Membrane Potential Dependent Inhibition of the Na,K-ATPase by *para*-Nitrobenzyltriethylammonium Bromide.

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Molecular Pharmacology

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,

$$[E]_{\mathrm{T}} = [E^{\circ}_{\circ}] + [E^{\bullet}_{\circ}] + [E^{\bullet}_{\bullet}] \tag{A1}$$

and the definition:

$$K_{\mathrm{a1}} = \frac{1}{K_{\mathrm{d1}}} = \frac{[E^{\bullet}_{\circ}]}{[E^{\circ}_{\circ}][Q]} \qquad \text{where } [Q] = p\text{NBTEA concentration} \tag{A2}$$

then,

$$\frac{1}{[E]_{\mathrm{T}}}\left(\frac{d[E^{\bullet}_{\circ}]}{dt}\right) = -\left[k_2[Q] + k_{-2}\left(1 + \frac{K_{\mathrm{d1}}}{[Q]}\right)\right]\frac{[E^{\bullet}_{\circ}]}{[E]_{\mathrm{T}}} + k_{-2} \tag{A3a}$$

and

$$\frac{1}{[E]_{\mathrm{T}}}\left(\frac{d[E^{\bullet}_{\bullet}]}{dt}\right) = -\left(k_{-2} + \frac{k_2[Q]}{1 + \frac{K_{\mathrm{d1}}}{[Q]}}\right)\frac{[E^{\bullet}_{\bullet}]}{[E]_{\mathrm{T}}} + \frac{k_2[Q]}{1 + \frac{K_{\mathrm{d1}}}{[Q]}} \tag{A3b}$$

The time-dependent solution for (A3b)

$$[E^{\bullet}_{\bullet}]_{(t)} = \frac{C_3}{C_2}[E]_{\mathrm{T}}\left[1 + \frac{(br+s)\exp(-at) - (ar+s)\exp(-bt)}{(a-b)}\right] \tag{A4}$$

was obtained as described in Peluffo (2004) with

$$a = \frac{C_1 + \sqrt{C_1^2 - 4C_2}}{2}$$

$$b = \frac{C_1 - \sqrt{C_1^2 - 4C_2}}{2}$$

$$C_1 = k_1[Q] + k_{-1} + k_2[Q] + k_{-2}$$

$$C_2 = k_1 k_2 [Q]^2 + k_1[Q]k_{-2} + k_{-1}k_{-2} \qquad \text{(A5)}$$

$$C_3 = k_1 k_2 [Q]^2$$

$$r = 1 - w(C_2/C_3)$$

$$s = (C_2/C_3)(wk_{-2} - vk_2[Q])$$

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

In this case, b = k_{tot} so that:

$$k_{\text{tot}} = \frac{1}{2}\left[(k_1 + k_2)[Q] + k_{-1} + k_{-2} - \sqrt{\left[(k_1 + k_2)[Q] + k_{-1} + k_{-2}\right]^2 - 4[k_1 k_2 [Q]^2 + (k_1[Q] + k_{-1})k_{-2}]}\right] \qquad \text{(A6)}$$

where $k_{\text{x}} = k_{\text{x}}^{\text{o}} e^{-\delta_{\text{x}} \lambda_{\text{x}} U}$; $k_{\text{-x}} = k_{\text{-x}}^{\text{o}} e^{(1-\delta_{\text{x}})\lambda_{\text{x}} U}$ for x=1,2. k_{x}^{o} and $k_{\text{-x}}^{\text{o}}$ 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

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

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 $q\left(V_{\mathrm{M}};[Q]\right) = zF[E^{\bullet}_{\circ}]_{\mathrm{ss}}$.

Considering that

$$\frac{[E^{\bullet}_{\circ}]_{\mathrm{ss}}}{[E]_{\mathrm{T}}} = \frac{k_1 k_{-2}[Q]}{k_1 k_2 [Q]^2 + k_1 k_{-2}[Q] + k_{-1} k_{-2}} \tag{A7}$$

then:

$$q\left(V_{\mathrm{M};}[Q]\right) = \frac{q_{\mathrm{TOT}}}{1 + \dfrac{K_{\mathrm{d1}}}{[Q]}\exp(-\lambda_1 U) + K_{\mathrm{a2}}[Q]\exp(-\lambda_2 U)} \tag{A8}$$

where: $K_{\mathrm{d1}} = k_{-1}^{0}/k_{1}^{0}$ and $K_{\mathrm{a2}} = k_{2}^{0}/k_{-2}^{0}$. Addition of a term defined at the holding potential leads to

$$\Delta q = \frac{q_{\mathrm{TOT}}}{1 + \dfrac{K_{\mathrm{d1}}}{[Q]} e^{-\lambda_1 U} + K_{\mathrm{a2}}[Q] e^{-\lambda_2 U}} - \frac{q_{\mathrm{TOT}}}{1 + \dfrac{K^{h}_{\mathrm{d1}}}{[Q]} + K^{h}_{\mathrm{a2}}[Q]} \tag{A9}$$

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

$$\begin{aligned}
q_{\mathrm{TOT}} &= 14.5 \pm 0.4 \text{ fC/pF} \\
K_{\mathrm{d1}} &= (7.9 \pm 1.3) * 10^{-6} \text{ M} \\
\lambda_1 &= 0.31 \pm 0.05 \\
K_{\mathrm{a2}} &= 3{,}900 \pm 300 \text{ M}^{-1} \\
\lambda_2 &= 0.70 \pm 0.04
\end{aligned}$$

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,

$$[E]_{\mathrm{T}} = [E^{\circ}_{\circ}] + [E^{\bullet}_{\circ}] + [E^{\circ}_{\bullet}] + [E^{\bullet}_{\bullet}] \tag{B1}$$

and the definitions:

$$K_{\mathrm{a}1} = \frac{[E^{\bullet}_{\circ}]}{[E^{\circ}_{\circ}][Q]}; \quad K_{\mathrm{a}2} = \frac{[E^{\bullet}_{\bullet}]}{[E^{\circ}_{\bullet}][Q]} \tag{B2}$$

replacing (B2) in (B1) and rearranging:

$$[E]_{\mathrm{T}} = \left(1 + \frac{1}{K_{\mathrm{a}1}[Q]}\right)[E^{\bullet}_{\circ}] + \left(1 + K_{\mathrm{a}2}[Q]\right)[E^{\circ}_{\bullet}] \tag{B3}$$

The reaction scheme yields the following system of differential equations:

$$\begin{cases} \dfrac{d[E^{\bullet}_{\circ}]}{dt} = -k_2[E^{\bullet}_{\circ}] + k_{-2}[E^{\circ}_{\bullet}] \\ \dfrac{d[E^{\circ}_{\bullet}]}{dt} = k_2[E^{\bullet}_{\circ}] - k_{-2}[E^{\circ}_{\bullet}] \end{cases} \tag{B4}$$

with the steady-state solution: $k_2[E^{\bullet}_{\circ}] = k_{-2}[E^{\circ}_{\bullet}]$ (B5)

Thus, replacing in (B3) and rearranging:

$$\frac{[E^{\bullet}_{\circ}]}{[E]_{\mathrm{T}}} = \frac{k_{-2}}{\left(1 + \dfrac{1}{K_{\mathrm{a}1}[Q]}\right)k_{-2} + \left(1 + K_{\mathrm{a}2}[Q]\right)k_2} \tag{B6a}$$

$$\frac{[E^{\circ}_{\bullet}]}{[E]_{\mathrm{T}}} = \frac{k_2}{\left(1 + \dfrac{1}{K_{\mathrm{a}1}[Q]}\right)k_{-2} + \left(1 + K_{\mathrm{a}2}[Q]\right)k_2} \tag{B6b}$$

Introducing expressions (B6) into system (B4):

$$\frac{1}{[E]_{\mathrm{T}}}\left(\frac{d[E^{\bullet}_{\circ}]}{dt}\right)=-\left[k_2+k_{-2}\left(\frac{1+K_{\mathrm{a}1}[Q]}{1+K_{\mathrm{a}2}[Q]}\right)\frac{1}{K_{\mathrm{a}1}[Q]}\right]\frac{[E^{\bullet}_{\circ}]}{[E]_{\mathrm{T}}}+\frac{k_{-2}}{1+K_{\mathrm{a}2}[Q]} \tag{B7a}$$

$$\frac{1}{[E]_{\mathrm{T}}}\left(\frac{d[E^{\circ}_{\bullet}]}{dt}\right)=-\left[k_{-2}+k_2\left(\frac{1+K_{\mathrm{a}2}[Q]}{1+\dfrac{1}{K_{\mathrm{a}1}[Q]}}\right)\right]\frac{[E^{\circ}_{\bullet}]}{[E]_{\mathrm{T}}}+\frac{k_2}{1+\dfrac{1}{K_{\mathrm{a}1}[Q]}} \tag{B7b}$$

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

$$\frac{[E^{\circ}_{\bullet}]}{[E]_{\mathrm{T}}}=\frac{a}{b}[1-\exp(-bt)] \tag{B8a}$$

$$\frac{[E^{\bullet}_{\circ}]}{[E]_{\mathrm{T}}}=\left(1-\frac{d}{c}\right)\exp(-ct)+\frac{d}{c} \tag{B8b}$$

where:

$$a=\frac{k_2}{1+\dfrac{1}{K_{\mathrm{a}1}[Q]}}$$

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

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

$$d=\frac{k_{-2}}{1+K_{\mathrm{a}2}[Q]}$$

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

$$k_{\mathrm{tot}}=k_2\frac{\left(1+K_{\mathrm{a}2}[Q]\right)K_{\mathrm{a}1}[Q]}{1+K_{\mathrm{a}1}[Q]}e^{-\delta\lambda_Q U}+k_{-2}e^{(1-\delta)\lambda_Q U} \tag{B9}$$

where λ_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

$$k_2 = 506 \pm 19\ \mathrm{s}^{-1}$$
$$\delta = 0.32 \pm 0.01$$
$$\lambda_Q = 0.41 \pm 0.01$$
$$k_{-2} = 283 \pm 9\ \mathrm{s}^{-1}$$
$$K_{\mathrm{a}1} = 30{,}300 \pm 2{,}400\ \mathrm{M}^{-1}$$
$$K_{\mathrm{a}2} = 3{,}100 \pm 200\ \mathrm{M}^{-1}$$

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: $q\left(V_{\mathrm{M}};[Q]\right) = zF\left[E^{\bullet}\right]_{\mathrm{ss}}$ and recalling (B6a), then:

$$q\left(V_{\mathrm{M};}[Q]\right) = \frac{q_{\mathrm{TOT}}}{1 + \dfrac{1}{K_{\mathrm{a}1}[Q]} + K_2\left(1 + K_{\mathrm{a}2}[Q]\right)\exp(-\lambda_Q U)} \tag{B10}$$

where: $q_{\mathrm{TOT}} = zF[E]_{\mathrm{T}}$; $K_2 = k_2^0/k_{-2}^0$ where the superindex "0" indicates $V_{\mathrm{M}} = 0$; and $U = zFV_{\mathrm{M}}/RT$. Addition of a term defined at the holding potential to ensure that $q(V_{\mathrm{h}};[Q]) = 0$ leads to

$$\Delta q = \frac{q_{\mathrm{TOT}}}{1 + \dfrac{1}{K_{\mathrm{a}1}[Q]} + K_2(1 + K_{\mathrm{a}2}[Q])e^{-\lambda_Q U}} - \frac{q_{\mathrm{TOT}}}{1 + \dfrac{1}{K_{\mathrm{a}1}[Q]} + K_2^h(1 + K_{\mathrm{a}2}[Q])} \tag{B11}$$

where K_2^h is the value of $K_2 e^{-\lambda_Q U}$ at the holding potential. This equation was used to fit data in Fig. 2A to yield the following parameter values:

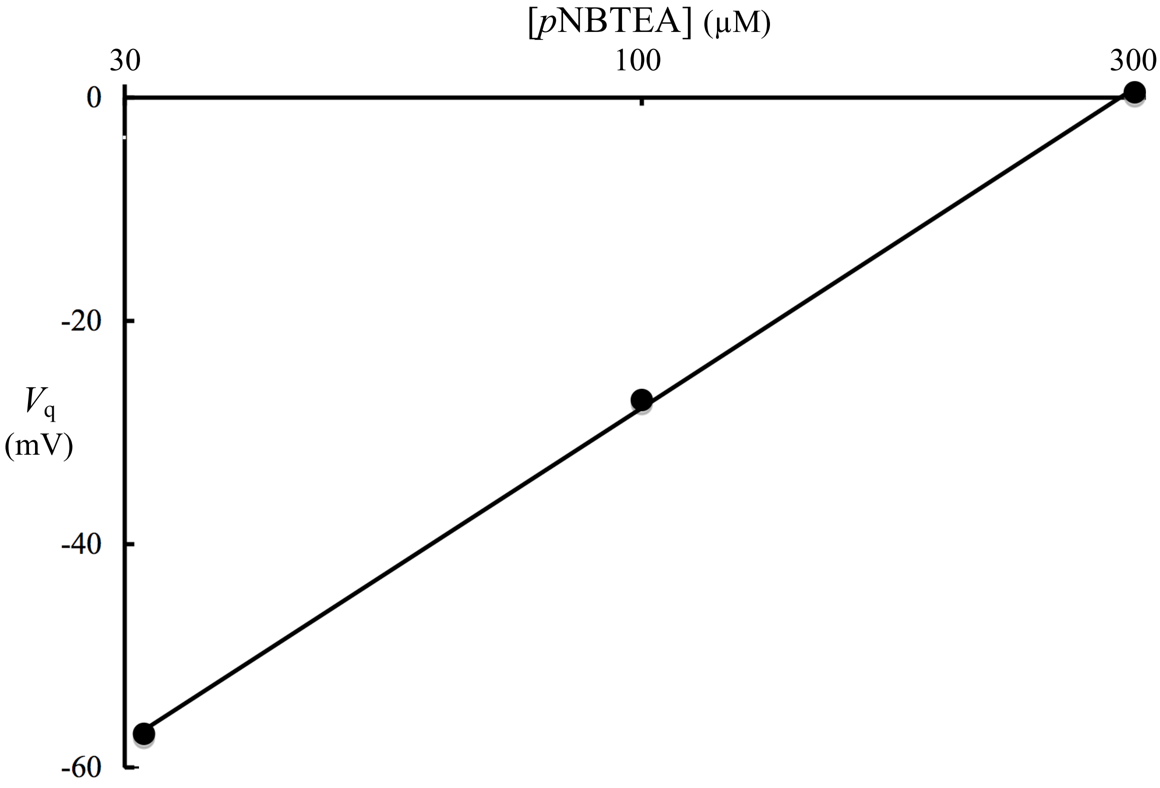
$q_{\mathrm{TOT}} = 15.5 \pm 0.4$ fC/pF
$K_{\mathrm{a1}} = 145{,}300 \pm 23{,}500\ \mathrm{M}^{-1}$
$K_2 = 0.20 \pm 0.02$
$K_{\mathrm{a2}} = 15{,}400 \pm 2400\ \mathrm{M}^{-1}$
$\lambda_Q = 0.64 \pm 0.03$

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

C. Comparison of the apparent affinity for *p*NBTEA 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 *p*NBTEA binds with an apparent affinity of 7 µM ($K_{\mathrm{D}} = 1/K_{\mathrm{a1}}$). By comparison, solving for the V_{M} dependence of charge kinetics with Scheme B suggested that the apparent affinity for *p*NBTEA binding was 33 µM. 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 *p*NBTEA concentration. If the binding affinity were actually 33 µM, then we should have observed a large increase in q_{TOT} between experiments using 33 and 300 µM concentrations of this amine. However, q_{TOT} was not significantly different at all concentrations tested, suggesting that binding affinity for *p*NBTEA is much less than 33 µM, the lowest concentration tested. Irrespective of the true value, it is clear that this simple molecule has a high enough inhibitory affinity that *p*NBTEA 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.