Grayanotoxin, Veratrine, and Tetrodotoxin-Sensitive Sodium Pathways in the Schwann Cell Membrane of Squid Nerve Fibers

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ABSTRACT The actions of grayanotoxin I, veratrine, and tetrodotoxin on the membrane potential of the Schwann cell were studied in the giant nerve fiber of the squid *Sepioteuthis sepioidea*. Schwann cells of intact nerve fibers and Schwann cells attached to axons cut lengthwise over several millimeters were utilized. The axon membrane potential in the intact nerve fibers was also monitored. The effects of grayanotoxin I and veratrine on the membrane potential of the Schwann cell were found to be similar to those they produce on the resting membrane potential of the giant axon. Thus, grayanotoxin I (1–30 μM) and veratrine (5–50 $\mu g \cdot ml^{-1}$), externally applied to the intact nerve fiber or to axon-free nerve fiber sheaths, produce a Schwann cell depolarization which can be reversed by decreasing the external sodium concentration or by external application of tetrodotoxin. The magnitude of these membrane potential changes is related to the concentrations of the drugs in the external medium. These results indicate the existence of sodium pathways in the electrically unexcitable Schwann cell membrane of *S. sepioidea*, which can be opened up by grayanotoxin I and veratrine, and afterwards are blocked by tetrodotoxin. The sodium pathways of the Schwann cell membrane appear to be different from those of the axolemma which show a voltage-dependent conductance.

Tetrodotoxin is considered a marker of the axon excitable membrane because it specifically blocks the sodium ionic current without altering its kinetics during the nervous impulse and the early phase of conductance increase in voltage clamped axons (Narahashi et al., 1964; Moore, 1965; Nakamura et al., 1965; Moore and Narahashi, 1967; Narahashi and Moore, 1968; Hille, 1968; Cuervo and Adelman, 1970). It also produces a slight increase in the membrane potential of the resting axon, which has been considered as indicating blockage of the sodium permeability of the resting membrane. Tetrodotoxin also reduces the increase in sodium permeability produced by veratrine, veratridine, and cevadine (Ulbricht, 1969; Moore et al., 1968; Ohta et al., 1973), batrachotoxin (Narahashi et al., 1971 *a*, *b*; Albuquerque et al., 1971; Albuquerque et al., 1973), and the grayanotoxins (Seyama and Narahashi, 1972, 1973 *a*, *b*; Narahashi and Seyama, 1974).

Recent studies carried out on the two plasma membrane fractions isolated from the walking-leg nerves of the lobster *Panulirus argus* (Barnola et al., 1973) indicate that both of them bind tetrodotoxin. Fraction I is considered to be an axolemma-enriched preparation and fraction II a periaxonal cell plasma membrane preparation formed mainly by Schwann cell plasma membrane. Similar results were obtained with the two plasma membrane fractions isolated from the olfactory nerve of the garfish *Lepisosteus osseus* (Chacko et al., 1974) and from the fin and stellar nerves of the squid *Sepioteuthis sepioidea* (Barnola et al., unpublished results). The binding of tetrodotoxin to the periaxonal cell plasma membrane preparations of lobster, garfish, and squid peripheral nerves (fraction II) might be partially explained by the presence of axolemma in them. However, the possibility exists that the plasma membrane of the periaxonal cells has tetrodotoxin receptors and sodium pathways which, although nonvoltage dependent (Villegas, 1972), can be opened up by grayanotoxin-I and veratrine, and afterwards are blocked by tetrodotoxin. Thus, it was considered worthwhile to further explore such a possibility utilizing living axons and Schwann cells of intact nerve fibers, and Schwann cells attached to axons cut lengthwise over several millimeters (axon-free Schwann cells).

The evidence presented here shows that the electrically unexcitable (Villegas et al., 1963) Schwann cells, both in the intact nerve fiber and in the axon-free nerve fiber sheaths, have sodium pathways sensitive to grayanotoxin-I (1–30 μM), veratrine (5–50 $\mu g \cdot ml^{-1}$), and tetrodotoxin (5–100 nM). A preliminary account of this work has been reported to the 19th Annual Meeting of the Biophysical Society (Villegas et al., 1974).

EXPERIMENTAL METHOD

General Procedure

Giant nerve fibers with a diameter of about 300–400 μM obtained from the hindmost stellar nerves of the squid *S. sepioidea* were used. Immediately after decapitation of the living squid, the nerve fiber was isolated from the mantle, placed in a Lucite holder containing artificial seawater, and freed from all adherent nerve fibers. The giant nerve fiber was kept under slight tension by means of threads tied to both ends. In those experiments involving the isolation of the nerve fiber sheaths, sharp microscissors were inserted into the axon and the nerve fiber was cut lengthwise over several millimeters. The Schwann cell and axon electrical potentials were measured intracellularly in each single nerve fiber immersed in the flowing test solutions which could be selected by means of a six-way tap with a small dead space.

In all experiments, the electrical potentials of several of the Schwann cells surrounding a single giant axon were successively measured by brief impalements from inside the axon, or from the axonal surface of the isolated nerve fiber sheaths, before and after the drugs at a given concentration were added to the bathing solutions. The axon membrane potential in the intact nerve fibers was also monitored. All the experiments were carried out at room temperature (20–22°C).

The experimental procedure utilized to measure the electrical potential differences across the Schwann cell membrane and across the axon membrane was similar to that described and discussed in previous works (Villegas et al., 1963; Villegas et al., 1968; Villegas, 1972).

Experimental Solutions

(a) Artificial seawater was used as normal medium. The concentration of its components was as follows (mM): NaCl, 442; KCl, 10; $CaCl_2$, 11; $MgCl_2$, 46; Tris-chloride buffer pH 8.0, 10. *(b)* Seawater solution containing 1 mM sodium was prepared by replacing sodium chloride with Tris-chloride, mole per mole. *(c)* Test solutions containing grayanotoxin-I or veratrine were prepared by adding to the control seawater solutions aliquots of concentrated solutions in 95% ethanol. The final concentration of ethanol (1%) has no effect on the resting membrane potentials of axons and Schwann cells. Grayanotoxin-I was kindly supplied by Dr. Masaiti Yasue of Nagoya City University, Japan, and by Dr. Toshio Narahashi of Duke University, Durham, N.C. *(d)* Test solutions containing tetrodotoxin were similarly prepared. Tetrodotoxin in citric acid-sodium citrate buffer (lyophilized, A grade; Calbiochem, San Diego, Calif.) was used. The final concentration of citrate buffer in the test solutions has no effect on the resting membrane potentials of axons and Schwann cells.

RESULTS

Effect of Grayanotoxin-I on the Electrical Potentials

Fig. 1 shows the results of one experiment in which the membrane potential of the axon was continuously monitored during the immersion of the intact nerve fiber in the different test solutions. This figure shows that, as previously found by Seyama and Narahashi (1973 *b*, 1974) for the axons of *Loligo pealii*, the external application of grayanotoxin-I causes the axon membrane to depolarize.

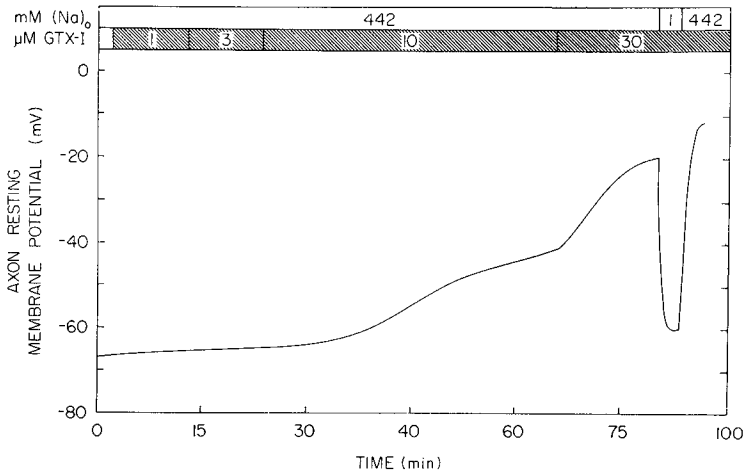

FIGURE 1. Effect of different external concentrations of grayanotoxin I (GTX-I) on the axon resting membrane potential. The tracing corresponds to the continuous intracellular recording of the axon electrical potential. During the interval indicated by the striped horizontal bar, grayanotoxin was present in the bathing solution at the millimolar concentration shown in the graph. The sodium concentration in each test solution is indicated in the upper part of the graph. Grayanotoxin I causes the resting axon to depolarize. Decreasing the external sodium concentration to 1 mM reverses the axon depolarization produced by the toxin. Similar results were obtained in six additional nerve fibers.

Decreasing the external sodium concentration to 1 mM resulted in almost complete recovery of the membrane potential. Readmission of the normal sodium concentration again brought about a depolarization. The magnitude of the axonal depolarization appears to be related to the concentration of grayanotoxin-I in the external seawater medium. To eliminate any possible effects of the axon membrane potential changes on the neighboring Schwann cells (Villegas, 1972, 1973, 1975), axon-free nerve fiber sheaths were used to explore the effect of grayanotoxin-I, low external sodium, and tetrodotoxin on the Schwann cell electrical potential. Fig. 2 shows the results of one experiment in which the electrical potentials of different Schwann cells, attached to a single axon cut lengthwise over several millimeters, were measured during immersion of the slit nerve fiber in the different test solutions. This figure shows that the Schwann cells become depolarized within 10 min after the external application of grayanotoxin-I (10 μM), and that they gradually return to the initial potential level within the next 10 min after the replacement of the test solution by toxin-free medium. Fig. 3 shows the results of a similar experiment in which the effect of tetrodotoxin (50 nM), externally applied, was tested before and after the exposure to grayanotoxin-I. This figure shows that, as in the previous experiment, the axon-free Schwann cells become depolarized after the addition of grayanotoxin-I to the external medium. It also shows that this depolarization is reversed in less than 5 min by the diminution of the external sodium concentration to 1 mM or by the treatment with tetrodotoxin (50 nM). It should also be pointed out that in the absence of grayanotoxin-I, both low external sodium and tetrodotoxin have only a small effect on the Schwann cell membrane potential. Table I summarizes the effect of tetrodotoxin on the grayanotoxin-induced depolarizations of the Schwann cells.

The effects of grayanotoxin-I, low external sodium, and tetrodotoxin on the

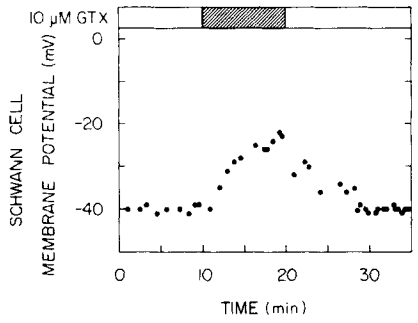

FIGURE 2. Effect of grayanotoxin I (GTX) on the membrane potential of the Schwann cell in axon-free nerve fiber sheaths obtained by cutting the giant axon lengthwise. The Schwann cell electrical potentials have been plotted as a function of time. During the interval indicated by the striped horizontal bar, grayanotoxin I (10 μM) was present in the external seawater bath. Each point corresponds to the potential difference recorded in a different Schwann cell in the same nerve fiber. Grayanotoxin I depolarizes the Schwann cell in a reversible way. Similar results were obtained in four additional nerve fibers.

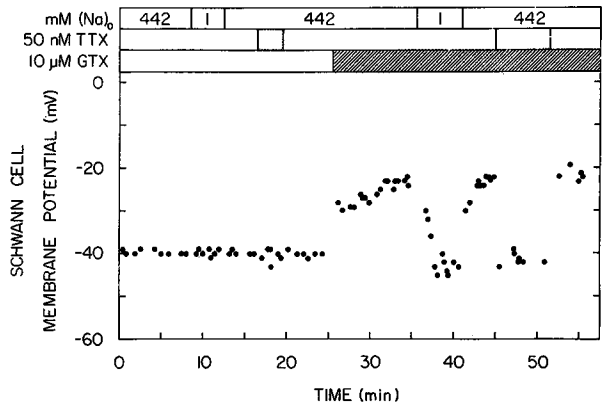

FIGURE 3. Effects of tetrodotoxin (TTX) and low external sodium on the Schwann cell depolarization induced by grayanotoxin I in axon-free nerve fiber sheaths. The Schwann cell electrical potentials have been plotted as a function of time. During the interval indicated by the striped horizontal bar, 10 μM grayanotoxin (GTX) was present in the seawater medium. Each point corresponds to the potential difference recorded in a different Schwann cell in the same nerve fiber. During the intervals indicated by the shaded bars, 50 nM tetrodotoxin (TTX) was also applied externally. The sodium concentration in each test solution appears indicated in the upper part of the graph. The Schwann cell depolarizations induced by grayanotoxin I are reversed by decreasing the external sodium concentration to 1 mM or by externally applying tetrodotoxin (50 nM). Similar results were obtained in three additional nerve fibers.

Schwann cells of intact nerve fibers, in which the membrane potential of the resting axon was also monitored, were similar to those described above. The changes in resting potential of the axon observed in these nerve fibers are in agreement with those previously reported by Seyama and Narahashi (1973 *b*, 1974) for the axons of other squid species.

Effect of Veratrine on the Electrical Potentials

Fig. 4 shows the effect of veratrine (5 $\mu g \cdot ml^{-1}$) on the resting membrane potential of the intact axon. Veratrine, as previously found by Moore et al. (1968) for the axons of other squid species, causes the axon membrane to depolarize. During the initial stage of depolarization, the axon membrane potential became oscillatory. The oscillatory behavior subsided in about 5 min, and then the depolarization of the axon continued at a smaller rate. Decreasing the external sodium concentration to 1 mM resulted in a marked recovery of the axon membrane potential. Readmission of the normal sodium concentration again brought about a depolarization. Subsequent application of tetrodotoxin (50 nM) also has a repolarizing effect on the axon membrane potential. Prolonged washing of veratrine tends to restore the membrane potential but at a much slower rate than the depolarizing effect of this drug.

Fig. 5 shows the effect of veratrine (5 $\mu g \cdot ml^{-1}$) on the membrane potential of axon-free Schwann cells. At this concentration, veratrine caused the Schwann

TABLE I

EFFECT OF TETRODOTOXIN (TTX) ON THE MEMBRANE POTENTIAL OF THE SCHWANN CELL IN AXON-FREE NERVE FIBER SHEATHS TREATED WITH DIFFERENT CONCENTRATIONS OF GRAYANOTOXIN-I (GTX)

GTX concentration	Number of nerve fibers	Schwann cell electrical potential (mean ± SEM of 30–44 different cells)		
		Control	GTX solution	GTX + TTX solution
M		*mV*	*mV*	*mV*
1×10^{-6}	4	−40 ± 1	−33 ± 1	−37 ± 3*
3×10^{-6}	4	−40 ± 1	−30 ± 1	−36 ± 1*
1×10^{-5}	4	−40 ± 1	−23 ± 2	−35 ± 2*
3×10^{-5}	3	− 40 ± 0	−19 ± 3	−31 ± 1*
1×10^{-5}	4	−40 ± 0	−24 ± 2	−40 ± 2‡

* 5 nM.
‡ 50 nM.

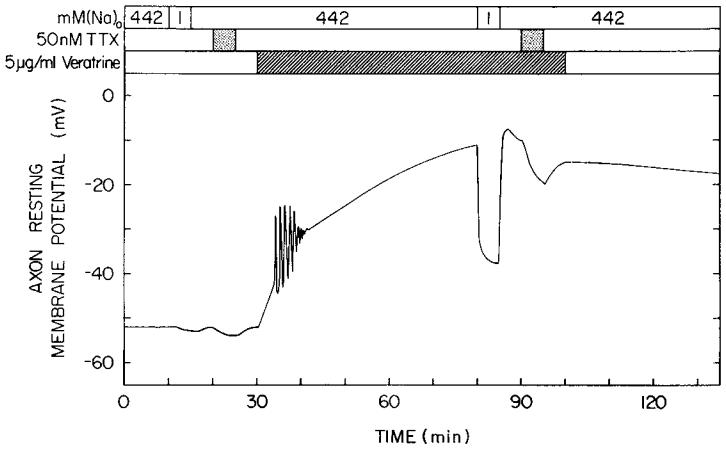

FIGURE 4. Effect of veratrine (5 $\mu g \cdot ml^{-1}$) on the axon resting membrane potential. The tracing corresponds to the continuous intracellular recording of the axon electrical potential. During the interval indicated by the striped horizontal bar, veratrine was added to the bathing solutions. During the intervals indicated by the shaded bars, 50 nM tetrodotoxin (TTX) was externally applied to the resting nerve fiber. The sodium concentration in each test solution is indicated in the upper part of the graph. Decreasing the external sodium concentration to 1 mM or applying tetrodotoxin externally reverses the axonal depolarization produced by veratrine. Similar results were obtained in two additional nerve fibers.

cell membrane to depolarize to −20 mV in about 5 min. Decreasing the external sodium concentration to 1 mM resulted in a complete recovery of the Schwann cell membrane potential, followed by depolarization upon readmission of the normal sodium concentration. Subsequent application of tetrodotoxin (100 nM) restored the Schwann cell membrane potential to the initial control level. The washing of both veratrine and tetrodotoxin is accompanied by a complete recovery of the membrane potential within 10 min of reimmersion in toxin-free seawater.

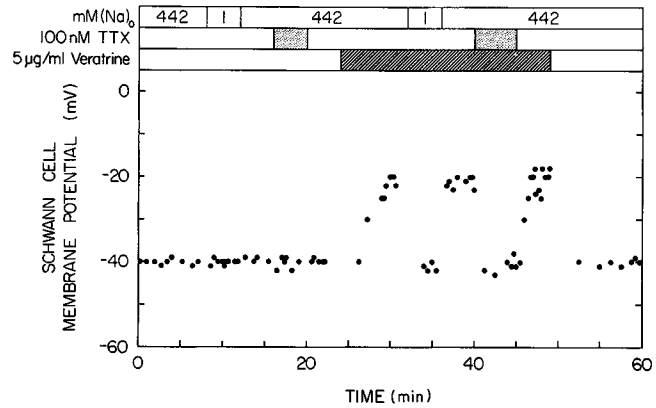

FIGURE 5. Effect of veratrine (5 $\mu g \cdot ml^{-1}$) on the membrane potential of the Schwann cells in axon-free nerve fiber sheaths. The Schwann cell electrical potentials have been plotted as a function of time. During the interval indicated by the striped horizontal bar, veratrine was added to the bathing solutions. Each point corresponds to the potential difference recorded in a different Schwann cell in the same nerve fiber. During the intervals indicated by the shaded bars 100 nM tetrodotoxin (TTX) was applied externally to the slit nerve fiber. The sodium concentration in each test solution appears indicated in the upper part of the graph. Veratrine depolarizes the Schwann cell in a reversible way. Decreasing the external sodium concentration to 1 mM or externally applying tetrodotoxin reverses the Schwann cell depolarization produced by veratrine. Similar results were obtained in two additional nerve fibers.

Fig. 6 shows the results of one experiment on the repolarizing effect of different concentrations of tetrodotoxin on veratrine-treated axon-free Schwann cells. The concentration of veratrine (50 $\mu g \cdot ml^{-1}$) used in this experiment is 10 times that used in the previous one, and it causes the Schwann cell membrane to depolarize to about the 0-mV level within 10 min after the immersion in the test solution. Again, tetrodotoxin has a repolarizing effect on the Schwann cell membrane potential, which appears to be related to the concentration of this toxin in the external solution. Readmission of the tetrodotoxin-free test solution of veratrine again brought about a depolarization.

DISCUSSION

The effects of grayanotoxin-I and veratrine on the membrane potential of the Schwann cell in *S. sepioidea* nerve fibers were found to be similar to those they produce on the resting membrane potential of the giant axon in these nerve fibers and in those of other squid species (Seyama and Narahashi, 1973 *b*; Narahashi and Seyama, 1974; Moore et al., 1968; Ohta et al., 1973). Thus grayanotoxin-I (1-30 μM) and veratrine (5–50 $\mu g \cdot ml^{-1}$), externally applied to the intact nerve fiber or to axon-free nerve fiber sheaths, produce a Schwann cell depolarization which can be reversed by decreasing the external sodium concentration or by external application of tetrodotoxin.

Previous studies carried out on the same nerve fibers have shown that the

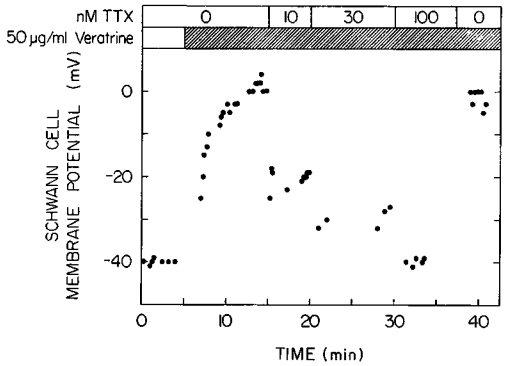

FIGURE 6. Effect of different concentrations of tetrodotoxin (TTX) on the Schwann cell depolarizations induced by veratrine (50 $\mu g \cdot ml^{-1}$) in axon-free nerve fiber sheaths. The Schwann cell electrical potentials have been plotted as a function of time. During the intervals indicated by the striped horizontal bar, veratrine was added to the bathing solutions. Each point corresponds to the potential difference recorded in a different Schwann cell in the same nerve fiber. The tetrodotoxin concentration in each test solution is indicated in the upper part of the graph. The repolarizing action of tetrodotoxin on the veratrine-treated Schwann cells appears to be related to its concentration in the external medium. Similar results were obtained in two additional nerve fibers.

Schwann cell membrane potential is determined by the ionic concentration gradients and permeabilities, mainly of potassium and to a minor extent of other ions including sodium (Villegas et al., 1965; Villegas et al., 1968; Villegas, 1968). A decrease in the external sodium concentration, or the addition of tetrodotoxin to the external seawater medium does not appreciably modify the Schwann cell potential and produces only a reversible 2-mV hyperpolarizing change in the axon resting membrane potential (Villegas et al., 1968; Villegas, 1974). Similar results were obtained in the present series of experiments (Figs. 3 and 5). Therefore, the marked dependence of the Schwann cell electrical potential on the sodium concentration gradient, observed in the grayanotoxin- and veratrine-treated nerve fibers, appears to indicate that these drugs increase the relative permeability of the Schwann cell membrane to sodium ions.

Working on perfused giant axons of *L. pealii*, Seyama and Narahashi (1973 *b*, 1974) and Ohta et al. (1973) have shown that axonal depolarization by grayanotoxin-I and veratridine is completely lacking when sodium ions are eliminated from both the external and the internal perfusion media. Performing such an experiment, instead of just decreasing only the external sodium concentration, allows excluding the possibility that an increase in leakage permeability, or a decrease in potassium permeability, or both, may be partly responsible for the cell membrane depolarization. Thus, it was concluded that the depolarizing action of grayanotoxin-I and veratridine is primarily due to a selective increase in membrane permeability to sodium ions. Whether the Schwann cell depolarization induced by these drugs is also due to a similar selective increase in sodium permeability could not be tested in the same way in the present experiments

because of the existing limitations to changing the Schwann cell sodium concentration. Although other explanations may not be ruled out at present, the repolarizing action of low external sodium and tetrodotoxin suggest that the Schwann cell depolarization caused by grayanotoxin-I and veratrine is mainly due to an increase in its relative sodium permeability.

Although the presence of sodium pathways in the Schwann cell membrane is not surprising, their sensitivity to tetrodotoxin raises several questions. The sensitivity of membrane potentials and currents to tetrodotoxin is currently considered as indicating the presence of voltage-dependent sodium channels in the cell membrane (Narahashi et al., 1964; Moore, 1965; Nakamura et al., 1965; Moore and Narahashi, 1967; Narahashi and Moore, 1968; Hille, 1968; Cuervo and Adelman, 1970). Thus, the sensitivity of the Schwann cell potential to tetrodotoxin may indicate either that the sodium pathways are also voltage dependent, or that tetrodotoxin can also block sodium pathways that have no voltage-sensing mechanisms. The existing evidence indicates that the Schwann cells in *S. sepioidea* nerve fibers are electrically unexcitable (Villegas, 1972). It has been found that the potential changes measured across the Schwann cell membrane during current injection in the cell are linearly related to the intensity of both hyper- and depolarizing pulses (Figs. 3 and 4 of Villegas, 1972). Furthermore, no regenerative responses have been observed in the Schwann cell, even with displacements of 30 mV in membrane potential produced by depolarizing current pulses (Villegas, 1972). These experimental findings have been considered to indicate that the Schwann cell membrane conductance is not voltage dependent. However, it cannot be ruled out that upon current injection the sodium conductance is turned on but inactivated so quickly that with the available techniques no conductance change is observed. Another possibility would be that both the sodium and potassium conductances respond to potential changes with identical time-courses and so no impulse-like responses are detected. However, if this were so, then the voltage/current relationship measured across the Schwann cell membrane would be different for the depolarizing and the hyperpolarizing current pulses which, as indicated above, was found not to be the case. Therefore, the most likely explanation is that tetrodotoxin can block sodium pathways that are not voltage dependent, at least in the plasma membrane of periaxonal cells.

The tetrodotoxin sensitivity of the Schwann cell membrane potential in the present series of experiments is associated with the activation of sodium pathways by grayanotoxin-I and veratrine. The possibility exists that such sodium pathways, and the tetrodotoxin receptors associated with them, are membrane entities created by the action of these depolarizing drugs. However, as indicated in the introduction of the present work, in Schwann cell plasma membrane preparations isolated from lobster, garfish, and squid nerve fibers not exposed to grayanotoxin-I and veratrine, there are sites which bind [^{3}H]tetrodotoxin with high specificity (Barnola et al., 1973; Chacko et al., 1974; Barnola et al., unpublished results). However, at present it is not possible to rule out that patches of axolemma attached to the Schwann cell fragments isolated by the method of

Camejo et al. (1969) may be responsible for at least part of the [^{3}H]tetrodotoxin binding to the Schwann cell membrane fraction.

It is unlikely that the sensitivity of the Schwann cell membrane potential to the drugs used in the present study is mediated by their action on the neighboring axolemma. Both the Schwann cells of intact nerve fibers and those attached to axon-free nerve fiber sheaths are sensitive to grayanotoxin-I, veratrine, and tetrodotoxin. In addition, the propagation of the action potential in the axon, which is accompanied by a large increment in the permeability of the axolemma to the sodium ion, produces only a small variation (within 2 mV) in the potential of the neighboring Schwann cells (Villegas et al., 1963; Villegas, 1972). Thus the results of the present work indicate the existence of specific sodium pathways in the Schwann cell membrane which are opened up by grayanotoxin-I and veratrine, and afterwards are blocked by tetrodotoxin.

Whether the sodium pathways of the Schwann cell membrane are closely similar to those in the axon excitable membrane, except that they are not responsive to changes in membrane potential, is not known at present. Nevertheless, the similarities observed between the sensitivity of the axon resting potential and that of the Schwann cell membrane potential to the drugs appear to favor such a possibility. Thus, it is tempting to suggest that the voltage-sensing mechanism, if it exists in the Schwann cells, is not in operating condition. Whichever interpretation proves to be right, the results reveal the presence in the Schwann cell membrane of sodium channels sensitive to grayanotoxin-I, veratrine, and tetrodotoxin.

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