ABSTRACT

Liston Anderson Orr. THE IONIC BASIS FOR ELONGATED ACTION POTENTIALS INDUCED IN NERVE BY PLATINIZED-TUNGSTEN METAL. (Under the direction of Dr. Edward M. Lieberman and Dr. Gerhard W. Kalmus), Departments of Physiology and Biology, January, 1985.

Crayfish giant axons were studied using axial wire, space and current clamp techniques. Following cannulation of an axon with a platinized-tungsten wire electrode both propagated and space-clamped action potentials (AP) elongated. AP duration was typically 200-300 mSec (maximum to 4 secs.) A slight slowing of the AP rise was noted. The process was reversible upon removal of the electrode from the axon. The effect was not dependent on the passing of current through the platinized-tungsten metal nor on any component of the platinizing solution. Neither pure tungsten or platinum nor platinized-platinum electrodes were effective. Membrane resistance (Rm) during the AP plateau was 50-300% higher than Rm at rest. The steady state I-V plot of the altered axon exhibited anomalous rectification for depolarizing steps. In the absence of an inward sodium current (TTX or low external Na^{+}), anomalous rectification was abolished, while an inhibition of normal rectification remained. Ca^{++} channel blockers verapamil and La^{3+} as well as Low Ca^{++} were antagonistic to plateau formation. It is concluded that a product of the platinum-tungsten:axoplasm interaction produced elongated AP's by altering the membrane by: 1) block of normal K^{+} outward rectifying current; 2) decrease of steady-state outward K^{+} current; and 3) accentuation of an inward Ca^{++} current during AP generation.

THE IONIC BASIS FOR ELONGATED ACTION POTENTIALS INDUCED IN NERVE BY TUNGSTEN-PLATINUM METAL

A Thesis

Presented to

The Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

Liston A. Orr

January 1985

THE IONIC BASIS FOR ELONGATED ACTION
POTENTIALS INDUCED IN NERVE
BY TUNGSTEN-PLATINUM METAL

by

Liston A. Orr

APPROVED BY:

DIRECTOR OF THESIS

Dr. Gerhard W. Kalmus

COMMITTEE MEMBER

Dr. Edward M. Lieberman

COMMITTEE MEMBER

Dr. Takeru Ito

COMMITTEE MEMBER

Dr. W. James Smith

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

Dr. Charles E. Bland

DEAN OF THE GRADUATE SCHOOL

Dr. Joseph G. Boyette

ACKNOWLEDGMENTS

I would like to thank Dr. Edward M. Lieberman for his invaluable teaching, support and encouragement throughout the research and writing of this thesis. I would also like to thank my thesis advisor, Dr. Gerhard W. Kalmus, plus thesis committee members Dr. Takaru Ito and Dr. W. James Smith for their helpful suggestions and review of the manuscript. Many thanks to the faculty and staff of the Department of Physiology, East Carolina School of Medicine for their support. Special thanks goes to Mr. Jonathan Pascarella, who many times provided useful information and to Dr. Richard H. Ray for proofreading the manuscript. I would like to especially thank Ms. Brenda Elks, whose skill and patience in typing the manuscript are very much appreciated.

The work was supported in part by a graduate research grant from the Department of Biology, East Carolina University and a Grant-in-Aid of Research from Sigma Xi, The Scientific Research Society.

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Abbreviations

uA - microampere

Ag-AgCl - silver-silver chloride

AP - action potential

Ca^{++} - calcium ion

Cl^{-} - chloride ion

cm - centimeter

El_{c} - center electrode

El_{g} - guard electrode

El_{rec} - recording electrode

El_{ref} - reference electrode

El_{s} - stimulation electrode

E_{m} - membrane potential

g - conductance

i - current

I_{m} - membrane current

K^{+} - potassium ion

um - micron

mV - millivolts

Na^{+} - sodium ion

NCS - Normal Crayfish Solution

Pt - platinum

Pt-W - platinized tungsten

TEA - tetraethylammonium

TTX - tetrodotoxin

W - tungsten

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INTRODUCTION

In the last 50 years, important advances have been made in the area of cellular neurophysiology. These advances have ranged from the development of intracellular recordings from axons (Hodgkin and Huxley, 1939; Curtis and Cole, 1939), to voltage clamp studies of membrane currents (Hodgkin, et al., 1952), to the discovery of ionic channel gating currents (Armstrong and Bezanilla, 1973; Schneider and Chandler, 1973). Much of this progress can be attributed to the discovery of agents which alter excitable membranes in specific ways, thus providing an expanded range of parameters to be investigated. In the early 1960's, the puffer fish poison, tetrodotoxin (TTX), was found to selectively block the transient inward sodium (Na^+) current which is responsible for the initiation of the action potential (Narahashi et al., 1960; 1964). The fact that TTX selectively blocked the Na^+ current allowed investigators to study remaining ionic currents without any masking influence from the Na^+ channel current (Hille, 1968; Koketsu and Nishi, 1968). It was possible to suggest models for the structure of the Na^+ channel by comparing possible binding sites and the structure of TTX (Smythies et al., 1974). Selective blockers of other channels were also discovered. Tetraethylammonium (TEA) was found to block potassium channels in excitable membranes (Hille, 1967), while polyvalent cations were found to depress calcium currents in nerve (Hagiwara and Nakajima, 1966). Thus, any agent which alters nerve in unique ways could provide new insights into the biophysics of excitable membranes.

The present study arose from an observation made while using tungsten (W) wire as a substitute for platinum (Pt) for electrophysiological measurements on crayfish giant axons. Tungsten appeared to be a good alternative to Pt because it has superior mechanical properties and equivalent electrical properties. Tungsten wire electrodes, prepared in a manner similar to Pt electrodes, were electroplated with a thin, rough coat of Pt to improve their electrical properties by increasing surface area. When crayfish giant axons were cannulated with a platinized tungsten (Pt-W) wire, modification of the action potential (AP) of the axon was noticed within 15 minutes. Whereas the normal crayfish AP has a duration of approximately 1 millisecond (msec), action potentials with durations of up to 4 seconds were observed in axons when recording with the Pt-W wire. The rising phase of the AP was largely unaffected, while the falling phase developed a plateau similar to that seen in cardiac muscle APs. The effect was not a mechanical artifact caused by cannulation, for this technique has been in use for decades using Pt wire with no unusual results. No current was passed through the wire before and during the development of the effect. Some component of the Pt-W wire or product of a chemical reaction between the Pt-W wire and axoplasm was evidently altering the membrane ionic permeability control mechanisms responsible for the production of the AP. The primary purpose of this study was to identify and characterize the ionic currents altered by the Pt-W wire and secondarily, if possible, to identify the component responsible for the effect.

LITERATURE REVIEW

Long duration APs similar in appearance to those seen in Pt-W altered crayfish axons occur naturally in cardiac muscle cells. In nerve preparations, long duration APs are not the norm. However, elongated APs can be produced in nerve by a variety of different agents which act by altering the membrane bound channels responsible for AP production.

Cardiac Muscle

The ionic currents which give rise to cardiac muscle APs have been studied since the early 1950s. The theoretical descriptions of these early studies consisted largely of modifications of the Hodgkin and Huxley (1952) equation which described the ionic currents responsible for APs in squid giant axons. Classically, for the squid giant axon, the rapid depolarization at the onset of the AP is initiated by a sudden increase in the membrane sodium conductance (gNa^+). The repolarization of the membrane potential (E_m) back to rest level is caused by an inactivation of the gNa^+ plus an increase in the membrane conductance to potassium (gK^+) (Figure 1). To explain the elongated AP of the cardiac cells, several different theoretical modifications of the Hodgkin and Huxley equation were introduced, all of which provided a satisfactory solution (Brady and Woodbury, 1960; Fitzhugh, 1960; Noble, 1960). A delayed activation of the gK^+ and a delayed inactivation of the gNa^+, together or separately, could create a prolonged AP. The background K^+ conductance, which occurs whether the membrane is active or at rest, could decrease as the E_m is depolarized. This would result in a lengthened AP duration.

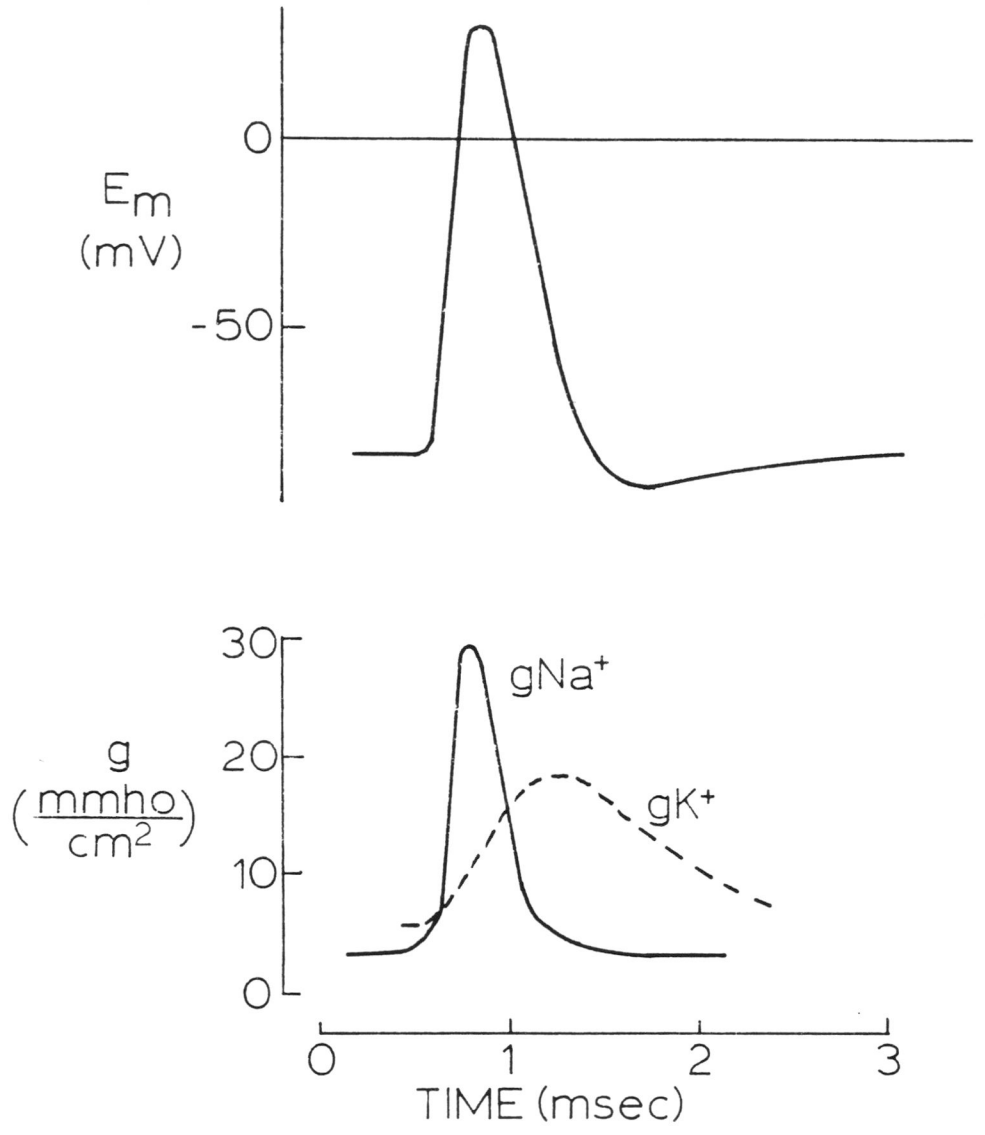


Figure 1. Typical nerve action potential and associated conductances. (from Noble, 1979)

Experimental work has shown that the cardiac AP is shaped by a combination of conductances: Na^+, K^+ and calcium (Ca^{++}) (Figure 2). In the cardiac AP, like the nerve AP, the initial depolarization of E_m is caused by a sharp increase in gNa^+. While the gNa^+ in nerve completely inactivates within a few msec, the gNa^+ in cardiac muscle exhibits incomplete inactivation; the remainder continues for the duration of the AP (Gettes and Reuter, 1974) (Figure 2). The long duration plateau has been at least partially attributed to the maintained residual gNa^+ (Attwell et al., 1979).

The existance of an inward Ca^{++} current during the cardiac AP, not predicted by the early theoretical studies, was discovered using voltage clamp analysis (Reuter, 1967). The conductance of calcium (gCa^{++}) was found to proceed with a time course significantly slower than the initial gNa^+. While a large portion of the gNa^+ is inactivated within a few msec, the Ca^{++} current takes approximately 300 msec (Figure 2). Na^+ and Ca^{++} components of the cardiac AP have been separated using Na^+ or Ca^{++} free bathing solutions and agents which selectively block Na^+ or Ca^{++} channels (Rougier et al., 1969; Beeler and Reuter, 1970). The results of these studies indicate that in addition to Na^+, an inward current of Ca^{++} plays a role in maintaining the depolarized state of the cardiac AP plateau.

In 1951, Weidmann studied total membrane conductance during cardiac APs. Small negative current pulses were injected across the membrane while an AP was initiated. The resulting voltage deflections situated on the AP plateau were of a larger amplitude than those seen while the E_m was at rest, indicating that conductance was less (or resistance was greater) during the plateau. The low conductance plateau of the cardiac

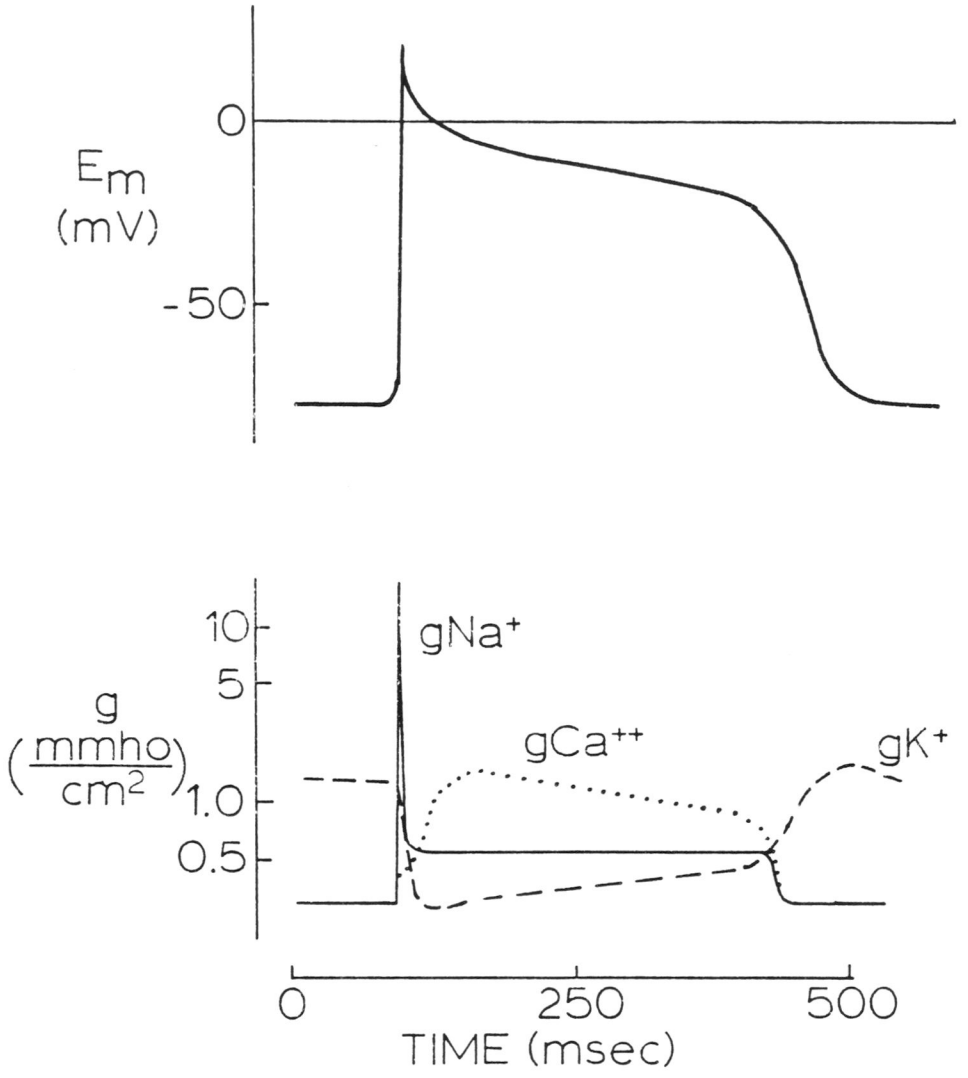


Figure 2. Typical cardiac muscle action potential and associated conductances. (from Noble, 1979)

AP was later found to be largely due to the property of the gK^+ in which it decreases as the E_m is depolarized. This conductance, which is made up of a voltage dependent component plus a steady state background component, shows an initial drop below rest level at the beginning of the AP, followed by a gradual increase as the AP plateau repolarized (Noble, 1979) (Figure 2). The property of a membrane which exhibits decreased gK^+ as the E_m is depolarized is termed inward-going rectification (Noble, 1965). It is produced by K^+ channels which reduce their outward flow of K^+ as E_m is depolarized. It is a mechanism by which the cardiac cell maintains the depolarized state of the AP plateau with a minimum expenditure of energy. The inward rectifying channels reduce the total amount of outward current during the AP. This reduction of outward current lowers the level of inward current (e.g. $I_{Na}{}^+$) necessary to maintain the depolarization. The result is a total conductance which is smaller than if the membrane had no inward rectifying channels (Noble, 1979).

Nerve

In 1957, Tasaki and Hagiwara demonstrated that injection of TEA into the interior of a squid giant axon caused the AP falling phase to form a plateau with durations up to 50 msec. The altered axon exhibited inward rectification. TEA was determined to have its effect by blocking the outward K^+ currents which normally repolarize the E_m during the falling phase of the AP (Armstrong and Binstock, 1965). TEA was also found to increase the duration of the crayfish giant axon AP twofold and cause repetitive APs when placed on the exterior of the axon (Suzuki,

1976). 4-aminopyridine was found to produce elongated APs in nerve by blocking voltage gated K^+ channels (Yeh et al., 1976).

Application of proteases inside the squid giant axon was found to increase the duration of the AP up to several seconds (Rojas and Luxoro, 1963; Shrager et al., 1969). The rising phase was unaffected while the falling phase developed a plateau. The mechanism of the protease effect is thought to be a combination of an inhibition of gK^+ activation and gNa^+ inactivation (Terakawa and Watanabe, 1976).

Toxins from the sea anemone have been found to prolong the falling phase of crayfish giant axon APs when applied externally (Rathmayer and Beress, 1976). AP durations were increased to more than one second. The anemone toxins act by inhibiting inactivation of the gNa^+ during the AP. Condylactis gigantea toxin has been found to operate in a similar manner (Narahashi et al., 1969). Scorpion (Androctomus australis) toxin produces elongated APs by inhibiting both gNa^+ inactivation and gK^+ activation (Romey et al., 1975).

Altering certain ion concentrations internally and externally can produce prolonged APs in nerve. When a squid giant axon was intracellularly perfused with a solution containing K^+ as the only cation species and placed in a bath containing only a Ca^{++} salt, an AP with a plateau duration of up to 300 msec was produced. The AP was due solely to a Ca^{++} conductance (Terakawa, 1981a; 1981b). Bathing an intact crayfish giant axon in a solution containing no K^+ and elevated levels of Ca^{++} produces low amplitude plateaus of approximately 5 msec in duration. This increased "after-depolarization" was determined to be caused by an increased Ca^{++} influx (Yamagishi and Grundfest, 1971).

In summary, the prolonged cardiac AP is apparently due to an increase of gNa^{+} and gCa^{++} combined with a decrease in gK^{+}. Elongated APs have been produced in nerve preparations by the use of pharmacological or toxic agents which block Na^{+} inactivation and/or K^{+} activation. The decrease of the steady state gK^{+} which is characteristic of cardiac muscle has not been previously seen in nerve.

MATERIALS AND METHODS

Animal Preparation

The nerve preparation used in the study was the crayfish (*Procambarus clarkii*) medial giant axon. Crayfish with an average length of 11 centimeters (cm) were obtained commercially from California, Louisianna, and South Carolina. The ventral nerve cord, which contains two medial giant axons with diameters of 100 to 350 micrometers (um) each, was removed from the live animal by the method described by Wallin (1967). A schematic of the nerve cord is shown in Figure 3. The nerve cord was held at each end by metal clips in a lucite chamber (Figure 4). The chamber was designed to provide a constant gravity flow of Normal Crayfish Solution (NCS), an external bathing solution approximating the normal ionic environment of the nerve cord (modified from Van Harreveld, 1936) (Table 1). The chamber design allowed a rapid (10 second) change of the external solution. The nerve cord was viewed with an Olympus binocular microscope. The diameters of the medial giant axons were measured using an occular micrometer. Axons with diameters of greater than 160 um were used for experiments. Excess tissue was trimmed from the nerve cord using fine forceps and micro-scissors. The sheath surrounding the nerve cord was removed between thoracic ganglia pairs 2 and 6 to facilitate impalement by glass microelectrodes and to increase the rate at which changes in the external solution would effect the preparation.

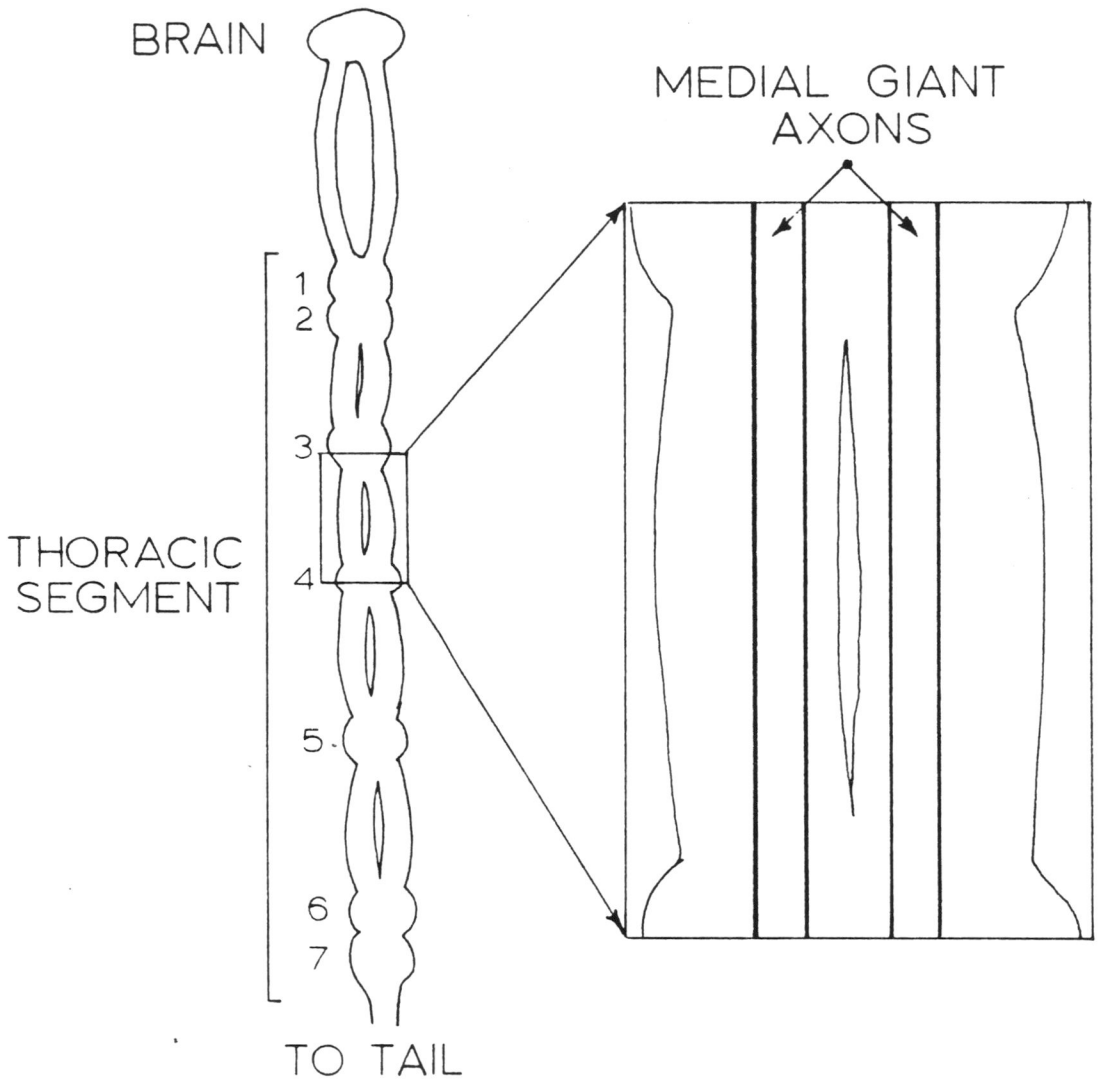


Figure 3. Schematic of crayfish ventral nerve cord. Numbers indicate thoracic ganglia pairs. Typical giant axon diameter 225 μm.

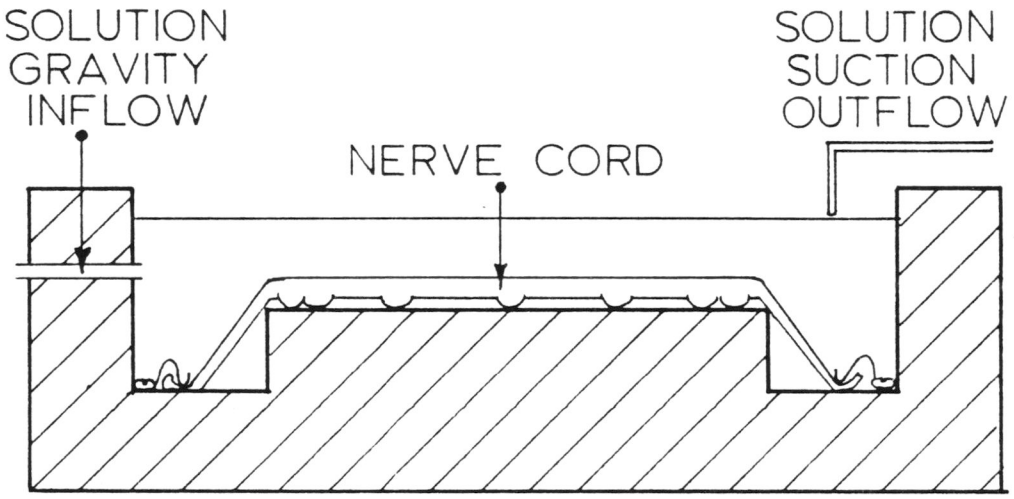


Figure 4. Nerve cord bathing chamber.

Table 1. Normal Crayfish Solution Composition (in millimoles)

NaCl	190
KCl	5.4
$CaCl_2$	13.5
$MgCl_2$	2.6
Tris	20

All external solutions at pH 7.3 - 7.5 and at osmolarity 425-445 milliosmoles

Table 2. Na_2WO_4 K^+ Isethionate Solution Composition (in millimoles)

K^+ Isethionate	280
NaCl	15
Tris	15
Na_2WO_4	1

pH = 7.3 - 7.5

Table 3. Altered Na^+ Crayfish Solution Compositions (in millimoles)

	5mM Na^+	25mM Na^+	52mM Na^+	73mM Na^+	97.5mM Na
NaCl	5.0	25.0	52.0	73.0	97.5
KCl	5.4	5.4	5.4	5.4	5.4
$CaCl_2$	13.5	13.5	13.5	13.5	13.5
$MgCl_2$	2.6	2.6	2.6	2.6	2.6
Tris	205.0	185.0	158.0	137.0	112. 5

Table 4. Altered Ca^{++} Crayfish Solution Compositions (in millimoles)

	1/4N Ca^{++}	1/2N Ca^{++}	3XN Ca^{++}
NaCl	164.0*	164.0	164.0
KCl	5.4	5.4	5.4
$CaCl_2$	3.4	6.8	40.5
$MgCl_2$	2.6	2.6	2.6
Tris	35.2	30.0	0.0

* NaCl reduced to 164mM to allow 3XN Ca^{++} while maintaining normal osmolarity. This concentration of NaCl was found to have no effect on normal and Pt-W altered membrane properties.

Table 5. Altered K^+ Crayfish Solution Composition (in millimoles)

	1/4N K^+	4 X N K^+
NaCl	190.0	190.0
KCl	1.4	21.6
$CaCl_2$	13.5	13.5
$MgCl_2$	2.6	2.6
Tris	24.0	3.8

Table 6. Zero Cl^- Crayfish Solution Composition (in millimoles)

Na Isethionate	190
K Isethionate	5.4
Ca Isethionate	13.5
Mg Isethionate	2.6
Tris	20

Electronics

a) Membrane Potential

The electronics used for measuring membrane potential (E_m) and passing current are diagramed in Figure 5. The E_m of the axon was the difference in potential measured between a recording electrode (El_{rec}) placed inside the axon and another El_{rec} in the external solution. The external potential was subtracted from the axonal potential by a WPI Model 750 dual amplifier acting as a differential amplifier to give the resultant E_m. The E_m was displayed on a Brush 440 chart strip recorder receiving a signal from a Tektronix type 3A3 differential amplifier, on a Hewlett Packard 120113 storage oscilloscope, a Tektronix D12 oscilloscope, and a digital voltmeter.

b) E_m Electrodes

The microelectrodes used for recording the axonal membrane potential, were produced from glass pipettes with diameters of 0.19 cm. These were heated and pulled on a David Kopf model 700C vertical pipette puller with final tip diameters of 0.1 - 0.5 um. The microelectrodes were filled with 2.5 M potassium chloride (KCl) solution by placing them in a boiling KCl solution under a partial vacuum. Electrical connection between the KCl solution of a microelectrode and the amplifier lead was made by a silver-silver chloride (Ag-AgCl) wire. Microelectrode tips were beveled in a swirling solution of 2.5 M KCl containing a silica polishing powder. When the microelectrode was held at the correct angle, the suspended polishing powder beveled the tip of the electrode in such a way to resemble the tip of a hypodermic needle (Corson et al., 1979). This had the dual function of sharpening the electrode tip and

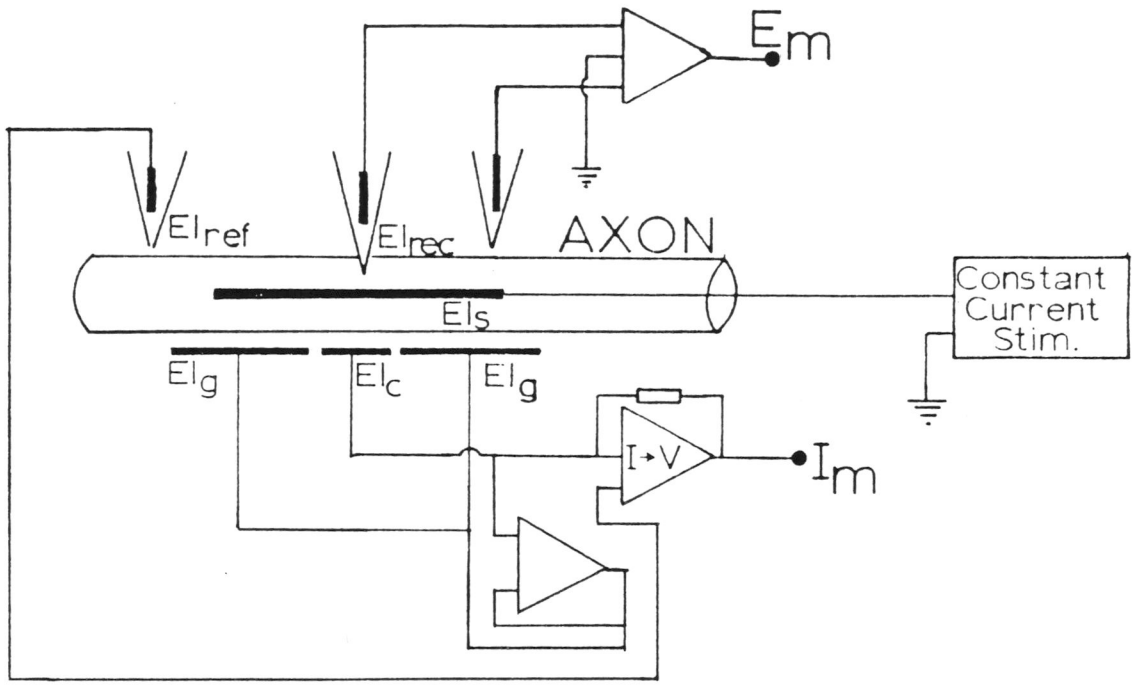


Figure 5. Electrophysiological organization for passage of current and recording of axon membrane potential. (See text for explanation)

lowering its electrical resistance. Electrode resistance was determined by passing a known current pulse through the electrode and observing the magnitude of the resulting voltage drop. Best results were obtained with microelectrodes with resistances between 10 and 20 mega ohms. The electrode which recorded the potential of the external solution consisted of a short glass pipette which was filled with 2.5 M KCl suspended in agar gel. Connection between the gel and the amplifier lead was by a Ag-AgCl wire in a 2.5 M KCl solution. Resistances of the agar electrodes were between 2 and 10 kilo ohms.

c) Current and space clamping

Current and space clamping was performed by passing current across the axonal membrane from an intracellular, axially placed wire stimulating electrode (El_s in Figure 5), to extracellular center (El_c) and guard (El_g) electrodes (Lieberman, 1976). The axial wire electrode consisted of a 25 um diameter wire threaded into a micro-pipette and allowed to extend beyond its tip for several cm. The small hole through which the wire had been passed was sealed with an insulating material, completing the insulation of the wire except for that length which was to be placed within the axon, thus preventing a short circuit between the axon interior and external solution. The exposed length of wire was 7 to 8 cm. Electroplating of the wire with Pt was carried out in a platinizing solution containing 3% platinum chloride and 0.025% lead acetate in 30 mM HCl. Plating was carried out at a current level of 1.5 milliamps for approximately 5 seconds. The external center and

guard electrodes consisted of 0.28 mm thick Ag-AgCl plates. The center electrode was 0.2 cm in length while each guard electrode was 0.4 cm in length. For clarity, Figure 5 shows these electrodes on only one side of the axon; a set of these electrodes was situated on either side of the axon. The function of the guard electrodes were to insure that current passing from El_s to El_c was parallel, allowing an accurate measurement of membrane current density. Current through El_s was supplied by a voltage to current converter driven by a Tektronix TM506 pulse generator (labelled Constant Current Stim. in Figure 5). Membrane current (I_m) was measured as a voltage and displayed on the oscilloscopes. The current generator was grounded relative to a reference electrode (El_{ref}). The El_{ref} consisted of an agar bridge electrode identical to that used in measuring the external potential for determination of E_m.

Axon Impalement and Cannulation Procedure

Both impalement and axial wire microelectrodes were mounted on micro-manipulators which provided precise directional control. The potential difference between the internal and external recording electrodes was set to zero in order to obtain an accurate measure of E_m. Impalement was performed by advancing the impalement electrode at an angle of approximately 30° to the axon until the electrode tip contacted the axon. A slight additional advancement of the electrode caused the axon beneath the tip to dimple. Light tapping of the advance knob of the micromanipulator advanced the electrode tip through the membrane and into the axon interior. Successful impalement was confirmed both

visually and by a sudden drop in the potential difference between recording electrodes from 0 mV to approximately -85 mV. APs were initiated by small stimulating pulses produced by a Grass S48 stimulator and delivered by two Pt wires in contact with the nerve cord. The Grass stimulator also supplied trigger pulses for the oscilloscopes and the current generator. Axons with membrane potentials of -80 to -90 mV and APs with amplitudes of 110 to 120 mV were used for experiments. The recording electrode was removed and the axon was prepared for cannulation. Under microscopic observation, a small hole was cut in the axon between ganglia pairs 5 and 6, and widened until three-quarters of the axon diameter was cut. The axon was cannulated with the axial wire until its tip was situated between ganglia pairs 4 and 5. Care was taken not to scrape the axon membrane. The axon was then reimpaled with the recording electrode between ganglia pairs 3 and 4, which were situated directly between the El_C pair. Following reimpalement, the axial wire was further advanced until an equal length of exposed wire was present on either side of the recording electrode.

Data Collection

The E_m could be adjusted to different potentials by injecting constant amounts of current through the axial wire. APs could be initiated by injecting short duration depolarizing pulses across the membrane. Characteristics of the axon steady-state membrane properties were monitored using current-voltage (I-V) plots. The plots were produced by injecting 100 msec current pulses of various amplitudes in both depolarizing and hyperpolarizing directions through the axon

membrane. I_m and resulting E_m amplitudes were plotted on the storage oscilloscope with E_m on the X axis versus I_m on the Y axis.

A completed I-V plot provided information on membrane resistance (R_m) at different potentials, and thus the nature of the rectification that was occurring in the membrane. Once a plot was displayed on the storage oscilloscope, it was photographed with a Hewlett Packard oscilloscope camera using Polaroid type 667 black and white film. The R_m at any E_m was determined by first drawing a tangent to the curve at the selected potential. From the slope of this line, the change in I_m was determined from the rise, and the resulting change in E_m was determined from the run. This information was used to calculate R_m by employing the equation

$$R_m = E_m / \frac{Im \times CF}{dl}$$

where R_m is in ohms/cm^2, E_m is in volts, I_m is the total current in amps/cm^2, CF is the conversion factor to give center current, d is the axon diameter in cm, and l is the length of axon being clamped by the El_c (0.2 cm).

Experimental Procedures

a) Measurement of electrical properties

Changes in AP kinetics and E_m were recorded with oscilloscope photographs and strip chart recordings as the Pt-W wire began to affect the axon. Steady-state current-voltage plots were evaluated for axons before and after AP elongation had begun in order to study membrane resistance in the Pt-W altered axon. Changes in membrane resistance

during the AP plateau was determined by injecting a train of small negative current pulses across the membrane while an AP was being recorded. The amplitude of the resulting voltage deflections on the AP plateau and at rest were compared, and could be related to changes in R_m.

b) Toxic substance

Attempts were made to identify the putative toxic substance of the Pt-W wire. This was done by cannulating axons with W wires which had been plated with individual components of the plating solution. W wire alone was also studied. To determine if the Pt-W wire had its effect by releasing a substance into the axoplasm, a procedure was carried out where a Pt-W wire was inserted into a suction/injection pipette. After the axon was cannulated with the pipette, axoplasm was pulled into the pipette and allowed to interact with the Pt-W wire for 20 minutes. The axoplasm was injected back into the axon and the AP and E_m were observed. To determine if a salt of W was responsible for the effect, a K^+ Isethionate solution containing 1 mM Na_2WO_4 (Table 2) was injected into the interior of the axon and the AP kinetics were monitored.

c) Ionic Channel Blockers

The Na^+ channel blocker TTX was used to study the role of the Na^+ current in the Pt-W effect. The Ca^{++} channel blockers verapamil and lanthanum were used to study the role of the Ca^{++} current. These specific channel blockers were added to the external solution after the Pt-W wire had begun to take effect. Observations were made of any changes in plateau duration and amplitude. I-V plots were also used to compare membrane resistances before and after application of the channel blockers.

d) Ionic Alteration

Changes were made in the ionic composition of NCS in order to study any changes caused by different levels of external ions on the Pt-W effect. Altered solutions were applied to the exterior of the axon either before or after the effect had begun. Plateau kinetics and I-V plots were studied. Ionic concentrations of NCS with altered Na^+, Ca^{++}, K^+ and Cl^- are shown in Table 3, Table 4, Table 5 and Table 6, respectively. Changes in osmolarity caused by altered ionic levels were compensated for by appropriate changes with Tris buffer.

Reagents

Tetrodotoxin was obtained from Sigma Chemical Company. Verapamil powder was obtained from Knoll Pharmaceutical Company. Lanthanum chloride was obtained from Alfa Products. All other reagents were obtained from Fisher Scientific Co.

RESULTS

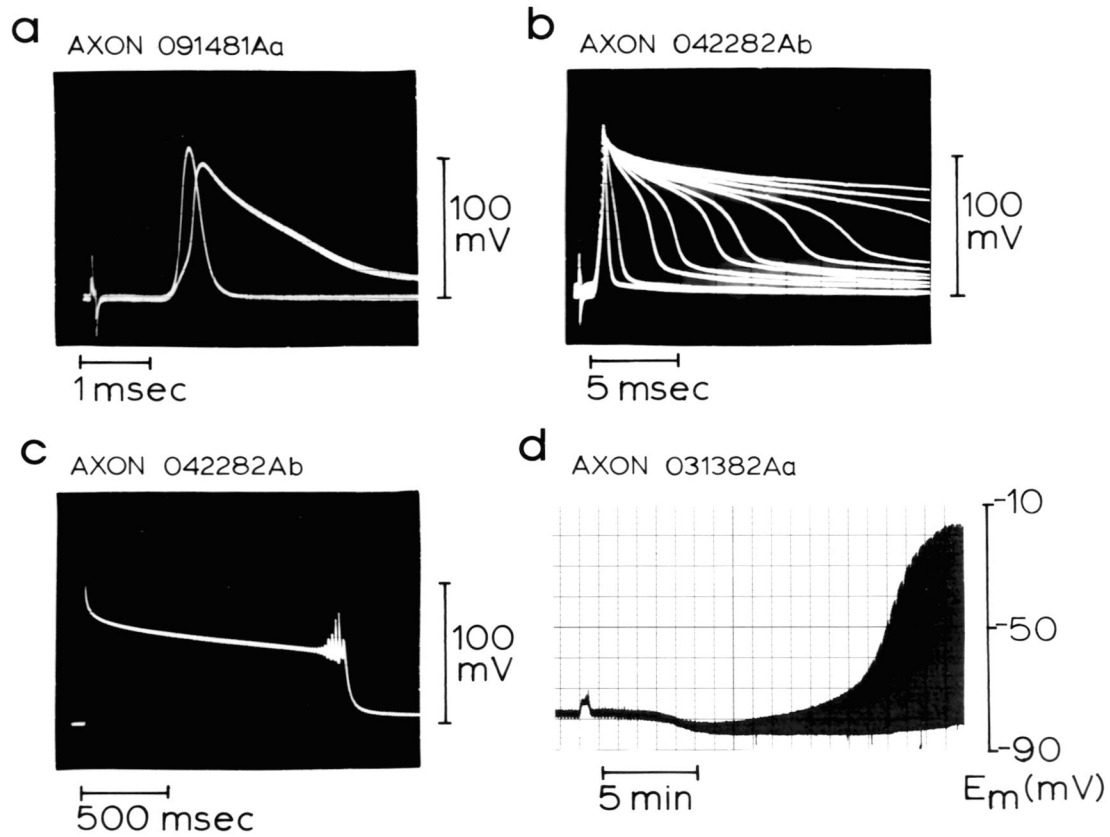
Elongated Action Potential: initial observations

Changes were usually noticed in the crayfish giant axon AP within 20 minutes of cannulation with a Pt-W wire (Figure 6). The initial change involved a prolonging of the AP falling phase, causing its slope to decrease. This was accompanied by a slight slowing of the rising phase and a small reduction in AP amplitude (Figure 6A). The falling phase continued to slow, developing into a plateau usually within 10 minutes of the start of AP elongation (Figure 6B). AP duration increased rapidly from this point, reaching durations of 20 to 100 msec within 5 minutes. Maximal durations typically ranged from 100 msec to 1 second, although durations of up to 4 seconds were observed. Oscillations in the E_m were often seen near the end of longer duration action potentials (Figure 6C).

The change in E_m which typically occurred is shown in Figure 6D. The E_m hyperpolarized approximately 5 mV prior to the beginning of AP elongation. The E_m began to slowly depolarize as plateau formation began. Without the application of a hyperpolarizing current, the depolarization would continue until the axon lost its excitability. Holding the E_m at 80 mV caused the axon to remain excitable for 20 minutes to 1 hour. The AP retained its long duration until the E_m would suddenly drop, resulting in a complete and irreversable loss of excitability.

The ability of the Pt-W wires to cause the effect was very sensitive to the level and duration of the current used to electroplate platinum onto the W wire. Any large variation from the 5 seconds at 1.5 milliamps

Figure 6. Pt-W effect. **A.** Oscilloscope photograph showing the initial change in AP kinetics. Note slight slowing of AP rise and small reduction of AP amplitude. **B.** Oscilloscope photograph exhibiting the characteristic rapid plateau formation over a 10 minute period. **C.** Oscilloscope photograph showing the typical maximal duration AP. Note the E oscillations at the termination of the plateau. **D.** Strip chart record exhibiting the typical hyperpolarization of the rest E_m prior to AP elongation. Artifact at beginning of the trace marks the point of axon cannulation with the Pt-W wire.


a
AXON 091481Aa
100 mV
1 msec
b
AXON 042282Ab
100 mV
5 msec
c
AXON 042282Ab
100 mV
500 msec
d
AXON 031382Aa
-10
-50
-90
5 min
Em (mV)

protocol resulted in a wire which would produce no effect. Electroplating the W wire for a longer period at a higher amperage produced a smooth even coat of Pt which completely covered the W. A shorter plating period resulted in a sparse coating of Pt not sufficient to produce the effect. Apparently both metals must be exposed in significant amounts in order for the effect to occur. Different wires showed different levels of effect strength. A more potent wire would cause the effect to begin more rapidly and proceed at a faster rate. The amplitude of plateau would also be higher. Plateau amplitudes ranged between 20 and 80 mV and averaged 65 to 70 mV. Each Pt-W wire had an effective lifespan of several days to 3 weeks. After they no longer produced the effect, they could often be recharged by an additional plating with Pt for 2 to 3 seconds. A wire could usually be recharged in this way no more than three times, after which it was no longer useful. This was probably due to the W being completely covered by Pt.

The rate of growth of AP duration during the formation of a plateau was found to be affected by two factors. Significantly increasing the rate of AP production temporarily decreased the duration of an elongating plateau for approximately 15 seconds. This was followed by continued elongation at a slower rate. Similar results were obtained by significantly increasing the flow rate of the external bathing solution.

In the normal crayfish axon, an external stimulating pulse or a depolarizing current pulse normally produces a single AP per pulse. Occasionally several APs were produced by a single pulse in a Pt-W altered axon. This repetitive firing was seen both before and during AP elongation.

The Pt-W effect was found to be reversable upon removal of the wire from the axon during the early stages of AP elongation. If the AP duration was less than 50 msec, removal of the wire usually caused the duration to return to normal within one minute. Removal of the wire after the duration had reached 100 msec usually had no affect on AP duration. AP elongation and E_m depolarization would continue as if the axon was still cannulated.

It was found that hyperpolarizing or depolarizing the axon E_m by passing a continuous current through the axial wire caused an elongated AP to decrease in its duration and plateau amplitude. It appeared that the normal resting potential of the axon was the optimal potential for the Pt-W effect.

Components of the Pt-W wire not responsible for the elongated AP.

A series of experiments was conducted to determine what component or combination of components of the Pt-W wire was responsible for the effect. Unfortunately this preliminary series could only identify what was not responsible. Axons were cannulated with a plain W wire for up to two and a half hours with no change in AP kinetics. Injection of the K^+ Isethionate solution containing 1 mM Na_2Wo_4 into the axon also had no effect. The influence of lead in the platinizing solution was investigated by cannulating axons with a W wire which had been plated in a 30 mM HCl solution containing .025% lead acetate as the sole solute. No change in AP kinetics was observed. Because plain Pt wires and Pt wires plated with Pt have been used for decades with no unusual effects, the results suggested that the combination of both W and Pt was necessary

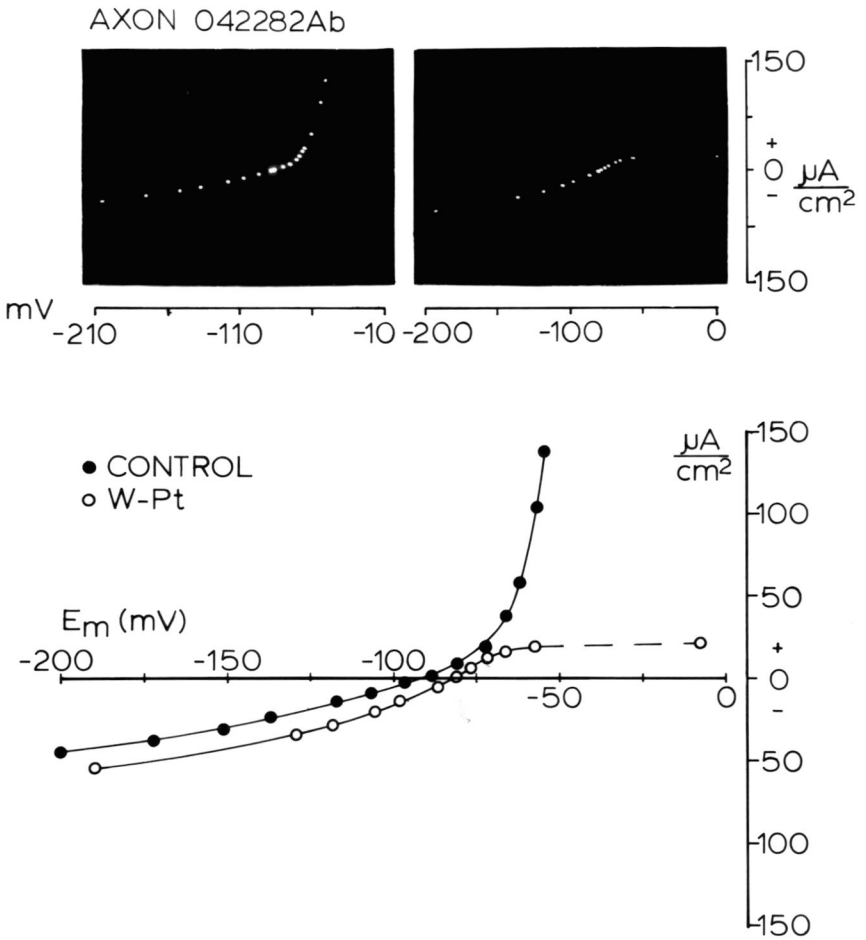
for the effect to occur. This idea was tested with a W wire electroplated in a 30 mM HCl solution containing 3% Pt but with no lead acetate. When cannulated into an axon, this wire was successful in producing elongated APs.

A suction/injection pipette containing a Pt-W wire was placed inside an axon and axoplasm was pulled in and allowed to interact with the wire for up to 30 minutes. Injection of this axoplasm back into the axon produced no change in AP kinetics.

Effect of Pt-W electrode on R_m

A comparison of the I-V relationship for a normal axon and for the same axon after AP elongation had occurred is shown in Figure 7. The most obvious difference in the curves occurs in the region where positive current flow caused a depolarization of E_m. The normal control curve exhibits outward rectification, that is, E_m begins to change only slightly for increasing positive current pulses. In contrast, the Pt-W plot exhibits inward or anomalous rectification for positive current pulses. This is caused by very large changes in E_m produced by very small positive current pulses. The large jump in E_m indicated by the dashed line represented a continuation of the AP plateau. The plateau formation could be explained by two mechanisms: (1) The plateau could result from a large sustained inward current (i.e., a Na^+ current with a delayed inactivation or a sustained Ca^{++} current); or (2) The plateau could result from a decreased potassium conductance relative to rest conductance, similar to what is seen in cardiac muscle. In order to

Figure 7. Oscilloscope photographs of current-voltage plots from an axon before and after the Pt-W wire has taken effect. The two plots are presented together graphically.


AXON 042282Ab
150
+
0 μA/cm²
-
150
mV
-210
-110
-10
-200
-100
0
μA/cm²
150
100
50
+
0
-
50
100
150
● CONTROL
○ W-Pt
Em (mV)
-200
-150
-100
-50
0

evaluate the I-V property of the axon membrane in the dashed area, the R_m during an AP plateau needed to be determined. This was done by injecting a train of small, negative current pulses across the membrane as an elongated AP was initiated (Figure 8). The deflections in the E_m are approximately 200% larger near the end of the AP plateau relative to those seen while the E_m is at rest. Because $E_m = I_m \times R_m$ and I_m is constant, the increase in the E_m deflections represents a corresponding rise in R_m. The increase of R_m was variable in different axons, ranging from almost no increase to a 300% increase. It should be noted that an increase in R_m was usually not seen in plateaus with durations less than 50 msec. As seen in Figure 8, the deflections in plateau E_m remain smaller than those prior to the AP until approximately 125 msec after AP initiation. In the majority of plateaus with durations less than 50 msec, the plateau R_m was less than rest R_m throughout the whole plateau. The large increases in R_m of 100% and more usually occurred in plateaus with durations greater than 100 msec.

Effect of Ionic Channel Blockers

The effect of the Na^+ channel blocker TTX in the external solution is shown in Figure 9A. Addition of 100 mM TTX into the bathing solution after a plateau had been formed caused the plateau duration to shorten progressively until an AP of normal duration remained. This was followed by the abolishment of the AP. A current pulse injection resulting in a E_m deflection of the same amplitude and duration of a normal AP would not reinstate a plateau. I-V plots from axons exposed

Figure 8. Illustration of the experiment performed to determine the R_m of the AP plateau. Injection of a train of small negative current pulses across the membrane during AP propagation provided a relative measure of plateau R_m. Note the approximately 200% increase of R_m at the end of the plateau relative to the resting R_m.

AXON 062382Ab

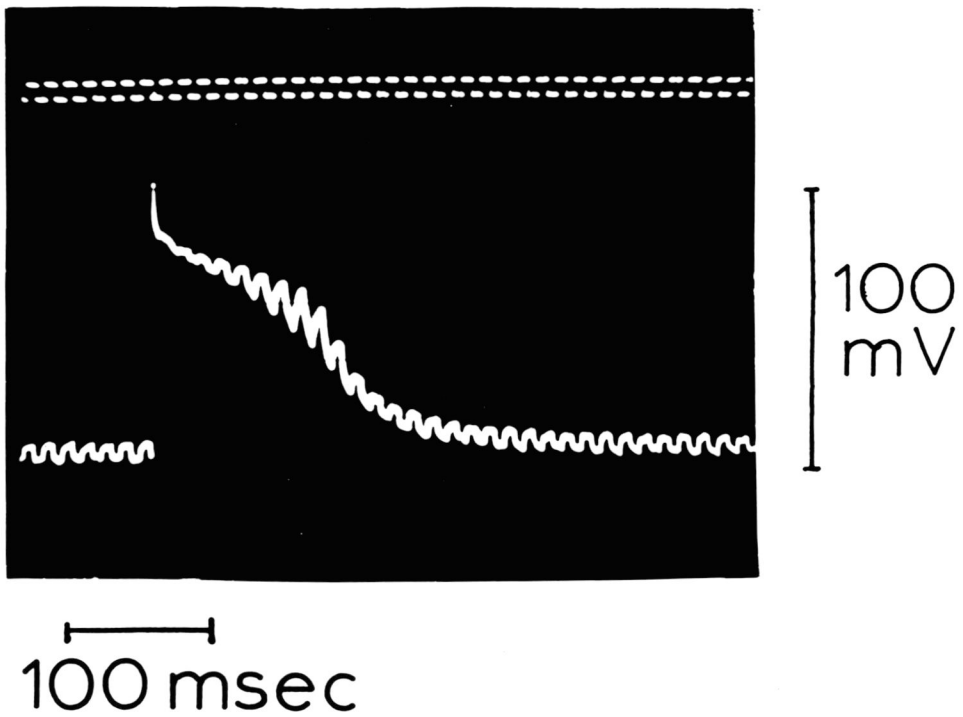
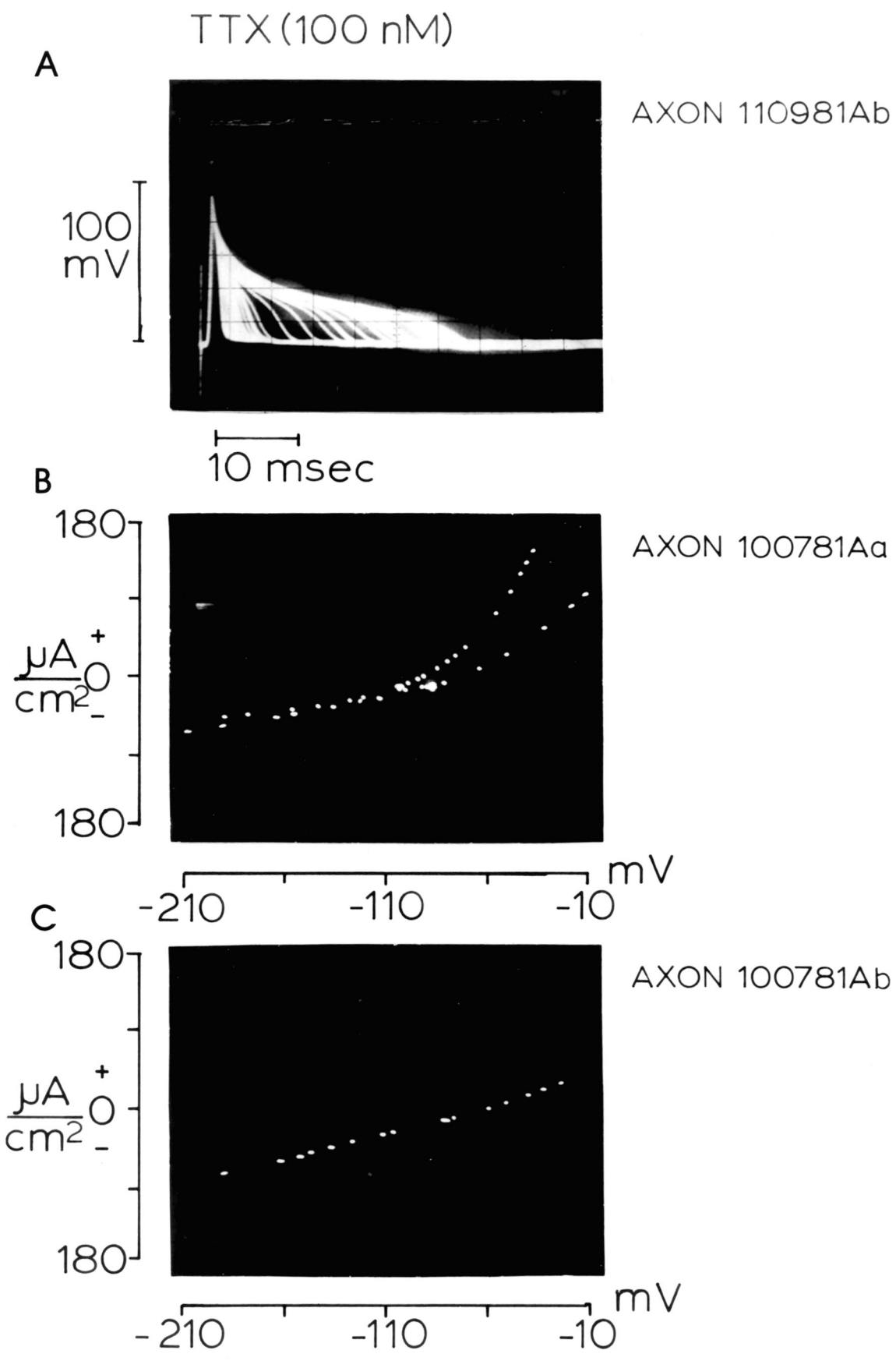


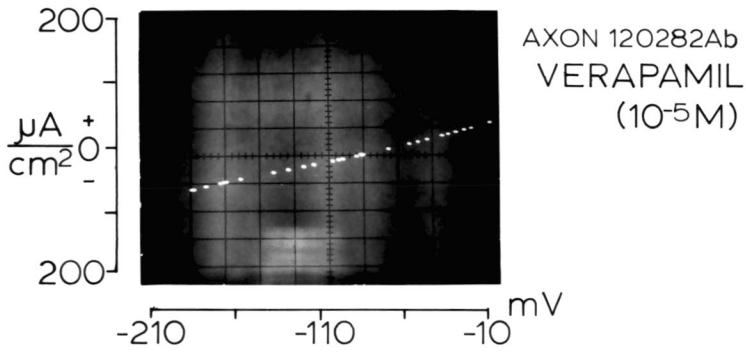
Figure 9. The effect of TTX on a Pt-W altered axon. **A.** The reduction of a Pt-W induced plateau prior to the elimination of the AP by 100 nM TTX. **B.** Two superimposed I-V plots from TTX treated axons showing the reduction of outward rectification during the onset of the Pt-W effect. **C.** I-V plot from a TTX treated axon 30 minutes after cannulation with a Pt-W wire. Note the absence of outward rectification plus the absence of the typical Pt-W induced inward rectification. No further change was seen with additional exposure time.


TTX (100 nM)
A
AXON 110981Ab
100
mV
10 msec
B
AXON 100781Aa
180
μA/cm²
+
0
-
180
mV
-210
-110
-10
C
AXON 100781Ab
180
μA/cm²
+
0
-
180
mV
-210
-110
-10

to TTX were produced immediately after the axons were cannulated with a Pt-W wire, and before any change in AP kinetics had occurred. These plots exhibited normal outward rectification, since this rectification is due to the opening of K^+ channels and is not affected by any alteration of Na^+ channels. As the Pt-W wire began to affect the axon, the outward rectification seen during depolarizing steps began to decrease (Figure 9B). The curve soon became completely linear, but did not go on to rectify in an inward manner (Figure 9C).

When the Ca^{++} channel blocker Verapamil was placed in the bathing solution in a concentration of 10^{-5}M, it prevented plateau formation and abolished plateaus which had already been formed. Under the influence of the Pt-W wire and Verapamil, APs were elongated slightly up to 10 msec, but no strong plateaus were formed. An I-V plot from such an axon exhibited an absence of normal outward rectification and also showed no anomolous inward rectification. The result was a straight, ohmic I-V plot similar to that seen with the Pt-W effect plus TTX (Figure 10). When the Ca^{++} channel blocker lanthanum was placed in the bathing solution in concentrations of 1 and 5 mM, it abolished plateaus and prevented inward rectification in a manner similar to Verapamil. Both Ca^{++} blocking agents abolished previously formed plateaus and exhibited similar characteristics. Upon application of either Verapamil or lanthanum, plateau durations began to decrease. The AP durations soon began to alternate; one to ten relatively short APs of several msec duration would be followed by a single large AP with a duration of several hundred msec to greater than 1 second. The number of short APs

Figure 10. I-V plot from a Pt-W altered axon treated with the Ca^{++} channel blocker Verapamil. Note the absence of normal outward rectification plus the absence of the typical Pt-W induced inward rectification.


200
μA/cm²
+
0
-
200
AXON 120282Ab
VERAPAMIL
(10⁻⁵M)
-210
-110
-10
mV

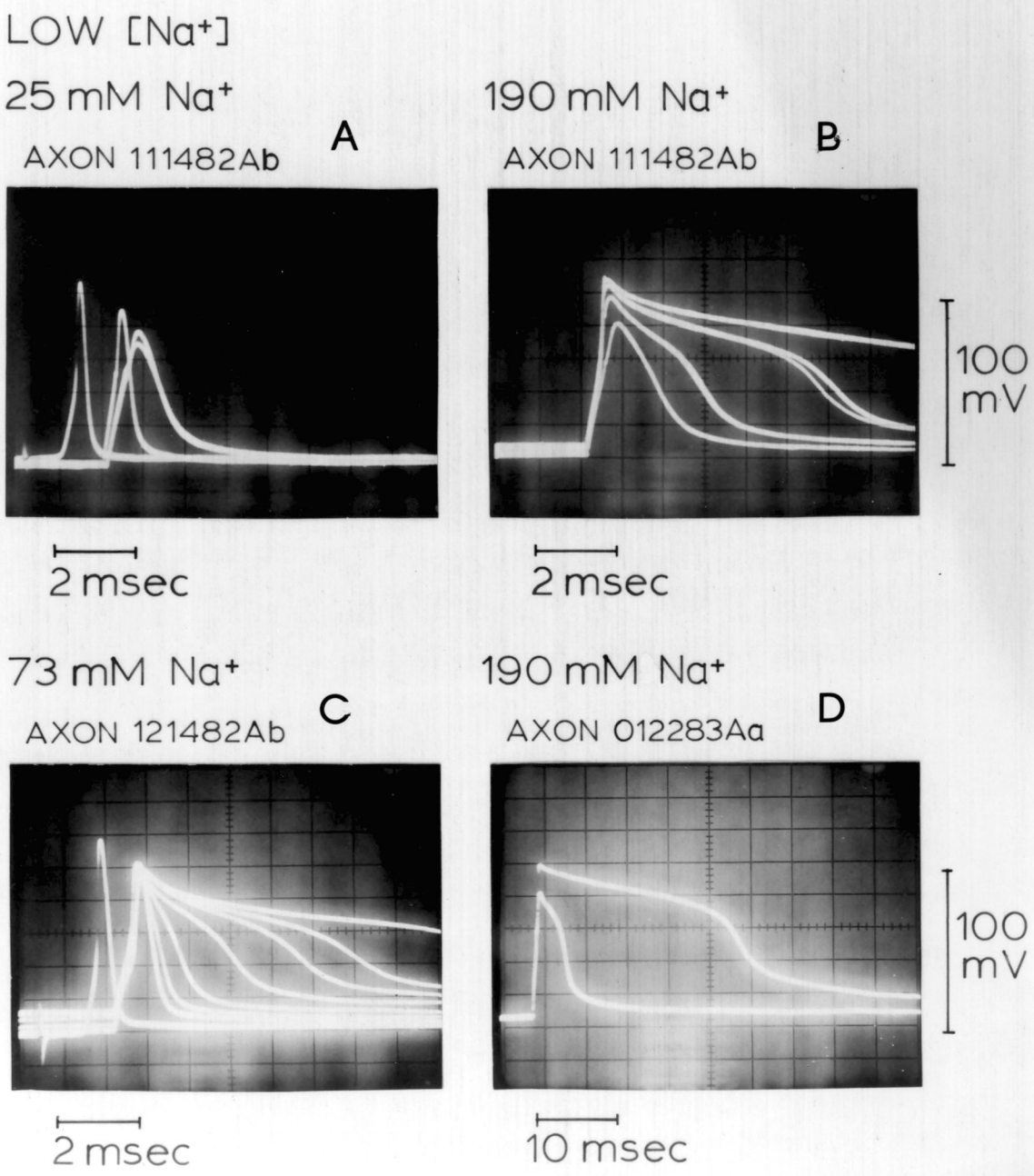
occurring between the long potentials increased until where the long duration APs no longer occurred. All that remained were APs with relatively short durations (5 to 10 msec). Verapamil is known to have a similar effect on plateaus of cardiac muscle APs (Cranefield, 1974).

In experiments in which plateau durations were studied, only large and very obvious changes were considered to be significant. This was due to the dynamic nature of the Pt-W effect, in which plateau duration was always gradually increasing. Useful quantitative measurements and statistical analysis were therefore not possible. Changes considered significant were so dramatic that there was no doubt of their validity.

Effects of Ionic Substitutions on the Elongated AP and Membrane Electrical Properties

When the Na^+ concentration in NCS was reduced to 5 mM, the AP amplitude of a normal axon was reduced approximately one third. No plateau formation occurred after the axon was cannulated with a Pt-W wire. The Pt-W wire did cause a slight slowing of both the rising and falling phases of the AP, and slightly reduced the AP amplitude. When the normal concentration of 190 mM Na^+ was returned to the external solution, AP amplitude increased to normal and was rapidly accompanied by the rapid formation of a plateau. Similar results were obtained with an external solution containing 25 mM Na^+ (Figure 11A,B). In 52 mM Na^+ NCS, the Pt-W wire produced a slightly more elongated falling phase than that seen with 25 mM Na^+. Addition of normal Na^+ concentration resulted in plateau formation. Although there was still substantial reduction in the AP amplitude in 73 mM Na^+ NCS, the Pt-W wire was able to produce definite plateaus with durations up to 15 msec. Addition of normal

Figure 11. Low Na^+ experiments. **A.** Three superimposed APs are shown left to right under respective conditions of normal control, 25 mM Na^+, and 25 mM Na plus the maximal Pt-W effect. **B.** Several superimposed APs showing the increase in AP duration over a period of two minutes upon addition of normal external Na^+. **C.** Several superimposed APs are shown which exhibit a normal control AP plus APs which exhibit the formation of a Pt-W plateau in 73 mM Na^+. **D.** Two superimposed APs showing the increase in plateau amplitude and duration which occurs upon increasing external Na^+ from 73 mM to normal 190 mM.

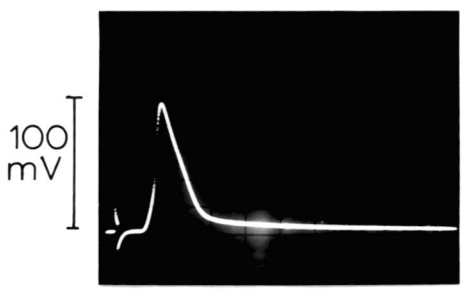

LOW [Na+]
25 mM Na+
AXON 111482Ab
A
2 msec
190 mM Na+
AXON 111482Ab
B
2 msec
100 mV
73 mM Na+
AXON 121482Ab
C
2 msec
190 mM Na+
AXON 012283Aa
D
10 msec
100 mV

levels of Na^+ increased the amplitude and duration of the AP (Figure 11C,D). Similar results were obtained with 97.5 mM Na^+ NCS. I-V plots from Pt-W altered axons in 5, 25, and 52 mM Na^+ NCS exhibited normal outward rectification. Altered axons in 73 and 97.5 mM Na^+ NCS exhibited typical Pt-W induced inward rectification.

Experiments similar to those carried out for low Na^+ were performed in 1/4 and 1/2 normal (N) Ca^{++}. Plateaus did not develop in Pt-W altered axons bathed in 1/4 N Ca^{++} NCS. Figure 12 exhibits an example of the maximal AP duration achieved in such an axon. When the opposite axon of the same nerve cord was cannulated with the same wire and normal Ca^{++} was placed in the bathing solution, a plateau with a duration greater than 1 second was formed. Pt-W altered axons bathed in 1/2 N Ca^{++} NCS exhibited a marked reduction in both plateau duration and amplitude. Raising the Ca^{++} level back to normal caused the plateau duration and amplitude to increase dramatically. The 1/4 and 1/2 Ca^{++} results exhibited a titratable effect of external Ca^{++} on plateau size similar to that seen for Na^+.

The possibility that increased levels of Ca^{++} cause an enlargement of the plateau was investigated. Plateaus with large amplitudes and durations were seen in 3 x N Ca^{++}. However, this change was possibly too small to be significant due to the continuously changing nature of the effect. Plateau resistance studies did not demonstrate any noticable differences between plateau resistances in normal and 3 x N Ca^{++}.

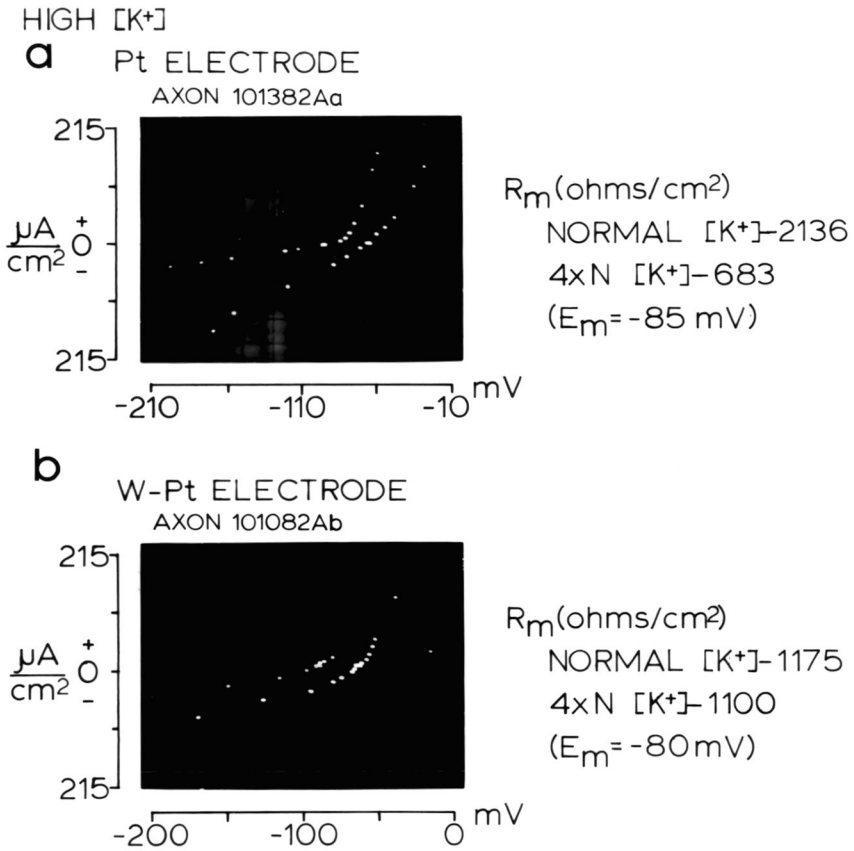
Figure 12. Maximum Pt-W effect in axon bathed in 1/4 N Ca^{++}. Pt-W wire cannulation of opposite axon bathed in normal Ca^{++} resulted in an AP with a duration of over 1 second.



AXON 042282Aa
1/4 N [Ca^{++}]

Initial experiments to determine the role of K^+ involved increasing external K^+ while maintaining the same E_m via the current clamp. This resulted in a decrease in AP duration. Maintaining the E_m at the same level insured that the reduction in AP duration was not due to any effects of depolarization on voltage sensitive channels. An example of the experiments carried out to further investigate the role of K^+ is shown in Figure 13. The initial part of the experiments involved the study of steady-state R_m of normal axons bathed in normal and 4 x N K^+. This was carried out by cannulating axons with an inert Pt wire and producing I-V plots while the axons were bathed in normal and 4 x N K^+. The E_m at which R_m was measured was always the resting E_m of the normal K^+ I-V plot. In 4 x N K^+, R_m of a control axon exhibits a dramatic drop from the normal R_m. This drop in R_m represents an opening of steady state K^+ channels. When the same procedure was carried out in a Pt-W altered axon, a significant drop in R_m at 4 x N K^+ was not seen. This suggests that the Pt-W "product" causes closure of K^+ channels at times that they would normally be open.

Figure 13. K^+ experiments. **A.** Two I-V plots from axons bathed in normal or 4 X N K^+ and cannulated with an inert platinum wire are compared. Note the large drop in resting R_m in the 4 X N K^+ axon. **B.** Two I-V plots from axons bathed in normal or 4 X N K^+ and cannulated with a Pt-W wire are compared. Note the minimal change in the 4 X N K^+ rest R_m.


HIGH [K+]
a
Pt ELECTRODE
AXON 101382Aa
215
μA/cm² + 0 -
215
-210
-110
-10
mV
Rm(ohms/cm²)
NORMAL [K+]-2136
4xN [K+]-683
(Em=-85 mV)
b
W-Pt ELECTRODE
AXON 101082Ab
215
μA/cm² + 0 -
215
-200
-100
0
mV
Rm(ohms/cm²)
NORMAL [K+]-1175
4xN [K+]-1100
(Em=-80mV)

DISCUSSION

This study was undertaken to investigate the mechanisms by which the short duration (1 msec) AP of the crayfish medial giant axon was prolonged to 50 msec or more by an axially placed Pt-coated W electrode.

This study emphasizes an electrophysiological description of the modified axon plus experiments designed to describe the modified ionic conductances that can produce an elongated cardiac-like AP.

Preliminary attempts were also made to identify the agent or agents released from the metal electrode or generated by a chemical reaction between the metal and axoplasm.

It was recognized in the initial experiments that the Pt-W altered AP was very similar in appearance to cardiac muscle APs, particularly those recorded from Purkinje fibers. The Purkinje fiber AP is characterized by an initial positive deflection from the resting potential of -90 mV to approximately +30 mV. This positive potential quickly changes to approximately 0 mV, at which time the potential begins a negative phase of slow repolarization. This slow repolarization forms the characteristic plateau. A relatively quick repolarization to rest marks the end of the AP, which typically has a duration of 500 msec.

The experiments designed to determine what component of the Pt-W wire or reaction product was causing the abnormal AP elongation revealed that plain W, sodium tungstate, Pt, or lead acetate had no effect separately. The only effective combination was platinum and tungsten.

In the initial studies, several characteristics of the Pt-W effect seemed to indicate that the active agent was released from the Pt-W

wire. This agent would be released into the axoplasm and react with the axonal membrane. The effect takes time to develop after cannulation of the axon, and axons with greater diameters (and greater volume of axoplasm for the substance to move through) took longer to exhibit the effect. The reversibility of the effect in its early stages upon removal of the Pt-W wire indicates that if a substance is responsible, it is of a rather labile nature. This would also explain the results of the experiment in which the injection of Pt-W treated axoplasm into an axon had no effect on AP kinetics. A constant source seems necessary in order to reach an effective concentration. After a time, the membrane became permanently altered; removal of the wire would not cause a reversal of the effect. An example of a possible active agent is ionic hydrogen. Decrease of intracellular pH in squid giant axons has been found to decrease outward K^+ current (Wanke et al., 1979; Carbone et al., 1981).

The results of the remaining experiments provide a good understanding of the ionic basis of the Pt-W effect. The first evidence was supplied by a study of steady-state I-V characteristics of control and Pt-W altered axon membranes. Outward rectification, normally seen in control axons, represents a decrease in R_m as the membrane is depolarized. The decrease in R_m is caused by an increased membrane permeability to K^+ due to the opening of voltage sensitive K^+ channels (Hodgkin and Huxley, 1952). The I-V relationship of Pt-W altered axons indicates that normal outward rectification is absent, and is replaced with rectification in the opposite direction (inward or anomalous rectification). The large jump in potential in the anomolous region (dashed

region - Figure 7) was due to the AP plateau. The characteristics of the curve in the region of membrane potential jump could not be directly evaluated using current clamp techniques. There are at least two possible explanations for the elongated AP and apparent anomolous rectification. Firstly, a decrease in an outward K^+ current relative to resting current could cause the R_m to be greater during the plateau (true anomolous or inward rectification) and generate the elongated AP by increasing the membrane IR drop as a result of the residual inward Na^+ and/or Ca^{++} currents. The second possibility could be related to a large and prolonged inward current (Na^+ and/or Ca^{++}) that would depolarize the membrane in a plateau-like fashion but would not generate a low R_m during the plateau. Certain toxins act in this fashion (Rathmayer and Beress, 1976).

The experimental procedure originally used by Weidman (1951) in the study of cardiac muscle APs was employed to investigate these two possibilities. Injection of a train of small depolarizing current pulses across an AP plateau confirmed that the AP plateau was due to an abnormal decrease in the outward K^+ current during depolarization, resulting in a true anomalous rectification.

The observed decrease in K^+ current induced by Pt-W in this preparation is a normal occurance in several cell types. Anomolous or inward rectification has been well documented in skeletal muscle cells (Katz, 1949; Standen and Stanfield, 1978; Hestrin, 1981) and cardiac muscle cells (Noble and Tsien, 1968; Cleemann and Morad, 1979). Anomolously rectifying K^+ channels have also been described in starfish eggs (Hagiwara and Yoshii, 1979). The mechanism of this channel has not

been determined with certainty. A model supported by several investigations represents the channel as a K^+ selective pore which has a blocking particle near the pore's opening into the inner surface of the membrane (Standen and Stanfield, 1978; Cleemann and Morad, 1979). The blocking particle is forced into the channel by outflow of K^+ ions initiated by a significant depolarization of the membrane. The pore remains impermeable to K^+ until a K^+ ion from outside of the membrane competes with the blocking particle's binding site and removes it from the pore. This model is supported by experimental results which show that K^+ current through the anomolously rectifying channel increases with increasing external K^+ concentration (Ohmoru et al., 1981). The increase of an outward K^+ current with increasing external K^+ appears to be a paradox until this phenomenon is viewed in the light of the effect of external K^+ on the blocking particle. Knowing this, it would be logical to assume that increasing external K^+ concentration would shorten the duration of a cardiac muscle AP. This has been observed experimentally (Cleemann and Morad, 1979).

Influence of $[K^+]$

Results similar to those seen with cardiac muscle APs have been observed in Pt-W induced plateaus of the crayfish giant axon. Increasing external K^+ while maintaining the same E_m via the current clamp resulted in a decreased AP duration. Maintaining the E_m at the same level insured that the reduction in AP duration was not due to any effects of depolarization on voltage sensitive channels.

When the E_m was allowed to depolarize in increased external K^+, it was possible to study another aspect of the Pt-W altered K^+ channels. In normal crayfish axons, K^+ induced depolarization will cause R_m at normal rest E_m to decrease (Lieberman, 1979). This decrease in R_m is due to voltage sensitive K^+ channels which are activated by the depolarization and are left in an open conducting position. In the Pt-W altered crayfish axon, a K^+ induced depolarization causes almost no change in rest R_m. This indicates that under a condition when K^+ channels are normally open, they remain closed under the influence of the Pt-W wire. However, under these conditions anomolous rectification does not occur for depolarizing steps. These results suggest that at least two K^+ channel types are involved in the Pt-W effect.

Cardiac muscle cells have been found to contain up to three different K^+ channel types whose currents make important contributions to AP kinetics (McAllister et al., 1975). The first current, iK_1, maintains the resting potential and helps repolarize the AP at the end of the plateau. It exhibits inward going rectification. The current termed iK_2 is found only in cardiac Purkinje fibers. It is slowly activated only at very negative potentials where its function is to control the pacemaker activity of the fiber. It also exhibits inward going rectification. The third current, i_x, is composed mainly of K^+ current, although it appears to contain other currents such as Na^+. It does not show inward going rectification. Of these three currents, iK_1 is a time independent "background" current which is not controlled by voltage sensitive gates of the type which control iK_2 and i_x. iK_2 and i_K take time to operate and are therefore termed time dependent. The normal or usual voltage sensitive K^+ current in nerve cells is most

similar to the i_x component of cardiac cell currents; they both control the repolarization of the AP. In nerve the K^+ current is activated within .5 msec. after the initiation of the AP, while in cardiac cells the i_x activation occurs much more slowly. One component of the Pt-W effect could be to delay the activation of the voltage sensitive K^+ current so that it would have kinetics similar to the cardiac i_x current (voltage gated outward going rectification). The time independent background current in nerve cells plays a relatively small role in the kinetics of the nerve AP when compared to the voltage sensitive currents (Noble, 1979). The K^+ background current in cardiac cells, iK_1, plays a large role in AP kinetics. While contributing to the repolarization of the AP at the end of the plateau, its inwardly rectifying nature at the initial part of the AP helps create the plateau. In the Pt-W altered axon, it appears that an inwardly rectifying background current is present, for a simple decrease and delay of the voltage activated K^+ current could not explain a plateau R_m which is greater than the R_m while the E_m is at rest. It appears that the background or "leakage" current in the crayfish axon has been transformed by the Pt-W "product" into a current which resembles iK_1 of cardiac cells.

Influence of $[Na^+]$ and Na^+ Channel Blockers

The experiments using TTX or low external Na^+ indicate that an inward flux of Na^+ is necessary for AP plateau formation and inward rectification. The effect of external Na^+ on plateau duration is titratable, with approximately 50 mM external Na^+ as the minimum concentration needed for plateau formation. I-V plots of Pt-W altered axons

bathed in TTX show an abolishment of normal outward rectification with no formation of inward rectification. The result is a straight ohmic plot. This lends support to the idea that two different K^+ channel types are involved in the Pt-W effect. In the absence of an inward Na^+ flux, the Pt-W wire appears to be able to inhibit the opening of the voltage sensitive K^+ channels which produce normal outward rectification. The wire is not, however, able to alter the membrane so that it rectifies in an inward manner. This dependence on Na^+ for the occurrance of inward rectification has been observed in cardiac muscle cells (Noble, 1979). The mechanism for this dependence in cardiac cells is not clear.

Influence of Ca^{++} and Ca^{++} Channel Blockers

The experiments using verapamil, lanthinum, and low external Ca^{++} indicate that, like Na^+, an inward flux of Ca^{++} is necessary for AP plateau formation and inward rectification. Ca^{++} has a titratable effect on plateau duration similar to that seen for Na^+, with a minimum concentration of approximately 1/2 N necessary for plateau formation. I-V plots for Pt-W axons bathed in verapamil exhibit straight ohmic plots identical to those seen in Pt-W altered axons bathed in TTX. The abolishment of the outwardly rectifying K^+ currents does not require a Ca^{++} influx. However, a dependence on Ca^{++} influx for the creation and/or operation of inwardly rectifying K^+ channels is indicated. In normal crayfish axons, Ca^{++} has been found to play only a minor role in AP kinetics (Yamagishi and Grundfest, 1971). In cardiac muscle, a slowly activated Ca^{++} current plays a major role in producing and maintaining the AP plateau (Rougier et al., 1969). It seems logical to

assume that one effect of the Pt-W product is to increase the normally insignificant Ca^{++} influx during AP propagation to the point that it makes a significant contribution to plateau formation. An increased Ca^{++} conductance could be possible in a low conductance plateau, because although the increased Ca^{++} conductance would be sufficient to help maintain the plateau, it would be relatively small compared to the decrease in K^{+} conductance. This situation occurs in cardiac cells (Noble, 1979). The observation that a block of Ca^{++} influx inhibits inward K^{+} steady state rectification suggests that Ca^{++} may be involved in an indirect mechanism, whereby a K channel type is chemically modulated by calcium. Ca^{++} modulated K^{+} channels have been described in neurons of the snail (Westerfield and Lux, 1982) and sea hare (Eckert et al., 1981). While these channels are affected by membrane potential, their action is primarily regulated by the internal Ca^{++} concentration. In order for these outward K^{+} currents to be activated, the channels must bind with internal Ca^{++} which gains entrance to the cell by way of voltage activated channels. Eckerd and Ewald (1982) have described a mechanism in which the buildup of residual free Ca^{++} ions inside a neuron can actually cause a decrease in the Ca^{++} modulated K^{+} current. This occurs by the inactivation of the voltage modulated Ca^{++} channels by residual internal Ca^{++}. Without the sufficient large influx of Ca^{++} necessary to activate the Ca^{++} modulated K^{+} channels, the K^{+} current through these channels is decreased. A Ca^{++} modulated channel has been described in cultured cardiac cells (Colquhoun et al., 1981). The channel carries an inward flux of all cations while excluding anions. This inward current is activated by internal Ca^{++}

ions but is not as voltage dependent as the currents described in nerve. The most likely effect of the Pt-W product on axonal Ca^{++} kinetics is an overall increase in the voltage sensitive Ca^{++} current coupled with the creation of a Ca^{++} modulated current similar to the type described either by Colquhoun et al. (1981) or Eckerd and Ewald (1982). Although this cannot be proven without voltage/patch clamp techniques, it is clear that AP plateau formation and inward rectification is dependent on an inward Ca^{++} influx.

The dependence of the Pt-W effect on Na^{+} and Ca^{++} influx can be presented schematically as seen in Figure 14. While the Pt-W product can inhibit the opening of the voltage modulated K^{+} channels with or without an influx of Na^{+} and Ca^{++}, an influx of both ions is necessary for the closing of the passive K^{+} channels so that they rectify anomolously. A blockage of either Na^{+} or Ca^{++} influxes by TTX or Verapamil respectively will prevent the Pt-W induced anomolous rectification and associated long duration APs.

Effects of Chloride

An aspect of the Pt-W effect that is not seen in cardiac muscle is the slow but continuous depolarization which usually begins soon after plateau formation. The most likely explanation of this involves the relative chloride permeabilities in nerve and muscle membrane. Crayfish axon membrane has a relatively low chloride permeability (Strickholm and Clark, 1977) compared to muscle membrane, which has a relatively high chloride permeability (Adrian, 1969). Adrian states that a K^{+} permeability which decreases during depolarization is a liability because it can cause an unstable resting potential, and the high

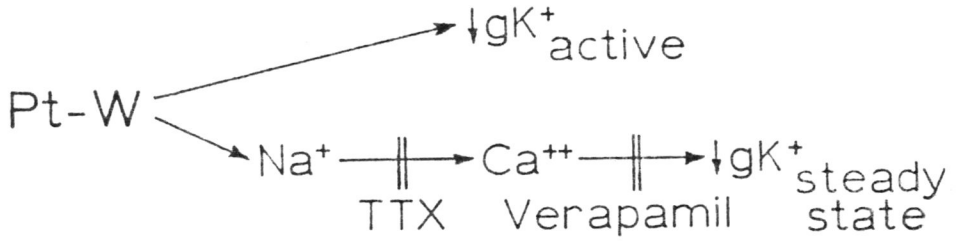


Figure 14. Pt-W mode of action suggested by experimental results. The Pt-W product appears to reduce the voltage modulated active gK^+ in a manner independant of any influx of Na^+ or Ca^{++}. In order for the background passive gK^+ to be reduced during AP propagation, inward fluxes of both Na^+ and Ca^{++} appear to be necessary. Blockage of either of these conductances by TTX or Verapamil will prevent the Pt-W induced reduction of steady-state gK^+. Inward rectification would therefore not be seen in the presence of either of these two drugs.

chloride permeability of muscle fibers may be necessary to make depolarization less likely. The Pt-W altered axon contains K^+ channels which decrease their permeability during depolarization. If the normally low chloride permeability of the axon is not increased to levels seen in muscle, an unstable resting potential could result. Removing chloride from external solution of Pt-W altered axons has no effect on AP kinetics nor does it have any effect on the rate of depolarization. This indicates that the chloride permeability of the Pt-W altered axon remains relatively small and thus provides a plausible explanation for the Pt-W induced depolarization.

SUMMARY AND CONCLUSION

The effect of the Pt-W product on the crayfish axon membrane can be summarized by comparing the altered membrane channels to those present in cardiac muscle cells. The K^+ channels of the normal crayfish axon consists of two channel types: a voltage sensitive channel whose outward current repolarizes the E_m at the end of an AP, and a passive leakage channel whose constant outward current is largely responsible for the formation of the resting E_m. The Pt-W product slows the activation of the voltage sensitive K^+ current so that it resembles the delayed rectifying current (i_x) of cardiac cells. At least part of the passive leakage conductance of the normal axon appears to become an inwardly rectifying conductance which is similar to the current that produces inward going rectification in muscle (iK_1). The results of this study suggest that this inwardly rectifying current is dependent upon intracellular Ca^{++} and Na^+ ions. A similar dependence has been observed in cardiac cells (Nobel, 1979; Colquhoun et al., 1981). It is not known if a residual Na^+ current persists throughout the AP as it does in muscle. The results indicate that the normally small Ca^{++} current (Yamagishi and Grundfest, 1971) in normal crayfish axons increases in the Pt-W altered axon. It may resemble the large Ca^{++} inward current described in muscle cells. The creation of a Ca^{++} modulated K^+ channel is also suggested. All of the above changes would contribute to the formation of an elongated AP. The small chloride permeability in the normal axon appears to remain relatively small in

the Pt-W altered axon. This deviation from the currents seen in cardiac cell membrane, which has a relatively high chloride permeability, explains the Pt-W induced depolarization of the resting E_m.

The Pt-W induced alteration of a crayfish axon which causes its currents to resemble cardiac cell currents provides some insights into the relationship between different types of excitable membranes. It is unlikely that the Pt-W product creates channels de novo; all channels responsible for the Pt-W effect are assumed to be channels already present which have been structually modified. An agent which alters ionic currents in one membrane type so that they resemble ionic currents of another membrane type suggests that all excitable membranes possess similar ionic channels. Evidence exists that unitary Ca^{++} currents in nerve of three different species have similar kinetics (Brown et al., 1982). The difference between different classes of excitable membranes (i.e., nerve vs. muscle) could be due to slight modifications or "masking" influences of the membrane channels. The Pt-W product may add or remove such a masking influence from a particular channel type, so that it responds in a manner characteristic of a different class of membrane. Such a possibility suggests a common evolutionary basis for all excitable membranes.

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