Separation of the Action Potential into a Na-channel Spike and a K-channel Spike by Tetrodotoxin and by Tetraethylammonium Ion in Squid Giant Axons Internally Perfused with Dilute Na-salt Solutions

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ABSTRACT Squid giant axons internally perfused with a 30 mM NaF solution and bathed in a 100 mM CaCl₂ solution, which are known to produce long lasting action potentials in response to pulses of outward current, were investigated. The effects of tetrodotoxin (TTX) and of tetraethylammonium ion (TEA⁺) on such action potentials were studied. The results are summarized as follows: (a) An addition of 1-3 μM TTX to the external solution altered but did not block the action potentials; it increased the height of the action potential by ~15 mV, and it decreased the membrane conductance at the peak of excitation by about two-thirds. (b) Voltage-clamp experiments performed with both NaCl and TTX in the external CaCl₂ solution revealed that the TTX-insensitive action potential does not involve a rise in gNa, whereas the experiments performed without TTX showed that the action potential is accompanied by a large rise in gNa. (c) Internally applied TEA⁺ was shown to selectively block the TTX-insensitive action potential, but it did not block the other component of the action potential, which is accompanied by a rise in gNa, and which is selectively suppressed by TTX. (d) The addition of a small amount of KCl to the external CaCl₂ solution containing TTX greatly increased both the maximum peak inward current under voltage clamp and the maximum slope conductance. Furthermore, it was shown that K⁺ applied on both sides of the axon plays a dominant role in producing the membrane potential in the active state in the presence of TTX, even though a large amount of Ca²⁺ is presented in the bathing medium. These observations have led me to conclude that the sodium channel is responsible for the production of the TTX-sensitive component of the action potential under the ionic conditions of these experiments, and the potassium channel for the TTX-insensitive component of the action potential.
INTRODUCTION

The generation of a normal action potential in nerve tissues is characterized by a fast transient rise in the sodium conductance followed by a relatively slow increase in the potassium conductance (Hodgkin and Huxley, 1952). Tetrodotoxin (TTX) is known to selectively block the sodium conductance without influencing the potassium conductance (cf. Narahashi et al. [1964] and Moore et al. [1967]). On the other hand, the quaternary ammonium base tetraethylammonium (TEA) has a selective effect on the potassium permeability channel (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965; Hagiwara and Saito, 1959; Nakajima, 1967; Koppenhöfer, 1967; Koppenhöfer and Vogel, 1969; Hille, 1967).

Under internal perfusion with a dilute sodium-saline solution, squid giant axons bathed in a CaCl₂ solution can produce prolonged action potentials in response to pulses of outward current applied to the membrane (Watanabe et al., 1967). The direction of the action potential is the same as that of the normal action potential, even though the concentration gradient of Na⁺ across the membrane is reversed. Considerable effort has been made to characterize the ionic processes underlying this anomalous action potential observed under conditions far from physiological (cf. Tasaki et al. [1967], Watanabe et al. [1967], Meves and Vogel [1973], Inoue et al. [1973], and Inoue et al. [1974]). Tasaki and his colleague explained the phenomenon in terms of a cooperative cation exchange taking place in only one species of macromolecular complex (site) in the membrane (cf. Tasaki [1968]). On the other hand, Meves and Vogel (1973) considered that even such an abnormal action potential could be explained by means of the sodium theory.

Quite recently, we found that a Ca²⁺ current component that is insensitive to TTX is involved in the long lasting action potential elicited from squid axons internally perfused with a sodium-saline solution.¹ The present investigation was undertaken to characterize the site responsible for production of the TTX-insensitive action potential, as well as that associated with development of the TTX-sensitive action potential. Experiments were performed on squid axons internally perfused with a 30 mM NaF solution and bathed in a 100 mM CaCl₂ solution, in the absence and in the presence of TTX. The effects on the electrical properties of these axons of externally applied Na⁺, K⁺, or TEA⁺, and of internally applied TEA⁺ or K⁺ were studied. This paper describes experimental results that reveal the roles of the sodium and potassium channels in the production of the two kinds of action potentials.

METHODS

Materials and Internal Perfusion

Giant axons of squid, Doryteuthis bleekeli, caught at the Tomo-ura Fishermen's Cooperative Association, Kaifu-cho, Shikoku-island, Japan, were used throughout the experiments. The diameter of the axons used was between 400 and 600 μm. The major portion of small nerve fibers and connective tissues surrounding the giant fiber were

¹ Inoue, I., T. Hirosye, and Y. Kobatake. Unpublished observation.
removed under a dissecting microscope. An axon thus prepared was then transferred into a Lucite chamber filled with natural seawater, where internal perfusion was performed. The technique of internal perfusion with two glass cannulae was basically the same as that reported previously (Inoue et al., 1976). The outer diameter of the inlet cannula was 130 μm and that of the outlet cannula was 320 μm. Internal perfusion was initiated with a 30 mM NaF solution containing 0.05 mg/ml of Pronase for a period of 2–3 min. During this period the external seawater was replaced with a 100 mM CaCl₂ solution. The enzyme solution was then replaced with an enzyme-free NaF solution. The length of the perfusion zone was 18–20 mm. The rate of flow of the internal perfusion fluid was kept at between 10 and 20 μl/min by placing a reservoir of the internal perfusion fluid at an appropriate height.

Solutions
A solution containing 20 mM NaF, 10 mM sodium phosphate, and 11.4% (by volume) glycerol was used as the standard internal perfusion fluid. The pH of the solution was adjusted to 7.3 ± 0.1 with the sodium phosphate. This solution is referred to as 30 mM NaF solution in this paper. The standard external solution contained 100 mM CaCl₂, 5 mM Tris-HCl, and 9% (by volume) glycerol, pH 7.9 ± 0.1. When an addition of NaCl, KCl, or TEA-Cl to the external medium was required, the nonelectrolyte (glycerol) of the 100 mM CaCl₂ solution was isoosmotically replaced with a salt. KF or TEA-phosphate was added to the internal perfusion fluid in a similar manner.

1 mg of TTX was dissolved in 10 ml of distilled water. The TTX solution was added to the external aqueous solution when treatment of the axons with TTX was required. An addition of 30 nM TTX (final concentration) to the external seawater completely blocked the sodium current in unperfused axons.

The TTX was provided by the Sankyo Chemical Co., Japan. The Pronase was obtained from the Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. The salts and acids were purchased from the Nakarai Chemical Co., Japan.

Electrical Instruments
The following instruments were used for studying the electrical properties of the axon membrane: a high input-impedance preamplifier (Nihon Kohden, Japan, model MEZ 7101), a dual-beam oscilloscope (Nihon Kohden, model VC-9), an oscillograph camera (Nihon Kohden, model PC-2B), an electric stimulator (Nihon Kohden, model SEN-7130), and a homemade feedback amplifier (gain, 60–80 db).

Measurement of the Membrane Potential
A glass pipette electrode (~70 μm in diameter) filled with a 3 M KCl solution in contact with an Ag-AgCl wire was used for measurement of the intracellular potential. The size of the hole at the tip of the electrode was reduced by heating to reduce outflow of the KCl solution. A platinum wire (30 μm in diameter) was inserted into the electrode to lower the electric resistance of the electrode. A calomel electrode immersed in the external solution was used as a reference point for the potential measurements. The internal electrode was introduced into the axon through the outlet cannula. Shortly before insertion of the internal electrode into the axon, the tip of the electrode was put into the external fluid, and the output potential level through the preamplifier was adjusted to zero by controlling a DC offset level of the instrument. After measurement of the membrane potential, the internal electrode was immersed again in the external fluid, and the magnitude of a change of the tip potential was checked. The change was not more than 2–3 mV during an experiment lasting ~1 h.
Measurements of the Membrane Current

A platinized platinum wire (50 μm in diameter) was used as an internal current supplying electrode. Platinized platinum wires (0.5 mm in diameter) immersed in the external fluid were used as a current measuring electrode with a guard system (Hodgkin et al., 1952). The rise time of voltage pulses in the voltage-clamp experiments was <30 μs. The membrane current was recorded through a high frequency cut-off filter (f > 30 kHz) in most of the experiments, in which only a slow and small peak current was detectable (see Results). The voltage-current curves give the maximum current (which could be either a peak or a steady current) at various voltages following steps from a holding potential of (usually) −35 to −40 mV.

All the experiments carried out at room temperature, 19° ± 2°C.

RESULTS

Demonstration of the TTX-insensitive Action Potential

The axons were internally perfused with the 30 mM NaF solution and bathed in the 100 mM CaCl₂ solution. The average value of the resting potential obtained from 25 axons was −37.1 mV, with a standard deviation (SD) of 5.0 mV. The amplitude of action potential elicited from these axons was 47.1 ± 5.8 mV (mean ± SD). Thus, the action potential overshoot was ~10 mV on the average. The duration of the action potentials was distributed in a wide range, 0.5–10 s. These observations are in agreement with those reported previously (Watanabe et al., 1967; Meves and Vogel, 1973; Inoue et al., 1974).

When a small amount of TTX (final concentration, ~1 μM) was added to the bathing medium, there was an immediate rise in the height of the action potential in all axons examined (10 of 25), whereas the resting potential was entirely unchanged. Excitability was maintained, and the axon continued to produce all-or-none action potentials of virtually the same size for at least 30 min, even when the TTX concentration was raised to 3 μM. The average height of the action potentials obtained from the TTX-treated axons was 62.1 mV with an SD of 8.5 mV. Therefore, TTX increased the action potential by ~15 mV on the average. Fig. 1 exhibits an example of oscillographic records showing such an effect of TTX on the action potential. The records on the left and on the right were obtained before and after an addition of 1 μM TTX to the external CaCl₂ solution, respectively. TTX increased the height of the action potential from 46.5 to 56.0 mV, but no appreciable effect of TTX on the rate of rise in the action potential was observed.

Voltage-clamp experiments revealed that externally applied TTX does not block peak inward current flow, but it reduces both the magnitude of the peak current and the maximum slope conductance. Fig. 2 illustrates an example of such an effect of TTX on the voltage-current characteristic of the membrane (left) and that on the time-course of the maximum peak current (right). (Records A and B represent the membrane current at the points marked by A and by B in the curves.) The holding potential was kept at −39 mV throughout the experiment. The open circles represent the data obtained before an application of TTX. Values of the maximum slope conductance and the leakage conductance are 2.86 and 0.17 mmho/cm², respectively. The
separation of the two zero-current intercepts, which correspond to threshold and to the action potential peak, is 44 mV in this case. The data shown by the solid circles were obtained after an addition of 3 μM TTX to the external solution. TTX decreased the maximum slope conductance to 0.91 mmho/cm², whereas it increased the reversal potential by ~23 mV. On the other hand, TTX had no effect at all on the leakage conductance.

In record A (Fig. 2), which displays the time-course of the maximum peak current before TTX treatment, one may notice that the rate of fall in the peak current is very slow in comparison with that in normal axons; the time required for the current to reach the peak is 14 ms. No earlier peak was detected under these conditions, even when the current recording was performed without the high frequency cut-off filter. Record B (Fig. 2) was obtained after treatment with TTX. It seems that TTX does not alter the rate of fall of the peak current but reduces the maximum peak current to some extent under these circumstances.

Intracellularly applied TTX had no effect on the electrical properties of the membrane. A replacement of fluoride ions in the internal perfusion fluid with phosphate ions produced no change in the electrical properties, either.

**Effect of Externally Applied Na⁺**

The TTX effect was more dramatic in the presence of external Na⁺. As has been reported (Cf. Inoue et al., 1974), an addition of NaCl to the external CaCl₂ solution enormously increases both the height and the maximum rate of rise in the action potential in axons internally perfused with a sodium-salt solution when they are not treated with TTX. These changes reflect in the membrane current under voltage clamp. Fig. 3a illustrates an example of such
an effect of Na⁺ on the time-course of the maximum peak current (right) and on the voltage-current characteristic of the membrane (left). In this experiment, 200 mM NaCl was added to the external 100 mM CaCl₂ solution. As can be seen from the data, externally applied Na⁺ brings about a large increase in the maximum slope conductance, in the separation of the two zero current intercepts, in the magnitude of the maximum peak inward current, and in the rate of fall in the peak current (see Fig. 3, record B). In record B,

![Diagram](image)

**Figure 2.** Voltage-current relations before (○) and after (●) an addition of 3 μM TTX to the external 100 mM CaCl₂ solution. The internal perfusion fluid was the 30 mM NaF solution. The oscillographic records A and B represent the time-courses of the maximum peak current marked A and B in the curves, respectively.

the time required for the current to reach the peak is approximately 0.6 ms, which is close to that of the sodium current in normal axons. The leakage conductance increased by ~50%.

When Na⁺ at the same concentration was added to the external CaCl₂ solution in the presence of TTX, the results obtained were quite different from those shown in Fig. 3a. One of the experiments is presented in Fig. 3b. An axon internally perfused with the 30 mM NaF solution was initially immersed
in a 100 mM CaCl₂ solution containing 3 μM TTX. The voltage-current curve represented by the solid circles was obtained under these conditions. The oscillographic record C in Fig. 3 represents the time-course of the maximum peak current. The external solution was then replaced by one

![Graph and oscillograms](image)

**Figure 3.** Effects of externally applied 200 mM Na⁺ on the membrane current under voltage clamp in the absence of TTX (a), and in the presence of 3 μM TTX in the external media (b). The axons were internally perfused with the 30 mM NaF solution, and the external media contained 100 mM CaCl₂. The oscillographic records A through D display the time-courses of the maximum peak current marked A through D in the V-I curves, respectively. For further details, see the text.
containing 100 mM CaCl₂, 200 mM NaCl, and 3 μM TTX. The voltage-current curves represented by the solid squares and by the solid triangles were taken 1 and 10 min after the application of Na⁺ externally. The oscillographic record D in Fig. 3 displays the time-course of the maximum peak current 1 min after the NaCl addition. In contrast to the effect of Na⁺ in the absence of TTX, externally applied Na⁺ in the presence of TTX produced no increase in the maximum slope conductance, in the separation of the two zero current intercepts, in the maximum peak inward current, or in the rate of fall in the peak current (record D); instead, there were small decreases in these parameters. On the other hand, the leakage conductance rose by ~50%, which was similar to the results in the absence of TTX.

![Graph](image)

**Figure 4.** Effects of internally applied TEA⁺ on the voltage-current characteristic of the membrane under three different external salt compositions. The cation compositions of both internal and external solutions are given in the figure.

When TTX was added to the bathing medium that contained both 100 mM CaCl₂ and 200 mM NaCl, there was an immediate fall in the excitability to a level close to that seen before applying Na⁺ externally, followed by a further gradual decline. The gradual decline of the excitability was encountered only when both TTX and Na⁺ were applied externally and was influenced not only by the concentrations of TTX and Na⁺ but also, to a certain extent, by the sequence of applications of TTX and of Na⁺; the decline was somewhat faster when Na⁺ was applied first.

These results can be interpreted as demonstrating that externally applied TTX completely blocks the entry of Na⁺ associated with excitation. This means that the TTX-insensitive action potential does not involve a rise in the
sodium conductance; nevertheless, it is accompanied by a rise in the membrane conductance.

Effect of TEA$^+$

As a first step in characterizing the sites responsible for production of the TTX-insensitive action potential, the effect of TEA$^+$ upon the voltage-current characteristic of the membrane was studied. Experiments were first performed without TTX. The axons studied were initially internally perfused with the 30 mM NaF solution, and subsequently with one containing both 30 mM NaF and 10–20 mM TEA-phosphate (pH 7.3). The effect of adding KCl or NaCl to the external 100 mM CaCl$_2$ solution was also studied.

Fig. 4a illustrates the effect of internally applied TEA$^+$ and that of externally applied K$^+$. The open circles show the control data obtained from an axon with the 30 mM NaF solution inside and with the 100 mM CaCl$_2$ solution outside. The maximum peak inward current observed under these conditions was $\sim -20 \mu$A/cm$^2$. When 10 mM TEA$^+$ was applied, there was a rapid fall in the peak current to almost zero, but the N-shaped characteristic was still detectable (see the data shown by the open triangles in Fig. 4a). On the other hand, TEA$^+$ did not affect the leakage conductance. The data shown by the crosses were obtained after the addition of 3 mM KCl to the external CaCl$_2$ solution in the presence of TEA$^+$ in the internal perfusion fluid. These data indicate that an application of such a small amount of K$^+$ does not alter the voltage-current characteristic of the membrane under these circumstances. (Note that this is in contrast to the effect shown in Fig. 7, in which the influence of externally applied K$^+$ in the absence of TEA$^+$ is exhibited.)

Fig. 4b shows the effect of externally applied Na$^+$ in the presence of TEA$^+$ in the internal NaF solution. The concentration of TEA-phosphate was 20 mM. The data shown by the open triangles were obtained when the axon was immersed in the 100 mM CaCl$_2$ solution. The curve is very similar to that shown by the same symbols in Fig. 4a. When 200 mM Na$^+$ was applied externally, a large earlier peak appeared in the membrane current. As shown by the open squares, the maximum peak inward current attained a level below $-150 \mu$A/cm$^2$. At the same time, the maximum slope conductance increased to a level above 9.0 mmho/cm$^2$. These values correspond to those obtained in the absence of TEA$^+$ (see Fig. 3a).

Experiments were then carried out in the presence of TTX. Fig. 5 shows one of the experiments. An axon bathed in the 100 mM CaCl$_2$ solution containing 1 $\mu$M TTX was initially internally perfused with the 30 mM NaF. The open circles in the figure display the control voltage-current curve obtained under these conditions. The maximum slope conductance and the leakage conductance are 0.53 and 0.20 mmho/cm$^2$, respectively. Then TEA$^+$ was applied internally by introducing a 30 mM NaF solution containing 20 mM TEA-phosphate to the axon interior. This caused an immediate suppression of the action potential. No sign of excitability could be elicited from the axon, as shown by the solid triangles. This suppression of excitability by TEA$^+$ was reversible, however. Reintroduction of the original NaF solution into the
axon could wholly restore excitability within 5 min. When full recovery of excitability was attained, the external solution was replaced by one containing 100 mM CaCl₂, 20 mM TEA-Cl, and 1 μM TTX. As shown by the solid circles, externally applied TEA⁺ had no effect at all upon the electrical properties of the membrane. The leakage conductance was unchanged throughout the experiment.

The experiments described above have revealed that the action potential developed under the experimental conditions described is composed of only two components; one is selectively suppressed by TTX applied externally, and the other, by TEA⁺ applied internally. One component of the action potential that is suppressed by TTX involves a large increase in the sodium conductance,

\[
\text{TTX treated}
\]

\[
\begin{align*}
&
\begin{array}{c}
30 \text{ Na} \\
100 \text{ Ca}
\end{array} \\
&
\begin{array}{c}
30 \text{ Na, 20 TEA} \\
100 \text{ Ca}
\end{array} \\
&
\begin{array}{c}
30 \text{ Na} \\
100 \text{ Ca, 20 TEA}
\end{array}
\end{align*}
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\[
\text{mV}
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\[
\text{μA/cm}^2
\]

\[
\text{FIGURE 5. Effects of internally applied 20 mM TEA}^+ (\blacktriangle) \text{ and of externally applied 20 mM TEA}^+ (\bullet) \text{ on the voltage-current characteristic of the membrane in the presence of 1 μM TTX in the external media. The open circles show the control data obtained with the 30 mM NAF solution internally and with the 100 mM CaCl}_2 \text{ solution containing TTX externally.}
\]

whereas the other does not. On the basis of the high sensitivity of sodium channels in nerve membranes in general to TTX applied extracellularly, and from the available data, it seems safe to conclude that the sodium channel is responsible for the TTX-sensitive component of the action potential. On the other hand, the selective effect of TEA⁺ upon the site responsible for the TTX-insensitive component of the action potential led me to presume this site to be the potassium channel, although the TTX-insensitive action potential was demonstrated in the CaCl₂ solution. This idea was confirmed in the experiments described in the following sections.

**Effect of Externally Applied K⁺**

In squid axons internally perfused with a dilute sodium-salt solution, an addition of potassium salt to an external CaCl₂ solution brings about a jump
in the membrane potential of ~50 mV (Inoue et al., 1973). The critical concentration of K\(^+\) for inducing such abrupt depolarization is usually <10 mM when the internal solution is the 30 mM NaF solution and the external solution contains 100 mM CaCl\(_2\). The oscillographic record on the top of Fig. 6 shows an example of abrupt depolarization at 3 mM K\(^+\). The membrane stays in its depolarized (active) state unless the external K\(^+\) concentration is lowered.

![Oscillographic record demonstrating abrupt depolarization of the membrane induced by an addition of 3 mM KCl to the external 100 mM CaCl\(_2\) solution. No electric shock was applied to the axon.](image)

![Effect of changing the external KCl concentration on the membrane potential in the absence of TTX (open circles) and in the presence of 1 μM TTX in the external solution (solid circles).](image)

**Figure 6.** *(Top)* Oscillographic record demonstrating abrupt depolarization of the membrane induced by an addition of 3 mM KCl to the external 100 mM CaCl\(_2\) solution. No electric shock was applied to the axon. *(Bottom)* Effect of changing the external KCl concentration on the membrane potential in the absence of TTX (open circles) and in the presence of 1 μM TTX in the external solution (solid circles). The same symbols show the data obtained from the same axon. The abrupt depolarization which took place at 3 mM KCl is shown by the arrow.

Similar abrupt depolarization by K\(^+\) was observed in axons internally perfused with the 30 mM NaF solution in the presence of TTX in the bathing medium. Furthermore, the magnitude of potential jump was larger than that observed in the absence of TTX. On the bottom of Fig. 6, the membrane potential recorded in the presence of 1 μM TTX (solid circles) and that in the absence of TTX (open circles) are plotted against the logarithm of KCl.
concentration added to the external 100 mM CaCl₂. (Only the experiments in which abrupt depolarization took place at 3 mM KCl are presented in this figure.) In both TTX-treated and untreated axons, the membrane potential in the resting state was ~−35 mV, and was only slightly influenced by an alteration of the external KCl concentration. At 3 mM KCl, there was a jump in the membrane potential of ~70 mV in the TTX-treated axons, whereas the membrane potential in the untreated axons jumped only ~45 mV. These magnitudes of potential jump roughly correspond to the height of the action potential in the respective axons. After abrupt depolarization, the membrane

![Diagram showing the voltage-current characteristic of the membrane in the presence of 1 µM TTX in the external solution. The salt compositions of both the internal and external solutions are given in the figure. The oscillographic record represents the time-course of the maximum peak current at 10 mM KCl.](image)

**Figure 7.** Effect of externally applied K⁺ on the voltage-current characteristic of the membrane in the presence of 1 µM TTX in the external solution. The salt compositions of both the internal and external solutions are given in the figure. The oscillographic record represents the time-course of the maximum peak current at 10 mM KCl.

continuously depolarized, with a further elevation of the external KCl concentration, showing a potassium-sensitive characteristic.

Attempts were made to study the effect of externally applied K⁺ on the voltage-current characteristic of the membrane. In this series of experiments, the membrane potential was held at the original resting level before K⁺ was applied externally. Fig. 7 illustrates one of the experiments. This experiment was conducted in the presence of 1 µM TTX in the bathing medium that contained 100 mM CaCl₂. The internal perfusion fluid was the 30 mM NaF solution. The holding potential was kept at −35 mV. When no KCl was
added to the external solution, the maximum slope conductance and the leakage conductance were 1.43 and 0.70 mmho/cm², respectively. The maximum peak inward current was \(-12\ \mu A/cm²\). When 3 mM KCl was applied externally, the maximum slope conductance increased to 2.39 mmho/cm², while the leakage conductance was unchanged. The maximum peak inward current reached a level of \(-28\ \mu A/cm²\). When the KCl concentration was raised to 10 mM, the values of the maximum slope conductance and the maximum peak inward current increased to 4.00 mmho/cm² and \(-60\ \mu A/cm²\), respectively. The leakage conductance kept its original value as long as the membrane potential was held at \(-35\) mV. As shown by the oscillographic record which represents the time-course of the maximum peak current at 10 mM KCl, externally applied K⁺ did not alter the time-course of membrane current but simply increased its magnitude. When voltage was unclamped, the membrane immediately underwent abrupt depolarization at 3 mM KCl or at 10 mM KCl. The magnitudes of the potential jump roughly corresponded to the separations of the two zero-current intercepts obtained from the curves at the respective KCl concentrations. (Though the magnitudes of the potential jump observed in this experiment were smaller than the average values obtained under the same environmental conditions, similar effects of K⁺ were recognized in all the experiments in this series. Similar results were also obtained without TTX.)

These results demonstrate that the TTX-insensitive action potential is accompanied by a large increase in the potassium conductance instead of the sodium conductance, and that it is selectively blocked by internally applied TEA⁺.

**Effect on the Membrane Potential of K⁺ Applied Both Internally and Externally**

To study further the role of K⁺ on the TTX-insensitive action potential, axons internally perfused with a solution containing both 30 mM NaF and a certain amount of KF were immersed in a solution containing 100 mM CaCl₂, 1 μM TTX, and KCl. The effect on the membrane potential of changing the external KCl concentration was tested. In Fig. 8, the average values of membrane potential with SD are plotted against the logarithm of external KCl concentration. Fig. 8 a illustrates the data obtained from seven axons internally perfused with a 30 mM NaF solution containing 3 mM KF. Abrupt depolarization, which is shown by the arrows, took place at 1 mM KCl in two axons, and at 3 mM KCl in the other five. The resting potential was \(~30\) mV and was slightly influenced by an alteration of the external KCl concentration. After abrupt depolarization, the membrane potential rose monotonously with log[KCl]. The maximum gradient of the potential variation of the active membrane, which is seen between 3 and 10 mM KCl, was 50 ± 3 mV per 10-fold increase in the KCl concentration. The axons could produce all-or-none action potentials when they were in the resting state; the height of the action potential was nearly equal to the magnitude of the abrupt depolarization. The data shown in Fig. 8 b were obtained from seven other axons internally perfused with a 30 mM NaF solution containing 10 mM KF. When the internal K⁺ concentration was raised to this level, it became difficult to
demonstrate all-or-none action potentials; the responses were graded. The magnitude of potential jump associated with abrupt depolarization also became considerably less. The results as a whole were, however, similar to those of Fig. 8a. Three axons underwent an abrupt depolarization of ~20 mV at 3 mM KCl, and the other four depolarized by ~25 mV at 5 mM KCl. The resting potential was ~−35 mV, which was about 5 mV more negative than that shown in Fig. 8a. The maximum gradient of the potential variation in the active membrane was 44.4 ± 4 mV per 10-fold increase in the KCl concentration.

![Graph](image.png)
It is important to point out that the membrane potential in the active state became zero when the $K^+$ concentrations on both sides of the axon were equal. This strongly suggests that $K^+$ plays a dominant role in determining the membrane potential in the active state in the presence of TTX, even though a large amount of $Ca^{2+}$ is present in the bathing solution.

Fig. 8c shows the data obtained from three axons internally perfused with the solution containing 30 mM NaF, 3 mM KF, and 20 mM TEA-phosphate. Under these circumstances, no abrupt depolarization was induced by externally applied $K^+$. The membrane potential rose monotonously with a slope of $-38 \text{ mV}$ per 10-fold elevation of the external KCl concentration in the region above 5 mM KCl. While the membrane was sensitive to $K^+$ in the high KCl concentration region, the membrane potential was not the equilibrium potential for $K^+$. This characteristic of the membrane was quite distinct from that shown in Fig. 8a. Obviously, the difference was due to TEA$. Internally applied TEA$ thus seems to block not only a transient rise in the potassium conductance but also the steady potassium conductance under the present experimental conditions.

### Table 1

**EFFECT OF $Ca^{2+}$ ON $E_{m}$ IN THE ACTIVE STATE**

| Outside $[Ca^{2+}]$ (mM) | Outside $[K^+]$ (mM) | Inside $[Na^+]$ (mM) | Inside $[K^+]$ (mM) | $E_{m}$ (mean ± SD) (mV) |
|--------------------------|----------------------|---------------------|---------------------|-------------------------|
| 40                       | 10                   | 30                  | 10                  | 5.0 ± 1.8               |
| 80                       | 10                   | 30                  | 10                  | 1.2 ± 2.7               |
| 100                      | 10                   | 30                  | 10                  | 0.5 ± 3.0               |
| 160                      | 10                   | 30                  | 10                  | -1.3 ± 0.5              |
| 320                      | 10                   | 30                  | 10                  | -0.4 ± 1.6              |

External media contained 1 $\mu$M TTX.

**Effect of External $Ca^{2+}$**

As has been pointed out above, a small amount of $K^+$ applied externally can play a dominant role in the production of the TTX-insensitive action potential, even though a large amount of $Ca^{2+}$ is present in the external solution. The role of $Ca^{2+}$ on such an action potential thus seems only minor. In fact, as can be seen from Table I, alterations in the external CaCl$_2$ concentration in a wide range, between 40 and 320 mM, produced no significant change in the membrane potential that had been depolarized by an addition of 10 mM KCl to the external solution containing 1 $\mu$M TTX.

Based on the data shown in Figs. 4–8 and in Table I, it is reasonable to conclude that the potassium channel is responsible for producing the TTX-insensitive component of the action potential, and thus the TTX-insensitive action potential developed in the CaCl$_2$ solution is due to a small calcium permeability of the potassium channel of the squid axon membrane.
DISCUSSION

It has been shown that the action potential elicited from squid axons internally perfused with a sodium-salt solution is composed of two spike components, i.e., a sodium-channel spike and a potassium-channel spike. Under certain environmental conditions, the reversal potential of the potassium channel becomes positive, so that the potassium-channel spike can be demonstrated by blocking the sodium channel. The present investigation supports the suggestion of Meves and Vogel (1973) that action potentials observed under such nonphysiological conditions can fundamentally be explained in terms of the sodium theory.

The effect of TEA + on the potassium channel under the present experimental conditions was somewhat greater than that observed in unperfused squid axons internally injected with TEA +. (According to Armstrong and Binstock (1965), K + antagonizes block by TEA + but may not overcome it even for inward current unless K + is very high. On the other hand, in the present study, TEA + applied by internal perfusion totally blocked a rise in the ionic permeabilities of the potassium channel.) Perhaps, the intensification of TEA + action was due to the removal of K + by internal perfusion. One can imagine that the potassium channels were more fully occupied by TEA + because of a removal of competing K +, so that the ionic permeabilities of the potassium channel were blocked more effectively.

The TTX-insensitive Ca 2+ influx through calcium channels in the squid axon membrane has been reported by Baker et al. (1973), using the Ca 2+-sensitive photoprotein aequorin. I first imagined that the calcium channel might play some important role in producing the long lasting action potential seen in a CaCl 2 solution. In the present experiments, however, I could not identify Ca 2+ current through calcium channels; and the major portion of the TTX-insensitive Ca 2+ current during the action potential was shown instead to flow through potassium channels. It seems, therefore, unlikely that the calcium channel plays a crucial role in producing prolonged, heartlike action potentials in the squid axon membrane.

As can be seen from the oscillographic record B in Fig. 2, there is a clear inactivation of the Ca 2+ current through the potassium channels. Although it is not yet certain that this inactivation represents an essential time-and-potential-dependent characteristic of the potassium channel, the time-course of the inactivation is somewhat similar to that of the slow inactivation of the K + outward current observed in normal squid axons (Ehrenstein and Gilbert, 1966). Further quantitative study of the potassium-channel spike is expected to lead us to a deeper understanding of the nature of the potassium channel.

Another interesting physiological problem arising from the present study is the question of what is the main factor that determines the resting potential of ~35 mV when the reversal potential of the potassium channel becomes inside positive. The results shown in Fig. 8 might give us some idea: If both of the two different behaviors of the membrane potential, i.e., the resting and active potentials (see Fig. 8 a and b), display the characteristics of the potassium channel, the potassium channel itself must possess two different
potential states under the same environmental salt conditions. On the other 
hand, if the behavior of the membrane potential shown in Fig. 8 c, which 
somewhat resembles that in normal axons, represents the characteristics of 
some other ionic channel or channels, we should reconsider the general concept 
that the potassium channel plays a leading role in determining the resting 
potential.

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