

Relation between Veratridine Reaction Dynamics and Macroscopic Na Current in Single Cardiac Cells

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ABSTRACT Veratridine modification of Na current was examined in single dissociated ventricular myocytes from late-fetal rats. Extracellularly applied veratridine reduced peak Na current and induced a noninactivating current during the depolarizing pulse and an inward tail current that decayed exponentially ($\tau = 226$ ms) after repolarization. The effect was quantitated as tail current amplitude, I_{tail} (measured 10 ms after repolarization), relative to the maximum amplitude induced by a combination of 100 μM veratridine and 1 μM BDF 9145 (which removes inactivation) in the same cell. Saturation curves for I_{tail} were predicted on the assumption of reversible veratridine binding to open Na channels during the pulse with reaction rate constants determined previously in the same type of cell at single Na channels comodified with BDF 9145. Experimental relationships between veratridine concentration and I_{tail} confirmed those predicted by showing (a) half-maximum effect near 60 μM veratridine and no saturation up to 300 μM in cells with normally inactivating Na channels, and (b) half-maximum effect near 3.5 μM and saturation at 30 μM in cells treated with BDF 9145. Due to its known suppressive effect on single channel conductance, veratridine induced a progressive, but partial reduction of noninactivating Na current during the 50-ms depolarizations in the presence of BDF 9145, the kinetics of which were consistent with veratridine association kinetics in showing a decrease in time constant from 57 to 22 and 11 ms, when veratridine concentration was raised from 3 to 10 and 30 μM , respectively. As predicted for a dissociation process, the tail current time constant was insensitive to veratridine concentration in the range from 1 to 300 μM . In conclusion, we have shown that macroscopic Na current of a veratridine-treated cardiomyocyte can be quantitatively predicted on the assumption of a direct relationship between veratridine binding dynamics and Na current and as such can be successfully used to analyze molecular properties of the veratridine receptor site at the cardiac Na channel.

INTRODUCTION

The alkaloid veratridine is a lipophilic, reversible activator of the voltage-dependent Na channel of excitable cells. Two different hypotheses have been advanced to

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explain its molecular mechanism of action (see Ulbricht, 1990). Originally, the modified macroscopic Na currents during and after a depolarizing pulse were attributed to Na channels with drug-imposed slow gating kinetics (Ulbricht, 1969). However, the interrelationship between slow Na current and veratridine binding, known to be rapidly reversible (Meves, 1966), was not accounted for. Subsequent studies elaborated the view that use-dependent binding of veratridine to open Na channels during the depolarization and unbinding after repolarization underlie the alkaloid-induced Na current modification (Leibowitz, Sutro, and Hille, 1986; Sutro, 1986; Hille, Leibowitz, Sutro, Schwarz, and Holan, 1987; Barnes and Hille, 1988). This hypothesis equates the lifetime of the veratridine–Na channel complex with the lifetime of the modified open state of the Na channel.

We have recently confirmed this view at the level of single cardiac Na channels comodified with BDF 9145, an allosteric inhibitor of Na channel inactivation (Wang, Dugas, Armah, and Honerjäger, 1990).

The relatively low and state-dependent affinity to Na channels of veratridine makes electrophysiological, rather than direct chemical or tracer ion flux analysis of its reaction important. Direct binding studies using [³H]veratridine have only revealed nonsaturable and nonspecific binding to lipid constituents of the excitable membrane (Balerna, Fosset, Chicheportiche, Romey, and Lazdunski, 1975).

Studies of veratridine-activated ²²Na uptake into neuroblastoma cell cultures (Catterall, 1977; Jacques, Fosset, and Lazdunski, 1978) were instrumental for defining the alkaloid's competitive interaction with batrachotoxin, grayanotoxin, or aconitine and allosteric synergistic interaction with *Anemonia sulcata* toxin II. However, such flux studies cannot resolve activator binding to the transient open state of the Na channel, and they cannot resolve reaction dynamics of ligands residing less than a second on the Na channel.

Veratridine effects on macroscopic Na current of a cardiomyocyte should conform to the microscopic rate constants that we determined on single Na channels of the same cell type (Wang et al., 1990). These data allow the prediction of veratridine concentration–effect relationships for cells with normal Na channels and cells with noninactivating Na channels during defined pulse protocols. They predict saturation of the veratridine effect at micromolar concentrations on noninactivating Na channels and practically no saturation for normally inactivating Na channels. Further, they predict the time course of the veratridine-modified Na current during depolarization and after repolarization. We have tested these predictions in the present paper. Establishing full concentration–effect relationships is a prerequisite for characterizing the veratridine receptor. The active form of veratridine, the sidedness of its action, and its interaction with cevadine are examined in the following paper (Honerjäger, Dugas, and Zong, 1992).

METHODS

Hearts were removed under sterile conditions from 14–24 fetuses of two Sprague-Dawley albino rats after 15–20 d of gestation. Atria and surrounding tissue were cut away from ventricles in ice-cold nominally Ca- and Mg-free Hanks' solution. The ventricles were cut into small pieces and placed in 50 ml of Hanks' solution containing 0.12% (wt/vol) trypsin and stirred with a magnetic bar at 37°C. Supernatants from two initial digestion periods of 15 min were

discarded. Supernatants from four subsequent 10-min digestions in 12 ml solution were filtered through cotton gauze and collected in 12 ml ice-cold Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum (DMEM-20FCS) to stop enzyme action. Cells were pelleted and resuspended in 40 or 60 ml DMEM-20FCS. After incubating 30 or 40 ml in a cell culture flask (75 cm²) at 37°C for 2 h to allow for attachment of fibroblasts, the supernatant myocardial cell suspension was collected and stored for up to 3 d at 4°C. Cells in 2–3-ml suspension were plated on 35-mm Petri dishes, cultured overnight or for a minimum of 4 h at 37°C in a water-saturated atmosphere of air/CO₂ (95:5), and used for experiments during the subsequent day.

Na current was recorded from single spherical cells adhering to the bottom of a 35-mm Petri dish with an L/M-EPC 7 amplifier (List-Electronic, Darmstadt, Germany) using the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). Fire-polished borosilicate glass micropipettes with 0.8–2 MΩ resistance were used. Membrane potentials are reported as the intracellular voltage with respect to the extracellular side. A Na current under good voltage control fit the following two criteria: graded activation during pulses to between –60 and –40 mV and absence of notches in the current records. The experiments were carried out in the Petri dish used for culturing and filled with 2–3 ml solution kept at 19.5–20.5°C by a Peltier element device (npi Advanced Electronic Systems, Eching a. A., Germany). The pipette contained the following intracellular solution (mM): 108 CsCl, 14 NaCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 10 glucose, 10 HEPES, 25 CsOH, and 0.3 NaOH (pH 7.3). Extracellular Na was reduced to improve the quality of the voltage clamp, extracellular Ca was reduced to minimize Ca current and contractions, and Cs was used to block K currents. The extracellular bath solution contained (mM): 69 NaCl, 69 CsCl, 5.4 KCl, 2 MgCl₂, 0.1 CaCl₂, 10 glucose, 10 HEPES, 1.6 NaOH, and 1.6 CsOH (pH 7.3). The cell under investigation was studied either after equilibration with the bathing solution or during continuous superfusion by a microsperfusion device consisting of a double-barreled glass tube mounted on a hydraulic micromanipulator and connected to two syringes in an infusion pump. The flow rate of 3 μl min⁻¹ resulted in a flow velocity of 1 cm s⁻¹ through each 80-μm orifice. One orifice was positioned as close as possible to the cell (diameter, 12–20 μm). The solution was changed by displacing the superfusion barrels rapidly under visual control with a video camera. This arrangement allowed us to study a maximum of three veratridine concentrations in a single experiment, the lowest being added to the solution in the Petri dish, the two others to that in the barrels of the microsperfusor.

Whole-cell transmembrane current, filtered to 3 kHz, was obtained from the L/M-EPC 7 amplifier, stored on videotape after processing with a PCM digital audio processor, and subsequently evaluated by computer. An MC 68020 processor-based system (Hewlett-Packard 9000/330) in combination with a fast A-D converter (AD200; Infotek Systems, Anaheim, CA) were used. The program for data acquisition and analysis was written in H-P Basic 5.1 by M. Dugas. Exponential functions describing the modified Na current were fitted by a least-squares method (Van Mastright, 1977). The cells were kept at a holding potential of –100 mV and pulsed to –30 mV for 50 ms every 5 s. Leakage current was typically <3% of peak Na current. Stability of leakage current throughout the experiment was verified by measuring the level of holding current at –100 mV. The amplitude of the veratridine-induced tail current, I_{tail} , was measured 10 ms after repolarization.

Veratridine base (Sigma, Munich, Germany) was converted into the hydrochloride by addition of HCl and dissolved in extracellular medium. BDF 9145, obtained from Beiersdorf AG (Hamburg, Germany), is 4-(3-(4-(4-cyanomethoxyphenyl)phenylmethyl)-1-piperazinyl)-2-hydroxypropoxy)-1H-indole-2-carbonitrile and was dissolved in dimethylsulfoxide (1 mM; final concentration, 1 μM).

Where appropriate, results are presented as mean ± SEM.

RESULTS

Veratridine Effect on Cardiac Cell Na Current

Na current elicited from -100 mV holding potential by 50-ms pulses to -30 mV, applied every 5 s, in a rat embryonic ventricular myocyte at 20°C is shown in Fig. 1 *A*. This pulse protocol was used throughout the study. Na current is seen to activate and inactivate within the first 10 ms of the pulse, in agreement with earlier investigations on the same cell type at similar temperature (Kunze, Lacerda, Wilson, and Brown, 1985). As in other cell types, superfusion of this cell with veratridine ($30\ \mu\text{M}$) reduced peak Na current slightly and induced a small noninactivating current during the pulse that remained as an inward tail current after repolarization. The tail current decayed slowly over ~ 1.5 s (Fig. 1 *B*). The interpulse interval of 4.95 s ensured complete decay of veratridine-induced tail current between pulses.

The veratridine effect illustrated by Fig. 1, *A* and *B*, is the steady-state effect of the chosen alkaloid concentration. A steady state was reached only ~ 1.5 min after the start of microsuperfusion with veratridine-containing extracellular solution (Fig. 1 *B*, *inset*) when switching the barrels of the microsuperfusor, although the superfusate exchange time at the cell surface was < 0.1 s. Evidence obtained in the subsequent paper (Honerjäger et al., 1992) suggests that veratridine acts from inside the sarcolemmal membrane. Hence, we attribute the delayed onset during extracellular application to the time necessary for membrane penetration and intracellular veratridine accumulation. All effects shown in this paper are steady-state effects.

The tail current which reflects the current of Na ions through veratridine-modified Na channels subjected to a larger driving force (-100 mV) than during the preceding depolarization (-30 mV) is the most conspicuous veratridine effect and was therefore used for its quantification. In line with the hypothesis developed by Sutro (1986), we interpret the initial amplitude of the tail current to reflect the number of Na channels that became modified during the preceding depolarization by use-dependent veratridine binding to open Na channels. To estimate the fraction of Na channels modified during a pulse in a given cell, we measured the effect of a saturating veratridine concentration in that cell. This would have required a very high (millimolar) veratridine concentration at normal Na channels. Therefore, we facilitated veratridine binding by allosteric pharmacological removal of Na channel inactivation with the cyanindol BDF 9145 (see Fig. 1 *C*, *inset*). Fig. 1 *C* shows slow Na currents from a different veratridine-treated cell. The tail current increased when alkaloid concentration was raised from 30 to $100\ \mu\text{M}$, and it grew further when $1\ \mu\text{M}$ BDF 9145 was added to the $100\ \mu\text{M}$ veratridine in the extracellular superfusate. We found the latter drug combination to elicit a maximum tail current amplitude (see below, Fig. 3) and used it to scale the effect of submaximally effect veratridine concentrations in each cell.

Fig. 2 reproduces the tail currents of Fig. 1 *C* together with monoexponential fits. The decay of these currents is well described as an exponential process. Furthermore, the three time constants did not differ by more than 12% despite a fourfold change of amplitude. Notably, Na channel comodification with BDF 9145 did not affect the time constant of the tail current. These results are consistent with the hypothesis that the decaying tail current directly reflects the dissociation of veratridine bound during the preceding pulse.

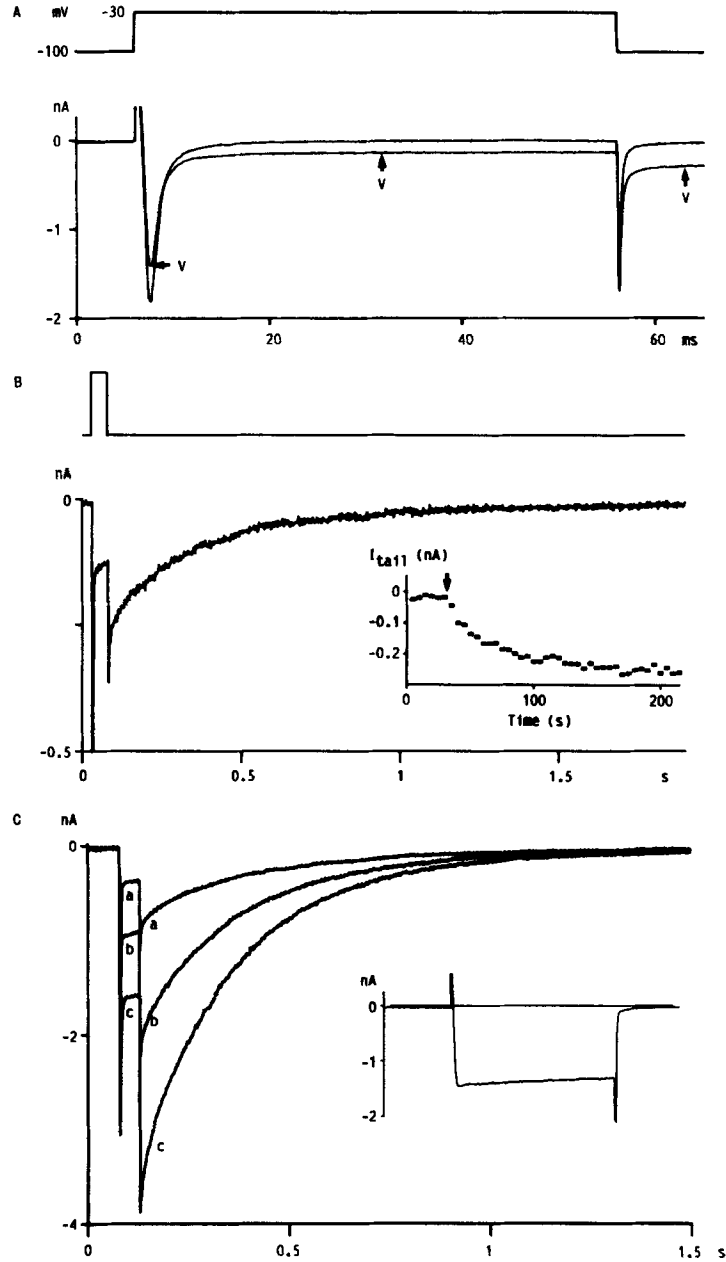


FIGURE 1. Quantification of Na channel modification by veratridine in single embryonic rat cardiomyocytes. (A) Na current was elicited every 5 s by 50-ms pulses to -30 mV from a holding potential of -100 mV. Trace V was recorded 3 min after the control trace and after start of microsuperfusion with $30 \mu\text{M}$ veratridine. (B) Veratridine trace of A on longer time scale to show time course of veratridine-induced tail current. Amplitude of the tail current, I_{tail} , was measured 10 ms after repolarization and is shown to stabilize within 2 min after the start of veratridine superfusion marked by the arrow of the inset. (C) Different cell equilibrated with $30 \mu\text{M}$ (a) and $100 \mu\text{M}$ veratridine (b) and subsequently with a mixture of $100 \mu\text{M}$ veratridine and $1 \mu\text{M}$ BDF 9145 (c) in order to determine the maximum veratridine effect on this cell. As shown by the inset, $1 \mu\text{M}$ BDF 9145 removes Na channel inactivation during a 50-ms depolarization to -30 mV nearly completely (different cell).

Theoretical and Experimental Concentration–Effect Relationship

We have previously analyzed the molecular reaction dynamics of veratridine at single Na channels in outside-out patches of the same cell type (Wang et al., 1990). Individual veratridine binding and unbinding reactions were detected at a constant depolarized membrane potential of -30 mV as current transitions between two open states: one, a low-conductance state, imposed by veratridine, and the other, a fully open state, imposed by BDF 9145. These data were quantitatively consistent with the bimolecular reaction scheme:

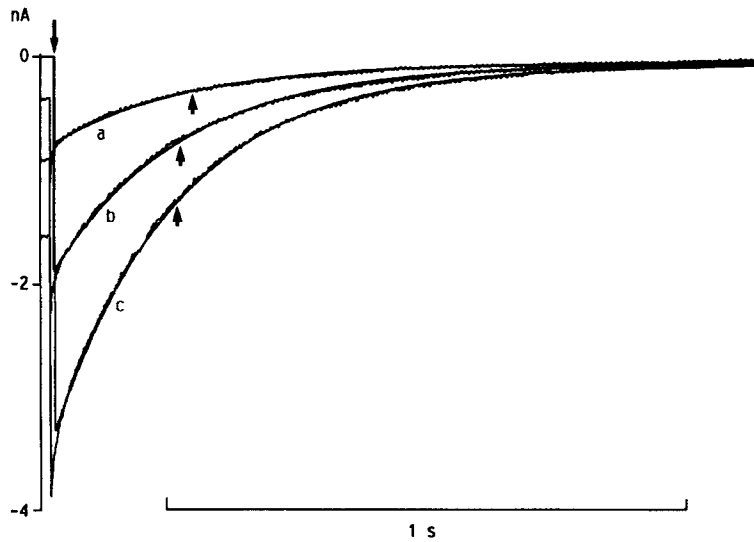
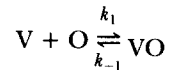


FIGURE 2. Magnification of the tail currents of Fig. 1 C together with monoexponential fits. The long arrow marks the start of the fit, and the short arrows point to time constants 269 (a), 244 (b), and 236 ms (c) of the fitted curves.

where one molecule of veratridine, V, binds reversibly to a Na channel, O, that is stabilized in the open conformation by BDF 9145. The ternary complex between veratridine, Na channel, and BDF 9145, VO, exhibits the veratridine-induced, low-conductance open state.

Experiments covering a 100-fold range of veratridine concentrations yielded the microscopic association rate constant $k_1 = 4.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and dissociation rate constant $k_{-1} = 2.2 \text{ s}^{-1}$ (Wang et al., 1990). With these rate constants known, the fractional veratridine occupancy y of the population of Na channels in a cell develops according the equation

$$y = \frac{V}{V + k_{-1}/k_1} \{1 - \exp [-(k_{-1} + k_1V)t]\} \quad (1)$$

where t is the time after a step increase of veratridine concentration from zero to the level V . The depolarization-induced opening of Na channels, i.e., their rapid conversion into a veratridine-binding conformation, in the presence of veratridine is equivalent to a step increase in veratridine concentration. Hence, parameter t of Eq. 1 can be equated with the open time of the Na channels. The loss of veratridine occupancy upon dissociation, assuming an initial value of one, should proceed according to the following equation:

$$y = \exp(-k_{-1}t) \quad (2)$$

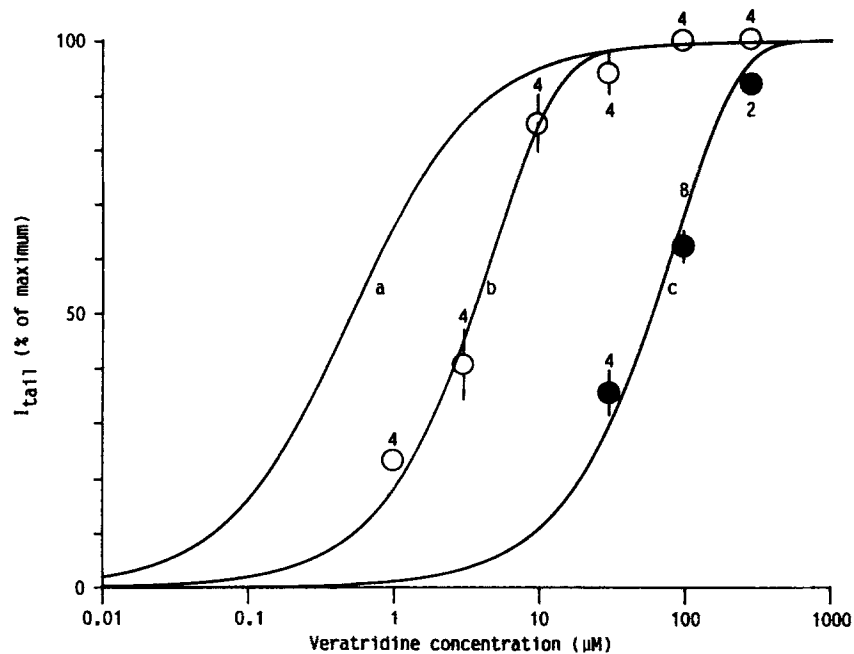


FIGURE 3. Relation between veratridine concentration and relative tail current amplitude, I_{tail} , as determined in Fig. 1. Symbols are mean values (\pm SEM as vertical bar) of indicated number of cells in the absence (filled circles) or presence of $1 \mu\text{M}$ BDF 9145 (open circles). Cells were equilibrated for at least 2 min with each of three different veratridine concentrations including the mixture of 100 or $300 \mu\text{M}$ veratridine with $1 \mu\text{M}$ BDF 9145 to obtain a maximum I_{tail} . The smooth curves represent the saturation function defined by Eq. 1 in the text with $t = \infty$ (a), 50 ms (b), or 2.7 ms (c).

We have calculated y as a function of veratridine concentration for three different values of t (smooth curves in Fig. 3) using Eq. 1. Curve *a* represents equilibrium binding to permanently activated Na channels ($t = \infty$). Curve *b* simulates pulse experiments with BDF 9145, where Na channels are available for veratridine binding nearly throughout the depolarizing pulse ($t = 50$ ms). Curve *c* simulates experiments without BDF 9145, where Na channels have their normal brief open time ($t = 2.7$ ms). A mean open time of 2.7 ± 0.2 ms was obtained by dividing the time integral of Na current by its amplitude in records from eight cells.

Fig. 3 compares the theoretical curves with experimental values of relative tail current amplitude, I_{tail} , after 50-ms pulses to -30 mV. As expected, tail currents did not reach the level predicted by curve *a* because veratridine binding does not reach equilibrium during exposure for 2.7 or 50 ms. Predicted fractional veratridine occupancies for binding periods of 50- and 2.7-ms duration are very close to the experimental points for macroscopic Na current with and without BDF 9145, respectively. Half-maximum occupancy is predicted to occur at $60 \mu\text{M}$ veratridine in cells with normally inactivating Na channels, and at $3.5 \mu\text{M}$ in cells with noninactivating Na channels subjected to 50-ms depolarization.

Theoretical and Experimental Time Course of Veratridine-modified Na Current

The onset of binding in a bimolecular reaction is a monoexponential process governed by the time constant $\tau = 1/(k_1 + k_1V)$ as shown by Eq. 1. It is difficult to

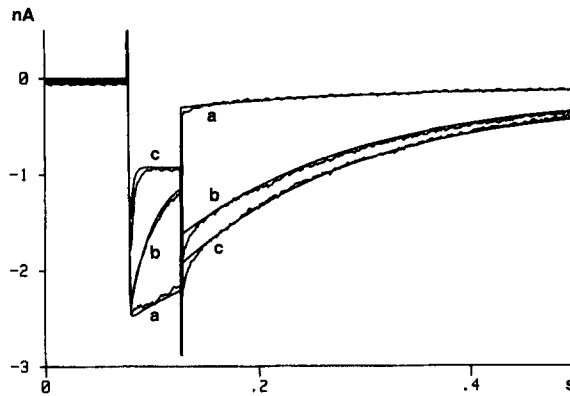


FIGURE 4. Simulation of the time- and concentration-dependent veratridine effect on noninactivating Na channels during and after a 50-ms depolarization to -30 mV. The noisy traces are original current records. Current record *a* was associated with upward and downward capacitive artifacts marking the start and end, respectively, of the depolarizing pulse. Currents were recorded from a cell equilibrated successively

with a mixture of $1 \mu\text{M}$ BDF 9145 and 1 (*a*), 10 (*b*), or $100 \mu\text{M}$ veratridine (*c*). The smooth curves were calculated on the time scale of the experimental record according to Eq. 3 during the 50-ms depolarization and Eq. 2 after repolarization using the same three veratridine concentrations (see text for details).

model veratridine binding during the brief and kinetically complex time course of normal Na current. We have therefore restricted analysis to the case of noninactivating Na channels. For the predictions shown by the smooth curves in Fig. 4, we have modeled noninactivating Na current by assuming that it turns on with the activation variable $[1 - \exp(-t/\tau_m)]^3$ and $\tau_m = 0.5$ ms (Kunze et al., 1985) and then remains stable (i.e., lacks inactivation). Veratridine is known to reduce current through the Na channel (Leibowitz et al., 1986; Sigel, 1987*a, b*; Barnes and Hille, 1988; Schreibmayer, Tritthart, and Schindler, 1989; Wang et al., 1990). Consequently, veratridine binding to noninactivating Na channels should reduce Na current at most to the level resulting from veratridine modification of all channels. Na current, I_{Na} , modified by progressive veratridine binding was therefore modeled by subtracting a current component corresponding to receptor occupancy (Eq. 1) and receptor availability (i.e., fraction of open Na channels) from the noninactivating Na current according to

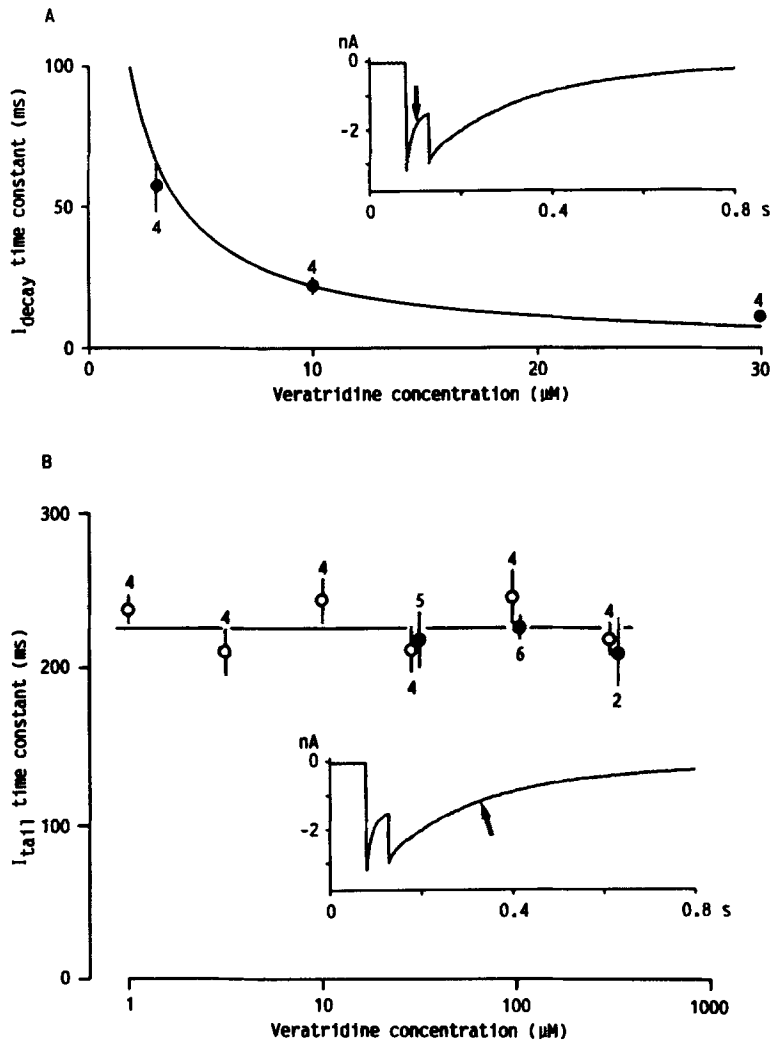


FIGURE 5. Relation between veratridine concentration and Na current kinetics. (A) Symbols show mean values (\pm SEM as vertical bars) of the I_{decay} time constant which was determined by monoexponential fits of the Na current decay during 50-ms depolarizations in the combined presence of 1 μM BDF 9145 and veratridine. One or two veratridine concentrations for each cell are shown (number of cells indicated). The smooth curve represents the function $\tau = 1/(k_{-1} + k_1V)$ as defined by Eq. 1 in the text. (B) Mean values (\pm SEM as vertical bars) of I_{tail} time constants in the absence (filled symbols) or presence of 1 μM BDF 9145 (open symbols). Up to three veratridine concentrations are shown for each cell (number of cells indicated). The regression line corresponds to a time constant of 226 ms for the 1–300 μM veratridine concentration range. The two identical insets show a current record in the presence of 1 μM BDF 9145 and 30 μM veratridine with the arrow pointing to the respective current segment used for exponential fitting.

the following equation:

$$I_{\text{Na}} = [1 - \exp(-t/\tau_m)]^3 - 0.65[1 - \exp(-t/\tau_m)]^3 y_{(t,V)} \quad (3)$$

where $y_{(t,V)}$ is defined by Eq. 1. To fit the experimental results, we arbitrarily assumed that the maximum reduction induced by veratridine was 65% of control peak Na current. This value is close to the 75% reduction of normal open-channel current induced by veratridine in single Na channels of the same cell type (Wang et al., 1990).

As shown by the original (noisy) traces in Fig. 4, veratridine caused the noninactivating Na current of this BDF 9145-treated cell to decay during the 50-ms depolarization. The decay rate was enhanced when veratridine concentration was raised from 1 to 10 or 100 μM . In addition, the amplitude of the Na current was reduced. This is expected if veratridine binds to Na channels during the rising phase of Na current, thus preventing some channels from maintaining their normal conductance at the time of peak Na current.

The experimental records in 1, 10, and 100 μM veratridine are in good agreement with the predicted currents shown in Fig. 4. The maximum predicted tail current amplitude was arbitrarily adjusted to the experimental record and drawn with the time constant of a monoexponential fit to the experimental trace in 100 μM veratridine. The relative initial tail current amplitudes for 1 and 10 μM veratridine were then calculated according to Eq. 1 for $t = 50$ ms. The decay time constants are the same as that in 100 μM veratridine. The predicted curves of Fig. 4 thus assume veratridine association during a 50-ms pulse with the k_1 and k_{-1} values for -30 mV given above and dissociation with a different k_{-1} value (5.8 s^{-1}) after repolarization to 100 mV. An increase of k_{-1} from 2.2 s^{-1} at -30 mV to 5.8 s^{-1} at -100 mV is consistent with the known voltage dependence of the tail current time constant in this potential range (Sigel, 1987b; Barnes and Hille, 1988) and indicates a slowing of dissociation with depolarization.

The veratridine-induced decay of noninactivating Na current (Fig. 4) was well described by a monoexponential function, and the time constants from exponential fits to records from eight cells in three different veratridine concentrations are shown in Fig. 5A. The experimental time constants are in reasonable agreement with their predicted value $\tau = 1/(k_{-1} + k_1V)$ and show the expected decrease with increasing veratridine concentration. In contrast, the time constants for the decay of tail currents were insensitive to a 300-fold change in veratridine concentration (Fig. 5B) as predicted for a dissociation process (Eq. 2).

DISCUSSION

We interpret the present results to show that macroscopic Na current elicited by a depolarizing pulse in a single cardiac cell equilibrated with veratridine reflects directly the alkaloid's use-dependent association with and subsequent dissociation from its receptor at the cardiac Na channel. This hypothesis of veratridine action was originally developed by Sutro (1986) for neuronal and skeletal muscle Na channels (see also Hille, 1968; Leibowitz et al., 1986; Hille et al., 1987). We extend their concept to the cardiac Na channel type and test, for the first time, quantitative predictions for whole-cell Na current behavior based on microscopic veratridine

reaction dynamics that we determined earlier (Wang et al., 1990). Within this hypothesis, each transient activation of Na channels in a veratridine-treated cell could be visualized as exposure of Na channel receptors to a veratridine concentration pulse resulting in time- and concentration-dependent receptor occupancy during the pulse and dissociation thereafter. If this view is correct, whole-cell Na current recording would be a convenient method to elucidate properties of the veratridine binding site with high time resolution and specific access to both extra- and intracellular aspects of the cardiac Na channel macromolecule. We have used this method to demonstrate competitive interactions between veratridine and cevadine in the subsequent paper (Honerjäger et al., 1992).

Extrapolation from Microscopic to Macroscopic Veratridine Reaction Dynamics

The most direct information on the molecular mechanism of veratridine action has come from studies on single Na channels. In records from single Na channels of muscular or neuronal origin expressed in oocytes (Sigel, 1987*a, b*), neuroblastoma cells (Barnes and Hille, 1988), and adult or embryonic rat cardiomyocytes (Schreibmayer et al., 1989; Wang et al., 1990) the veratridine effect manifests itself as a sudden reduction of normal open-channel current by ~75%. The modified Na channel then remains activated at low conductance for an exponentially distributed duration with $\tau = 0.48$ s at -30 mV (Wang et al., 1990) or 1.6 s at about -45 mV (Barnes and Hille, 1988) and also after repolarization to -100 mV with $\tau = 0.5$ s (Sigel, 1987*a*) or 0.35 s (Sigel, 1987*b*) in different types of Na channels.

The veratridine-induced, low-conductance state is characterized by a predominant open state (mean duration, 71 ms) alternating with brief closures (mean duration, 8 ms) in rat embryonic cardiomyocytes at -30 mV (Wang et al., 1990) and is thus better described as a "burst" of current. Bursts originating from closed (i.e., resting or inactivated) channel states are very rare in heart cells (Wang et al., 1990), which is consistent with the lack of effect of up to 300 μM veratridine on holding current at -100 mV (Figs. 1 and 4). We have recently directly monitored both veratridine-associated and veratridine-free states of a single cardiac Na channel by comodifying it with the allosteric activator BDF 9145 that imposes the normal open channel conductance (i.e., fully activated bursts of Na current) before association and after dissociation of veratridine (Wang et al., 1990). These data were quantitatively consistent with the interpretation that veratridine-induced bursts correspond to the random times that a veratridine molecule resides on the Na channel, while fully activated bursts correspond to the random waiting times of the unoccupied channel before it binds another alkaloid molecule.

Microscopic reaction rate constants were $k_1 = 4.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $k_{-1} = 2.2 \text{ s}^{-1}$ for an open conformation of the Na channel stabilized at -30 mV with BDF 9145 (20°C). In the present experiments we have pulse-activated normal or BDF 9145-modified Na channels of single cardiac myocytes using 50-ms depolarizations to -30 mV. Veratridine was superfused extracellularly and allowed to equilibrate, whereas it was applied intracellularly in the outside-out patches (Wang et al., 1990) anticipating an intracellular site of action (Honerjäger et al., 1992). Veratridine is a weak base with a reported pK_a of 9.7 (Büch, 1976) or 9.5 (McKinney, Chakraverty, and DeWeer, 1986). We have used the same pH value of 7.3 for extra- and intracellular solutions to

ensure symmetrical veratridine concentrations across the cell membrane after equilibration. The only other difference between the present whole-cell experiments and the outside-out patches in our previous study concerned the Na concentrations: 71 vs. 140 mM extracellularly and 14 vs. 0 mM intracellularly.

Using the microscopic rate constants, we have predicted the fractional receptor occupancy for the population of Na channels in a single cardiac cell as it develops during a reaction time of 50 ms for noninactivating Na channels or 2.7 ms for normally inactivating Na channels (Fig. 3). At the macroscopic level, the fractional receptor occupancy should correspond to the relative tail current amplitude because the tail current is the sum of veratridine-modified elementary Na currents at the end of the depolarizing pulse and therefore directly proportional to their number. As shown by Fig. 3, microscopic rate constants predicted relative tail current amplitudes successfully both for BDF 9145-treated and normal cells.

Veratridine showed a 20-fold higher binding affinity to BDF 9145-treated cells corresponding to an ~20-fold longer binding time during the 50-ms pulse. While the effect hardly saturated at 300 μM in normal cells, there is clear theoretical and experimental saturation near 30 μM veratridine in BDF 9145-treated cells. We conclude that a single 50-ms depolarization in the presence of 100 μM veratridine and 1 μM BDF 9145 drives veratridine receptor occupancy of a rat cardiomyocyte from zero to saturation. The accurate prediction for normally inactivating cells (Fig. 3) suggests that the BDF 9145-induced open conformation (on which our microscopic data are based) is very similar to the normal open state with respect to veratridine binding dynamics.

Veratridine Association

It has been extensively documented that veratridine binds preferentially to the open conformation of the Na channel both at the macroscopic (Leibowitz et al., 1986; Sutro, 1986; Hille et al., 1987; Barnes and Hille, 1988) and microscopic level (Sigel, 1987*a, b*; Barnes and Hille, 1988; Schreibmayer et al., 1989; Wang et al., 1990) in skeletal and cardiac muscle as well as brain and neuroblastoma Na channels. Frog node of Ranvier Na channels differ in that there is significant additional veratridine binding to (and opening of) channels in the inactivated state (Ulbricht, 1972; Rando, 1989). The latter type of association is not as apparent in cardiac cells and is not accounted for by our model. Batrachotoxin has also been shown to bind predominantly to the open conformation of the Na channel in the squid giant axon, and the association rate constant for binding to this state has been estimated as $2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at 8–10°C (Tanguy and Yeh, 1991).

Veratridine reduces single Na channel conductance in intact cells, and Na channels inserted into lipid bilayers display a lower conductance after modification with veratridine than with batrachotoxin (Garber and Miller, 1987; Recio-Pinto, Duch, Levinson, and Urban, 1987; Duch, Recio-Pinto, Frenkel, Levinson, and Urban, 1989). Reduction of Na channel conductance provides a qualitative explanation for the reduction of peak Na current observed by many authors (Sutro, 1986; Sigel, 1987*a, b*; Barnes and Hille, 1988; Rando, 1989) and in Fig. 1 of this study. Since veratridine binds to open Na channels and reduces their conductance, veratridine association is manifested as a reduction in Na current. We have shown in Figs. 4 and

5 that this effect, if quantitated on cells with noninactivating Na channels, showed concentration-dependent kinetics that are quantitatively consistent with veratridine association dynamics. Previous estimates of the veratridine association rate based on macroscopic and microscopic Na current measurements on skeletal muscle and neuroblastoma cells, respectively (Leibowitz et al., 1986; Barnes and Hille, 1988), have been of the same order as ours.

Veratridine Dissociation

Sutro (1986) first suggested that the tail current following a depolarizing pulse reflects the slow unbinding of veratridine from the Na channel. Although this interpretation has been questioned for the frog node of Ranvier by Rando (1989), who proposed slow inactivation of veratridine-modified channels, there is compelling evidence in its favor from other cell types. Perhaps the strongest evidence comes from single-channel experiments which have shown that the veratridine-induced burst is not followed by a closure indicative of inactivation but can be equated with the lifetime of the drug-channel complex (Wang et al., 1990). The correlation between burst length and macroscopic tail current likewise supports the dissociation hypothesis (Barnes and Hille, 1988). We have confirmed the monoexponential nature of the tail current and the lack of dependence of its time constant on veratridine concentration over a 300-fold range (Fig. 5 B), which is in line with a dissociation process. Independent evidence is provided in the following paper (Honerjäger et al., 1992), which shows a marked slowing of the tail current in response to an increase of intracellular viscosity. This suggests that the tail current reflects veratridine dissociation from an intracellular binding site into the intracellular space.

Extreme hyperpolarizations (e.g., to -170 mV) close the veratridine-modified Na channel and apparently trap veratridine at its binding site (Leibowitz et al., 1986; Hille et al., 1987). Clearly, the veratridine dissociation process can only be monitored in a potential range, at -100 mV and more positive levels, where the modified channel is also predominantly open.

In conclusion, we have shown that macroscopic Na current of a veratridine-treated cardiomyocyte can be largely predicted on the assumption of a direct relationship between veratridine binding dynamics and Na current. Association is manifested as a reduction in Na current, and dissociation as a tail current following repolarization. Whole-cell voltage clamp is thus a powerful tool to characterize the specific binding dynamics of this lipophilic and relatively low-affinity Na channel ligand.

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