

ANALYSIS OF VERATRINE EFFECTS ON HELIX NEURONS: A POSSIBLE EPILEPSY MODEL

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INTRODUCTION

It has been known for more than 100 years that extracts from *Veratrum* species have strong physiological effects. This extract, veratrine, contains a group of steroid alkaloids, veratridine and cevadine being the major constituents (6). The experimental study of veratrine effects on excitable tissues commenced in the twenties of this century. Riesser (12) found that veratrine at low concentrations altered the muscle contractions in preparations from a wide variety of invertebrate species. They became longer and/or stronger and in certain cases repetitive twitches occurred. As judged from the nature of electro-mechanical coupling in smooth muscles, these phenomena indicate altered electrical properties of the muscle membrane. Similar effects of veratrine on vertebrate skeletal muscle and heart have been described (7). Contractures lasting several minutes or tens of minutes were observed. More recent findings concerning the electrical behaviour of the muscle membrane have been published by the Varga group (3), using mainly cevadine. They found a massive depolarization lasting for tens of minutes accompanied by a strong sodium influx (4).

The action of *Veratrum* alkaloids on neural elements has also been investigated by several authors. Squid axons under the effect of 20 $\mu\text{g/ml}$ veratridine were found to produce lasting depolarizations triggered by a spike (8). Alternatively, spike potentials were followed by strong and slowly decaying after-depolarizations, which, in certain cases, gave rise to repetitive discharge. Ranvier nodes of frog sciatic nerve responded to veratridine in a similar manner. Ulbricht (15) described steady membrane depolarizations and depolarizing afterpotentials following spike discharges. The current-

voltage characteristic changed to an N-shaped type. Both this author and Straub (13) noted that all the effects of veratridine were bound to the presence of sodium.

The influence of veratridine on snail neurons has been studied by Leicht et al. (8). According to their findings, 10 μ g/ml veratridine evoked long-lasting depolarization and sometimes oscillations of the membrane potential. Action potentials were followed by long after-depolarizations. The current-voltage curve displayed a region of negative slope resistance. These phenomena turned out to be sodium-dependent but were contrary to findings on muscle membranes, insensitive to tetrodotoxin. Slowly developing, long-lasting inward currents were described under effect of veratridine in voltage clamp conditions (9).

Considering the results reviewed above, we thought it promising to examine the effects of veratrine on *Helix* neurons from the point of view of experimental epilepsy. On the basis of their hyperactivity- and burst-inducing properties, these alkaloids can be considered as convulsants and may be suitable for creating models of epileptic phenomena at cellular level.

METHODS

Preparation

The experiments were done on the suboesophageal ganglion group of *Helix pomatia*. The ganglion was dissected out of the animal and the connective tissue was removed under Ringer's solution. The preparation was fixed with steel needles to the Sylgard bottom of a translucent chamber, being continuously perfused at a rate of 1 ml/min. It was illuminated from below and viewed through a stereomicroscope. The electrode was positioned by means of a mechanical manipulator.

Registration

A single microelectrode with resistance of 2-6 MOhm, filled with KCl-K-citrate 1:1 M, was introduced into the cell. The electric activity was led to a single-electrode device suitable for current- and voltage clamp operation. Command pulses were given by a square- and ramp-pulse generator. Output signals were fed into a Tektronix storage oscilloscope and an X-Y

plotter (for drawing current-voltage curves). The sampling cycle of the voltage clamp was continuously monitored on a separate scope. For optimal clamping and time resolution, sampling frequency, time constant and gain had to be set for each neuron. Drugs were applied to the neurons by continuous perfusion. Experiments were done at room temperature.

The neuronal activity was visualized, stored and photographed on the Tektronix screen. Current-voltage curves were drawn by the X-Y plotter in voltage clamp mode, using slow (20-25 mV/s) ramp pulses. Part of the records was corrected for leakage. Currents obtained with equal de- and hyperpolarizations from holding potential were evaluated and summed algebraically. The leakage was supposed to be linear. At I-V curves, the correction was done at every 10 mV on the active, depolarizing limb and the curve was redrawn according to the original.

Solutions and agents used were as follows:

Ringer	Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺	Tris ⁺	(in mM)
normal	80	4	7	5	5	
Na-free	0	4	8	5	85	(all in Cl ⁻ form)

Agents

veratrine 5, 10, 15, 20, 30 μ g/ml
 tetraethylammonium (TEA) 30 mM (in Br⁻ form)
 tetrodotoxin (TTX) 10 μ M
 Co²⁺ 15 mM (in Cl⁻ form)
 Ni²⁺ 15 mM (in Cl⁻ form)

Veratrine, TTX and Tris were from Sigma, TEA was from Aldrich, all other compounds were from Reanal. The pH was set to 7.5. No osmolarity compensation was made.

RESULTS

Studies in current clamp

On the basis of previous experience (Erdélyi, unpublished), veratrine was applied at a concentration of 30 μ g/ml and its effect was examined in current clamp mode. It soon turned out, however, that at this dose neurons soon go to the final state of veratrine effect and remain permanently depo-

larized. Thus, lower concentrations were applied, too (see Methods). It is noteworthy that, after some time, the activity of the neurons studied reached the same final state at any veratrine concentration and only the speed of development of effects was concentration dependent. In Fig. 1 the effects of 10 and 20 $\mu\text{g/ml}$ veratrine on the neuronal activity and basic membrane parameters are demonstrated. According to chromatographic analyses (6) these concentrations are equivalent to 6 and 12 μM pure veratridine, respectively.

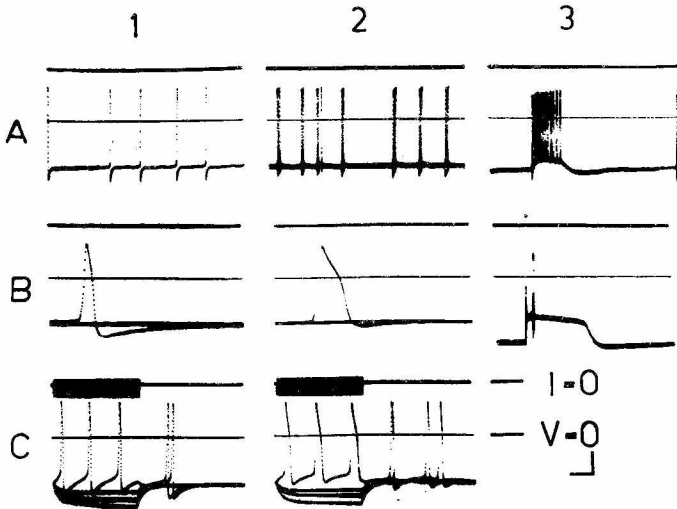


Figure 1. Effects of veratrine on the neuronal activity and basic membrane parameters.

1. Control. A: spontaneous activity. B: a single spike. C: effects of de- and hyperpolarizing current pulses from -1 to $+3$ nA.
2. As in 1, but recorded after 15 min of 10 $\mu\text{g/ml}$ veratrine application. A: The activity turned into bursting and a small after-depolarization appeared following the bursts. B: the spike has been broadened and the negative afterpotential reduced in length and amplitude. C: action potentials became broader without appreciable change in resting potential, membrane resistance and time constant (as judged from the onset, steady level and decay of hyperpolarizations).
3. Further development of veratrine effect in time. Concentration was raised to 20 $\mu\text{g/ml}$ in the 35th min. A: spontaneous PDS taken in the 55th, B: another one in the 135th min. The amplitude and length of plateaus increase with lapse of time.

Calibration: 20 mV voltage, 5 nA current for each part. Time: 1 s for 1/A, 20 ms for 1/B and 2/B, 100 ms for 1/C and 2/C, 5 s for others. Zero potential and current levels are indicated by continuous lines.

The neuron was a pacemaker one beating with some irregularity, its resting potential was about -45 mV (1/A). The spike, moderately wide (10 ms at

half amplitude) had a very little plateau and a considerable after-hyperpolarization (1/B). In the 15th min of veratrine action (column 2) the spike activity markedly changed (2/A), short bursts instead of single spikes occurred. The hyperpolarizing afterpotential decreased within the bursts and a weak after-depolarization appeared at the end of them. The membrane potential did not change. (Note different time scales between 1/A and 2/A.) Spike potentials also showed alterations (2/B): their width was more than twice the control value. The plateau was broadened and the decaying limb slowed down. Decrease of the after-hyperpolarization is clearly seen. Records in row C show the effects of current steps. Neither time course nor steady value of the potential changes caused by hyperpolarizing current steps were altered by veratrine. Values of membrane potential, resistance and time constant are summarized in Table 1.

Table 1. Membrane parameters of the neuron in Fig. 1 and the effect of veratrine

	resting potential (mV)	resistance (MOhm)	time constant (ms)
control	46	8.1	36
veratrinized	46	8.3	41

Further development of veratrine effect can be seen in column 3, Fig. 1. After 35 min the concentration was raised to 20 $\mu\text{g/ml}$. The small after-depolarization seen in 2/A grew to a typical paroxysmal depolarization shift by the 55th min (3/A). It was of moderate amplitude yet, as no spike inactivation occurred during its plateau phase. Only the first spikes showed some after-hyperpolarization. The PDS occurred and ceased spontaneously and had some negative afterpotential. A next PDS was triggered by a short current pulse in the 145th min (3/B). Its plateau was higher and more rectangular in shape than the previous one. Its decay had two phases: the plateau was slowly falling and this turned abruptly into a fast decay continuing in an after-hyperpolarization. Here, even the first spike is without negative afterpotential. The ones riding on the PDS seem to have it, but, as they do not reach the resting level, are not really negative. The burst-type activity induced by veratrine had always a time course like that described above. At the beginning, the bursts were short and their after-depolarization wave was small. With lapse of time this wave increased

in height and length. At first this produces a prolonged burst with spikes riding on the wave. Finally the wave amplitude exceeds spike inactivation level and a pure, square-shaped PDS appears, occasionally without any spike even at the beginning. Such a sequence is demonstrated also in Fig.3.

In accordance with findings of others (8, 10, 15), neurons were sometimes permanently depolarized by veratrine. This required more than half an hour to set in. The regenerative spike mechanism was not destroyed in these cells. Applying a constant hyperpolarizing current we could bring the membrane potential close to the normal resting level. With brief current pulses from here spikes and bursts or PDSs could be elicited. Fig. 2/E and F shows such records.

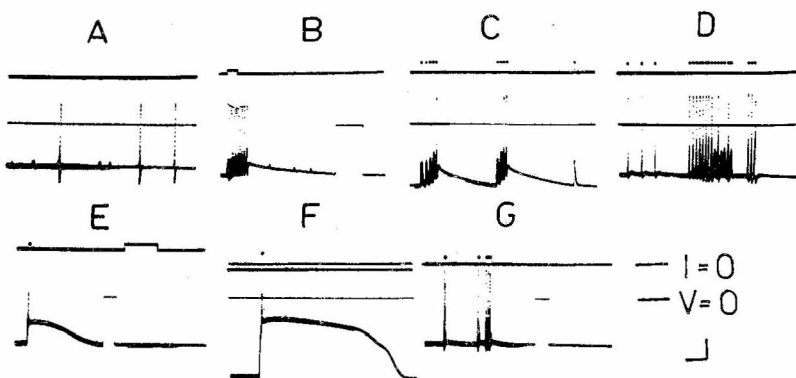


Figure 2. Development of veratrine action and its ionic dependence.

A: Control activity. B: Evoked burst after 4 min in 30 µg/ml veratrine. C: In Na-free Ringer (5 min), spikes are triggered only by stronger currents, bursting is absent and plateaus decay rapidly. D: 10 min later in Na-free Ringer, single spikes but no bursts and plateaus are evoked even by frequent trains. E: Returning to normal Ringer, the neuron got depolarized. It was kept at normal resting potential by continuous current injection and a PDS is triggered by a brief depolarization. F: Application of 15 mM Co^{2+} . The neuron still had to be kept hyperpolarized. A depolarizing pulse evoked a rudimentary spike and a huge, lengthy PDS. G: Again in Na-free Ringer. Spontaneous depolarization ceased. Full-sized spikes could be evoked but they did not trigger a PDS. Calibration of voltage and current as in Fig. 1. Time: 1 s for A to D, 5 s for E, F, G. Zero levels indicated by continuous lines or brief jumps to that level (in B, E, G).

It is generally assumed that Veratrum alkaloids affect sodium channels. Using Na-free Ringer we tested if the action of veratrine is in relation to them in *Helix* neurons. Bursting activity was induced in several cells by veratrine and then Na was replaced by Tris in the perfusion fluid.

Bursts and PDSs seen under veratrine effect disappeared or were strongly reduced in Na-free Ringer, while spike potentials remained. Fig. 2 (A-D and G) and Fig. 3 (1/A, 2/C) show the effect of Na-removal. Preservation of spikes in Na-free Ringer means that they are carried by ions other than Na. This component (probably Ca-current) was apparently not affected by veratrine.

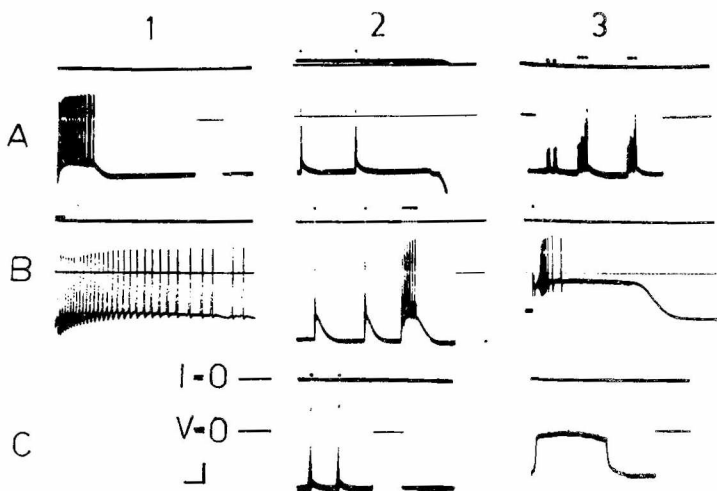


Figure 3. Ionic dependence of veratrine effect.

1. A: PDS from a neuron at the 20th min of 20 $\mu\text{g/ml}$ veratrine action. B: Beginning of PDS recorded with faster speed. Spikes on the plateau are characteristically broadened.
 2. A: Effect of 15 mM Ni^{2+} at 15 min of application. The neuron got hyperpolarized and was kept at normal resting level by current injection. Further depolarizations (short jumps on the current line) elicit a weak spike and a minimal after-depolarization. B: Ni^{2+} washed out. Spike discharges and plateaus could be elicited by current pulses. No spontaneous activity. C: In Na-free Ringer only spikes could be evoked without plateaus.
 3. A: Simultaneous effect of Na-deprivation and Ni^{2+} . Neither spikes nor depolarizing waves could be evoked even by impulse trains. B: Na concentration restored, a current pulse evoked a typical PDS with small spikes. C: Spontaneous late type PDS recorded in the wash out period.
- Indication of zero levels as in Fig. 2, calibration of voltage and current as in Fig. 1. Time: 0.5 s for 1/B, 5 s for all others.

In order to elucidate the site of action of veratrine on the neuron membrane, it was of interest to know by what means the veratrine-induced current could be blocked. The current being Na-dependent, the first choice would have been TTX, but it has been published (8) that on snail neurons TTX did not block the slow current induced by veratridine. Therefore we

decided to test the effect of both TTX and some divalent cations known as Ca-channel blockers. In accordance with findings referred to above, TTX was not able to block the veratrine-current in our experiments. Fig. 5 shows that the current did not vanish or decrease but even went on growing. It is important to note that normal spikes of Helix neurons are also TTX resistant (11). Two divalent cations, Co^{2+} and Ni^{2+} were tested as possible blockers. Co^{2+} reduced or blocked the spikes but was largely ineffective in blocking the depolarization waves. Fig. 2 shows that in a medium with 15 mM Co^{2+} veratrine still can cause a PDS. Also steady depolarization continued, indicating that the non-inactivating part of the veratrine-current is also resistant to this concentration of Co^{2+} . Ni^{2+} is known to be a more potent blocker of TTX resistant inward current than Co^{2+} . In 15 mM concentration it greatly reduced both the spikes and the depolarizing waves triggered by them. The neuron in Fig. 3 went over into a deep hyperpolarization on application of Ni^{2+} . It had to be kept at a normal level by current injection; without that the potential went to more negative (part 2/A, hyperpolarization out of screen). Spikes evoked under Ni^{2+} effect were markedly reduced in height and after-depolarizations following them were short. This blocking effect was not significantly increased by simultaneous Na-removal (3/A in Fig. 3). Ni^{2+} was thus very effective in blocking both the normal and the veratrine-induced inward current.

Studies in voltage clamp

By means of single-electrode voltage clamp we analysed the I-V curves of the neurons in normal and veratrinized state. The first curve in Fig. 4 represents the control state of Helix neurons. A value close to the normal resting level was chosen as holding potential; here it was -50 mV. On hyperpolarization a purely ohmic leakage current developed. It was mostly small, correction for leakage was not usually necessary. On depolarization, the slow outward current could be seen. Due to the time dependence of this potassium-current, the curve had a hysteresis. Sharp distortions on its upstroke are spike artifacts from poorly clamped axonal area. Application of veratrine (15 $\mu\text{g}/\text{ml}$ for 7 min) caused no major changes. The hysteresis moderately grew and the intersections of the curve (both at upstroke and downstroke) with the abscissa shifted to more positive direction. These phenomena indicate that a voltage-dependent inward current began to develop, and, on the other hand, the membrane without voltage clamp would have

been depolarized, probably due to a persistent inward current. To obtain the veratrine-induced current in pure form, voltage-dependent K-current was suppressed by TEA. To have a rapid onset, but slow development of the veratrine effect, its concentration was lowered to 10 $\mu\text{g/ml}$. The third curve shows how suppression of K-current unmasks the veratrine effect. To prevent a partial persistent inactivation of the veratrine-current the holding potential was raised to -80 mV. Now (fourth curve) the slow inward current was the only active one. It was first activated at -45 mV. Its maximum was at zero potential and inactivated fully, reaching $+20$ mV. The downstroke of the curve shows small if any inward current indicating that inactivation is removed slowly. This holds also for the persistent part of the inward current shown by the difference between the leakage lines (leftmost) of upstroke and downstroke.

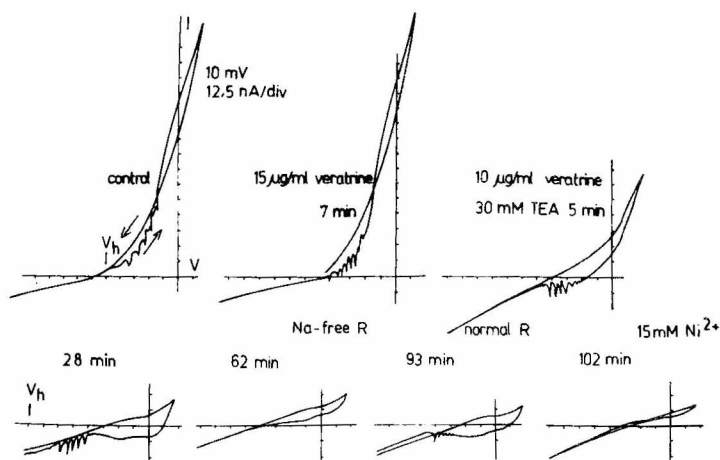


Figure 4. The effect of veratrine on the I-V curves alone and in combination with TEA. Records not corrected for leakage. Holding potential -50 mV for the first 3 curves, -80 mV for the others. Rate of ramp command: 20 mV/sec.

The control I-V curve was not much altered by $15 \mu\text{g/ml}$ veratrine in 7 min. On depression of the slow K-current by 30 mM TEA a slow inward current appears, rendering the curve N-shaped. The inward current disappears in Na-free Ringer or in 15 mM Ni^{2+} . A continuous shift of the resting membrane potential (where holding current is zero) towards 0 mV can be seen. Continuous time marks are for veratrine, other effects recorded when fully developed.

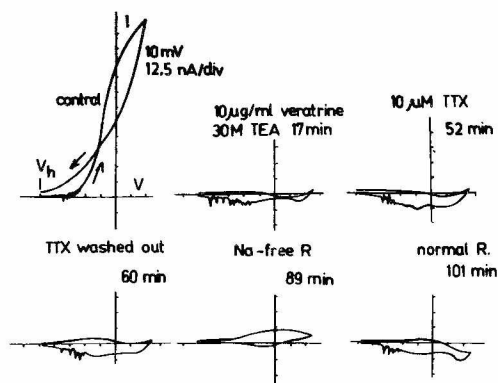


Figure 5. The veratrine-induced slow inward current is Na-dependent but resistant to TTX. Curves corrected for leakage (see Methods). Veratrine induced a negative slope conductance region on the I-V curve. The slow inward current remained also at a relatively high dose of TTX and became even stronger. In Na-free Ringer the current disappeared almost completely, on restoring Na concentration it reappeared. Holding potential is -50 everywhere. Time indication and other parameters as in Fig. 4.

In sodium free Ringer the veratrine-induced inward current disappeared also under these circumstances. In the fifth curve the hysteresis is greatly reduced due to its almost total absence. Returning to normal Ringer the current rapidly reappeared. During the experiment presented in Fig. 4 and several others, we found a continuous shift of the resting potential level in positive direction. The current caused by veratrine seemed to have a persistent part which slowly increases and makes the membrane depolarize. This explanation is supported by the fact that the shift was stopped when the inward current had no carrying ion or was blocked. The I-V curves taken under veratrine effect cross the abscissa usually twice, with positive slope at both points. This means that the neurons can be bistable. It was possible indeed to shift the membrane between the stable points, that is, to trigger or break a PDS.

Fig. 5 shows I-V curves from a different neuron treated with veratrine and TEA. After correction for leakage the net active currents are demonstrated. The slow rate of development of the veratrine-induced current can

be clearly seen. In the 17th min the inward current flows mostly between the holding level and 0 mV. At about the 60th min a considerable part of it is in the positive potential range and at 100 min it is hardly inactivated at the peak level of the ramp pulse, +20 mV. It looks like that, as time lapses, the sodium current induced by veratrine was not only activated at lower and lower potentials but it also remained activated at far positive levels. A change in the proportion between the time dependent and persistent component of the veratrine-induced current may underlie this phenomenon. This Figure also shows the sodium-dependence and TTX-resistance of the current (third and sixth curve). Neither the current itself nor its growth in time is affected by TTX. In Na-free Ringer practically no inward current remains. The small outward current usually seen (also here) was apparently insensitive to TEA at the concentration used, but it did not interfere with the properties of the veratrine-current studied by us.

DISCUSSION

The results presented here indicate that Veratrum alkaloids have a strong effect on the activity of Helix central neurons. The central issue is a novel ionic current not present in untreated cells. This current proved to be Na-dependent in accordance with observations on neural elements (1, 8, 10) and muscle fibres (3, 15). Veratrine induces this current most probably by affecting the channels of fast inward current. It has been demonstrated (2, 16) that veratridine attaches directly to the Na-channel and deeply alters its kinetics and voltage dependence. Channels affected open at lower depolarizations and inactivate very slowly, or, maybe, not at all. Thus a voltage-dependent slow inward current can flow which has been separated in our experiments. Its development takes a long time. It is supposed that veratridine binds mostly to open channels (5, 14). This may explain the broadening of the spikes within a burst.

The phenomena caused by veratrine or its purified alkaloids are similar to those caused by known epileptogenic substances. Veratrine can induce bursts, PDSs and N-shaped I-V curves like pentylenetetrazol on molluscan neurons or penicilline and aminopyridine on mammalian brain. An inward current with slow kinetics may underlie the action of known convulsants and veratrine. Thus it seems that veratrine or its alkaloids may help in constructing new experimental models of the epileptic state.

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DISCUSSION

AKAIKE, N.: What kind of mechanism have you in mind regarding the suppression of veratrine response by Na?

PAPP, A.: It has been demonstrated that veratrine (and its alkaloids) act on the channels of fast inward current. In case of muscle and nerve fibres these are sodium channels which can be blocked by TTX in intact or veratrinized state. In *Helix* neurons the first inward current is a mixed, Na-Ca one and it is sensitive to Ca-blockers, like Ni^{2+} but not to TTX. We suppose that veratrine affects the inward current channels also in *Helix* but the altered channels remain Na^{2+} -sensitive.

WINLOW, W.: 1. Are you aware that general anaesthetics such as halothane can produce paroxysmal depolarizing shifts in *Lymnaea*? (Girdlestone and Winlow, 1987). Could there be a common mode of action with veratrine?

2. Potassium channel blockers such as TEA and 4AP can also produce PDS (Holden, Winlow and Maydon, 1982). How does this fit in with your explanation of the actions of veratrine?

PAPP, A.: 1. We did not know anything about that. Veratrine is supposed - and on other preparations proved - to act on sodium channels, or other channels of fast inward current. If anaesthetics do the same, there may be a common mechanism.

2. I think that if a substance is potassium-blocker, it is not necessarily a convulsant. There can exist, however, "hidden faster" neurons, in which a slow inward current is covered by the slow outward current. A channel blocker can thus unmask the bursting character.