

# Membrane Potential-Dependent Inhibition of the Na<sup>+</sup>,K<sup>+</sup>-ATPase by *para*-Nitrobenzyltriethylammonium Bromide<sup>S</sup>

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## ABSTRACT

Membrane potential ( $V_M$ )-dependent inhibitors of the Na<sup>+</sup>,K<sup>+</sup>-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<sup>+</sup>,K<sup>+</sup>-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<sup>+</sup>,K<sup>+</sup>-ATPase inhibitor, *para*-nitrobenzyltriethylamine (*p*NBTEA), binds to the enzyme by measuring the extracellular *p*NBTEA 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 *p*NBTEA binding differ from those for extracellular Na<sup>+</sup> and K<sup>+</sup> binding, even though inhibitor binding is competitive with extracellular K<sup>+</sup>. The data were also fit to specific models for *p*NBTEA binding to show that *p*NBTEA binding is a rate-limiting  $V_M$ -dependent reaction that, in light of homology models for the Na<sup>+</sup>,K<sup>+</sup>-ATPase, we interpret as a transfer reaction of *p*NBTEA from a peripheral binding site in the enzyme to a site near the known K<sup>+</sup> coordination sites buried within the transmembrane helices of the enzyme. These models also suggest that binding occurs with an apparent affinity of 7  $\mu$ M. This apparent binding affinity suggests that high-affinity  $V_M$ -dependent Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors should be feasible to design and test as specific enzyme inhibitors.

## Introduction

Interest in small-molecule inhibitors of the P-type Na<sup>+</sup>,K<sup>+</sup>-ATPase has reemerged with the realization that pharmacological interventions targeting this enzyme might be relevant in a diverse array of disease processes. The Na<sup>+</sup>,K<sup>+</sup>-ATPase is well known for its critical role in developing and maintaining electrochemical gradients for Na<sup>+</sup> and K<sup>+</sup> across the plasma membrane (Glynn, 1985). In this role, the Na<sup>+</sup>,K<sup>+</sup>-ATPase controls contractility of cardiac muscle cells by regulating cell Ca<sup>2+</sup> loading via the sarcolemmal Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (Eisner et al., 1984). Manipulating the Na<sup>+</sup> electrochemical gradient, and thereby cellular Ca<sup>2+</sup>, by Na<sup>+</sup>,K<sup>+</sup>-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 (Gheorghide 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<sup>+</sup>,K<sup>+</sup>-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<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors as potential chemotherapeutic agents (for review, see Newman et al., 2008) and suggest that the Na<sup>+</sup>,K<sup>+</sup>-ATPase might be an attractive drug target beyond its established roles in inotropic and antiarrhythmic therapeutics.

Cardiac glycosides are the most widely studied Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitors. Mutagenesis studies have identified amino acids in the enzyme's  $\alpha$  subunit that are important for ouabain binding (for review, see Lingrel, 2010) and recent high-resolution structures of the Na<sup>+</sup>,K<sup>+</sup>-ATPase include bound ouabain (Ogawa et al., 2009; Yatime et al., 2011). Cardiac glycosides have been synthesized and tested in more

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**ABBREVIATIONS:** BTEA, benzyltriethylamine; *p*NBTEA, *para*-nitrobenzyltriethylamine, *para*-nitrobenzyltriethylammonium bromide.

than five decades of drug discovery efforts (Glynn, 1964; Güntert and Linde, 1981; Gobbini and Cerri, 2005). In addition, other high-affinity  $\text{Na}^+, \text{K}^+$ -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  $\text{Na}^+, \text{K}^+$ -ATPase located in different cellular or tissue compartments in which the enzyme resides. Selectivity in  $\text{Na}^+, \text{K}^+$ -ATPase inhibition, however, may provide advantages in treatment of congestive heart failure and is certainly relevant if  $\text{Na}^+, \text{K}^+$ -ATPase inhibitors are to be developed as chemotherapeutic agents. For this reason, the search for novel  $\text{Na}^+, \text{K}^+$ -ATPase inhibitors is a promising area of drug discovery.

In a previous study, we identified a compound, benzyltriethylamine (BTEA), that inhibits the  $\text{Na}^+, \text{K}^+$ -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 seems to bind at a  $\text{K}^+$  coordination site within the enzyme although, unlike  $\text{K}^+$ , it is neither occluded nor transported. What is intriguing about BTEA is that it is the first reported  $V_M$ -dependent blocker of the  $\text{Na}^+, \text{K}^+$ -ATPase, although 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  $\text{Na}^+, \text{K}^+$ -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 under *Discussion*, this property might be useful for the treatment of congestive heart failure. In addition,  $V_M$ -dependent  $\text{Na}^+, \text{K}^+$ -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  $\text{Na}^+, \text{K}^+$ -ATPase, but at a much lower concentration (Peluffo et al., 2009). These results were promising for development of similar compounds as high-affinity  $\text{Na}^+, \text{K}^+$ -ATPase inhibitors, but two important questions remained unanswered in previous work. First, although BTEA and *p*NBTEA seem to bind at a  $\text{K}^+$  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  $\text{Na}^+, \text{K}^+$ -ATPase. Second, we have not previously determined the apparent affinity for inhibition of the  $\text{Na}^+, \text{K}^+$ -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  $\text{Na}^+, \text{K}^+$ -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 M $\Omega$ ) as published previously (Ishizuka et al., 1996). Animals were treated according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) 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  $\text{Na}^+$ - and  $\text{K}^+$ -free superfusion solutions containing 145 mM tetramethylammonium chloride, 2.3 mM  $\text{MgCl}_2$ , 0.2 mM  $\text{CdCl}_2$ , 5.5 mM dextrose, and 10 mM HEPES (pH 7.4 with Tris). The bromide salt of *p*NBTEA was added to this solution at the indicated concentrations. The patch electrode solution contained 130 mM  $\text{K}^+$ , 20 mM tetraethyl ammonium chloride, 9.1 mM  $\text{MgCl}_2$ , 0.7 mM ATP- $\text{Mg}^{2+}$  salt, 30 mM phosphate ( $\text{H}_2\text{PO}_4^- + \text{HPO}_4^{2-}$ ), 1 mM EGTA, 66 mM aspartic acid, and 10 mM HEPES, pH 7.3 (20°C). The magnesium phosphate concentration was calculated with MaxChelator to be 1.3 mM.

The experimental procedure to measure extracellular *p*NBTEA transient charge movements was to first superfuse a voltage-clamped myocyte with a *p*NBTEA-containing  $\text{Na}^+$ - and  $\text{K}^+$ -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 that 2 min of 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 a ouabain-sensitive charge ( $q$ ; in units of femtocoulombs per picofarad). A more complete description of the experimental protocol is found in Peluffo et al. (2009).

**Analysis, Curve-Fitting, and Model Selection.** Data are presented as means  $\pm$  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 nonlinear least-squares algorithm available in commercial software (SigmaPlot, Systat Software Inc., San Jose, CA) 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 ( $\text{AIC}_c$ ) with a limited data set was calculated for each model as

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

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

$$w_i = \frac{\exp(-0.5\text{AIC}_i)}{\sum_{r=1}^N \exp(-0.5\text{AIC}_r)} \quad 0 \leq w_i \leq 1$$

where  $\text{AIC}_i$  is the  $\text{AIC}_c$  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  $\mu\text{M}$  *p*NBTEA have been reported in Peluffo et al. (2009); however, analysis of the data set at all tested *p*NBTEA concentrations is presented here for the first time.

**Reagents.** Reagents were from Sigma-Aldrich (St. Louis, MO) 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  $^{86}\text{Rb}^+$  occlusion by the  $\text{Na}^+, \text{K}^+$ -ATPase. In the same study, *p*NBTEA was found to be a  $V_M$ -dependent inhibitor by

examining the effects of  $100 \mu\text{M}$   $p\text{NBTEA}$  on steady-state  $\text{Na}^+, \text{K}^+$ -pump current and  $p\text{NBTEA}$ -dependent pre-steady-state transient charge movements at different membrane potentials. To gain greater insight into the mechanism of enzyme block by  $p\text{NBTEA}$ , we have also conducted measurements of transient charge movements with  $33$  and  $300 \mu\text{M}$  concentrations of the quaternary amine added to the perfusion solution in patch-clamped rat ventricular myocytes (see *Materials and Methods*). Figure 1, A to C, shows representative  $1 \text{ mM}$  ouabain-sensitive difference currents recorded when the membrane potential was stepped from the holding potential,  $-40 \text{ mV}$ , to  $0 \text{ mV}$  for  $100 \text{ ms}$ , before returning to the holding potential. Our previous work (Peluffo and Berlin, 1997; Peluffo et al., 2009) has shown that  $1 \text{ mM}$  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  $\text{Na}^+, \text{K}^+$ -ATPase. In the absence of  $p\text{NBTEA}$ , ouabain-sensitive transient currents were not observed (Peluffo et al., 2009). Thus, these currents show that the inhibition of the  $\text{Na}^+, \text{K}^+$ -ATPase by the positively charged  $p\text{NBTEA}$  involves at least one  $V_M$ -dependent reaction.

Ouabain-sensitive charge movements reflect a change of ion binding by the  $\text{Na}^+, \text{K}^+$ -ATPase in response to a change in  $V_M$  (Gadsby et al., 1993; Peluffo and Berlin, 1997). With transported cations (i.e.,  $\text{Na}^+$  and  $\text{K}^+$ ), the kinetics of these charge movements are limited by subsequent electroneutral reactions involved in ion occlusion by the enzyme (Gadsby et

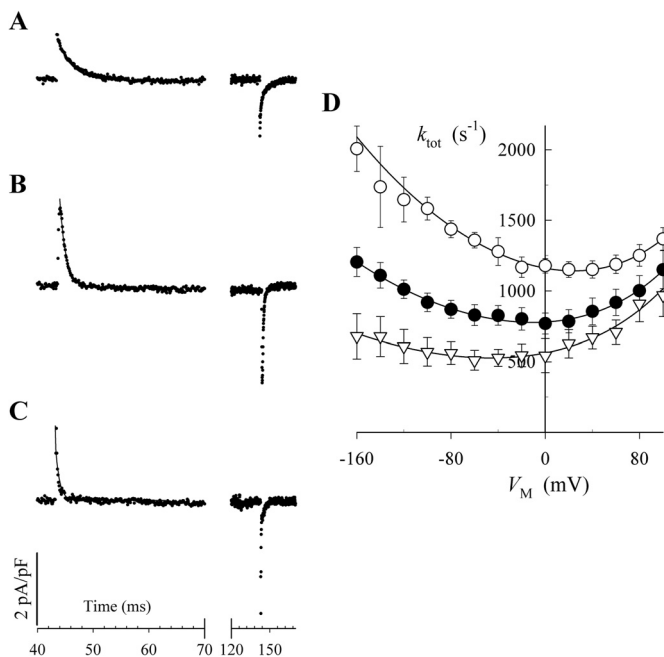
al., 1993; Hilgemann, 1994; Wuddel and Apell, 1995). However, as we have previously shown, organic quaternary amine inhibitors of the  $\text{Na}^+, \text{K}^+$ -ATPase, such as  $p\text{NBTEA}$ , are not occluded (Peluffo et al., 2009). Therefore, the kinetics of  $p\text{NBTEA}$ -dependent transient charge movements are a direct measure of the rate of quaternary amine binding reactions.

As shown in Fig. 1, A to 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. 1, A to C, indicating a pseudo-first-order process. This fitting allowed us to derive a rate ( $k_{\text{tot}}$ ) for the change in  $p\text{NBTEA}$  binding after a  $V_M$  jump. The rate of decay of the transient current increased at higher  $p\text{NBTEA}$  concentrations (Fig. 1D), as would be expected for a pseudo-first-order ligand binding reaction. Thus, the transient currents shown here result from the  $V_M$ -dependent binding of the quaternary amine as it inhibits the  $\text{Na}^+, \text{K}^+$ -ATPase.

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

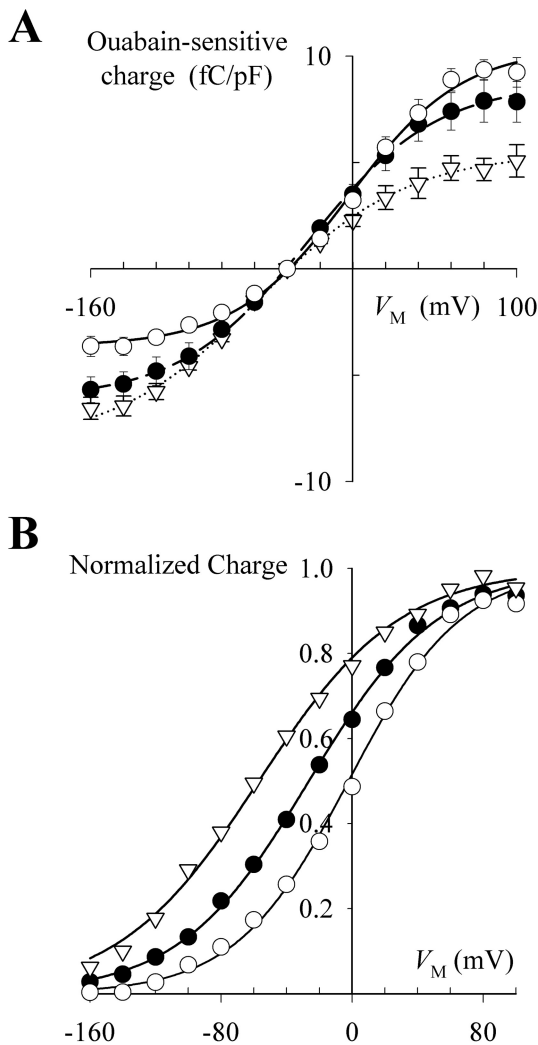
$$k_{\text{tot}} = k_f^0 [Q]^n \exp(-\delta \lambda_Q F V_M / RT) + k_r^0 \exp((1-\delta) \lambda_Q F V_M / RT) \quad (1)$$

where  $k_f^0$  and  $k_r^0$  are the forward and reverse rate constants for  $p\text{NBTEA}$  binding at  $0 \text{ mV}$ ,  $[Q]$  is the extracellular  $p\text{NBTEA}$  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  $F$ ,  $R$ , and  $T$  are the Faraday constant, the gas constant, and the absolute temperature, respectively. The calculated values for  $k_f^0$  and  $k_r^0$  are given in the legend to Fig. 1. This calculation showed that the apparent charge ( $\lambda_Q$ ) 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 from the 37% of the electric field dissipated by extracellular  $\text{K}^+$  during its activation of ion transport by the  $\text{Na}^+, \text{K}^+$ -ATPase (Peluffo and Berlin, 1997). This similarity along with the competitive nature of  $p\text{NBTEA}$  enzyme inhibition led us to conclude that these amines were binding at a  $\text{K}^+$  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  $k_{\text{tot}}$  at positive potentials and the shift in the  $V_M$  at which  $k_{\text{tot}}$  reaches a minimum with increased  $p\text{NBTEA}$  concentration



**Fig. 1.** Ouabain-sensitive pre-steady-state currents with various  $p\text{NBTEA}$  concentrations. Currents were recorded in response to a 100-ms depolarization to  $0 \text{ mV}$  in the presence and absence of  $1 \text{ mM}$  ouabain in superfusion solutions containing  $33 \mu\text{M}$  (A),  $100 \mu\text{M}$  (B), and  $300 \mu\text{M}$   $p\text{NBTEA}$  (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  $V_M$  (D) for  $33 \mu\text{M}$  ( $\nabla$ ),  $100 \mu\text{M}$  ( $\bullet$ ), and  $300 \mu\text{M}$   $p\text{NBTEA}$  ( $\circ$ ). The solid curves were determined by fitting the entire data set to eq. 1. The calculated values of  $k_f^0$  and  $k_r^0$  were  $58,600 \pm 2000 \text{ s}^{-1} \text{ M}^{-n}$  and  $282 \pm 9 \text{ s}^{-1}$ , respectively.





**Fig. 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  $V_M$  ( $q_{ON}$ ) divided by cell capacitance during superfusion with solutions containing 33 ( $\nabla$ ), 100 ( $\bullet$ ), and 300  $\mu\text{M}$  *pNBTEA* ( $\circ$ ). A, charge. B, charge normalized to  $q_{TOT}$  calculated by fitting the data at each *pNBTEA* concentration with the following Boltzmann equation:  $q = q_{min} + q_{TOT}/(1 + \exp[-(V_M - V_q)/z_q])$ . Values for the best fit parameters are given in Table 1.

(see eq. 1). The Hill coefficient was  $0.52 \pm 0.01$ , a value indicative of negative cooperativity in *pNBTEA* binding to the enzyme, which implies that more than one amine molecule binds to the enzyme.

To further describe the  $V_M$  dependence of *pNBTEA* 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-two-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  $\text{Na}^+, \text{K}^+$ -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 the *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 the *pNBTEA* concentration is increased (i.e., at a higher amine concentration, a smaller fraction of total  $\text{Na}^+, \text{K}^+$ -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 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 seems 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). Because the Hill coefficient for transient currents does not equal 1 (Fig. 1), the molecularity of the reaction is unknown, although 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-two-state model used for analysis of data in Figs. 1 and 2 accommodates neither the molecularity nor the type of binding reaction.

To understand more about *pNBTEA* binding to the  $\text{Na}^+, \text{K}^+$ -ATPase, different ligand binding models were evaluated to determine which provides the best fit to the data. In evaluating potential binding models for *pNBTEA*, random schemes were excluded because  $\text{Rb}^+$  binding to the  $\text{Na}^+, \text{K}^+$ -ATPase is an ordered process (Forbush, 1987; González-Lebrero et al., 2002). Given that *pNBTEA* seems to bind at an extracellular  $\text{K}^+$  coordination site, the assumption of ordered binding is also likely to be valid. Furthermore, the effect of BTEA on  $\text{Rb}^+$  occlusion (Peluffo et al., 2009) is well described by postulating two amine binding sites in the enzyme. Although a greater number of sites cannot be excluded, there is no obvious rationale to include more. For this reason, only models with two *pNBTEA* binding sites were evaluated. The monoexponential decay of *pNBTEA*-activated transient charge movements also suggests that either 1) rate-limiting  $V_M$ -dependent reactions have similar kinetics or 2) multiple  $V_M$ -dependent reactions have very different kinetics. In both cases,

**TABLE 1**  
Pseudo-two-state Boltzmann function parameters for the  $V_M$  dependence of  $q$

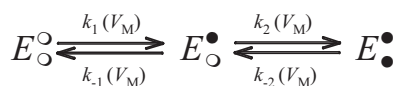
Parameter	[ <i>pNBTEA</i> ]		
	33 $\mu\text{M}$	100 $\mu\text{M}$	300 $\mu\text{M}$
$q_{TOT}$ (fC/pF)	$13.7 \pm 0.6$	$14.9 \pm 0.4$	$14.0 \pm 0.5$
$q_{MIN}$ (fC/pF)	$-8.3 \pm 0.5$	$-6.1 \pm 0.2$	$-3.6 \pm 0.2$
$V_q$ (mV)	$-57.0 \pm 3.4$	$-27.1 \pm 1.8$	$0.5 \pm 2.9$
$z_q$	$0.56 \pm 0.04$	$0.65 \pm 0.03$	$0.72 \pm 0.06$

these schemes simplify to an ordered reaction with a single  $V_M$ -dependent reaction.

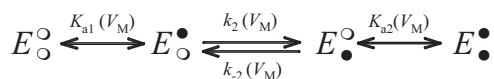
In a broad sense, ordered reaction schemes fall into two categories shown as Schemes A and B. Scheme A is a classic sequential reaction mechanism for a two-site enzyme in which  $p\text{NBTEA}$  binding at site 1 and/or site 2 can be  $V_M$ -dependent. The rates of the binding reactions are described by pseudo-first-order forward ( $k_x$ ) and reverse ( $k_{-x}$ ) rate constants (where  $x = 1, 2$ ). Because the decay of  $p\text{NBTEA}$ -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 on the work of Glynn et al. (1985), Forbush (1987), and González-Lebrero et al. (2002), whose analysis of  $\text{Rb}^+$  occlusion kinetics by the  $\text{Na}^+, \text{K}^+$ -ATPase suggested that  $\text{Rb}^+$  binding is in rapid equilibrium with the  $\text{Na}^+, \text{K}^+$ -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. Because BTEA is not occluded (Peluffo et al., 2009), we have instead substituted a rate-limiting reaction of the  $p\text{NBTEA}$ -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 models representative of Scheme A and Scheme B are presented in Fig. 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 ( $\text{AIC}_c$ ).

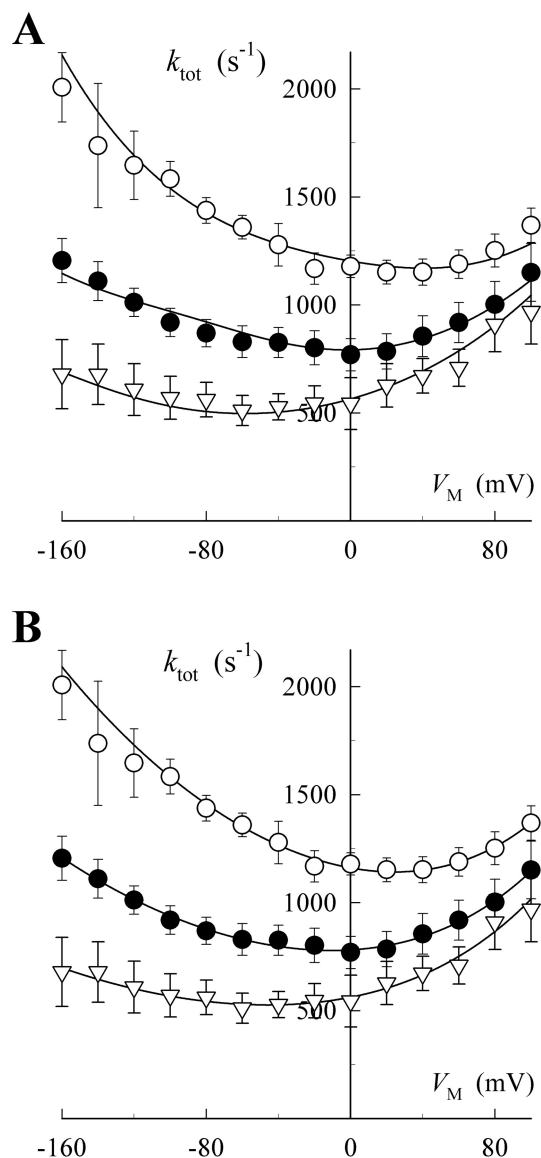
The best-fitting models for Schemes A and B both tracked the experimental data well (see supplemental data for the fitted functions and best-fit parameter values). However, it is clear that the Scheme B model provided the better fit. This graphical impression is substantiated by the  $\text{AIC}_c$  for Scheme A ( $\text{AIC}_c = 321.2$ ) and for Scheme B ( $\text{AIC}_c = 226.8$ ), which provide an Akaike weight ( $w_i$ ) for Scheme A that approaches a value of 0, meaning that the model is highly unlikely, whereas the  $w_i$  for Scheme B approaches a value of 1, meaning that this model is quite likely to be the best one describing the  $V_M$  and  $p\text{NBTEA}$  concentration dependence of  $k_{\text{tot}}$  (see *Materials and Methods*). From this analysis, we conclude that the  $V_M$ -dependent step in  $p\text{NBTEA}$  inhibition of the  $\text{Na}^+, \text{K}^+$ -ATPase occurs as a rate-limiting reaction of the



Scheme A



Scheme B



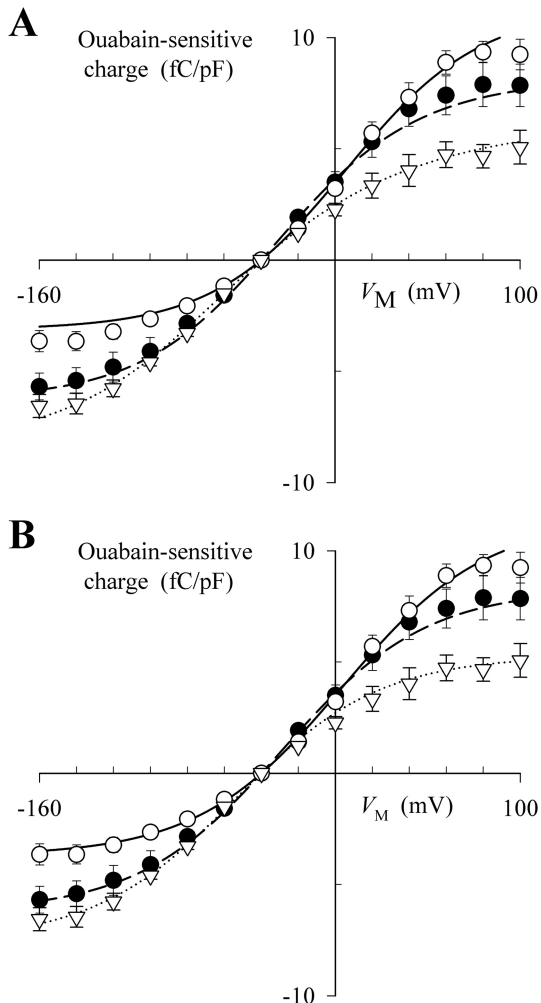
**Fig. 3.** Fitting charge movement kinetics with specific  $p\text{NBTEA}$  binding models. Data points are reproduced from Fig. 1D. Curves represent the functions solved for the best fit models based on Scheme A (A) and Scheme B (B) using eqs. A6 and B9, respectively. These equations and their best fit parameter values are listed in the supplemental data. Symbols are as indicated in Fig. 1D.

amine-bound enzyme. As presented under *Discussion*, we believe that this  $V_M$ -dependent step represents the transfer of bound  $p\text{NBTEA}$  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 seem to fit these steady-state data quite well; however, the model based on Scheme B seems 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-two-state model is overcome by using a specific model that accounts for the molecularity of amine binding to the  $\text{Na}^+, \text{K}^+$ -ATPase.

What is interesting about the best-fit functions for Scheme B is the prediction that the first *p*NBTEA binds to the  $\text{Na}^+, \text{K}^+$ -ATPase with high affinity. From the  $V_M$  dependence of steady-state charge distribution, the model calculates an apparent affinity of  $7 \mu\text{M}$  ( $K_{D1} = 1/K_{a1} = 6.9 \mu\text{M}$ ). Consistent with the negative cooperativity shown in Fig. 1, the second amine molecule binds with lower apparent affinity (e.g.,  $K_{D2} = 1/K_{a2} = 65 \mu\text{M}$  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 showed that binding of one molecule of *p*NBTEA inhibits the  $\text{Na}^+, \text{K}^+$ -ATPase (Peluffo et al., 2009). Thus, 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.



**Fig. 4.** Fitting the  $q$ - $V_M$  relationship with specific *p*NBTEA binding models. Data points are reproduced from Fig. 2A. Curves show the functions solved for the best-fit models based on Scheme A (A) and Scheme B (B) using eqs. A9 and B11, respectively. These equations and their best fit parameter values are listed in the Supplemental Data. Symbols are as indicated in the legend to Fig. 2.

## Discussion

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

First, what is the mechanism of *p*NBTEA  $V_M$ -dependent  $\text{Na}^+, \text{K}^+$ -ATPase inhibition? BTEA and *p*NBTEA seem to bind at an extracellularly accessible  $\text{K}^+$  coordination site; however, they are neither occluded nor transported by the enzyme (Peluffo et al., 2004, 2009). *p*NBTEA binding also does not conform to the properties of ion binding in an ion well.  $V_M$ -dependent binding of  $\text{Na}^+$  and  $\text{K}^+$  in the  $\text{Na}^+, \text{K}^+$ -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 seem to move little charge in the membrane electric field. For this reason, the proportionality coefficient “ $\delta$ ” in eq. 1 is approximately equal to 1 for  $\text{K}^+$ , meaning  $k_{\text{tot}}$  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  $k_{\text{tot}}$ , 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  $\text{K}^+$  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  $p\text{NBTEA}$ -bound enzyme. First,  $V_M$  dependence can arise from movement of intrinsic charges in the protein during a conformational change, and second,  $p\text{NBTEA}$  could move within the electric field even after binding. With  $\text{Na}^+$ - and  $\text{K}^+$ -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  $\text{Na}^+, \text{K}^+$ -ATPase  $\alpha$  subunit [with sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$ -ATPase structure 3B9B as a template] suggested 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 and 6 at a  $\text{K}^+$  coordination site. We have postulated that the first site is outside the membrane electric field, whereas the second site is in the field such that extracellular  $\text{K}^+$  or BTEA binding dissipates approximately one-third of the total electric field. This postulate could explain why most quaternary amine blockers of the  $\text{Na}^+, \text{K}^+$ -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 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  $\text{K}^+$  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  $\text{K}^+$  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 toward the transmembrane helices, deeper into a binding pocket in the enzyme.

The second question is whether  $p\text{NBTEA}$  inhibits the  $\text{Na}^+, \text{K}^+$ -ATPase with high enough affinity that these compounds warrant further investigation as possible enzyme blockers. Amine-containing compounds were first shown to inhibit the  $\text{Na}^+, \text{K}^+$ -ATPase in a manner that is competitive with extracellular  $\text{K}^+$  in the 1960s (Sachs and Conrad, 1968). In that article 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  $\text{Na}^+, \text{K}^+$ -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 whether an amine-containing compound could inhibit the  $\text{Na}^+, \text{K}^+$ -ATPase at lower concentrations, and, in our initial report (Peluffo et al., 2009), its  $V_M$ -dependent inhibi-

tion 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  $\text{Na}^+, \text{K}^+$ -ATPase is trapped in phosphorylated  $E_2$  states (Peluffo and Berlin, 1997). Therefore, 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 seems to be independent of the model used.

A final question is, could a  $V_M$ -dependent inhibitor of the  $\text{Na}^+, \text{K}^+$ -ATPase be a beneficial pharmacologic agent? To date,  $V_M$ -dependent  $\text{Na}^+, \text{K}^+$ -ATPase inhibitors contain ammonium moieties, as in  $p\text{NBTEA}$ , and thus 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 with more depolarized tissue, such as ischemic heart muscle whose membrane potential is reported to be as much as 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% having 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 nonischemic heart failure. Likewise, digoxin was found to be an independent risk factor for excess mortality in patients surviving myocardial infarction (Leor 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 compared with 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  $\text{Na}^+, \text{K}^+$ -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 those of  $p\text{NBTEA}$  will have such a preference to inhibit  $\text{Na}^+, \text{K}^+$ -ATPase in highly polarized cells.

Conceptually, it should also be possible to develop a  $\text{Na}^+, \text{K}^+$ -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  $\text{Na}^+, \text{K}^+$ -ATPase inhibitors can be developed remains to be deter-

mined; however, the present results suggest that the development of such agents should be feasible.

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#### Authorship Contributions

*Participated in research design:* Berlin and Peluffo.

*Conducted experiments:* Berlin and Peluffo.

*Contributed new reagents or analytic tools:* Berlin.

*Performed data analysis:* Berlin and Peluffo.

*Wrote or contributed to the writing of the manuscript:* Berlin and Peluffo.

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