Membrane Potential Dependent Inhibition of the Na,K-ATPase by

para-Nitrobenzyltriethylammonium Bromide.

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Non-standard abbreviations:

BTEA - benzyltriethylamine

$p$NBTEA – para-nitrobenzyltriethylamine, para-nitrobenzyltriethylammonium bromide
Abstract

Membrane potential (V_M)-dependent inhibitors of the Na,K-ATPase are a new class of compounds that may have inherent advantages over currently available drugs targeting this enzyme; however, two questions remain unanswered regarding these inhibitors; (1) what is the mechanism of V_M-dependent Na,K-ATPase inhibition, and (2) is their binding affinity high enough to consider them as possible lead compounds? To address these questions, we investigated how a recently synthesized V_M-dependent Na,K-ATPase inhibitor, para-nitrobenzyltriethylamine (pNBTEA), binds to the enzyme by measuring the extracellular pNBTEA concentration and V_M dependence of ouabain-sensitive transient charge movements in whole-cell patch-clamped rat cardiac ventricular myocytes. By analyzing the kinetics of charge movements and the steady state distribution of charge, we show that the V_M-dependent properties of pNBTEA binding differ from those for extracellular Na^+ and K^+ binding, even though inhibitor binding is competitive with extracellular K^+. The data were also fit to specific models for pNBTEA binding to show that pNBTEA binding is a rate-limiting V_M-dependent reaction that, in light of homology models for the Na,K-ATPase, we interpret as a transfer reaction of pNBTEA from a peripheral binding site in the enzyme to a site near the known K^+ coordination sites buried within the transmembrane helices of the enzyme. These models also suggest that binding occurs with an apparent affinity of 7 µM. This apparent binding affinity suggests that high affinity V_M-dependent Na,K-ATPase inhibitors should be feasible to design and test as specific enzyme inhibitors.
**Introduction**

Interest in small molecule inhibitors of the P-type Na,K-ATPase has reemerged with the realization that pharmacological interventions targeting this enzyme might be relevant in a diverse array of disease processes. The Na,K-ATPase is well known for its critical role in developing and maintaining electrochemical gradients for Na⁺ and K⁺ across the plasma membrane (Glynn, 1985). In this role, the Na,K-ATPase controls contractility of cardiac muscle cells by regulating cell Ca²⁺ loading via the sarcolemmal Na/Ca exchanger (Eisner et al, 1984).

Manipulating the Na⁺ electrochemical gradient, and thereby cellular Ca²⁺, by Na,K-ATPase inhibition, is believed to be the basis for the inotropic actions of the cardiac glycoside, digoxin (Akera and Brody, 1977), a classic pharmacological agent and, until recently, a first-line treatment for congestive heart failure (Gheorghiade et al., 2006). Mutations in the enzyme’s alpha subunit have been linked to autosomal dominant forms of migraine (De Fusco et al., 2003) and Parkinson’s disease (Blanco-Arias et al., 2009). A signaling function has also been postulated for the Na,K-ATPase via Src-dependent pathways (Haas et al., 2000; Tian et al., 2006). For example, the cardiac glycoside ouabain has been shown to regulate cell junctional contacts (Larre et al., 2010) and cell proliferation (Tian et al., 2009) at concentrations that have little or no significant effect on net ion transport. Such reports have generated interest in Na,K-ATPase inhibitors as potential chemotherapeutic agents (reviewed in Newman et al., 2008) and suggest that the Na,K-ATPase might be an attractive drug target beyond its established roles in inotropic and antiarrhythmic therapeutics.

Cardiac glycosides are the most widely studied Na,K-ATPase inhibitors. Mutagenesis studies have identified amino acids in the enzyme’s alpha subunit that are important for ouabain binding (reviewed in Lingrel, 2010) and recent high-resolution structures of the Na,K-ATPase...
include bound ouabain (Ogawa et al., 2009; Yatime et al., 2011). Cardiac glycosides have been synthesized and tested in over five decades of drug discovery efforts (Glynn, 1964; Güntert and Linde, 1981; Gobbini and Cerri, 2005). In addition, other high affinity Na,K-ATPase inhibitors, such as cassaine (Tobin et al., 1975), have been reported and have served as the basis for recent efforts to synthesize novel enzyme inhibitors (Gobbini and Cerri, 2005). However, none of these inhibitors can distinguish between Na,K-ATPase located in different cellular or tissue compartments in which the enzyme resides. Selectivity in Na,K-ATPase inhibition, however, may provide advantages in treatment of congestive heart failure and is certainly relevant if Na,K-ATPase inhibitors are to be developed as chemotherapeutic agents. For this reason, the search for novel Na,K-ATPase inhibitors is a promising area of drug discovery.

In a previous study, we identified a compound, benzyltriethylamine (BTEA), that inhibits the Na,K-ATPase in a membrane-potential ($V_M$)-dependent fashion such that enzyme block is more pronounced at negative potentials (Peluffo et al., 2004). This compound appears to bind at a K$^+$ coordination site within the enzyme though, unlike K$^+$, it is not occluded nor transported. What is intriguing about BTEA is that it is the first reported $V_M$-dependent blocker of the Na,K-ATPase, though several other $V_M$-dependent inhibitors have since been reported (Peluffo et al., 2009; Ratheal et al., 2010). These compounds offer the possibility of a class of Na,K-ATPase inhibitors that distinguish, to some degree, enzyme located in electrically polarized cells versus enzyme in cells with depolarized membrane potentials. As will be presented in the Discussion, this property might be useful for the treatment of congestive heart failure. In addition, $V_M$-dependent Na,K-ATPase inhibitors might also be useful as chemotherapeutic agents.
BTEA is a low affinity blocker, used in the millimolar concentration range (Forbush, 1988; Peluffo et al., 2004). For this reason, the para-nitro derivative of BTEA, $p$NBTEA, was synthesized and found to have similar $V_M$-dependent inhibitory effects on the Na,K-ATPase, but at a much lower concentration (Peluffo et al., 2009). These results were promising for development of similar compounds as high affinity Na,K-ATPase inhibitors, but two important questions remained unanswered in previous work. First, though BTEA and $p$NBTEA appear to bind at a $K^+$ site in the enzyme, their mechanism of $V_M$-dependent inhibition was not studied. Using pre-steady state binding kinetics, we have compared different models for $V_M$-dependent binding in the present work to determine which best describes $p$NBTEA inhibition of the Na,K-ATPase. Second, we have not previously determined the apparent affinity for inhibition of the Na,K-ATPase by $p$NBTEA. Our analysis here shows it is a much more potent inhibitor than the parent BTEA. These results suggest that further development of $V_M$-dependent Na,K-ATPase inhibitors is warranted.
Materials and Methods

Whole-cell patch clamp: Ventricular myocytes were enzymatically isolated from rat hearts and voltage-clamped with patch electrodes (1.0-1.5 MOhms), as published previously (Ishizuka et al., 1996). Animals were treated according to the Guide for the Care and Use of Laboratory Animals in a protocol approved by our Institutional Animal Care and Use Committee. For measurements of transient charge movements, protocols were followed as outlined previously (Peluffo and Berlin, 1997; Peluffo et al., 2009). Experiments were performed at 18-20°C with Na\(^+\) and K\(^+\)-free superfusion solutions containing (in mM): 145 TMA chloride, 2.3 MgCl\(_2\), 0.2 CdCl\(_2\), 5.5 dextrose, 10 HEPES (pH 7.4 with Tris). The bromide salt of pNBTEA was added to this solution at indicated concentrations. The patch electrode solution contained (in mM): 130 mM K\(^+\), 20 TEA chloride, 9.1 MgCl\(_2\), 0.7 ATP - Mg\(^{2+}\) salt, 30 phosphate (H\(_2\)PO\(_4\)\(^-\) + HPO\(_4^{2-}\)), 1 EGTA, 66 Aspartic acid, 10 HEPES pH 7.3 (20°C). Magnesium phosphate concentration was calculated with MaxChelator to be 1.3 mM.

The experimental procedure to measure extracellular pNBTEA transient charge movements was to first superfuse a voltage-clamped myocyte with a pNBTEA-containing Na\(^+\) and K\(^+\)-free solution and determine the current-voltage relationship between -160 and +100 mV two times, separated by 1 min. The myocyte was then perfused for 2 min with the same solution that also included 1 mM ouabain, and another current-voltage relationship was determined before switching back to the ouabain-free solution. Our previous experiments (Peluffo et al., 2009) have shown 2 min exposure to 1 mM ouabain is sufficient to completely block quaternary amine-dependent transient charge movements. Currents measured before and 2 min after addition of ouabain were then subtracted to yield ouabain-sensitive transient currents. These currents were
integrated and divided by cell capacitance to yield ouabain-sensitive charge ($q$; in units of fC/pF). A more complete description of the experimental protocol is in Peluffo et al. (2009).

**Analysis, Curve-fitting and Model Selection:** Data are presented as mean ± S.E.M. for the indicated number of replicates or cells. Cell capacitance was calculated as the integral of current elicited with 5 mV pulses. Indicated functions were fit to the data with a non-linear least-squares algorithm available in commercial software (SigmaPlot, SPSS) using statistical weights proportional to $(\text{S.E.M.})^{-1}$. Different binding models were evaluated by applying a second order Akaike information criterion and Akaike weights (Yamaoka et al. 1978; Burnham and Anderson, 2002; González-Lebrero et al., 2002; Montes et al., 2004). The Akaike information criterion ($AIC_c$) with a limited data set was calculated for each model as

$$AIC_c = -2 \ln(L(\theta|y)) + 2K \left( \frac{n}{n - K - 1} \right)$$

where $L(\theta|y)$ is the root mean square of the residuals between the data and fitted model function, $n$ is the sample size, and $K$ is the number of estimated parameters. To determine the weight of evidence that a particular model is actually the best model to describe the data, Akaike weights ($w_i$) were calculated as

$$w_i = \frac{\exp(-0.5AIC_i)}{\sum_{r=1}^{N} \exp(-0.5AIC_r)}$$

$0 \leq w_i \leq 1$

where $AIC_i$ is the $AIC_c$ for the $i^{th}$ function out of the total number ($N$) of functions tested. The likelihood that the $i^{th}$ model is the best model for the data increases as $w_i$ approaches a value of 1 (Burnham and Anderson, 2002). Experiments using 100 µM $p$NBTEA have been reported in
Peluffo et al. (2009); however, analysis of the data set at all $p$NBTEA concentrations is presented here for the first time.

**Reagents:** Reagents were from Sigma-Aldrich Chemical Co. and were of analytical grade or higher. $p$NBTEA was synthesized as outlined previously (Peluffo et al., 2009).
Results

Peluffo et al. (2009) showed that BTEA competitively inhibits $^{86}$Rb$^+$ occlusion by the Na,K-ATPase. In the same study, $p$NBTEA was found to be a $V_M$-dependent inhibitor by examining the effects of 100 µM $p$NBTEA on steady state Na,K-pump current and $p$NBTEA-dependent pre-steady state transient charge movements at different membrane potentials. To gain greater insight into the mechanism of enzyme block by $p$NBTEA, we have also conducted measurements of transient charge movements with 33 and 300 µM of the quaternary amine added to the perfusion solution in patch-clamped rat ventricular myocytes (see Methods). Figure 1A-C shows representative 1 mM ouabain-sensitive difference currents recorded when the membrane potential was stepped from the holding potential, -40 mV, to 0 mV for 100 ms, before returning to the holding potential. Our previous work (Peluffo and Berlin, 1997; Peluffo et al., 2009) has shown that 1 mM ouabain is sufficient to completely block extracellular ion-dependent charge movements in rat ventricular myocytes. The ouabain sensitivity of these currents demonstrates that they arise from a $V_M$-dependent process in the Na,K-ATPase. In the absence of $p$NBTEA, ouabain-sensitive transient currents were not observed (Peluffo et al., 2009). Thus, these currents show that the inhibition of the Na,K-ATPase by the positively charged $p$NBTEA involves at least one $V_M$-dependent reaction.

Ouabain-sensitive charge movements reflect a change of ion binding by the Na,K-ATPase in response to a change in $V_M$ (Gadsby et al., 1993; Peluffo and Berlin, 1997). With transported cations, i.e. Na$^+$ and K$^+$, the kinetics of these charge movements are limited by subsequent electroneutral reactions involved in ion occlusion by the enzyme (Gadsby et al., 1993; Hilgemann, 1994; Wuddel and Apell, 1995). However, as we have previously shown, organic quaternary amine inhibitors of the Na,K-ATPase, like $p$NBTEA, are not occluded
(Peluffo et al., 2009). Therefore, the kinetics of \( p \)NBTEA-dependent transient charge movements are a direct measure of the rate of quaternary amine binding reactions.

As shown in Figure 1A-C, depolarization produced an outward current transient and hyperpolarization an inward current transient, consistent with the movement of a positive charge in the membrane electric field. An exponential function provided a good fit to the decaying portion of the current traces in Fig. 1A-C, indicating a pseudo-1\(^{st}\) order process. This fitting allowed us to derive a rate (\( k_{\text{tot}} \)) for the change in \( p \)NBTEA binding after a \( V_{\text{M}} \) jump. The rate of decay of the transient current increased at higher \( p \)NBTEA concentrations (Fig. 1D), as would be expected for pseudo-1\(^{st}\) order ligand binding reaction. Thus, the transient currents shown here result from the \( V_{\text{M}} \)-dependent binding of the quaternary amine as it inhibits the Na,K-ATPase.

The calculated \( k_{\text{tot}} \) is plotted as a function of \( V_{\text{M}} \) for all \( p \)NBTEA concentrations tested in Fig. 1D. This \( k_{\text{tot}}-V_{\text{M}} \) relationship has several interesting features. The relationship is “U”-shaped for all \( p \)NBTEA concentrations tested. This feature distinguishes quaternary amine binding kinetics from those for ions that are bound, occluded and transported by the Na,K-ATPase (see Discussion). Interestingly, the \( V_{\text{M}} \) at which \( k_{\text{tot}} \) was a minimum shifted to more positive \( V_{\text{M}} \) with increasing \( p \)NBTEA concentration, and the kinetics appeared to be more \( V_{\text{M}} \)-dependent at positive potentials. To describe the \( p \)NBTEA- and \( V_{\text{M}} \)-dependent properties of \( k_{\text{tot}} \), the entire data set was fitted simultaneously with eq. 1 that is derived assuming that 1\(^{st}\) order kinetics of \( p \)NBTEA-dependent transient currents reflect a system in which Na,K-ATPase is amine-bound or -free, i.e. a pseudo-2 state model,

\[
k_{\text{tot}} = k_{f}^{0} [Q]^{n} \exp\{-\delta \lambda_{Q} F V_{M} /RT\} + k_{r}^{0} \exp\{(1-\delta) \lambda_{Q} F V_{M} /RT\}
\]  

(1)
where $k_f^0$ and $k_r^0$ are the forward and reverse rate constants for $p$NBTEA binding at 0 mV, $[Q]$ is the extracellular $p$NBTEA concentration, $n$ is the Hill coefficient for the reaction, $\lambda_Q$ is the apparent charge moved in the $V_M$-dependent reaction, $\delta$ is a proportionality coefficient that distributes the effect of the electric field between the forward and backward reactions, and $F$, $R$, $T$ have their usual meanings. The calculated values for $k_f^0$ and $k_r^0$ are given in the legend of Fig. 1. This calculation showed that the apparent charge equaled $0.41 \pm 0.01$, equivalent to a full charge dissipating 41% of the electric field during enzyme inhibition. This value was not significantly different than the 37% of the electric field dissipated by extracellular K$^+$ during its activation of ion transport by the Na,K-ATPase (Peluffo and Berlin, 1997). This similarity along with the competitive nature of $p$NBTEA enzyme inhibition led us to conclude that these amines were binding at a K$^+$ coordination site in the enzyme (Peluffo et al., 2009). The proportionality constant $\delta$ was $0.32 \pm 0.01$. This value reflects the steeper $V_M$ dependence of $k_{tot}$ at positive potentials and the shift in the $V_M$ at which $k_{tot}$ reaches a minimum with increased $p$NBTEA concentration (see eq. 1). The Hill coefficient was $0.52 \pm 0.01$, a value indicative of negative cooperativity in $p$NBTEA binding to the enzyme that implies more than one amine molecule binds to the enzyme.

To further describe the $V_M$ dependence of $p$NBTEA binding, charge ($q$) moved in response to step changes in $V_M$ was calculated by integrating the area under ouabain-sensitive difference currents. The current integral at the beginning of the $V_M$ step, $q_{ON}$, was the same as the current integral, $q_{OFF}$, measured after returning to the holding potential. Equality of $q_{ON}$ and $q_{OFF}$ is indicative of a reversible binding process for the quaternary amine (Peluffo and Berlin, 1997; Peluffo et al., 2009).
The values for $q_{ON}$ were plotted against $V_M$, before (Fig. 2A) and after normalization to $q_{TOT}$, the total amount of charge moved (Fig. 2B). The data at each $pNBTEA$ concentration were also fitted with a Boltzmann equation that describes the probability of the enzyme being in a $pNBTEA$-bound state as a function of $V_M$. Best-fit parameter values are shown in Table 1.

This analysis revealed $V_M$-dependent properties of $pNBTEA$ binding reactions as well as limitations of the pseudo-2 state model analysis. $q_{TOT}$ was unchanged with $pNBTEA$ concentration, as if $V_M$ could drive $pNBTEA$ into or out of a fixed number of Na,K-ATPase binding sites over the range of concentrations tested; however, the quantity of charge moved by a large negative step in $V_M$ ($q_{MIN}$) from the holding potential decreased as $pNBTEA$ concentration was increased. This shift in the $V_M$ dependence of charge movement, shown more clearly in Fig. 2B, is consistent with increased amine binding at the holding potential as $pNBTEA$ concentration is increased, i.e. at higher amine concentration, a smaller fraction of total Na,K-ATPase remains to be driven into the bound state at negative $V_M$. The magnitude of the shift in charge movement, 26 mV per e-fold change in $pNBTEA$ concentration (Supplemental Data, Fig. 1), would be consistent with one charge dissipating the equivalent of the entire electric field across the cell membrane during $pNBTEA$ binding. Thus, $V_M$ dependence of binding calculated in this manner appears to be much stronger than that determined by the value for $\lambda_Q$, the apparent charge calculated from the kinetics of transient current decay (Fig. 1).

Eyring rate theory suggests that the shift in charge movement depends on the effective charge of the $V_M$-dependent reaction and the molecularity of that reaction (Peluffo, 2004). Since the Hill coefficient for transient currents does not equal one (Fig. 1), the molecularity of the reaction is unknown, though clearly more than one $pNBTEA$ molecule is involved in these reactions. Thus, there is no a priori expectation that $V_M$-dependent parameters derived from
analyses of kinetics and quantity of charge movement will be consistent because the pseudo-2 state model accommodates neither the molecularity nor the type of binding reaction.

To understand more about \( p \)NBTEA binding to the Na,K-ATPase, different ligand binding models were evaluated to determine which provides the best fit to the data.

In evaluating potential binding models for \( p \)NBTEA, random schemes were excluded because Rb\(^+\) binding to the Na,K-ATPase is an ordered process (Forbush, 1987; González-Lebrero et al., 2002). Since \( p \)NBTEA appears to bind at an extracellular K\(^+\) coordination site, the assumption of ordered binding is also likely to be valid. Furthermore, the effect of BTEA on Rb\(^+\) occlusion (Peluffo et al., 2009) is well described by postulating two amine binding sites in the enzyme. While a greater number of sites cannot be excluded, there is no obvious rationale to include more. For this reason, only models with two \( p \)NBTEA binding sites were evaluated. The mono-exponential decay of \( p \)NBTEA-activated transient charge movements also suggests that either (a) rate limiting \( V_M \)-dependent reactions have similar kinetics or (b) multiple \( V_M \) -dependent reactions have very different kinetics. In both cases, these schemes simplify to an ordered reaction with a single \( V_M \)-dependent reaction.

In a broad sense, ordered reaction schemes fall into two categories shown as schemes A and B. Scheme A is a classic sequential reaction mechanism for a two site enzyme in which

\[
E^0 \xrightleftharpoons[k_{1}V_M]{k_{-1}V_M} E^0 \xrightleftharpoons[k_{2}V_M]{k_{-2}V_M} E^0
\]

Scheme A

\[
E^0 \xrightleftharpoons[K_{a1}V_M]{K_{a1}V_M} E^0 \xrightleftharpoons[k_{2}V_M]{k_{-2}V_M} E^0 \xrightleftharpoons[K_{a2}V_M]{K_{a2}V_M} E^0
\]

Scheme B
pNBTEA binding at site 1 and/or site 2 can be \( V_M \)-dependent. The rates of the binding reactions are described by pseudo first order forward \( (k_x) \) and reverse \( (k_{-x}) \) rate constants (where \( x = 1,2 \)). Since the decay of pNBTEA-dependent transient currents is exponential, it can be assumed that, for scheme A, either the sum of \( k_1 + k_{-1} \) is similar to the sum of \( k_2 + k_{-2} \) or these sums are very different. Scheme B is based upon the work of Glynn et al. (1985), Forbush (1987) and González-Lebrero et al. (2002) whose analysis of \( \text{Rb}^+ \) occlusion kinetics by the \( \text{Na,K-ATPase} \) suggested that \( \text{Rb}^+ \) binding is in rapid equilibrium with the \( \text{Na,K-ATPase} \) but the sequential binding of each \( \text{Rb}^+ \) is limited by the accessibility of the \( \text{Rb}^+ \) binding sites, as if a gate with low open probability (“leaky gate”) limited access to the ion coordination sites. Since BTEA is not occluded (Peluffo et al., 2009), we have instead substituted a rate-limiting reaction of the pNBTEA-bound enzyme, illustrated in Scheme B as transfer of the bound amine between the first and second binding sites (see Discussion). Any or all of these reaction steps can be \( V_M \)-dependent.

Given these two reaction schemes, there are a total of 10 possible model systems (3 for scheme A and 7 for scheme B) that derive from assigning \( V_M \) dependence to any one or combination of reaction steps. Some of these model systems are mathematically identical so that only eight model systems were fitted to the data for the \( k_{\text{tot}}-V_M \) relationship. Most models could not be fitted to the data without collapsing to a simpler system. Of the remaining models, the best fitting model representative of Scheme A and Scheme B are presented in Figure 3 for the \( k_{\text{tot}}-V_M \) relationship. Because these models have different numbers of free parameters, evaluations of goodness-of-fit were made using an Akaike Information Criterion (\( AIC_c \)).

The best-fitting models for Schemes A and B both tracked the experimental data well (see Supplemental Data for the fitted functions and best fit parameter values). However, it is
clear that the Scheme B model provided the better fit. This graphical impression is substantiated by the $AIC_c$ for Scheme A ($AIC_c=321.2$) and Scheme B ($AIC_c=226.8$) which provide an Akaike weight ($w_i$) for Scheme A that approaches a value of 0, meaning the model is highly unlikely, whereas the $w_i$ for Scheme B approaches a value of 1, meaning this model is quite likely to be the best one describing the $V_M$ and $p$NBTEA concentration dependence of $k_{tot}$ (see Methods).

From this analysis, we conclude that the $V_M$-dependent step in $p$NBTEA inhibition of the Na,K-ATPase occurs as a rate-limiting reaction of the amine-bound enzyme. As presented in the Discussion, we believe that this $V_M$-dependent step represents the transfer of bound $p$NBTEA from site 1 to site 2 within the enzyme.

Figure 4 shows the functions fitted to the $q$-$V_M$ data for the same Scheme A and B models (see Supplemental Data). Again, both models appear to fit these steady state data quite well; however, the model based on Scheme B appears to provide the better fit. In addition, both models show that the membrane electric field has a similar effect on the $V_M$ dependence of charge kinetics and quantity. With Scheme A, binding of the first and second amine molecule is $V_M$-dependent, together dissipating between 80 and 100% of the electric field, for both charge kinetics (Fig. 3A) and quantity (Fig. 4A). Scheme B is also somewhat successful with the single $V_M$-dependent reaction predicted to dissipate between 41 and 64% of the membrane electric field (see Supplemental Data for values of $\lambda_Q$), for charge kinetics (Fig. 3B) and quantity (Fig. 4B).

Thus, to a large degree, the apparent contradiction regarding the effectiveness of the membrane electric field on charge movement parameters derived from using a more generalized pseudo-2 state model is overcome by using a specific model that accounts for the molecularity of amine binding to the Na,K-ATPase.
What is interesting about the best-fit functions for Scheme B is the prediction that the first \( p \)NBTEA binds to the Na,K-ATPase with high affinity. From the \( V_M \) dependence of steady state charge distribution, the model calculates an apparent affinity of 7 µM (\( K_D^1 = 1/K_a^1 = 6.9 \) µM). Consistent with the negative cooperativity shown in Figure 1, the second amine molecule binds with lower apparent affinity (e.g. \( K_D^2 = 1/K_a^2 = 65 \) µM from the \( V_M \) dependence of steady state distribution of charge. See Supplemental Data for parameter values when eq. B11 is solved). Our previous experiments show that binding of one molecule of \( p \)NBTEA inhibits the Na,K-ATPase (Peluffo et al., 2009). As such, we conclude that the effect of negative \( V_M \) is to increase apparent affinity by driving the amine into the second binding site within the enzyme.
Discussion

In this report, pNBTEA reaction kinetics with the Na,K-ATPase were examined. We reported the first $V_M$-dependent Na,K-ATPase inhibitor, BTEA (Peluffo et al., 2004) and subsequently an initial characterization of its para-nitro derivative, pNBTEA (Peluffo et al., 2009). These compounds are competitive inhibitors of $K^+$ activation of the Na,K-ATPase hydrolytic activity, extracellular $K^+$ activation of steady state ion transport and $^{86}$Rb$^+$ occlusion. Their $V_M$ dependence of inhibition, dissipating approximately 40% of the membrane electric field, is similar to that for extracellular $K^+$ activation of the enzyme (Peluffo and Berlin, 1997), when analyzed using a pseudo-2 state model to describe binding. Our interpretation of these data is that these compounds bind at a $K^+$ coordination site in the enzyme. Recently, guanidinium, acetamidinium and formamidinium ions have also been reported to be $V_M$-dependent inhibitors of the Na,K-ATPase (Ratheal et al., 2010). With the discovery of these $V_M$-dependent inhibitors, we have begun to consider whether similar compounds can be developed into a useful class of Na,K-ATPase inhibitors. To explore this question, a more in-depth investigation of pNBTEA was undertaken to answer two questions relevant to its utility as a $V_M$-dependent enzyme inhibitor.

First, what is the mechanism of pNBTEA $V_M$-dependent Na,K-ATPase inhibition? BTEA and pNBTEA appear to bind at an extracellularly accessible $K^+$ coordination site; however, they are not occluded nor transported by the enzyme (Peluffo et al., 2004; 2009). pNBTEA binding also does not conform to the properties of ion binding in an ion well. $V_M$-dependent binding of $Na^+$ and $K^+$ in the Na,K-ATPase are diffusion-limited processes accompanied by slow conformational changes of the enzyme (Gadsby et al., 1993; Wuddel and Apell, 1995; Peluffo and Berlin, 1997) in which ion diffusion to its coordination site is $V_M$-
dependent, whereas accompanying enzyme conformational changes appear to move little charge in the membrane electric field. For this reason, the proportionality coefficient “δ” in equation 1 is approximately equal to 1 for K⁺, meaning $k_{tot}$ is an asymptotic function of $V_M$ (Peluffo and Berlin, 1997). In contrast, the properties of transient charge movements and our modeling calculations show that $V_M$-dependent $p$NBTEA binding must be much slower than would be expected for a diffusion limited process. Kinetics of $p$NBTEA-dependent transient charge movements, measured as $k_{tot}$, have a “U”-shaped dependence on $V_M$ that is reflected in the value of $\delta = 0.32$, a value showing that the effect of the membrane electric field on forward and reverse binding reaction kinetics is experimentally measurable. This condition cannot occur when a $V_M$-dependent diffusion-limited process is kinetically limited by a slow electroneutral reaction, i.e. a conformational change. The fact that model scheme B yields a far better fit to the data than scheme A is also consistent with the $V_M$-dependent step being rate limiting. Scheme B also stipulates that a rapid binding process occurs without movement of charge in the membrane electric field. This initial binding step is then followed by a slow charge moving reaction step. Thus, $p$NBTEA binding is clearly distinct from the mechanism involved in $V_M$-dependent extracellular K⁺ binding.

What is the $V_M$-dependent reaction that occurs during $p$NBTEA binding? Our present and previous data suggest a possible mechanism. Two general types of $V_M$-dependent reaction mechanisms are possible with a $p$NBTEA bound enzyme. First, $V_M$-dependence can arise from movement of intrinsic charges in the protein during a conformational change, and second, $p$NBTEA could move within the electric field even after binding. With Na⁺ and K⁺-dependent reactions, significant movement of intrinsic protein charges has not been observed during ion binding (Gadsby et al., 1993; Hilgemann, 1994; Wuddel and Apell, 1995; Peluffo and Berlin,
1997). There is no obvious reason to infer that such movements would become apparent after 
\( p\text{NBTEA} \) binding, so this first mechanism seems unlikely. As to the second possibility,
simulations of BTEA binding to a homology model of the Na,K-ATPase \( \alpha \) subunit (with SERCA
structure 3B9B as a template) suggest that there are two amine sites in the enzyme (Peluffo et al.,
2009). One of those sites is located at the extracellular surface of the protein in a wide vestibule
with an electronegative surface charge. The second site is located within the transmembrane
domain between helices 4-6 at a K\(^+\) coordination site. We have postulated that the first site is
outside the membrane electric field, while the second site is in the field such that extracellular K\(^+\)
or BTEA binding dissipates approximately one third of the total electric field. This postulate
could explain why most quaternary amine blockers of the Na,K-ATPase are not \( V_M \)-dependent
inhibitors (Peluffo et al., 2009), i.e. they would have low affinity for the second quaternary
amine site located within the enzyme’s transmembrane domain. Thus, data and modeling are
consistent with the \( V_M \)-dependent reaction occurring when \( p\text{NBTEA} \) transfers from its initial
binding site to the second site in the transmembrane domain. The reason that this reaction would
not be diffusion limited is that the bulky \( p \)-nitrobenzyl moiety would have to be accommodated
in the space between the transmembrane helices. The possibility that an enzyme conformational
change could move bound \( p\text{NBTEA} \) in the electric field cannot be ruled out; however, that
movement would have to dissipate the same fraction of the membrane electric field as
extracellular K\(^+\) binding. This possibility also seems to be unlikely. Thus, a scenario to explain
\( V_M \)-dependent inhibition by \( p\text{NBTEA} \) is that it moves to a K\(^+\) coordination site in the enzyme’s
transmembrane domain from its initial peripheral binding site. The effect of the membrane
electric field is then to bias the binding site towards the transmembrane helices, deeper into a
binding pocket in the enzyme.
The second question is whether \( p\text{NBTEA} \) inhibits the Na,K-ATPase with high enough affinity that these compounds warrant further investigation as possible enzyme blockers. Amine containing compounds were first shown to inhibit the Na,K-ATPase in a manner that is competitive with extracellular \( K^+ \) in the 1960s (Sachs and Conrad, 1968). In that paper and subsequent reports (Kropp and Sachs, 1977; Forbush, 1988), amine concentrations were generally in the millimolar range, much too high to consider for pharmacological applications. Even BTEA, the first of the \( V_M \)-dependent inhibitors to be reported, inhibited Na,K-pump current (at 0 mV) by 50% at a concentration of 4 mM (Peluffo et al., 2004). Therefore, we synthesized \( p\text{NBTEA} \) to determine if an amine-containing compound could inhibit the Na,K-ATPase at lower concentrations, and, in our initial report (Peluffo et al., 2009), its \( V_M \)-dependent inhibition was studied at a concentration of 100 \( \mu \text{M} \). We did not, however, investigate the apparent binding affinity for \( p\text{NBTEA} \). To determine that value, we have examined the concentration and voltage dependence of charge movements that, as shown previously (Peluffo et al., 2004; 2009), represent the binding steps for BTEA and \( p\text{NBTEA} \). In our experiments, the Na,K-ATPase is trapped in phosphorylated \( E_2 \) states (Peluffo and Berlin, 1997). As such, charge movements represent net redistribution of enzyme conformations between two steady state conditions and thereby provide an approximation of equilibrium \( p\text{NBTEA} \) binding. Thus, steady state distribution of charge \( (q_{\text{ON}}) \) was used to estimate the apparent affinity of \( p\text{NBTEA} \) binding as 7 \( \mu \text{M} \). In fact, even the Scheme A model which fit the data with greater error also predicted that binding occurred with similar affinity (8 \( \mu \text{M} \)). Thus, the prediction of high affinity binding appears to be independent of the model employed.
A final question is whether a $V_M$-dependent inhibitor of the Na,K-ATPase could be a beneficial pharmacologic agent? To date, $V_M$-dependent Na,K-ATPase inhibitors contain ammonium moieties, as in $p$NBTEA, so all are likely to inhibit more strongly at negative membrane potentials. With these compounds, highly polarized tissues such as healthy cardiac ventricular muscle would be preferentially inhibited compared to more depolarized tissue, such as ischemic heart muscle whose membrane potential is reported to as much 35 mV less negative than surrounding well-perfused muscle (Wit and Janse, 1993). In fact, myocardial ischemia is a frequent complication in heart failure according to the Digitalis Investigation Group (1997), the largest clinical study of digoxin therapy for treatment of congestive heart failure, with 65% of patients having a history of myocardial infarction and 27% on-going angina. This same study documented the finding that, given digoxin, patients with ischemic heart disease showed worse outcomes (higher rate of hospitalization and mortality) than patients with non-ischemic heart failure. Likewise, digoxin was found to be an independent risk factor for excess mortality in patients surviving myocardial infarction (Goldbourt et al., 1995). These clinical data suggest that myocardial ischemia increases the risk of digoxin-induced toxicity, i.e. arrhythmogenesis.

The reason for digoxin’s excess morbidity and mortality with ischemic heart disease may well reflect the propensity for ischemic heart tissue to develop cardiac glycoside-induced arrhythmias when compared to normal tissue (Lynch et al., 1986; Mazur and Anderson, 1998). Thus, one approach to minimizing arrhythmogenesis with ischemic tissue is to use a $V_M$-dependent Na,K-ATPase inhibitor that preferentially inhibits highly polarized, i.e. well perfused, heart muscle while being relatively less effective in depolarized, ischemic cardiac tissue. A $V_M$-dependent inhibitor with properties similar to $p$NBTEA will have such a preference to inhibit Na,K-ATPase in highly polarized cells.
Conceptually, it should also be possible to develop a Na,K-ATPase inhibitor that has the opposite $V_M$ dependence, inhibiting depolarized tissues preferentially over highly polarized tissue. Such an inhibitor might be useful for a glycoside developed as a chemotherapeutic agent that would spare muscle and nerve tissue from unwanted drug actions. Whether clinically useful $V_M$-dependent Na,K-ATPase inhibitors can be developed remains to be determined; however, the present results suggest that the development of such agents should be feasible.
Acknowledgments

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Authorship Contributions

Participated in research design: J.R. Berlin, R.D. Peluffo
Conducted experiments: R.D. Peluffo
Contributed new reagents J.R. Berlin
Performed data analysis: J.R. Berlin, R.D. Peluffo
Wrote or contributed to the writing of the manuscript: J.R. Berlin, R.D. Peluffo
References


**Footnotes**

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Figure legends

Figure 1: Ouabain-sensitive pre-steady state currents with various pNBTEA concentrations. Currents were recorded in response to a 100 ms depolarization to 0 mV in the presence and absence of 1 mM ouabain in superfusion solutions containing 33 (A), 100 (B) and 300 µM pNBTEA (C). Ouabain-sensitive difference currents are displayed without any capacitance correction or leak subtraction. The solid curve through the decaying phase shows the exponential function fit to the data. Note the change in the time scale. The rate of current decay ($k_{\text{tot}}$) is displayed as a function of $V_M$ (Panel D) for 33 ($\nabla$), 100 (●) and 300 µM pNBTEA (○). The solid curves were determined by fitting the entire data set to equation 1. The calculated values of $k_f^0$ and $k_r^0$ were 58,600 ± 2000 s⁻¹M⁻¹ and 282 ± 9 s⁻¹, respectively.

Figure 2: pNBTEA concentration and $V_M$ dependence of charge movement. Charge ($q$) was calculated as the integral of the ouabain-sensitive difference current recorded in response to a step change in $V_M$ ($q_{\text{ON}}$) divided by cell capacitance during superfusion with solutions containing 33 ($\nabla$), 100 (●) and 300 µM pNBTEA (○). A. Charge. B. Charge normalized to $q_{\text{TOT}}$ calculated by fitting the data at each pNBTEA concentration with the following Boltzmann equation:

$$q = q_{\text{min}} + q_{\text{tot}} / \left[ 1 + \exp \left( - \frac{(V_M - V_q) / z_q}{k_B T} \right) \right]$$

Values for the best fit parameters are given in Table 1.

Figure 3: Fitting charge movement kinetics with specific pNBTEA binding models. Data points are reproduced from Figure 1D. Curves represent the functions solved for the best fit models based on Scheme A (A) and Scheme B (B) using eqs. A6 and B9, respectively. These equations
and their best fit parameter values are listed in Supplemental Data. Symbols are as indicated in Fig. 1D.

Figure 4: Fitting the $q$-$V_M$ relationship with specific $p$NBTEA binding models. Data points are reproduced from Figure 2A. Curves show the functions solved for the best fit models based on Scheme A (A) and Scheme B (B) using equations A9 and B11, respectively. These equations and their best fit parameter values are listed in Supplemental Data. Symbols are as indicated in Fig. 2.
Table 1: Pseudo-2 state Boltzmann function parameters for the $V_M$ dependence of $q$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$[p\text{NBTEA}]$ (µM)</th>
<th>33</th>
<th>100</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td>$q_{\text{TOT}}$ (fC/pF)</td>
<td></td>
<td>13.7 ± 0.6</td>
<td>14.9 ± 0.4</td>
<td>14.0 ± 0.5</td>
</tr>
<tr>
<td>$q_{\text{MIN}}$ (fC/pF)</td>
<td></td>
<td>-8.3 ± 0.5</td>
<td>-6.1 ± 0.2</td>
<td>-3.6 ± 0.2</td>
</tr>
<tr>
<td>$V_q$ (mV)</td>
<td></td>
<td>-57.0 ± 3.4</td>
<td>-27.1 ± 1.8</td>
<td>0.5 ± 2.9</td>
</tr>
<tr>
<td>$z_q$</td>
<td></td>
<td>0.56 ± 0.04</td>
<td>0.65 ± 0.03</td>
<td>0.72 ± 0.06</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3
Figure 4

A  
Ouabain-sensitive charge (fC/pF)

B  
Ouabain-sensitive charge (fC/pF)
Membrane Potential Dependent Inhibition of the Na,K-ATPase by

\textit{para}-Nitrobenzyltriethylammonium Bromide.

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**Supplemental Data**

A. Scheme A: Derivation of equations and best fit parameter values for kinetics and steady state distribution of charge

B. Scheme B: Derivation of equations and best fit parameter values for kinetics and steady state distribution of charge

C. Comparison of the apparent affinity for \( p \)-NBTEA binding calculated from transient charge movement kinetics and steady state charge distribution

D. Supplemental Figure 1
The functions fitted to the data for Scheme A and B binding models are derived below. The parameter values providing the best fit to the data for $V_M$ dependence of charge kinetics (i) and steady state charge distribution (ii) are also shown.

A. Scheme A: Sequential binding steps

i. Transient charge movement kinetics

Considering mass balance,

$$[E]_T = [E^o] + [E^*] + [E^{\bullet\bullet}] \quad \text{(A1)}$$

and the definition:

$$K_{a1} = \frac{1}{K_{d1}} = \frac{[E^*]}{[E^o]Q} \quad \text{where } [Q] = pNBTEA \text{ concentration} \quad \text{(A2)}$$

then,

$$\frac{1}{[E]_T} \left( \frac{d[E^*]}{dt} \right) = - \left[ k_2[Q] + k_{-2} \left( 1 + \frac{K_{d1}}{[Q]} \right) \right] [E^*]_T + k_{-2} \quad \text{(A3a)}$$

and

$$\frac{1}{[E]_T} \left( \frac{d[E^{\bullet\bullet}]}{dt} \right) = - \left( k_{-2} + \frac{k_2[Q]}{1 + \frac{K_{d1}}{[Q]}} \right) [E^*]_T + \frac{k_2[Q]}{1 + \frac{K_{d1}}{[Q]}} \quad \text{(A3b)}$$

The time-dependent solution for (A3b)

$$[E^{\bullet\bullet}]_t = \frac{C_3}{C_2} [E]_T \left[ 1 + \frac{(br + s) \exp(-at) - (ar + s) \exp(-bt)}{(a - b)} \right] \quad \text{(A4)}$$

was obtained as described in Peluffo (2004) with
\[
\begin{align*}
a &= \frac{C_1 + \sqrt{C_1^2 - 4C_2}}{2} \\
b &= \frac{C_1 - \sqrt{C_1^2 - 4C_2}}{2} \\
C_1 &= k_1[Q] + k_{-1} + k_2[Q] + k_{-2} \\
C_2 &= k_1k_2[Q]^2 + k_1[Q]k_{-2} + k_{-1}k_{-2} \\
C_3 &= k_1k_2[Q]^2 \\
r &= 1 - w(C_2/C_3) \\
s &= (C_2/C_3)(wk_{-2} - vk_2[Q])
\end{align*}
\]

where \( w \) and \( v \) are also described in the Appendix of Peluffo (2004).

In this case, \( b = k_{\text{tot}} \) so that:

\[
k_{\text{tot}} = \frac{1}{2} \left[ (k_1 + k_2)[Q] + k_{-1} + k_{-2} - \sqrt{[(k_1 + k_2)[Q] + k_{-1} + k_{-2}]^2 - 4(k_1k_2[Q]^2 + (k_1[Q] + k_{-1})k_{-2})} \right]
\]

where \( k_x = k_x^0 e^{-\delta_x \lambda x U}; \) \( k_{-x} = k_{x}^0 e^{(1-\delta_x) \lambda x U} \) for \( x = 1, 2 \). \( k_x^0 \) and \( k_{-x}^0 \) are the rate constants at 0 mV. \( U = FV_{\text{m}}/RT \).

This equation was used to fit the data in Fig. 1D. The resulting parameters values were

\[
\begin{align*}
k_1^0 &= 4,303,000 \pm 493,500 \text{ s}^{-1}\text{M}^{-1} \\
k_2^0 &= 819,800 \pm 108,200 \text{ s}^{-1}\text{M}^{-1} \\
k_{-1}^0 &= 442 \pm 26 \text{ s}^{-1} \\
k_{-2}^0 &= 1,162 \pm 72 \text{ s}^{-1} \\
\delta_2 \lambda_2 &= 0.28 \pm 0.02 (\lambda_2 = 0.36; \delta_2 = 0.78) \\
(1 - \delta_2) \lambda_2 &= 0.08 \pm 0.01 \\
\delta_1 \lambda_1 &= 0.25 \pm 0.02 (\lambda_1 = 0.45; \delta_1 = 0.54) \\
(1 - \delta_1) \lambda_1 &= 0.21 \pm 0.02
\end{align*}
\]

The curves in Fig. 3A are the solution to this best-fitting function for Scheme A.
ii. Steady-state charge distribution

In this scheme, the expression for the mobile charge is defined as $q(V_M; [Q]) = zF[E^*_s]_{ss}$.

Considering that

$$\frac{[E^*_s]_{ss}}{[E]_T} = \frac{k_1 k_{-2} [Q]}{k_1 k_2 [Q]^2 + k_1 k_{-2} [Q] + k_{-1} k_{-2}}$$  \hspace{1cm} (A7)

then:

$$q(V_M; [Q]) = \frac{q_{TOT}}{1 + \frac{K_{d1}}{[Q]} \exp(-\lambda_1 U) + K_{a2} [Q] \exp(-\lambda_2 U)}$$  \hspace{1cm} (A8)

where: $K_{d1} = k_{10} / k_{10}$ and $K_{a2} = k_{20} / k_{-20}$. Addition of a term defined at the holding potential leads to

$$\Delta q = \frac{q_{TOT}}{1 + \frac{K_{d1}}{[Q]} e^{-\lambda_1 U} + K_{a2} [Q] e^{-\lambda_2 U}} - \frac{q_{TOT}}{1 + \frac{K_{d1}^h}{[Q]} + K_{a2}^h [Q]}$$  \hspace{1cm} (A9)

where $K_x^h$ is the value of $K_x e^{-\lambda_x U}$ ($x=1,2$) at the holding potential. This equation was used to fit data in Fig. 2A. The resulting parameter values were

$q_{TOT} = 14.5 \pm 0.4 \text{ fC/pF}$

$K_{d1} = (7.9 \pm 1.3) \times 10^{-6} \text{ M}$

$\lambda_1 = 0.31 \pm 0.05$

$K_{a2} = 3900 \pm 300 \text{ M}^{-1}$

$\lambda_2 = 0.70 \pm 0.04$

The curves in Fig. 4A are the solution to this best-fitting function for scheme A.
B. Scheme B: Two rapid-equilibrium binding reactions separated by a rearrangement step

i. Transient charge movement kinetics

Considering mass balance,

\[ [E]_T = [E^+_o] + [E^+_o] + [E^+_o] + [E^+_o] \]  \hspace{1cm} (B1)

and the definitions:

\[ K_{a1} = \frac{[E^+_o]}{[E^+_o][Q]}; \quad K_{a2} = \frac{[E^+_o]}{[E^+_o][Q]} \]  \hspace{1cm} (B2)

replacing (B2) in (B1) and rearranging:

\[ [E]_T = \left(1 + \frac{1}{K_{a1}[Q]}\right)[E^+_o] + \left(1 + K_{a2}[Q]\right)[E^+_o] \]  \hspace{1cm} (B3)

The reaction scheme yields the following system of differential equations:

\[
\begin{align*}
\frac{d[E^+_o]}{dt} &= -k_2[E^+_o] + k_{-2}[E^+_o] \\
\frac{d[E^+_o]}{dt} &= k_2[E^+_o] - k_{-2}[E^+_o]
\end{align*}
\]  \hspace{1cm} (B4)

with the steady-state solution:

\[ k_2[E^+_o] = k_{-2}[E^+_o] \]  \hspace{1cm} (B5)

Thus, replacing in (B3) and rearranging:

\[
\frac{[E^+_o]}{[E]_T} = \frac{k_{-2}}{\left(1 + \frac{1}{K_{a1}[Q]}\right)k_{-2} + \left(1 + K_{a2}[Q]\right)k_2}
\]  \hspace{1cm} (B6a)

\[
\frac{[E^+_o]}{[E]_T} = \frac{k_2}{\left(1 + \frac{1}{K_{a1}[Q]}\right)k_{-2} + \left(1 + K_{a2}[Q]\right)k_2}
\]  \hspace{1cm} (B6b)

Introducing expressions (B6) into system (B4):
\[
\frac{1}{[E]_T} \left( \frac{d[E^*_o]}{dt} \right) = \left[ k_2 + k_{-2} \left( \frac{1 + K_{a_1}[Q]}{1 + K_{a_2}[Q]} \right) \frac{1}{K_{a_1}[Q]} \right] \frac{[E^*_o]}{[E]_T} + \frac{k_{-2}}{1 + K_{a_2}[Q]} \tag{B7a}
\]

\[
\frac{1}{[E]_T} \left( \frac{d[E^*_e]}{dt} \right) = -k_{-2} + k_2 \left( \frac{1 + K_{a_2}[Q]}{1 + K_{a_1}[Q]} \right) \frac{[E^*_e]}{[E]_T} \tag{B7b}
\]

Equations (B7) where solved to give the following time-dependent solutions:

\[
\frac{[E^*_o]}{[E]_T} = \frac{a}{b} [1 - \exp(-bt)] \tag{B8a}
\]

\[
\frac{[E^*_e]}{[E]_T} = \left( 1 - \frac{d}{c} \right) \exp(-ct) + \frac{d}{c} \tag{B8b}
\]

where:

\[
a = \frac{k_2}{1 + \frac{1}{K_{a_1}[Q]}}
\]

\[
b = \frac{k_{-2} \left( 1 + K_{a_1}[Q] \right) + k_2 \left( 1 + K_{a_2}[Q] \right) K_{a_1}[Q]}{1 + K_{a_1}[Q]}
\]

\[
c = \frac{k_{-2} \left( 1 + K_{a_1}[Q] \right) + k_2 \left( 1 + K_{a_2}[Q] \right) K_{a_1}[Q]}{\left( 1 + K_{a_2}[Q] \right) K_{a_1}[Q]}
\]

\[
d = \frac{k_{-2}}{1 + K_{a_2}[Q]}
\]

The parameter \( b \) above was defined as \( k_{tot} \) so that

\[
k_{tot} = k_2 \frac{\left( 1 + K_{a_2}[Q] \right) K_{a_1}[Q]}{1 + K_{a_1}[Q]} e^{-\beta \lambda_Q U} + k_{-2} e^{\left( 1-\beta \right) \lambda_Q U} \tag{B9}
\]

where \( \lambda_Q \) is the apparent charge of the rate limiting \( V_M \)-dependent reaction in Scheme B. Notice that the amine concentration dependence is associated with the forward reaction \( (k_2) \).
This equation was used to fit the data in Fig. 1D to yield the following parameter values

\[ k_2 = 506 \pm 19 \text{ s}^{-1} \]
\[ \delta = 0.32 \pm 0.01 \]
\[ \lambda_Q = 0.41 \pm 0.01 \]
\[ k_{-2} = 283 \pm 9 \text{ s}^{-1} \]
\[ K_{a1} = 30,300 \pm 2,400 \text{ M}^{-1} \]
\[ K_{a2} = 3,100 \pm 200 \text{ M}^{-1} \]

The curves in Fig. 3B are the solution to this best-fitting function for Scheme B.

ii. Steady-state charge distribution

Considering that the mobile charge is: \( q(V_M;[Q]) = zF [E^*_\text{ss}] \) and recalling \((B6a)\), then:

\[ q(V_M;[Q]) = \frac{q_{\text{TOT}}}{1 + \frac{1}{K_{a1}[Q]} + K_2(1 + K_{a2}[Q])\exp(-\lambda_Q U)} \]

where: \( q_{\text{TOT}} = zFE_T \); \( K_2 = k_2^0/k_{-2}^0 \) where the superindex “0” indicates \( V_M = 0 \); and \( U = zFV_M/RT \). Addition of a term defined at the holding potential to ensure that \( q(V_h;[Q]) = 0 \) leads to

\[ \Delta q = \frac{q_{\text{TOT}}}{1 + \frac{1}{K_{a1}[Q]} + K_2(1 + K_{a2}[Q])e^{-\lambda_Q U}} - \frac{q_{\text{TOT}}}{1 + \frac{1}{K_{a1}[Q]} + K_2^h(1 + K_{a2}[Q])} \]

where \( K_2^h \) is the value of \( K_2e^{-\lambda_Q U} \) at the holding potential. This equation was used to fit data in Fig. 2A to yield the following parameter values:
\[ q_{TOT} = 15.5 \pm 0.4 \text{ fC/pF} \]
\[ K_{a1} = 145,300 \pm 23,500 \text{ M}^{-1} \]
\[ K_2 = 0.20 \pm 0.02 \]
\[ K_{a2} = 15,400 \pm 2400 \text{ M}^{-1} \]
\[ \lambda_Q = 0.64 \pm 0.03 \]

The curves in Fig. 4B are the solution to this best-fitting function for scheme B.

C. **Comparison of the apparent affinity for pNBTEA binding calculated from transient charge movement kinetics and steady state charge distribution**

Solving for the steady state charge distribution in Scheme B leads to the conclusion that pNBTEA binds with an apparent affinity of 7 \( \mu \text{M} \) \( (K_D = 1/K_{a1}) \). By comparison, solving for the \( V_M \) dependence of charge kinetics with Scheme B suggested that the apparent affinity for pNBTEA binding was 33 \( \mu \text{M} \). We doubt the validity of this value because this Scheme B model predicts that the total amount of charge \( (q_{TOT}) \) is a saturating function of pNBTEA concentration. If the binding affinity were actually 33 \( \mu \text{M} \), then we should have observed a large increase in \( q_{TOT} \) between experiments using 33 and 300 \( \mu \text{M} \) concentrations of this amine. However, \( q_{TOT} \) was not significantly different at all concentrations tested, suggesting that binding affinity for pNBTEA is much less than 33 \( \mu \text{M} \), the lowest concentration tested. Irrespective of the true value, it is clear that this simple molecule has a high enough inhibitory affinity that pNBTEA might serve as a precursor structure to design high affinity \( V_M \)-dependent Na\(^+\),K\(^+\)-ATPase blockers.
D. Supplemental Figure 1

The dependence of $V_q$ on $p$NBTEA concentration. Values of $V_q$ taken from Table 1.