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Tetrapentylammonium block of chloramine-T and veratridine modified rat brain type IIA sodium channels

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Tetrapentylammonium (TPeA) block of rat brain type IIA sodium channel α subunit was studied using whole cell patch clamp. Results indicate that TPeA blocks the inactivating brain sodium channel in a potential and use-dependent manner similar to that of the cardiac sodium channel. Removal of inactivation using chloramine-T (CT) unmasks a time-dependent block by TPeA consistent with slow blocking kinetics. On the other hand, no time dependence is observed when inactivation is abolished by modification with veratridine. TPeA does not bind in a potential-dependent fashion to veratridine-modified channels and does not significantly affect gating of veratridine-modified channels suggesting that high affinity binding of TPeA to the brain sodium channel is lost after veratridine modification.
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Keywords: Sodium channels; veratridine; chloramine-T; tetrapentylammonium ions; gating mechanisms; kinetic analysis; patch clamp

Abbreviations: CT, chloramine-T; QA, quaternary ammonium; TAA, tetraalkylammonium; TEA, tetraethylammonium; TPeA, tetrapentylammonium; Vh, holding potential; VTD, veratridine

Introduction

Quaternary ammonium (QA) compounds have been extensively used as models for understanding local anaesthetic and antiarrhythmic drug action on sodium and potassium channels (e.g. Strichartz, 1973; Snyders & Yeola, 1995). Another fruitful use of these compounds has been as biophysical probes of the lining of the cytoplasmic vestibule of channels (e.g. Armstrong, 1966; Yellen *et al*., 1991; Stotz & Haynes, 1996).

Tetra-alkylammonium (TAA) compounds are pore blockers that bind to sodium channels with affinities correlated to increasing lengths of alkyl chains (Rojas & Rudy, 1976; O'Leary & Horn, 1994). These TAA compounds exhibit a gradation in their blocking kinetics. Small molecules like tetraethylammonium (TEA) block channels with kinetics that are immeasurably fast. Larger compounds like tetrapentylammonium (TPeA) are slow blockers with measurable blocking kinetics (O'Leary *et al*., 1994).

KIFMK, a synthetic open-channel blocking peptide, and VTD are mutually exclusive in binding to the Na^+ channel. QX-314 interacts with VTD-modified channels in a more complicated fashion (Ghatpande & Sikdar, 1997). Here we report a study of the interactions of VTD-modified channels with TPeA ions that belong to the class of QA blocking compounds, along with a study of interactions of TPeA with channels made inactivation-deficient with chloramine-T.

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Methods

Chemicals and reagents

All chemicals were purchased from Sigma. TPeACl (Aldrich) was a kind gift from Dr Richard Horn of the Institute of Hyperexcitability, Jefferson Medical College, Pennsylvania, U.S.A.

Whole cell voltage clamp, data acquisition, and analysis

CNa18 cells (Sarkar *et al*., 1995) stably expressing the RIIA sodium channel α subunit were maintained as described previously (Ghatpande & Sikdar, 1997). Cells were divided into 35-mm dishes, grown to 40–60% confluence and used for whole-cell patch-clamp recordings. Bath solution contained (mM) NaCl 137.5, HEPES 5, $MgCl_2$ 1, $CaCl_2$ 1.5. Osmolarity was maintained at 290 mosmol kg^{-1} by adjusting the glucose concentration. The pH was adjusted to 7.4 using NaOH. Chloramine-T (CT) was dissolved in water at a concentration of 10 mM. CT was directly added near the cell at a final concentration of approximately 30–50 μM, during the recording.

Appropriate amounts of 1 mM VTD stock solution, prepared using the bath solution, were added to the bath to give final concentrations of 100 or 200 μM. The pipette solution contained (mM): NaCl 137.5, HEPES 5, EGTA 5, and $CaCl_2$ 0.5, pH 7.4. Osmolarity was maintained at 290 mosmol kg^{-1} by adding CsF to the solution. TPeACl was dissolved in pipette solution to give a stock concentration of 10 mM. Appropriate amounts of this stock was added to the pipette solution to give final concentrations of 1, 20, 50 or 100 μM. Bath and pipette solutions were filtered through a 0.22 μm filter before use. Micropipettes were pulled from thin-walled, omega dot capillaries (Clarke Electromedical

Instruments) and had an average resistance of 1.15–1.25 MΩ. Average cell capacitance was 20 pF, as read from the slow capacitance meter of the EPC 7 patch clamp amplifier after series resistance compensation of 65–70%. To minimize series resistance effects we recorded from cells only if the uncompensated slow capacitative decay was around 200 μs. Data acquisition was as described previously (Ghatpande & Sikdar, 1997). Data were filtered at 2 kHz. Curve fitting and data plotting were done in WCP (John Dempster, Strathclyde) and SigmaPlot v 1.02. (Jandel Scientific, CA, U.S.A.). Results are described as mean ± s.e.mean.

The holding potential (Vh) was usually kept at −90 mV unless mentioned otherwise. Experiments done with TPeA in the pipette were recorded 15 min after going to the whole cell configuration to allow for diffusion. In experiments with CT-treated cells, TPeA effects were observable immediately after going to the whole-cell configuration, therefore, these recordings were done 1–3 min after going whole-cell. Depolarizing pulses were applied at a frequency of 1/30 Hz to minimize use dependence, except those shown in Figure 1, and those after CT treatment. Experiments where the depolarizing pulse frequency is other than 1/30 Hz, the respective frequency is mentioned in the figure legend.

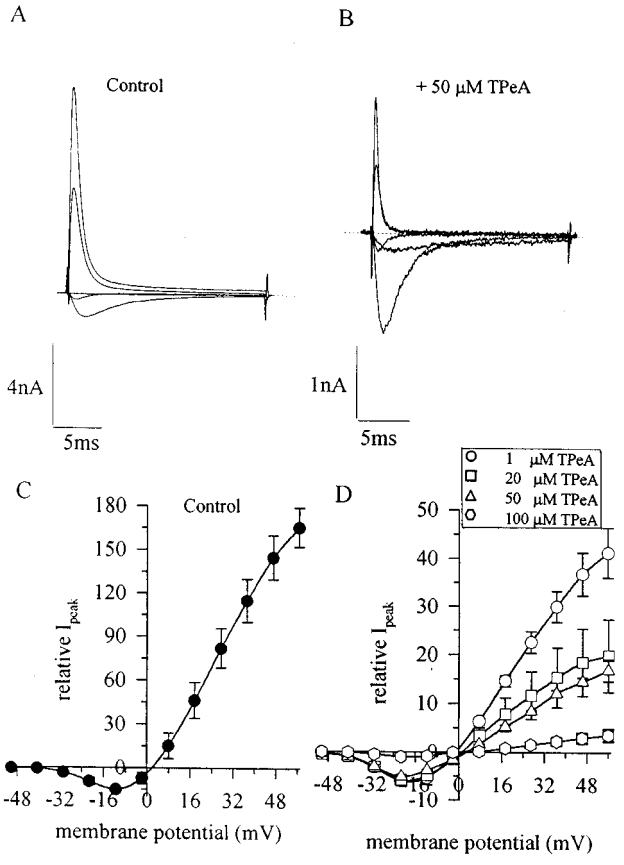

Figure 1 TPeA block of RIIA sodium channels. (A) Current traces recorded from a CNa18 cell in absence of TPeA. (B) Current traces with 50 μM TPeA in the pipette solution. In (A) and (B) currents are shown at representative potentials (mV) of −40, −20, −2, +17 and +36 of 20 ms duration each; Vh = −80 mV; (C) Control I–V relation. (D) Peak current-voltage relations of cells perfused for 15 min with 0, 1, 20, 50, and 100 μM TPeA in the pipette. Plots (C) and (D) are normalized with respect to current at −40 mV, since no use-dependence was obvious at that potential ($n=3$ each; $n=2$ for 100 μM TPeA). Depolarizing frequency, 1 Hz.

In experiments designed to measure TPeA block of VTD-modified channels, the transient currents in the pre-pulse show a use-dependent block by TPeA. This, along with intrinsic variation in VTD modification (±4%) makes the number of VTD-modified channels variable. This variability was corrected by dividing the current in the test pulse by the steady-state current within the associated pre-pulse. The rationale for this correction is as follows. Macroscopic current at any potential through VTD-modified channels is given by $I = N_v * p_o * i$, where N_v is the number of channels modified in each pulse; p_o is the open probability of the channel at that potential; i, is the single channel current. At potentials more depolarizing than −90 mV, the open probability of VTD-modified channels is close to one (Ghatpande & Sikdar, 1999). The number of channels (N_v) remains the same in the test pulse and its associated pre-pulse. Therefore, the ratio of macroscopic current amplitudes in the test pulse and pre-pulse gives a ratio of the single channel currents at the two potentials. The data was further scaled by taking current at −84.4 mV in the test pulse as one, i.e. assuming there is negligible block by TPeA of VTD-modified channels at −84.4 mV.

Analysis for the steady-state activation was done in the following manner. The tail currents (marked with an asterisk in Figure 4A) at the end of the hyperpolarizing voltage pulse were fit to a biexponential function of the type:

$$I(t) = a_1 * \exp(-t/\tau_1) + a_2 * \exp(-t/\tau_2) + c \qquad (1)$$

The sum $m = a_1 + a_2 + c$ was taken as a measure of the steady-state fraction of channels open at the end of the hyperpolarizing pulse. The normalized m was plotted against the hyperpolarizing pulse potential and fit with a Boltzmann function of the type:

$$f = 1/\{1 + \exp[(V_{1/2} - V)/k]\} \qquad (2)$$

where $V_{1/2}$ is the potential of half activation and k is a slope factor. Due to a fraction of channels remaining open at −170 mV, the steady-state activation showed a pedestal of 10%. We, therefore, fixed the value of m at −170 mV to zero.

Results

Effect of TPeA on RIIA sodium currents

TPeA has two obvious effects on macroscopic RIIA Na^+ currents: rate of current decay is increased along with suppression of peak current. Both effects are concentration and potential dependent. Figure 1A,B shows a family of sodium currents recorded from CNa18 cells with and without 50 μM TPeA in the pipette. TPeA speeds up current decay of RIIA channels and peak currents are suppressed. This suppression is more, at more depolarizing potentials as is evident from a comparison of inward and outward currents in Figure 1A, B. In our recording conditions, outward currents (at more depolarizing potentials) are much greater in magnitude with respect to inward currents in control. In presence of TPeA, the inward and outward currents become comparable in magnitude due to greater suppression of

current at more depolarized potentials. Inactivation is complete in presence of TPeA and there is no evidence of tail currents at the end of depolarizing pulses, indicating that blocked channels can inactivate. TPeA also produces a use-dependent block of RIIA Na^+ currents (data not shown).

Plots of peak I–V relations for control and various concentrations of TPeA are shown in Figure 1C,D. The I–V relations have been normalized with respect to current at −40 mV because no use and time-dependent block was evident at that potential. These I–V relations can be understood in terms of the properties of TPeA block described above. TPeA, at concentrations from 1 μM onwards, suppresses peak currents. The potential at which maximum inward current is seen shifts from −15 to −24 mV, indicating the block is more potent at −15 mV than at −24 mV. Reduction of peak currents with a shift in the peak of the I–V plots is consistent with a voltage-dependent block by TPeA. The drastic suppression of outward peak currents is expected if blocked channels have accumulated due to use-dependent block.

O'Leary *et al.* (1994) have hypothesized, based on similar effects on cardiac sodium channels, that TPeA might interact with the fast inactivation gate of the channel. We wanted to study this in detail by removing fast inactivation. We used CT (a mild oxidant) and VTD to remove fast inactivation and studied TPeA block on these inactivation-deficient channels.

Time-dependent block by TPeA of chloramine-T modified channels

A time-dependent block of channels lacking fast inactivation should be seen if TPeA is a slow blocker of RIIA sodium channels (Armstrong, 1971). Figure 2A shows a representative family of Na^+ currents recorded from a CNa18 cell treated with CT at Vh of −80 mV. CT treatment removed fast inactivation almost completely. At potentials more depolarizing than −30 mV some residual fast inactivation is visible.

Steady-state inactivation for RIIA channels is best described by a double Boltzmann fit with $V_{1/2}$ of −50 and −52 mV respectively (Sarkar *et al.*, 1995). At these potentials, about 50% of channels that remain unmodified even after CT treatment will be in an inactivated state. Since the number of channels with intact fast inactivation is very low after CT treatment, holding at −50 mV will effectively make the contribution of inactivating channels negligible. Figure 2B shows another representative family of currents recorded at a Vh of −50 mV. No inactivation is apparent at this holding potential indicating that contribution from channels with intact fast inactivation is negligible as expected.

Figure 2C,D show superimposed current traces recorded at −10 and 66 mV respectively from two cells each, one exposed to CT and the other with identical conditions but perfused internally with 10 μM TPeA. The Vh was kept at −50 mV in these recordings. The inward currents in Figure 2C show very little fast inactivation confirming that CT treatment indeed removes fast inactivation. No decay is apparent in current from cell perfused with TPeA. The outward current from the cell internally perfused with TPeA in Figure 2D shows a decay that has distinctly different kinetics from that of fast inactivation (compare Figure 1A to

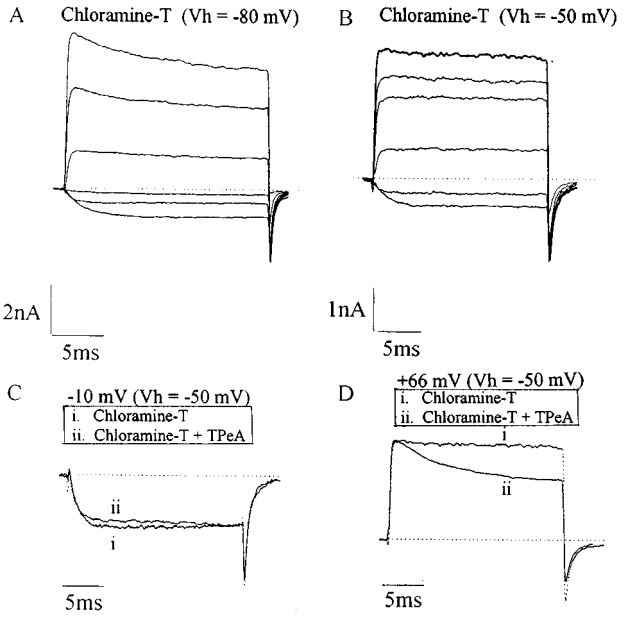

Figure 2 Time-dependent decay and use-dependence by TPeA in CT treated channels. (A) Family of current traces at representative potentials (in mV) of −48, −29, −10, +10, +29 and +48 of 20 ms duration each. Inactivation removed with 30 μM CT added to the bath solution. Vh = −80 mV. (B) Family of current traces at potentials (in mV), −20, −1, 18, +38, +47 and +67. Vh = −50 mV. Other conditions similar as above. (C) Superimposed normalized current traces recorded from CT treated control, and cell with 10 μM TPeA perfused inside + CT in the bath. Voltage pulse to −10 mV and Vh = −50 mV. (D) Superimposed normalized current traces recorded at voltage pulse to +66 mV, other conditions identical to (C). Depolarization frequency, 1 Hz.

Figure 2D) suggesting TPeA is a slow blocker of inactivation deficient RIIA channels and that TPeA block is potential dependent. The results shown in Figure 2 are typical of three similar and independent experiments done in different cells with similar results.

TPeA block of VTD-modified channels

To investigate whether TPeA also blocks VTD-modified channels in a time-dependent manner, VTD (100 or 200 μM) was added to the bath of cells perfused internally with TPeA. VTD modifies sodium channels in a use-dependent manner (Sutro, 1986; Ghatpande & Sikdar, 1997). In a single depolarizing pulse only a fraction of the total number of channels are modified, resulting in a partial removal of inactivation in macroscopic recordings. Therefore, a two-pulse protocol was used to study TPeA block of VTD-modified channels. As shown in Figure 3A, a pre-pulse to +30 mV lasting 45 ms was applied to modify sodium channels by VTD and to inactivate unmodified channels. Following the pre-pulse, a test pulse varying between −158.2 to 92 mV in 14.74-mV steps, lasting 10 ms, elicited currents through VTD-modified channels (alternate traces shown for clarity). At potentials up to −100 mV, the currents show deactivation. Beyond −100 mV, the current varies as a function of the driving force, equal to the membrane potential in this case. As seen in Figure 3B, no time-dependent block by TPeA is evident in a 10 ms pulse at a concentration 5 fold of that evoking a time-dependent block

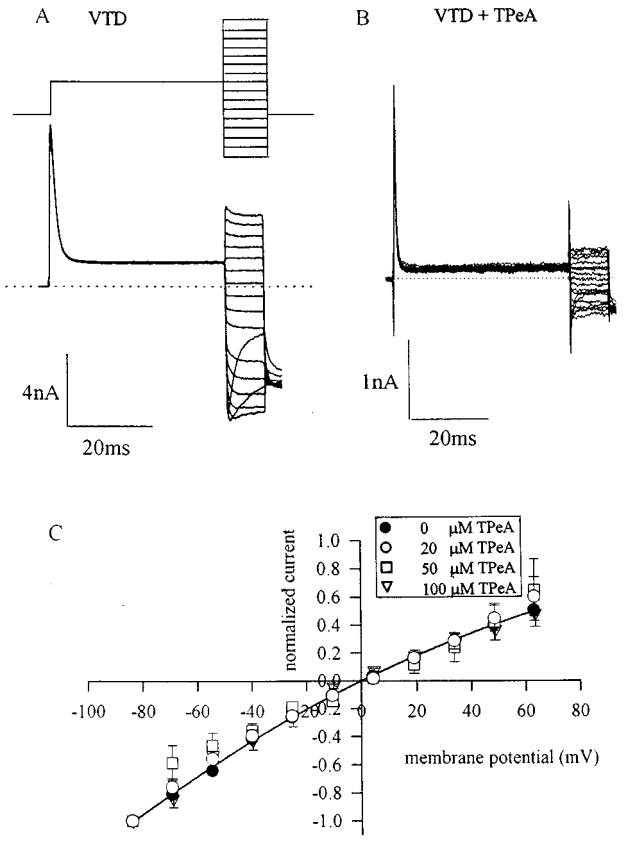

Figure 3 TPeA effect on VTD modified channels. (A) Two-pulse protocol from a cell equilibrated in 200 μM VTD. Vh = −90 mV. Pre-pulse to +30 mV for 45 ms followed by test pulses of 10 ms from −157.96 to +107.4 mV in steps of 14.74 mV. (B) Currents elicited from a cell equilibrated in 200 μM VTD and perfused internally with 100 μM TPeA for 15 min. Pulse protocol identical to that in (A). (C) I–V relations from cells equilibrated in 100/200 μM VTD and perfused internally with 0 (n = 3); 20 (n = 6); 50 (n = 5); and 100 (n = 3) μM TPeA for at least 15 min. Pulse protocol identical to that in (A) and (B). Data beyond 63 mV not shown.

in CT treated channels. Note that peak currents show accelerated inactivation and use-dependent block, indicating TPeA has reached the channel. We saw no time-dependent block by TPeA in an experiment with a 10-ms pre-pulse and a 45 ms long test pulse (data not shown). Lack of time-dependent block indicates that either TPeA dissociation has become rapid (i.e. VTD-modification of the channel makes TPeA a fast blocker) or, TPeA is unable to bind to VTD-modified channels. The transient currents in the pre-pulse show accelerated inactivation. Both the transient currents and the steady state currents within the pre-pulse, show use-dependent inhibition while the currents in the test pulse are markedly suppressed. Note the lack of time-dependent block of the steady state current in the pre-pulse. The use-dependence in the pre-pulse was corrected for as described in Methods, and normalized currents in the test pulse were plotted against test pulse potential. Plots of such I–V relations are shown in Figure 3C, for 0, 20, 50 and 100 μM TPeA. The I–V relations for all concentrations superimpose indicating that TPeA is unable to block VTD-modified RIIA sodium channels in a potential-dependent manner, at concentrations 100 fold higher than that required for block of unmodified channels. These results indicate two possibilities: (1) TPeA blocks VTD-modified channels through a fast but voltage-independent mechanism. This type of block will not be apparent in the experiments of Figure 3B and the plots in Figure 3C since in that analysis it is assumed that there is negligible block at −84.4 mV; or (2) TPeA is unable to bind to VTD-modified channels.

If TPeA binds to VTD-modified channels by a fast, voltage-independent mechanism, its effects might be apparent on the gating of these channels. Therefore we tested this possibility by studying the gating of VTD-modified channels in the presence of TPeA.

Gating of VTD-modified sodium channels in presence of TPeA

Activation and deactivation gating is modified profoundly by VTD (Leibowitz *et al*., 1986; Ghatpande & Sikdar, 1999). Figure 4A shows deactivation of VTD-modified currents in presence of 20 μM TPeA recorded using a two-pulse protocol as shown. VTD-modified channels deactivate with a potential dependence and reach a steady state. Equation 1 was fit to the tail current at the end of the deactivating pulse and the fit-amplitude was used to estimate the number of channels open at the end of the pulse. The normalized data is plot in Figure 4B. The data was fit with Equation 2 and the parameters estimated from the fit were $V_{1/2}$ = −133 mV and slope k = 8.8 mV. In presence of 20 μM TPeA the parameters were $V_{1/2}$ = −130 mV and k = 9.8 mV.

The kinetics of deactivation was analysed in presence of TPeA as described in Methods. Deactivation of VTD-modified RIIA channels is biexponential (Ghatpande & Sikdar, 1999). Figure 4C shows the plots of amplitude factors, steady state current and time constants as a function of potential. Superimposed are the values of these parameters taken from data recorded in the absence of TPeA. Most of the parameters are well superimposed on each other, but the slow time constants of deactivation appear to be faster in presence of TPeA. This is a puzzling result and the explanation is not obvious. We cannot be sure whether this is an effect of TPeA or just an apparent effect arising out of poor resolution of the slow component.

The fast component is well resolved and no effect of TPeA is seen as judged by kinetic as well as equilibrium data. Since TPeA at concentrations 100 fold of that producing use-dependent block in unmodified channels has no effect on deactivation kinetics of the fast, voltage-dependent, major component of VTD-modified channels, it seems reasonable to infer that TPeA cannot bind to this component.

Discussion

An earlier study by O'Leary *et al*. (1994) investigated in detail TPeA block of cardiac sodium channels with intact inactivation. Our results with TPeA, shown in Figure 1, on RIIA currents with intact inactivation are quite similar to theirs and indicate that TPeA is a slow, potential dependent blocker of RIIA channels.

The rate of current decay increases with TPeA in the pipette and at depolarizing potentials. This increase in rate of current decay suggests that the on rate of TPeA increases at more depolarizing potentials with the off rate being slow.

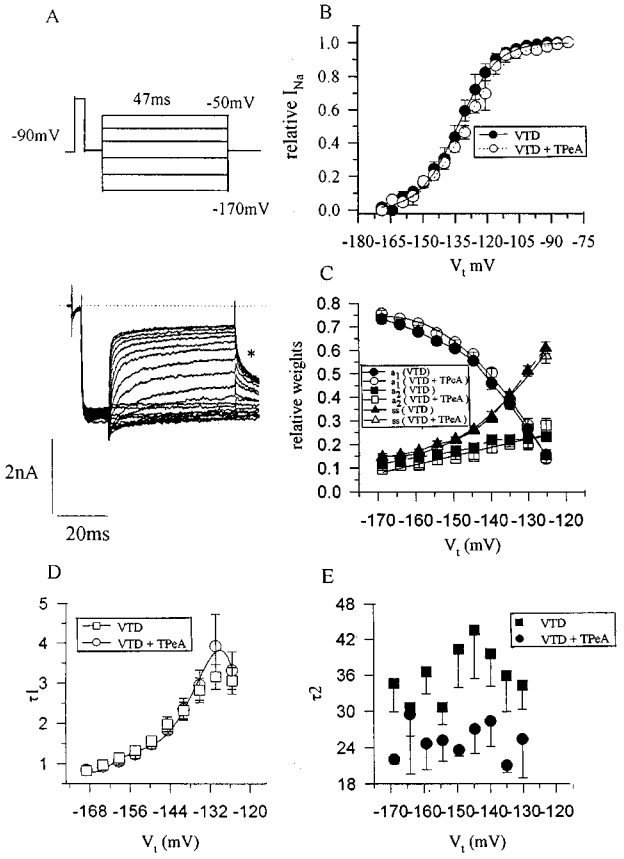

Figure 4 Gating of VTD-modified channels in presence of TPeA. (A) Deactivation. Cell equilibrated in 200 μM VTD and perfused internally with 20 μM TPeA for >15 min. Vh = −90 mV; Prepulse to 0 mV for 4 ms was followed by a hyperpolarization to Vh for 10 ms. Test pulses varying from −168.9 to −72 mV in 4.8-mV steps of 50 ms duration were applied after the step to holding potential. The channels deactivate till −90 mV as seen, at more depolarizing potentials the currents vary with the driving force and are seen as overlapping steady-state currents on the deactivating current traces. The tail currents (asterisk) at the end of the test pulses were fit by double exponential functions to estimate the steady-state activation of channels. (B) Voltage dependence of steady-state activation of VTD-modified channels in absence and presence of 20 μM TPeA. Each point calculated as described in Methods ($n=3$ each). Data corrected for persistent current remaining at −169 mV. Line through data points is Boltzmann function of Equation 2 with $V_{1/2}$ = −133 mV (control); −130 mV (TPeA) and slope factors $k=8.8$ mV (control) and $k=9.8$ mV (TPeA). (C) Plot of amplitude factors, fast a_1, slow a_2, and steady-state current, ss, from double exponential fits of deactivating currents, as a function of membrane potential, for control (VTD) and VTD + TPeA. (D) Plot of fast time constant $\tau 1$ as a function of membrane potential obtained from double-exponential fit to data in Figure 3, for VTD and VTD + TPeA ($n=3$). (E) Plot of slow time constant $\tau 2$ as a function of membrane potential for VTD and VTD + TPeA ($n=3$).

This gives rise to a time-dependent block of the current that adds to the intrinsic inactivation thereby increasing the rate of current decay. The absence of tail currents suggests that blocked channels might inactivate unlike other inactivation simulating drugs like thiazin dyes (Armstrong & Croop, 1982). The observations described above suggest a slow, open channel block by TPeA. Evidence for the pore blocking nature of intracellular TPeA is well documented for channels as diverse as neuronal chloride channels (Sanchez & Blatz, 1995), cyclic GMP-gated cation channels (Stotz & Haynes, 1996), cardiac sodium (O'Leary *et al*., 1994), and potassium channels (Snyders & Yeola, 1995).

O'Leary *et al*. (1994) proposed a three-state model involving block from the open state leading to an absorbing inactivated state. Based on this model they have calculated voltage-dependent on and off rates for TPeA and have proposed that use-dependence due to TPeA arises by a direct interaction of TPeA with the inactivation gate of the channel.

CT is known to remove fast inactivation of Na^+ currents (Wang *et al*., 1987). Our results demonstrate that TPeA produces a time-dependent block of such inactivation-deficient channels at high depolarizing potentials and indicates that it is a slow blocker with a potential dependent on rate. This behaviour is best explained as a pore block by a charged impermeant ion. These results indicate that CT treatment does not modify the QA ion-binding site of the channel. In contrast, the lack of a potential-dependent and concentration dependent block of VTD-modified RIIA channels indicates that the TPeA binding site is modified by VTD.

To summarize, our data shows that TPeA blocks CT treated inactivation deficient channels with slow blocking kinetics along with use-dependence whereas TPeA does not seem to bind to VTD modified channels either in potential or time dependent manner. The latter result seems surprising given that QX-314 and VTD do not seem to be mutually exclusive (Ghatpande & Sikdar, 1997). QX-314 and TPeA differ in the length of their alkyl chains and the presence of an aromatic ring. Work done earlier to determine structural determinants of quaternary ammonium and amine blockers (Wang *et al*., 1993; Zamponi & French, 1994) shows increased blocking affinity due to presence of an aromatic ring in these blockers. Wang (1990) suggested that there are two separate hydrophobic domains, one binding aromatic rings and the other interacting with amino terminal alkyl chains of local anaesthetics. The presence of such distinct sites may be responsible for the seemingly contradictory behaviour of QX-314 and TPeA on VTD-modified channels. Similarly, N-propyl ajmaline, another aromatic QA compound, blocks both normal and VTD-modified channels in a time and use-dependent fashion (Khodorov & Zaborovskaya, 1983). These findings support the existence of two distinct sites within the pore, one of which binds to aromatic compounds and another binding to alkyl chains of QA compounds and local anaesthetics.

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