Comparison of Tetrodotoxin and Procaine in Internally Perfused Squid Giant Axons

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ABSTRACT Squid giant axons were internally perfused with tetrodotoxin and procaine, and excitability and electrical properties were studied by means of current-clamp and sucrose-gap voltage-clamp methods. Internally perfused tetrodotoxin was virtually without effect on the resting potential, the action potential, the early transient membrane ionic current, and the late steady-state membrane ionic current even at very high concentrations (1,000–10,000 nM) for a long period of time (up to 36 min). Externally applied tetrodotoxin at a concentration of 100 nM blocked the action potential and the early transient current in 2–3 min. Internally perfused procaine at concentrations of 1–10 mM reversibly depressed or blocked the action potential with an accompanying hyperpolarization of 2–4 mv, and inhibited both the early transient and late steady-state currents to the same extent. The time to peak early transient current was increased. The present results and the insolubility of tetrodotoxin in lipids have led to the conclusion that the gate controlling the flow of sodium ions through channels is located on the outer surface of the nerve membrane.

Selective blockage of the early transient ionic conductance increase by tetrodotoxin (TTX), the active component of the toxins from the puffer fish and California newt, has been well established in giant axons of lobster (Narahashi, Moore, and Scott, 1964; Takata, Moore, Kao, and Fuhrman, 1966) and squid (Moore, Blaustein, Anderson, and Narahashi, 1967; Nakamura, Nakajima, and Grundfest, 1965 *a*) and in electroplaques of eel (Nakamura, Nakajima, and Grundfest, 1965 *b*), since this mechanism was first suggested from studies on frog muscle fibers (Narahashi, Deguchi, Urakawa, and Ohkubo, 1960; Nakajima, Iwasaki, and Obata, 1962). Because of its very strong affinity for the early transient channel, TTX has now become a powerful tool for characterizing the conductance changes responsible for excitation.

In contrast, procaine blocks both the early transient and the late steady-state conductance changes (Taylor, 1959; Shanes, Freygang, Grundfest, and

Amatniek, 1959; Blaustein and Goldman, 1966). Procaine differs from TTX not only in this nonselective blockage but also in its much higher threshold concentration and its effect on the kinetics of the early transient conductance increase (Takata et al., 1966).

In the meantime the internal perfusion technique has been developed for squid axons (Baker, Hodgkin, and Shaw, 1961; Oikawa, Spyropoulos, Tasaki, and Teorell, 1961), giving us a straightforward approach for study of symmetry or asymmetry of the nerve membrane with respect to the action of various agents (Narahashi, 1963, 1965; Baker, Hodgkin, and Shaw, 1962; Tasaki, Watanabe, and Takenaka, 1962; Tasaki and Takenaka, 1964; Rojas, 1965). Since TTX is insoluble in most organic solvents (Mosher, Fuhrman, Buchwald, and Fischer, 1964) and hence most probably so in lipids, it would be expected to block the early transient conductance mechanism only if that site is located on the surface of the membrane and only when TTX is applied to that surface. Contrary to this, because of procaine's penetrability through the lipid membrane (Goodman and Gilman, 1956; Dettbarn, 1962; Rosenberg, Higman, and Bartels, 1963) procaine might be expected to execute its block of both the early transient and late steady-state conductance mechanisms whether these sites were surface-located or imbedded in the membrane.

A preliminary experiment showed that the early transient conductance increase of a squid axon was not blocked by internally perfused TTX at a concentration of 60 nM which was effective in blocking when applied externally (Moore, 1965). However, Nakamura et al. (1965 *a*) were able to block the action potential of squid axons following injection of TTX at an estimated concentration of 300 nM. The experiments to be reported here have been performed in an effort to settle this discrepancy about the action of internally perfused TTX and to characterize and localize the sites of ionic channels in the nerve membrane.

A preliminary report of this work has been given (Narahashi, Anderson, and Moore, 1966).

METHODS

Internal Perfusion Giant axons of the squid, *Loligo pealii*, available at Marine Biological Laboratory in Woods Hole, Mass., were used; the axon diameters ranged from 400 to 500 μ. The axoplasm of uncleaned nerve preparation was squeezed out by a small roller and the axon preparation was inflated and internally perfused by the method of Baker et al. (1961). Potassium glutamate or potassium fluoride solutions were used as internal perfusate, and either 10 mM or 50 mM sodium was added for voltage-clamp experiments to define the equilibrium potential for sodium. The compositions of the solutions are given in Table I. Filtered natural see water was used as bathing medium.

Current Clamp The perfused preparation, after partial cleaning, was mounted

in a chamber horizontally, and internally perfused through a cannula at one end. A potential and a current electrode were inserted longitudinally from the opposite end of the axon through an incision which also served as the outlet for the internal perfusate. The internal solution flowed continuously at a rate of 10–30 μl/min and was changed by a switch near the cannula. The dead space for changing the solution was measured by using a dye.

A glass capillary of about 100 μ in diameter, filled with 0.6 M KCl, was used as the potential electrode. A silver wire 25 μ in diameter was inserted in the capillary to reduce the high frequency impedance (Hodgkin and Katz, 1949). In some experiments, the tip of the capillary was made narrower (of the order of 5 μ), or the tip was filled with 0.6 M KCl agar, to slow down the diffusion of solution. The capillary electrode was connected to the input of a high input impedance preamplifier by means of a calomel electrode. Another calomel electrode dipped into the bathing medium was used as the reference electrode.

TABLE I

COMPOSITIONS OF INTERNAL SOLUTIONS

Solution	K	Na	Glutamate	F	Cl	H_2PO_4*	Sucrose
	mM	mM	mM	mM	mM	mM	mM
A	400	0	370	0	0	15	333
B	410	0	370	0	10	15	316
C	400	10	0	380	0	15	316
D	400	50	0	420	0	15	250

pH, 7.3; A and B, for current clamp. C and D, for voltage clamp.
* In the form of KH_2PO_4.

A silver wire 50 μ in diameter, with the insulation removed and silver chloride-coated for a length of about 10 mm from the tip, was used as the current electrode. This was twisted around the potential capillary electrode in such a way as to locate the tip of the latter at the middle of the 10 mm Ag–AgCl region.

The junction potential, between the tip of the potential electrode and the solution, was measured in each external and internal solution with a calomel electrode for reference and used to correct the apparent membrane potential. The current-clamp experiments were performed at a room temperature of 20–22°C.

Voltage Clamp A perfused squid axon, after cleaning, was mounted in a sucrose-gap chamber similar to that described previously (Moore, Narahashi, and Ulbricht, 1964) and modified as in the preceding paper (Moore et al., 1967). The voltage-clamp experiments were performed at a temperature of about 8°C.

Tetrodotoxin and Procaine Crystalline TTX, supplied by Sankyo Company, Tokyo, Japan, was dissolved in deionized water to make up a stock solution. The stock solution was kept in a refrigerator and diluted with internal or external solutions to give desired concentrations shortly before use. Procaine hydrochloride solutions were prepared daily.

RESULTS

Tetrodotoxin

CURRENT CLAMP Unlike the very high potency of TTX in blocking action potentials by external application, internal perfusion of TTX had virtually no effect on excitation even at very high concentrations. An example of experiments is illustrated in Fig. 1, in which the resting potential, action

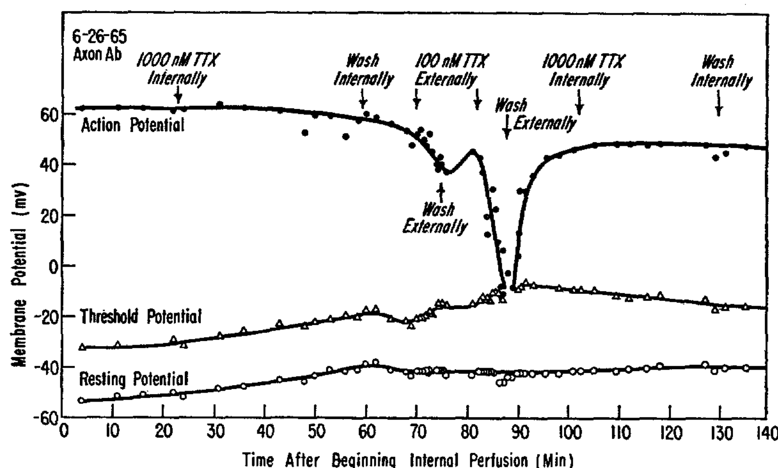

FIGURE 1. The effects of internal perfusion of 1,000 nM tetrodotoxin (TTX) and of external perfusion of 100 nM tetrodotoxin on resting potential, action potential, and threshold potential. The threshold potential refers to the absolute membrane potential at which regenerative activity occurs by depolarizing pulse of current. Internal solution, Table I A.

potential, and threshold potential are plotted against time. The threshold potential refers to the absolute value of the membrane potential at which regenerative activity begins. The maximum rate of rise of the action potential was also observed. No change occurred in these four parameters except for slow gradual natural declining for a period of 36 min after introducing 1,000 nM TTX internally. On the contrary, external application of 100 nM TTX caused a rapid decline of the action potential without accompanying change in resting potential. The threshold potential slightly decreased during blockage; i.e., the critical depolarization for excitation increased. Almost complete recovery occurred after washing with normal sea water. Following this, 1,000 nM of TTX was again applied internally, and its ineffectiveness was confirmed (Fig. 1).

Internal perfusion was also tried with 10,000 nM TTX in other nerve preparations for a period of up to 17 min with no change in resting and action

potentials. Since the threshold concentration of externally applied TTX to block the action potential is around 30 nM, the results indicate that the inner surface of the nerve membrane is less sensitive than the outer surface by a factor of more than 300.

Numerical data on the resting and action potentials before and after application of TTX internally are given in Table II.

VOLTAGE CLAMP In spite of this dramatic insensitivity of the action potential to internally applied TTX, there still remained the possibility that the early transient and late steady-state conductance mechanisms were affected in a manner which produced little change in the configuration of

TABLE II

THE RESTING AND ACTION POTENTIALS BEFORE AND AFTER APPLICATION OF 1,000 nM OR 10,000 nM TETRODOTOXIN (TTX) INTERNALLY

Date	Axon	TTX		Resting potential		Height of action potential	
		Concentration	Time	Control	TTX	Control	TTX
		mM	*min*	*mv*	*mv*	*mv*	*mv*
6-26-65	Aa	1,000	15	−43	−43	73	78
6-26-65	Ab	1,000	36	−50	−41	111	98
		1,000*	27	−42	−39	88	87
6-28-65	Aa	1,000	24	−60	−43	108	89
6-29-65	Aa	1,000	12	−40	−39	53	52
6-29-65	Ab	1,000	16	−49	−46	78	73
7-3-65	Ba	10,000	12	−46	−43	No change‡	
7-3-65	Ca	10,000	10	−60	−57	No change‡	

* Second application after washing with normal internal perfusate.

‡ Action potentials were not recorded on films.

the action potential (Moore, Ulbricht, and Takata, 1964). For this reason, voltage-clamp experiments were performed with internal perfusion of TTX.

The early transient current and the late steady-state current are plotted as a function of membrane potential in Fig. 2. There was virtually no change in these current-voltage relations after introducing 1,000 nM TTX internally. The observation could be extended up to 25-30 min without detecting any change in these currents. Fig. 3 shows an example of the change in the peak early transient current with time. Although no significant change occurred during internal perfusion of 1,000 nM TTX for 27 min, an introduction of 100 nM TTX in the bathing medium blocked the early transient current in 2–3 min. No change was observed in the time to peak early transient current during internal perfusion of TTX or during the course of blockage following external application of TTX.

The sodium conductance, g_{Na}, is usually defined by the following equation:

$$g_{Na} = \frac{I_{Na}}{E - E_{Na}} \quad (1)$$

where I_{Na} is the sodium current, E is the membrane potential, and E_{Na} is the sodium equilibrium potential. Here it is assumed that the early transient current (total current minus "leakage current") is entirely carried by sodium ions and that the membrane potential at which the early transient current becomes zero is the sodium equilibrium potential. In Fig. 4 the early tran-

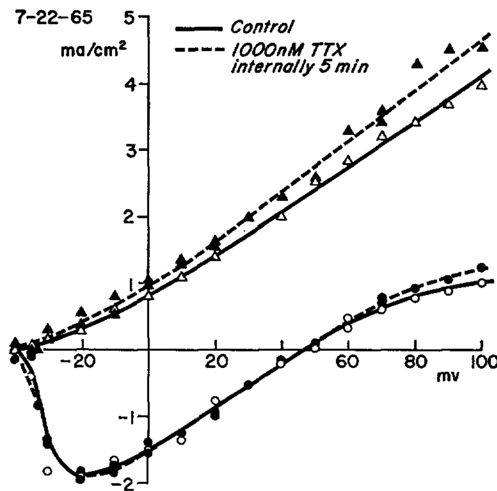

FIGURE 2. Current-voltage relations for the early transient current (circles) and the late steady-state current (triangles) in a perfused voltage-clamped axon before and 5 min after application of 1,000 nM tetrodotoxin (TTX) internally. Holding potential −80 mv. Internal solution, Table I D.

sient (sodium) conductance is plotted against the membrane potential in semilogarithmic scale. The slight decrease of the early transient conductance with strong depolarizations is probably a result of difficulties in resolving the capacitative, leakage, and increasingly faster early transient currents. However, the ineffectiveness of internally perfused 1,000 nM TTX is again apparent.

Table III gives numerical data on the maximum value for the early transient (sodium) conductance computed by equation 1 and on the maximum value for the late steady-state (potassium) slope conductance before and after application of 1,000 nM TTX internally.

Procaine

CURRENT CLAMP In contrast to the ineffectiveness of internally perfused TTX to block excitability, procaine was able to block action potentials from inside the nerve membrane as easily as it did from the outside. A typical example of experiments is illustrated in Fig. 5, in which resting potential

and action potential are plotted against time. Internal application of procaine at a concentration of 1 mM caused a quick hyperpolarization (of the order of 1 min) by an amount of 2–4 mv. The action potential progressively declined, but usually no complete blockage occurred with this concentration

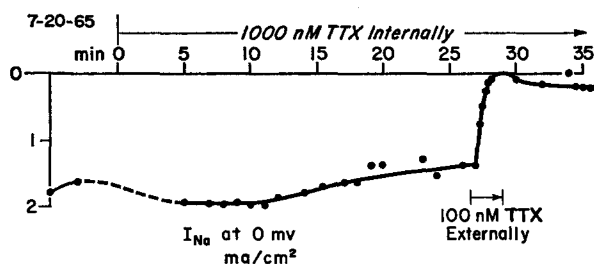

Figure 3. Changes in the early transient current (I_{Na}) in a perfused voltage-clamped axon before and after application of 1,000 nM tetrodotoxin (TTX) internally and of 100 nM tetrodotoxin externally. The early transient currents associated with depolarizations from the −90 mv holding potential to 0 mv are plotted against time. Internal solution, Table I C.

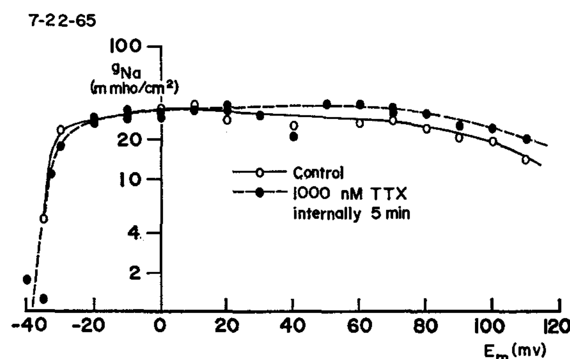

Figure 4. The membrane conductance at the peak early transient (sodium) current as a function of the membrane potential in a perfused voltage-clamped axon before and 5 min after application of 1,000 nM tetrodotoxin (TTX) internally. Holding potential −80 mv. Internal solution, Table I D.

of procaine. Upon washing with normal internal medium, the action potential was restored almost completely. The recovery of resting potential from the hyperpolarized level was slow in contrast to the rapid change on application of procaine inside. Internal procaine at a concentration of 10 mM caused a similar quick hyperpolarization associated with a faster and greater reduction of action potential (Fig. 5). Complete blockage and complete recovery upon washing occurred in most cases.

Numerical data on the resting and action potentials before and after application of procaine internally are given in Table IV.

TABLE III

THE MAXIMUM VALUES FOR THE EARLY TRANSIENT (SODIUM) CONDUCTANCE (g_{Na}) DEFINED BY EQUATION 1 AND FOR THE LATE STEADY-STATE (POTASSIUM)SLOPE CONDUCTANCE (g_K) IN PERFUSED VOLTAGE-CLAMPED AXONS BEFORE AND AFTER APPLICATION OF 1,000 nM TETRODOTOXIN (TTX) INTERNALLY

		g_{Na}		g_K	
Date	Time in TTX	Control	TTX	Control	TTX
	min	*mmho/cm²*	*mmho/cm²*	*mmho/cm²*	*mmho/cm²*
7-20-65	11	*	11	*	23
	16		10		20
	19		11		18
7-20-65	6	32	36	42	48
	14		35		39
	24		25		33
7-22-65	5	37	50	40	57
	13		52		68
	20		50		80
	28		45		75
7-22-65	5	30	35	33	40
	15		34		42

* No data are available before applying TTX.

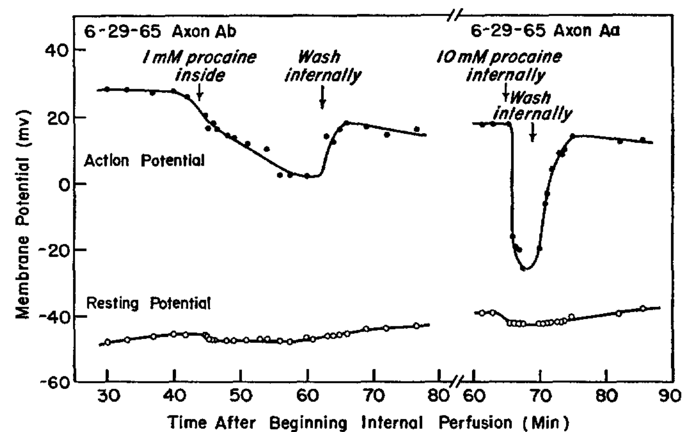

FIGURE 5. The effects of internal perfusion of 1 mM and 10 mM procaine on resting potential and action potential. Internal solution, Table I A.

VOLTAGE CLAMP Voltage-clamp experiments revealed that internally applied procaine blocked both the early transient and late steady-state conductance increase mechanisms as it did from outside the nerve membrane (Taylor, 1959; Shanes et al., 1959; Blaustein and Goldman, 1966). Typical

TABLE IV

THE RESTING AND ACTION POTENTIALS BEFORE AND AFTER APPLICATION OF PROCAINE INTERNALLY

Date	Axon	Procaine Concentration	Procaine Time	Resting potential Control	Resting potential Procaine	Height of action potential Control	Height of action potential Procaine
		mM	*min*	*mv*	*mv*	*mv*	*mv*
6-29-65	Ab	1	18	−45	−47	71	49
7-3-65	Aa	1	8	−48	−51	66	Block
7-10-65	Ab	1	9	−48	−49	79	67
7-3-65	Ca	3	4	−55	−58	*	Block
6-28-65	Aa	10	20	−44	−47	83	Block
6-29-65	Aa	10	4	−39	−43	57	Block
6-29-65	Ab	10	4	−43	−47	59	Block
7-10-65	Aa	10	5	−53	−52	82	Block

* Action potentials were not recorded on films.

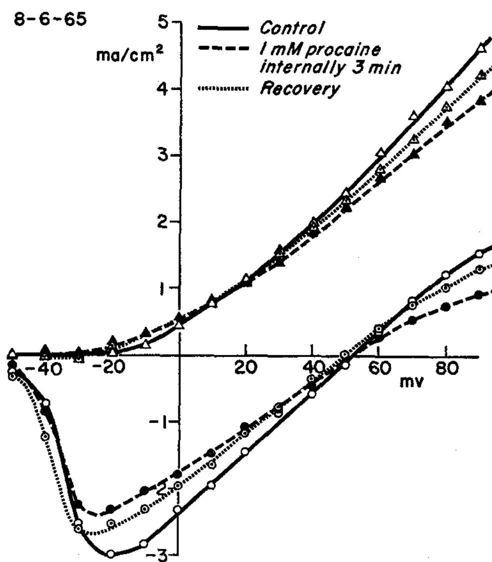

FIGURE 6. Current-voltage relations for the early transient current (circles) and the late steady-state current (triangles) in a perfused voltage-clamped axon before and 3 min after application of 1 mM procaine internally and after washing with normal internal solution. Holding potential −70 mv. Internal solution, Table I D.

current-voltage relations for the early transient current and the late steady-state current are illustrated in Figs. 6 and 7. Internal application of procaine at a concentration of 1 mM depressed both currents and partial recovery occurred upon washing (Fig. 6). There was no shift in the membrane potential at which the early transient current reversed its direction. Fig. 7 shows the result with 10 mM internal procaine. Depression of the early transient and late steady-state currents was greater and the recovery was poorer in this particular case.

The time to peak early transient current was prolonged by internal perfusion of procaine as it was from outside the nerve membrane. Fig. 8 shows the relationship between the time and the membrane potential, before, during, and after 10 mM internal procaine.

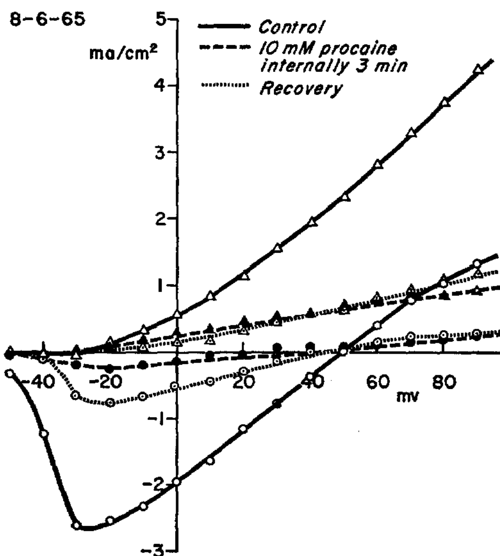

FIGURE 7. Current-voltage relations for the early transient current (circles) and the late steady-state current (triangles) in a perfused voltage-clamped axon before and 3 min after application of 10 mM procaine internally, and after washing with normal internal solution. Holding potential −70 mv. Internal solution, Table I D.

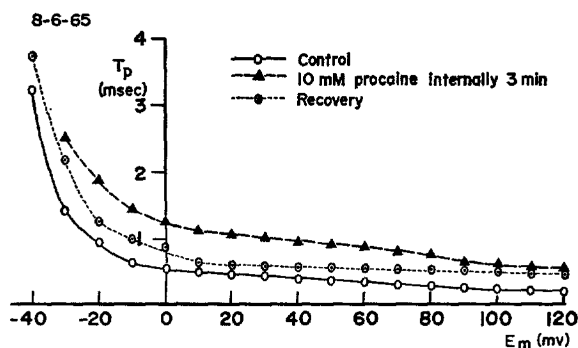

FIGURE 8. The time to peak early transient current (T_p) plotted as a function of the membrane potential in a perfused voltage-clamped axon before and 3 min after application of 10 mM procaine internally, and after washing with normal internal solution. Holding potential −70 mv. Internal solution, Table I D.

The early transient (sodium) conductance at the peak initial current was calculated as in TTX by equation 1, and plotted as a function of membrane potential in Fig. 9. It will be seen that internal procaine, both at 1 mM and at 10 mM, brings the conductance curve downward along the conductance axis. In the experiment of Fig. 9, there was no shift of the conductance curve

along the potential axis for 1 mM and 10 mM procaine when the curves were normalized. In two other experiments, there were shifts of 6 and 10 mv for 1 mM internal procaine toward more positive inside membrane potentials. Taylor (1959) reported a slight shift (3–6 mv) in the same direction for an external application of 0.1% (3.7 mM) procaine. Recalculations of the data of Blaustein and Goldman (1966) for an external application of 4 mM procaine showed shifts of 2 and 12 mv in the same direction in two cases (their Figs. 5 and 7) but little or no shift in another case (Fig. 1). Thus it can be said that the shift of the early transient conductance curve along the potential axis, if present, is in the same direction for external and internal applications of procaine.

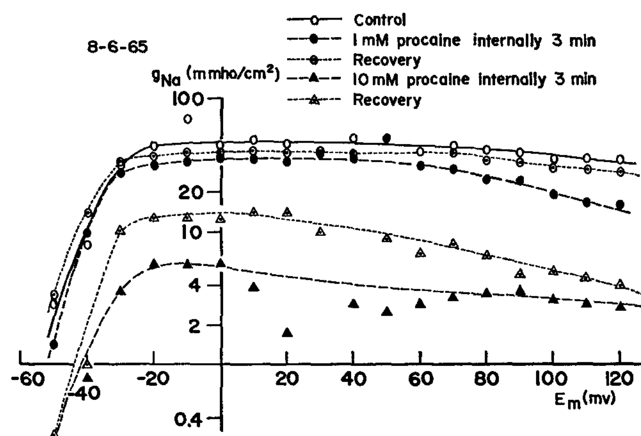

FIGURE 9. The membrane conductance at the peak early transient (sodium) current as a function of the membrane potential in a perfused voltage-clamped axon with and without procaine internally at concentrations of 1 mM and 10 mM. The measurements were made in the order of: control, 1 mM internal procaine, recovery after internal washing, 10 mM internal procaine, recovery after internal washing. Holding potential −70 mv. Internal solution, Table I D.

The maximum value for the early transient (sodium) conductance computed by equation 1 and the maximum value for the late steady-state slope (potassium) conductance are given in Table V. It is clear that both conductances are inhibited to the same extent by 1 mM internal procaine. This is in contrast to the effect of external procaine, which inhibits the early transient conductance more effectively than the late steady-state conductance (Taylor, 1959).

DISCUSSION

The results of present experiments confirm and extend those reported previously (Moore, 1965), but are in sharp contrast with those obtained by Naka-

mura et al. (1965 *a*). Although the latter could obtain blockage by injection of TTX at an estimated concentration of 300 nM, the present results may be taken as well established, because TTX was certainly in contact with the internal surface of the nerve membrane at higher concentrations for long periods of time. One of the possible reasons for blockage by TTX injection by Nakamura et al. (1965 *a*) would be diffusion of injected TTX to the outside surface of the nerve membrane through branches of the axon.

In view of reasonably stable properties of TTX molecules in aqueous solution, it is highly unlikely that TTX was degraded to inactive forms shortly after dissolving in K glutamate or KF internal perfusate. It is also most

TABLE V

THE MAXIMUM VALUES FOR THE EARLY TRANSIENT (SODIUM) CONDUCTANCE (g_{Na}) DEFINED BY EQUATION 1 AND FOR THE LATE STEADY-STATE (POTASSIUM) SLOPE CONDUCTANCE (g_K) IN PERFUSED VOLTAGE-CLAMPED AXONS BEFORE AND AFTER APPLICATION OF 1 mM PROCAINE INTERNALLY

	g_{Na}			g_K		
Date	Control	Procaine	Inhibition	Control	Procaine	Inhibition
	mmho/cm²	*mmho/cm²*	%	*mmho/cm²*	*mmho/cm²*	%
7-23-65	103	45	57	109	48	56
7-28-65	83	29	65	70	16	77
8-6-65	54	18	67	48	17	65
8-6-65	48	39	19	55	43	22
Mean			52			55

likely that internally perfused TTX did reach the inner surface of the nerve membrane, because no diffusion barrier has been found even in the perfused axon in which greater amounts of axoplasm remained (Tasaki et al., 1962).

TTX is not soluble in most organic solvents (Mosher et al., 1964), so that it is safe to assume that it is not soluble in lipids of the nerve membrane either. A corollary of this property and of the present experimental results is that the site of action of TTX is located on the outer surface of the nerve membrane rather than on the inner surface or within the membrane itself. The site of action can be visualized at the "gate" of the early transient channel. It is reasonable to assume that this gate is open at the outer surface of the nerve membrane in view of the insolubility of TTX in lipids. The gate is probably controlled by membrane potential and calcium (Goldman, 1964). High external calcium concentration has been shown to interact with the blocking action of TTX (Takata et al., 1966), and this is consistent with the above mentioned notion.

A guanidinium group is contained in the TTX molecule (Mosher et al.,

1964). Sodium can be replaced by guanidine to produce action potentials in squid axons under certain conditions (Tasaki, Singer, and Watanabe, 1965), in frog spinal ganglion cells (Koketsu, Cerf, and Nishi, 1959), and in frog nerve fibers (Larramendi, Lorente de Nó, and Vidal, 1956). This is probably due to the ability of guanidine to pass through the early transient channels of the nerve membrane. It then appears possible that the guanidinium of the TTX molecule becomes lodged in the gate of the early transient channel on the nerve membrane surface thereby blocking the movement of sodium ions as suggested for saxitoxin (Kao and Nishiyama, 1965). However, the blocking ability of TTX cannot merely be ascribed to the presence of the guanidinium group, since one of the TTX derivates fails to show the ability despite the presence of the guanidinium group in the molecule (Narahashi, Moore, and Poston, 1966; Deguchi, personal communication).

The observation that procaine blocks excitability from inside the nerve membrane was rather to be expected in view of its ability to penetrate through the lipid membrane. Thus when procaine is applied to either side of the membrane, it can reach the sites of action no matter where they may be located.

In contrast to the selective blockage of the early transient channel by TTX, cesium and tetraethylammonium (TEA) appear to block the late steady-state channel only from inside the squid nerve membrane. Externally applied cesium apparently does not penetrate the squid nerve membrane either through the early transient or through the late steady-state channel (Pickard, Lettvin, Moore, Takata, Pooler, and Bernstein, 1964). When applied internally, cesium blocks the late steady-state conductance change (Chandler and Meves, 1965; Adelman, Cuervo, Dyro, and Senft, 1966). Tasaki et al. (1965) have shown that externally applied TEA is carried through the early transient channel with great difficulty. TEA can block the late steady-state conductance change only when it is applied internally (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965). These observations are compatible with the notion that the gate of the late steady-state channel is open on the inner surface of the nerve membrane, if cesium and TEA block the late steady-state current by plugging in the channel. Alternatively, it is also possible for TEA and cesium to block the late steady-state current by dislocating the crystal lattice of the membrane structure, but this is less likely because nonspecific disturbance of the ionic currents would be expected under this condition. However, the situation appears to be different in the node of Ranvier of frog, because TEA on the outside blocks the late steady-state conductance mechanism without affecting the early transient conductance mechanism (Hille, 1966).

The hypothesis of the outside early transient gate and the inside late steady-state gate might explain the ability of procaine to inhibit the late steady-state

conductance mechanism more strongly from inside than it does from outside. The average percentage inhibitions of the early transient and late steady-state conductances for the external procaine concentration of 0.1% (3.7 mM) were calculated from Tables 2 and 5 of Taylor (1959), and turned out to be 62% for the early transient conductance and 24% for the late steady-state conductance. For internal application of 1 mM procaine, the percentage inhibitions were 52% for the early transient conductance and 55% for the late steady-state conductance (Table V). In spite of the lower concentration, procaine causes higher inhibition of the late steady-state conductance mechanism from inside the nerve membrane than it does from outside. This is in favor of the notion that the late steady-state gates are located on the inner surface of the nerve membrane. However, we cannot exclude the possibility that procaine disturbs the early transient and late steady-state channels through dislocation of the crystal lattice of the membrane structure, because it causes nonselective blockage of both the early transient and late steady-state mechanisms and also because the threshold concentration is very high compared with that of TTX.

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