation of the enzyme (see APPENDIX):

$$K_{0.5} = K_{0.5}^0 \exp(\lambda_K U) \times \left\{ 1 + \left(\frac{[Q]}{K_Q^0} \right)^{\gamma_Q} \exp(-\gamma_Q \lambda_Q U) \right\}^{\frac{1}{\gamma_K}} \quad (2)$$

where [Q] and K_Q⁰ are the quaternary amine concentration and the amine concentration for half-maximal inhibition at 0 mV, respectively, and γ_Q and λ_Q are the Hill coefficient and the portion of the membrane dielectric dissipated during Na,K pump current inhibi-

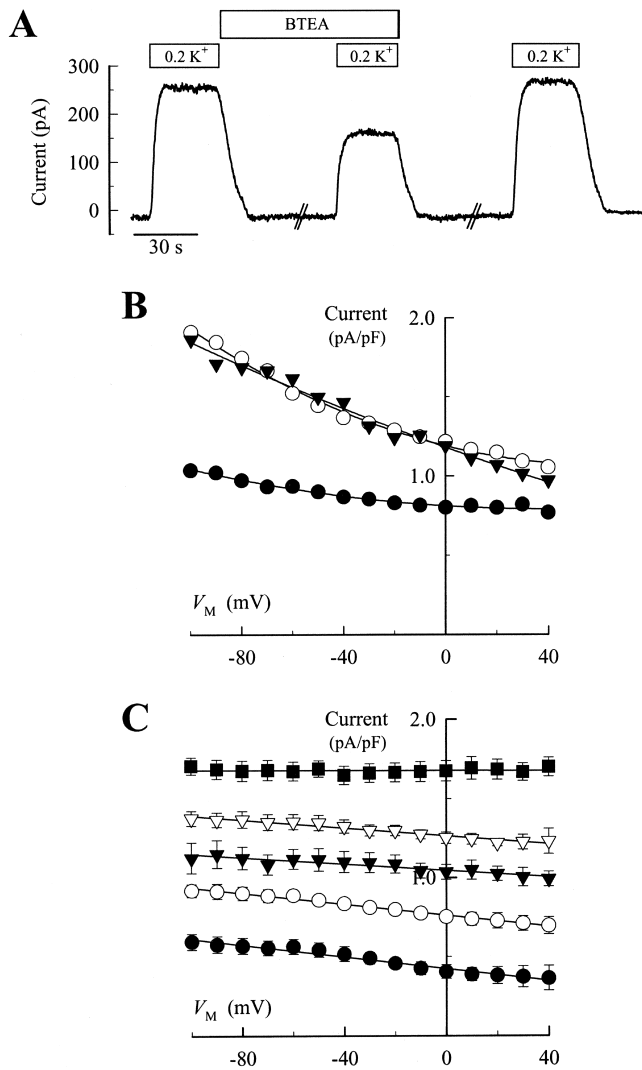


FIGURE 5. Effect of BTEA on Na,K pump current. (A) Current record showing the reversibility of BTEA inhibition. BTEA (2 mM) and 0.2 mM K_o⁺ were added to Na_o⁻- and K_o⁺-free superfusion solution as indicated. Note the breaks in the current record. In this experiment, the myocyte was incubated with BTEA-containing, K_o⁺-free solution for 1.5 min before current activation with 0.2 mM K_o⁺. Likewise, 0.2 mM K_o⁺-activated current was measured 1.5 min after washout of BTEA-containing solution. (B) Effect of BTEA on the V_M dependence of 0.2 mM K_o⁺-activated Na,K pump current. Displayed current-V_M relationships were determined before (○), during (●), and after superfusion with 2 mM BTEA-containing solution (▼). (C) V_M dependence of Na,K pump current in the presence of 5 mM BTEA. Na,K pump current densities are plotted as in Fig. 2 A for superfusion solutions containing 0.05 (●), 0.2 (○), 0.5 (▼), 1.0 (▽), and 5.0 mM K_o⁺ (■). Curves were fit to the data by eye.

tion, respectively. This equation has six parameters that can be varied to optimize the fit to the data. However, information regarding one of these parameters, γ_K , is not available in the plotted data. Even so, as mentioned above and shown in Fig. 3 C, γ_K is independent of V_M and TEA concentration. This finding allows γ_K to be

fixed, in this case, at its average value of 0.55, while the other five parameters are varied to fit Eq. 2 to the data in Fig. 4 as a function of V_M and amine concentration.

Solid curves in Fig. 4 are the best-fit functions produced by simultaneously fitting Eq. 2 to the entire displayed dataset. The calculated values of $K_{0.5}^0$ and λ_K in the presence of TEA were $201 \pm 6 \mu\text{M}$ and 0.37 ± 0.02 , respectively. These parameter values were not significantly different than those calculated in the absence of TEA with Eq. 1 (in Fig. 2 C). Furthermore, these calculations showed that K_Q^0 for TEA was $26.6 \pm 0.7 \text{ mM}$. The value of λ_Q , 0.04 ± 0.02 , was not significantly different than zero, so that it is clear that TEA inhibition does not occur in the membrane electric field, i.e., TEA inhibition is V_M independent. These results are consistent with those of Eckstein-Ludwig et al. (1998), who also found that TEA block of Na,K pump currents in oocytes is not V_M dependent. The results in Fig. 4 are also consistent with the predictions of a pseudo three-state model for the Na,K-ATPase in the presence of a V_M-independent blocker that inhibits the enzyme in a competitive manner with K_o⁺ (see APPENDIX and Fig. 9).

The value of γ_Q calculated with Eq. 2 was equal to 0.45 ± 0.02 . This value of γ_Q is significantly less than one, suggesting that negative cooperativity, possibly between multiple TEA molecules, occurs during inhibition of the Na,K-ATPase. This value is also similar to that for γ_K (0.55).

Effect of Extracellular Benzyltriethylammonium Ions on Na,K Pump Current

In studying the effects of organic amines on Rb⁺ occlusion by the Na,K-ATPase, Forbush (1988) noted that quaternary amines containing a benzyl side chain had the highest apparent affinities for slowing release of occluded Rb⁺ from the enzyme. Therefore, to determine if these benzyl-containing quaternary amines inhibited Na,K pump current in a similar manner to TEA, we examined the effects of a benzylic derivative of TEA, BTEA, on Na,K pump current. At concentrations where TEA had little effect on current, BTEA decreased Na,K pump current amplitude by nearly 50% (Fig. 5 A). Like TEA, current inhibition by BTEA was rapidly reversible at all V_M (Fig. 5, A and B). Most interestingly, the V_M dependence of current in the presence of BTEA looked quite different than current in the presence of TEA. Inspection of Figs. 3 A and 5 B shows that BTEA appeared to inhibit current more effectively at negative V_M, whereas TEA more effectively blocked current at positive V_M. Thus, the effect of V_M on Na,K pump current in the presence of BTEA appears to be different than that observed with TEA, a V_M-independent blocker of the Na,K-ATPase.

To examine the properties of BTEA block more fully, experiments in Figs. 1 and 2 were repeated in the presence of various BTEA concentrations from 0.5 to 5

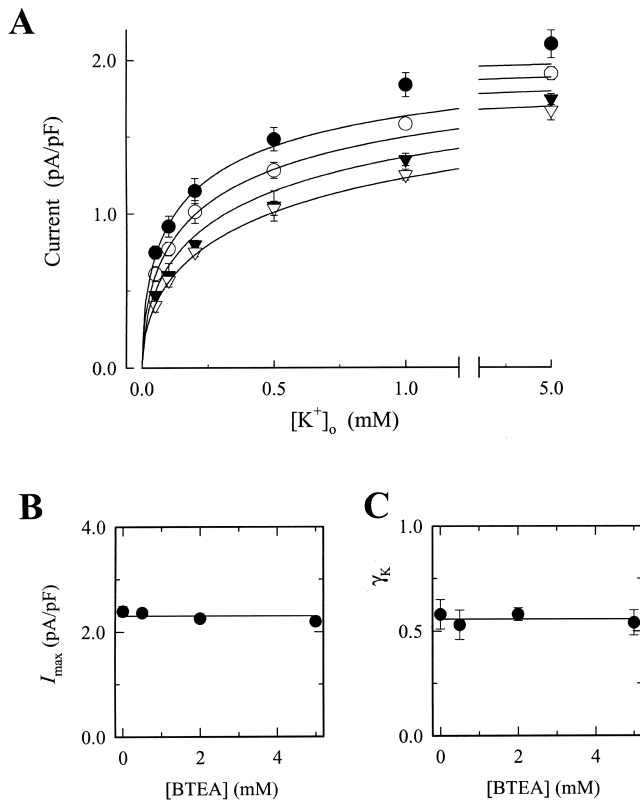


FIGURE 6. Effect of BTEA on the K^+_o concentration dependence of Na,K pump current at 0 mV. (A) K^+_o dependence of Na,K pump current at 0 mV in the presence of 0 (\bullet), 0.5 (\circ), 2 (\blacktriangledown), and 5 mM BTEA (∇). Data were collected from 14, 8, 12, and 10 cells, respectively. Curves result from the solution of Eq. 5 (APPENDIX) at each BTEA concentration using the average I_{max} and γ_K values calculated for the entire BTEA dataset (see B and C, respectively) along with $K^0_{0.5}$, K^0_Q and γ_Q values in the text. (B) Effect of BTEA on maximal Na,K pump current density. Data points are the average I_{max} and SEM determined at each BTEA concentration. For some points, SEM is smaller than the symbol. The line is the average value of the displayed data points. (C) Effect of BTEA on the Hill coefficient for K^+_o activation of Na,K pump current. Data points are the average γ_K and SEM determined at each BTEA concentration. The line is the average value of the displayed data points. See the text for details.

mM. Fig. 5 C shows the K^+_o and V_M dependence of Na,K pump current measured in the presence of 5 mM BTEA. The notable feature of Fig. 5 B, i.e., the relatively shallow slope of the current- V_M relationship in the presence of BTEA, is observed at all K^+_o examined, quite unlike the current- V_M relationship of Na,K pump current in the absence of BTEA (Fig. 2 A). The concentration dependence of BTEA effects on Na,K pump is summarized in Fig. 6 A, which shows current density measured at 0 mV in the presence of different BTEA concentrations and plotted as a function of K^+_o concentration. BTEA reduced Na,K pump current density in a concentration-dependent manner.

Similar to the analytical procedure shown in Fig. 3,

the data at each V_M and BTEA concentration were fit with a Hill equation. This fitting procedure showed that both I_{max} and γ_K were V_M independent at all BTEA concentrations tested (not depicted). As a result, average values of I_{max} and γ_K for the 15 V_M tested were calculated and plotted in Fig. 6, B and C, respectively. Over the concentration range tested, BTEA had no significant effect on I_{max} or γ_K , an indication that this quaternary amine is a competitive inhibitor of Na,K pump current. These average values of I_{max} and γ_K were, therefore, averaged again to yield values of 2.30 pA/pF and 0.56, respectively.

Fitting the Na,K pump current data obtained at each V_M with Hill equations also showed the V_M and BTEA dependence of $K^0_{0.5}$ values (Fig. 7). BTEA produced a concentration-dependent increase in $K^0_{0.5}$ at all V_M tested. The unusual feature of the data was the apparent parallel shift of $K^0_{0.5}$ to higher values with BTEA, irrespective of V_M . This type of behavior is actually predicted for a V_M -dependent inhibitor of the Na,K-ATPase (see APPENDIX). By simultaneously fitting Eq. 2 to all data in Fig. 7 with γ_K fixed at 0.56, $K^0_{0.5}$ and λ_K for current activation, $200 \pm 10 \mu\text{M}$ and 0.35 ± 0.02 , were found to be unchanged in the presence of BTEA. On the other hand, the K^0_Q for block of current activation was calculated to be $4.0 \pm 0.3 \text{ mM}$, a value almost sevenfold lower than that for TEA. Thus, consistent with the results of Forbush (1988), the benzylic side chain increased the potency for block of ion transport. These same calculations showed that λ_Q for BTEA was 0.40 ± 0.05 , a demonstration that a sizable fraction of the membrane dielectric was dissipated during BTEA block. In other words, BTEA inhibited the Na,K-ATPase in a V_M -dependent manner. In addition, λ_Q was not significantly different than λ_K . The implications of this result are presented in the DISCUSSION.

To demonstrate that the calculated parameters provided a reasonable description of the data, the values of these parameters were used to solve Eq. 5 (APPENDIX) at each BTEA concentration. The resulting curves (Fig. 6 A) showed that the predicted values were very close to the experimental data points.

Given the average value of γ_K above, 0.56, Eq. 2 predicts that the value of $K^0_{0.5}$ (at any V_M) will increase monotonically in a parabolic-like manner with BTEA concentration if γ_Q equals 1. The inset of Fig. 7 does show that $K^0_{0.5}$ (at 0 mV) increases monotonically with BTEA; however, the slope of the relationship between $K^0_{0.5}$ and BTEA becomes somewhat less steep at the higher amine concentrations tested. Such a nonlinear relationship between $K^0_{0.5}$ and amine concentration is predicted by Eq. 2 if $\gamma_Q < 1$. In fitting Eq. 2 to the data, the value of γ_Q was found to be 0.56 ± 0.03 . By plotting Na,K pump current density versus BTEA concentration at each K^+_o , γ_Q was found to be independent of K^+_o .

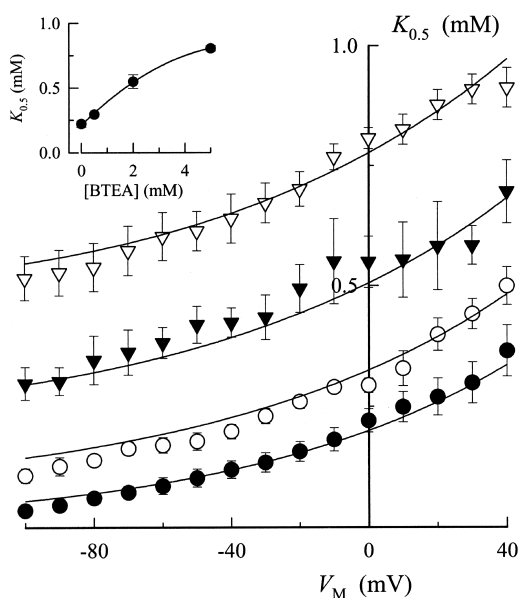


FIGURE 7. Effect of BTEA on the V_M dependence of apparent K_o affinity for activation of Na,K pump current. $K_{0.5}$ values calculated at each V_M are plotted for Na,K pump current measured in the presence of 0 (●), 0.5 (○), 2 (▼), and 5 mM BTEA (▽). Curves are the functions determined by simultaneously fitting all the data to Eq. 2 with $\gamma_K = 0.56$. The resulting parameter values for $K_{0.5}^0$, K_{Q}^0 , γ_Q , λ_K , and λ_Q are listed in the text. (Inset) $K_{0.5}$ at 0 mV plotted as a function of BTEA concentration. The curve through the data points was fit by eye.

concentration (not depicted). These results indicate that BTEA inhibits the Na,K-ATPase with negative cooperativity.

DISCUSSION

The main finding of this study is that the organic quaternary amine, BTEA, inhibits Na,K pump current in a V_M -dependent manner, whereas tetraethylammonium ion blocks Na,K pump current in a V_M -independent manner. To the best of our knowledge, V_M -dependent block has not been reported previously for a Na,K-ATPase inhibitor. Therefore, to understand the mechanism of such block, we first need to review the effects of quaternary amines on ion binding and occlusion by the Na,K-ATPase as well as the V_M dependence of ion binding reactions themselves. In brief, the basis of our conclusion that quaternary amines produce V_M -dependent block resides in the fitting of experimental data to a pseudo three-state model that has two premises: (1) K_o binding to the Na,K-ATPase is V_M dependent, and (2) quaternary amine inhibition is competitive with K_o activation. Evidence supporting these premises is reviewed below.

K_o binding is the V_M -dependent reaction during ion transport by the Na,K-ATPase: Evidence for the V_M dependence of a K_o -dependent reaction step during ion

transport by the Na,K-ATPase is shown by experiments, similar to those in Fig. 1, which display a K_o -dependent negative slope in the current- V_M relationship of Na,K pump current (Bielen et al., 1991; Rakowski et al., 1991; Sagar and Rakowski, 1994; Berlin and Peluffo, 1997; Peluffo et al., 2000). To attribute this V_M dependence of Na,K pump current to K_o -dependent reactions, experiments were performed under conditions that prevent other V_M -dependent reaction steps from regulating the kinetics of the ion transport cycle. The concentration of cytosolic Na^+ in this study, set by the patch-electrode solution containing 115 mM Na^+ , is high enough to saturate cytosolic-facing Na^+ binding sites of the Na,K-ATPase whose apparent Na^+ affinity, under turnover conditions similar to our own, is ~ 10 mM (Nakao and Gadsby, 1989). With saturating concentrations, cytosolic Na^+ binding reactions are not rate limiting. Therefore, the V_M -dependent properties of these reactions cannot be observed. In addition, experiments were also performed in the absence of extracellular Na^+ , conditions that render extracellular Na^+ release reactions by the Na,K-ATPase irreversible so that enzyme turnover will not depend on the V_M dependence of these reactions (Lauser, 1991). Our observation that Na,K pump current density measured in the presence of saturating K_o concentrations (5 mM) is almost independent of V_M (Figs. 1 C and 2 A) also confirms the point that V_M -dependent reaction steps do not control enzyme turnover under these conditions, thereby allowing us to conclude that any V_M dependence of intracellular K^+ -dependent reactions (e.g., Hansen et al., 2002) is not apparent under our experimental conditions. Thus, a K_o -dependent step is the only V_M -dependent reaction that can limit enzyme turnover in our experiments.

The mechanism of the K_o - and V_M -dependent reaction was identified in our previous work (Peluffo and Berlin, 1997) by trapping the Na,K-ATPase in K^+ -transporting conformations, i.e., those that promote K^+-K^+ exchange, and then measuring the Tl_o^+ dependence of presteady-state charge movements during perturbations of V_M . The disappearance of these charge movements at saturating Tl_o^+ concentrations demonstrated that the V_M -dependent reaction is extracellular Tl^+ (or K^+) binding.

Quaternary Amines Competitively Block K^+ Transport by the Na,K-ATPase

The competitive nature of quaternary amine inhibition of Na,K pump current is demonstrated by our data showing that maximal Na,K pump current density is unchanged (Figs. 3 C and 6 B) while the apparent affinity for K_o activation of current is significantly decreased (Figs. 4 and 7). Previous studies have also shown comparable effects of extracellular TEA (Sachs

and Conrad, 1968) and tetrapropylammonium ion (TPA) on Na^+ and K^+ transport mediated by the Na,K-ATPase in red blood cells (Kropp and Sachs, 1977) and TEA on Na,K pump currents in oocytes (Eckstein-Ludwig et al., 1998). Thus, we conclude that our results are consistent with previous reports that quaternary amines inhibit K^+ -dependent activation of ion transport by the Na,K-ATPase in a competitive manner.

Our own data and previous reports, therefore, suggest that the premises of the pseudo three-state model (see APPENDIX) are valid. As a result, we concluded that BTEA inhibits Na,K pump current in a V_M -dependent manner. On the other hand, TEA appears to block Na,K pump current in a V_M -independent manner. The occurrence of V_M -dependent block and the effect of quaternary amines on ion occlusion by the Na,K-ATPase provide evidence to distinguish between the two alternative mechanisms posited in the literature (Hilgemann, 1994b) to explain V_M -dependent ion binding in the Na,K-ATPase. The basis for distinguishing between these alternative mechanisms is discussed below.

The most detailed study of quaternary amine block of ion transport (Kropp and Sachs, 1977) suggested that TPA is a dead-end inhibitor with highest affinity for the Na,K-ATPase without bound K^+ , slightly decreased affinity when the enzyme is bound with a single K^+ and a very much lower affinity for the doubly K^+ -bound enzyme. This behavior was characterized in Na^+ -free conditions, similar to the present study, and a hyperbolic relationship between K^+ concentration and ion transport rate was observed in the presence and absence of amine. A linear Dixon plot of ion transport rate versus TPA concentration was interpreted as showing that binding of a single quaternary amine was sufficient to inhibit the Na,K-ATPase. In the present experiments, the Hill coefficient (γ_K) for K^+ activation of Na,K pump current in the absence of TEA or BTEA was 0.58, an indication of negative cooperativity for K^+ activation of enzyme turnover. Interestingly, the Hill coefficient (γ_Q) for quaternary amine inhibition of current had a similar value, whether the amine was TEA or BTEA. Negative cooperativity suggests that more than one amine may be involved in inhibiting Na,K pump current. Furthermore, since the Hill coefficient for TEA and BTEA inhibition of current is independent of K^+ , this negative cooperativity is unlikely to be due to a quaternary amine- K^+ interaction.

Effect of Amines on K^+ Occlusion by the Na,K-ATPase

Binding and occlusion of extracellular K^+ by the Na,K-ATPase appears to be an ordered process in which only one of the two K^+ binding sites is accessible from the extracellular medium (Forbush, 1987; González-Lebrero et al., 2002). This postulate rests principally on

data showing that, in the presence of Mg^{2+} and inorganic phosphate (P_i), K^+ and K^+ congeners slow the release kinetics of $\sim 50\%$ of the Rb^+ occluded by the Na,K-ATPase, without affecting the rapid release of the remaining occluded Rb^+ (Glynn et al., 1985; Forbush, 1987). Similar results were obtained whether Rb^+ was occluded without other ligands or in the presence of Na^+ , Mg^{2+} , and micromolar ATP concentrations (González-Lebrero et al., 2002). Forbush (1987) interpreted these data as showing that Rb^+ bound to one K^+ site, the “fast” site, that could rapidly release Rb^+ to the extracellular medium and to a second K^+ site, the “slow” site, that could only be occupied by Rb^+ that had first bound to the fast site. As a result, when the first K^+ binds at the fast site, the second K^+ ion cannot bind until the first bound K^+ shifts to the second slow site. Ordered binding of Rb^+ to the fast and slow K^+ sites also led to ordered release from these sites. The conditions of these experiments, i.e., including Mg^{2+} and P_i during Rb^+ release, suggested that release was to the extracellular side of the enzyme.

The accessibility of the fast K^+ site to the extracellular medium also appears to be restricted by a flickering or leaky gate (Forbush, 1987; González-Lebrero et al., 2002). The strongest data supporting this concept is that the affinity with which extracellular Rb^+ blocks release of bound Rb^+ is similar to the affinity for Rb^+ occlusion by the Na,K-ATPase (Forbush, 1987). Since the kinetics of extracellular K^+ binding reactions are very rapid (Peluffo and Berlin, 1997), such a similarity in Rb^+ blocking and occlusion affinities could only occur if access to K^+ binding sites is rate limiting. In other words, the gate is poised strongly toward the closed position. This gating process might be analogous to the rapid opening and closing kinetics observed in ion channels (Hille, 2001) and with palytoxin-modified Na,K-ATPase (Kim et al., 1995; Artigas and Gadsby, 2003).

Organic quaternary amines, and in fact a variety of amine-containing compounds, are interesting because, like extracellular K^+ congeners, they can block the release of Rb^+ from the slow K^+ site without affecting release of Rb^+ from the fast K^+ binding site (Forbush, 1988). Quaternary amines, however, differ from K^+ in one important respect. The amine concentrations required to block Rb^+ occlusion are far higher than those required to block release of occluded Rb^+ (Forbush, 1988). These data have been interpreted as showing that extracellular quaternary amines have access to the fast K^+ binding site in the enzyme, but that they do not become occluded like K^+ . Forbush (1988) envisioned the action of quaternary amines as getting a “foot in the door”, thereby preventing the closing of the occlusion gate. Thus, quaternary amines appear to bind to sites in the Na,K-ATPase without becoming occluded.

The experiments of Forbush (1988) showing that quaternary amines slow the release of $^{86}\text{Rb}^+$ from Na,K-ATPase, do not necessarily demonstrate that the amines bind to the fast K^+ binding site in the enzyme. In a similar manner, competitive inhibition of enzyme activation is not sufficient to demonstrate that amines and K^+ bind at the same site (Segel, 1975).

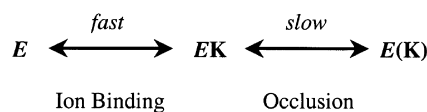
Our data show that inhibition of Na,K pump current by TEA or BTEA displays a Hill coefficient significantly less than 1. One implication of this result is that the quaternary amines are capable of interacting at two binding sites. Certainly, our finding that TEA inhibits Na,K pump in a V_M -independent manner while BTEA produces V_M -dependent block shows that the amines can act at sites with different locations in the membrane electric field; however, the relationship between electrical and physical distances is unknown. Even so, inhibition of Na,K pump current by BTEA dissipates 40% of the membrane electric field, and activation of Na,K pump current by extracellular K^+ dissipates a similar fraction of the membrane electric field (35–39%) in our experiments. Given that BTEA is a competitive inhibitor of the enzyme (Fig. 6), the simplest explanation for our data is that this amine is blocking at or very near a K^+ binding site located within the membrane dielectric.

With the Hill coefficient for current block by BTEA being less than one, we can also speculate that BTEA is capable of interacting with a second site, possibly the same site located outside of the membrane electric field at which TEA inhibits Na,K pump current. However, if BTEA interacts at two sites in the Na,K-ATPase, an interesting problem arises as to why TEA does not also produce V_M -dependent block of the enzyme. In fact, we cannot rule out this possibility because TEA may interact with the K^+ site located in the electric field with much lower affinity than BTEA. In this regard, we performed a few experiments using 100 mM extracellular TEA (unpublished data), and the data gave some indication that V_M -dependent block occurred, specifically, a small decrease in the slope of the $K_{0.5}V_M$ relationship in the presence of 100 mM TEA. We did not investigate this point because any V_M -dependent effects of TEA do not change our mechanistic interpretation of V_M -dependent inhibition of the Na,K-ATPase, and we were concerned that high TEA concentrations were having additional effects on the cells under study. In any case, if the amines are interacting at two sites in the Na,K-ATPase, one located in the membrane electric field, the other outside of the field, this result does not itself demonstrate that the amines are binding at the slow and fast K^+ sites identified by Forbush (1987), since we cannot conclude whether an amine binding site located outside of the membrane

electric field is a K^+ binding site; however, such a possibility is not incompatible with our data.

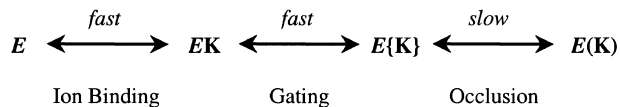
Implications of V_M -dependent Block by BTEA for the Mechanism of V_M -dependent K^+ Binding by the Na,K-ATPase

As mentioned above, our previous work has demonstrated that a V_M -dependent step during K^+ transport by the Na,K-ATPase is extracellular K^+ binding, and that this reaction is followed by a slower electroneutral reaction (Peluffo and Berlin, 1997). The Albers-Post reaction model for the Na,K-ATPase (Glynn, 1985) would suggest this slow electroneutral reaction is probably occlusion and enzyme dephosphorylation. Scheme I summarizes this reaction sequence without specifying the stoichiometry of K^+ binding or the phosphorylation state of the



SCHEME I

enzyme. Given Rb^+ occlusion data suggesting the existence of a flickering gate, the underlying reason for slow occlusion kinetics is that the gate opens with low probability (Forbush, 1987; González-Lebrero et al., 2002). Data showing rapid gating of the palytoxin-modified enzyme (Kim et al., 1995; Artigas and Gadsby, 2003), analogous to the opening and closing of the gate in an ion channel, might suggest that Scheme I could be modified so that rapid ion binding is followed by a rapidly reversible gating reaction and then slower ion occlusion (Scheme II). Hilgemann (1994b) predicted a similar reaction sequence and suggested that the gating step (to form $E\{\text{K}\}$) would be a microscopic occlusion reaction that precedes the formation of a stable ion-occluded enzyme intermediate. With such a reaction scheme, Hilgemann (1994b) also predicted that as long as the fraction of K^+ -bound but not -occluded enzyme (EK conformation) is small, it would not be practical to distinguish whether the V_M -dependent reaction is ion binding or rapid gating of the ion-bound enzyme.



SCHEME II

The first issue then is whether the concentration of EK in Scheme II is likely to be small during K^+ binding and occlusion. In Rb^+ occlusion conditions, González-Lebrero et al. (2002) calculated the equilibrium constants for ion binding and occlusion to show that the reaction equilibrium is displaced almost entirely toward the occluded state ($E(K)$ conformation), a point made quite obvious by the ability to occlude Rb^+ in the

presence of Na^+ , Mg^{2+} , and micromolar concentrations of ATP (Glynn and Richards, 1982). Therefore, at least in the forward direction, fast gating must very much favor the closed gate conformation.

In the reverse direction, there is no direct information about the equilibrium position of the ion binding reaction under the conditions of our experiments. Indirectly, one could infer that the bound conformation would rapidly decay to the unbound state because the $K_{0.5}$ for equilibrium Rb^+ occlusion in the absence of other ligands is much smaller than the Rb^+ concentration that produces half-maximal rate of occlusion (González-Lebrero et al., 2002). In Scheme II, this difference in $K_{0.5}$ values would suggest that either the formation of the Rb^+ -bound but not-occluded state and/or the closed gate conformation of the enzyme is disfavored. However, as pointed out above, the gating reaction is strongly poised in the closed direction (González-Lebrero et al., 2002), so that only the binding reaction can realistically be disfavored. Thus, a reasonable conclusion is that the K^+ -bound but not-occluded conformation of the enzyme exists only in very small quantities.

Organic quaternary amines make it possible to distinguish whether the ion binding or gating reactions are V_M dependent. Forbush (1988) showed that these amines bind to the Na,K-ATPase but are not occluded, and he interpreted these data as showing that closing of the flickering gate is blocked. If this is the case, the enzyme can accumulate to a significant degree in the amine-bound (but not -occluded) conformation. Under these conditions, it should be possible to distinguish whether binding or gating is the V_M -dependent reaction. If gating is V_M dependent then amine block of enzyme cycling should be V_M independent. Conversely, if binding is V_M dependent, one could anticipate that amine block could also be V_M dependent. The present data clearly demonstrate that the latter is possible. In addition, we found that the fraction of the membrane dielectric dissipated during BTEA inhibition is similar to that dissipated during K^+ activation of enzyme cycling. These data suggest that the binding step is the major V_M -dependent reaction while conformational changes associated with ion binding move little charge in the membrane electric field. This V_M dependence of ion binding will occur as K^+ moves from the extracellular medium to its binding sites in the enzyme, implying that a high-field access channel exists in the Na,K-ATPase. Thus, these data provide strong evidence for an ion channel-like structure in the Na,K-ATPase.

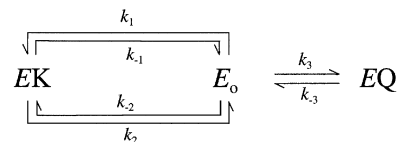
Summary

In this study, we have demonstrated that the organic quaternary amine, BTEA, is a competitive, V_M -depen-

dent blocker for K^+ activation of Na,K pump current under conditions where K^+ binding is the only V_M -dependent reaction controlling enzyme turnover. Given previous data concerning amine inhibition of ion transport and occlusion by the Na,K-ATPase as well as our understanding of V_M -dependent K^+ binding by this enzyme, the present results indicate that BTEA is binding at a K^+ site located in the membrane electric field. Furthermore, these data demonstrate that this binding site must be located in an access channel-like structure in the enzyme. Thus, quaternary amines should provide a valuable tool to probe the structure of K^+ binding sites and the access channel of the Na,K-ATPase.

APPENDIX

In the absence of quaternary amines, Na,K pump current measured in Na^+ -free solution and saturating cytosolic Na^+ concentrations can be described with a pseudo two-state model in which a single reaction step is V_M dependent (Sagar and Rakowski, 1994). To model the effect of quaternary amines on Na,K pump current, the following properties of quaternary amines are assumed: (a) inhibition is competitive with K^+ activation, (b) inhibition prevents the enzyme from supporting ion transport, i.e., no current arises from inhibited enzymes, (c) the amine binds with unknown stoichiometry, and (d) inhibition can be V_M dependent. With these assumptions, Scheme III describes possible reactions of the Na,K-ATPase where the enzyme is assumed to occur in three forms, a K^+ -bound form (EK), an ion-free form equivalent to E_2P (E_o), and an amine-bound form (EQ). Stoichiometry of K^+ binding to the enzyme is not shown in this scheme.



SCHEME III

Voltage dependence is assigned arbitrarily to the binding steps to E_o (k_1 for K^+ and k_3 for Q) rather than dissociation steps (k_{-1} and k_{-3} , respectively). If the backward running of the enzyme under our conditions is assumed to be negligible, i.e., $k_{-2} \sim 0$, Scheme III can be solved for the concentration of EK under steady-state conditions to be as in Eq. 3

$$[EK] = N \left(1 + \frac{(k_{-1} + k_2)}{k_1 [\text{K}^+]^{\gamma_K} \exp(-\gamma_K \lambda_K U)} \right) + \quad (3)$$

$$\left(\frac{(k_{-1} + k_2) k_3 [\text{Q}]^{\gamma_Q} \exp(-\gamma_Q \lambda_Q U)}{k_{-3} k_1 [\text{K}^+]^{\gamma_K} \exp(-\gamma_K \lambda_K U)} \right)^{-1},$$

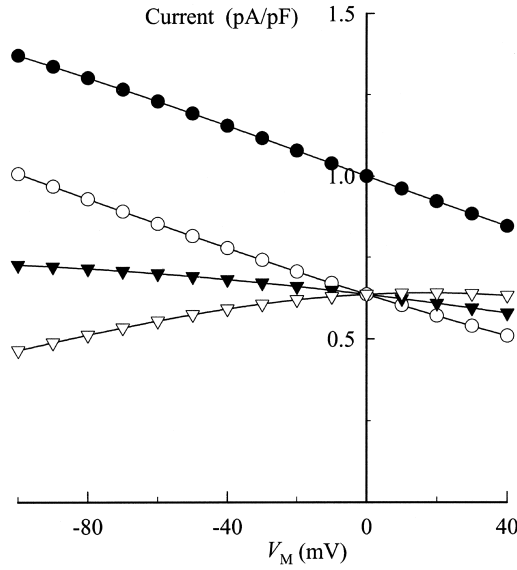


FIGURE 8. Simulations showing the effect of quaternary amine inhibition on the V_M dependence of Na,K pump current. Eq. 4 was solved with the following parameter values: $K_{0.5}^0 = 0.2$ mM, $K_Q^0 = 4$ mM, $[Q] = 5$ mM, $\lambda_K = 0.37$, $\gamma_K = 0.56$, $\gamma_Q = 0.6$, and $\lambda_Q = 0$ (○), 0.4 (▼), and 0.75 (▽). The control curve (●) was calculated with $[Q] = 0$ mM.

where N is the total concentration of Na,K-ATPase proteins involved in ion transport, i.e., $N = [E_o] + [EK] + [EQ]$, and $[K^+]$ is the K^+ concentration. Assuming that Na,K pump current (I) is equal to $zFk_2[EK]$, where $z = 1$ charge per cycle, Eq. 3 can be substituted into this expression to yield:

$$I = I_{\max} \left(1 + \left(\frac{K_{0.5}^0}{[K^+]} \right)^{\gamma_K} \exp(\gamma_K \lambda_K U) \times \left(1 + \left(\frac{[Q]}{K_Q^0} \right)^{\gamma_Q} \exp(-\gamma_Q \lambda_Q U) \right)^{-1} \right) \quad (4)$$

where

$$K_{0.5}^0 = \left(\frac{k_{-1} + k_2}{k_1} \right)^{\frac{1}{\gamma_K}} \quad (4a)$$

$$K_Q^0 = \left(\frac{k_{-3}}{k_3} \right)^{\frac{1}{\gamma_Q}} \quad (4b)$$

$$I_{\max} = zFk_2N. \quad (4c)$$

Na,K pump current (I) in Eq. 4 depends on $[K^+]$, the amine concentration ($[Q]$) and V_M , all experimentally controllable parameters. Fig. 8 shows the results of a simulation in which the amine is assumed to act in a V_M -independent fashion, i.e., $\lambda_Q = 0$. Simulated

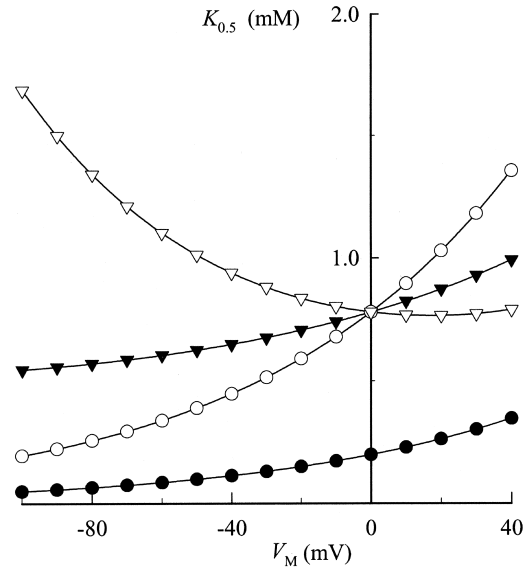


FIGURE 9. Simulations showing the effect of quaternary amine inhibition on the V_M dependence of $K_{0.5}$. Eq. 2 was solved with the parameter values used in Fig. 8 where $\lambda_Q = 0$ (○), 0.4 (▼), and 0.75 (▽). The control curve (●) was calculated with $[Q] = 0$ mM.

current is activated at constant $[K^+]$ (0.2 mM) in the absence and presence of the inhibitory quaternary amine. Note the similarity to Fig. 3 A, inhibition of Na pump current by TEA. Like the experimental data, the V_M -independent inhibitor appears to block simulated Na,K pump current more effectively at positive V_M . However, when the simulation is run with a V_M -dependent blocker ($\lambda_Q = 0.4$ and 0.75), the V_M dependence of the current progressively changes so that the inhibitor appears to block current more effectively at negative V_M , similar to the data shown in Fig. 5 B.

Eq. 4 can also be expressed in the more familiar format of a Hill equation:

$$I = I_{\max} \left(1 + \left(\frac{K_{0.5}}{[K^+]} \right)^{\gamma_K} \right)^{-1}, \quad (5)$$

where $K_{0.5}$ is expressed as in Eq. 2. Simulations for the effect of V_M -independent and -dependent blockers on the $K_{0.5}$ - V_M relationship are shown in Fig. 9. If inhibition of Na pump current is V_M independent ($\lambda_Q = 0$), the $K_{0.5}$ for K^+ will simply be scaled up from the curve obtained in the absence of inhibitor. On the other hand, with V_M -dependent inhibition, the curves progressively flatten out and change curvature as the ion-well depth for the inhibitor is increased ($\lambda_Q = 0.4$ and 0.75). Again, the simulations with $\lambda_Q = 0$ and 0.4 are qualitatively similar to the V_M dependence of $K_{0.5}$ values observed in the presence of TEA (Fig. 4) and BTEA (Fig. 7), respectively.

In the absence of organic amines, Scheme III reduces to a pseudo two-state model. In this case, Eq. 4 simplifies to

$$I = I_{\max} \left(1 + \left(\frac{K_{0.5}^0}{[K^+]} \right)^{\gamma_K} \exp(\gamma_K \lambda_K U) \right)^{-1}, \quad (6)$$

and the expression for $K_{0.5}$ becomes Eq. 1, which is used to fit the data in Fig. 2 C.

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