EFFECT OF TETRODOTOXIN ON THE EARLY OUTWARD CURRENTS IN PERFUSED GIANT AXONS*

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The ionic currents that flow during a voltage clamp of the membrane of the squid axon in sea water were measured\(^1\) and analyzed\(^2\) into an early transient current of Na\(^+\) and a later outward current of K\(^+\). In 1952, Hodgkin et al.\(^2\) observed a reversal in the early current when the axonal membrane was strongly depolarized. The characteristics of the early current led Hodgkin and Huxley\(^3\) to propose that, whether inward or outward, it was carried by the same ion, sodium. The potential at which the early current reversed was called the sodium potential and it was shown to vary with the log of the external sodium concentration.

Recently, Chandler and Meves\(^4\) studied the effects of internal ions on the early voltage clamp outward current with internally perfused squid axons. By varying the concentrations of monovalent cations inside and outside the axon and measuring the potentials at which the early current reversed direction, they found that internal K, Rb, and Cs ions contribute to the early outward current. The discovery that tetrodotoxin blocks the early inward current of Na\(^+\) without affecting the later outward current of K\(^+\) in the excitable membrane\(^5\) has naturally led to the question: Is tetrodotoxin interfering directly with the particular ion carrying this current or, more generally, with a fast mechanism by which various ions cross in the membrane?

Moore et al.\(^7\) found that the inward current was blocked by tetrodotoxin whether carried by sodium or by lithium, and that the later outward current was not affected whether carried by potassium or rubidium. The evidence, then, points to tetrodotoxin as a blocking agent specific for the early mechanism, regardless of the ions involved. The results presented here provide additional support for this hypothesis, by demonstrating that externally applied tetrodotoxin is as effective in blocking the early outward current when the current is carried by potassium ions as when it is carried by sodium or lithium ions.

Methods.—The experiments were carried out on the giant axon of the Chilean squid, Dosidicus gigas. These squid were caught and killed a few miles offshore. The mantles were kept in iced sea water during the trip to shore. Within 2 hr of capture of the animal, the mantle nerves were removed and the giant axons were then dissected from these bundles and prepared for experiment.

An axon was suspended in a temperature-controlled bath of K\(^+\)-free artificial sea water (430 mM NaCl, 10 mM CaCl\(_2\), 50 mM MgCl\(_2\), 5 mM Tris at pH 7.5). Internal perfusions of the axons were performed by means of a suction perfusion procedure.\(^8\) A glass outlet cannula was introduced into one end and the axoplasm was gently removed by suction as the cannula was slowly advanced. A glass inlet cannula, connected to a perfusion solution reservoir, was introduced into the other end of the axon until the tip of the cannula was inside the outlet cannula. The axoplasm was then washed out of the outlet cannula with the perfusion solution and the outlet cannula was drawn back until about 15 mm of the axon was under perfusion. After a steady perfusion rate was obtained, an internal electrode was introduced so that its tip was at a point midway along the perfused region. The membrane potential was taken between this point and an external reference electrode. A platinum wire was used to supply current along the part of the axon under perfusion. The wire was insulated except for the 15-mm length in the perfused zone where it had been plated with platinum black. However, instead of introducing the current wire through the
outlet cannula as described previously, which often led to damage of the axonal membrane, the wire was fixed to the inlet cannula and introduced at the same time. The 15-mm platinumized length extended beyond the end of the cannula along the axis of the axon, and its tip was brought to rest just within the opening of the outlet cannula.

In these experiments, the point control voltage-clamp system developed by Moore and Cole was used. As the early outward current rises within 200 μsec after the start of a potential step in the voltage clamp, one of the major difficulties in resolving this current was the initial capacity current surge that may mask even the first millisecond of membrane current. To reduce the duration of the capacitive transient, and thus improve the time resolution of the system, the impedance of the internal voltage-measuring electrode was reduced by inserting a bright platinum wire inside the electrode and filling the electrode with 600 mM KCl solution. This low-resistance salt bridge was connected to a Ag, AgCl electrode, as was the external reference salt bridge.

Before beginning a series of voltage-clamp experiments, the concentration of tetrodotoxin required to reversibly block the membrane action potential was determined. The poison was added to the external K-free artificial sea water. It was found that an external concentration of \(10^{-4} \text{M}\) tetrodotoxin reversibly blocked the action potential in about 2 min without altering the resting potential, whereas a \(10^{-5} \text{M}\) solution produced no measurable effect within 20 min after application. In all experiments, tetrodotoxin was applied externally at the concentration of \(10^{-4} \text{M}\).

To determine the effect of tetrodotoxin on voltage-clamp currents, a standard protocol was followed. Axons were subjected to five voltage-clamp runs (in one case three) under different conditions, the first and last being controls run for comparison.

First, the fibers were perfused externally in K-free artificial sea water and internally for 5–25 min with potassium solution (500 mM KF, 50 mM KCl, 5 mM Tris, pH 7.3). During the first voltage clamp run, the membrane was pulsed from the resting potential (about −60 mv) to a series of depolarizing values up to +130 mv. Reversal in the early inward current was apparent when the membrane was depolarized to beyond approximately +80 mv.

After replacing about 20% of the K in the internal perfusion solution with Na (110 mM NaF, 11 mM NaCl, 389 mM KF, 39 mM KCl, 5 mM Tris, pH 7.3), or Li, three voltage-clamp series were run: first, for bathing externally with K-free artificial sea water, second, for external bathing with K-free artificial sea water containing \(10^{-4} \text{M}\) tetrodotoxin, and third, allowing 15–20 min for recovery, again for bathing externally with K-free artificial sea water.

In most cases, a final series was run, perfusing with the original potassium solution. In one case, the internal solution did not contain partial replacement of potassium with other cations before blocking the axon with tetrodotoxin.

The oscilloscope records were made on film, projected, and traced. The membrane currents shown in the figures were not corrected for membrane capacity or leakage. Resting and action potentials were recorded for each change in the composition of the internal and external solutions. The resting potentials were corrected for junction potentials and the action potentials were measured directly from the films.

Results.—Effect of tetrodotoxin on voltage-clamp currents: The inhibition of the early outward current by tetrodotoxin when potassium was the only internal cation is shown in Figure 1. In the first run (A), the early current reversed at an absolute membrane potential of about +77 mv. This run was taken after 16 minutes of internal perfusion with the potassium solution at a rate of 15 μl/minute. As the volume of the perfused zone was less than 15 μl, the internal solution was changed at least once a minute. As potassium was the only internal cation, it is considered as carrying this current. Record (1B) shows that both the early inward current and the early outward current were blocked by tetrodotoxin. Measurements over the complete voltage-clamp run showed that while the early current was blocked, the later outward steady current was not appreciably affected (only a very slight increase in the later outward current was noticed). That the blocking effect was reversible is shown in record (1C), which was taken 17 minutes after the removal of the toxin.
Figure 1.—Voltage-clamp currents obtained with axon 1, diameter 1050 μ, intracellularly perfused with potassium solution. External solution, potassium-free artificial sea water. Pulse duration 30 msec, no pre-pulse. The numbers and the underlines give the clamped membrane potential in mv and the base line. (A) Control run after 16 min of perfusion; (B) second run after 20 min with tetrodotoxin in the external solution; (C) recovery run 17 min after removal of tetrodotoxin. Temperature of the external solution was 8.5°C after the addition of tetrodotoxin.

Figure 2 illustrates the last three runs of a typical experiment in which the internal perfusion solution contained sodium. Record (2A), presenting the third voltage-clamp run performed on axon 3, shows blockade of the early outward current. Record 2B shows the recovery with sodium still inside. The last run, record (2C), obtained after perfusing 22 minutes with potassium as the only internal cation, shows the decrease in the early outward current. One experiment was performed with lithium instead of sodium, at the same concentration, and the results obtained were almost identical to those with sodium.

Effect of tetrodotoxin on potentials: Potential values before and after treatment with tetrodotoxin are listed in Table 1. Also given in Table 1 are the apparent equilibrium potentials obtained from the initial membrane current null-points. These potentials were computed after correcting the initial current component for leakage current. As tetrodotoxin blocks both inward and outward early currents, the remaining current visible in record (1B), persisting for about 400 microseconds after the pulse onset, was considered to be most probably due to leakage current. A plot of this leakage current against absolute membrane potential up to +130 mv was linear and had a positive slope. In each experiment, the early current was measured at each absolute membrane potential pulse value, and was then corrected for the leakage current obtained from the corresponding voltage-clamp run with tetrodotoxin. Thus the corrected early currents were plotted against absolute membrane potential, and the intercepts, at zero current, were taken as the apparent equilibrium potential, $V_e$. 

FIG. 2.—Voltage-clamp currents obtained with axon 3, diameter 1000 μ intracellularly perfused with sodium (expt. 3b) and potassium solution (expt. 3a). External solution, potassium-free artificial sea water. Pulse duration 30 msec, no pre-pulse. The numbers and the underlines represent the clamped-membrane potential in mv and the base line. (A) Third run after 22 min with tetrodotoxin in the external solution, perfusing with sodium solution; (B), recovery run 20 min after removal of tetrodotoxin, perfusing with sodium solution; (C) final run after 22 min of perfusing with potassium solution. Temperature of the external solution was 6.5°C before the last run.

Table 1 shows that with potassium as the only internal cation (expts. 1, 2a, 3a) the average apparent equilibrium potential, $V_e$, is +72 mv. To compare these results with those of Chandler and Meves, for $V_e = +72$ mv, a $P_{Na}/P_K$ ratio of 25 was computed, in contrast to the $P_{Na}/P_K$ ratio of 12 computed by them. Chandler and Meves reported a decrease in the calculated $P_{Na}/P_K$ ratio when the internal perfusion solution was diluted, so that some of the discrepancy may have been caused by the lower ionic strength internal solution that they used. Table 1 also shows that when we internally perfuse with a solution containing 20 per cent of the K replaced by Na (expts. 2b, 3b), the ap-

### Table 1

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Cation</th>
<th>R.P.</th>
<th>Before</th>
<th>V</th>
<th>After</th>
<th>A.P.</th>
<th>V</th>
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<tr>
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<td>73</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
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<td>K</td>
<td>61</td>
<td>106</td>
<td>69</td>
<td>61</td>
<td>106</td>
<td>69</td>
<td></td>
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<tr>
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<td>96</td>
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<td>—</td>
<td>61</td>
<td>104</td>
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</tbody>
</table>

Index, a or b, of the axon number represents different experimental conditions with the same axon. R.P., resting potential corrected for a junction potential of +7 mv measured between the internal solution and the external solution through a 3 M KCl agar-bridge; A.P., action potential height; $V_e$, apparent sodium equilibrium potential determined after correcting for leakage currents.
parent equilibrium potential decreases to an average value of +47.7 mv. Using
a value of 25 for the ratio $P_{Na}/P_{K}$, we calculate a $V_e$ value of +27 mv, indicating
either that the equation is invalid for these data or that the $P_{Na}/P_{K}$ ratio is altered
by the internal concentrations of cations.

Discussion and Conclusions.—The results presented here for data obtained from
giant axons of Dosidicus gigas confirm the results previously found in other excitable
membranes,\(^5\)\(^-\)\(^7\) namely that externally applied tetrodotoxin blocks the early inward
current of Na\(^+\) without appreciably affecting the later outward current of K\(^+\).
Furthermore, we have confirmed that the early outward current is also blocked by
externally applied tetrodotoxin. In neither case does tetrodotoxin block the de-
layed outward current carried by potassium. However, what is most striking is
that external tetrodotoxin blocks the early current irrespective of the type of ion
carrying the charge. As tetrodotoxin blocks the early current regardless of the ion
carrying that current or the direction of the flow, it seems quite evident that this
poison is specific for the fast conductance mechanism in the membrane shown\(^11\) to be
responsible for the normal rising phase of the action potential.

Tetraethylammonium (TEA) has been shown to be an inhibitor of the later out-
ward potassium current in the giant axons.\(^12\)\(^-\)\(^13\) TEA and tetrodotoxin reflect the
asymmetry of the axonal membrane in that TEA is only effective when applied
internally,\(^13\) and tetrodotoxin is only effective when applied externally.\(^14\) One
might expect that these two inhibitors of membrane conductance mechanisms would
act in a similar manner, namely by competing with the ions carrying the currents.
However, if one considers the TEA-blocking mechanism proposed by Armstrong
and Binstock,\(^13\) the results reported here are somewhat curious. According to
them, TEA, in competing with K, effectively blocks the later current in only one
direction—outward. A strong hyperpolarizing pulse causes an instantaneous re-
versal of the outward current and temporarily “sweeps off” the blocking molecules,
thus allowing some potassium current to flow in through the membrane. If this
were the case for the blocking mechanism of tetrodotoxin, one would expect to see,
in a tetrodotoxin-blocked axon, some early outward current, at least at the very high
depolarizations. However, for depolarizing pulses of as much as 220 mv (some 80
mv above the sodium equilibrium potential), no hint of an early outward current
was seen. Therefore, tetrodotoxin either competes much more efficiently for the
ions than TEA, or the blocking mechanism is completely different. In view of the
tremendous difference in the effective concentrations of these two inhibitors—TEA
blocks\(^15\) at more than $10^{-3} M$ and tetrodotoxin at $10^{-8} M$—it seems more plausible
that the blocking mechanism is in fact different. The present results have also
shown that the magnitude of the early outward current depends on the internal
cations and their concentrations.

It is now clear that the early outward current is in fact a flow of monovalent
cations carried by the fast mechanism, as has been proposed by Chandler and
Meves.\(^4\) It should be possible to calculate the permeability ratios of the cations for
this system of the membrane using the early outward current and the changes in the
apparent equilibrium potential. However, from the data presented here, pre-
liminary calculations of the sodium and potassium permeability ratios are not in
accordance with those reported by Chandler and Meves\(^4\) for different cation con-
centrations. Although we still believe that such calculations are feasible, we find
that the theoretical treatment available at present is not adequate to determine permeability ratios for the initial conductance system of the axonal membrane.

Recently we have observed that $10^{-4} M$ TEA, internally applied, reversibly inhibits the later outward potassium current but that does not interfere with the early outward current carried by potassium ions.

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