Mutually Exclusive Action of Cationic Veratridine and Cevadine at an Intracellular Site of the Cardiac Sodium Channel

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ABSTRACT Veratridine modification of Na current was examined in single dissociated ventricular myocytes from late-fetal rats by applying pulses to −30 mV for 50 ms every 2 or 5 s from a holding potential of −100 mV (20°C) and measuring amplitude, $I_{\text{tail}}$, and time constant, $\tau_{\text{tail}}$, of the post-repolarization inward tail current induced by the alkaloid. Increasing the pH of a 30 μM veratridine superfusate from 7.3 to 8.3 (which increases the fraction of uncharged veratridine molecules from 0.5 to 5% while decreasing that of protonated molecules from 99.5 to 95%) increased $I_{\text{tail}}$ by a factor of 2.5 ± 0.5 (mean ± SEM; n = 3). Switching from 100 μM veratridine superfusate at pH 7.3 to 10 μM at pH 8.3 did not affect the size of $I_{\text{tail}}$ (n = 4). Intracellular (pipette) application of 100 μM veratridine at pH 7.3 or 8.3 produced small $I_{\text{tail}}$'s suggesting transmembrane loss of alkaloid. If this was compensated for by simultaneous extracellular application of 100 μM veratridine at a pH identical to intracellular pH, $I_{\text{tail}}$ (measured relative to the maximum amplitude induced by a combination of 100 μM veratridine and 1 μM BDF 9145 in the same cell) at pH 7.3 did not significantly differ from that at pH 8.3 (84 ± 4 vs. 70 ± 6%; n = 3 each). Results from six control cells and five cells subjected to extracellularly increased viscosity by the addition of 0.5 or 1 molal sucrose showed that increasing extracellular viscosity 1.6- and 2.5-fold increased $\tau_{\text{tail}}$ 1.5- and 2.3-fold, respectively, while a selective 2.5-fold increase of extracellular viscosity did not significantly affect $\tau_{\text{tail}}$. Superfusion with the related ceveratrum alkaloid cevadine (1–100 μM), in cells additionally treated with 1 μM BDF 9145 to remove inactivation, induced a tail current whose amplitude was half-maximal at 4 μM and saturating at 30 μM, while $\tau_{\text{tail}}$ (mean value 49 ms; eight cells) was not affected by concentration. The addition of 100 or 30 μM cevadine to the superfusate containing a saturating concentration of veratridine (30 μM) had little effect on the initial amplitude of the veratridine-induced tail current, but converted its monoeXponential decay into a biexponential decay characterized by time constants corresponding to cevadine and veratridine, respectively. The veratridine-induced component of $I_{\text{tail}}$ was reduced to 25% by 100 μM cevadine (n = 1) and to 60 ± 7% (n = 3) by 30 μM cevadine.

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cevadine. We conclude on the basis of the extracellular and intracellular pH and viscosity effects that extracellularly applied veratridine diffuses in its free base form through the sarcolemma and activates the Na channel at an intracellular site in its protonated form. This action is mutually exclusive with that of cevadine, the C-3 angelic acid ester analogue of veratridine, suggesting a competitive interaction. Chemical complementarity of veratridine to its binding site suggests the intracellular presence of a negative charge and adjacent hydrophobic amino acid residue as essential components of the veratridine recognition site at the Na channel macromolecule.

**INTRODUCTION**

Recent biochemical and electrophysiological work on the voltage-dependent Na channel has led to the identification of individual amino acids within the channel macromolecule that are involved in the binding of scorpion toxin, tetrodotoxin or saxitoxin, and Zn$^{2+}$ ions (Tejedor and Catterall, 1988; Thomsen and Catterall, 1989; Noda, Suzuki, Numa, and Stühmer, 1989; Schild and Moczydlowski, 1991).

Detailed characterization of such binding sites, including their localization to the extra- or intracellular side, structure–activity relationships, and resolution at the individual amino acid level in conjunction with knowledge of the biophysical effects of the ligand may help to define structure–function relationships of the Na channel macromolecule. The location and chemical properties of the binding site for a widely used class of Na channel activators, veratridine, and other ceveratrum alkaloids, is still largely unknown. Analysis of this topic is complicated by the low affinity of these alkaloids, by their ability to diffuse through the plasmalemma, thereby reaching the membrane side opposite to that of application, and by their existence in two molecular forms, protonated and free base.

In this study on whole-cell Na current in rat ventricular cardiomyocytes, we determine the active form of veratridine, the sidedness of its action, and its interaction with a closely related ceveratrum alkaloid, cevadine. The results are consistent with an intracellular binding site that is exposed only during activation of the Na channel and to which veratridine binds reversibly in its protonated form. Na channel modification by veratridine will be shown to be mutually exclusive with cevadine. Complementarity to the positive charge on veratridine and to the hydrophobicity of the C-3 veratric acid ester group suggest an intracellular anionic amino acid side chain in proximity to a hydrophobic residue as essential components of the veratridine binding site. With the primary structure of the rat heart Na channel known (Rogart, Cribbs, Muglia, Kephart, and Kaiser, 1989; Cribbs, Satin, Fozzard, and Rogart, 1990; Kallen, Sheng, Yang, Chen, Rogart, and Barchi, 1990), experiments can be designed to test these conclusions at the molecular level.

**METHODS**

Whole-cell Na current was recorded from cultured rat ventricular cardiomyocytes as described in the preceding paper (Zong, Dugas, and Honerjäger, 1992).

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). Additional CsOH was used for alkalinization to pH 8.3. 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, and 1:1 NaOH and CsOH as required for pH 7.3 or 8.3. The viscosity of the medium in contact with the extra- or intracellular side of the cell membrane was increased by addition of sucrose, and osmotic effects of sucrose were avoided by addition of equimolar urea, which does not increase viscosity, to the solution at the opposite side of the cell membrane. Since high concentrations of these nonelectrolytes were required, molal (m) rather than molar concentrations were prepared. The cell under investigation was either studied after equilibration with the bathing solution or during continuous superfusion by a microsuperfusion device described in the preceding paper (Zong et al., 1992). The amplitude of the veratridine-induced tail current, I_{tail}, was measured 10 ms after repolarization.

Veratridine base (Sigma, München, Germany) and cevadine base (a gift from the late Dr. O. Krayer) were converted into the hydrochlorides by addition of HCl and dissolved in extracellular medium (their chemical structures are shown in Fig. 1). 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. Student’s t test for unpaired values was used in Table II.

RESULTS

Increase of Extracellular pH

Veratridine is a weak base with pKₐ determined as 9.7 (Büch, 1976) or 9.54 (McKinney, Chakraverty, and De Weer, 1986). Taking pKₐ as 9.6, the Henderson-Hasselbalch equation yields the following ratios of protonated charged to uncharged (free base) molecules at different pH values:

pH 7.3: 200
pH 8.3: 20
Increasing the pH of a veratridine solution from 7.3 to 8.3 thus increases the fraction of uncharged veratridine molecules 10-fold, from 0.5 to 5%, while that of protonated molecules decreases only slightly, from 99.5 to 95%. The term ionization will be used to describe the degree of protonation of veratridine, while the term dissociation will denote the unbinding of veratridine from its receptor at the Na channel.

In the absence of veratridine, changing the extracellular pH between 7.3 and 8.3 did not affect peak Na current elicited by our standard pulse protocol (50-ms depolarization to $-30$ mV from $-100$ mV holding potential every 5 s; $n = 3$) in line with the insensitivity of frog node of Ranvier Na current over this range of pHo (Hille, 1968).

We first tested the possible role of uncharged veratridine molecules by applying 30 μM veratridine at the standard pH of 7.3 and then switching the pH of the veratridine superfusate to 8.3 (Fig. 2). The superfusate exchanges the environment of the cell within ~0.1 s. In contrast, the veratridine effect developed much more slowly, reaching a steady state only after ~1.5 min. This slow development of $I_{\text{tail}}$ does not represent slow association of veratridine to its receptors. Rather, each $I_{\text{tail}}$ point is proportional to the number of Na channels to which veratridine associated during the immediately preceding 50-ms depolarization (Sutro, 1986; Zong et al., 1992). The tail current itself reflects veratridine dissociation, and the dissociation is complete long before the next pulse (Zong et al., 1992). $I_{\text{tail}}$ is thus a measure of the "effective" veratridine concentration prevailing during each 50-ms pulse. Clearly, this effective concentration does not reflect the extracellular concentration, which reaches its steady-state level within 0.1 s. The delayed response to extracellular veratridine application argues against an extracellular site of action of this alkaloid.

As shown by Fig. 2, increasing pHo to 8.3 after stabilization of the veratridine effect at pH 7.3 resulted in a delayed and eventually 3.4-fold increase of $I_{\text{tail}}$, which was reversible upon reacidification. Results of three experiments are summarized in Table I A. The tail current time constant was not consistently affected by the pHo change (Fig. 2, Table I A). The increase of $I_{\text{tail}}$ by a factor of $2.5 \pm 0.5$ upon elevation of pHo to 8.3 is consistent with the augmentation induced by a 10-fold increase in veratridine concentration (from 30 to 300 μM) at a constant pHo of 7.3 (Fig. 3 in Zong et al., 1992). This result suggests that uncharged veratridine, which increases from 0.15 to 1.5 μM if pH is increased from 7.3 to 8.3 at 30 μM total veratridine, is responsible for inducing the tail Na current during extracellular application. The possible additional contribution of extracellular protonated veratridine molecules, although unlikely because of the slow onset, was tested by a maneuver that alters the concentration of charged veratridine without affecting that of the free base. A concentration of 100 μM veratridine was superfused at pH 7.3, followed by simultaneous reduction of veratridine concentration to 10 μM and an increase of pH to 8.3. As shown by Fig. 3, there was no change of $I_{\text{tail}}$ during this solution change where extracellular charged veratridine is reduced from 99.5 to 9.5 μM at constant uncharged veratridine concentration (0.5 μM). This lack of effect was confirmed in three additional experiments performed in reversed sequence (Table I B). We conclude that the cardiac Na channel is insensitive to extracellular protonated veratridine molecules although this molecular species is present in 200-fold excess over the free base form at physiological pH.
FIGURE 2. Increasing extracellular pH augments effect of superfused veratridine on Na current in a single embryonic rat cardiomyocyte. (A) Plot of $I_{\text{Na}}$ elicited by 50-ms depolarizations to $-30$ mV at 0.2 Hz from $-100$ mV holding potential (filled symbols) and $I_{\text{hold}}$ (open symbols) as a function of time immediately before and during superfusion with 30 µM veratridine as indicated in the upper bar. After veratridine application at the standard extracellular pH of 7.3 until a steady-state effect was established, the microsuperfusion was switched to solution containing the same veratridine concentration at pH 8.3 and later back to the initial veratridine solution. Note the threefold increase in $I_{\text{Na}}$ at the higher pH value, its reversibility, and the delayed response to extracellular pH changes which were completed within $<0.1$ s. (B) Na tail current records from the same experiment before veratridine application (a) and after equilibration with 30 µM veratridine at pH 7.3 (b) and pH 8.3 (c). Peak $I_{\text{Na}}$ was truncated.
Increase of Intracellular pH

Because the foregoing experiments excluded an extracellular site of action of either form of veratridine, we next examined the possibility of an intracellular site of action. Fig. 4A, trace a, shows the tail current induced by intracellular application of 100 μM veratridine at pH 7.3 via the patch pipette. \( I_{\text{tail}} \) was quantified as 27% of the maximum \( I_{\text{tail}} \) produced later in the same cell by additional extracellular superfusion with 100 μM veratridine and 1 μM BDF 9145 (trace c), a condition that modifies all Na channels of the cell as described in the preceding paper (Zong et al., 1992). In three experiments, \( I_{\text{tail}} \) amounted to 36 ± 6% of maximum \( I_{\text{tail}} \). Fig. 4B, trace a,

\[
\text{T A B L E I}
\]

Effect of Extracellular pH on Veratridine-induced Tail Current

(A) Change of pH from 7.3 to 8.3 during superfusion with 30 μM veratridine

| Cell | \( I_{\text{tail}} \) at pH 7.3 | \( \tau_{\text{tail}} \) | \( I_{\text{tail}} \) at pH 8.3 | \( \tau_{\text{tail}} \) | \( I_{\text{tail}} \) at pH 8.3 | \( \tau_{\text{tail}} \) |
|------|---|---|---|---|---|---|
| Z93  | -0.14 ms | -0.14 ms | 0.20 ms | 1.6 ms | 0.20 ms | 1.0 ms | 183 |
| Z94  | 0.19 156 | 0.46 2.4 | 0.17 0.9 | 152 |
| Z95  | 0.25 229 | 0.86 3.4 | 1.6 233 |
| Mean ± SEM | 194 ± 21 | 25 ± 0.5 | 205 ± 30 | 1.2 ± 0.2 | 196 ± 30 |

(B) Simultaneous change of pH and veratridine concentration

| Cell | \( I_{\text{tail}} \) at pH 8.3 | \( \tau_{\text{tail}} \) | \( I_{\text{tail}} \) at pH 8.3 | \( \tau_{\text{tail}} \) |
|------|---|---|---|---|
| Z71  | 0.68 206 | 0.56 1.2 | 214 |
| Z72  | 0.22 242 | 0.25 1.0 | 211 |
| Z73  | 0.54 210 | 0.60 0.9 | 210 |
| Z70* | 0.55 221 | 0.56 1.0 | 230 |
| Mean ± SEM | 220 ± 82 | 1.0 ± 0.1 | 216 ± 5 |

*Sequence reversed in this experiment.

shows the tail current induced by intracellular application of 100 μM veratridine at pH 8.3. While maximum \( I_{\text{tail}} \) was comparable in cells A and B, sole application of intracellular veratridine at pH 8.3 produced a smaller \( I_{\text{tail}} \) (17% of maximum) as compared with that at pH 7.3. This difference, amounting to a factor of 2.3 on average, was statistically significant (Table II B).

The pH dependence of veratridine action during intracellular application was thus opposite to the pH dependence during extracellular application. While extracellular tests pointed to an exclusive role of the free base form, but excluded an extracellular site of action, the intracellular results rule out that the free base form is active either from inside the membrane or after diffusion into the lipid phase of the membrane,
**Figure 3.** Increasing extracellular pH compensates for reducing extracellular veratridine concentration. (A) Plot of \( I_{\text{out}} \) elicited by 50-ms depolarizations at 0.5 Hz to \(-30\) mV from \(-100\) mV holding potential (filled symbols) and \( I_{\text{inj}} \) (open symbols) as a function of time immediately before and during superfusion with veratridine as indicated in the upper bar. The first few pulses in veratridine were not recorded. After application of 100 \( \mu \)M veratridine at the standard extracellular pH of 7.3 until a steady-state effect was established, the microsuperfusion was switched to solution containing one-tenth of the veratridine concentration at a pH of 8.3. (B) \( Na^+ \) tail current records from the same experiment before veratridine application (a) and after equilibration with 100 \( \mu \)M veratridine at pH 7.3 (b) and subsequently 10 \( \mu \)M veratridine at pH 8.3 (c). Peak \( I_{\text{Na}} \) was truncated. Record a contaminated by veratridine leakage.
because Na channel modification decreased despite a 10-fold increase of the intracellular free base form (from 0.5 μM at pH 7.3 to 5 μM at pH 8.3). Hence, uncharged veratridine molecules mediate the action during cell superfusion, but they are not the active molecular species. We propose that veratridine acts from inside the membrane in its protonated form.

The loss of activity resulting from intracellular alkalinization during patch pipette application (Fig. 4, Table II) despite a nearly unchanged concentration (99.5 μM at pH 7.3, 95 μM at pH 8.3) can be explained by pH-dependent loss of veratridine via its free base form across the cell membrane. For a typical 20-μm-diam spherical cell, the cell membrane area (1,260 μm²) is 250-fold larger than the tip opening area of

![Figure 4](image)

**Figure 4.** Increasing intracellular pH has little effect on intracellularly applied veratridine. Na current records from two different cells (A, B) depolarized by 50-ms pulses at 0.2 Hz to -30 mV from -100 mV holding potential. (A) Record a was obtained 1 min after establishing the whole-cell clamp configuration with a pipette filled with 100 μM veratridine at the standard intracellular pH of 7.3. The veratridine-induced tail current had remained stable at the level shown since the initial amplifier adjustments. Record b was obtained 1.5 min later after additional extracellular superfusion with 100 μM veratridine at pH 7.3 and stabilization of the tail current. Record c was taken another 1.5 min later after addition of 1 μM BDF 9145 to the extracellular veratridine-containing superfusate. The resulting tail current represents the maximum veratridine effect on this cell. (B) The protocol for obtaining records a, b, and c was identical to that described for A except that intra- and extracellular pH was 8.3.

the patch pipette (∼5 μm²) through which veratridine diffuses into the cell, thus providing a large area for diffusional loss. Transmembrane loss of veratridine via its free base form can be compensated for by additional extracellular application of veratridine at the same pH and concentration chosen for the pipette filling solution. If loss occurs, the additional extracellular application should increase the veratridine effect. As shown by Fig. 4 and Table II, veratridine superfusion did in fact markedly augment *I*ₘ₉₉₉ by a factor of 2.3 at pH 7.3 and 4.4 at pH 8.3. Relative *I*ₘ₉₉₉ during simultaneous intra- and extracellular application of veratridine at pH 7.3 was not significantly different from that at pH 8.3 (Fig. 4, Table II). This is consistent with an intracellular site of action of the protonated veratridine molecule.
Increase of Intracellular Viscosity

Altering the viscosity of the reaction medium is a valuable tool to identify and localize diffusion-controlled reactions (Schurr, 1970; Miller, 1990). The rationale of this approach is based on the Stokes-Einstein relation, which states that the diffusion coefficient \( D \) of a solute of effective radius \( r \) varies inversely with the medium viscosity \( \eta \):

\[
D = \frac{kT}{6\pi r \eta}
\]

where \( k \) is the Boltzmann constant and \( T \) is temperature. Since an increase in viscosity impedes both association and dissociation (Schurr, 1970), our concept of veratridine action predicts that an increase of intracellular viscosity should slow dissociation of veratridine from its putative intracellular binding site into the intracellular medium and therefore slow down the decay of veratridine-induced tail current, while extracellular viscosity changes should not affect its time constant. Fig. 5 compares tail currents from four different cells, each of which was superfused with 100 \( \mu \)M veratridine plus 1 \( \mu \)M BDF 9145 for 1.5–3 min. Cell A served as control and \( \tau_{\text{tail}} \) was 0.24 s. The superfusate on cell B contained 1 m sucrose to increase extracellular viscosity, and the pipette was filled with 1 m urea to prevent osmotic shrinkage of the cell. The resulting \( \tau_{\text{tail}} \) was identical to that of the control cell. Cells C and D were treated identically, the superfusate containing 1 m urea and the pipette solution containing 1 m sucrose to increase intracellular viscosity. Evidently, this treatment resulted in a slowing of tail current decay. The tail currents of both cells were well described by a monoexponential function with \( \tau_{\text{tail}} = 0.51 \) s. Results from six control

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**TABLE II**

|                  | Veratridine intracellularly | Plus veratridine and BDF 9145 extracellularly |
|------------------|-----------------------------|-----------------------------------------------|
|                  | \( I_{\text{tail}} \)     | \( I_{\text{tail}} \)                        | \( I_{\text{tail}} \)                        |
|                  | \( -nA \)                  | \% of max.                                   | \( -nA \)                                   | \% of max. |
| Z159             | 0.45                       | 32.8                                         | 1.04                                        | 76.5       |
| Z160             | 0.63                       | 47.6                                         | 1.18                                        | 89.4       |
| Z161             | 0.39                       | 26.6                                         | 1.27                                        | 87.6       |
| Mean ± SEM       | —                          | 36 ± 6                                       | —                                           | 84 ± 4     |

**TABLE II**

|                  | Veratridine intracellularly | Plus veratridine and BDF 9145 extracellularly |
|------------------|-----------------------------|-----------------------------------------------|
|                  | \( I_{\text{tail}} \)     | \( I_{\text{tail}} \)                        | \( I_{\text{tail}} \)                        |
|                  | \( -nA \)                  | \% of max.                                   | \( -nA \)                                   | \% of max. |
| Z156             | 0.22                       | 16.5                                         | 1.09                                        | 82.0       |
| Z157             | 0.18                       | 2.9                                          | 1.08                                        | 60.3       |
| Z158             | 0.43                       | 21.1                                         | 1.37                                        | 67.5       |
| Mean ± SEM       | —                          | 16 ± 3*                                      | —                                           | 70 ± 6'    |

Veratridine concentration = 100 \( \mu \)M; BDF 9145 = 1 \( \mu \)M.

*Significantly different from pH 7.3 (\( P < 0.05 \)).

*Not significantly different from pH 7.3 (\( P > 0.1 \)).
Veratridine-induced tail current is slowed by increased intracellular, but not extracellular, viscosity. Na tail current records from four different cells (A–D) depolarized by 50-ms pulses at 0.2 Hz to −30 mV from −100 mV holding potential. Each cell was superfused with 100 μM veratridine for 2–3 min in the presence of 1 μM BDF 9145. (A) Control. (B) Superfusate contained 1 m sucrose and the intracellular solution contained 1 m urea. (C, D) Intracellular solution contained 1 m sucrose and the superfusate contained 1 m urea.

Cells and five cells subjected to extra- and/or intracellularly increased viscosity are summarized in Table III. Increasing the intracellular viscosity 1.6- and 2.5-fold increased $\tau_{\text{tail}}$ 1.5- and 2.3-fold, respectively.

**Cevadine Effect on Cardiac Cell Na Current**

The concept of saturable veratridine binding to the Na channels of single cardiac cells (Zong et al., 1992) would be further strengthened if similarly structured ligands
were shown to compete with veratridine for a common binding site. We therefore characterized the effect of cevadine (Fig. 1) on cardiac cell Na current (Fig. 6A). This cell was also treated with 1 μM BDF 9145 to remove inactivation. Similar to veratridine (cf. Fig. 4 of Zong et al., 1992), cevadine induced (a) a progressive decay of the noninactivating Na current during the 50-ms depolarization and (b) a tail current whose amplitude increased with cevadine concentration. As in the case of veratridine, the tail current decayed monoexponentially, but with a distinctly shorter time constant, 41–44 ms in the cell of Fig. 6A.

The dependence on cevadine concentration of $I_{\text{tail}}$ and $\tau$ determined in eight cells is shown in Fig. 6, B and C. Half-maximally effective concentration for $I_{\text{tail}}$ was

| \(n\) | \(\tau\) (ms) | \(\eta_0/\eta_i\) | \(\eta_0/\eta_c\) |
|---|---|---|---|
| 206 | 333 | 1.5 | 1.6 | 1.6 |
| 210 | 245 | 1.1 | 1.0 | 2.5 |
| 211 | 268 | 1.2 | 1.0 | 2.5 |
| 212 | 510 | 2.3 | 2.5 | 1.0 |
| 209 | 505 | 2.3 | 2.5 | 1.0 |

All experiments were done in the presence of 100 μM veratridine and 1 μM BDF 9145 applied extracellularly. i, intracellular; e, extracellular; t, experiments with sucrose-containing solutions; c, control experiments. Relative viscosity, \(\eta\), for sucrose-containing solutions was taken from Weast (1988).

*Intracellular solution contained 1 M urea.

Extracellular solution contained 1 M urea.

4 μM $\tau_{\text{tail}}$ did not change with cevadine concentration (1–100 μM) and the average was 49 ms (i.e., 22% of $\tau_{\text{tail}}$ induced by veratridine [226 ms, Zong et al., 1992]). In three cells examined in the absence of BDF 9145, cevadine (10–30 μM) induced a tail current with similar time constant ($\tau = 44 \pm 1$ ms).

If veratridine and cevadine compete for a common intracellular site, the effect of extracellularly applied cevadine, which has the same pKₐ value as veratridine (Shanes and Gershfeld, 1960), should also increase with an elevation of pH₀. Two experiments analogous to Fig. 2A using 10 μM (n = 1) or 30 μM cevadine (n = 1) gave a 2- and 1.6-fold augmentation of $I_{\text{tail}}$, respectively, upon elevating pH₀ from 7.3 to 8.3.
FIGURE 6. Effect of cevadine on cardiomyocyte Na current. All data from cells additionally equilibrated with 1 μM BDF 9145. (A) Na currents elicited by 50-ms depolarizations to −30 mV from −100 mV holding potential (0.2 Hz) in the presence of 1 μM cevadine (bath-applied, a) and after 2-min superfusion with 10 μM (b) and 30 μM cevadine (c). Tail currents were fitted by monoexponential functions with τ = 0.041 s (a), 0.043 s (b), and 0.044 s (c). (B) Relative I_{tail} (mean ± SEM) as a function of cumulatively increasing cevadine concentrations (3, 30, 100 μM or 1, 10, 30 μM) from the indicated number of cells. EC_{50} is 4 μM. (C) Plot of τ_{tail} (mean ± SEM) from the experiments shown in B; the straight line corresponds to the average of all τ_{tail} values (49 ms).

Competition between Veratridine and Cevadine

If veratridine and cevadine interact with the same site or overlapping sites of the Na channel, two easily testable predictions can be made for whole-cell Na current.

(a) Combining veratridine and cevadine at low concentrations such that each drug binds to a small, e.g. 20%, fraction of the Na channels should induce a tail current
with an initial amplitude corresponding approximately to the sum of amplitudes produced by each alkaloid component alone and with a biexponential decay in which the short time constant should correspond to cevadine dissociation ($\tau \approx 50 \text{ ms}$) and the long time constant to veratridine dissociation ($\tau \approx 230 \text{ ms}$).

(b) Adding a high (saturating) concentration of cevadine to a saturating concentration of veratridine should have little effect on the initial amplitude of the tail current, because cevadine cannot recruit additional Na channels but can only replace veratridine at a fraction of the Na channels. This argument implies that the conductance and open probability of veratridine- and cevadine-modified Na channels are identical. In previous experiments we found the elementary current level of cevadine-modified Na channels at $-30 \text{ mV}$ to be indeed identical to that of veratridine-modified channels (Honerjäger, Dugas, and Wang, 1990) and the gating kinetics to be similar. Furthermore, cevadine should speed the decay of the veratridine-induced tail current because the cevadine molecules which bind to a fraction of Na channels at the expense of veratridine-modified channels will dissociate about five times faster than veratridine upon repolarization. As in prediction (a), the tail current should then exhibit a biexponential time course with $\tau_{\text{tail}}$'s corresponding to cevadine and veratridine, respectively.

As shown by Fig. 7 and Table IV, both predictions were experimentally confirmed. Since saturation with veratridine or cevadine is difficult to achieve at normally inactivating Na channels, all experiments were performed in the presence of 1 $\mu$M BDF 9145.

(a) Superfusion with a mixture of 1 $\mu$M veratridine and 2 $\mu$M cevadine produced a biexponentially decaying tail current with time constants (60 and 204 ms) corresponding closely to those observed with cevadine or veratridine alone (Fig. 7A). The initial amplitude of the tail current was 63% of that observed with a saturating cevadine concentration. This is in good agreement with the 50% resulting from adding the effects of 1 $\mu$M veratridine (Fig. 3 in Zong et al., 1992) and 2 $\mu$M cevadine (Fig. 7B) alone.

(b) The addition of 100 $\mu$M cevadine to a saturating concentration of veratridine (30 $\mu$M) increased the decay rate of the veratridine-induced tail current markedly with little effect on its initial amplitude (Fig. 7B). The monoexponential decay in the presence of veratridine ($\tau \approx 212 \text{ ms}$) was converted into a biexponential decay with an additional fast component corresponding to cevadine dissociation ($\tau \approx 40 \text{ ms}$) and a nearly unchanged slow component ($\tau \approx 226 \text{ ms}$) corresponding to veratridine dissociation. The ratio of fast to slow components of tail current decay was 3.0 in good agreement with the ratio of molar cevadine to veratridine concentration (3.3). This agreement is predicted by the near equipotency of cevadine with respect to their effect on $I_{\text{tail}}$ in our experiments. Table IV lists this experiment together with three trials using 30 $\mu$M cevadine. These experiments yielded results also in accordance with the prediction for combining saturating alkaloid concentrations. Adding 30 $\mu$M cevadine to 30 $\mu$M veratridine had little effect on the initial amplitude of tail current, but induced a component of fast decay (amounting to 40% of total decay) at the expense of the slow veratridine-induced decay. The protection against veratridine modification provided by cevadine is clearly visualized by the cevadine-induced decrease of tail current amplitude measured 86 ms after repolarization, at which
FIGURE 7.
Table IV

| Cell   | \(I_v\)       | \(\tau_v\)  | \(I_c\) | \(\tau_c\) | \(I_v + I_c\) | \(\tau_v + \tau_c\) | \(I_v + I_c\) | \(\tau_v + \tau_c\) |
|--------|----------------|-------------|---------|-------------|---------------|----------------------|---------------|----------------------|
|        | \(nA\)        | ms          | \(nA\)  | ms          | \(nA\)        | ms                   | \(nA\)        | ms                   |
|        |                |             |         |             |               |                      |               |                      |
| Z141   | 2.39           | 308         | 1.22    | 1.46        | 0.54          | 46.5                 | 314           | 1.12                 |
| Z154   | 0.94           | 256         | 0.26    | 0.75        | 0.74          | 63.6                 | 278           | 1.08                 |
| Z155   | 0.53           | 193         | 0.51    | 0.35        | 0.53          | 28.4                 | 217           | 1.26                 |
| Mean ± SEM | —             | —           | —       | 0.60 ± 0.07 | —             | —                   | 1.15 ± 0.06   | —                   |
|        |                |             |         |             |               |                      |               |                      |
| Z121   | 1.69           | 212         | 1.17    | 0.39        | 0.25          | 40.0                 | 226           | 0.93                 |
| Mean ± SEM | 242 ± 26      | —           | —       | —           | 44 ± 7        | 259 ± 23             | (\(n = 4\))  |                      |

Tail currents for veratridine only were fitted by the function \(-I_v \cdot \exp(-t/\tau_v)\), for veratridine + cevadine by the function \(-I_v \cdot \exp(-t/\tau_v) - I_c \cdot \exp(-t/\tau_c)\).

Discussion

This study has defined in further detail the veratridine recognition site of the cardiac Na channel by experimental results that are consistent with the protonated form of veratridine being the active molecular species and the intracellular aspect of the channel macromolecule being the site at which the alkaloid acts. In the accompanying paper (Zong et al., 1992) we have shown the saturable nature of the veratridine effect on whole-cell Na current. Our present evidence that cevadine reduces the veratridine-induced tail current in the presence of a saturating veratridine concentration strengthens the concept that veratridine binds to a limited number of sites on the point most of the fast cevadine-induced component has decayed and veratridine-modified channels determine the tail current (Fig. 7 B, inset).

Figure 7. (opposite) Interaction between low concentrations (A) and high concentrations (B) of veratridine and cevadine. Records are from two different cells, both of which were additionally treated with 1 \(\mu M\) BDF 9145. (A) Na current elicited by 50-ms depolarization to \(-30\) mV from \(-100\) mV holding potential (0.2 Hz) after 2-min equilibration with a mixture of 1 \(\mu M\) veratridine and 2 \(\mu M\) cevadine. Tail current amplitude was 63% of maximum \(I_{\text{tail}}\) determined subsequently in the presence of 1 \(\mu M\) BDF 9145 and 30 \(\mu M\) cevadine (not shown). Tail current (nanoamperes) as a function of time \(t\) in seconds was fitted by the biexponential function \(-1.24 \exp(-t/0.050) - 0.47 \exp(-t/0.204)\). The second term (veratridine component) is shown as a dotted line. (B) Na current elicited by identical pulse protocol after 1-min equilibration with 30 \(\mu M\) veratridine (a) and after 2-min subsequent exposure to a mixture of 30 \(\mu M\) veratridine and 100 \(\mu M\) cevadine (b). Note the reduction of tail current by cevadine. Tail current a was fitted by the monoexponential function \(-1.69 \exp(-t/0.212)\) and tail current b by the biexponential function \(-1.17 \exp(-t/0.040) - 0.39 \exp(-t/0.226)\). Smooth lines are fitted functions and vertical lines mark start of fit. (Inset) Tail current amplitude measured 86 ms after repolarization plotted for the time interval immediately before and after addition of cevadine.
the cardiac cell and suggests a competitive interaction between these two ceveratrum alkaloids which differ only in the nature of their C-3 ester substituent. Although previous studies did not explicitly attempt to identify the active form and site of action of veratridine, we shall discuss related previous experimental contributions on the mode of action of veratridine and the three other activator ligands thought to share their site of action, called site 2, with veratridine: batrachotoxin, grayanotoxins, and aconitine (Catterall, 1977). We conclude by describing our current view of the molecular mechanism of action veratridine and other ceveratrum alkaloids at the cardiac Na channel.

**Effect of pH on the Action of Veratridine and Batrachotoxin**

Our results are in agreement with Ulbricht's (1969) finding of a stronger veratridine effect at pHo 8.1 as compared with pHo 7.2, where the effect was measured as depolarization of the resting membrane in the frog node of Ranvier. Warnick, Albuquerque, Onur, Jansson, Daly, Tokuyama, and Witkop (1975) investigated the pHo dependence of action of batrachotoxin, a Na channel activator thought to share its site of action with veratridine (Catterall, 1977). Increasing pHo in the range 6.0–9.0 increased the extent and rate of the depolarization induced by batrachotoxin in rat skeletal muscle, the depolarization reflecting persistent activation of Na channels. To Warnick et al. (1975) these results suggested "that the most active form of batrachotoxin is the free base."

Our results confirm this interpretation for extracellularly applied veratridine, but the true active form of batrachotoxin, like that of veratridine, could be the cationic form acting intracellularly with the free base form providing transmembrane access for the alkaloid during extracellular application.

Could pH-dependent alterations of the veratridine receptor rather than changes of the ionization of veratridine explain our results? The slow increase of the veratridine effect in response to a fast alkalinization of the extracellular superfusate (Fig. 2), which would immediately affect the ionization of extracellular residues on the channel, argues against sensitivity to pHo of the veratridine binding site in the range 7.3–8.3.

During intracellular veratridine application (and simultaneous extracellular application in order to compensate the loss of veratridine across the sarcolemma) the veratridine effect on cells with a pHi of 7.3 did not differ from that on cells with a pHi of 8.3. Since the concentration of protonated veratridine molecules changes by <5% over this pH range, we propose that this form of veratridine is the active form. If, contrary to our view, intracellular veratridine were active in its free base form, the increase of pHi by one unit, which increases uncharged veratridine concentration 10-fold, should have blocked 90% of the veratridine receptors in order to account for the observed unchanged effect. A block by this extent seems very unlikely since ionization of most amino acids changes by <1% over this pH range and that of the most sensitive residue, cysteine (pKa = 8.5), by only 40%.

**Kinetics of the Veratridine Effect**

We have shown that the effect of superfused veratridine on a single cardiomyocyte develops over 1–2 min, although the extracellular veratridine concentration was
established within 0.1 s by the microsuperfusion device. The effect, measured as tail
current amplitude every 5 s, monitors the number of Na channels that react with
veratridine during the immediately preceding 50-ms pulse (Sutro, 1986; Hille,
Leibowitz, Sutro, Schwarz, and Holan, 1987; Zong et al., 1992). The slow onset of the
effect thus shows that veratridine has to diffuse to and accumulate in a space other
than the extracellular space in order to be reactive.

This conclusion confirms a kinetic analysis on the frog node of Ranvier Na current
(Ulbricht, 1972) which showed that the onset of veratridine action during alkaloid
superfusion is too slow to be accounted for by diffusion to the extracellular surface of
the axolemma.

**Defining the Intracellular Site of Action**

During extracellular application, we have shown a close correlation between the free
base form of veratridine and its effect on Na current when pH was changed at
constant veratridine concentration (Fig. 2) or when pH and total veratridine
concentration were altered such that the free base concentration remained un-
changed (Fig. 3). During intracellular application, a route that has previously been
shown in the squid giant axon to be effective for ceveratrum alkaloids (Meves, 1966;
Honerjäger, 1973), batrachotoxin (Narahashi and Deguchi, 1971), and grayanotox-
ins (Seyama and Narahashi, 1973) and for aconitine in neuroblastoma cell outside-
out patches (Negulyaev, Vedernikova, and Savokhina, 1990), we have seen a close
correlation between the charged and presumably membrane-impermeant form of
veratridine and its effect on Na current (Fig. 4; Table I), suggesting that veratridine
acts in its protonated form at the intracellular side of the Na channel.

Evidence from earlier work points to an intracellular site of action of site 2 ligands.
The effect on Na current of the semisynthetic ceveratrum alkaloid germine-3-acetate,
applied extracellularly to an intracellularly perfused squid axon, is inversely related
to the rate of intracellular perfusion, suggesting that the latter effectively dilutes the
alkaloid concentration at its intracellular site of action (Honerjäger, 1973). This
relationship has been confirmed for the depolarizing effects of veratridine, batracho-
toxin, aconitine, and a grayanotoxin (Seyama, Yamada, Kato, Masutani, and Ha-
mada, 1988). These authors conclude that these toxins "may reach a site only
accessible from the internal surface of the membrane" in accordance with our
conclusion concerning germine-3-acetate (Honerjäger, 1973) and the conclusion
derived in the present work for veratridine.

Defining the active form and site of action of weak bases, such as veratridine, is
complicated by their transmembrane diffusion and subsequent protonation which
occurs irrespective of extra- or intracellular application. Principally, veratridine could
act in both its molecular forms, each either binding to one common site or to its
separate site (see Howe and Ritchie, 1991). However, the fact that veratridine-
modified Na currents are well described by assuming a single association and
dissociation rate constant at both the macroscopic (Zong et al., 1992) and micro-
scopic levels (Wang, Dugas, Armah, and Honerjäger, 1990) makes these theoretical
possibilities very unlikely. For defining the single active molecular species, variation
of extracellular or intracellular pH is a valuable approach, and pH effects other than
those on the degree of veratridine ionization seem unlikely. Nevertheless, an
independent method to define the site of action of membrane-permeant channel ligands seems desirable.

The rate of ligand association to its binding site from the aqueous solution surrounding the target protein is controlled by its diffusion coefficient, and the rate of ligand dissociation into the aqueous medium is also affected by the diffusion coefficient (Schurr, 1970). Since the diffusion coefficient is inversely related to viscosity, an increase in viscosity of the reaction medium slows both ligand association and dissociation rates (Schurr, 1970). Miller (1990) has exploited these principles in an electrophysiological study on the possible diffusional control of charybdotoxin binding to single Ca-activated K channels.

Inspired by the article of Miller (1990) we have adopted the viscosity test to determine the sidedness of veratridine action in single cardiomyocytes in the whole-cell clamp configuration. The monoexponential decay of the veratridine-induced tail current, which is particularly suitable for quantitative evaluation, is thought to reflect dissociation of veratridine molecules from Na channels to which it bound during the preceding activating pulse (Sutro, 1986; Hille et al., 1987; Wang et al., 1990; Zong et al., 1992). If this view is correct and if veratridine dissociation occurs from an intracellular binding site of the Na channel into the cytoplasmic microenvironment, then $t_{\text{tail}}$ should be sensitive to intracellular, but not to extracellular, viscosity.

Both predictions were found to be correct, and the sensitivity to intracellular viscosity was quantitatively consistent with a direct relation between $t_{\text{tail}}$ and intracellular veratridine dissociation rate (Fig. 5; Table III). The results suggest that veratridine does not dissociate into the extracellular space or into the lipid microenvironment of the Na channel but exclusively into the intracellular space.

In addition to defining the sidedness of veratridine action, the viscosity effect is valuable in identifying tail current decay as a direct consequence of the chemical process of veratridine dissociation from its receptor. This view, originally developed by Sutro (1986), has recently been challenged by Rando (1989). After a conditioning pulse that activates Na channels and induces a veratridine modification in the frog node of Ranvier, he observed peak Na current to recover more slowly than would be expected if veratridine unbinds in parallel to tail current decay making Na channels immediately reavailable for voltage activation. According to Rando (1989), the tail current therefore reflects transition of veratridine-modified open Na channels into closed ("inactivated"), veratridine-bound channels from which veratridine would dissociate much more slowly and would be undetectable by electrical measurement.

Two independent arguments obtained on cardiac cells are at variance with this interpretation: the viscosity effect shown here and the behavior of single Na channels comodified with the allosteric activator BDF 9145 (Wang et al., 1990). The latter study has shown that a given single Na channel, after going through the typical low-conductance, burst-like modification induced by veratridine, does not enter a closed ("inactivated") state but is immediately available for another veratridine modification with a latency inversely related to the veratridine concentration bathing the intracellular membrane surface. The termination of the veratridine-induced Na current can therefore be equated with veratridine dissociation and its onset with veratridine association (Wang et al., 1990).
Intracellular Action of Veratridine and Cevadine

We have now provided four independent lines of evidence that support the concept of an intracellular site of action of the protonated form of veratridine: (a) the slow onset of action during extracellular application; (b) the close association of the effect with the free base and presumably membrane-permeant form during extracellular application and with the protonated presumably membrane-impermeant form during intracellular application; (c) the increase of \( \tau_{\text{tail}} \) in response to an increase of intracellular, but not extracellular, viscosity; and (d) in a separate study on veratridine reaction dynamics at activated single cardiac Na channels, we determined a veratridine association rate of \( 4.3 \times 10^6 \, \text{M}^{-1}\text{s}^{-1} \) with the drug applied intracellularly (Wang et al., 1990), a value similar to that observed in single neuronal Na channels (Barnes and Hille, 1988). If calculated for the free base form rather than for total veratridine concentration, and disregarding the possibility of free base accumulation in a hydrophobic space, a value of \( 8.6 \times 10^5 \, \text{M}^{-1}\text{s}^{-1} \) is obtained. This value is close to the maximum of a diffusion-controlled encounter frequency and would imply that the free base form of veratridine binds to the receptor site during each diffusional collision irrespective of its conformation and its orientation relative to that site, and irrespective of the momentary conformation of the veratridine-binding site. This seems very unlikely for a recognition site that interacts specifically with at least one group of the alkaloid, the C-3 ester substituent, as shown by the difference between veratridine and cevadine dissociation rates (see below). Thus, we conclude that the association rate constant is consistent with the protonated form of veratridine being the reactive molecular species.

**Effect of Cevadine**

In addition to the protonated nitrogen, the veratric ester group of veratridine appears to play a decisive role in stabilizing the veratridine–Na channel complex. This interpretation is based on the shorter \( \tau_{\text{tail}} \) induced by cevadine, indicating a five times faster dissociation from the Na channel of this alkaloid (Fig. 6) which carries a less lipophilic ester group (angelic acid) at C-3 and probably binds to the same site as veratridine as suggested by their mutually exclusive effects (Fig. 7 B; Table IV). Veracevine, the unesterified alkamine, shows a still faster dissociation rate in the millisecond range (Honerjäger et al., 1990). The lipophilicity provided by the C-3 substituent decreases in the order veratridine > cevadine > veracevine as determined by the partitioning between water and chloroform phases (Büch, 1976). The shorter \( \tau_{\text{tail}} \) induced by cevadine in comparison to veratridine (49 vs. 226 ms) in the cardiomyocytes confirms the kinetic difference found for frog muscle Na current (Leibowitz, Schwarz, Holan, and Hille, 1987) and is also in line with earlier work showing that cevadine induces shorter-lasting afterdepolarizations in various nerve preparations than in veratridine (Shanes, 1952; Honerjäger, 1973). The possibility of competitive interactions between ceveratrum alkaloids has not been previously examined by electrophysiological experiments. In Na flux studies on neuroblastoma cells, where cevadine itself does not increase Na influx, cevadine has been shown to reduce Na channel activation by veratridine in line with a competitive interaction (Honerjäger, Frélin, and Lazdunski, 1982).
**Molecular Aspects**

The most detailed hypothesis for the molecular mechanism of action of veratridine has been developed by Hille et al. (1987). According to these authors, the conformational changes associated with the opening of the Na channel macromolecule expose a receptor for veratridine "that we suppose faces the hydrophobic interior of the lipid bilayer... When agonist binds to the exposed receptor, it could form a wedge between domains and stabilize the open state while distorting the macromolecule sufficiently to enlarge the selectivity filter. This stabilization hinders channel closing by either of the two normal mechanisms, deactivation or inactivation. If the modified channel does close, the receptor pocket narrows and presses on the drug. This strain favors reopening of the modified channel even at potentials more negative than usual."

Our experiments suggest that the veratridine receptor faces the intracellular aqueous phase rather than the hydrophobic interior of the cell membrane and that it reacts with positively charged intracellular veratridine rather than intramembraneous nonionized alkaloid molecules. By analogy with other cation-binding receptors, the veratridine receptor is likely to contain a negatively charged residue to form a salt bridge with the ammonium group of veratridine. In addition, we propose an adjacent lipophilic group that forms a hydrophobic bond with the veratric acid ester group in veratridine or, in less stable fashion, with the angelic acid ester group in cevadine.

These proposed structural features of the veratridine binding site could be verified and identified at the amino acid level by probing the veratridine effect on Na channels exposed to appropriate site-directed antibodies or modified by site-directed mutagenesis. The intactness of the intracellular peptide loop connecting segment 6 of domain III with segment 1 of domain IV, probed by liganding it with a site-directed antibody (Vassilev, Scheuer, and Catterall, 1988) or by cutting the coding mRNA for this loop (Stühmer, Conti, Suzuki, Wang, Noda, Yahagi, Kubo, and Numa, 1989), has been shown to be essential for the fast inactivation gating process in native skeletal muscle Na channels and a cloned rat brain sodium channel, respectively. It is therefore possible that cationic veratridine binds to one of the known carboxylates on this loop (Asp-1486, Glu-1491, or Glu-1492 in the rat heart Na channel), which have been shown to be highly conserved in a wide range of Na channel subtypes in different species (Rogart et al., 1989). A possible role of these two glutamates in inactivation gating has recently been considered on the basis of negative results when positive charges of the III-IV linker were eliminated by site-directed mutagenesis (Moorman, Kirsch, Brown, and Joho, 1990). Molecular identification of the veratridine recognition site should reveal a transiently accessible region of the Na channel macromolecule, exposed by Na channel opening, that is directly or allosterically coupled to several functions including inactivation, deactivation, conductance, and ion selectivity.

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