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Effect of Copper and Penicillamine on the Electrical Properties of Frog Atrial Fibers

by

Zofia Jarmoc

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THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Effect of copper and penicillamine on the electrical properties of frog atrial fibers" submitted by Zofia Jarmoc in partial fulfillment of the requirements for the degree of Master of Science.

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Date October 6, 1987

ABSTRACT

Copper is an essential trace element in mammalian life, but if accumulation occurs, damage to liver and brain can ensue (Wilson's disease). The effect may be relieved by penicillamine. The effect of Cu^{++} at different concentrations, and also when followed by administration of penicillamine, on the electrical properties of frog atrial fibers has been studied to obtain information in transmembrane ionic currents under these conditions. > 5 μM Cu^{++} produced a concentration and time dependent depolarization of resting potential and a decrease in peak amplitude of action potential. The effect on duration was biphasic: there was a temporary increase at ≤ 30 μM Cu^{++}, and a decrease at > 30 μM Cu^{++}. In the presence of copper there was a marked reduction in magnitude of the slow inward current and a negative shift of the reversal potential. Voltage clamp experiments revealed a transient decrease in delayed rectification current for concentrations up to 30 μM Cu^{++} and a small increase for concentrations higher than this. Washout failed to restore normal properties; rather further deteriorization occurred.

Following the administration of 40 μM Cu^{++}, 0.3 mM penicillamine restored resting potential, amplitude and duration of the action potential. It also produced partial recovery of the amplitude of the slow inward current, together with a recovery of the reversal potential to its initial value and an increase of the delayed outward current. Penicillamine concentration was critical: > 0.3 mM increased greatly the duration of the action potential which did not return to the control value even after extended washout; < 0.3 mM produced only partial recovery of the action potential.

The results suggested that copper interacts with membrane either by blocking active ionic transport (inhibition of ionic pumps) or by changing membrane permeabilities (inhibition of ionic channels) or both. Whatever mechanism, effect of Cu^{++} was irreversible. Penicillamine at 0.3 mM concentration was found to be capable of reversing the toxic effect of 40 μM Cu^{++} on the frog atrial fibers. It is suggested that penicillamine reactivates ionic pumps previously suppressed by copper and maybe also increases membrane permeability to different ions.

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CHAPTER 1 INTRODUCTION

1.1 Introduction

Cardiac muscle, like skeletal muscle and nerve, is electrically excitable. An electric stimulus in a specific region produces a temporary transmembrane depolarization which travels in a regenerative manner throughout the cell surface. This electrical impulse is governed by membrane permeability changes and is called an action potential.

Important steps in the understanding of cardiac electrical activity at the cellular level were made in 1950. Transmembrane potentials were recorded by Woodbury et. al. (1950) and Draper and Weidmann (1951). Brady and Woodbury (1960) first proposed alterations in the selective permeability of the cell membrane to specific ions to be the basis of the mechanism of transmembrane potential changes, leading to the cardiac action potential. Noble (1962) published a mathematical model based on the model of Hodgkin and Huxley (1952) for nerve fibers, together with measurements of the various ionic transmembrane currents in cardiac preparations.

Sodium, calcium and potassium are the major ions involved in the cardiac action potential. Their concentration gradients across the membrane generate the resting potential, and changes in permeability which occur as a function of (imposed) transmembrane potentials are responsible for the action potential (Hille, 1984). The cell membrane presents a barrier to the free movement of these ions and by the development of ionic pumps in the membrane (Haas 1972, Thomas 1972, Luttgan and Glitsch 1976), concentration differences are established across the membrane.

The specific ionic permeability of the membrane gives rise to a situation in which the extracellular concentration of sodium ions in cardiac cells is much higher than intracellularly. In the case of potassium ions, the situation is reversed. In the resting state the cell membrane is more permeable to potassium ions than to any others and thus there is a tendency for potassium ions to move out of the cell making the cytoplasm negative with respect to the extracellular fluid. Sodium inward permeability is low in the resting membrane, so diffusion of these ions counterbalances the outward flow of potassium only to a small extent. The same applies to other ions such as calcium and chloride. The combined effect of all transmembrane ionic distributions is to generate a transmembrane potential with the interior of the cell approximately -90 mV with respect to the exterior.

Under normal conditions the passive currents of K^+, Na^+, and Ca^{++} ions across the membrane do not undergo any change in direction. This situation would result in a progressive accumulation of Na^+ and Ca^{++} in the cells and a depletion of K^+. Consequently there must be an opposite active transfer in order to maintain the respective internal ion concentrations. With regard to the equilibrium of sodium and potassium ions, the most favored concept is that of a sodium-potassium exchange pump. An active sodium extrusion was first postulated for skeletal muscle by Dean (1941) and Krogh (1946), and was later shown to be linked to potassium uptake in a number of tissues (Harris and Maizels, 1951; Steinbach, 1951; Desmedt, 1953; Hodgkin and Keynes, 1955; Shaw 1955).

In most tissues studied so far, ATP has been found to be the immediate source of energy for the sodium pump. The flux measurements on frog atria (Haas et al., 1967) do not suggest a fixed stoichiometric

relation between sodium extrusion and potassium uptake. The data favor the idea of a loose linkage between sodium and potassium transport or two independent transport systems. Blockage of an electrogenic Na^+ pump can account for the depolarization of the frog atrial resting potential observed during metabolic inhibition.

The ionic and energetic requirements of active calcium extrusion from cardiac muscle have been studied by Reuter and Seitz (1968) and Glitsch et al. (1970). These studies revealed an intimate linkage between calcium and sodium exchange. The immediate source of energy of calcium extrusion may be a physical process rather than a chemical reaction.

When depolarization occurs beyond a critical value (the threshold value) the membrane permeability to different ions undergoes striking changes. Permeability to sodium ions increases greatly, causing a rapid influx of these ions into the cell. Then calcium inflow follows, which generates the secondary depolarization thereby producing and regulating the plateau height and length. Finally the cell repolarizes to the resting level by outward flow of potassium ions. The ions cross the membrane through specific channels (Mullins 1959, Fozzard and Gibbons 1973).

In order to analyze quantitatively the ionic currents associated with the action potential it is essential to control and measure accurately the membrane potential. The development of the voltage clamp technique (Deck et al. 1964) provided a method for accurately controlling membrane potential, but for a long time its application was restricted to the giant squid axon. For atrial or ventricular myocardium the sucrose gap method developed by Stampfli (1954, 1963)

can be used in two ways, (1) to pass a current into the preparation (Reuter and Scholz, 1968; Mascher and Peper, 1969), or (2) in a double sucrose gap system, to measure membrane potential also (Rougier et al., 1968). Spatial homogenity of the voltage clamp cannot be complete, because the preparations used contain a great number of thin cardiac muscle cells in a bundle. The voltage clamp achieved in cardiac muscle preparations is thus only an approximation, and the records must always be interpreted with this in mind.

There are distinct ionic currents in cardiac muscle. These include:

- a rapid inward current, carried mainly by sodium ions, which is responsible for the primary depolarization,
- a slow inward current, carried mainly by calcium ions, which maintains depolarization and produces the plateau,
- two delayed outward currents, carried mainly by potassium ions, which are responsible for the repolarization process.

Rougier et al. (1968) first studied the inward ionic currents in frog atrial muscle.

The fast current is:

a) dependent on extracellular sodium ionic concentration;

b) abolished by tetrodotoxin (5.0×10^{-7} g/ml);

c) rapidly activated and inactivated.

Use of the double sucrose gap technique to analyse the fast inward current in cardiac preparation is difficult.

The slow inward current is:

a) not blocked by TTX (5.0×10^{-7} g/ml);

b) decreased in magnitude when either the external sodium or calcium

concentration is decreased. Rougier et al. (1968) suggested this slow inward current to be carried by both sodium and calcium ions;

c) blocked by application of manganese ions (1-2 mM), cobalt, nickel, lanthanium and drugs like verapamil and D600 (Kohlhardt et al. 1973, Reuter 1973, 1974b, Weidmann 1974).

Rougier et al. (1968) also first recorded the delayed outward currents in the frog atrium. They used this delayed rectification property together with the slow inactivation of the slow inward current to account for the repolarization phase of the action potential. Further voltage clamp experiments showed that there are two distinct components of the outward current present in delayed rectification (Brown and Noble 1969 a,b). A study of the kinetics of the delayed rectifier in atrial trabeculae from Rana catesbeiana (Giles, 1970; Noble, 1972) revealed:

- the time constant of the faster component $i_{x_{fast}}$ was of the order of 100 to 500 ms
- the time constant of the slower component $i_{x_{slow}}$ was approximately 1.5 - 2.1 s
- short duration voltage clamp pulses (less than 1 s) activated only the slowly decaying component of the outward current. During longer clamp pulses, both current components were invariably activated.

Many agents can alter normal electrical activity of cardiac tissue. Their effect may be to change the amplitude of the action potential, change its shape, and change the resting potential. Copper is one such agent.

Copper is one of the essential trace elements in the living system. Its vital function is participation in indispensable catalytic

reactions. Copper is present at the active site of a number of enzymes. Trace amounts of copper are essential for life, but amounts in excess of the needs are toxic and have highly deleterious effects on living tissues. It is now generally accepted that the clinical and morphological manifestations of Wilson's disease are due to an accumulation of copper in the tissues of liver, brain, kidney and cornea (Sigel, 1978).

Wilson's disease is one of the inborn errors of metabolism characterized by the classical triad of severe progressive liver disease (cirrhosis), a peculiar neurological syndrome, and the presence of Kayser-Fleisher rings of the cornea. The disease is inherited in an autosomal-recessive fashion. Copper concentration in the body apparently increases as the disease progresses.

Only a small quantity of copper is absorbed from the total amount ingested in the daily diet. Normally, almost all of the absorbed metal is incorporated into ceruloplasmin at the time this specific plasma protein is being newly synthesized. As a result, about 98% of the copper in plasma is present as a firmly bound part of the protein structure. And, in the normal state, the amount of copper incorporated into ceruloplasmin each day is counterbalanced by an equal daily elimination of the copper-containing protein. Thus, in normal individuals, plasma-water and tissues contain negligible amounts of copper in the free, ionic state. In Wilson's disease, however, the plasma-water contains a greater than normal quantity of free copper ions; there is insufficient synthesis of ceruloplasmin. As a result, free copper ions are distributed to the tissues, where they become bound to various components. It is the slow but progressive accumulation and

deposition of copper that produces the toxicity.

A drug which is clinically applied in the treatment of Wilson's disease is penicillamine (Karcioglu, Sarper 1980). It produces an increase in copper excretion in the urine.

Another piece of evidence on toxicity of Cu^{++} comes from work of Sir Rudolph A. Peters (Peisach et al., 1966) who found that minute amounts of Cu^{++} (21 μM) induced convulsions and death when injected into the subarachnoid pace in the pigeon's brain. Based on his experiments Peters concluded that Cu^{++} induces convulsions by interfering with an SH- containing group in a membrane ATPase and that the compound which Cu^{++} forms with the tissue constituent is irreversible. Effects of penicillamine on membrane ATPase were also tested. He established a 14-31% increase in activity induced by 2.24 mM penicillamine.

Penicillamine is an effective chelating agent of the choice for many heavy-metal (copper, iron, lead, mercury, zinc) poisons (Friberg et al., 1979). Most recently, it has become established as one of the most effective means of combating rheumatoid arthritis (Proc. Symp. on Fundamental Studies ...). The mode of action of penicillamine in the treatment of rheumatoid arthritis is a complicated one. The drug prevents connective tissue proliferation and the accumulation of rheumatoid components, but it is not known how it does so.

1.2 Aim of the Study

As discussed in the Introduction, accumulation of copper is known to produce damage to liver, brain and other tissues (Sigel, 1978). It was decided then to use cardiac tissue and study an effect of copper ions on its electrical activity.

Penicillamine is known to have high affinity to Cu^{++} (Doull et al., 1980) and is used in the treatment of Wilson's disease with satisfactory results (Karcioglu, Sarper, 1980; Sigel, 1978). However, the nature of its action is not well understood. But since it clearly has some effect it was felt that a study of the effects on the electrical parameters when penicillamine is introduced in the environment of fibers which are under the influence of copper, might provide additional information on the nature of the effect of copper, the nature of the mode of action of penicillamine, and the manner in which it relieves the toxic effects of copper.

Firstly, the aim was to study the effect of different concentrations of copper on the cardiac action potential and to determine the extent to which the induced changes in the electrical activity could be reversed upon returning the preparation to a normal environment.

Following this, the aim was to study the details of the changes in transmembrane ionic currents underlying the action potential (in particular the slow inward current and the total outward current) induced by introduction of copper ions.

Finally, the aim was to establish the concentration of penicillamine which would restore the action potential to its control value. This specific dosage of the drug would be then applied to the cardiac preparation in order to study its effect on transmembrane ionic currents. It was hoped that the results of these experiments would permit the suggestion of the mechanism by which copper ions affect the electrical activity of cardiac tissue, and also would demonstrate the nature of the effect of penicillamine on cardiac tissue in which

electrical activity was impaired by the presence of Cu^{++} ions.

1.3 Scheme of the Presentation

The double sucrose gap technique for voltage and current clamping has been used to study the action potential and the transmembrane ionic currents of the bullfrog atrial fibers. The techniques are described in Chapter 2.

The experimental results obtained using copper and penicillamine are represented in Chapter 3.

Chapter 4 contains the discussion of the results together with a proposed mechanism of copper interaction with the cell membrane.

CHAPTER 2 MATERIALS AND METHODS

2.1 Double Sucrose Gap Technique

In many studies of electrical activity of cardiac muscle cells, microelectrodes with a high resistance have been used (Deck et al. 1964a; Dudel et al., 1967; Giebisch and Weidmann 1967). However, atrial muscle is poorly suited to this technique. The extremely rapid spatial decrement of a subthreshold stimulation applied from a microelectrode produces a highly localized current flow, and consequently a point source of current is probably not able to produce uniform depolarization of the fiber (Woodbury and Crill, 1961). Moreover, contraction of the fiber tends to break the very delicate tip of the microelectrode, and the small size of atrial cells reduces the chance that the microelectrode will remain impaled for a sufficiently long time to allow an experiment to be completed. For these reasons, extracellular electrodes are desirable for studies of atrial preparations. The double sucrose gap technique offers a method for doing this (Stampfli, 1954; Rougier et al., 1968).

The limitations of this technique are due, in part, to the structural complexity of cardiac tissues, the effect of which must be taken into account in evaluating the measurements. Since transmembrane currents change with membrane voltage, one of the basic requirements of the voltage clamp is that the voltage be the same in each part of the membrane in which the current is being measured. This means that, ideally, the test region should be uniformly polarized. If the length of the fiber under investigation is small compared with the space constant of the preparation, the degree of spatial homogenity is

improved to an acceptable level.

For a frog atrial muscle fiber, the resting space constant has been measured to be about 690 μm (Brown et al., 1975). The width of the test compartment of the chamber used in this study was 150 - 200 μm, i.e., about 0.2 - 0.3 of the space constant. Assuming that nearly all of the intracellular current leaves the cells within the test compartment, this portion of the preparation may be treated as an open-circuited short cable (Adrian and Freygang, 1962). If this cable is linear, an effective length of the test compartment corresponding to 0.2 - 0.3 of the space constant would be subject to voltage drop of 6% and 9% respectively.

Haas et al. (1970) using an intracellular microelectrode directly tested the spatial uniformity of the potential across a 200 μm test gap. They found that the voltage decrement was only about 2 mV, even during the activation of the fast inward current. This indicates that the axial decrement of the potential is not significant within a 200 μm test compartment. However, since a typical preparation has a diameter of approximately 100 μm, the radial voltage decrement must also be considered.

According to Baldwin (1970), the tight packing of fiber bundles in the trabeculae, and the narrow clefts between cells where there are no cardiac adhesion plaques or close junctions, could present a high resistance to radial current flow. This resistance is in series with the membrane resistance (Beeler and Reuter, 1970a) and is believed to introduce appreciable error in voltage clamp measurements when the transmembrane currents are large (for example, at the peak of the fast inward sodium current).

It has been shown by De Hemptinne (1973) that the error factor resulting from the presence of the series resistance was relatively small in frog atrial fibers studied by the double sucrose gap technique when a vaseline-sealing method was used to separate the fluid compartments. Transmembrane microelectrode recordings made during voltage clamp experiments while using this method indicated satisfactory voltage control even during the flow of the peak inward current. In the present study, however, no attempt was made to measure the fast inward current.

To measure the true value of a potential difference between two regions of a biological conductor the extracellular current flow between these regions must be made very small. The measured potential can be calculated according to the equation:

$$E = E_m \frac{R_e}{R_e + R_i}$$

where E - potential recorded across the external resistance,

E_m - true membrane potential,

R_e - longitudinal resistance per unit length of the external medium, and

R_i - longitudinal resistance per unit length of the internal medium

When the external resistance (R_e) is very large, the short circuit factor $\frac{R_e}{R_e + R_i}$ approaches one and the measured potential closely approximates the actual transmembrane potential.

Application of the double sucrose gap technique requires the preparation to be perfused with isotonic sucrose in two insulating regions along its length. These sucrose gaps present an extremely high

resistance to extracellular current flow. Thus it can be assumed that the current entering the test compartment originates from the actual preparation and not from leakage of current across the sucrose gap. Therefore, using extracellular electrodes, it is possible (in principle) to:

(i) measure the transmembrane potential across either gap or

(ii) inject current into the preparation.

The chamber used in this study (Figure 1) was made of perspex and contained five compartments which could be separated by vaseline seals. Compartments 2 and 4, of approximately 200 μm width, were perfused with isotonic sucrose solution. Compartment 3, the test compartment of 150 - 200 μm width, was perfused with test solution. Compartments 1 and 5 contained Ringer solution or isotonic KCℓ solution (117 mM KCℓ). Ag - AgCℓ electrodes, with a resistance of less than 500 Ω, were inserted in compartments 1, 3 and 5, and were used to pass current and to record membrane current and voltage. The upper flat surface of the chamber was sealed with transparent "magic" tape except for the small region where the preparation was to be placed. The atrial muscle fiber was placed perpendicular to the gaps along a narrow shallow groove in the upper part of each partition.

2.2 Electronic Apparatus

The electronic equipment permitted two types of measurements:

a) using current-clamp: changes in the membrane potential across the muscle fiber plasma membrane due to an imposed constant current;

b) using voltage-clamp: changes in the transmembrane ionic current in the muscle fiber due to an imposed constant potential across the

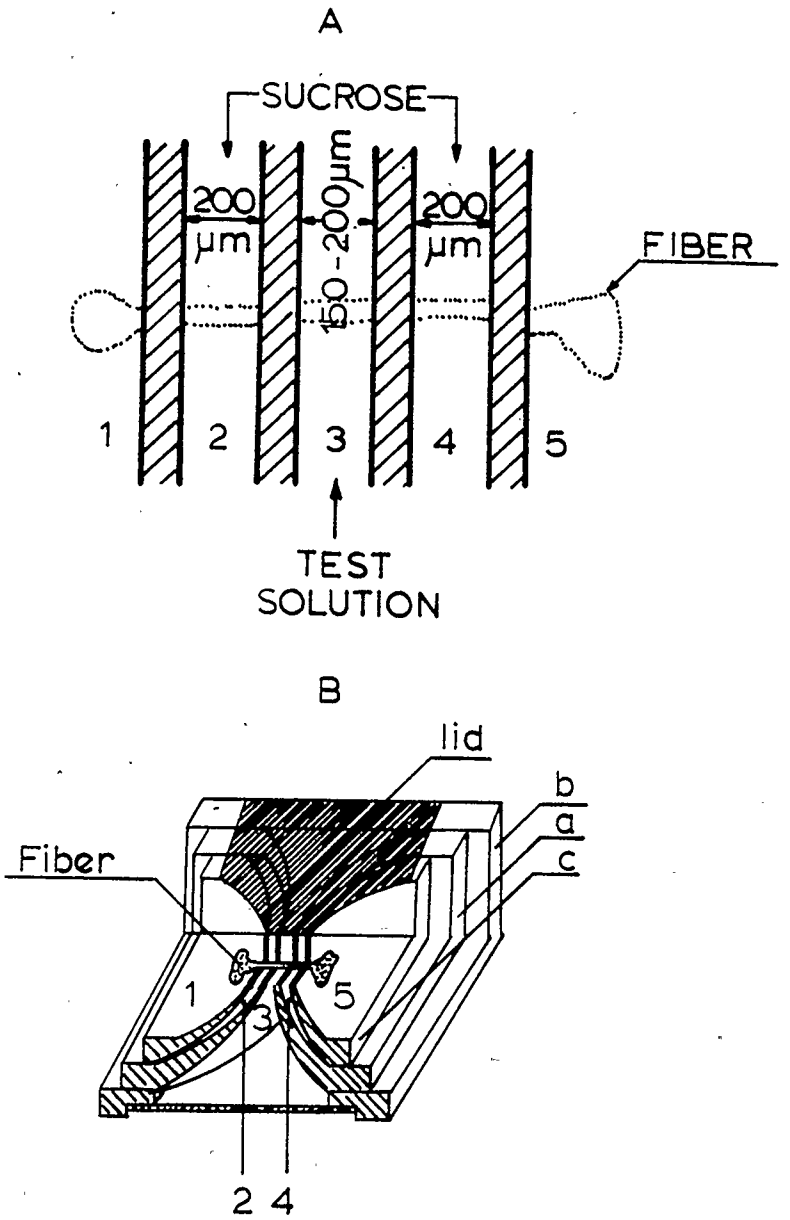


Figure 1. Double sucrose gap technique apparatus (chamber).

A - close-up look of three gaps to be perfused with appropriate solutions

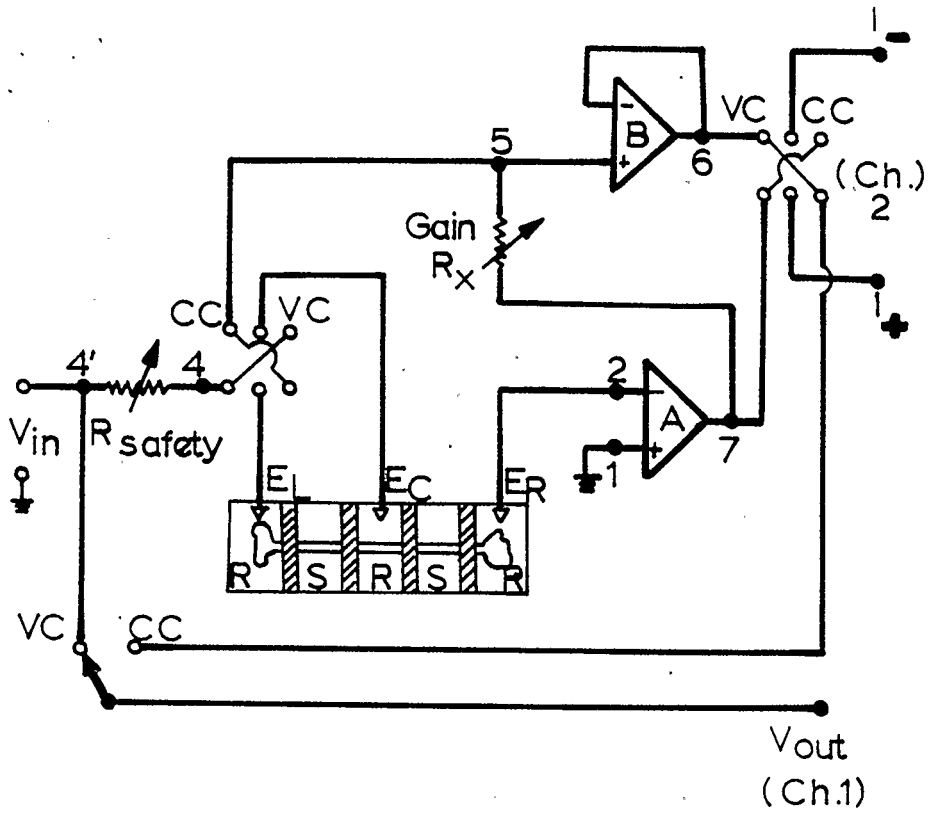
B - longitudinal section of the central region of the chamber showing the location of the fiber

membrane at the artificial node.

For both measurements, the same DC feedback operational amplifier (Analog 45K) was used. Figure 2 is a block diagram of the electronic apparatus used in current and voltage clamp techniques. The membrane potential of each preparation was individually measured as the potential difference between an electrode in the central compartment and a second electrode in either of the end compartments. This potential difference was then applied to the vertical input of the differential amplifier (No. 1) of a dual beam storage oscilloscope (Tektronix 7313). The potential difference due to the transmembrane ionic current was connected to the vertical input of the differential amplifier (No. 2) of the same oscilloscope. The stimulation was generated by a Grass Instrument S48 stimulator and was applied via an electrode in one of the end compartments or the central one. Two operational amplifiers, A and B (Analog 45K), each with high input impedance ($> 10^{11}$ Ω), low output impedance ($\sim 10^{3}$ Ω), and large open-loop gain (5×10^{4}), were used, both connected with inverted input, but with amplifier B operating to produce unity gain. The resting potential between two electrodes E_R and E_c was counterbalanced by the DC output of the differential amplifier A, which thus delivered zero current. For reference purposes this potential was made the zero point, making subsequent measurements of potential by the amplifier to represent changes of potential from this reference level.

2.2.1 Current clamp

Figure 3 is an equivalent circuit for current clamp. Terminal 1 was grounded, making V_1 the reference potential, i.e., $V_1 = 0$. The



CC	current clamp	R	Ringer solution
VC	voltage clamp	S	sucrose solution
E_L	left electrode	V_{in}	input voltage
E_R	right electrode	V_{out}	output voltage
A	operational amplifier A	Ch.1	Channel 1
B	operational amplifier B	Ch.2	Channel 2
R_{safety}	safety resistor	I_+	ionic current +ve input
		I_-	ionic current −ve input

Figure 2. Block diagram of the electronic apparatus used in current and voltage clamp techniques.

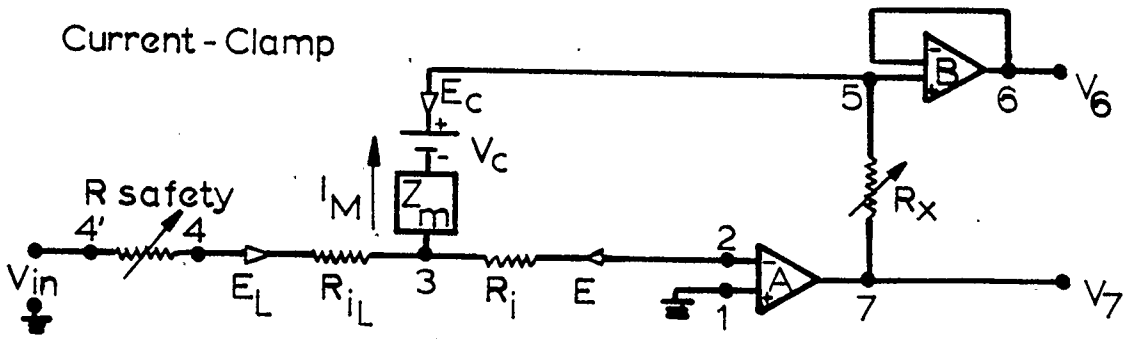


Figure 3. Equivalent circuit for current clamp.

potential at point 2, V_2, was equal to V_1, i.e., $V_1 = V_2 = 0$. Point 3, being within the intracellular space of the fiber in the test compartment, had a potential V_3 which, because no current was flowing, produced zero potential also, i.e., $V_3 = 0$. A safety series resistance R_{safety} of 10 MΩ was inserted to limit the current which could pass through the fiber. This resistance was progressively reduced to zero, making the input voltage V_{in} the same as the voltage at point 4. Point 5 represents the exterior of the fiber in the test compartment and the potential at point 6 thus was giving the experimental membrane potential. When a stimulating pulse was applied, a constant current I_M flowed through the membrane impedance Z_{vM}. The same current flowed through the known resistance R_X, i.e., $I_M = \frac{V_7 - V_5}{R_X}$. This current induced a change in the membrane potential which could be measured at point 5. But due to unity gain of the operational amplifier B, $V_5 = V_6$.

2.2.2 Voltage clamp

Figure 4 represents an equivalent circuit for voltage clamp. As in the current clamp $V_1 = V_2 = V_3 = 0$. Point 4 represents the location of the central electrode, outside the fiber in the test compartment. When $R_{safety} = 0$, the potential at point 4 became equal to the input potential V_{in} which produced a current toward the transmembrane potential (i.e., the "battery", V_c). When a stimulating pulse was applied (V_{in}), a current i_m was generated from the amplifier equal and opposite to the current generated by the active membrane in such a way as to maintain the potential drop across Z_M equal to V_{in}. The same current i_m flow through R_X. Since $V_6 = V_5$ thus:

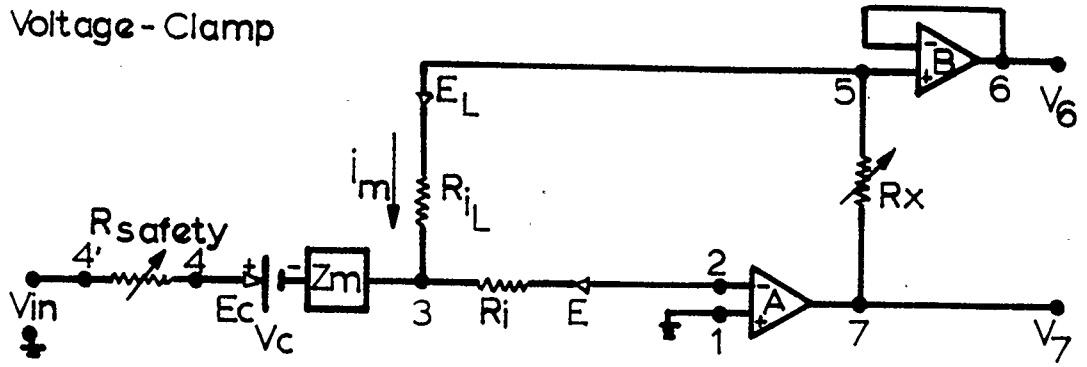


Figure 4. Equivalent circuit for voltage clamp.

$$V_7 - V_5 = i_m R_X = V_7 - V_6$$

or

$$i_m = \frac{V_7 - V_6}{R_X}$$

The potential difference $V_7 - V_6$ could be measured on the oscilloscope by means of the differential input. One could then calculate the transmembrane current using the known value of R_X. In both current and voltage clamp, R_X was usually set to 250 kΩ, although this value could be varied as desired.

2.3 Materials

In these studies the atrial fibers isolated from frog hearts (Rana catesbeiana) were used. Frogs were killed by severing the spinal column at the neck. The beating heart was removed and transferred through a series of beakers, each containing normal Ringer's solution. It was left for a short time in each beaker while the blood it contained was exchanged for the bathing solution. The heart was then pinned to the bottom of a dissection dish containing normal Ringer's solution. The dissection was performed with the aid of a dissecting microscope. The atrium was opened exposing the entire surface of the inner wall. The inner atrial wall is composed of bundles of muscle fibers or trabeculae. One of these with a diameter of about 100 μm and length of about 2 - 4 mm was dissected and placed in the double sucrose gap chamber. For the experiments only quiescent fibers were chosen. The experiments were performed at room temperature (20 - 22°C).

2.4 Physiological Solutions

All solutions were made from Fisher chemicals and distilled water. (Sybron Barustead Nano-pure II, 18.3 megaohm-cm).

The composition of solutions was as follows:

Normal Ringer's solution;

NaCl 110 mM, KCl 2.5 mM, $CaCl_2$ 1.8 mM,

$MgCl_2$ 2.0 mM, Tris 5.3 mM, glucose 5.0 mM

pH was adjusted to 7.3

Isotonic Sucrose Solution:

sucrose 80.8 g/l, distilled water.

Sucrose solution was passed through a deionizing column just before entering the sucrose gap

2 MΩ/cm specific resistivity value was obtained for every preparation

Drug solutions:

- Tetrodotoxin (TTX; Sigma Chemical Company) was added to the normal Ringer's solution to a final concentration of 5×10^{-7} g/ml.
- Cupric chloride ($CuCl_2 \cdot 2H_2O$; Fisher Scientific Company) was made as stock solution in Ringer's solution about 1 hour before the experiment, then was diluted with Ringer's solution to the desired concentration immediately before use. pH was readjusted to 7.3.
- Penicillamine (Pen.; Sigma Chemical Company) 1 mg/ml. Stock solution in Ringer's solution was prepared about 1 hour before the experiment, then was diluted with Ringer's solution before use. pH was readjusted to 7.3.

All solutions were prepared fresh before each experiment.

2.5 Measurement of the Action Potential and Membrane Currents

After each preparation was placed into the chamber, control measurement was performed. The size and the shape of the action potential could vary as a result of disection, sealing technique or speed of perfusion. Only fibers with action potential amplitude higher than 90 mV and the duration longer than 150 ms were accepted for experiments. The experimental results were recorded with use of the photographic camera. The drawings or the superimposed drawings from those oscilloscope photos are presented in this thesis.

The peak amplitude of the action potential was measured from the resting potential to the maximum depolarization of the membrane potential.

The duration of the action potential was taken as the width of the trace measured at 50% of peak amplitude (see Figure 5).

The slow inward current was measured in Ringer-TTX solution by taking the maximum current with respect to the current at the end of the pulse (see Figure 6a) (Coraboeuf, 1978).

The delayed outward current was obtained by taking the current value at the end of long duration pulses (5s) (see Figure 6b).

2.6 Convention

V[mV] - is a deviation of the membrane potential from its resting value, defined as zero. Depolarizations are positive.

I[A] - is a transmembrane ionic current. Outward currents are positive.

2.7 Abbreviations

TTX - tetrodotoxin

Pen - penicillamine

Cu^{++} - cupric ions

Ringer-TTX - normal Ringer's solution containing $5x10^{-7}$ g/ml TTX

Ringer-Cu^{++} - normal Ringer's solution containing Cu^{++}

Ringer-Pen - normal Ringer's solution containing Pen

Ringer-TTX-Cu^{++} - normal Ringer's solution containing $5x10^{-7}$ g/ml TTX and Cu^{++}

Ringer-TTX-Cu^{++}-Pen - normal Ringer's solution containing $5x10^{-7}$ g/ml TTX, Cu^{++} and Pen

APA - peak amplitude of the action potential

$D_{50\%}$ - duration of the action potential measured at 50% of the peak amplitude

AP - action potential

I_S - slow inward current

I_T - delayed outward current

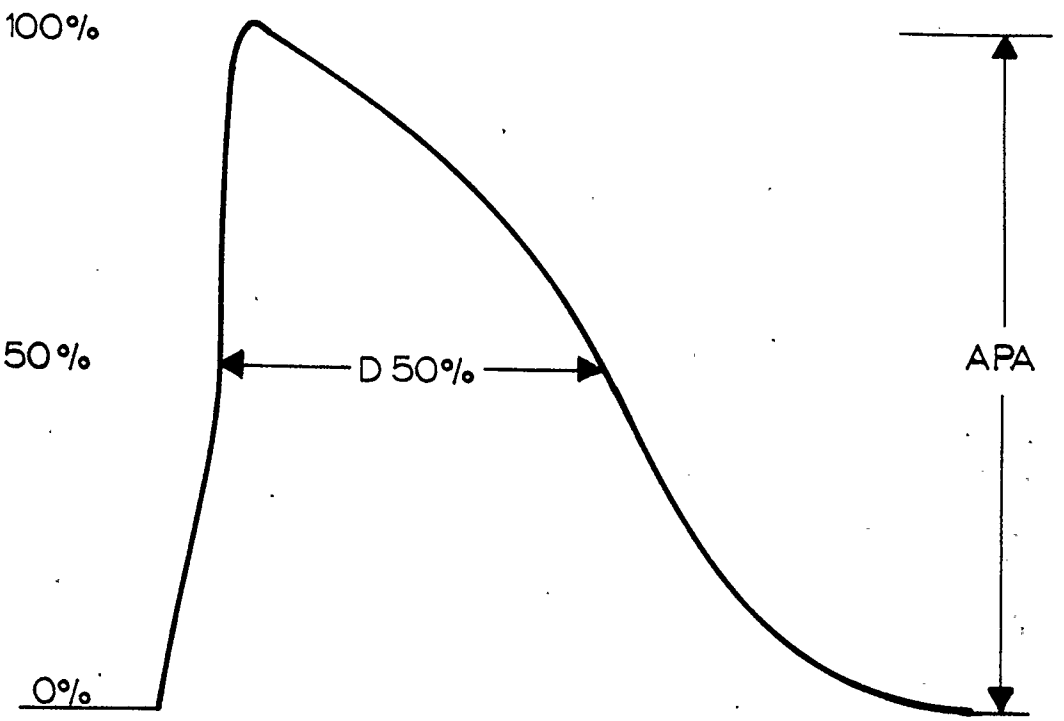


Figure 5. Sketch of the typical action potential of frog atrial fiber.
APA - peak amplitude of the action potential.
$D_{50\%}$ - duration of the action potential measured at 50% of the peak amplitude.

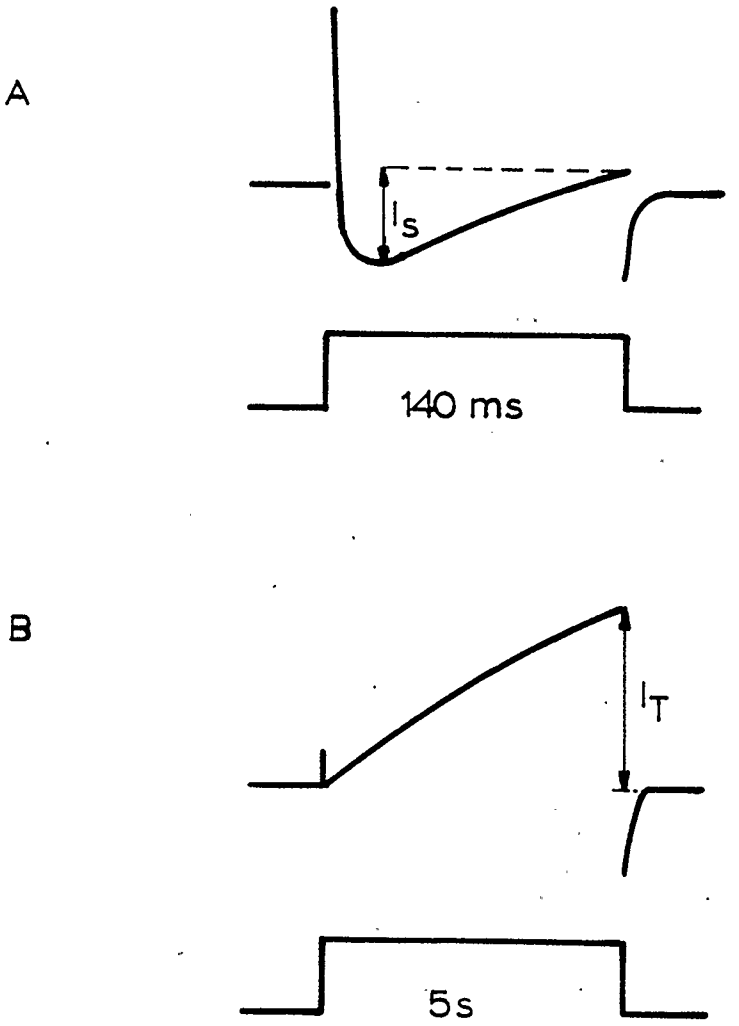


Figure 6. Sketch of the transmembrane ionic currents in frog atrial fiber.

a. Slow inward current (I_S).
b. Total outward current (I_T).

CHAPTER 3 RESULTS

3.1 Effect of Cu^{++}

3.1.1 Effect of Cu^{++} on Action Potential

Bullfrog atrial fibers were exposed to Cu^{++} at various concentrations (10, 20, 30, 40, 50, 60, 100 μM) for 30 min. and then returned to a normal physiological environment in order to study whether extended washout (60 min) in normal Ringer's solution would reverse the toxic effect of Cu^{++}. It was found that introduction of cupric ions to normal Ringer's solution produced a depolarization of the resting potential (5 - 10 mV). This effect was observed for every concentration tested and each preparation (N = 70). Peak amplitude of the action potential was decreased and continued to decrease during Cu^{++} treatment (Figure 7). However, the effect on the duration of the action potential was more complex. Cu^{++} at low concentrations (< 30 μM) produced a temporary increase in duration of the action potential. When Cu^{++} treatment was continued, the duration of the action potential was then progressively decreased to a value below that observed before the introduction of Cu^{++} (Figure 8). For concentrations greater than 30 μM Cu^{++}, the change in the duration of the action potential was monotonic. The duration always decreased with increasing time of Cu^{++} treatment (Figure 9). Washout with normal Ringer's solution following the application of Cu^{++} failed to restore the action potential to its control value. This was consistent for every concentration of Cu^{++} used in the present study. Figure 10 shows the effect of washout with normal Ringer's solution following application of Cu^{++} at 10, 40, 60 and 100 μM. Extended washout (60 min.) failed to restore either amplitude or

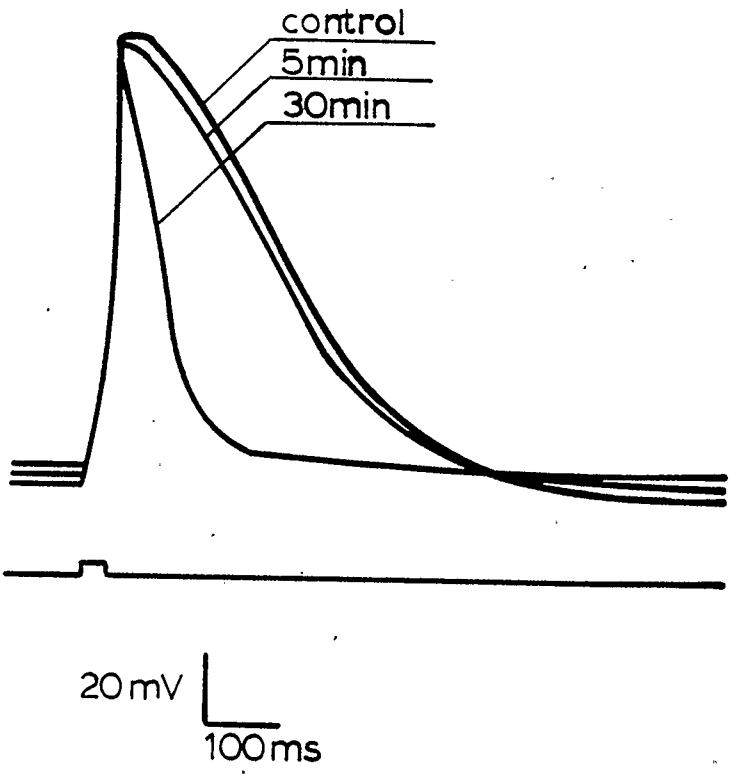


Figure 7. Time dependence of the effect of 40 μM Cu^{++} on the amplitude of the action potential (typical example from 12 preparations).

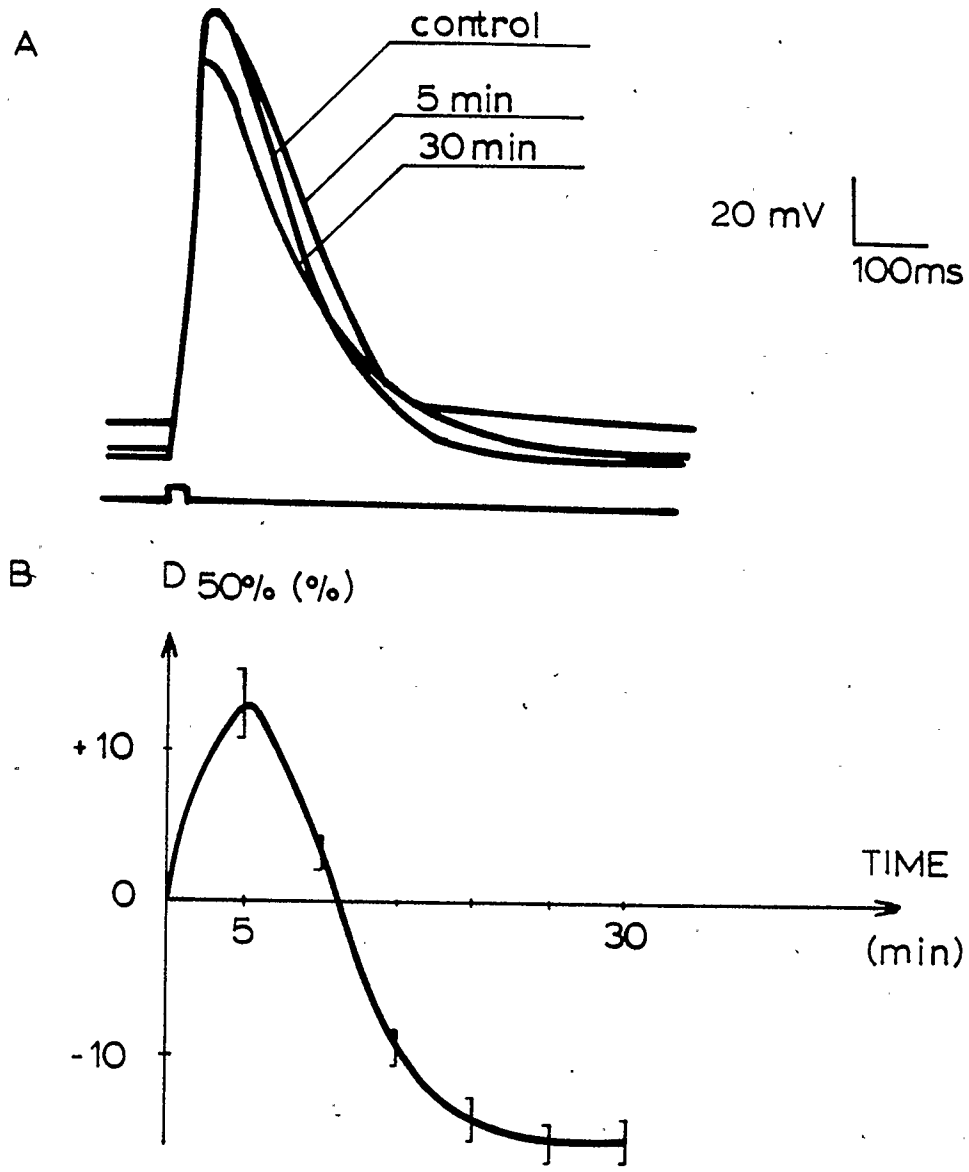


Figure 8. A. Time dependence of the effect of 10 μM Cu^{++} on the duration of the action potential (typical example from 6 preparations).

B. The percentage reduction of the duration of the action potential versus time following the application of 10 μM Cu^{++}.

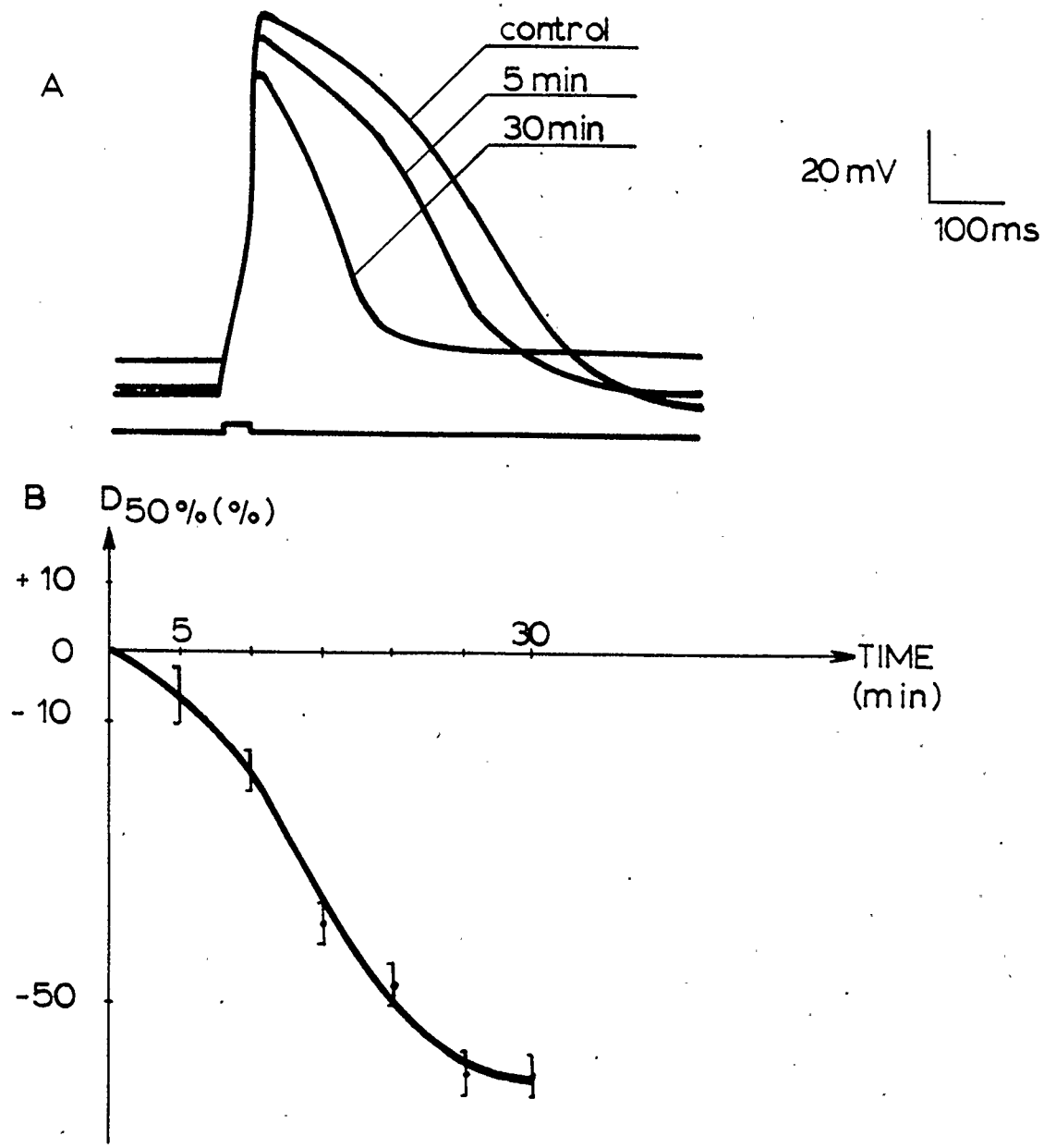


Figure 9. A. Time dependence of the effect of 100 μM Cu^{++} on the duration of the action potential (typical example from 6 preparations).

B. The percentage reduction of the duration of the action potential versus time following the application of 100 μM Cu^{++}.

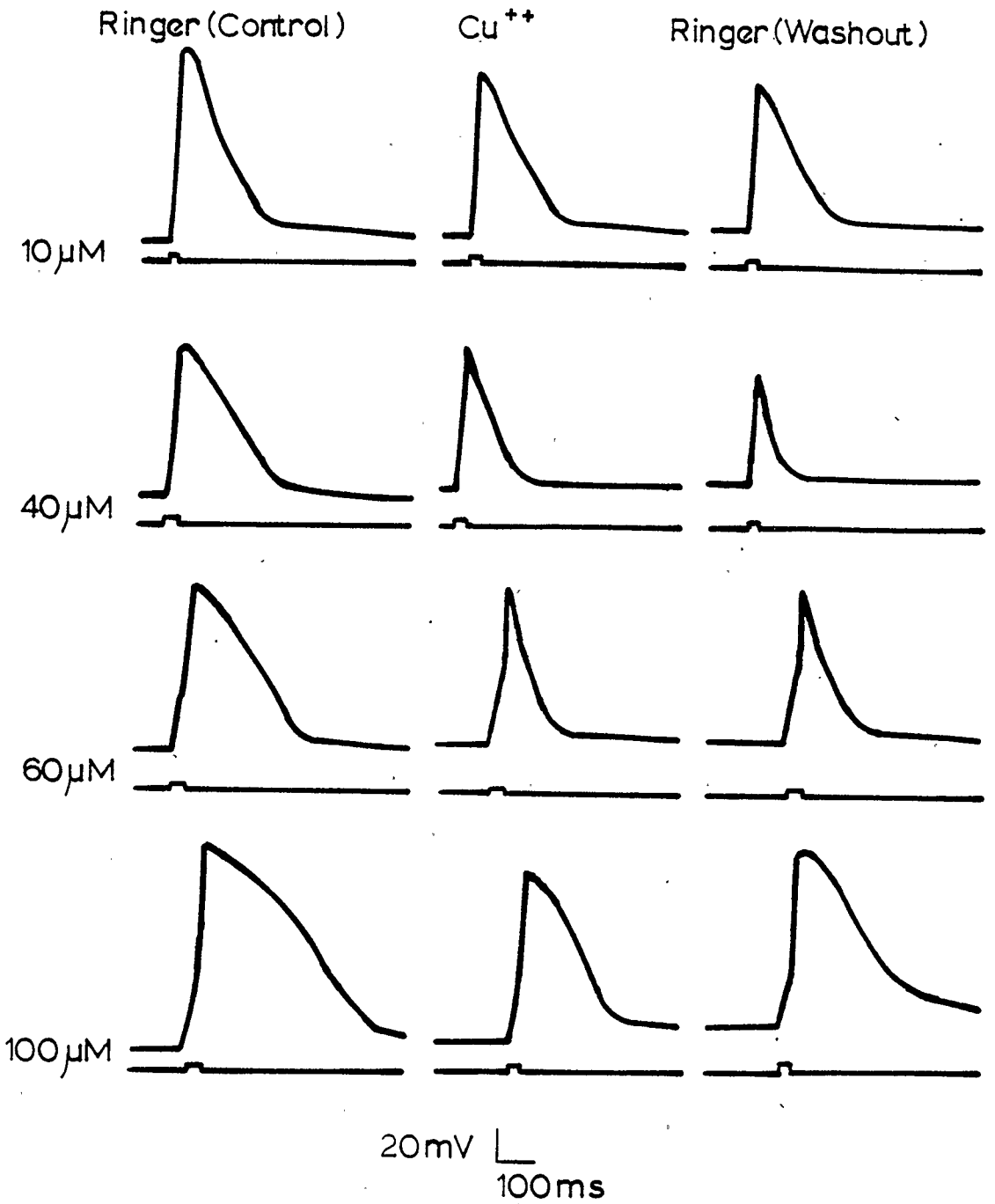


Figure 10. Effect of washout with normal Ringer's solution (60 min.) following application of different concentration of Cu^{++} on the action potential of a frog atrial fiber. (Typical example from: 6 preparations for 10 µM Cu^{++}
12 preparations for 40 µM Cu^{++}
8 preparations for 60 µM Cu^{++}
6 preparations for 100 µM Cu^{++}).

duration of the action potential of a frog atrial fiber. Rather, further deterioration occurred. Figure 11 summarizes the effect of different concentrations of Cu^{++} on amplitude and duration of the action potential of a frog atrial fiber. There was a slight decrease (2%) in amplitude following the application of Cu^{++} for 5 min. which progressed with time to reach about 15% level after 30 min. This tendency was independent of the concentration of Cu^{++} used in the study. Duration, on the other hand, was dependent on the concentration of Cu^{++} and the length of time of treatment. For concentrations < 30 μM, a transient increase in duration of the action potential was observed following the application of Cu^{++} for 5 min. When perfusion was continued, the duration fell below the control value. For concentrations > 40 μM there was a steady decrease of the duration with time.

3.1.2 Effect of Cu^{++} on Slow Inward Current

The slow inward current was isolated by introducing $5x10^{-7}$ g/ml TTX into the normal Ringer's solution, and was measured using depolarizing pulses of 140 ms duration, and 15 mV to 120 mV amplitude. The control value of the slow inward current was measured after 1 minute in Ringer-TTX solution. (TTX suppresses fast sodium current in a matter of seconds, unmasking slow inward current). After the introduction of Cu^{++} into the test compartment, reduction of the slow inward current amplitude was observed. Again, Cu^{++} at different concentrations (20, 30, 40, 50, 60, 100 μM) was used, and in each case the current-voltage relationship for the slow inward current was obtained (measured at 5 min. after the application of Cu^{++}).

Results indicated that at either low (see Figure 12) or high (see

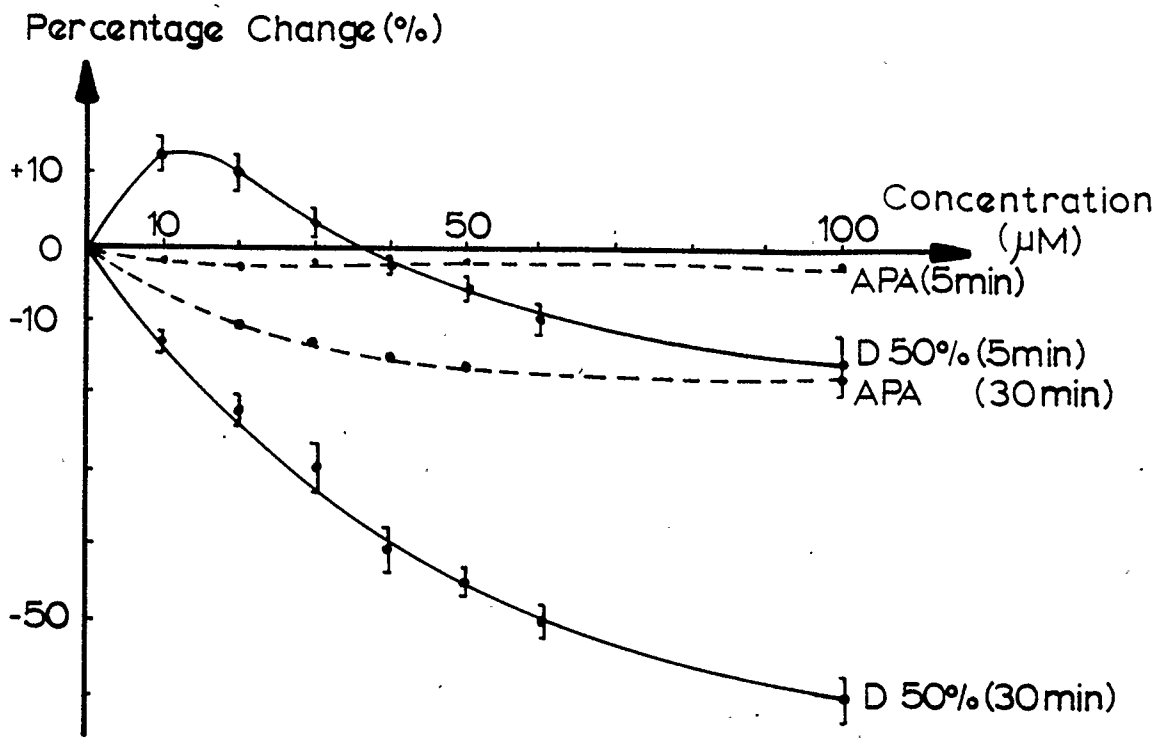


Figure 11. The percentage change of the peak amplitude and duration of the action potential as a function of Cu^{++} concentration and time of Cu^{++} treatment.

solid line $D_{50\%}$ (5 min.) - duration after treatment with Cu^{++} for 5 min.
solid line $D_{50\%}$ (30 min.) - duration after treatment with Cu^{++} for 30 min.
dashed line APA (5 min.) - amplitude after treatment with Cu^{++} for 5 min.
dashed line APA (30 min.) - amplitude after treatment with Cu^{++} for 30 min.

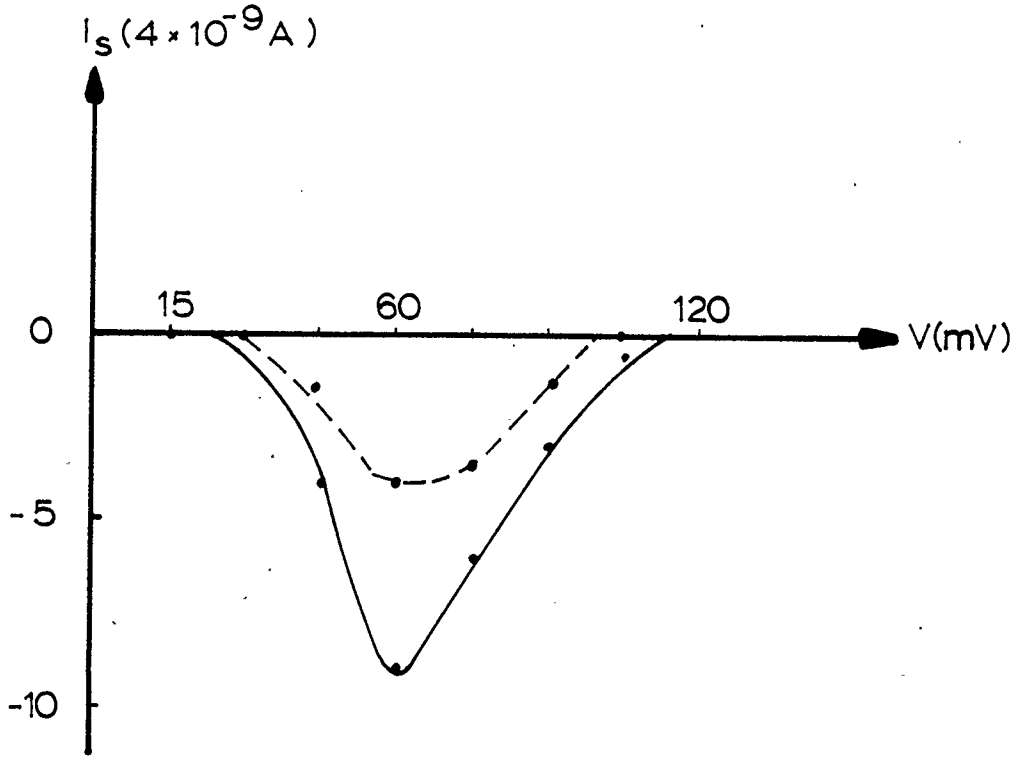


Figure 12. Effect of 20 μM Cu^{++} treatment on the slow inward current in frog atrial fiber.

solid line - Ringer-TTX
dashed line - Ringer-TTX-Cu^{++} (after 5 min.)
(typical example from 5 preparations)

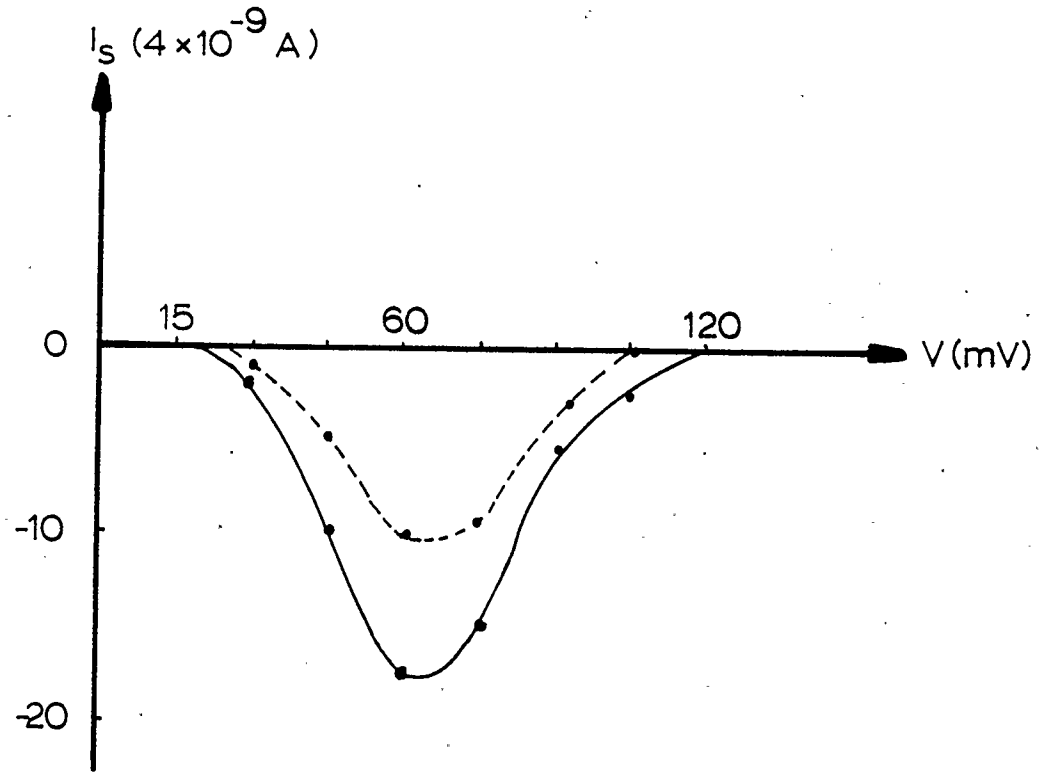


Figure 13. Effect of 60 μM Cu^{++} treatment on the slow inward current in frog atrial fiber.

solid line - Ringer-TTX
dashed line - Ringer-TTX-Cu^{++} (after 5 min.)
(typical example from 4 preparations)

Figure 13) concentrations, Cu^{++} reduced the amplitude of the slow inward current, and produced a negative shift (towards lower values) of the reversal potential (equilibrium potential for Ca^{++}). In Ringer-TTX solution, the slow inward current was activated at around +25 mV, with its reversed potential located around +120 mV. After introduction of Cu^{++} into the test compartment, the reduction of the peak amplitude of the slow inward current obtained from the current voltage relationship was about 40%, together with a negative shift of the reversal potential of about 15 mV (N = 30). Figure 14 shows the effect of different Cu^{++} concentrations on the slow inward current in a frog atrial fiber. Reduction of the slow inward current at each concentration was calculated as an average from all preparations (20 μM Cu^{++} - N = 5, 30 μM Cu^{++} - N = 4, 40 μM Cu^{++} - N = 12, 50 μM Cu^{++} - N = 5, 60 μM Cu^{++} - N = 4).

3.1.3 Effect of Cu^{++} on Delayed Outward Current

Long duration (5s) depolarizing and hyperpolarizing pulses were applied in the voltage clamp condition. The delayed rectification current was measured in Ringer's solution, and in Ringer's solution containing Cu^{++}. The effect of different Cu^{++} concentrations, and various times of treatment, on the delayed outward current were studied. It was found that Cu^{++} at concentrations up to 30 μM produced a transient decrease in delayed rectification current. When Cu^{++} treatment was continued, this current increased to a value greater than the control one (see Figure 15). At higher Cu^{++} concentrations there was a monotonic effect on the delayed outward current. An increase in the delayed current was observed after 5 min. in Ringer-Cu^{++} solution

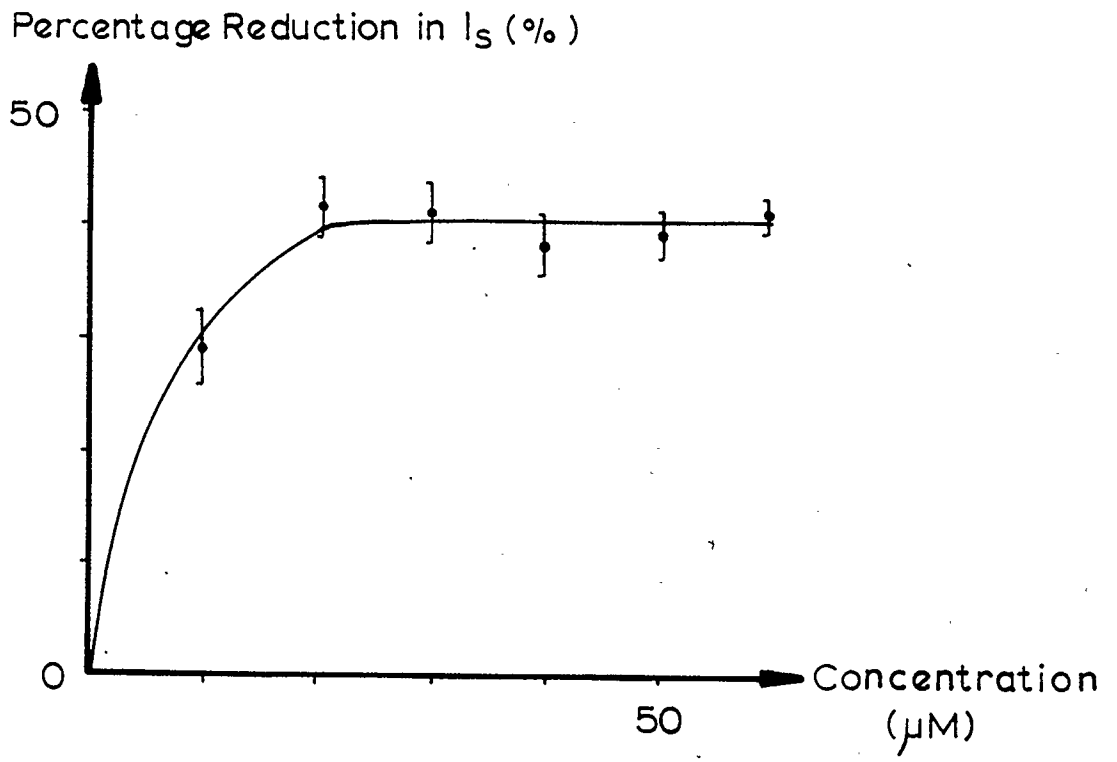


Figure 14. Effect of different concentrations of Cu^{++} on the average percentage reduction of the peak amplitude of the slow inward current.

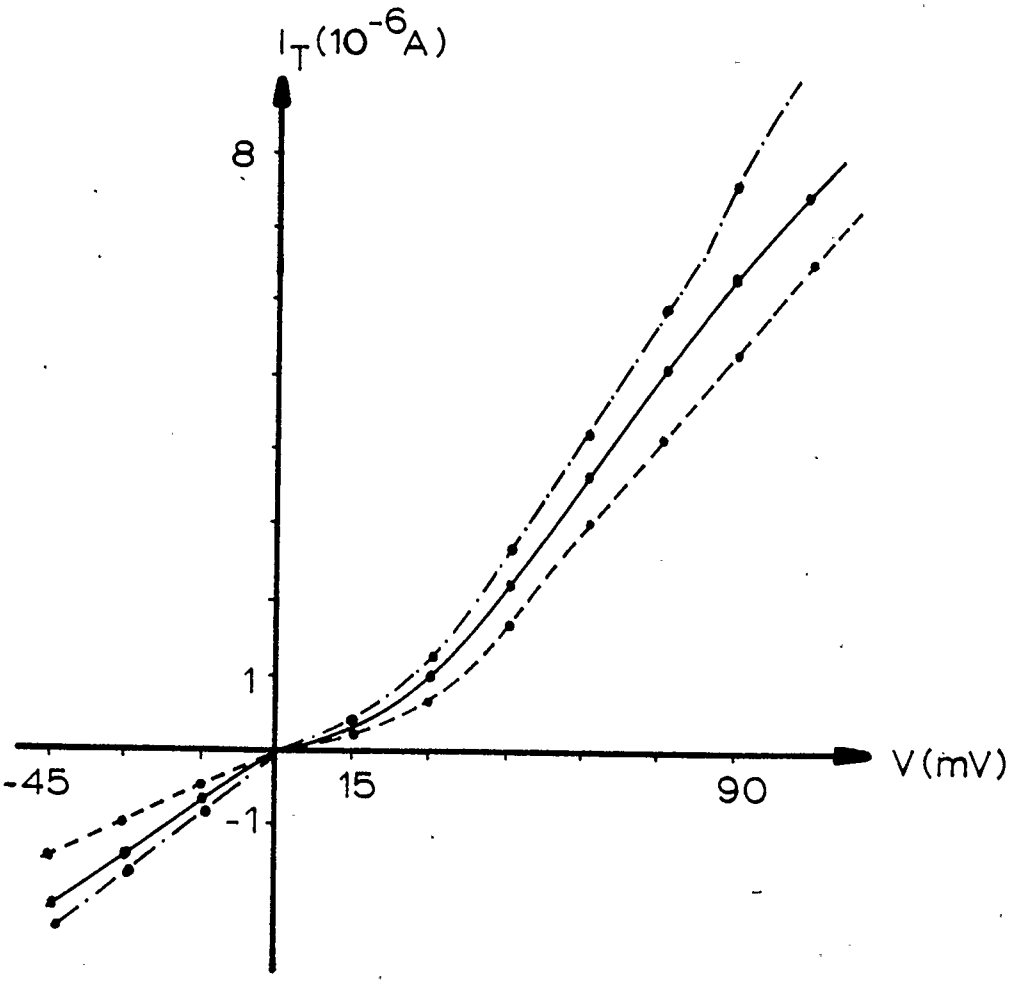


Figure 15. Effect of 30 μM Cu^{++} on the delayed outward current.

solid line - Ringer
dashed line - Ringer-Cu^{++} after 5 min.
dotted line - Ringer-Cu^{++} after 15 min.
(typical example from 6 preparations).

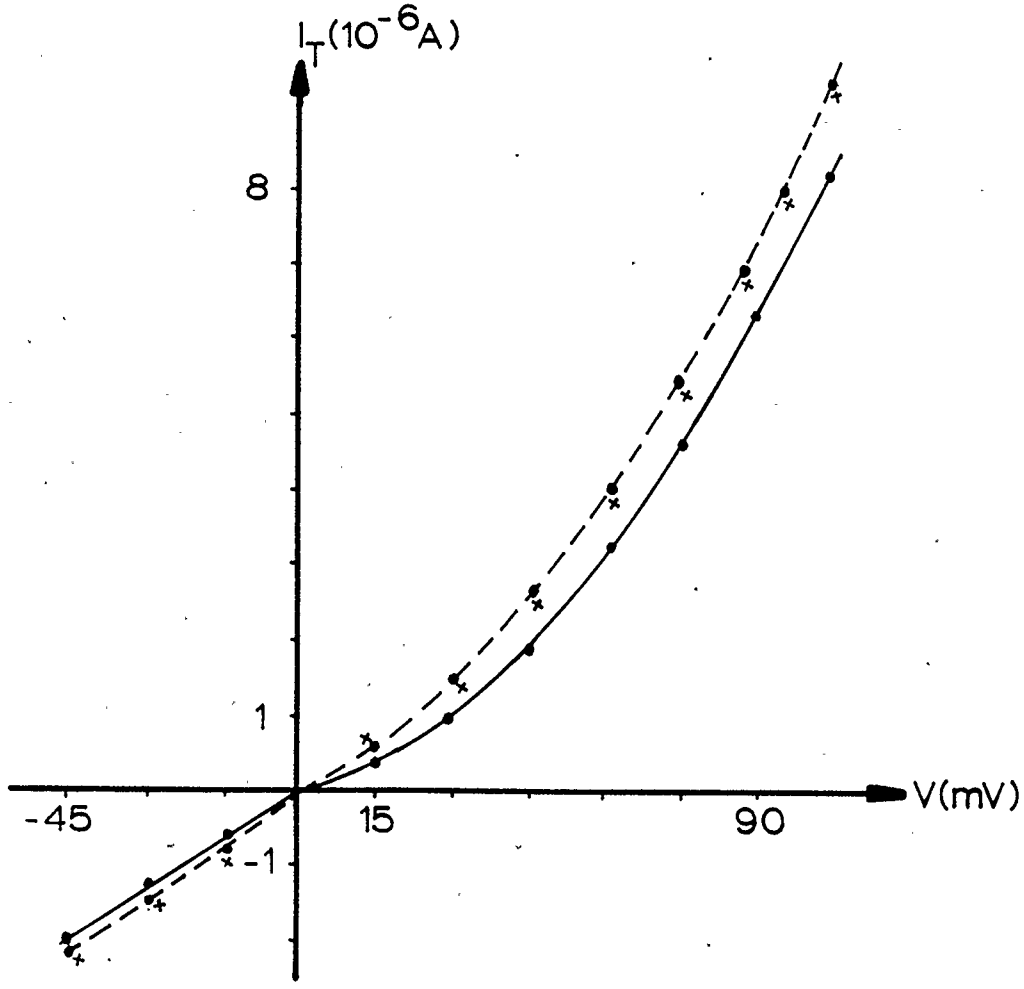


Figure 16. Effect of 50 μM Cu^{++} on the delayed outward current.

solid line - Ringer
dashed line - Ringer-Cu^{++}: (o) after 5 minutes
(x) after 15 minutes
(typical example from 5 preparations)

and this persisted at the same level for a further 10 minutes (see Figure 16).

The application of Cu^{++} did not induce a significant modification in the delayed outward current but a small increase (about 15%) was seen for all the preparations used (N = 25). It was also observed that washout with normal Ringer's solution following the application of Cu^{++} failed to restore the delayed outward current; rather further increase occurred. The change in the delayed outward current as a result of Cu^{++} treatment was then irreversible.

3.2 Effect of Penicillamine

For the investigation of the effect of penicillamine on toxicated atrial fibers it was decided to use a single concentration of Cu^{++}. 40 μM Cu^{++} was chosen because cupric ions at this concentration produced a monophasic effect on the amplitude and the duration of the action potential (see Figure 7 and 11).

3.2.1 Effect of Penicillamine on Action Potential During Cu^{++} Treatment

40 μM Cu^{++} produced changes in shape and size of the action potential which were not reversed when normal physiological conditions were restored (Figure 10). It was decided to study penicillamine, a drug clinically applied in the treatment of Wilson's disease, to find whether it would reverse the toxic effect of Cu^{++} on frog atrial fibers under experimental conditions. Cardiac tissue was treated for 30 minutes with Ringer-Cu^{++} solution. Typical changes in action potential were observed (depolarization of resting potential, reduction in peak amplitude and duration of the action potential). Penicillamine was then introduced

into the Ringer-Cu^{++} solution. Following 30 minutes treatment with penicillamine the atrial fiber was returned to normal Ringer's solution and washed out for one hour. The action potential following extended washout was then examined and compared with the control one. It was found that the degree of recovery was critically dependent on the concentration of penicillamine used in the experiment. Figure 17 shows the effect of 0.2 mM penicillamine on the action potential of a frog atrial fiber previously treated with 40 μM Cu^{++}. Application of penicillamine produced an increase in both peak amplitude and duration of the action potential. Both parameters were still less than the control values, and decreased slightly following extended washout with normal Ringer's solution (N = 8). When higher concentrations of penicillamine were used (0.5 mM) both amplitude and duration of the action potential were increased to above the control values following 30 minutes treatment (N = 5). Washout with normal Ringer's solution failed to restore the action potential to its control value; rather a further increase in amplitude and duration were observed (see Figure 18). Based on these results it was speculated that there may be some concentration of penicillamine which would produce total recovery. This specific dosage of penicillamine would be within the 0.2 - 0.5 mM range. In order to find this concentration, increments of 0.05 mM were tested (N = 3 in each case). Finally, it was established that penicillamine at concentration of about 0.3 mM produced a good degree of recovery of the action potential which had been suppressed by 40 μM Cu^{++}. Figure 19 illustrates this finding.

The control experiments were also performed to see the effects of penicillamine alone on the action potential. Atrial fibers were exposed

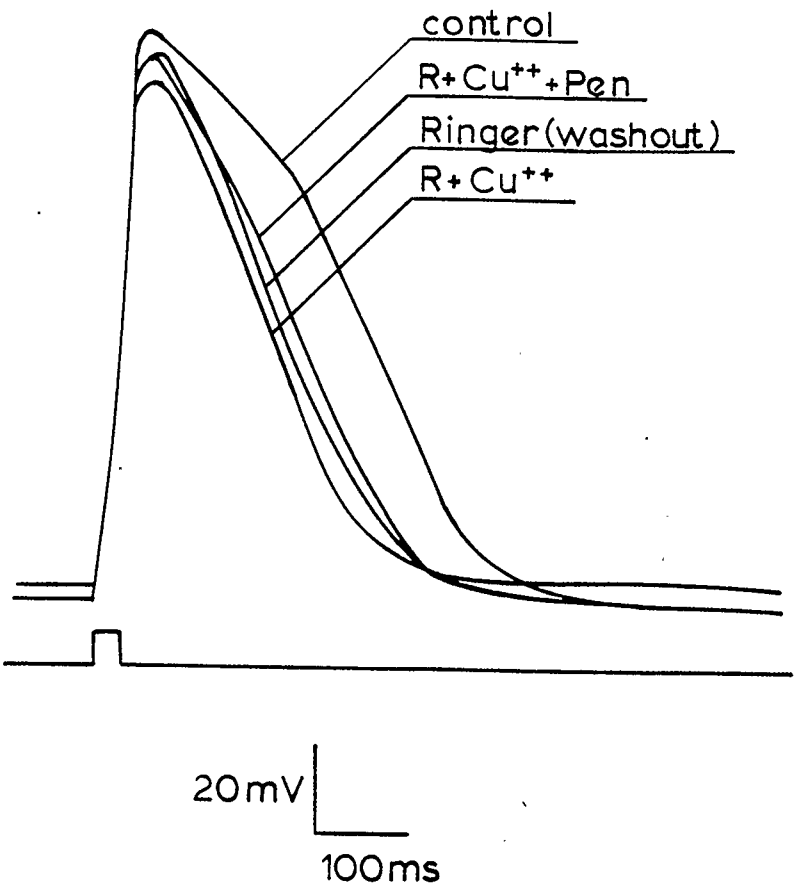


Figure 17. Effect of 0.2 mM penicillamine on the action potential of a frog atrail fiber treated with 40 μM Cu^{++}.

(typical example from 8 preparations)

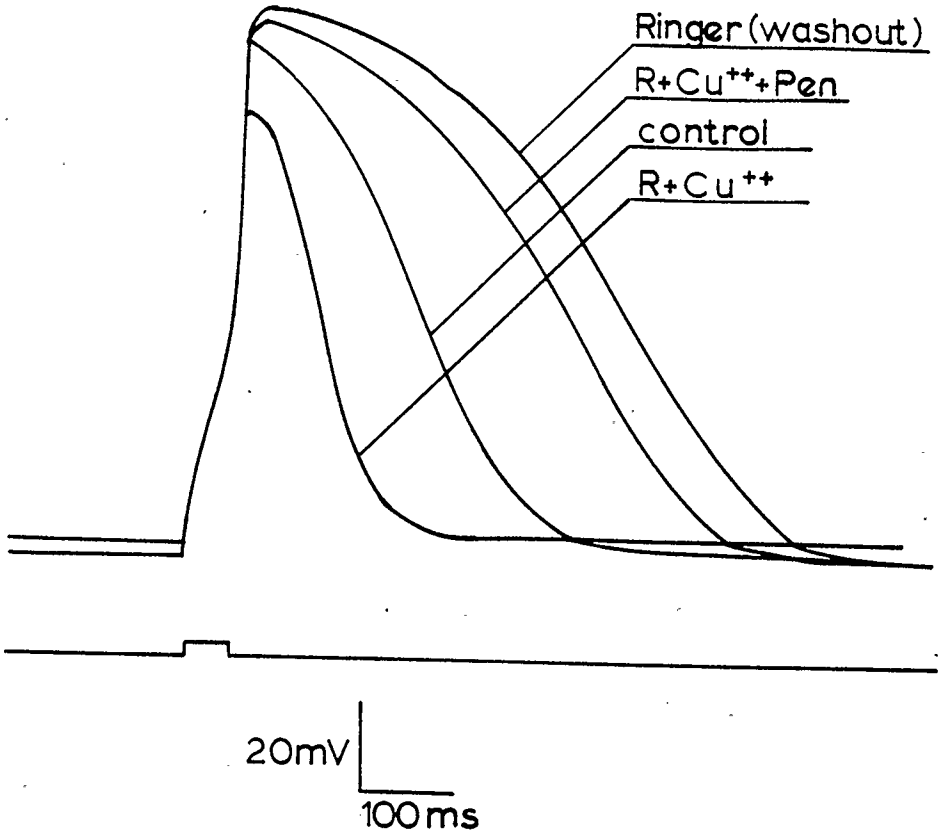


Figure 18. Effect of 0.5 mM penicillamine on the action potential of a frog atrial fiber treated with 40 μM Cu^{++}.

(typical example from 5 preparations)

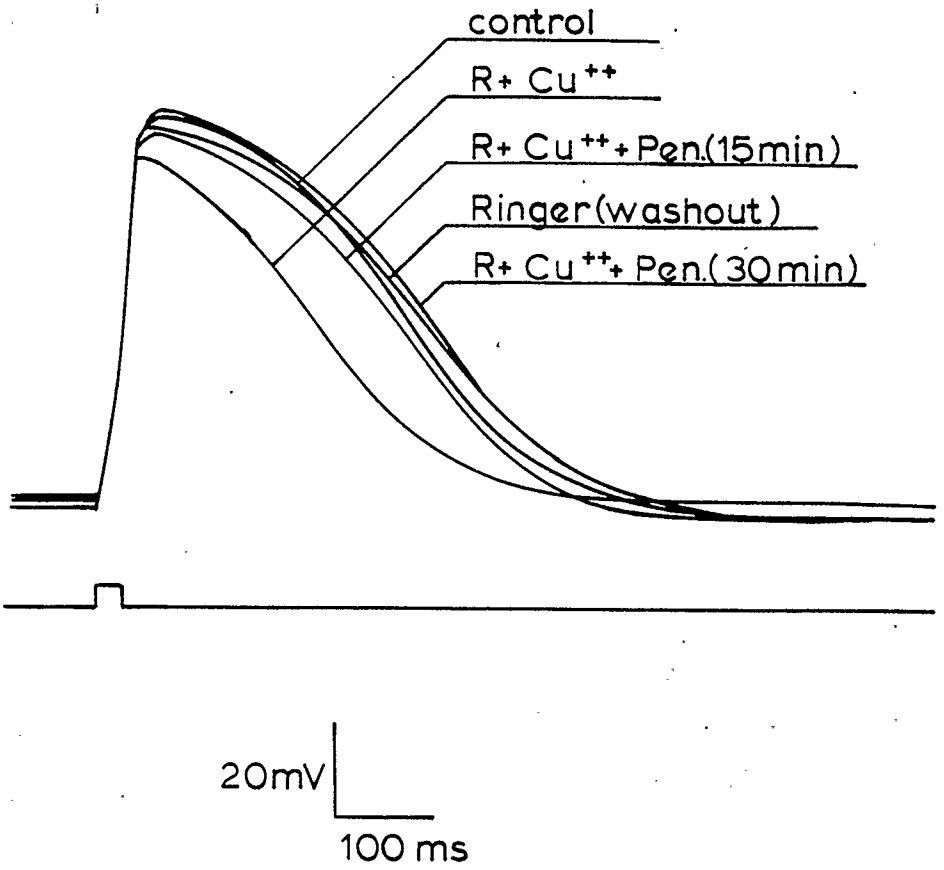


Figure 19. Effect of 0.3 mM penicillamine on the action potential of a frog atrial fiber treated with 40 μM Cu^{++}.

(typical example from 6 preparations)

to 0.3 mM penicillamine for 30 minutes and then returned to normal Ringer's solution for 30 minutes washout. Penicillamine produced hyperpolarization of the resting potential together with an increase in the amplitude and the duration of the action potential. Those parameters recovered to normal values following washout with normal Ringer's solution. However, there were not enough experiments run to allow statistic analysis (N = 2). Therefore, one can treat those observations only in a qualitative manner.

3.2.2 Effect of Penicillamine on Slow Inward Current During Cu^{++} Treatment

Cu^{++} at 40 μM concentration produced a reduction in the slow inward current, together with a negative shift of the reversal potential. These changes were irreversible. Washout with Ringer-TTX solution following administration of Cu^{++} not only failed to return the slow inward current to its control value (see Figure 20), but rather, further deterioration occurred.

As was described in the previous section, 0.3 mM penicillamine produced a recovery in the action potential of a frog atrial fiber which has been treated with 40 μM Cu^{++}. It was then decided to examine how the same concentration of penicillamine would influence the slow inward current.

An atrial fiber was treated with 40 μM Cu^{++} solution for 5 minutes (N = 6). Typical changes in slow inward current were observed. 0.3 mM penicillamine was then introduced into Ringer-TTX-Cu^{++} solution and perfusion was carried out for another 15 minutes. From current clamp studies it was known that after 15 minutes penicillamine treatment there

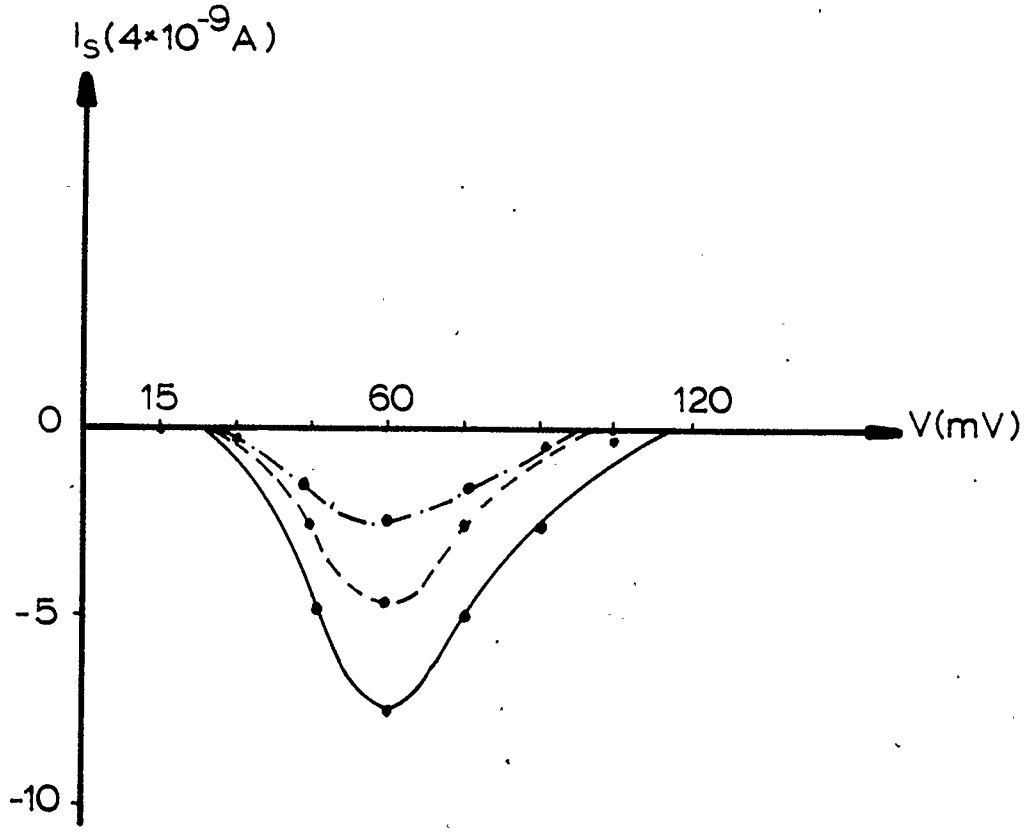


Figure 20. Effect of 40 μM Cu^{++} treatment on the slow inward current in frog atrial fiber.

solid line - Ringer-TTX
dashed line - Ringer-TTX-Cu^{++} (after 5 min.)
dotted line - Ringer-TTX (after 15 minute washout)

(typical example from 6 preparations)

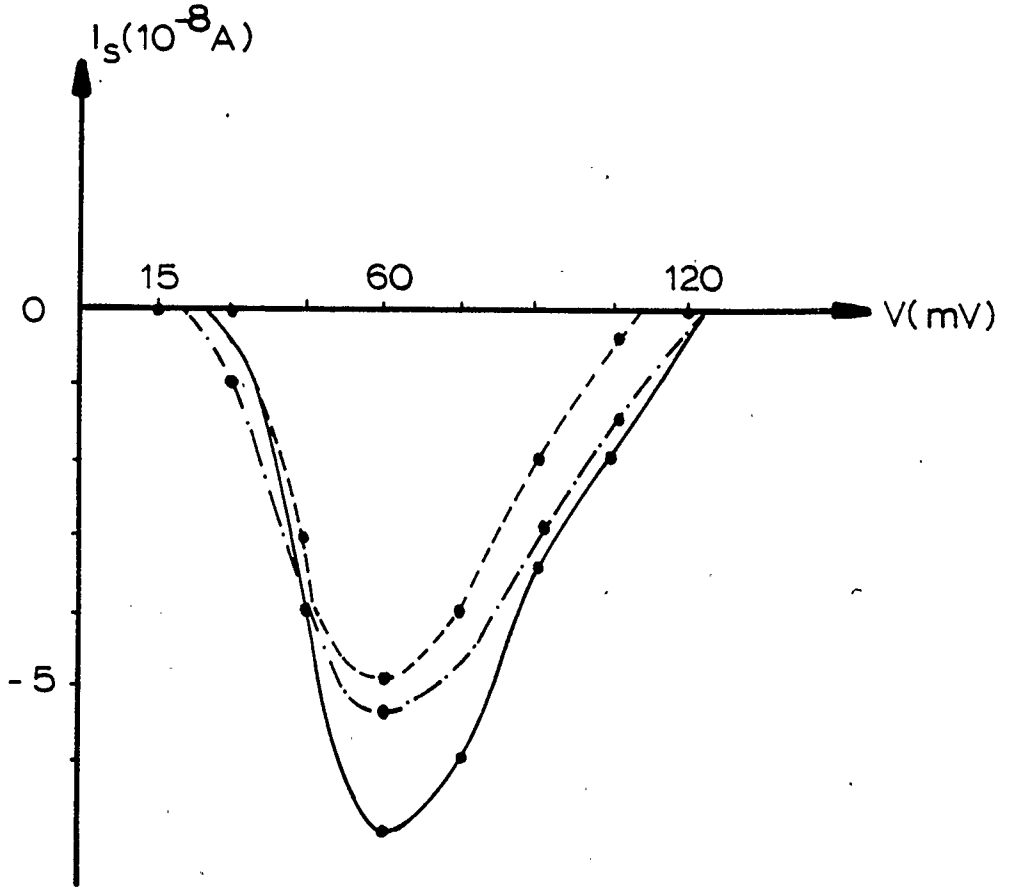


Figure 21. Effect of 0.3 mM penicillamine on slow inward current in frog atrial fiber toxicated with 40 μM Cu^{++}.

solid line - Ringer-TTX
dashed line - Ringer-TTX-Cu^{++} (after 5 min.)
dotted line - Ringer-TTX-Cu^{++}-Pen (after 15 min.)

(typical example from 6 preparations)

was partial recovery of the duration of the action potential (see Figure 19). It was anticipated that an increase of slow inward current toward its control value would occur. Figure 21 shows the current-voltage relationship obtained in this experiment. 0.3 mM penicillamine produced partial recovery of the amplitude of the slow inward current in all 6 preparations studied. In addition, the reversal potential of the slow inward current which had been shifted in the negative direction during Cu^{++} treatment was restored in the presence of the penicillamine.

3.2.3 Effect of Penicillamine on Delayed Outward Current During Cu^{++} Treatment

Cu^{++} at a concentration of 40 μM produced a small increase in delayed outward current (N = 6). Figure 22 represents the current-voltage relationship for the delayed outward current in frog atrial fiber treated with 40 μM Cu^{++} for 15 minutes. In current clamp studies it was observed that 0.3 mM penicillamine produced a recovery of the resting potential of the action potential of a frog atrial fiber treated with 40 μM Cu^{++} (see Figure 19). Based on this observation it was anticipated that an increase in delayed outward current during penicillamine treatment would occur. Figure 23 shows the effect of 0.3 mM penicillamine on the delayed outward current following perfusion with 40 μM Cu^{++}. The current-voltage relationship for the delayed outward current shows an increase of this current for all potentials. This was true for each preparation (N = 7) used in this study.

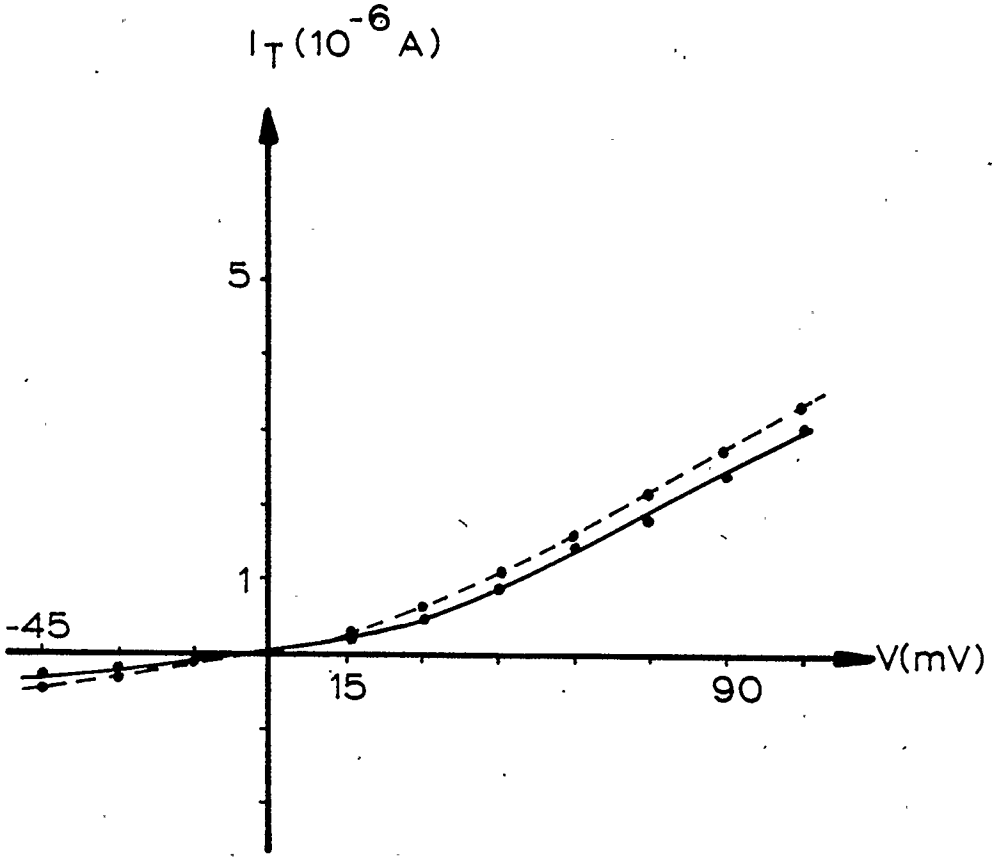


Figure 22. Effect of 40 μM Cu^{++} on the delayed outward current in frog atrial fiber.

solid line - Ringer
dashed line - Ringer-Cu^{++} after 15 minutes

(typical example from 6 preparations)

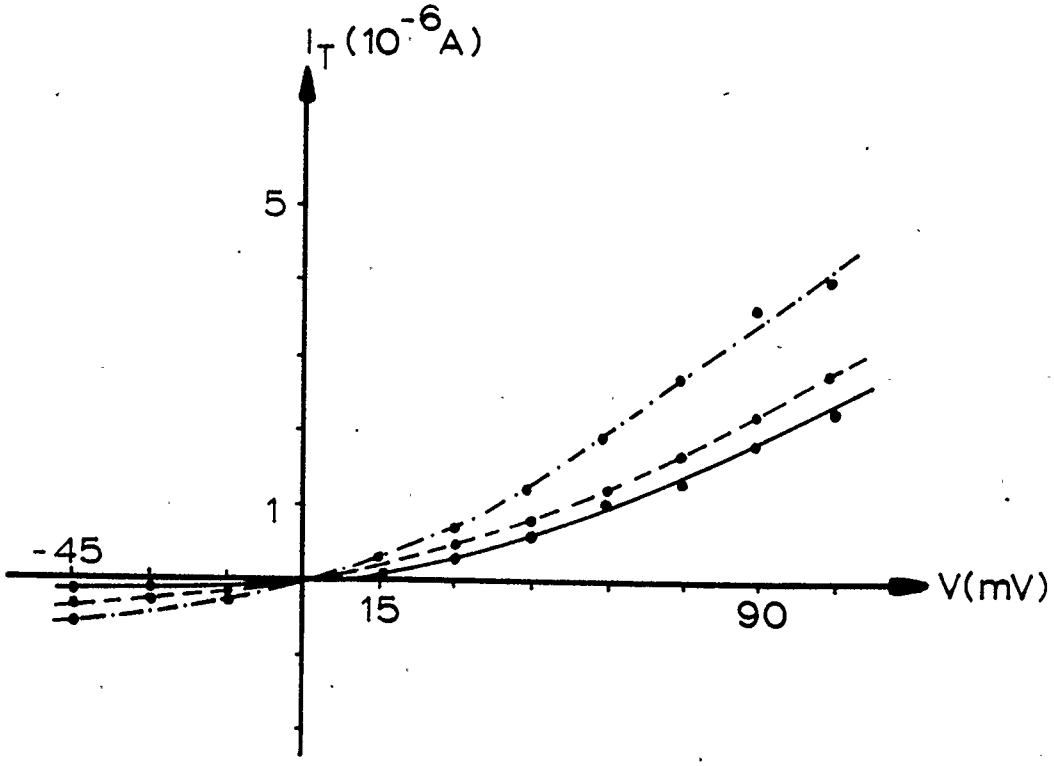


Figure 23. Effect of 0.3 mM penicillamine on delayed outward current in frog atrial fiber toxicated with 40 μM Cu^{++}.

solid line - Ringer
dashed line - Ringer-Cu^{++} after 5 minutes
dotted line - Ringer-Cu^{++}-Pen after 15 minutes

(typical example from 7 preparations)

CHAPTER 4 DISCUSSION

It is well established that electrical activity of cardiac cells is intimately related to the cellular metabolism (Haas et al. 1970). Any factor reducing the availability or utilization of metabolic energy leads to typical changes in the shape of the action potential (Trautwein and Dudel, 1956; De Mello, 1959; Kleinfeld et al., 1961; Haas et al., 1967). The most consistent early change is a progressive shortening of the action potential duration (a loss of plateau). Subsequent effects of metabolic inhibition are a decrease in amplitude of the action potential and a depolarization of the resting potential.

The simplest interpretation of the action potential changes observed during inhibition would be:

1. a change in passive permeabilities (or conductances) of the membrane caused by an alteration of energy-dependent membrane structures or
2. a change of ion concentrations on either side of the membrane due to a breakdown of active transport (i.e., a change in the driving force of passive ion movements).

The latter would imply an indirect influence of ion transport on electrical activity.

In this study, the effects of cupric ions on the electrical activity of the frog atrial fibers have been studied.

The membrane resting potential in the presence of cupric ions became depolarized by 5 - 10 mV (see Figure 7). This could be explained by the influence of Cu^{++} on the ionic pump. Partial blockage of the Na^{+} pump leads to an increase in intracellular free Na^{+} and thus to depolarization of the membrane resting potential. The next consistent

change produced by Cu^{++} was a reduction in the peak amplitude of the action potential. This effect was time and concentration dependent (see Figure 11). With more concentrated Cu^{++} solution, and longer times of treatment, the decrease became pronounced. The decreased amplitude of the action potential must be due to a reduction in the sodium current. The depression of peak sodium current could be explained by a partial block of the sodium channels caused by Cu^{++}, or by a decreased sodium concentration gradient after inhibition of active sodium expulsion. Although measurements of the fast sodium current were not undertaken in this project (due to the limitations of the double sucrose gap technique (McGuigan, 1974)) one could speculate that reduction of the peak amplitude of the action potential was produced by a depression of the sodium current due to one or both mechanisms discussed above. The effect of cupric ions on duration of the action potential was more complicated. Since the repolarizaton of the cardiac action potential is thought to be carried out, at least in part, by a potassium outward current, the effect of cupric ions on this ionic current will be discussed first. It was found that Cu^{++} at low concentrations (< 30 μM) produced a transient increase in the duration of the action potential (see Figure 8) paralleled by a transient decrease of the delayed outward current (see Figure 15). This could be explained in two ways. The cupric ions produced an inhibition of the potassium pump which led to a decrease in the transmembrane potassium concentration gradient. Decreased driving force for K^{+} movement would lead to a reduction in the delayed outward current. Another possibility was to assume that Cu^{++} at low concentrations decreased the membrane potassium permeability thus reducing the delayed outward current. Similar results have been

reported in the case of manganese ions. In very low concentrations manganese ions increase slightly the duration of the action potential. This experimental observation (which agrees with the observations of Hagiwara and Nakajima (1966) and of Coraboeuf and Vassort (1967, 1968)) could be caused by a lowering of the membrane potassium permeability.

When perfusion with a low concentration of Cu^{++} was continued, or higher concentrations were used, a decrease in the duration of the action potential and an increase in delayed outward current was observed (see Figures 8, 15, 9 and 16). A reduction of the duration of the action potential was thought to be related to an increase in potassium conductance (De Mello, 1959; Haas et al., 1970). The present study showed that there was only a small increase in the delayed outward current during Cu^{++} treatment which would be insufficient to account for the observed shortening of the action potential.

A blocking of the sodium ionic pump would produce an increase in the delayed outward current. Na^{+} - Ca^{++} exchange normally provides the mechanism for expelling Ca^{++} from the cells (Coraboeuf, 1978), but when the extracellular Na^{+} concentration is reduced, or intracellular Na^{+} concentration is increased, Ca^{++} accumulates within the cell. In a variety of tissues an increase in the intracellular Ca ion concentration has been shown to increase the K^{+} permeability of the cell membrane (red cells: Whittam, 1968; Lew, 1970; Romero and Whittam, 1971, Simons, 1975; Porzig, 1975; snail neurons: Meech and Strumwasser, 1970; Meech, 1970; Meech and Standen, 1975; spinal neurons: Krnjevic and Lisiewicz, 1972; squid axon: Tasaki, A. Watanabe and Lerman, 1967). A similar association has been suggested in the case of smooth muscle (Bulbring, 1973; Tomita and H. Watanabe, 1973) skeletal muscle (Fink

and Luttgan, 1973), and skate electroreceptor epithelium (Clusin, Spray and Bennett, 1974). In heart muscle a connection between intracellular Ca^{++} concentration and potassium permeability (manifested by an outward current) has been postulated by various authors (e.g., Morad and Greenspan, 1973; Reiter and Shickel, 1968; Prasad, 1974). Niedergerke and Orkand (1966) and McGuigan (1974) have suggested that intracellular Ca^{++} concentraton sets the level of potassium permeability and Colatsky and Hogan (1975) have suggested a similar relationship for Purkinje fibers. Evidence to support this idea has been obtained by Isenberg (1975), who injected Ca^{++} ions into short Purkinje fiber preparations and found a hyperpolarization of the membrane potential and a shortening of the action potential duration.

As has been mentioned, a small increase in the delayed rectifier was insufficient to account for the observed shortening of the action potential. However, there is a second ionic current which regulates the plateau phase of the action potential - the slow inward current. The effect of different Cu^{++} concentrations on this current was tested in the voltage clamp experiments, and the results were summarized in Section 3.1.2. Cu^{++} at all concentrations studied, produced approximately a 40% decrease in the slow inward current, together with a negative shift of the reversal potential of about 15 mV. These results could be attributed to a diminution in slow channel conductance, and/or the driving force for Ca ions (the shift of the reversal potential), the latter being due to an increase in intracellular free calcium produced by metabolic poisoning. The results illustrated in Figure 14 show saturation of Cu^{++} blockage of the slow inward current. It seems that copper at concentrations ranging from 20 μM to 60 μM produced the

constant reduction of the slow inward current most likely by suppression of a mechanism utilizing metabolic energy. As it was indicated by work of Peters on pigeon's brain (Peisach et al., 1966) the same level of reduction of the amount of phosphate split from ATP in 10 minutes by the membrane ATPase was produced by wide range of Cu^{++} doses (2 μg - 10 μg in 1.5 ml).

Present findings on the transmembrane electrical properties of frog atrial fibers during Cu^{++} treatment (decrease in Ca^{++} driving force, decrease in Ca^{++} membrane permeability, increase in K^{+} conductance, depolarization of the resting potential) were consistent with a hypothesis of metabolic inhibition. Cupric ions seemed to block an active ionic transport (i.e., alter the driving force of passive ions movements) and possibly to alter membrane permeabilities. These changes in the electrical activity produced by Cu^{++} were irreversible. Extended washout with normal Ringer's solution following Cu^{++} treatment failed to recover normal action potential (see Figure 10).

It was decided to use penicillamine, a drug used clinically in the treatment of Wilson's disease, and to study whether it would produce recovery of normal electrical activity in frog atrial fibers previously treated with cupric ions.

In current clamp experiments it was found that only a very specific concentration of the penicillamine could produce recovery of the normal action potential in frog atrial fibers treated with 40 μM Cu^{++}. This concentration was found to be about 0.3 mM. When concentrations smaller than 0.3 mM were used the amplitude and the duration of the action potential was partially restored toward control values (see Figure 17). On the other hand, penicillamine at concentrations higher than 0.3 mM

produced an increase in both amplitude and duration of the action potential far above control values.

An important observation was that penicillamine restored the resting potential which had been depolarized by the presence of cupric ions (see Figure 17, 18, 19). It was suggested that penicillamine activated ionic pumps which had been inactivated by Cu^{++} and/or increased the permeability to potassium ions. Both effects would result in increased delayed rectification (by an increase in driving force for passive potassium ion movement and by an increase in membrane permeability to K^{+}). Either of these two mechanisms would produce an increase in delayed outward current, which would contribute to the repolarization phase of the action potential in the atrium. Measurements of the delayed rectifier in the frog atrial fibers supported this theory. An increase in outward current was observed during penicillamine treatment in voltage clamp experiments (see Figure 23).

A very consistently observed change produced by penicillamine was an increase in peak amplitude of the action potential in the frog atrial fiber. Although no attempt was made to measure the fast inward current it could be assumed that penicillamine increased the amplitude of the sodium current either by increasing the driving force for Na^{+} movement (activation of sodium pump) or by increasing the membrane permeability to sodium ions.

Recovery of the duration of the action potential was also achieved by the application of penicillamine. Current clamp experiments showed a progressive increase in the duration of the action potential in the frog atrial fibers during penicillamine perfusion (see Figure 19). Because

the slow inward current regulates the plateau phase of the action potential, an increase in this transmembrane ionic current should be produced by the application of penicillamine. In the voltage clamp experiments it was demonstrated that penicillamine not only increased the peak amplitude of the slow inward current but also restored the reversal potential of the slow inward current to its control level (see Figure 21). It implied that penicillamine activated the Na^{+} - Ca^{++} exchange mechanism, which in turn increased the driving force for Ca^{++} (producing an increase of the slow inward current) and possibly increased slow channel conductance.

Penicillamine - Cu^{++} stoichiometry is quite high (7.5 : 1) which would suggest non-specific response or even unrelated response. As it was mentioned earlier penicillamine induced extra activity of membrane ATPase in pigeon's brain (Peisach et al., 1966). Preliminary experiments on the effects of penicillamine alone showed hyperpolarization of the cardiac membrane and increase in the amplitude and the duration of its action potential which could be attributed to activation of ionic pumps. It could be a case that cupric ions affect different ATPase component than penicillamine as it was demonstrated in pigeon's brain preparations (Cu^{++} sensitive and stimulated by penicillamine), therefore Cu^{++} and penicillamine acting at two different sites. However, since penicillamine is known to increase urinary secretion of copper in Wilson's disease patients (Friberg et al., 1979; Sigel, 1981) its mobilizing effect on Cu^{++} must be considered. Penicillamine could produce recovery by partial removal of excess copper from the tissue and by activation of ionic pumps previously inhibited by metal ions. Possibility of direct interaction of Cu^{++} and penicillamine

(reduction and increase in permeability of cardiac membrane, respectively) with ionic channels could not be ruled out at this point.

CHAPTER 5 CONCLUSION

Results obtained using the techniques of current and voltage clamp have documented the effects of the cupric ions and penicillamine on the electrical properties of frog atrial fibers. In the presence of cupric ions the amplitude and the duration of the action potential were reduced and depolarization of the resting potential occurred. However, at low Cu^{++} concentration (< 30 μM) a transient increase of the duration of the action potential was observed.

Present findings were consistent with the theory that Cu^{++} produces metabolic inhibition. Cu^{++} ions seemed to block active ionic transport (by inhibition of ionic pumps). This would affect the ionic concentration gradient across the membrane, decreasing the driving force of passive ion movements. As a result of accumulation of Na^{+} within a cell, the resting potential became depolarized, and due to decreased driving force for sodium ions, the fast inward current became reduced, manifesting itself in a decrease in the peak amplitude of the action potential.

Increased intracellular Na^{+} concentration also caused accumulation of Ca^{++} within the cell. This again would change the driving force for calcium ion movement, thus decreasing the slow inward current (producing loss of a plateau). The increased outward current observed during Cu^{++} treatment could be attributed to Ca^{++}-activated membrane permeability to potassium ions.

Another possible way of Cu^{++} interaction with the cell membrane was a change in membrane permeabilities (inhibition of ionic channels). Blockage of sodium and slow channels would also produce a decrease in the amplitude and the duration of the action potential.

However, inhibition of ionic pumps seemed to be more likely (depolarization of the resting potential together with increase of the delayed rectifier), but inhibition of ionic channels was a possibility which should be further investigated.

Penicillamine at 0.3 mM concentration was found to be capable of reversing the toxic effect of 40 μM Cu^{++}, on the frog atrial fibers. It was suggested that penicillamine reactivates ionic pumps previously suppressed by cupric ions, and maybe also increases membrane permeability to different ions. Concentration of the penicillamine which produces a recovery of the electrical activity in the frog atrial fiber was critical.

In future study, it would be suggested to use patch-clamp experiments to evaluate more accurately the role of Cu^{++} and penicillamine in altering the electrical activity of the cardiac membrane. The voltage clamp experiments with penicillamine alone should be done in order to evaluate its effect on transmembrane ionic currents. Also some known ionic pump inhibitors and selective channel blockers could be used in combination with penicillamine to establish the nature of its interaction with excitable membrane. The calcium channels blockers as manganese or cobalt could be used together with Cu^{++} to pin down its role on atrial tissue. Another possibility would be to replace Cu^{++} in normal Ringer's solution by other divalent cations such as strontium and barium which are also accepted by slow channels or to vary external concentrations of sodium, calcium and potassium ions and then study the effect of Cu^{++} and penicillamine on atrial fibers.

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