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THE EFFECT OF TEMPERATURE ON VERATRIDINE ACTION IN SQUID GIANT AXONS

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Resting membrane potential and intracellular sodium and potassium concentrations were determined at 5 and 21°C in normal and veratridine-treated axons of the squid *Doryteuthis plei*. 300 μM veratridine produced an increase in the intracellular sodium concentration, which changed from 52 to 284 mM in 10 min of exposure at 21°C, and from 76 to 260 mM at 5°C. Under the same treatment the intracellular potassium concentration changed from 357 to 221 mM (21°C) and from 334 to 194 mM (5°C). All the changes could be prevented by adding 1 μM tetrodotoxin. Veratridine (30, 100 and 300 μM) increased the resting sodium permeability of the giant axon, and the effect was greater at 21°C. The affinity of the membrane for veratridine increases when the nerves are cooled, the three concentrations tested produce maximum activation of the sodium channels at 5°C. But only the higher two concentrations are saturating at 21°C.

Introduction

Veratridine is a steroidal alkaloid well known for its ability to depolarize nerve fibers by opening sodium channels in nerve, muscle and neuroblastoma cell membranes [1–6]. All the actions of veratridine on excitable tissues are diminished when temperature decreases [7–14].

This communication describes experiments in which the effect of veratridine on nerve membrane potential, intracellular ionic concentrations and sodium permeability, were explored in giant axons of the squid *Doryteuthis plei* at 5 and 21°C. Our result indicate that the decrease in veratridine effect at low temperatures is due to a decrease in the maximum sodium permeability induced by saturating concentrations of the drug.

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Methods

Fine-cleaned axons from the posterior stellate nerve of freshly killed squid were used for all the experiments. Membrane potential was measured with an axial cannula (75 μm in diameter) connected to a high-impedance amplifier and a strip chart recorder (Model 3047, Yokogawa Electric Works, Tokyo). Temperature was controlled by causing the sea water to flow through a heat exchanger and it was monitored with an accuracy of 0.5°C with an electronic thermometer (Yellow Springs Instruments Co., Ohio) and a thermistor placed in the experimental chamber. The experiments were carried out in artificial sea water of the following composition (in mM): Na^+, 457; K^+, 10; Ca^{2+}, 3; Mg^{2+}, 53; Hepes, 6 and Cl^-, 576. The pH was adjusted to 7.5 and the osmolality routinely measured between 1000 and 1010 mosmol/kg. Ethanol was added to all solutions containing veratridine in a proportion of 1% (v/v).

Intracellular sodium and potassium concentrations were determined under conditions indicated in Table II. After this treatment the axons were carefully blotted with filter paper (Whatman No. 1) to remove the excess solution adhering to its surface, and axoplasm was extruded with a Teflon ® (DuPont Chemical Co.) roller after placing the axon with a cut end on a Parafilm ® sheet (American Can Co., Greenwich, CT). The extruded axoplasm was sucked into a preweighed polyethylene tubing. Since the axoplasm density in squid ranges between 1.03 and 1.04 g/ml [15,16] the difference in weight of the polyethylene tubing pieces before and after sucking the axoplasm was taken as the volume of the latter. The tubing pieces were exhaustively washed with distilled water into tightly capped test-tubes after adjusting their volume to 1 ml for determining Na^+ or to 3 ml for determining K^+. The ionic concentrations were determined by flame photometry (Evans Electroselenium, Ltd., Halstead, Essex, U.K.) comparing the emission with standard solutions of NaOH or KOH (Titrisol ®, Merck, Darmstadt). The concentrations of standard solution prepared for the calibration curves were 50, 100, 250, 500 and 700 μM.

The apparent sodium permeability (π') was measured in fine-cleaned giant axons of freshly killed squid, immersed in low-sodium artificial sea water with the following composition (in mM): Na^+, 5; K^+, 10; Ca^{2+}, 11; Mg^{2+}, 56; Tris, 471; and Cl^-, 548. The pH was 7.5. ^{22}Na was added to this solution to a specific activity of 22 μCi/ml. To load the axons with ^{22}Na they were incubated in the radioactive solution for 1 h at 5°C. The rate of discharge of ^{22}Na at either 5 or 21°C was then measured by soaking the nerves in 1 ml tracer-free low-sodium sea water containing 30, 100 or 300 μM veratridine. The axons were continuously agitated while being discharged, and remained 1 min in each of the first eight aliquots and 4 min in each of other eight aliquots. The radioactivity in the discharged aliquotes was determined by taking a 800 μl sample of each one, diluting the sample with 5 ml of emulsifier (Instagel, Packard Instruments Co.) and counted in a liquid scintillation counter (Tricarb Model 2202, Packard Instruments Co.).

Fine-cleaned axons with their ends tied with silk threads were recorded on film with an inverted microscope (Carl Zeis, Oberkochen, F.R.G.) to determine the axonal diameters. The diameters were measured by comparison with a micrometer slide photographed with the same magnification, in a Nikon 6C profile comparator (Nippon Kogaku K.K., Tokyo). The results are reported as median and their 95% confidence interval, both determined by the method of Hodges and Lehman [17]. The significance of differences between medians was determined with the Mann-Whitney (Wilcoxon) test. The minimum significance level required to discard the null hypothesis was 0.05.

Results

The action of veratridine on membrane potential

Fig. 1 presents the action of veratridine on

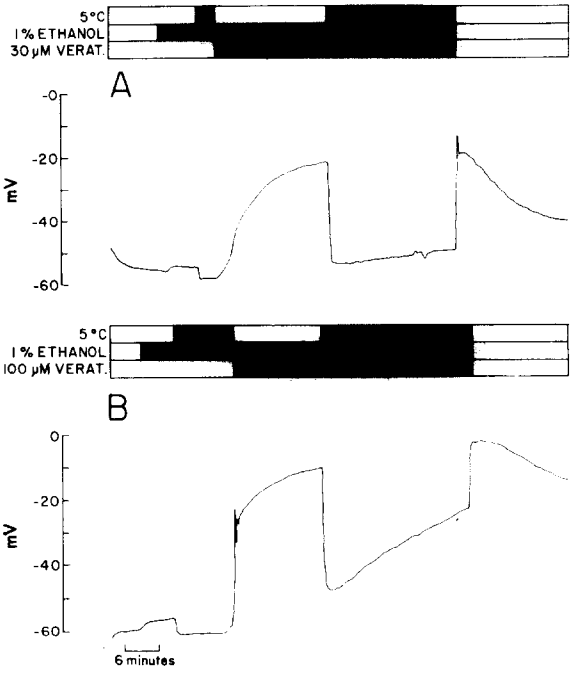

Fig. 1. Effect of veratridine on resting membrane potential at 5°C and 21°C. The ordinate of both halves on the figure is resting membrane potential in mV, the abscissa is the time elapsed after impalement with the recording electrode. A. Effect of 30 μM veratridine. B. Effect of 100 μM veratridine. The bars on top of each half on the figure indicate the conditions in effect at any given time. The light parts of the topmost bars in each half on the figure correspond to periods during which the axons were at 21°C.

TABLE I

PERCENTAGE OF DEPOLARIZATION PRODUCED BY VERATRIDINE IN *D. PLEI* GIANT AXONS

Data presented as median and its 95% confidence interval (in parentheses). Number of experiments in square brackets for each median. *P* calculated with the two-tailed Man-Whitney (Wilcoxon) test.

Veratridine concentration (μM)	Percentage of depolarization		Significance of the difference (*P*)
	5°C	21°C	
1	3 (1, 5) [6]	1 (0, 3) [6]	> 0.10
3	3 (2, 5) [5]	3 (1, 4) [5]	> 0.10
10	17 (11, 22) [5]	22 (17, 26) [5]	> 0.10
	19 (12, 30) [6]	51 (44, 57) [6]	< 0.01
100	40 (18, 56) [6]	73 (64, 77) [6]	0.01
300	49 (44, 53) [4]	77 (71, 80) [4]	0.02

membrane potential at 5 and 21°C. Two concentrations of the drug, 30 μM (Fig. 1A) and 100 μM (Fig. 1B), are presented. The depolarization produced by both concentrations diminishes when the temperature is lowered. The main difference between the effects of 30 and 100 μM veratridine, apart from the magnitude of the depolarizations, is the reversibility of the effects. While the effect of the lower concentration is almost completely reversible, the depolarization produced by 100 μM veratridine increases after the initial repolarization induced by cooling the nerve, and recovers only partially after the alkaloid is washed out. The depolarizations produced by 1, 3, 10, 30, 100 and 300 μM veratridine at 5 and 21°C are presented in Table I as percentage of the initial resting potential (−56.5 (−58.5; −55) mV, 5°C; −53 (−55; −51) mV, 21°C; paired difference 4 (3.5; 4.5) mV ($P < 0.0001$), 35 nerves (Wilcoxon Sign Test)).

The effect of veratridine on intracellular sodium and potassium concentrations

Tetrodotoxin antagonizes the depolarization produced be veratridine [2,4]. To test if the incomplete reversibility of veratridine effects at high drug concentrations is related to changes in intracellular Na^+ and/or K^+ concentrations, these were determined in normal sea water plus 1% ethanol (control), and in the same solution with the addition of 300 μM veratridine, alone or with 1 μM tetrodotoxin. These results are presented in Table II. The control values at 5 or 21°C are in agreement with the data in the literature: *Sepia officinalis* [16]; *Loligo pealii* [18]; *Sepioteuthis sep-*

TABLE II

EFFECT OF VERATRIDINE ON THE INTRACELLULAR SODIUM AND POTASSIUM CONCENTRATIONS IN *D. PLEI* GIANT AXONS AT 5 AND AT 21°C

Data presented as median and its 95% confidence interval (in parentheses). All drugs applied for 10 min before determining the ion concentrations.

Experimental condition	Intracellular ionic concentration (mM)		Number of nerves at each temp.
	5°C	21°C	
	Sodium		
Control	76 (55, 99)	52 (39, 69)	11
300 μM veratridine	260 (162, 318)	284 (261, 327)	6
300 μM veratridine + 1 μM tetrodotoxin	32 (24, 45)	52 (39, 61)	7
	Potassium		
Control	334 (309, 370)	357 (311, 391)	6
300 μM veratridine	221 (192, 252)	194 (163, 223)	6
300 μM veratridine + 1 μM tetrodotoxin	336 (245, 410)	331 (289, 364)	4

ioidea [19]; *Doryteuthis plei* [20]. The table shows that the 10 min of application of 300 μM veratridine increases the intracellular sodium concentration by 200 mM or more ($P < 0.01$) at either 21 or 5°C. At the same time, the intracellular K^+ concentration decreases by more than 100 mM ($P < 0.01$). All the changes of intracellular ionic concentrations produced by veratridine may be prevented if 1 μM tetrodotoxin is applied in combination with the alkaloid (300 μM veratridine + 1 μM tetrodotoxin in Table II).

Temperature effect on sodium permeability increases induced by veratridine

Fig. 2 presents a plot of the logarithm of the radioactivity of the discharge samples (cpm) divided by the duration of nerve immersion in the sample (cpm/min), versus the duration of the discharge. Two nerves exposed to 100 μM veratridine are presented, one discharged at 5°C (Fig. 2a) and the other one discharged at 21°C (Fig. 2b). The dotted lines were fitted to the data with a simplex algorithm [21] (minimizing the absolute value of the deviations of the experimental points to the line) and obeys the following equation:

$$y = (S_1/\tau_1)\exp(-t/\tau_1) + (S_2/\tau_2)\exp(-t/\tau_2) \quad (1)$$

where

y = cpm leaving the nerve per unit time (cpm/min);
$S_1 = {}^{22}Na$ cpm in the outer phase of the nerve at $t = 0$ (cpm);
τ_1 = time constant for difussion of ^{22}Na from the outer phase of the nerve (min);
S_2 = cpm ^{22}Na in the axoplasm at $t = 0$;
τ_2 = time constant for diffusion of ^{22}Na from the axoplasm (min).

Eqn. 1 represents the rate of diffusion of solute from two compartments in series if $\tau_1 \ll \tau_2$. The values of π' were calculated from τ_2 with the following relation derived from the first Fick law of diffusion.

$$\pi' = r/(2\tau_2) \quad (2)$$

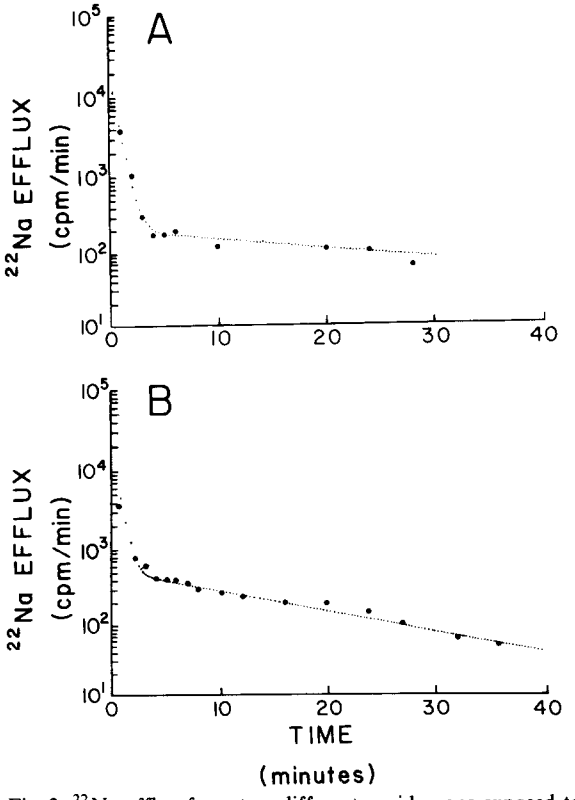

Fig. 2. ^{22}Na efflux from two different squid axons exposed to 100 μM veratridine. A. At 5°C; π' for the slow component is 41 nm/s. B. At 21°C; π' is 86 nm/s. The ordinate is the radioactivity leaving the nerves in 1 minute (cpm/min). Abscissa is time in min.

TABLE III

APPARENT ^{22}Na PERMEABILITY INDUCED BY VERATRIDINE IN *D. PLEI* GIANT AXONS AT 5 AND AT 21°C

Data presented as median and its 95% confidence interval (in parentheses). Number of experiments in square brackets by each median. P calculated with the two-tailed Mann-Whitney (Wilcoxon) test.

Veratridine concentration (μM)	Apparent permeability coeficient (π') (nm/s)		Significance of the differences between π' at 5 and at 21°C (P)
	5°C	21°C	
30	45 (32, 66) [4]	49 (29, 61) [4]	> 0.10
100	31 (21, 41) [5]	112 (83, 134) [6]	< 0.01
300	50 (39, 52) [6]	72 (57, 76) [5]	< 0.05

where r stands for the radius of the axon. Fig. 2 shows that the slow component of diffusion of ^{22}Na is further slowed when veratridine is applied at 5°C.

Table III sumarizes the values of π' calculated for several axons in presence of 30, 100 and 300 μM veratridine at either 5 or 21°C. As indicated in the table, the values of π' obtained in the presence of 100 and 300 μM veratridine at 21°C are higher than their counterparts at 5°C. It must be pointed out that the three values of π' obtained at 5°C are not distinct ($P > 0.05$), and that the value obtained with 100 μM veratridine at 21°C is higher than the effect of 30 or 300 μM veratridine at the same temperature.

Discussion

Our results confirm the reports by other authors in different preparations that low temperature antagonizes the depolarization produced by veratridine [8–14] and shows that this antagonism is due to decrease in the maximum effect produced by veratridine at low temperatures. Veratridine treatment modifies the intracellular sodium and potassium concentrations in squid nerve. This is remarkable, since the surface the volume ratio in this axon is small. The axons used for our experiments had a diameter of 299 (288, 309) μm when measured in 1% ethanol/sea water and 304 (291, 311) μm after 10 min of exposure to 300 μM veratridine. Since membrane potential was not controlled, the depolarization induced by veratridine increases the voltage gradient that moves potassium out of the axons. Whether this is an important factor in explaining the changes in intracellular cation concentrations is not clear, since the same depolarization decreases the voltage gradient that moves sodium into the cell. In any case, it is clear that the changes in intracellular ionic concentrations reported in this communication are large, and indicate that it is not possible to draw quantitative conclusions on dose-response relations of veratridine, if the intracellular composition is not controlled.

It may be shown that flux density (J), intracellular (C_i) and extracellular (C_e) sodium concentrations and π' are related by the following expression:

$$\pi' = -J/(C_i + C_e) \quad (3)$$

The normal sodium efflux density in *D. plei* is approx. 30 pmol/cm^2 per s (DiPolo, R., personal communication). Since the intracellular sodium concentration is approx. 50 mM (Refs. 16, 18–20 and this communication) it is poosible to estimate that under normal conditions ($C_e = 450$ mM) the resting value for apparent sodium permeability is 0.75 nm/s. Thus the resting apparent permeability coefficient in normal conditions is more than one order of magnitude smaller than any of the values presented in Table III and the possible reduction of active extrusion when the nerves are cooled is at least 10-times smaller than the smallest significant difference between values of π' in the table. Since all the values of π' in Table III at 5°C are equal, it is reasonable to conclude that at this temperature all the concentrations produce maximum effect, that is, they produce the maximum increment of sodium permeability possible at 5°C. From Table I, it is evident that the maximum increase of π' that can be produced by veratridine is at least 3-times greater at 21°C than at 5°C and that the concentration of the alkaloid must be raised over 30 μM to reach maximum effect. Since its customary to use the concentration that produces one-half of the maximum effect (i.e., the apparent dissociation constant) as an indication of affinity of the drug for its receptors, Table I suggests that the affinity increases when the nerves are cooled. Our result indicate that the antagonism by low temperature of veratridine effect on sodium permeability in *D. plei* is produced either by a decrease of intrinsic activity [22] or by a decrease in number of sodium channels that my be activated by the alkaloid at 5°C. It is interesting to notice that the value of π' observed in presence of 100 μM veratridine at 21°C is decreased by approx. 70% when the nerves are cooled to 5°C, in good agreement with the 80% reduction of electrically excitable sodium conductance observed when the nerve [23,24] is cooled from 21 to 5°C.

The decrease of π' produced at 21°C when the veratridine concentration increases from 100 to 300 μM, may be considered similar to the diminution of the ^{22}Na flux into liposomes (40 mg soybean

lipids/ml) with Na^+ channels incorporated (0.5 mg protein/ml) when they are treated with concentrations of veratridine higher than 1 mM (Villegas, R., personal communication). The difference in the concentrations required in each case may due to the relative ammounts of lipids in which the ammount of veratridine has to be dissolved. An unspecific anesthesic effect, such as the one produced by many nonpolar drugs at high concentrations [25–27] may be the cause of the results.

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