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Competition for Binding Between Veratridine and *KIFMK:* An Open Channel Blocking Peptide of the RIIA Sodium Channel

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Abstract. Veratridine, an alkaloid isolated from the rhizome of *V. album,* binds and slows the inactivation of the brain sodium channels. The synthetic pentapeptide *KIFMK* causes a voltage- and use-dependent open-channel block of the RIIA (rat brain type IIA) sodium channel (Eaholtz, Scheuer & Catterall, 1994). Our studies on the RIIA sodium channel expressed in CHO cells reveal that the fraction of veratridine modified sodium channels decreases linearly with increasing *KIFMK* concentration. However, the time constant for dissociation of veratridine from the channel remains unchanged in the presence of a high concentration of *KIFMK,* as opposed to that in the presence of QX314 where the dissociation appears to be more complex. These data are consistent with mutually exclusive binding of the open channel blocking peptide and veratridine to the brain sodium channel.

Key words: Veratridine — *KIFMK* — sodium channel — binding competition — Site 2 Receptor — Whole cell patch clamp

Introduction

Veratridine (VTD) is an alkaloid isolated from the rhizome of *V. album* or the seeds of *S. officinale.* VTD and other lipid-soluble ''alkaloid'' neurotoxins like batrachotoxin, aconitine and grayanotoxin are known to bind to the pharmacological site 2 (Catterall, 1980) on the sodium channel. On binding of VTD, the inactivation is slowed down, activation becomes exponential, shifts towards hyperpolarized potentials (Ulbricht, 1969; Sutro, 1986), and channel selectivity is altered (Scruggs & Landowne, 1978). The location of site 2 in relation to the structure of the sodium channel remains unknown although it is important from the structure-function point of view since it influences almost all the electrophysiological properties of the sodium channel (Strichartz et al., 1987; Brown, 1988). More recently it has been shown that VTD acts from the cytosolic side of the membrane and the active species is the protonated form of VTD (Honerjäger, Dugas & Zong, 1992).

KIFMK, a synthetic pentapeptide, and QX314 are open channel blockers of the sodium channel with distinct modes of action. The pentapeptide *KIFMK* has been shown to cause a voltage- and use-dependent open-channel block (Eaholtz et al., 1994; Tang, Kallen & Horn, 1996) of the rat type IIA sodium channel but has to dissociate before the channel can inactivate or close. QX314 binds deep inside the pore (Schwarz, Palade & Hille, 1977) and gets trapped within inactivated channels. These distinct modes of action imply distinct binding sites within the pore and we have used these open channel blockers to probe the binding of VTD to the channel.

In this paper we present evidence to show that VTD and the peptide compete for binding to the channel.

Materials and Methods

Cell Culture and Solutions

CHO cells stably expressing rat brain type IIA sodium channel α subunit, called CNa18 cells (Sarkar & Sikdar, 1994) were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 HAM mixture supplemented with 8% fetal bovine serum in the presence of 300 μg/ml G418 antibiotic in a humidified 5% CO_2 environment. These cells were split into 35-mm dishes, grown to 40–60% confluency and used for whole-cell patch clamp recordings. Bath solution contained (in mM): 52 NaCl (Figs. 1 and 5), 85 NaCl (Figs. 2–4), 85 choline chloride (Figs. 1 and 5), 52 choline chloride (Figs. 2–4), 5 HEPES, 1 $MgCl_2$, 1.5

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$CaCl_2$, 10 glucose. pH was adjusted to 7.4 using NaOH. Pipette solution contained (in mM): 12 NaCl, 130 CsCl, 5 HEPES, 5 EGTA, and 0.5 $CaCl_2$. For Fig. 5, the pipette solution contained 72.6 CsF, 48.4 CsCl, 10 HEPES, 5 EGTA and 0.5 $CaCl_2$; 1 mM stock VTD solution was made in the bath solution by acidifying to dissolve VTD and raising the pH back to 7.4 (Leibowitz, Sutro & Hille, 1986). VTD concentration was checked by $A_{263\,nm}$ (McKinney, Chakraverty & De Weer, 1986). QX314 was generously gifted by Dr. Rune Sandberg of Astra Pain Control, Sweden. 1 mM QX314 solution was made in the pipette solution used for Fig. 5. HPLC purified peptides were provided by A.J. Northrop (Cambridge, UK). The peptides were dissolved in the pipette solution. All chemicals except the peptides and QX314 were obtained from Sigma. Bath and pipette solutions were filtered through a 0.22 μm filter before use.

Data Acquisition and Analysis

Pipettes were pulled from thin-walled, omega-dot capillaries (1.5 mm o.d., Intracel, Royston, Herts, UK) in a two-stage pulling procedure and then fire polished. Pipettes filled with the pipette solution had resistances from 1.5–3MΩ. The pipette tip was filled with a small amount of blocker-free solution to promote seal formation and subsequently with the blocker (peptide or QX314) solution. After establishing the whole-cell mode, cells were voltage clamped to −80 mV using a List EPC-7 patch clamp amplifier. Capacitance and series resistance compensation (up to 70%) was done using built-in circuits of the EPC-7 amplifier. Application of pulse protocols, leak correction and data analysis were done using the WCP program provided by John Dempster (University of Strathclyde, UK) and by modified WCP programs developed in our laboratory.

For Figs. 1*a* and 5*a*, cells were held at −80 mV and depolarized to −3 mV for 20 msec. Leak records were collected by hyperpolarizing to −117.5 mV. Four test and 10 leak records were collected. For Fig. 1*b*, cells were held at −80 mV, a depolarizing pulse to −5 mV was applied for 1 msec and then held at −100 mV for 5 msec, the data were sampled at 20 μsec. For Figs. 2 and 3, cells were held at −80 mV and two depolarizing pulses to −5 mV, of 5 msec duration separated by 100 msec, were given. Five leak records were collected using hyperpolarizing pulses to −117.5 mV, after each test record. The cells were initially bathed in a 25 μM VTD solution and the first data point recorded. Another aliquot of a 1 mM VTD solution was added to the bath to achieve the desired concentration and the next data point recorded after 5 min. All the data points were recorded similarly. Data were filtered at 3kHz and sampled at 16kHz. It was digitized using a CED 1401 A/D D/A converter and stored in a PC-AT 286 computer.

For Figs. 4 and 5 the dissociation time constant was measured by holding the cell at −80 mV and giving a series of 30 depolarizing pulses of 5 msec to −5 mV at a frequency of 25 Hz and then recording the decay of the tail current at a sampling frequency of 400 Hz. Each record contained 4096 data points which gave a total record length of 10 sec. The decaying tail current was fitted to a monoexponential function of the form $y(t) = A\ exp\ (-t/\tau_{dissociation}) + b$ using the Levenberg-Marquardt algorithm. The sodium channel block by QX314 was removed by applying a train of 20-msec pulses to −3 mV preceded by a 50 msec prepulse to −140 mV at 0.5 Hz before the experiments shown in Figs. 5*a* and *b* commenced (Schwarz, Palade & Hille, 1977). All experiments were carried out at 15°C. Temperature was maintained using a TC-202 bipolar temperature controller (Medical Systems, USA).

Statistics

Points plotted and numerical values are given as mean ± SEM, with *n* being the number of experiments. The statistical significance of changes were evaluated with an unpaired Student's *t*-test, with probability, *P* which is the probability of being incorrect in stating that the two means are different.

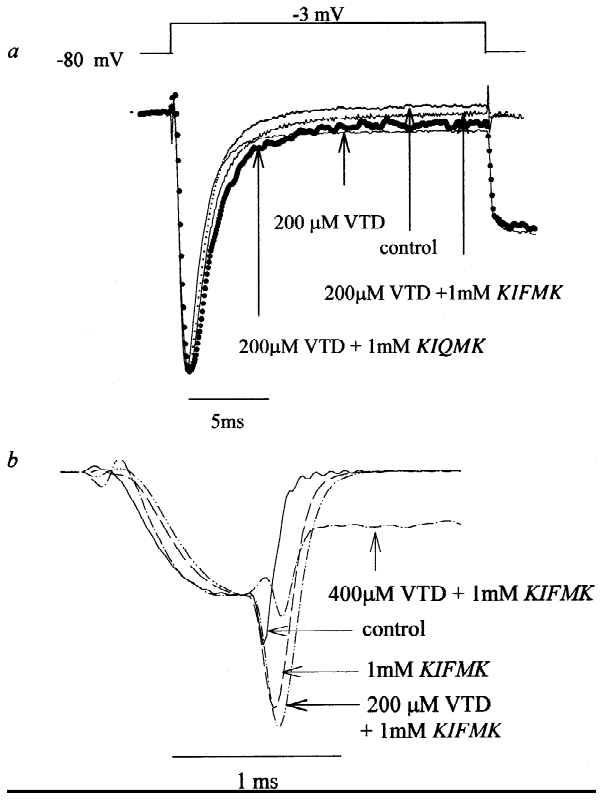

Fig. 1. *KIFMK* alters VTD modification of sodium channels. Normalized whole-cell recording of sodium currents (*a*) and tail currents (*b*) in the presence of VTD (*a*, 200 μM; *b*, 200 μM; 400 μM) in bath and *KIFMK* (1 mM) in pipette. (*a*) The currents with VTD and *KIQMK* show a steady-state current within the pulse and a large ''steady-state'' tail current at the end of the pulse. With 1 mM *KIFMK* in the pipette the current is qualitatively similar to native channels (control) and the tail current is absent. (*b*) With 1 mM *KIFMK* in the pipette a ''hooked'' tail current is seen. On addition of 400 μM VTD a second ''steady-state'' component is seen in the tail current. Currents are normalized with respect to the peak sodium current. The traces are recordings obtained from different cells.

Results

Figure 1*a* shows normalized sodium current traces from CNa18 cells, (Sarkar & Sikdar, 1994; Sarkar, Adhikari & Sikdar, 1995). With 200 μM VTD there is an increased steady-state current within the depolarizing pulse and a large tail current at the end of it as compared to the native channel current. With 1 mM *KIFMK* (acetyl-*KIFMK*-amide) in the pipette and VTD in the bath, the large tail current at the end of the pulse is not seen. A large and sustained tail current similar to VTD alone was seen when 1 mM of the ''mutant'' peptide *KIQMK* (Eaholtz et

al., 1994), which has a glutamine instead of phenylalanine, was used. Figure 1*b* shows a prominent ''hooked'' tail current in the presence of 1 mM *KIFMK* with a distinctly slower time course compared to the control trace. On addition of 400 μM VTD there is an appearance of a ''steady-state'' component in the tail current which is not apparent at an intermediate concentration (200 μM) of VTD. The tail current amplitude is variable in these experiments but cannot be emphasized since it varies in untreated cells too. These experiments suggest that *KIFMK* may prevent the binding of VTD to the channel as shown in scheme I below. Alternatively, *KIFMK* might restore inactivation to VTD modified channels.

$$\begin{array}{c} O_v \\ [\mathrm{VTD}]\ k_1 \uparrow\downarrow k_{-1} \\ C \leftrightarrow O \leftrightarrow I \\ [KIFMK] \downarrow\uparrow \\ O_I \end{array} \qquad \text{(Scheme I)}$$

Here, C, O, I are the closed, open and inactivated states of the channel, O_v is the VTD modified open state and O_I is the open blocked state with *KIFMK* bound to the channel.

This scheme assumes that VTD binds only to open channels (Sutro, 1986, Barnes & Hille, 1988) and predicts that k_1, i.e., the association rate constant for VTD should decrease as a function of increasing concentration of *KIFMK* according to the equation:

$$k_1' = k_1(1+[KIFMK]/K_I)^{-1} \qquad (1)$$

where K_I is the inhibition constant for *KIFMK*. The second prediction of the scheme is that the dissociation rate constant for VTD, k_{-1}, should remain unchanged in presence of *KIFMK*.

The modification of sodium channels by VTD was measured using a protocol developed by Leibowitz et al. (1986) as shown in Fig. 2*a*. The steady-state tail current remaining after the depolarizing pulse is directly proportional to the number of VTD-modified channels and the difference I_{ss} after the second, and I_i after the first pulse is therefore a measure of the number of channels modified by VTD in one pulse. Figure 2*b* shows this measure of the number of channels modified by VTD (I_{ss}–I_i) in each pulse normalized with the peak current amplitude, I_{pk}, at different concentrations of VTD. The linear rise in the fraction of modified channels at increasing concentrations of VTD indicates that VTD binds only to open channels and modification can be equated to binding.

Figure 3*a* shows the fraction of VTD-bound channels at two different fixed concentrations of *KIFMK*. The fraction of VTD-bound channels in each pulse still increases linearly but with a decreased slope at 400 μM *KIFMK* compared with Fig. 2*b* and VTD binding is almost completely inhibited at 1 mM *KIFMK*. Figure 3*b* shows the ''rate'' constant of VTD binding as measured by the parameter (I_{ss}–I_i) /I_{pk}, at various concentrations of *KIFMK* which decreases linearly as *KIFMK* concentration is increased. This observation is qualitatively consistent with the first prediction of scheme I.

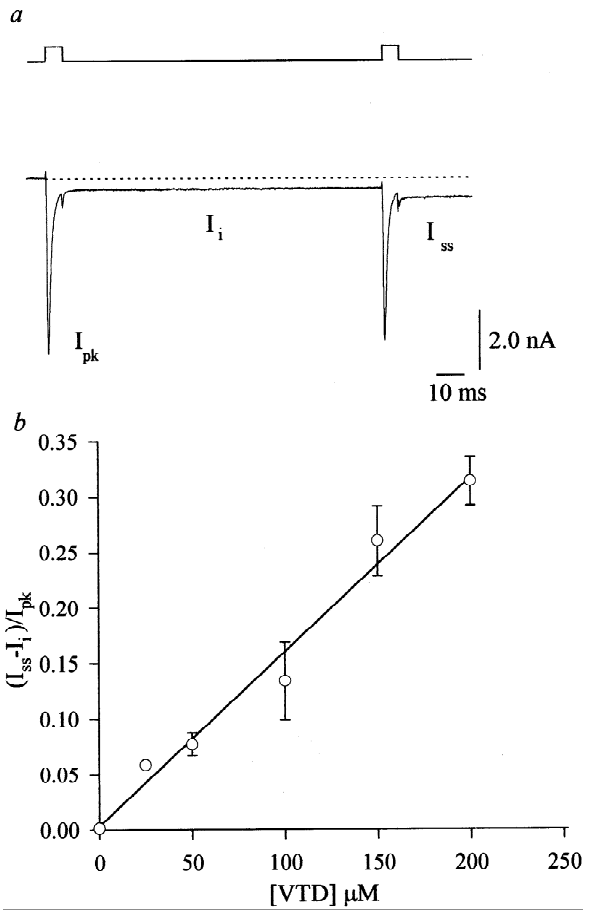

Fig. 2. Binding of VTD as measured by increase in ''steady-state''current (I_{ss}–I_i/I_{pk}) per pulse. (*a*) two depolarizing pulses to −5 mV of 5 msec duration, separated by 100 msec, were applied and the three parameters I_{pk} (peak current), I_i (tail current after first pulse) and I_{ss} (tail current after second pulse) as shown were measured. (VTD 200μM; *KIFMK* 800μM) *b*, the parameter (I_{ss}–I_i)/I_{pk} increases linearly with VTD concentration indicating that VTD binds only to open channels. Error bars (where bigger than the symbol) show standard errors of mean, n = 2–3 cells. The correlation coefficient r for the linearly regressed line is 0.98.

To test the validity of scheme I, we also measured the dissociation time constant of VTD in the presence and absence of *KIFMK* as shown in Fig. 4. The decaying phase of the current following accumulation of VTD-bound channels during the pulse train, is due to the dissociation of VTD and subsequent channel closure (Sutro, 1986). The currents elicited during the pulse train merge into one another at this sampling frequency. Dissociation time constants in absence of the peptide were re-

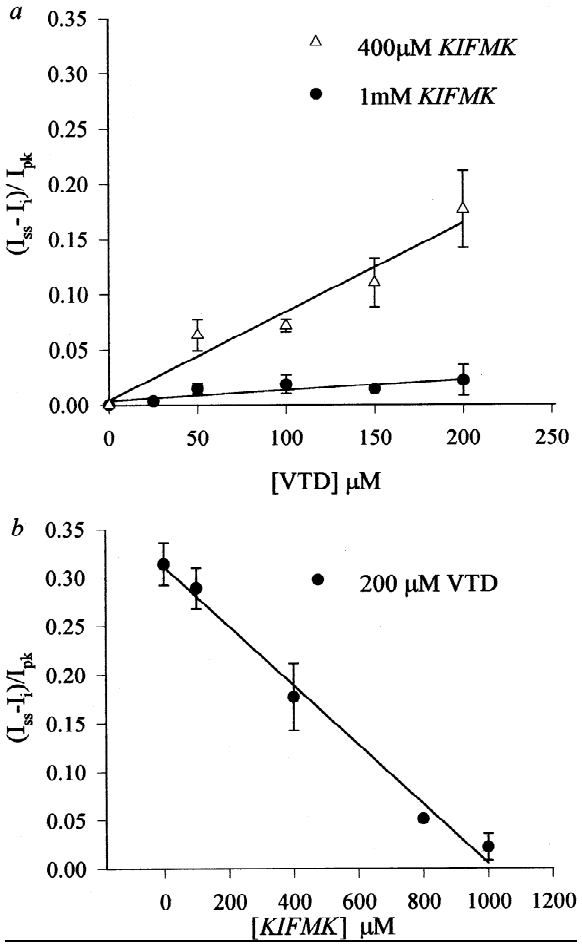

Fig. 3. Effect of *KIFMK* on VTD binding. (*a*) with 400 μM or 1 mM *KIFMK* present in the pipette, the binding increased linearly with VTD concentration but with decreased slopes. Standard errors of the mean are shown as bars, where bigger than the symbol, $n = 3$ cells for 400 μM *KIFMK*, correlation coefficient $r = 0.96$; $n = 1$–3 cells for 1 mM *KIFMK* and $r = 0.84$. (*b*) Plot of the fraction of channels modified by VTD *vs. KIFMK* concentration. Standard errors of the mean are shown as bars except for the point at 800 μM *KIFMK* which is from a single cell, for the other points $n = 3$ cells each and $r = 0.99$.

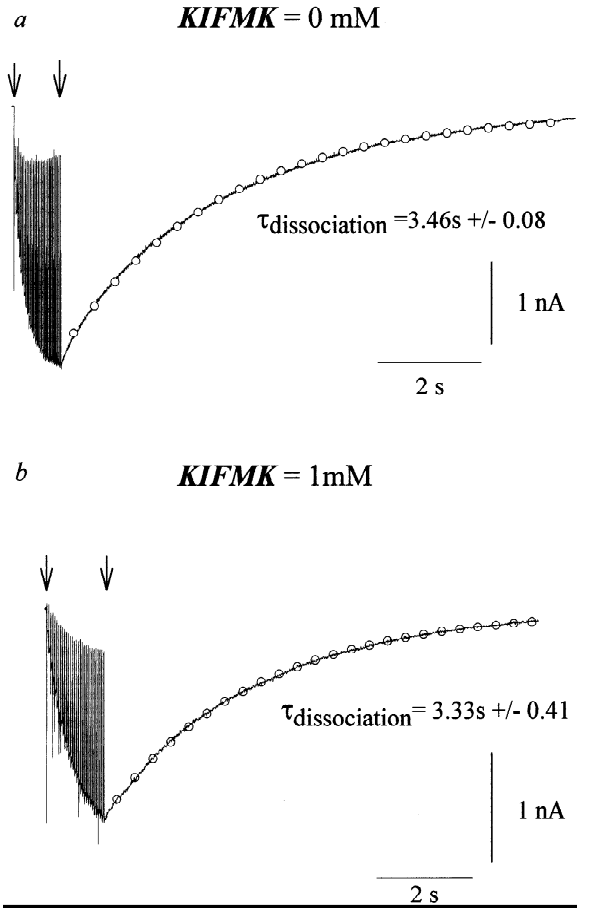

Fig. 4. Effect of *KIFMK* on the dissociation time constant for VTD. Dissociation time constant was measured by holding the cell at −80 mV and giving a series of 25–30 depolarizing pulses of 5-msec duration to −5 mV at a frequency of 25 Hz and then recording the decay of the tail current at a sampling frequency of 400 Hz. The currents elicited during the pulse train (indicated by arrows) merge into one another at this sampling frequency. The monoexponential fit is shown as hollow circles (one out of every 150 points are shown) superimposed on the decaying current. The fit did not improve using a double exponential function (not shown). Dissociation time constant ($\tau_{dissociation}$) in the absence of peptide, *a, (KIFMK* = 0 mM, $n = 9$) and *b*, 1 mM *KIFMK*, $n = 3$ obtained from the monoexponential fit for representative traces are shown. The bath contained 200 μM VTD (*see results*).

corded for nine cells with the VTD concentration varying from 5 to 200 μM (mean = 3.46 sec ± 0.08) since the dissociation is independent of VTD concentration (Sutro, 1986). The dissociation time constant in presence of 1 mM *KIFMK* was 3.33 sec ± 0.41 ($n = 3$) and at 400 μM *KIFMK* was 3.25 sec ± 0.11 ($n = 2$). In presence of 1 mM *KIFMK* the amount of binding of VTD as measured by the two pulse protocol (Fig. 2) is small but the modified channels accumulate in the train of pulses (Fig. 4) to give rise to a substantial tail current at the end of the train. The competition for binding between VTD and *KIFMK* is reflected in the number of pulses required to saturate the channels in the cells with VTD as can be seen in Fig. 4. The differences in the dissociation time constants in the presence and absence of the peptide are insignificant as judged by the unpaired Student's *t* test $P = 0.63$, deg. of freedom = 10 for 1 mM *KIFMK* and $P = 0.32$, deg. of freedom = 9 for 400 μM *KIFMK*. Thus, the dissociation rate of VTD remains unchanged at the highest concentration of *KIFMK* used, showing that *KIFMK* does not affect the dissociation reaction of VTD from the channel.

We used QX314, a charged quaternary ammonium analogue of lidocaine, to examine the effects of another open-channel blocker on the binding and dissociation of VTD. Hyperpolarizing prepulses were used to remove initial QX314 block for this experiment as described in the Data Acquisition and Analysis section. Figure 5*a*

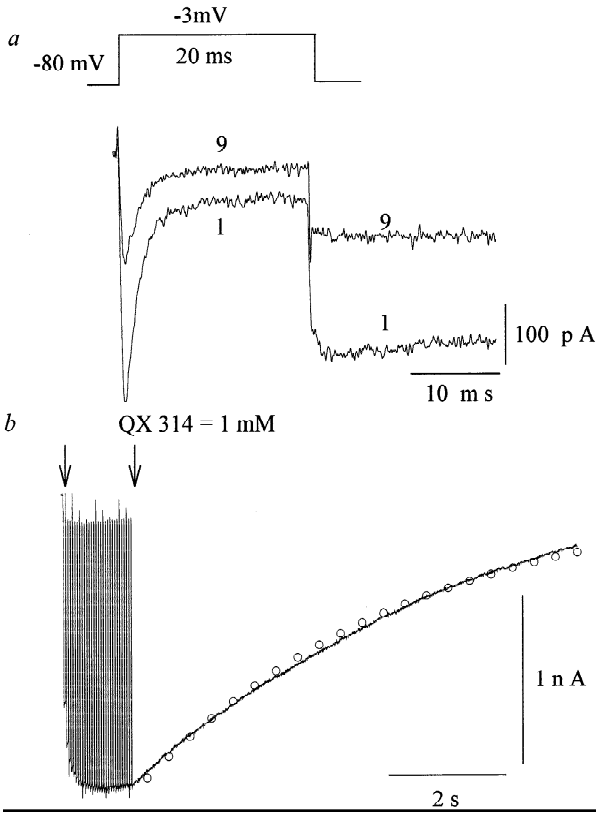

Fig. 5. Effect of QX314 on the binding (*a*) and dissociation of VTD (*b*). (*a*) In the presence of 1 mM QX314 in pipette the peak sodium currents and ''steady-state'' tail currents show a decrease in subsequent pulses. (*b*) Dissociation of VTD as measured by the protocol in Fig. 4. The monoexponential fit shown as hollow circles (one out of every 150 points) superimposed on the decaying current does not describe the decay well ($n = 3$).

shows currents recorded with 1 mM QX314 in the pipette and 200 μM VTD in the bath. In contrast to experiments with *KIFMK*, the transient peak current and the ''steady-state'' tail current in the first record are similar to that with 200 μM VTD alone but decrease with subsequent pulses (*see* record 1 and 9, Fig. 5*a*). Figure 5*b* shows the dissociation of VTD in presence of 1 mM QX314. The saturation of the channels with VTD occurs in fewer pulses as compared to VTD alone and VTD with 1 mM internal *KIFMK*. The decay of the current is not fit satisfactorily by a monoexponential or biexponential (*not shown*) function indicating that the dissociation of VTD is affected by QX314 in a complex manner.

Discussion

Figure 1*a* argues for the specificity in the competition between VTD and *KIFMK* even at the high concentrations of peptide used, since the control peptide *KIQMK* is unable to reverse the effects of VTD. It also suggests that charge repulsion between protonated VTD and the lysines of *KIFMK* is not the mechanism of the competition seen in our data. The ''hooked'' tail currents seen in the presence of *KIFMK* (Fig. 1*b*) indicate that the peptide blocks the channels in the open state and has to dissociate before the channel closes. Increasing the concentration of VTD to 400 μM results in the appearance of the ''steady-state'' tail current characteristic of VTD-modified channels. This is consistent with scheme I.

Contrary to scheme I if VTD were to bind to closed channels, then the parameter $(I_{ss}-I_i)/I_{pk}$ used in Fig. 2 should show a saturation at higher concentrations of VTD. As seen in Fig. 2, $(I_{ss}-I_i)/I_{pk}$ does not saturate at VTD concentrations well beyond the reported K_d of 0.51 μM (Wang et. al., 1989) for VTD. This result reproduces that of Leibowitz et al. (1986) and validates the assumption of scheme I that VTD binds only to open channels. It also gives us a measure of the association rate of VTD. The true second order association rate constant of VTD should decrease quantitatively with increasing peptide concentration as shown in Eq. 1. Unfortunately, because of the limitations of using the whole-cell mode we are unable to determine the true second order association rate constant for VTD but the linear decrease in $(I_{ss}-I_i)/I_{pk}$ as peptide concentration increases is qualitatively consistent with Eq. 1.

The time constant of decay of the VTD induced tail current after a train of depolarizing pulses in presence of 1 mM *KIFMK* and in absence of the peptide does not differ significantly (*see results*). The decay of the VTD-induced current is well described by a monoexponential fit in three independent experiments at 1 mM *KIFMK* and two experiments at 400 μM *KIFMK*. The monoexponential fit of the decay indicates *KIFMK* does not bind to VTD-bound channels, which is expected to give rise to a double exponential decay of the current. The second prediction of scheme I is thus directly confirmed.

Although a large number of sodium channel blockers like local anesthetics are known to inhibit batrachotoxin binding to site 2 (Postma & Catterall, 1984) they all do so by increasing the rate of dissociation of the alkaloid neurotoxin. This has been interpreted to mean an indirect allosteric mechanism of inhibition by binding of the local anesthetic to a site distinct from site 2. In the presence of 1 mM QX314 the transient peak and the ''steady-state'' tail currents decrease with subsequent pulses. QX314 is known to bind to open channels and get trapped within the channel as it inactivates. This gives rise to the apparent use-dependent decrease in peak currents. Therefore, if QX314 and VTD binding were mutually exclusive, fewer channels would be available for VTD binding leading to the decrease in ''steady-state'' tail currents. Alternatively, QX314 could bind to VTD modified channels. The dissociation of VTD is affected in presence of QX314 thus ruling out mutually

exclusive binding to the channel. *KIFMK* seems to inhibit VTD binding by reducing the on rate without affecting the off rate indicating that *KIFMK* and VTD bind in a mutually exclusive fashion to the sodium channel. It does not mean that they bind to the same site although it is one of the possibilities. The other possibility is that *KIFMK* binds to the channel and alters it's conformation such that VTD is unable to bind to the channel. We have been unable to measure the association rates for the peptide or VTD and are therefore unable to distinguish between these two possibilities. In conclusion, we feel that our data is consistent with Scheme I but noncompetitive binding cannot be excluded at this point.

One hypothesis about the location of the VTD receptor is that it lies in the inner mouth of the channel and these toxins then exert a ''foot in the door'' effect to block inactivation. This hypothesis also predicts that with alterations in the channel or the toxin structure these toxins could act as open-channel blockers (reviewed by Khodorov, 1985).

Honerjäger et al. (1992) showed that the protonated form of VTD is the active species. These authors also showed that cevadine, which differs from VTD only in the C-3 substituent moiety, competes with VTD for binding but has a distinctly faster dissociation rate. VTD is the veratric acid ester of veracevine while cevadine is the angelic ester. The authors suggest that the veratric acid group of VTD binds to a hydrophobic site stabilizing the VTD receptor interaction. The veratric acid group contains an aromatic ring similar to phenylalanine. We speculate that the veratric acid group binds to a hydrophobic site within the pore to which the aromatic ring of phenylalanine of *KIFMK* normally binds. As a corollary, our study also suggests a hydrophilic pathway for VTD receptor interaction.

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