THE EFFECT OF PHARMACOLOGICAL AGENTS ON *NITELLA* CELLS

by

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ABSTRACT

The electrical properties of the cell membrane of Nitella flexilis were investigated. Using a specially designed experimental system the movement of tracers from or into the cell was directly correlated with the electrical activity of the preparation. A new type of micromanipulator was designed so that electrical measurements could be made while the cell was in close contact with a radiation detector. The design and application of the micromanipulator in electrophysiological research are discussed.

Several pharmacological agents were shown to prolong the duration of the spike (action potential). In many cases their effect is identical to that observed in the excitable nerve and muscle cells of animals.

Three categories of agents that prolong the spike were found; a, Ions resembling Ca^{++} in size; b, Ca^{++} chelators; c, phosphorylation uncouplers.

The mode of action of each category appears to be different but all appear to affect cell bound Ca^{++}. This conclusion is supported directly by experiments on ^{45}Ca efflux and ^{131}Ba influx.

A common molecular mechanism is proposed to explain the action of these pharmacological agents on excitable plant and animal cells.

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GENERAL INTRODUCTION

The large internodal cells of the freshwater algae Nitella and Chara have many advantages over other cells for electrophysiological investigations. Their exceptional size and geometry, their ease of dissection, their relatively simple ionic requirements, and their ability to withstand experimental conditions for days and even weeks are some of the properties which are uncommon in other excitable cells.

Internodal cells of Nitella are basically hollow cylinders separated from the environment by a cell membrane or plasmalemma closely opposed to a cellulose cell wall some two micrometres (μ) in thickness. (Hough et al 1952)* Together with the osmotic pressure the cell wall gives rigidity to the cell (Diamond and Solomon, 1959). Solutes and solvents from the external solution can readily penetrate the cell wall into the so called free space (Diamond and Solomon, 1959). The protoplasm or cytoplasm fills the space between the plasmalemma and the tonoplast or vacuolar membrane which separates the sap of the large central vacuole from the cytoplasm. Cytoplasmic streaming occurs in a very regular fashion around the vacuole and appears to follow a helical pattern, similar to the arrangement of the chloroplasts on the inside of the plasmalemma.

These cells can be stimulated to produce a spike which spreads along the cell and neighbouring cells. During the impulse, cytoplasmic streaming is temporarily halted (Barry, 1968). Otherwise no known effector is associated with excitation (Mackie 1970).

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* references start at page 26

As early as 1938 the similarities of the electrical behavior of Nitella cells with animal cells was recognized (Osterhout 1938). Pacemaker activity, arrythmia and block in Nitella appeared to be similar to the behavior of thevertebrate heart under certain conditions (Osterhout 1938). Hyperexcitability analogous to fibrillary tremors in muscle cells has also been observed in Nitella (Osterhout 1942).

Hodgkin (1957) using voltage clamp data, successfuly related ionic movements to the electrical transients observed during the spike in nerve cells. It was felt that this ionic theory could be extended to all excitable systems. The generality of Hodgkin's ionic theory has, however, been questioned (see Grundfest, 1966) but, as Grundfest points out: "Most critics of the ionic theory are concerned only with a narrow range of phenomena". The complex electrical phenomena of highly specialized cells could therefore obscure the basic mechanism which the ionic theory attempts to explain.

The similarities between the electrical behavior of Nitella cells and animal cells, as observed by Osterhout (1938, 1942), could point to a very basic mechanism controlling the spike in all excitable membranes. It has been established that the spike in Nitella cells is brought about by depolarizing Cl^- activation (Mailman and Mullins 1966) and a repolarizing K^+ activation (Gaffey and Mullins 1958).

The following specific problems were identified:

1. What are the effects on Nitella of pharmacological agents that are thought to interfere with K^+ activation in other excitable cells?
2. Are the agents that prolong the spike of Nitella interfering with or replacing Ca^{++} on the excitable membrane?

3. Are any of these processes energy-dependent, and can spikes be prolonged by agents that block the production of ATP?
4. Can any evidence for the mode of action of these drugs, or for the replacement or removal of Ca^{++} from the excitable membrane be obtained from the behaviour of Nitella cells loaded with radioactive Ca^{++} and Ba^{++}.
5. The internodal cells of Nitella are large and their electrical activity is about 1000 times slower than that commonly studied in animal nerve and muscle cells. The final question is concerned with the problem of whether or not the basic mechanism that regulates depolarizing and repolarizing electrogenesis is common to these tissues. A simple model for these processes is postulated and discussed critically.

These problems are introduced in more detail in their respective Chapters.

CHAPTER I

The effects of pharmacological agents on the electrical responses of cells of *Nitella flexilis*

CHAPTER I

INTRODUCTION

Cells of freshwater algae of the Stonewort group, which include the genera Chara and Nitella, are electrically excitable and can conduct spikes similar in many features to those recorded from animal nerve and muscle cells.

Their environment is entirely different from that of nerve and muscle cells however, as it contains only a few millimoles of ions even in "hard" water. To reconcile this situation with the generally accepted ionic theory of bioelectric potentials, several groups of workers have investigated the electrochemistry of these giant plant cells over the last decade.

The most detailed analysis appears to be that by Kishimoto (1964, 1965) (summarised and simplified in Table I) using a Nitella species. Results are necessarily more complicated than those obtained from animal cells owing to the vacuole inside the cell which is surrounded by a second plasma membrane, the tonoplast, concentric with the outer plasmalemma, and to the negatively-charged cell wall outside the plasmalemma, which evidently acts as a cation exchanger (Dainty and Hope 1959).

Kishimoto's figures are in reasonable agreement both with chemical analysis carried out previously and with electrical measurements carried out by others (Spanswick and Williams 1965, Findlay and Hope, 1964, Williams and Bradley, 1968).

Table 1. Ionic concentrations (millimolar) of the artificial pond water (outside) and of the cytoplasm and vacuolar sap in the Nitella species used by Kishimoto (1964, 1965). His calculations for the vacuole/outside equilibrium potentials are included in the right hand column, his measurements of the potentials of the two intracellular phases in the bottom row. The cytoplasmic concentration of Ca^{++} is taken from an analysis of Nitella translucens.

	Plasmalemma		Tonoplast	
	Outside	Cytoplasm	Vacuole	Equilibrium potential $E_{ion}\frac{out}{vac}$
Na^+	0.2	13	50	-140 mV
K^+	0.08	119	100	-180 mV
Ca^{++}	0.5	8*	10	- 38 mV
Cl^-	1.2	82	100	+111 mV
Potential	0 (Wall-50 mV)	-170 mV	-160 mV	

* Spanswick and Williams (1965).

There is reasonably direct evidence that both the steady internal negativity and the spikes that can be recorded from these plant cells are for the most part set up across the outer plasmalemma (Findlay and Hope 1964). The potential differences measured between the vacuole and the outside medium are thus often called membrane potentials; a usage I shall follow.

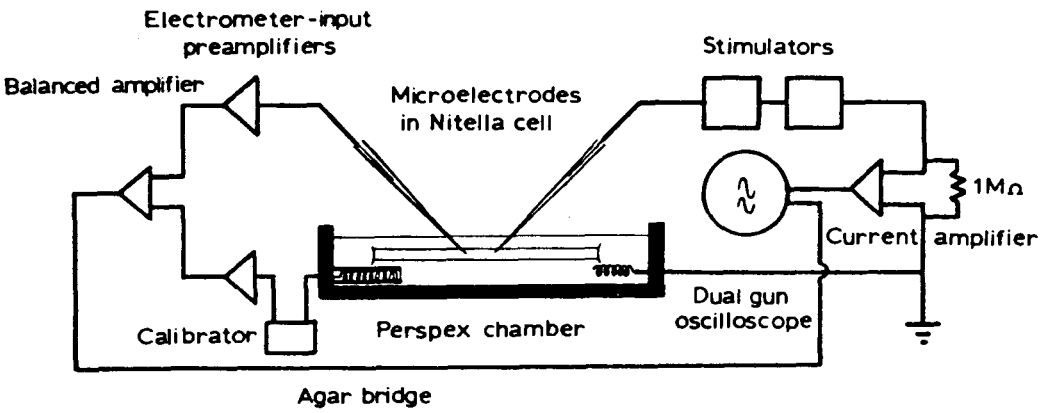
The weight of available evidence supports the hypothesis that depolarising electrogenesis is brought about mainly by Cl^- activation (Mailman and Mullins 1965) and a resultant efflux of this ion, although Ca^{++} may flow inward at the same time. Repolarising electrogenesis is thought to occur during the falling phase of the spike when an efflux of K^+ returns the membrane potential to its resting level (Gaffey and Mullins 1958).

In a recent review, Grundfest (1966) pointed out that many ionic activation and inactivation processes can be affected specifically by pharmacological agents, and as few of these appear to have been tested on plant cells, the following experiments were carried out to investigate their effects on cells of Nitella.

METHODS

The cells were obtained from a local biological supplier and kept in artificial pond water (APW) with the following composition (millimolar, mM) NaCl 1.0; $CaCl_2$ 1.0; KCl 0.10 and $MgCl_2$ 0.10, for a few days before experiments. Single internodal cells were isolated in fresh APW some hours before they were placed in a small bath made

Figure 1 Schematic diagram of the recording and stimulating arrangements. Metallic electrodes in the bath and in the glass electrodes are silver - silver chloride. The balanced amplifier and current amplifier are within the oscilloscope.


Electrometer-input
preamplifiers
Balanced amplifier
Microelectrodes
in Nitella cell
Stimulators
1MΩ
Current amplifier
Dual gun
oscilloscope
Calibrator
Perspex chamber
Agar bridge

from 'Perspex'.

The recording and stimulating arrangements are shown in Fig. 1. Intracellular glass capillary electrodes with tips about 10 μ in diameter filled with 150 mM KCl were inserted into the vacuole. One was used to record the potential difference while the other was used to pass current for stimulation and to apply hyperpolarising pulses, 100 milliseconds (msec) in duration, at 500 msec intervals.

At a particular setting of hyperpolarising current the amplitude of the voltage pulses is proportioned to the effective resistance between the vacuole and external fluid. This current was held constant by passing it through a 60 megohm resistor in series with the electrode. Potential and current traces were viewed on a Tektronix 565 oscilloscope, recorded on a Precision Instruments 6200 tape recorder and replayed at slower speed into a Riken Denshi SFH4 2 channel potentiometric recorder. When the cells were treated with a drug, spikes were elicited after short-term changes in membrane potential were completed. This was usually within 2 minutes.

All solutions were made up in APW and 10 ml of each was rinsed slowly over the cell and their effects investigated over a period of 15-30 min. The volume of the bath was less than 1 ml so that solutions were almost completely changed during this process. Each experiment was repeated on at least 3 different cells and the results were in all cases consistent. Changes were observed in membrane potentials under changing light conditions and therefore the ambient illumination was maintained constant at about 5 lux.

Figure 2 Normal responses

a, and d. Spikes evoked by prolonged (0.5 sec) depolarising pulses at the threshold for excitation.

b, Evoked by brief (5 msec) depolarising pulses at the threshold for excitation.

c, Depolarisation and a break response following 500 msec hyperpolarising pulse.

Vertical calibrations shown in d also apply to a, b, and c.

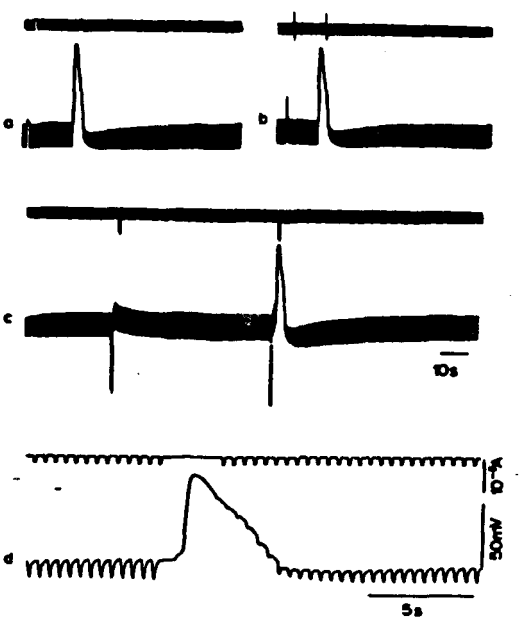
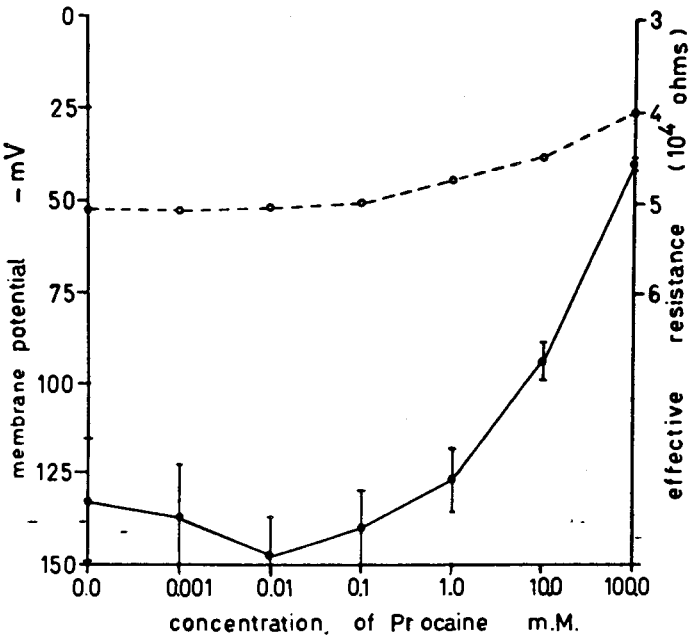

a
b
c
10s
d
10 nA
50mV
5s

Figure 3 Effect of different concentrations of procaine on Nitella cells. Data from three cells with comparable membrane potentials have been plotted. The standard error is shown on the potential curve (●). The standard error for the resistance did not exceed the amplitude of the experimental points (o).


0
25
50
75
100
125
150
membrane potential - mV
3
4
5
6
effective resistance (10^4 ohms)
0.0
0.001
0.01
0.1
1.0
10.0
100.0
concentration of Procaine m.M.

RESULTS

Normal spikes

For a particular cell, consistently reproducible spikes could be elicited using brief (5 msec) high voltage or long-lasting (up to 500 msec) depolarising pulses at the threshold for excitation. Responses could also be elicited at the "break" of large hyperpolarising current pulses. In all cases the spikes were similar (Fig. 2). In most cells the membrane potentials ranged between -75 and -140 mV and spikes between 60 and 100 mV. The spikes under these conditions did not overshoot the zero potential and were followed by a long-lasting (30 sec) after-hyperpolarisation during which the effective resistance of the cell was reduced. Hyperpolarising pulses just below threshold for break responses evoke a long lasting depolarisation with very little change in resistance. The effective resistance of the membrane during the spike was reduced by a factor of about ten initially but increased gradually during the falling phase (Fig. 2d).

Procaine

The effect of different concentrations of procaine hydrochloride on the membrane potential and effective resistance of the cell is shown in Fig. 3. Concentrations of procaine between 0.01 and 0.1 mM hyperpolarised the cells. Concentrations greater than 1 mM decreased the steady membrane potential but the duration and the potential reached at the peak of the spike changed little. The effective resistance decreased with increasing concentrations of procaine. The duration of

Figure 4 Effect of procaine.

a, a'. Normal spikes recorded at two different speeds in APW.

b, b'. Spikes recorded from the same cell with 50 mM procaine added.

c, c'. Spikes recorded from the same cell after washing with APW for 30 minutes.

a, b', c and a', b, c', same time scale, vertical calibration applies to all traces.

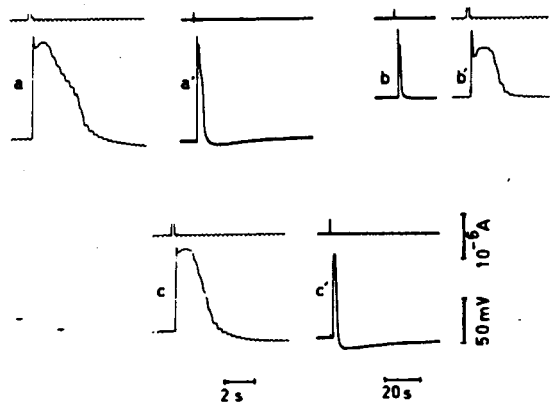

a
a'
b
b'
c
c'
10^{-6}A
50 mV
2 s
20 s

Figure 5 Effect of different concentrations of TEA on Nitella cells. Data from 3 cells with comparable membrane potentials have been plotted. The standard error is shown on the membrane potential curve (●). The standard error for the resistance (o) was smaller than the diameter of the open circles.

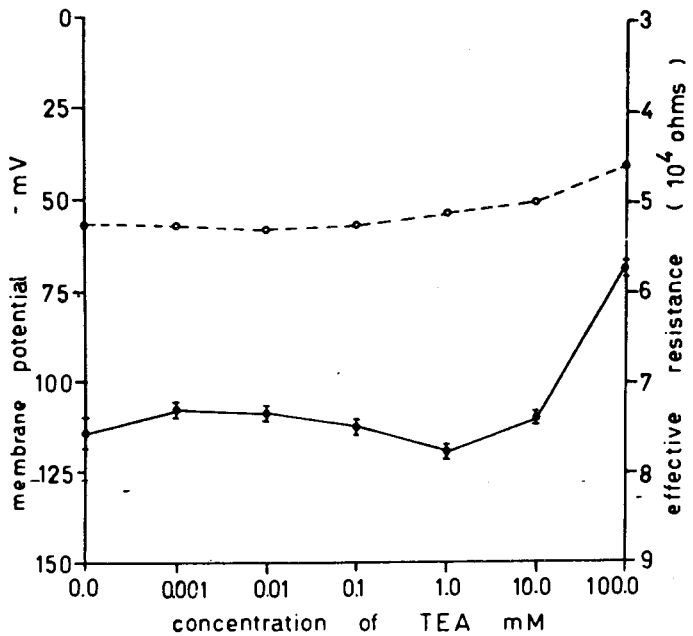

0
25
50
75
100
125
150
- mV
membrane potential
3
4
5
6
7
8
9
effective resistance (10^4 ohms)
0.0
0.001
0.01
0.1
1.0
10.0
100.0
concentration of TEA mM

Figure 6 Effect of TEA.

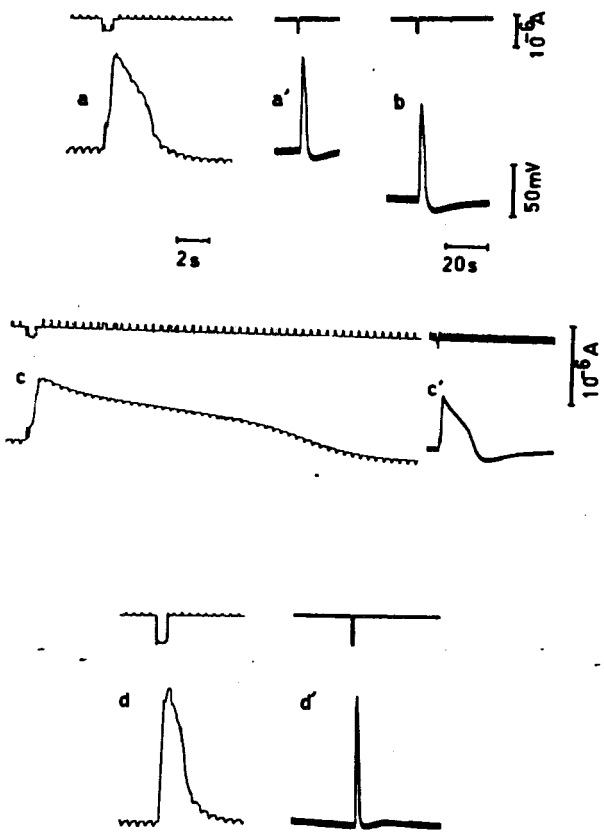
a, a'. Normal spikes recorded at two different speeds in APW.

b, Spikes recorded from the same cell after 2 min in 1 mM TEA in APW.

c, c'. Spikes recorded from the same cell after 2 min in 10 mM TEA in APW.

d, d'. From the same cell after 5 min in APW.

a, c, d, and a', b, c', d'. Same time and voltage calibrations. Current scale is the same in a, a', b, and d, d'.


a
a'
b
10^{-6}A
50mV
2s
20s
c
c'
10^{-6}A
d
d'

the spike changed little whereas after-hyperpolarisation following it disappeared completely in 50 mM procaine (Fig. 4b). After 5 min continuous washing with APW the after-hyperpolarisation reappeared (Fig. 4 c, c'). After a 30 minute wash period the effects of procaine were almost entirely reversed.

Tetraethylammonium (TEA)

The relationship between the concentration of TEA bromide and the potential and effective resistance of the membrane is complex as shown in Fig. 5. Above 1 mM concentration the spikes were reduced in amplitude but prolonged; in a typical cell in 10 mM TEA the amplitude was reduced from 90 to 50 mV whereas the duration increased from 4 to 20 sec (Fig. 6 c, c'). The after-hyperpolarisation never disappeared but increased in duration when associated with prolonged spikes (Fig. 6 c'). Continuous washing with APW reversed the effect of TEA on the duration of the spike within minutes (Fig. 6 d, d'). The membrane potential was left in a slightly hyperpolarised state which could not be brought back to normal even after a 2 hr washing period. During the experiment the peaks of the spikes did not remain at the same level, but became slightly more negative as shown in Fig. 6 d, d'.

Barium

The effect of different concentrations of $BaCl_2$ on membrane potential and effective resistance is shown in Fig. 7. Both membrane potential and effective resistance decreased with an increase in concentration. At

Figure 7 Effect of different concentration of Ba^{++} on *Nitella* cells. Data from 3 cells with comparable membrane potentials have been plotted. The standard error is shown on the membrane potential trace (•). The standard error for the membrane resistance (o) was smaller than the diameter of the open circles.

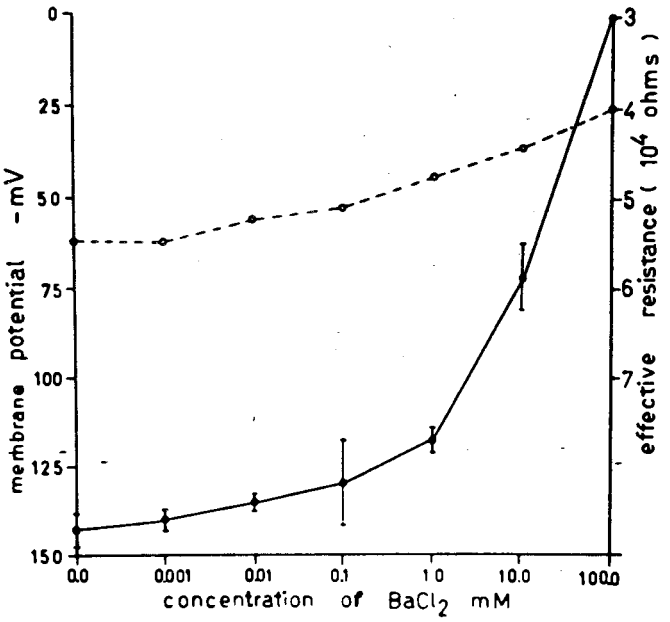

membrane potential -mV
0
25
50
75
100
125
150
effective resistance (10^4 ohms)
3
4
5
6
7
0.0
0.001
0.01
0.1
1.0
10.0
100.0
concentration of $BaCl_2$ mM

Figure 8 Effect of Ba^{++}

a, a'. Normal spikes recorded in APW.

b, b', b'', b'''. After 2 min in 1 mM Ba in APW.

c, After 1 hr in 1 mM Ba in APW. Cell was stimulated by pulses of increasing amplitude immediately following a prolonged spike. Initially the responses are brief but increase slightly in duration until another prolonged spike is elicited after a refractory period.

Time scale under c refers also to a, b, b' and b'', and that under b'' refers also to a'. Vertical calibration applies to all figures.

In this and the next figure the current traces are displaced to the right relative to voltage traces owing to misalignment of the pens inherent in this type of recorder.

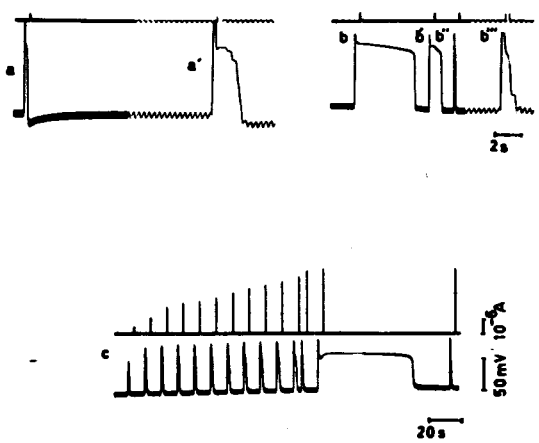
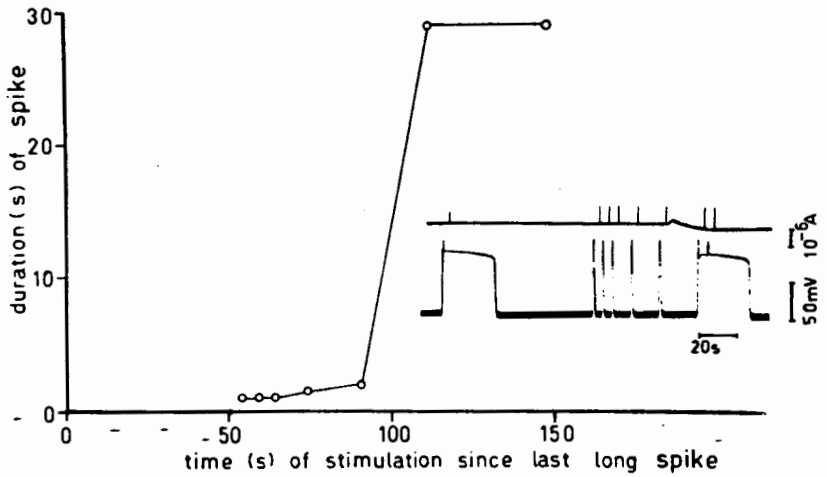

a
a'
b
b'
b''
b'''
2s
c
10pA
50mV
20s

Figure 9 Effect of a constant stimulus at different time intervals since the last prolonged spike in a cell, soaked for 30 min in 1 mM Ba in APW. A slight increase in duration can be observed between the prolonged spikes. Movement of the current trace after the sixth stimulus is an artefact not related to the experiment.


30
20
10
0
duration (s) of spike
0
50
100
150
time (s) of stimulation since last long spike
10^{-6}A
50mV
20s

concentrations greater than 0.1 mM, prolonged spikes first started to appear. Higher concentrations resulted in spikes of even greater duration, some lasting up to 1 1/2 min with 1 mM and showing a small peak just before the plateau (Fig. 8 c). In contrast to the effect of TEA, the amplitude of the spike changed very little and furthermore the after-hyperpolarisation following the spike was abolished in 1 mM Ba^{++}. These prolonged spikes also differed from those obtained in TEA in that the plateau was almost horizontal (a slope of about 0.5 mV/sec) and the falling phase after the plateau much more rapid. Prolonged spikes were reproducible at a particular concentration, even when they were occasionally generated spontaneously. Immediately after a prolonged spike only brief ones could be elicited. A constant relative refractory period, in the order of minutes, was observed during which no prolonged spike could be generated (Fig. 8 b'', b''', Fig. 9). The brief spikes that could be evoked during this relative refractory period were graded over several second's duration depending both on the stimulus interval and on its intensity (Figs. 8 c, 9). The effect of Ba^{++} was never reversible even after extensive washing with APW.

DISCUSSION

The electrical responses are similar to those recorded by others from Nitella and the corticated alga, Chara (Oda 1956; Findlay and Hope 1964; Kishimoto 1965). Membrane potentials and the amplitude and duration of the spikes are within the range reported by others in Nitella

species using intracellular electrodes. The large graded depolarisations following the spike that Gaffey and Mullins (1958) evoked by using extracellular electrodes were not seen with intracellular stimulation. On the contrary a phase of hyperpolarisation and reduced membrane potential was observed following spikes that was constant for a particular cell whatever the amplitude or polarity of the intracellular stimuli. The after-hyperpolarisation could be caused by activation of an ion or ions with equilibrium potentials near or below the resting level, and such activation could also explain the after-depolarisation seen in Chara (Oda 1956) and some Nitella species (Findlay 1959) if the equilibrium potential were more positive than the resting level. Outward diffusion of KCl through the cation perselective cell wall (Mailman and Mullins 1966) could not explain an after-hyperpolarisation.

Procaine has been shown to reduce both Na^+ and K^+ activation in squid axons and at 5 mM will block the spike of squid axons completely (Taylor 1959; Shanes et al. 1959). In arthropod muscle fibers, where depolarising activation is much less specific, 50 mM apparently delays K^+ activation, converting normally graded responses into all-or-none spikes and often prolonging them (Takeda 1967). In eel electroplaques as well as in frog muscle and nerve cells, the effect of procaine seems to be only on Na^+ activation (Nakamura et al. 1965; Kuperman 1968). The efflux of K^+ during repolarising electrogenesis evidently carries the membrane potential close to the equilibrium potential for this ion, which in our cells is evidently more negative than the resting potential. The effect of 50 mM procaine in abolishing this after-hyperpolarisation in

Nitella could therefore be due to a reduction of K^+ permeability. This explanation may appear to be inconsistent with the effect of procaine in reducing membrane resistance, but as both this drug and TEA have different effects at different concentrations they must be able to exert opposite actions. These actions probably occur independently on the mechanisms responsible for generating the steady membrane potential and the spike.

TEA evidently reduces K^+ permeability and also delays its activation in axons, neurons and in muscle fibers of the lobster (Werman and Grundfest 1961) whereas in insect muscles at a concentration of 1 mM or more membrane resistance is reduced and the cells are depolarised (Belton and Grundfest 1961; Wood 1958). At low concentrations, TEA depolarises Nitella cells but increases their effective resistance. At 1 mM these effects are reversed and the cells hyperpolarise with some loss of resistance. Prolonged spikes can be evoked in concentrations greater than 1 mM although both the steady membrane potential and effective resistance are reduced. Investigations of the neurons of the Mollusk Onchidium using a voltage clamp technique show that K^+ activation is blocked by TEA without an obvious change in the resting resistance of the cell (Hagiwara and Saito 1959). The action of TEA in lowering the effective resistance of the membrane in Nitella could like that of procaine be independent of its effect in prolonging the spike by reducing or delaying K^+ activation.

Barium increases the membrane resistance, prolongs, and often increases the amplitude of spikes of a wide variety of excitable cells,

these effects being more obvious if Cl^- is removed from the medium of cells like frog fast muscle fibers, which have a high resting Cl^- permeability (Sperelakis et al. 1967). Even though the effective resistance decreases in Nitella cells, the effect of Ba^{++} on the spike of Nitella is almost indistinguishable from its effect on muscle fibers of the lobster (Werman and Grundfest 1961) or of insects (Belton and Grundfest 1961). The amplitude of the spike in Nitella is, however, reduced by 10 mM Ba^{++}, unlike the situation in many arthropods where Ca^{++} Sr^{++} or Ba^{++} can enter muscle fibers during spikes (Fatt and Ginsborg 1959).

There is some indication that the rate of repolarisation during the plateau of prolonged spikes is higher in TEA than in Ba^{++} solutions in lobster muscle fibers (Werman and Grundfest 1961). In Nitella cells this is certainly the case, the rate of repolarisation being fourfold higher in TEA spikes than in Ba^{++} spikes of the same duration. Evidently these compounds have different effects on the repolarising process although both appear to delay it. Ba^{++} and TEA are also different in their effects on steady membrane potential and resistance and in general the effect of TEA is more complex than that of Ba^{++}.

The refractoriness of the prolonged spikes (Fig. 9) indicates that the changes giving rise to the plateau can be reversed by electrical, ionic or time-dependent processes going on during the plateau itself. Normal spikes elicited during the refractory period for prolonged spikes do not prevent Ba^{++} ions from re-asserting their effect after this period.

The pH of the medium is known to have effects on resting potential

(Kitasato 1968) but the pH of all the solutions used fell within the range of 6.5 -6.8 and even if the membrane behaved as a perfect H^+ electrode this would represent a change of only 19 mV. Changes of greater magnitude such as are observed with procaine, TEA and Ba^{++} must therefore be due to more than a change of pH.

CONCLUSIONS

Pharmacological agents that interfere with the K^+ activation process associated with repolarising electrogenesis in animal cells have almost identical effects in Nitella cells.

Ions such as Ba^{++} and TEA^+ that can flow into the muscle fibers of arthropods and give rise to spikes evidently cannot do so in Nitella cells.

The ionic mechanism for returning the membrane potential to its "resting" level in Nitella cells is evidently one of K^+ activation, as in all animal cells except a few electroplaques. Moreover, the similarity of effects of pharmacological agents that influence this process both in animal cells and in Nitella provides compelling evidence that the molecular events that cause K^+ activation are almost identical despite the relatively great chemical and anatomical differences between plant and animal cells.

Most of the effects investigated took place within seconds and it is felt that they can confidently be ascribed to changes in the outer plasma (cell) membrane. The possibility cannot be ruled out that during some

experiments the inner plasma (tonoplast) membrane was also affected. Further experiments are needed to investigate this possibility.

CHAPTER 1

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CHAPTER II

The effect of Calcium deficiency on the electrical activity of *Nitella flexilis*

CHAPTER II

INTRODUCTION

The effect of Ca^{++} deficiency on the electrical responses of single cells has been investigated in many organisms. Squid axons as well as amphibian nerve cells fire spontaneously in Ca^{++} deficient saline (Frankenhauser and Hodgkin 1957, Huxley 1959, and Kuperman, et al. 1967). Crayfish muscle fibers show spike like oscillation in the presence of 1.0 mM EDTA (Reuben 1967) and Nitella cells generate trains of repetitive spikes in the presence of 1 mM EDTA or 2 mM ATP (Kishimoto 1966). Of these, crayfish muscle fibers and Nitella cells show an increase in duration of the spike as well as repetitive activity. In Nitella cells, Kishimoto obtained prolonged responses when he changed the membrane potential. The shape of the spike obtained in this fashion was very similar to the responses recorded by Belton and van Netten (1971) in the presence of 1 mM Ba Cl_2. Kishimoto (1966) does not discuss this prolongation. It is my objective to clarify the effect of the absence of Ca^{++} on the electrical responses, especially spike duration, in the cells of Nitella flexilis.

METHODS

Single internodal cells of Nitella flexilis were isolated and kept in artificial pond water (APW) several days prior to the experiment. (Composition in mM, NaCl 1.0, $CaCl_2$ 1.0, KCl 0.10, and $MgCl_2$ 0.10).

Figure 1 Effects of different concentrations of EDTA on *Nitella* cells. Data from three cells have been plotted. The standard error is shown on the potential curve (●) and the resistance curve (o).

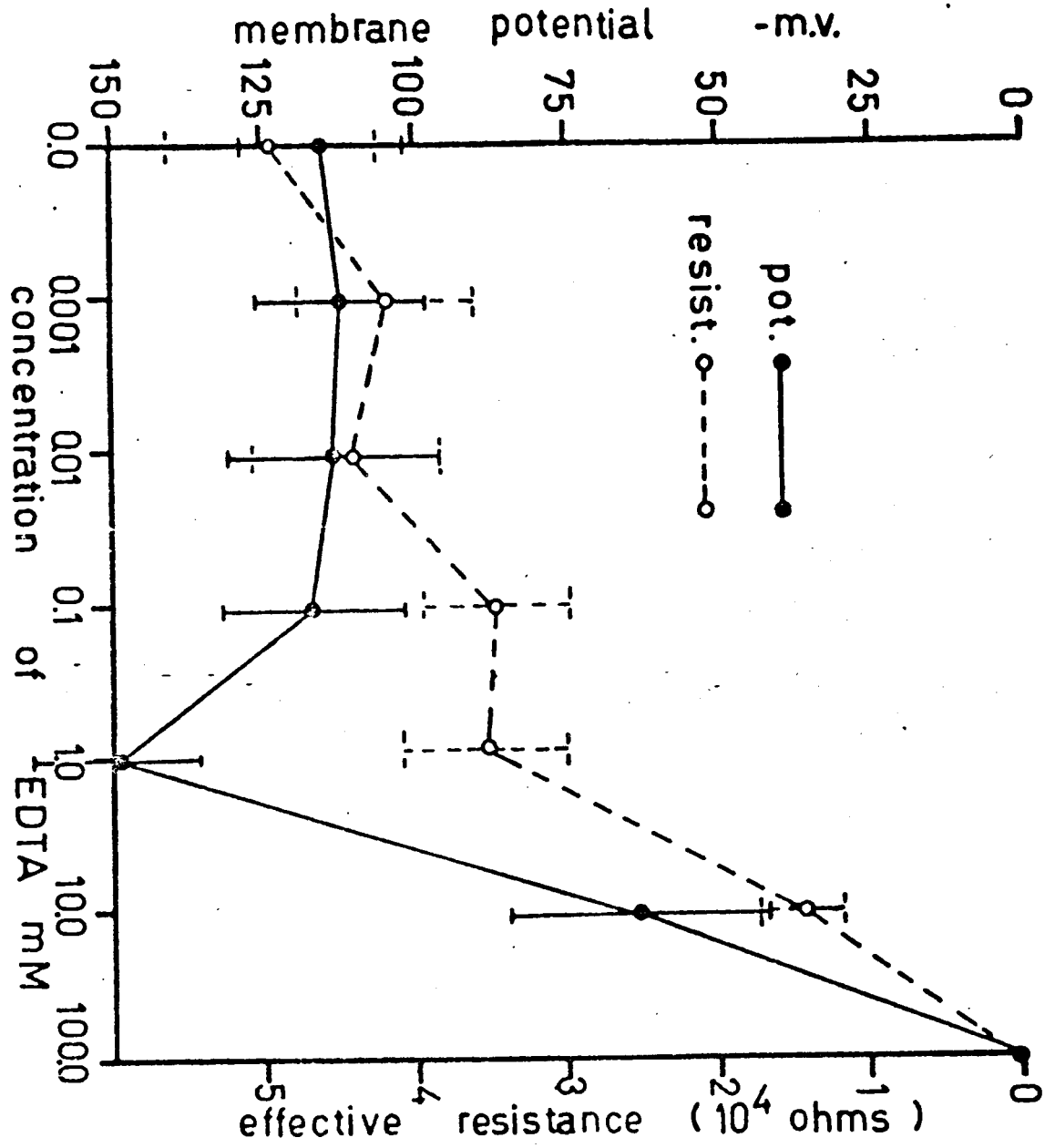
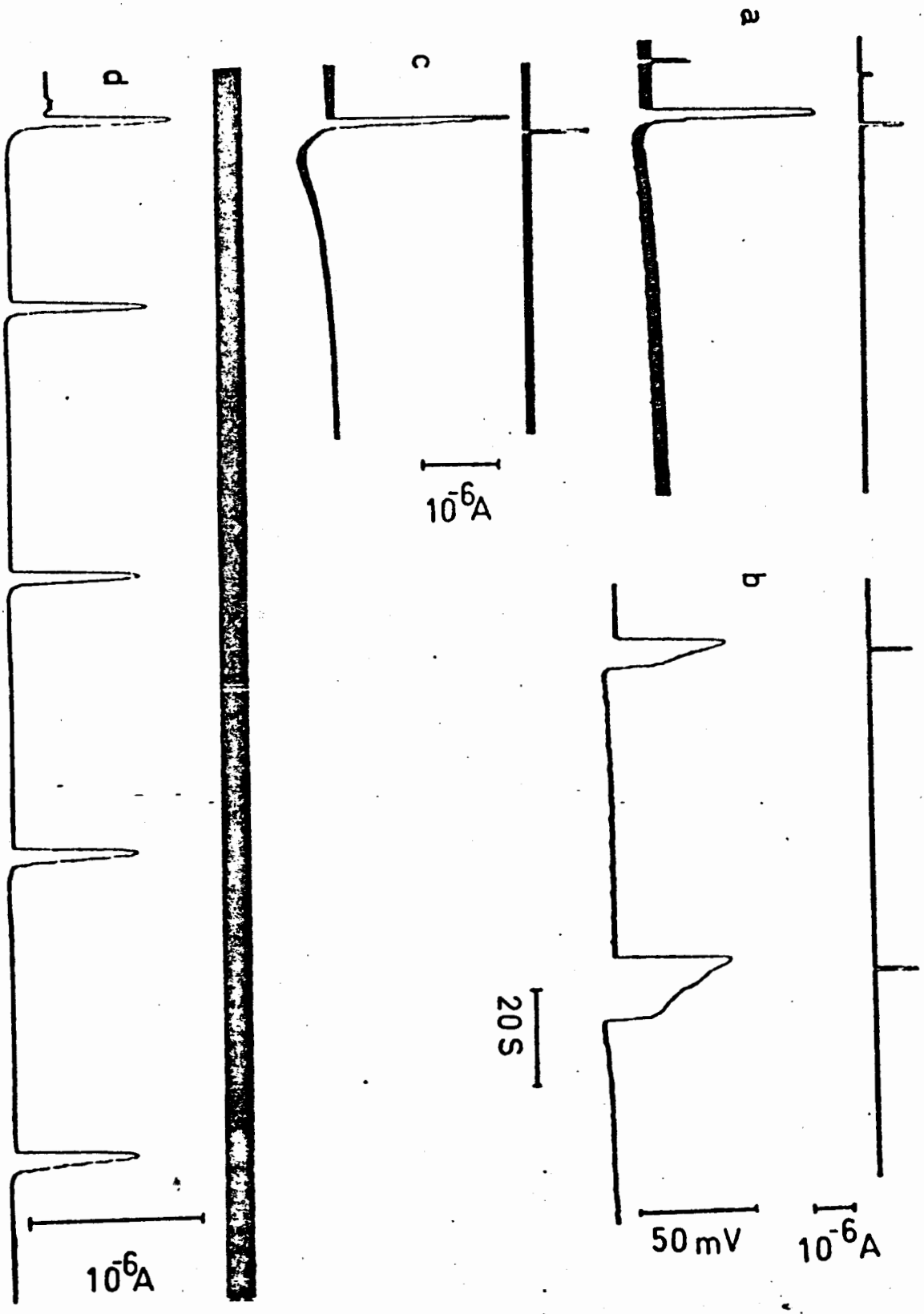

membrane potential -m.v.
150
125
100
75
50
25
0
0.0
0.0001
0.001
0.1
1.0
10.0
1000
concentration of EDTA mM
resist.
pot.
effective resistance (10^4 ohms)
5
4
3
2
1
0

Figure 2 Effects of EDTA. (a) normal spike, (b) spikes recorded from the same cell after 3 min in a 1 mM EDTA solution in APW, (c) normal spike of another cell, (d) repetitive spikes of increasing duration recorded directly after addition of 1 mM EDTA. a,b,c,d, same time and voltage calibrations. a,b, same current-trace calibrations, c,d, current trace calibration as shown. In this and similar figures the upper (current) trace represents zero potential and owing to the characteristics of the recorder is displaced to the right relative to the voltage trace.


a
b
c
d
20 s
50 mV
10^{-6}A
10^{-6}A
10^{-6}A

Intracellular glass electrodes were used to record potentials and to pass current. Effective membrane resistance was calculated as described in Chapter I. The TRIS chloride and propionate solutions were prepared by titrating a 2.25 mM solution of TRIS hydroxide in distilled water with 2 M HCl and 2 M Propionic acid respectively, with neutral red as an indicator.

For the washing out experiment, TRIS solutions were allowed to flow past the cell at a steady rate of 3 mls per min.

RESULTS

Ethylene diamine tetraacetic acid, Sodium salt (EDTA)

The effects of different concentration of EDTA on the membrane potential and effective resistance of the cells are shown in Fig. 1. Concentrations up to 0.1 mM decreased the membrane potential and its effective resistance slightly; concentration between 0.1 mM and 1 mM hyperpolarized the cell membrane while decreasing the effective resistance still further. The shape of the spike and the excitability of the membrane remained unaltered with the addition of up to 1 mM EDTA, concentration greater than 1 mM produced a few spontaneous spikes (Fig. 2, Fig. 2c is a normal response) and increased their duration from 3 (Fig. 2a) to 13 sec. (Fig. 2b). The absolute potential of the peak of the spikes did not remain constant during the experiment. Concentrations larger than 10 mM EDTA blocked the spike and reduced both the effective resistance and membrane potential to zero at the higher concentrations. At this

Figure 3. Effects of different concentrations of Na Citrate on Nitella cells. Data from three cells have been plotted. The standard error is shown on the potential curve (●) and the resistance curve (o).

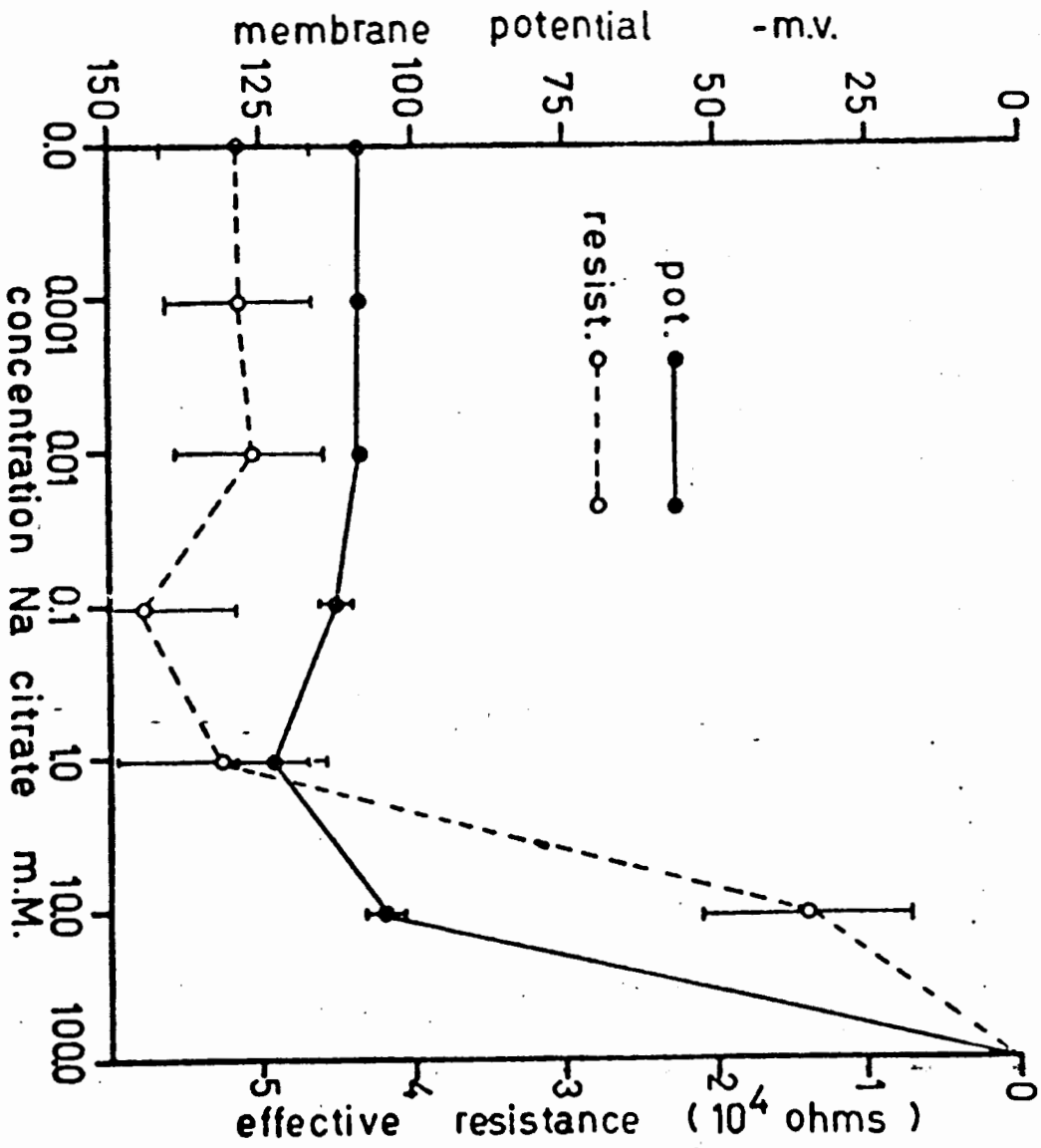
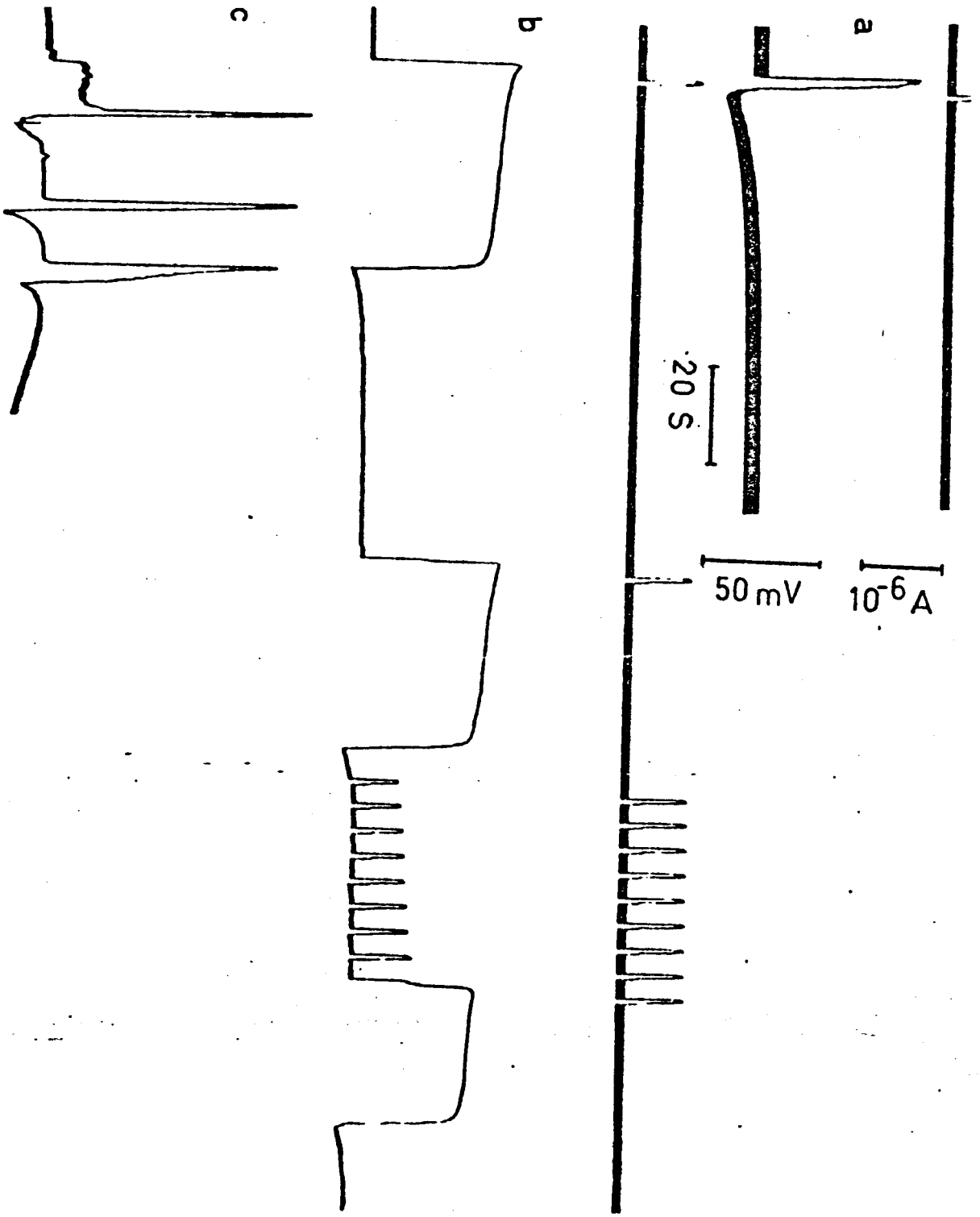

membrane potential -m.v.
150
125
100
75
50
25
0
resist.
pot.
0.0
0.001
0.01
0.1
1.0
10.0
100.0
1000
concentration Na citrate m.M.
5
4
3
2
1
0
effective resistance (10^4 ohms)

Figure 4 Effects of Na Cit. (a) normal spike in APW, (b) prolonged spikes recorded after 3 min in 10 mM Na Cit. in APW. The effect of a constant depolarization increases probably as a result of an increase in effective membrane resistance until the threshold for the spike is reached (relative refractory period) (c) the effect of 10 mM Na Cit. on a cell not previously exposed to lower concentrations. a,b,c, same time voltage calibrations. a,b, same current calibrations.


c
b
a
20 s
50 mV
10^{-6} A

point the cell collapsed physically. The effect of EDTA was readily reversible with APW when different concentrations up to 1 mM EDTA were used, i.e. before prolonged spikes were obtained.

Sodium Citrate (Na Cit.)

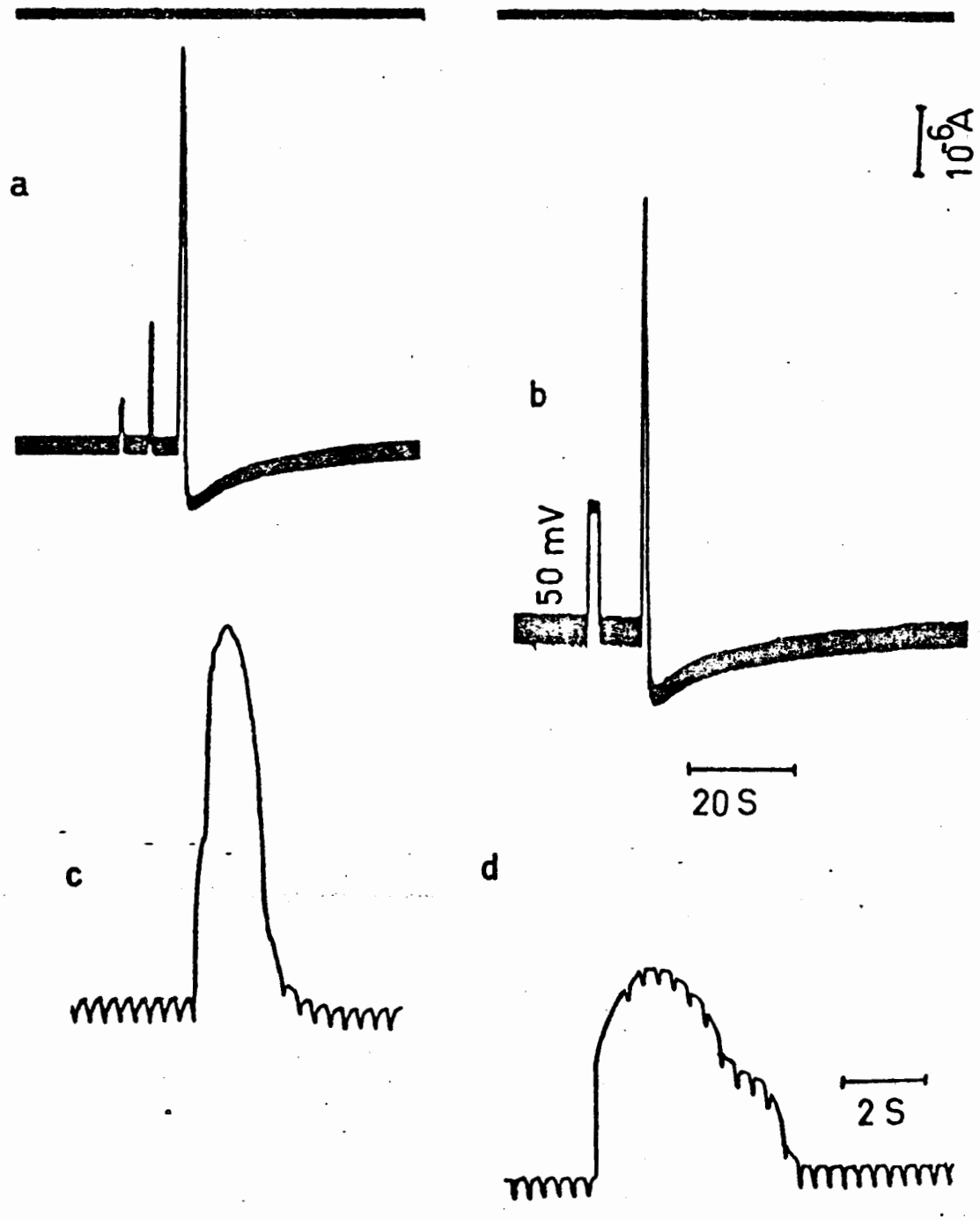
The effects of increasing concentrations of Na Cit. on the potential and effective resistance are shown in Fig. 3. Both the effective resistances and membrane potential increased when concentrations up to 1 mM Na Cit. were applied. Higher concentrations reduced both these parameters below their normal resting levels. At 10 mM Na Cit. spikes of increased duration were obtained (4 sec. Fig. 4a to 40 sec. Fig. 4b). Repetitive activity was also observed but was more pronounced when 10 mM Na Cit. was added to cells not previously exposed to lower concentration (Fig. 4c). The spike height remained relatively constant with concentrations up to 10 mM Na Cit. At 100 mM concentrations cytoplasmic streaming ceased and both the membrane potential and effective resistance fell to zero.

The effect of Na Cit. was readily reversible with APW when concentrations up to 10 mM were used, i.e. before prolonged spikes were obtained.

Tris (hydroxymethyl) aminomethane Chloride. (TRIS Cl)

The effects of 2.25 mM TRIS Cl in distilled water (replacing all inorganic cations in APW) are shown in Fig. 5 (normal responses Figs. 5a and c). The cell hyperpolarized, and spikes as shown in Fig. 5b were

Figure 5 Effects of Tris Cl. (a,c) normal spikes, (b) spike 2 min after washing with TRIS Cl, (d) spike after 10 min washing with TRIS Cl. same voltage, (e) spikes after 30 min washing with TRIS Cl., current calibrations for a,b,c,d and e. a,b,e, and c,d, use the same time scale, d is retouched.


a
b
10^{-6}A
50 mV
20 S
c
d
2 S

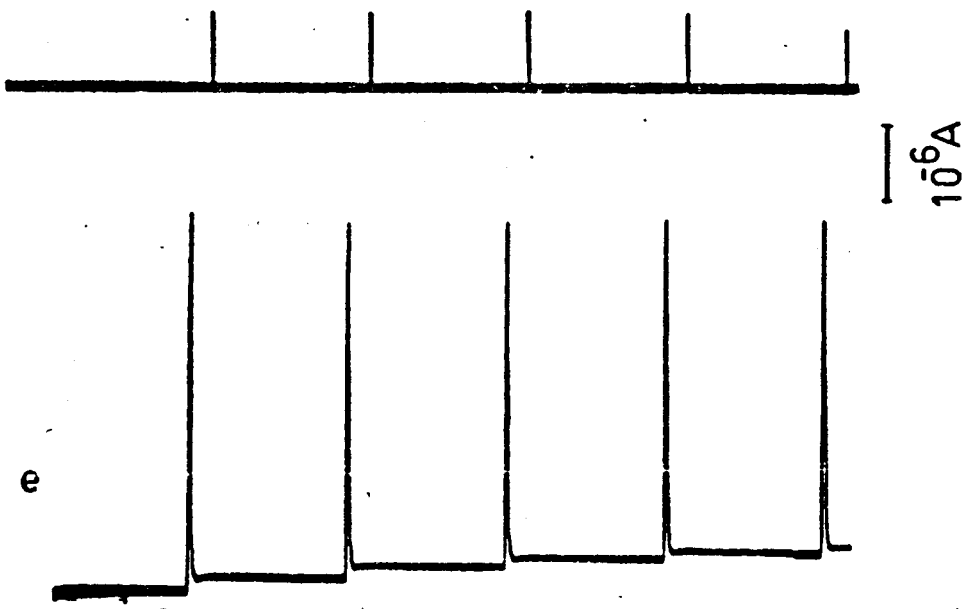
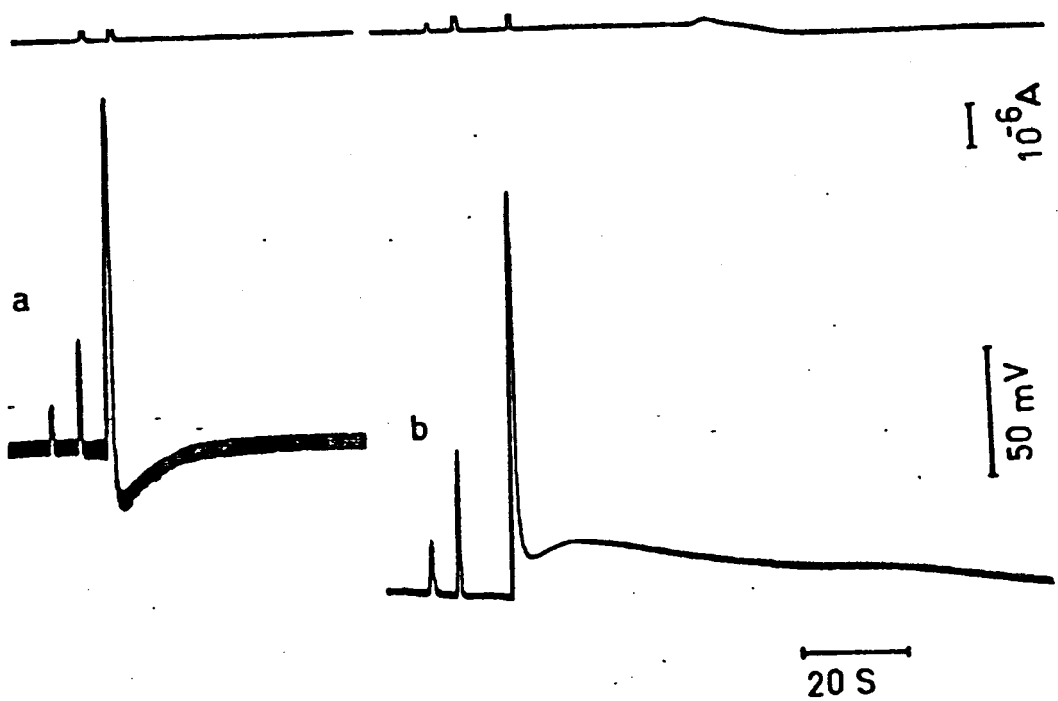

10^{-6}A
e

Figure 6 Effects of TRIS prop. (a) normal spike, (b) spike after 5 min washing with TRIS prop., a,b, same time, voltage and current calibrations. Notice the reversal of the after-potential associated with a shift in steady potential. Movement of the current trace in b is an artefact not related to the experiment.


10^{-6}A
a
b
50 mV
20 S

obtained. Spikes with slightly increased duration were obtained after 10 minutes of continuous washing with TRIS Cl (Fig. 5d). Continuous spiking after a 30 minute washing period, produced spikes which did not return to the normal resting potential (Fig. 5e). Much longer periods of continuous washing (1-2 hours) usually resulted in a loss of excitability which was not restored to normal by addition of 1-10 mM K Cl, NaCl, or $CaCl_2$ to the TRIS solution or replacement of the TRIS Cl by APW.

Tris (hydroxymethyl) aminoethane Propionate (TRIS prop.)

Continuous washing of a cell with 2.25 mM TRIS prop. made up in distilled water, resulted in an immediate hyperpolarisation and decrease in effective resistance. After a spike the membrane potential did not return to its previous resting level immediately, but took 1-2 minutes as shown in Fig. 6b, (Fig. 6a is the corresponding normal response). Long periods of washing with TRIS prop. (1-2 hours) resulted in a loss of excitability which could not be reversed by addition of 1-10 mM solution KCl, NaCl, $CaCl_2$ or by APW.

DISCUSSION

Cells of Nitella subjected to concentrations of EDTA up to 1 mM increase their normal resting potential and decrease their effective membrane resistance. The maximum increase in membrane potential (30 mv) is found at 1 mM concentration. This effect could be directly due to the chelation of Ca^{++} from the medium, thus lowering the external Ca^{++}

concentration and producing the observed hyperpolarization, if Ca^{++} is involved in a resting potential of the type described by the Goldman equation (Goldman 1943). This effect can also be obtained by lowering the external Ca^{++} concentration (Findlay 1961). The hyperpolarization could also be explained as the electrical consequence of an outward movement of Ca^{++} across the membrane. Intercellular Ca^{++}, especially from the cytoplasm, could diffuse to the external medium hyperpolarizing the cell, as there is no evidence for a Ca^{++} pump at the plasmalemma (Spanswick and Williams 1965). At concentration of EDTA higher than 1 mM the cells depolarize and lose membrane resistance. At these concentrations EDTA is probably chelating the bound Ca^{++} that, according to Spanswick and Williams (1965) is attached to the cell wall and cell membrane. Removal of cell bound Ca^{++}, as indicated by ^{45}Ca tracer experiments (see Chapter V), apparently changes the normal behaviour of the cell membrane by altering its permeability to some other ion or ions. In other organisms low $[Ca^{++}]$ increases the permeability of the membrane to K^+ (van Steveninck 1964, Huxley 1959).

EDTA also interferes with the mechanisms controlling the threshold for stimulation and the duration of the spike, i.e. those controlling Cl^- and K^+ activation and perhaps others as well, as indicated by the spontaneous activity and prolonged spikes. The mechanisms controlling threshold for stimulation are affected first, as the first spike during repetitive firing is normal and the spikes following it are progressively longer in duration. The peaks of the spontaneous spikes reach progressively

lower potentials, a situation similar to that observed in squid axons (Huxley 1954). The spontaneous activity recorded in the experiments described in this chapter did not last as long or occur as often as that observed by Kishimoto (1966). This discrepency could be due to the difference in composition of APW as well as the difference in recording techniques used.

The difference in membrane potential curves of cells treated with EDTA and Na Cit. can be explained by the different affinities of these compounds for Ca^{++}. The log of the stability constant $K_s = \frac{[Ca\text{-ligand}]}{[Ca^{++}]\ [ligand]}$ (Harrison and Long 1968) for Ca EDTA and Ca Cit. are respectively 10.59 and 3.3. Small increases in EDTA concentrations above 1 mM depolarize the cell very rapidly, whereas the same change in Na Cit. concentration causes only a slight depolarization. A 10 fold increase in EDTA concentration depolarizes the cell 85 mV while a similar increase in Na Cit. concentration produces a 22 mV depolarization.

The concentration range at which prolonged spikes are obtained is very specific and apparently depends on the K_s of the compound in question. The range is very narrow for EDTA and careful adjustment of the concentration is needed to obtain a spike of maximum duration. This is not always possible and a slight excess of EDTA damages the cell irreversibly. As Na Cit. is a much weaker chelator this concentration range is much broader and prolonged spikes are more easily obtained. This effect could explain the difference in spike duration but does not account for the difference in rate of repolarization during the plateau of prolonged spikes. Different rates of repolarization have also been observed between prolonged

responses of Nitella cells treated with Ba^{++} and TEA, (Chapter I). If Ca^{++} affects only the mechanism controlling the duration of spike one would not expect different chelators to affect the rate of repolarization during the plateau. As Na Cit. is the weaker Ca^{++} chelator but affects the rate of repolarization more than EDTA, increased spike duration is not entirely due to low Ca^{++} but probably also due to some other factor inherent in the difference between these two compounds. The prolonged spikes obtained with Na Cit., are similar in duration to those obtained in 1 mM BaCl and 10 mM TEA, (Chapter 1).

The slow increase in effective resistance seen during the plateau of the spike of $BaCl_2$-treated cells was not observed in the response from Na Cit.-treated cells (Figs. 4b). Brief spikes following the prolonged response after Ba^{++} treatment as shown in Chapter I, were not observed. This indicates a different action of these compounds on the mechanism regulating spike duration. Ba^{++} and TEA probably produce prolonged spikes, not by Ca^{++} chelation, but Ca^{++} displacement from the cell membrane. This is further supported by the similarity in size of the hydrated ionic nuclei of Ca^{++},Ba^{++} and TEA, which are respectively 3.0 A°, 2.80 A° and 2.89 A° (Werman and Grundfest 1961).

The initial increase in resting membrane potential in TRIS Cl and TRIS prop. treated cells is probably due to a mechanism similar to that responsible for the same changes in cell treated with EDTA. In this case not only is $[Ca^{++}]$ changed but also $[K^+]$, $[Na^+]$ and $[Mg^{++}]$. This may be the reason for the much larger hyperpolarization of cells treated with TRIS as compared to EDTA. An alternate explanation for the observed hyperpolarization of the cells treated with TRIS Cl and TRIS prop. could be a mechanism similar to that observed by Akiyama and Grundfest (1971) in frog skeletal muscle cells, where it was found that the induced hyperpolarization induced by K^+ free solution was directly

related to the activity of an ionic pump. No repetitive responses were obtained from cells treated with TRIS indicating that Ca^{++} ions which are not readily washed out have to be removed by a chelator before the cell will produce repetitive responses. This idea is further supported by a decrease in resting potential on addition of EDTA to TRIS treated cells. Experiments on crayfish muscle cells show a very similar effect; EDTA has to be present to produce spontaneous activity (Reuben et al. 1966). The slight increase in duration of the spike in TRIS Cl is probably due to prolonged Cl^- conductance as this effect was not obtained in TRIS prop.

The long lasting after depolarization of the spike recorded in TRIS prop., and the depolarization after continuous spiking in TRIS Cl can be explained in terms of changing K^+ concentration in the external solution. On stimulation, a K^+ efflux brings the membrane potential back to normal resting level (Gaffey and Mullins 1958). This will produce an increase in K^+concentration which is negligible with respect to the K^+ concentration in the medium (0.10 mM) and should not affect the membrane potential as calculated by the Nernst equation. The amount of K^+ efflux has been estimated by Grundfest (1967), to be approximately 10^{-12} mole/cm^2; for a 10 cm long and 1 mm diameter Nitella cell this value is 10^{-9} moles per cell (van Netten 1969). However if there is no K^+ in the medium this concentration change cannot be ignored and could, as calculated by the Nernst equation, account for a maximum change of 18 mv if the cell were a perfect K^+ electrode. A resting potential 5 mv less than before stimulation is therefore not unreasonable. Continuous spiking should increase the K^+ concentration in the outside medium even more, resulting in an even greater loss of membrane potential, as observed. The depolarizing electrogenesis in Nitella is brought about by Cl^- activation

(Mailman and Mullins 1966). The Cl^- increase in the external medium following a spike should not affect the resting membrane potential since Cl^- permeability is very low relative to the K^+ permeability, as is shown by their permeability coefficients K^+ 4.05×10^{-8} cm/sec, Cl^- 1.04×10^{-9} cm/sec. (Kitasato 1968). This is further supported by the failure of cells to depolarize when external Cl^- is replaced by propionate. The failure of cells, rendered inexcitable by washing with TRIS Cl or TRIS prop., to return to normal excitable behaviour following addition of Na^+, K^+, Ca^{++} or even APW indicates that during the washing out process, some important, unidentified, factor is lost which is either not available or not easily replaced even after several hours. After a 24 hour period in APW the cell behaves normally again. The metabolism of the cell may therefore be involved.

CONCLUSION

Gradual replacement of Ca^{++} by means of TRIS solutions does not remove the cell bound Ca^{++} which appears to control the threshold for stimulation and the duration of the spike as shown by the effect of Ca^{++} chelators. Ba^{++} and TEA produce long lasting spikes (Chapter II) probably by displacing Ca^{++} from the cell membrane. Na Cit. has other effects on the cell besides the chelation of Ca^{++}. The lack of cell-bound Ca^{++}, produced either by chelation or displacement, changes the normal behaviour of the cell during the spike by altering its permeability characteristics to some other ion or ions. Tracer experiments are in progress to clarify this.

CHAPTER II

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CHAPTER III

The effects of ammonium ions and 2, 4-dinitrophenol on the electrical activity of *Nitella flexilis*

CHAPTER III

INTRODUCTION

When electron flow is uncoupled from photophosphorylation, for instance by ammonium ions (NH_4^+), an inhibition of the normal K^+ influx is observed in cells of Nitella translucens "at rest" (MacRobbie, 1966). Apparently passive fluxes are unaffected.

Inhibition of phosphorylation by 2, 4-dinitrophenol (DNP) has been shown to decrease the resting potential in Nitella clavata which is consistent with the hypothesis that some energy consuming mechanism maintains the resting potential in these cells (Kitisato, 1968). In this Chapter evidence is presented that energy, probably in the form of ATP is required during the spike of Nitella cells.

METHODS

Single internodal cells were separated and kept in artificial pond water (APW) of the following millimolar (mM) composition NaCl 1.0, $CaCl_2$ 1.0, KCL 0.10 and $MgCl_2$ 0.10 for several weeks prior to the experiment.

Stimulating and recording arrangement were the same as used in Chapter I.

All solutions were made up in APW and were slowly rinsed over the cell. Data from at least three different cells was recorded for each experiment.

Figure 1 Effect of different concentrations of NH_4Cl on *Nitella* cells. Data from three cells with comparable membrane potentials have been plotted. The standard error is shown on the potential curve (•). The standard error for the resistance is shown on the resistance curve (o).

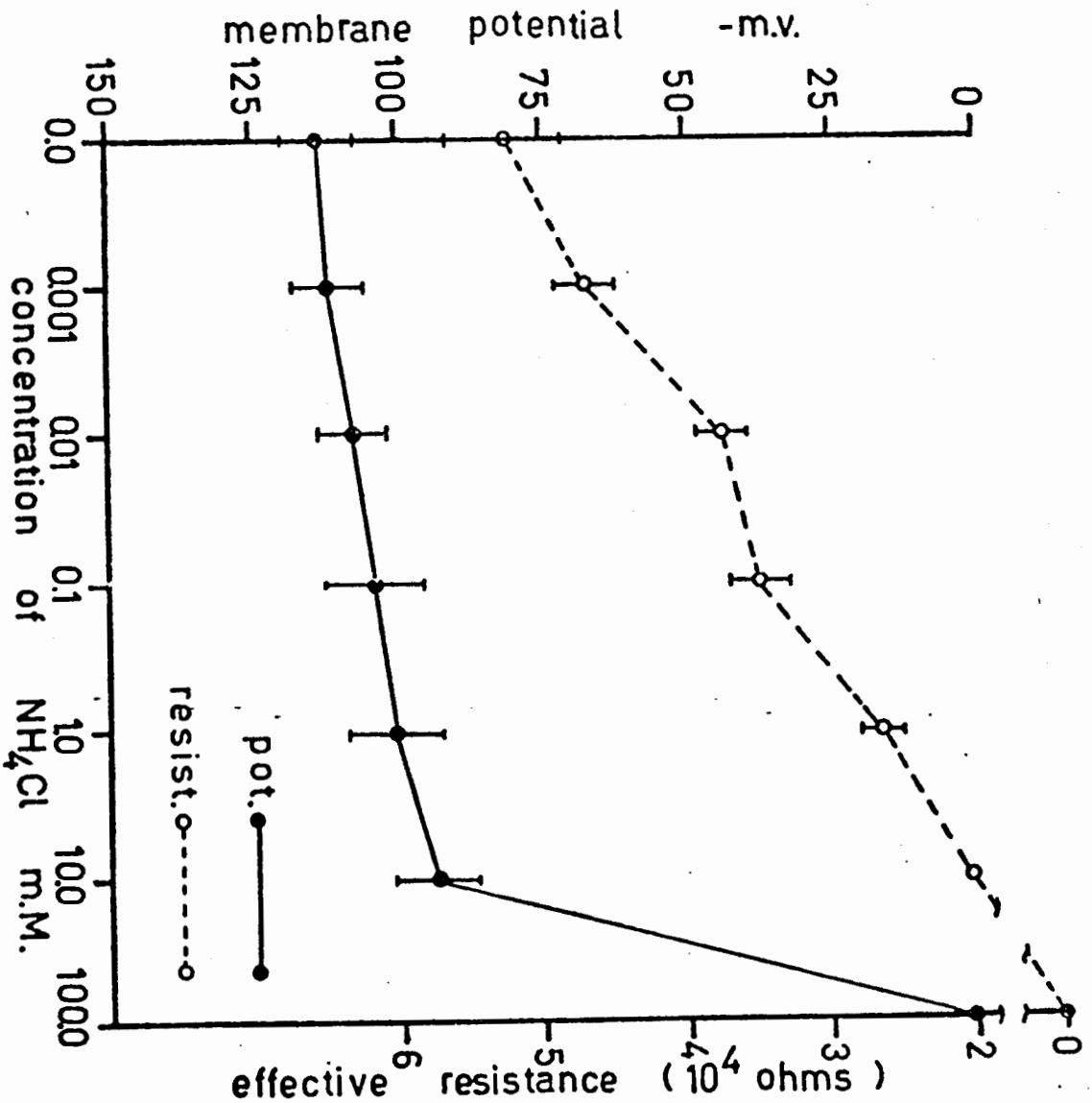
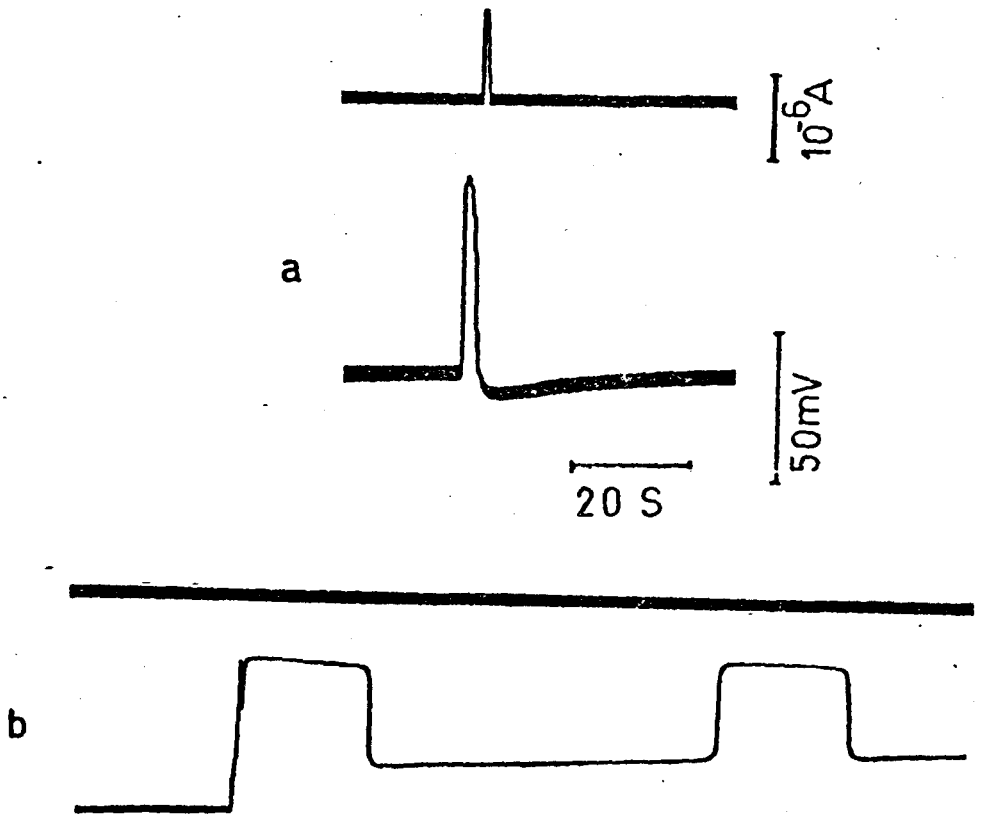

membrane potential -m.v.
150
125
100
75
50
25
0
0.0
0.001
0.01
0.1
1.0
10.0
100.0
concentration of NH4Cl m.M.
resist.o-----o
pot.
effective resistance (10^4 ohms)
6
5
4
3
2
1
0

Figure 2 Effects of NH_4Cl. a. normal spike. b. prolonged responses recorded in 10 mM NH_4Cl. Time, current and voltage calibrations as shown and are the same for both figures.


10^{-6}A
a
50mV
20 S
b

RESULTS

Ammonium chloride (NH_4Cl)

The effect of different concentrations of NH_4^+ on the membrane potential and effective resistance is shown in Fig. 1. Both these parameters decreased logarithmically with increasing NH_4^+ concentration to a concentration of 10.0 mM. The membrane potential showed a sharp change at a concentration of 10.0 mM NH_4^+. At this concentration prolonged spikes were obtained as shown in Fig. 2 (2a, a normal spike 2.5 sec, 2b, prolonged spike 15 sec). The slope of the plateau of these prolonged spikes is very small, showing no measurable change in the effective resistance.

2, 4-dinitrophenol (DNP)

The relationship between the concentration of DNP and the potential and effective resistance of the membrane is shown in Fig. 3. Up to 1.0 x 10^{-2} mM concentrations, only a slight decrease of both the membrane potential and effective resistance was observed.

At 1.0 x 10^{-2} mM concentration, prolonged spikes were obtained (4a, normal spike 2.5 sec, 4b, a prolonged spike 10 sec). Concentrations higher than 1.0 x 10^{-2} mM caused a sharp decrease in both the effective resistance and membrane potential.

DISCUSSION

Ammonium ions (NH_4^+)

Experiments on isolated chloroplasts show that NH_4^+ uncouple

Figure 3 Effect of different concentrations of 2,4-DNP on Nitella cells. Data from 3 cells have been plotted.

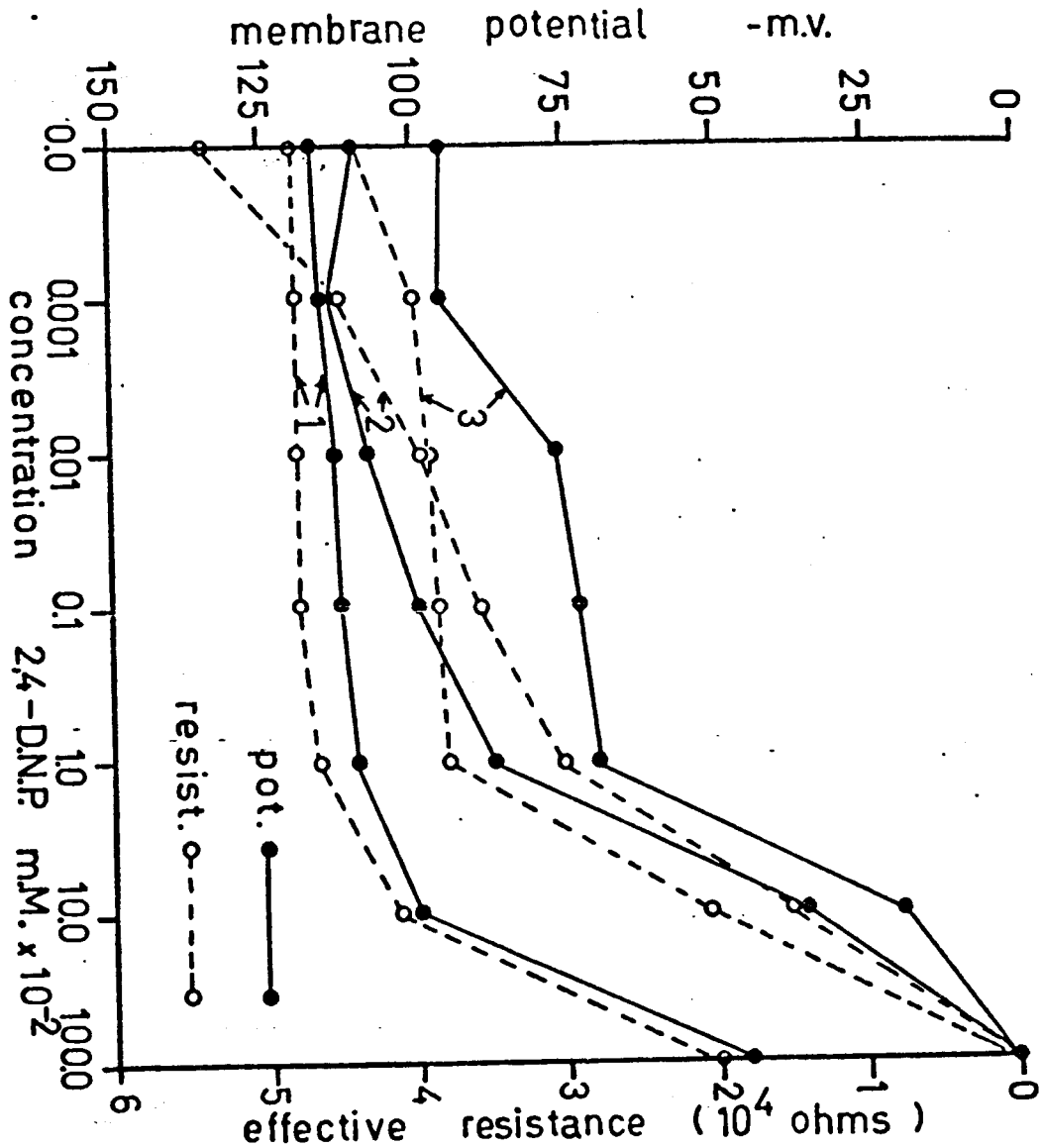
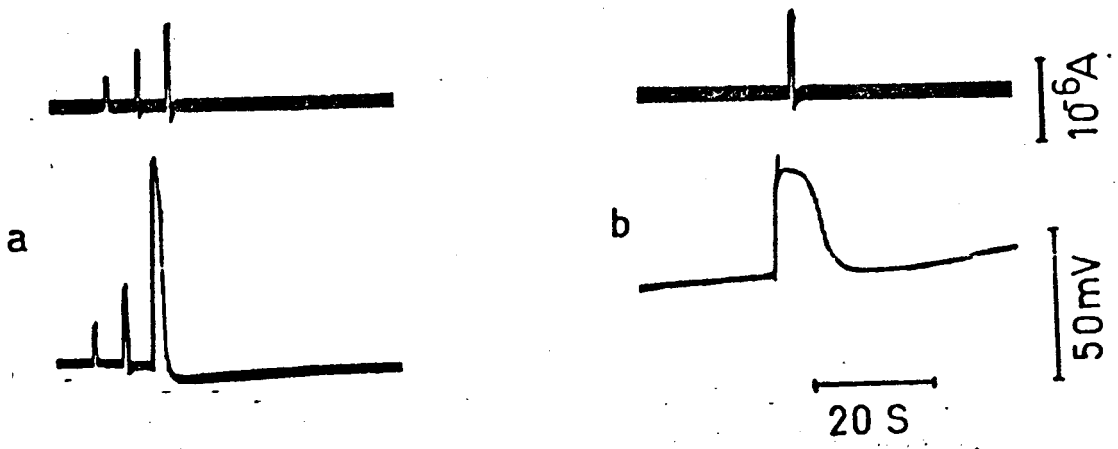

membrane potential -m.v.
150
125
100
75
50
25
0
0.0
0.001
0.01
0.1
1.0
10.0
100.0
concentration 2,4-D.N.P mM. x 10^{-2}
1
2
3
resist.
pot.
6
5
4
3
2
1
0
effective resistance (10^4 ohms)

Figure 4 Effects of 2,4-DNP. a. normal spike, b. prolonged spike recorded in 10^{-2} mM. Time, current and voltage calibrations are the same for both figures.


a
b
10^{-6}A
50mV
20 S

photophosphorylation (Good, 1960, Hind and Wittingham, 1963, Stokes and Walker, 1970). In squid axons NH_4^+ can substitute for Na^+ and K^+ (Binstock and Lecan, 1969). An efflux of K^+ occurs during the repolarizing phase of the spike in Nitella cells (Chapter I) and the effect of NH_4^+ on Nitella cells could therefore be due to a combination of effects on both phosphorylation and on the repolarizing phase of the spike. However, as the effect of NH_4^+ is very similar to the effect of imidazol on Nitella cells (MacRobbie, 1966), inhibition of photophosphorylation is definitely occurring.

Prolongation of the spike caused by NH_4^+ could be due to uncoupling of photophosphorylation or to a combination of this effect together with competition between K^+ and NH_4^+.

2,4-dinitrophenol (DNP)

DNP is a well known inhibitor of oxidative phosphorylation (Lardy, Witonski and Johnson, 1965). DNP apparently does so by stimulating respiration and preventing the synthesis of ATP from ADP.

Experiments on rabbit vagus nerves indicate that low concentrations of Ca^{++} speed up mitochondrial oxidative phosphorylation (Landowne and Ritchie, 1970). These authors speculate that the influx of Ca^{++} during the spike increases the activity of phosphorylase a phosphofructokinase, enhancing glycogenolysis, resulting in an increase of ATP which is needed for the Na^+ pump.

The concentration range of DNP at which prolonged spikes are obtained is narrow and careful adjustment of its concentration is required. Too

high a concentration of DNP causes irreversible damage to the cell. The difficulty in adjusting the critical concentration could explain the failure to observe similar effects in other organisms (Heneeus-Cox, et al. 1966). Perfusion studies with cyanide also show no inhibition of the spike (Heneeus-Cox, 1966). It has been argued that perfusion techniques produce abnormal situations becuase a new interface not normally present is created (Strunk, 1971) and therefore results obtained from such an experiment do not necessarily reflect what happens in intact cells. Studies on the effect of azide on nerve cells show that the configurational change of nerve proteins normally associated with stimulation is blocked (Ungar, et al., 1957). This probably indicates that the observed conformational change is energy dependent.

Prolonged spikes in Nitella cells can be produced by a delay in Cl^- inactivation or a delayed K^+ activation or both. It has been shown that Cl^- transport in Nitella cells is independent of ATP (MacRobbie, 1966). However, as the ion channels during excitation are not necessarily the same or controlled by the same mechanism as those active during the resting state (Yamagishi and Grundfest, 1971), prolongation of the spike could occur by both mechanisms.

Both phosphorylation inhibitors, NH_4^+ and DNP, prolong the spike, which would indicate that the prolonging effect is due, in the case of DNP and possibly of NH_4^+, to a limiting amount of energy in the form of ATP and is probably not due to Ca^{++} substitution (Chapter II). This is

further supported by the extremely low concentration of DNP required to obtain prolonged responses.

In Chapter II, it has been demonstrated that Ca^{++} is essential for normal repolarizing K^+ activation to produce a spike of normal duration. Now evidence is presented that K^+ activation or Cl^- inactivation or both also require energy in the form of ATP. It is highly probable that energy is needed for the removal of Ca^{++} from the membrane sites, changing the membrane configuration, initiating the appropriate activation or inactivation processes. When phosphorylation is almost entirely inhibited, a small, probably rate-limiting, amount of ATP is generated. More time would therefore be required to open and close ionic pores, resulting in the prolonged spikes observed. Ca^{++}, in this hypothesis, acts in a gating mechanism. Removal of the Ca^{++} from the gate allows K^+ to diffuse outward down the electrochemical gradient. The Ca^{++} thus released under normal conditions probably on the inside of the cell, could very well speed up mitochondrial oxidative phosphorylation as has been postulated by Landowne and Ritchie (1971) to occur in mammalian nerve cells.

CONCLUSIONS

DNP and probably NH_4Cl prolong spikes in Nitella by reducing the amount of available energy, probably in the form of ATP to a rate limiting amount. The energy appears to be required for repolarizing K^+ activation.

It is possible that NH_4^+ also produces prolonged spikes by being a less effective K^+ substitute.

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CHAPTER IV

Design, description and application of a micromanipulator used for ^{45}Ca efflux studies in *Nitella* cells

CHAPTER IV

INTRODUCTION

Commercially available micromanipulators cannot be used in restricted areas or in a closed system, so that, for example, radioactive solutions can be passed over a preparation without leakage with the system in any position. Attempts to make a closed system have been made for example, by floating a layer of parrafin oil over the saline covering and preparation (Keynes and Lewis 1951) for the measurement and exchange of radioactive potassium in crab nerve.

Several micromanipulators were built and tested for use in experiments designed to correlate electrical activity directly with other physical parameters. Some of these experiments must be carried out in extremely restricted areas such as the chambers of scintillating counters or spectrophotometers. A further requirement is that electrodes are inserted into cells under a dissecting microscope and the preparation with electrode inserted must then be moved into the restricted area. The most successful design and applications of the system are discussed.

DESCRIPTION

A diagram of the micromanipulator is shown in Fig. 1. The overall size is small, 3/4" x 1/2" (1.87 cm x 1.27 cm) yet many of the features of commercially available micromanipulators are present. Micromanipulation in a horizontal plane is achieved by use of eccentrics. The total area covered by the manipulator is that of

Figure 1 The design of the Micromanipulator

A. Two cylindrical elements with a threaded hole cut at their junction.

B. Captive set screw.

C. Electrode holder.

D. Barrel.

E. Locking screw.

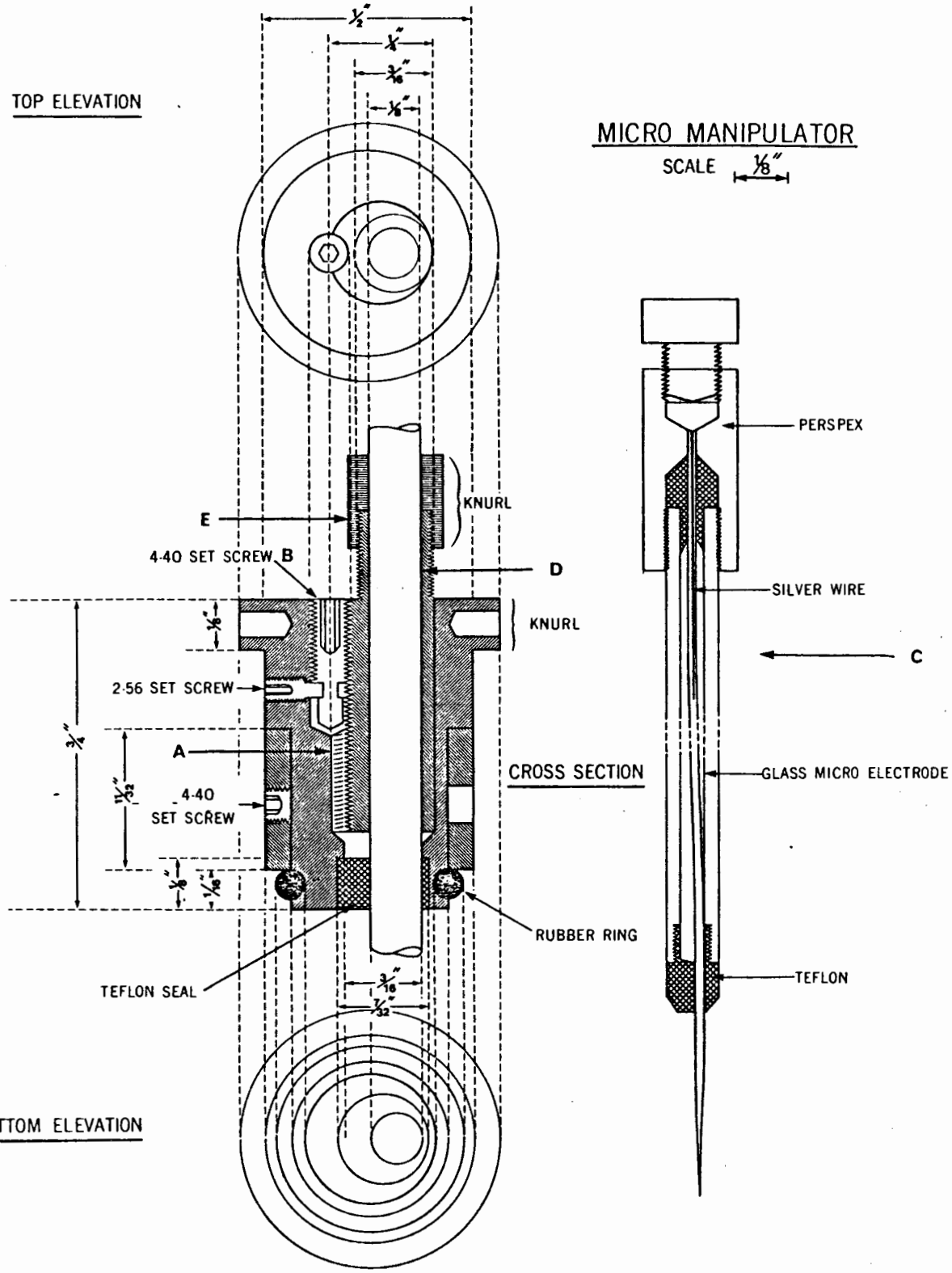
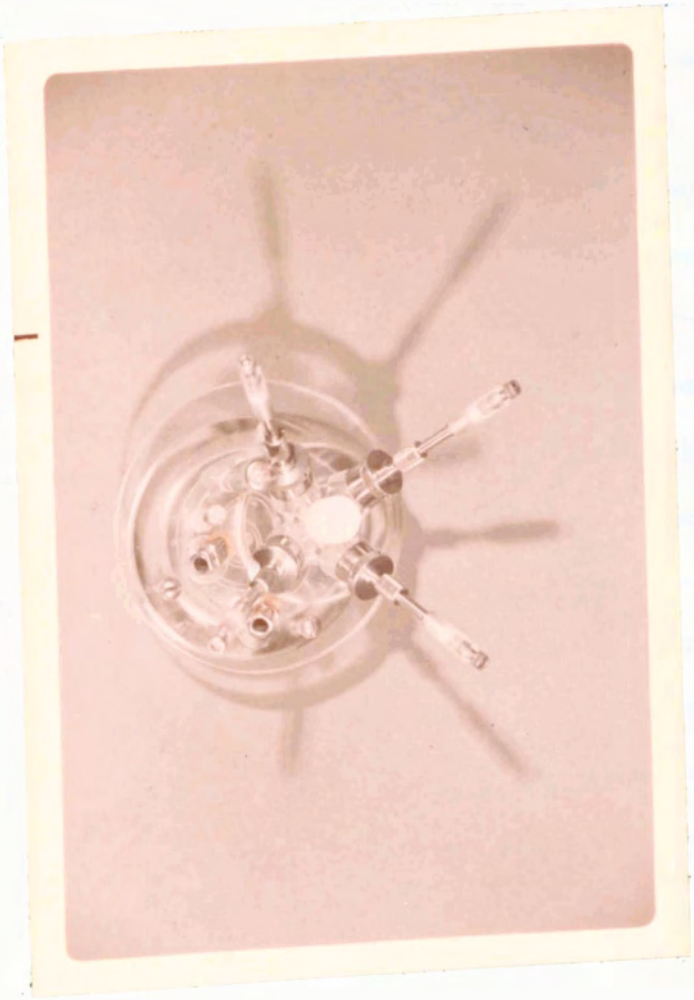

TOP ELEVATION
MICRO MANIPULATOR
SCALE 1/8"
PERSPEX
KNURL
E
4-40 SET SCREW B
D
KNURL
SILVER WIRE
C
2-56 SET SCREW
A
CROSS SECTION
GLASS MICRO ELECTRODE
4-40
SET SCREW
RUBBER RING
TEFLON SEAL
TEFLON
BOTTOM ELEVATION

Figure 2 Photograph of four micromanipulators positioned in a holder.



a circle 1/4" (.365 cm) in diameter which is large enough for most applications. Two cylindrical elements (A in Fig. 1) with a threaded hole cut at their junction is the mechanism used to convert the rotary movement of a captive set screw (see B in Fig. 1) into a vertical movement fine enough (0.025 inch or 635 μ per turn) to allow microelectrodes or microtools to be positioned inside single nerve, muscle or plant cells. The vertical fine movement is comparable with that of a compound microscope, usually about 200 μ per turn. The fine movement can be used at any desired depth by adjusting the electrode holder (C) up or down the barrel (D) in which it is held by a locking screw (E). This coarse range is 1 inch (2.54 cm) with the microelectrode holder shown in Fig. 1 (C). If access to a larger horizontal area is required, the micromanipulator holder can be rotated, increasing the total area to that of a circle one inch (2.54 cm) in diameter.

Positioned in an appropriate holder (Fig. 2) the micromanipulator is essentially leak-proof and can withstand moderate changes in pressure (± .3 atmospheres).

APPLICATION

The micromanipulator described has been successfully used in a closed system for recording and stimulating Nitella cells with continuous flow of certain pharmacological agents past these cells (Chapter II and IV).

The manipulator has also been used to correlate the electrical

activity of ^{45}Ca loaded Nitella cells to the efflux of ^{45}Ca (Chapter V).

Another application has been the continuous recording of electrical parameters of Nitella cells situated in a neutron beam, generated by a Cockcroft-Walton neutron generator. (van Netten, 1969).

As the apparatus lends itself freely to uses other than that as a microelectrode holder by interchanging the actual electrode holder (C) for any other appropriate instrument, its use is virtually unlimited in any branch of science and technology when the accurate positioning of small objects is essential.

An application to patent the device has been submitted to C.P.D.L. (case no. 5075).

CHAPTER IV

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CHAPTER V

The effects of TEA, Ba^{++}, Na Cit. and EDTA on cell bound Ca^{++}

CHAPTER V

INTRODUCTION

In Chapters I and II it was demonstrated that pharmacological agents such as barium ions (Ba^{++}), tetraethylammonium ions (TEA), ethylenediaminetetraacetic acid (EDTA) and sodium citrate (Na Cit.) produce prolonged spikes in internodal cells of Nitella flexilis. It was suggested that Ca^{++} chelating agents such as EDTA and Na Cit. produce such spikes by removing membrane bound Ca^{++}, and that Ba^{++} and TEA do so by removing and substituting for cell-bound Ca^{++}. In this Chapter a description is made of the measurements of the effects of these compounds on cell-bound Ca^{++} and correlate Ca^{++} movement directly with the electrical activity of the cell using ^{45}Ca tracers.

METHODS

Single internodal cells of Nitella flexilis were kept for 3 weeks prior to an experiment in 75 ml of artificial pond water (APW) (millimolar (mM) composition: 0:1 KCl, 1.0 NaCl, 1.0 $CaCl_2$, and 0.1 $MgCl_2$ to which 1 mCi of ^{45}Ca (specific activity 0.0324 mCi/ugm Ca (Amersham and Searle)) was added. The light conditions were held constant at 15 lux during this period. Immediately prior to an experiment a cell was washed with nonradioactive APW for 5 minutes and placed in a Lucite chamber (volume 0.3 ml). Intracellular glass electrodes were inserted to record potentials and to pass currents as described in Chapter I. For each

Figure 1 Experimental design of a continuous flow system used for ^{45}Ca efflux measurements.

A Electrode holder.

B 3 way stopcock.

C Plastifluor spiral scintillator.

D Photomultiplier tube.

E Lead shield.

F Black plastic envelope.

DIS Discriminator.

SC Scaler.

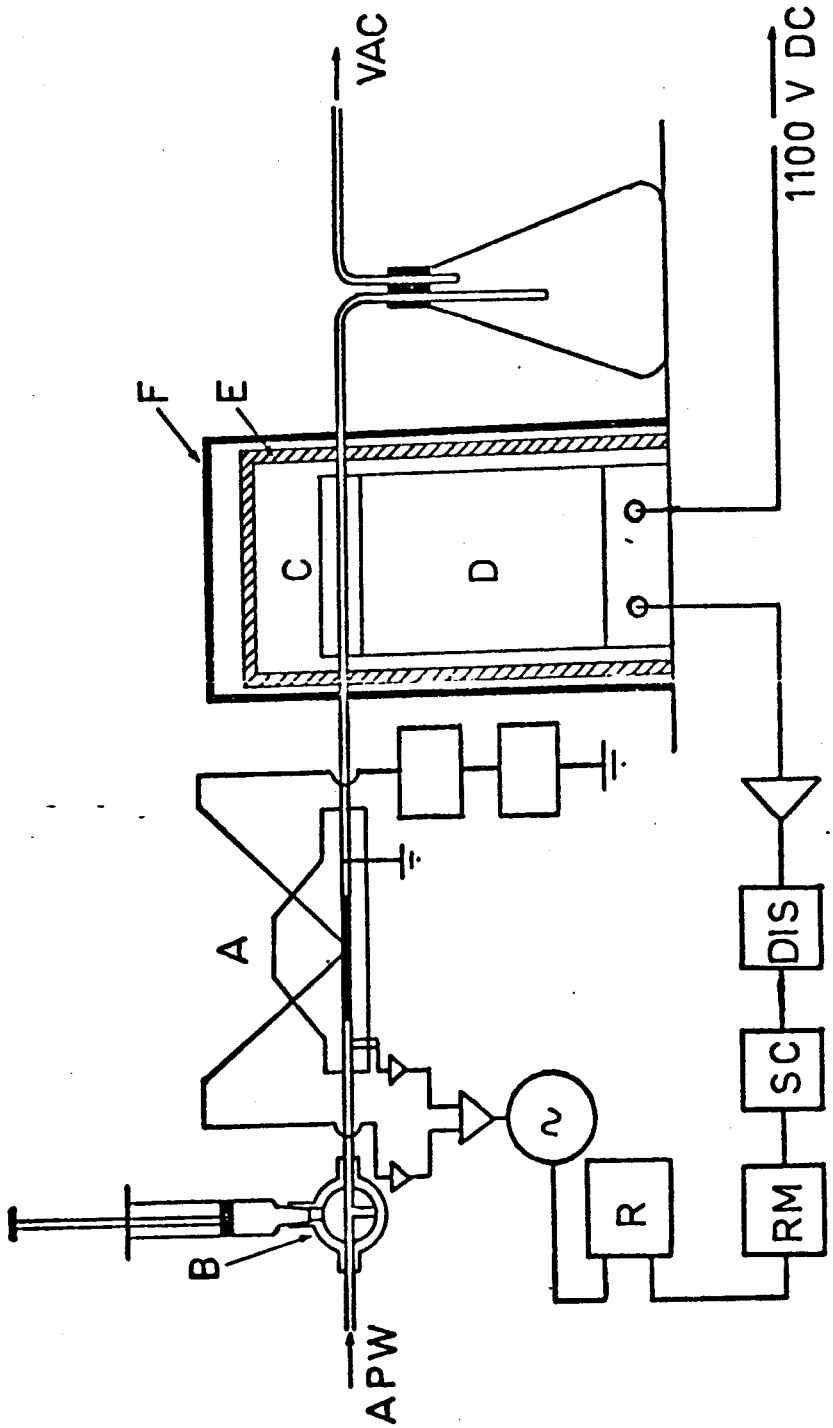
RM Rate meter.

R 2 Channel penrecorder.

APW Artificial pond water.

VAC Vacuum.

Stimulating and recording arrangement is the same as described in Chapter I.


VAC
1100 V DC
F
E
C
D
A
B
APW
DIS
SC
RM
R

experiment a new ^{45}Ca loaded cell was used. Experiments were repeated at least 3 times.

To measure ^{45}Ca release by the cell, an experimental arrangement as shown in Fig. 1 was used. The Nitella cell was contained in a closed system and two microelectrodes inserted into the cell by means of specially designed micromanipulators (Chapter IV). APW flowed over the cell (A in Fig. 1) at a constant rate of 0.05 ml/sec. Pharmacological agents were injected into the system through the stopcock (B in Fig. 1). The APW was led from the cell into a .94 cm x 5.1 cm polished Plastifluor spiral scintillator (Isomet corporation) with a volume of 0.2 ml. (c Fig. 1) This was placed on top of an RCA 8053 photomultiplier tube (D in Fig. 1). The photomultiplier was coupled to the Plastifluor scintillator with oil and was mounted in a lead chamber (E in Fig. 1) which in turn was enclosed by a thick light-proof black Lucite box (F in Fig. 1). A slight negative pressure was applied at the exit of the spiral scintillator to ensure an even flow through the system. The signal obtained from the photomultiplier was amplified in an oscilloscope and fed into a discriminator and scaler. The scaler output was fed into a rate meter and the resulting signal was recorded simultaneously with the membrane potential on a 2 channel Riken Denshi SFH4 potentiometric recorder (Fig. 1). The counting efficiency of this continuous flowing system was measured by injecting a sample of APW containing a known amount of ^{45}Ca activity into the flow of APW by means of the stopcock and measuring the total number of counts, which is given by the area under the curve as plotted by the penrecorder. Careful adjustment of activity and of the volume it was contained in,

Figure 2 Calibration of counting efficiency

a. addition of 4.11×10^4 dpm ^{45}Ca in .2 ml.

b. effect of EDTA, shown here for comparison. Calibrations as shown.

The delay between the potential and radioactivity traces in this and the following figures is due to the flow rate of the system.

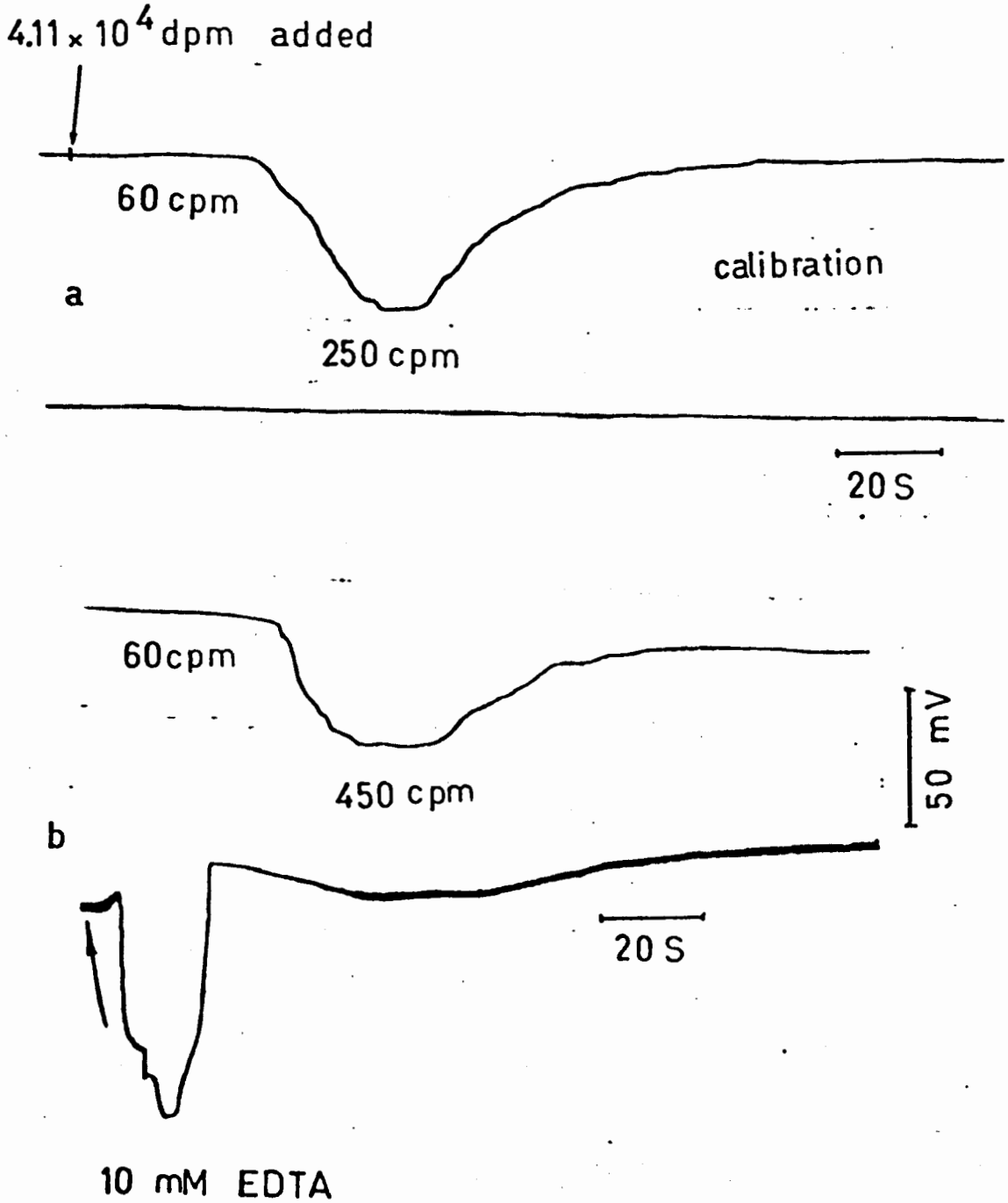
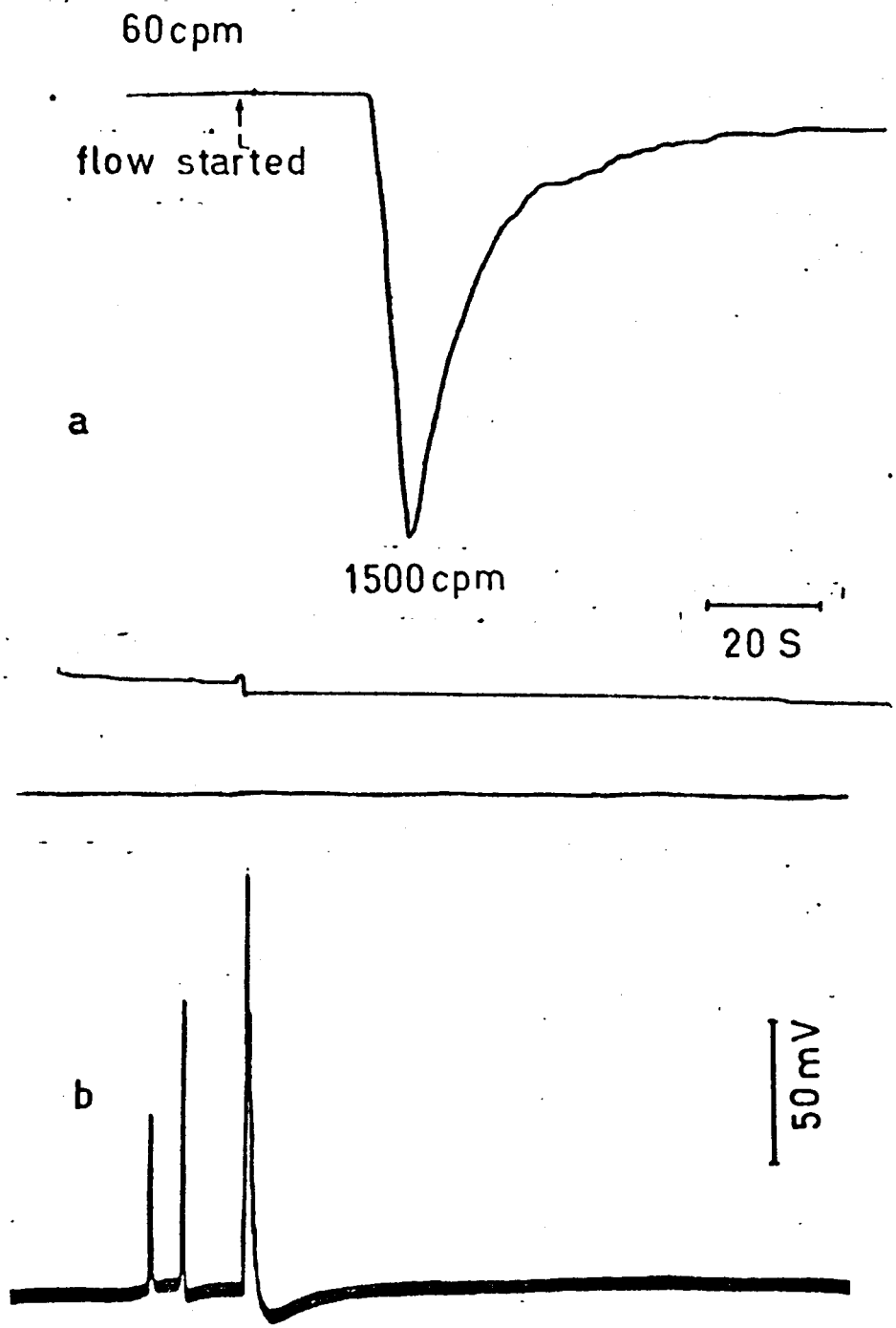

4.11 x 10^4 dpm added
60 cpm
a
250 cpm
calibration
20 S
60cpm
450 cpm
50 mV
b
20 S
10 mM EDTA

Figure 3a Washout curve of ^{45}Ca from a cell after two electrodes were inserted. Calibrations as shown.

b The effect of hyperpolarizing pulses, depolarizing pulses and of the spike on ^{45}Ca activity in effluent. Calibrations the same as for Fig. 3a.


60cpm
flow started
a
1500cpm
20 S
b
50mV

was made, in order to simulate the release of Ca^{++} by the cell. Flow rates were kept constant (0.5 ml/sec). Injection of ^{45}Ca into the system did not affect the background counting level as measured prior to injection so that ^{45}Ca ions were not bound to the spiral scintillator.

The total uptake of ^{45}Ca by Nitella cells at the end of the loading period was obtained by counting the activity of 10 cells in a liquid scintillation counter.

RESULTS

The activity measured by the Plastifluor scintillator and registered on the scales on injection of a .2 ml radioactive ^{45}Ca solution (total activity 4.11×10^4 d PM), was 250 counts, i.e. the area under the curve in Fig. 2a.

The mean activity of 10 cells loaded with ^{45}Ca for 3 weeks and washed for 5 min with APW was 2.347×10^5 cpm. as measured by the liquid scintillator counter (Efficiency ^{45}Ca, 60%). Handling and inserting two microelectrodes into these cells causes a release of approximately 10% of the total activity as shown in Fig. 3. Normal background readings were obtained after 5 min of continuous washing. Hyperpolarizing pulses, depolarizing pulses, spike generation or injection of 5 ml of APW into the system did not have any effect on the activity of the effluent (Fig. 3b).

Injection of a 1 ml sample of a 10 mM TEA solution in APW into the system raised the counting rate of the effluent from 60 cpm (normal

Figure 4 Effect of TEA on cell bound ^{45}Ca.
Calibrations as shown.

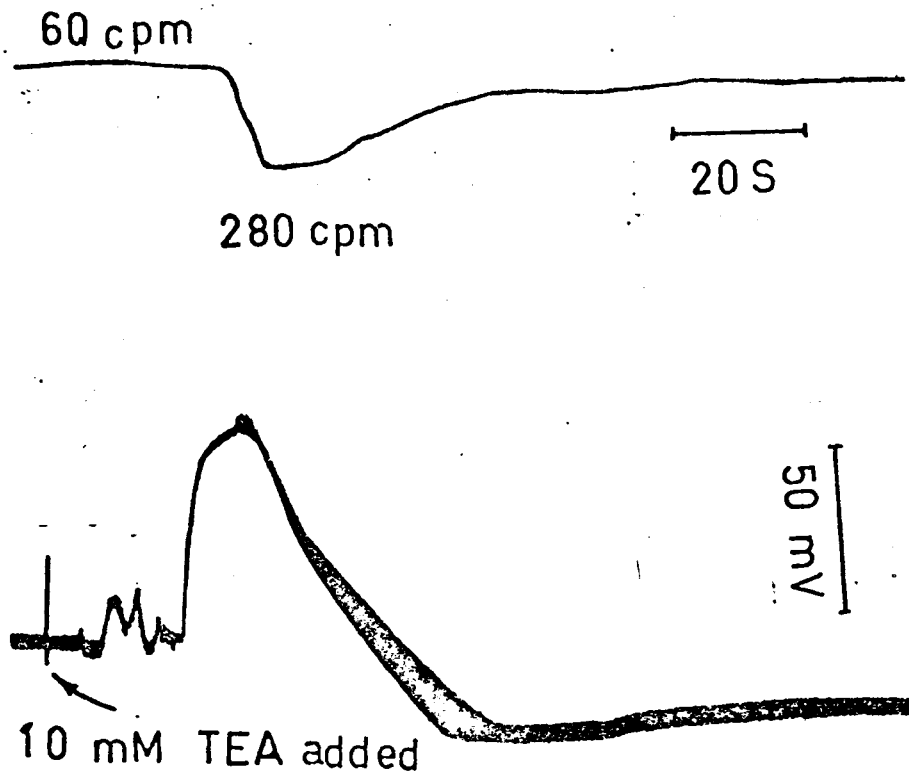

60 cpm
20 S
280 cpm
50 mV
10 mM TEA added

Figure 5 The effect of Ba^{++} on cell bound ^{45}Ca activity. Hyperpolarizing pulses were switched off. Calibrations as shown.

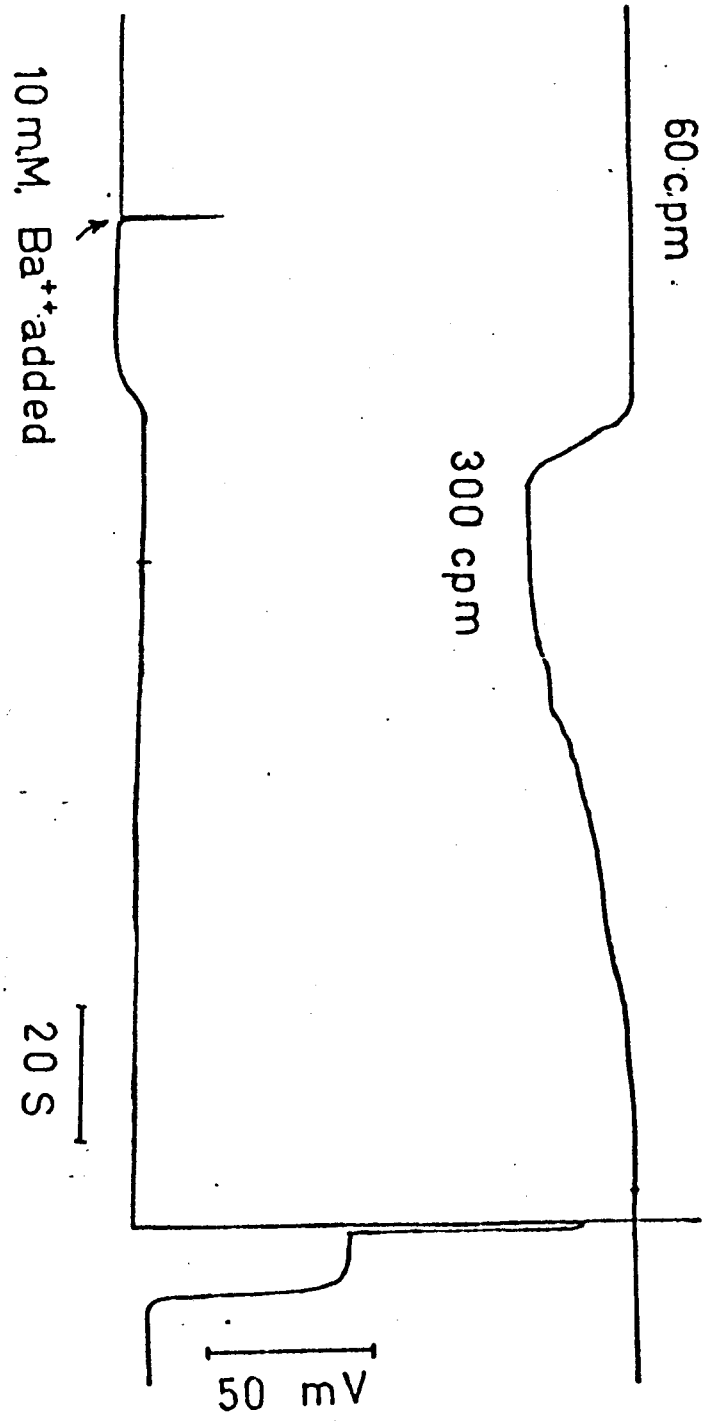

10 mM. Ba++ added
60 cpm
300 cpm
20 S
50 mV

Figure 6 The effects of Na Cit. on cell bound ^{45}Ca activity. Calibrations as shown.

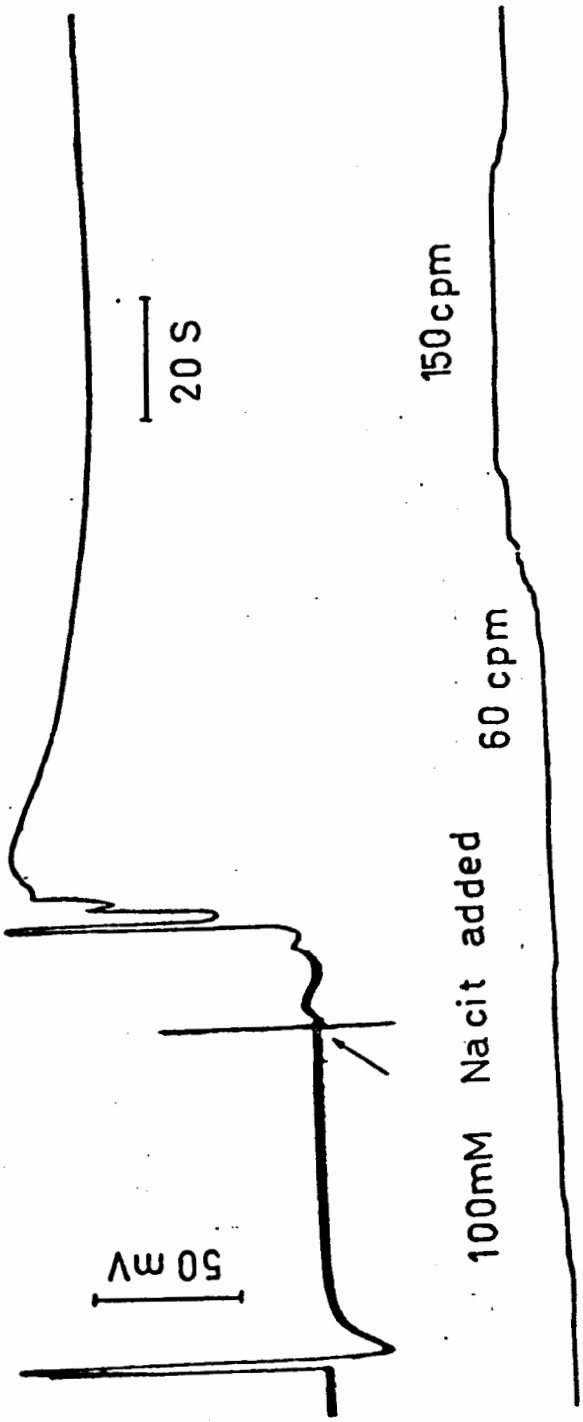
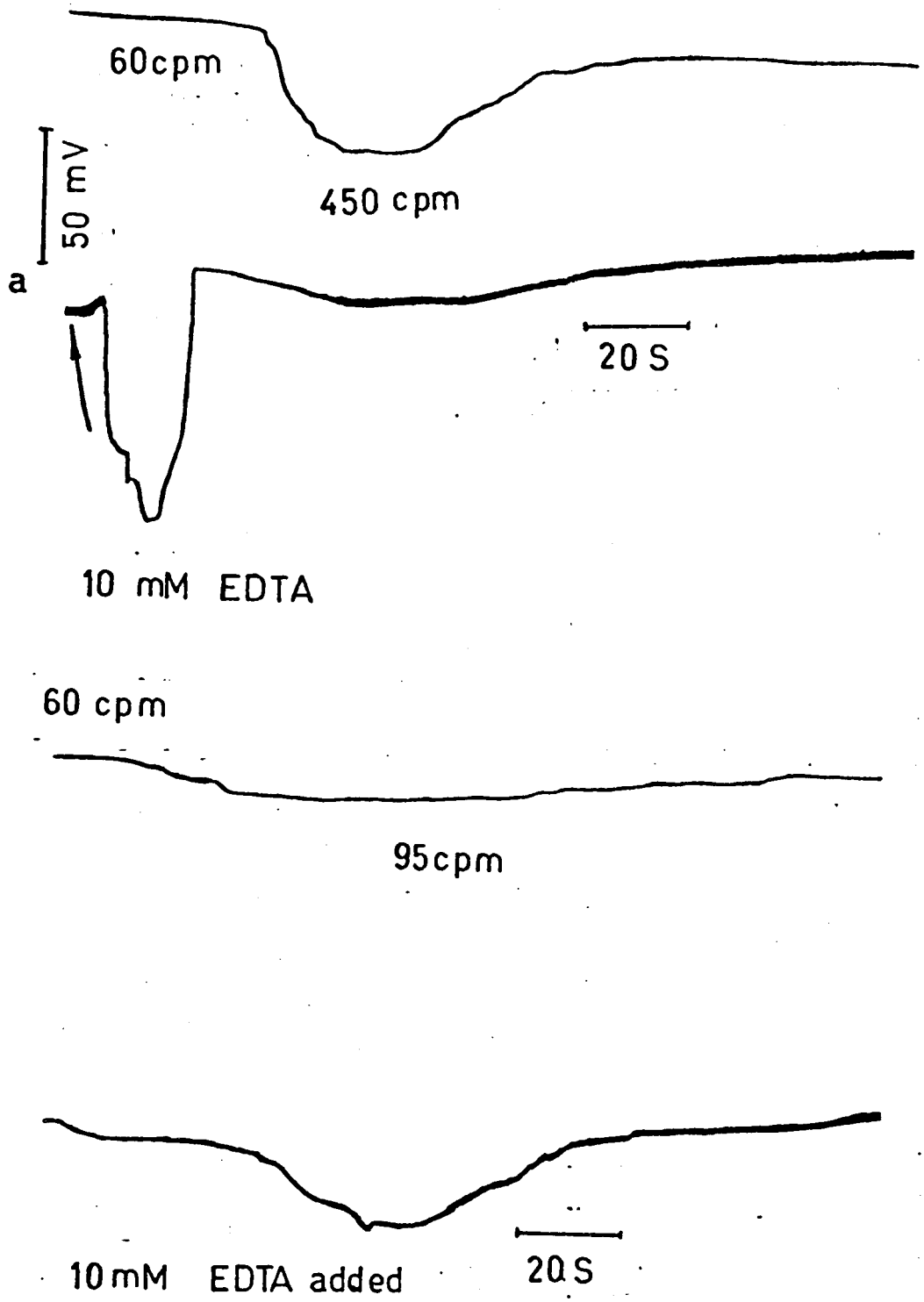

20 S
150cpm
100mM Na cit added
60 cpm
50 mV

Figure 7 The effects of EDTA on cell barium ^{45}Ca activity.

a. Activity released from a cell loaded with ^{45}Ca, not previously treated with pharmacological agents.

b. Activity released from a cell previously treated with 10 mM Ba^{++}- calibrations as shown.


60cpm
50 mV
450 cpm
a
20 S
10 mM EDTA
60 cpm
95cpm
b
10mM EDTA added
20 S

background) to 280 cpm as shown in Fig. 4. The total activity released by the cell during this period resulted in an increase of 93 counts above background as registered by the scaler and coincided with the area under the curve in Fig. 4. Normal background levels were obtained 1 minute later. Subsequent additions of 10 mM TEA had no effect. Prolonged spikes were always obtained after injection of 10 mM Ba^{++}. The counting rate changed from 60 cpm to 300 cpm as shown in Fig. 5 and 200 counts were above background were registered by the scaler (area under the curve in Fig. 5). During a prolonged spike no measurable amount of ^{45}Ca was lost by the cell (Fig. 5). Subsequent additions of 10 mM BaCl solutions did not change the activity of the effluent. Prolonged spikes were always obtained after ^{45}Ca activities were measured in the effuent. Addition of 10 mM Na Cit. had no measurable effect but 100 mM Na Cit. raised the counting rate of the effluent from 60 to 150 cpm as shown in Fig. 6 i.e. 90 counts above background were registered by the scaler (i.e. area under the curve in Fig. 6). At this time prolonged spikes were recorded. Very similar results were obtained with 10 mM EDTA. The counting rate changed from 60 to 450 cpm as shown in Fig. 7 and 270 counts above background were registered by the scaler, i.e. the area under the curve in Fig. 7a. Application of 10 mM EDTA to a cell which had already released some ^{45}Ca due to TEA, Ba^{++} or Na Cit. injection, resulted in an additional 150 counts measured by the scaler and was equal to the area under the curve in Fig. 7b.

DISCUSSION

In Fig. 2 two curves are shown of the activity in the effluent produced by a. injection of 0.2 ml of radioactive ^{45}Ca standard, activity 4.11×10^4 dpm b. injection of a 1 ml sample of 10 mM EDTA into the system containing a ^{45}Ca loaded cell. As mentioned before the volume of the standard solution was adjusted in order to simulate the rate of efflux of ^{45}Ca from the cell. It is clear from Fig. 2 that the two curves are almost identical.

Differences brought about by interfering factors such as rate of release of activity from the cell and hence possible different quenching effects, non laminar flow and adsorption are therefore compensated for.

The number of counts as registered by the scaler is now directly proportional to the original activity present in the cell using the data from the standard radioactive solution. The value of 250 counts measured correlates to 4.11×10^4 dpm injected into the flowing system. The conversion factor is therefore 166.5. The efficiency of the flowing system is 0.6%.

The minimum amount of Ca^{++} that can theoretically be measured using this counting arrangement is 5.67×10^{-8} mM using 10 counts as the minimum detectable number of counts above the background. Using the counting efficiency of .6% this corresponds to 27.7 disintegrations per second (dps). The amount of Ca^{++} in solution, including ^{45}Ca is 75.666×10^{-3} mM which has an activity of 3.7×10^7 dps. Therefore, 27.7 dps corresponds to 5.67×10^{-8} mM Ca.

Tracer studies

Approximately 10% of ^{45}Ca activity is lost when the electrodes are inserted (Fig. 3a). This is probably due to local injury of the cell and consequent leakage from the cell wall or the cytoplasm before the cell seals around the electrodes. As the amount of ^{45}Ca lost is not the same from cell to cell, no precise quantitative comparisons can be made. The experimental arrangement allows one to correlate the electrical behavior and ^{45}Ca release from the cell directly. In Fig. 3b no Ca^{++} efflux could be measured following stimulation of a ^{45}Ca loaded cell. Ca^{++} may be involved in spike generation (Chapter II) but it is evidently not released to the outside of the cell in amounts exceeding 5.67×10^{-8} mM, i.e., minimum detectable amount of Ca^{++}. Some influx of Ca^{++} could occur but this technique can only demonstrate an efflux.

TEA and Ba^{++} do not prolong normal spikes unless 10% of ^{45}Ca activity is lost by the cell. This is direct evidence that prolonged spikes caused by TEA and Ba^{++} are related to Ca^{++} substitution, as indicated by preliminary ^{131}Ba tracer studies (appendix). A very similar mechanism for the action of TEA on frog nerve muscle preparations has been postulated (Beaulieu and Frank, 1962). However, these authors suggested a Ca^{++} deficiency rather than substitution caused by TEA is responsible for the TEA effect. Ca^{++} effluxes have been measured in frog nerve cells after treatment with complex quaternary ammonium salts and appeared to be related to the neuromuscular blocking effect of these salts (Greenberg, 1972). Kuperman et al. (1967) have demonstrated that certain anasthetics

will competitively displace Ca^{++} from frog nerve and muscle cells. Greenberg and Kuperman did not relate the removal of Ca^{++} directly to the electrical activity of the nerve and muscle cell. Such a correlation is now possible and is demonstrated on Nitella cells.

The investigation and the data presented here allow us to make the following speculation on the effect of Ba^{++}, TEA, Na Cit. and EDTA on the membrane and consequently, the duration of the spike.

In Chapter II and III it was suggested that Ca^{++} is involved in a gating mechanism. Removal of the gate, presumably by a step involving ATP and Ca^{++}, allows K^{+} to diffuse outward down its electrochemical gradient.

As TEA and Ba^{++} are capable of substituting for Ca^{++} on the membrane, as shown in this paper and by preliminary ^{131}Ba tracer studies, they have a higher affinity for the membrane than Ca^{++}. If the opening of a K^{+} gate involves active removal of Ca^{++}, it is reasonable to suggest that it would be more difficult to remove the stronger binding ions, TEA and Ba^{++}, and that for the same rate of expenditure of energy, more time is required. Hence, the increase in spike duration.

The results of two types of experiment indicate that Ba^{++} is more strongly bound to the membrane than TEA. First, the effect of Ba^{++} is much more difficult to reverse than that of TEA and secondly, Ba^{++} increases the duration of normal spikes five fold more than does TEA.

Furthermore, the slope of the plateau of the prolonged spike should be an indication of the rate at which K channels are opened.

If K^+ channels were opened at a steady rate, one would expect the plateau to return linearly to the "resting" potential. If the opening of a certain number of K^+ channels leads to the opening of all the remaining channels, there would be a rapid repolarization at the end of the plateau. This implies that the K^+ activation is regenerative i.e., a local initiation of K^+ activation induces K^+ activation in neighbouring membrane regions. There is no direct evidence from Nitella cells to support this, but a regenerative K^+ activation has been observed under certain conditions in Onchidium nerve cells (Hagiwara, Kusano and Saito, 1961) and Tenebrio muscle fibres (Belton and Grundfest, 1962).

The prolonged spikes recorded from cells treated with Na Cit. and EDTA can also be explained by the previous hypothesis if it is postulated that membrane bound Ca^{++} and ATP are required to catalyze the reaction which opens the K^+ channels. Thus, the effect of Na Cit. and EDTA can be explained. A marginal amount of Ca^{++} present is capable of opening only a few K^+ channels and as, according to this hypothesis, a minimum number of K^+ channels have to be opened before all channels are induced to open, more Ca^{++} must diffuse from intracellular sources to meet the Ca^{++} requirement. Diffusion takes time and therefore the increase in spike duration. In view of this model, ions which can substitute for Ca^{++} are not as efficient in catalyzing the gate opening reaction and more time is required.

CONCLUSION

Ca^{++} is not released from Nitella cells during stimulation in amounts exceeding 5.67×10^{-8} mM. The prolongation of the spike by TEA, Ba^{++}, EDTA and Na Cit. is due to the interference of these compounds with cell bound Ca^{++}, releasing it to the outside medium. TEA and Ba^{++} prolong the spike apparently by substituting for Ca whereas EDTA and Na Cit. do so by reducing Ca^{++} to a rate limiting amount. A model explaining these results is postulated.

CHAPTER V

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GENERAL DISCUSSION

If it is postulated that Ca^{++} is removed selectively from Cl^- gating sites instead of K^+ gating sites by stimuli that evoke spikes and that mobilized Ca^{++} is required to stimulate the K^+ activation gating mechanism, a process which requires ATP, then one can, using the data presented, explain the threshold for stimulation, the initiation of Cl^- activation, the connection between Cl^- activation and K^+ activation, and the process of K^+ activation itself.

The threshold for stimulation and Cl^- activation.

Ca^{++} probably has to be removed from membrane pore blocking sites for Cl^- activation. This process requires energy in the form of an electrical or mechanical stimulus. Effective stimuli are probably capable of removing a certain number of Ca^{++} from their Cl^- pore blocking sites. Voltage clamp studies show that a certain minimum number of Cl^- channels must be opened in order for complete Cl^- activation to occur (Kishimoto 1968). Cl^- activation in one region of the membrane will initiate Cl^- activation in neighbouring regions. Cl^- activation therefore is regenerative.

Spontaneous activity, as observed in cell treated with Na Cit. and EDTA, can be explained by the removal of Ca^{++} and thus the opening of Cl^- channels. When a certain amount of Ca^{++} is removed from the membrane by means of chelation, a few Cl^- activation channels are opened and a smaller stimulus, indicating a lower threshold, is necessary to meet

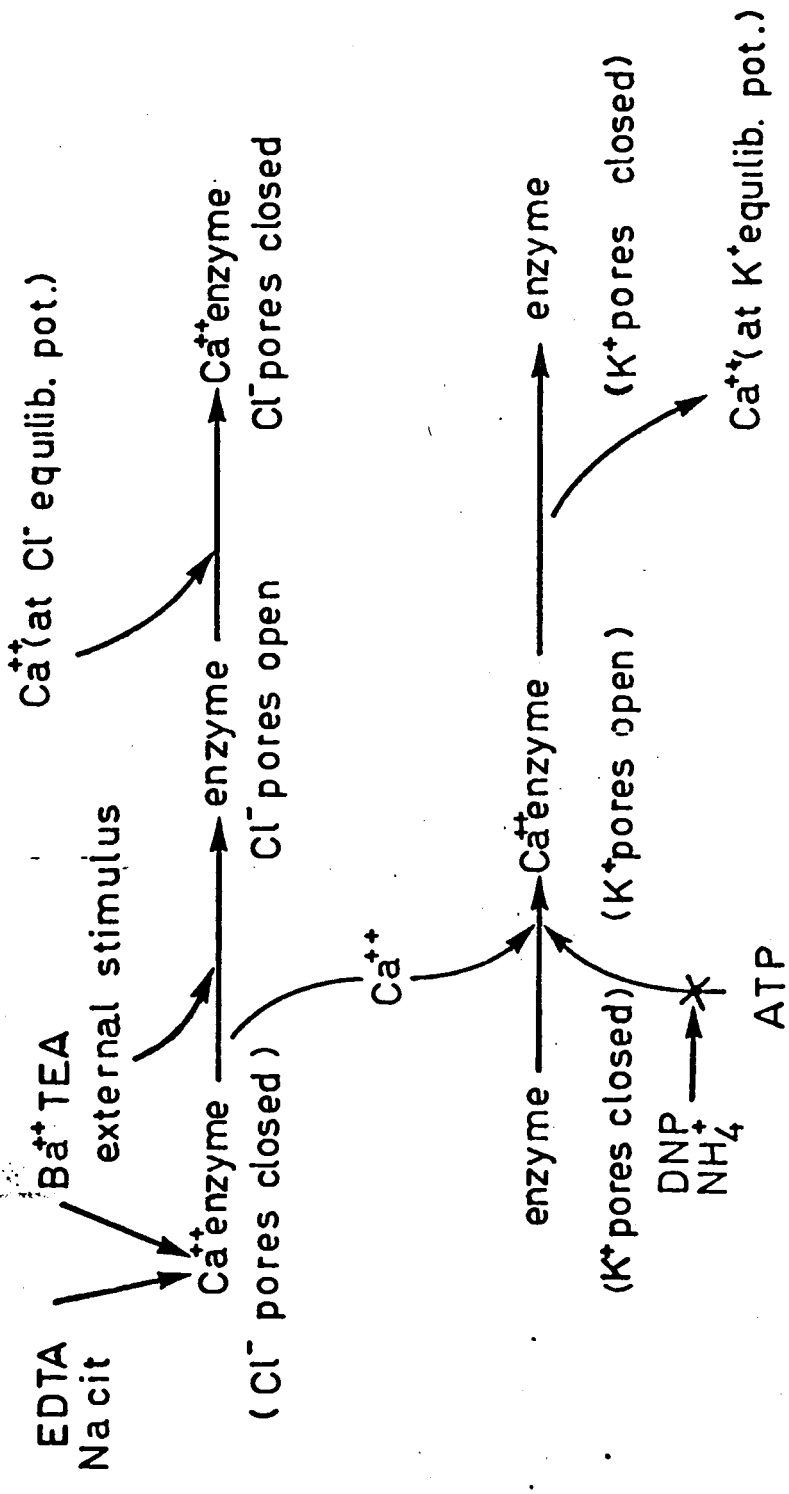
the minimum requirement of open Cl^- channels for regenrative Cl^- activation to occur. If more membrane Ca^{++} is removed by higher concentration of chelators a larger number of blocked Cl^- activation channels is opened and spontaneous activity is observed (Chapter III). As soon as the Cl^- pores are opened, Cl^- diffuses out of the cell down its electrochemical gradient until Cl^- equilibrium is reached. At this time Ca^{++} may have the reverse effect and close the Cl^- pores. In other words, once the Cl^- activation has started, it cannot be stopped until the Cl^- equilibrium potential is reached, i.e., the "all or nothing" principle.

Initiation of K^+ activation and the process of K^+ activation.

The Ca^{++} released from the Cl^- activation blocking sites by an external stimulus, may be freed to attach to sites on the membrane where they can control K^+ pores. This would require that the Cl^- and the K^+ activation sites are relatively close together, perhaps even part of the same mechano-enzyme complex.

The Ca^{++} released during the Cl^- activation could perhaps stimulate the opening of K^+ channels. However, as there is no external energy present, as for example, the external stimulus needed for Cl^- activation, energy in the form of ATP (Chapter III) is probably utilized at this time to open the K^+ channels. The presence of Ca^{++} could trigger this reaction as it does in many other enzymatic processes for example ATPase activity (Wolf 1972). After a number of K^+ channels are opened, the K^+ activation could spread over the entire membrane surface in a regenerative

Figure 1 Schematic representation of a model explaining the role of Ca^{++} during depolarizing Cl^- activation and repolarizing K^+ activation.


EDTA
Na cit
Ba++ TEA
external stimulus
Ca++enzyme
(Cl- pores closed)
enzyme
Cl- pores open
Ca++(at Cl- equilib. pot.)
Ca++enzyme
Cl- pores closed
Ca++
enzyme
(K+pores closed)
DNP
NH4+
ATP
Ca++enzyme
(K+pores open)
enzyme
(K+pores closed)
Ca++(at K+equilib. pot.)

fashion (Chapter V). K^+ will diffuse out of the cell down the electrochemical gradient until the K^+ equilibrium potential is reached, Ca^{++} is released from the K^+ blocking sites, closing the K^+ pores.

In view of this hypothesis, (Fig. 1) the effect of Ba^{++} and TEA can be more fully explained. As shown in Chapter V, Ba^{++} and TEA can substitute for Ca^{++} on the membrane sites because of their stronger affininty for these sites than Ca^{++}. Stronger binding would require more energy for removal from the blocking sites in order to initiate depolarizing Cl^- activation. This is directly supported by the increase in stimulus required to evoke spikes in cells treated with these compounds.

When 10 mM Ba^{++} or 10 mM TEA is directly applied to a cell, not previously exposed to lower concentrations of these compounds, a spontaneous spike is recorded, even though the threshold for subsequent spikes is increased (Chapter I). The phenomenon can be related directly to the substitution of Ca^{++} by Ba^{++} or TEA. During the substitution process the Ca^{++} is momentarily removed from the Cl^- activation blocking sites, before other ions take over the blocking action, initiating Cl^- activation and resulting in a spontaneous spike. This spike is of normal duration, providing direct evidence that the process of Ca^{++} substitution is not yet completed. The fact that high concentrations of Ba^{++} or TEA (10 mM) only produce spontaneous spikes in untreated cells can be explained in terms of Ca^{++} substitution. If lower concentrations of Ba^{++} or TEA (1 mM) are used, no spontaneous spikes are observed because, as explained before a certain minimum number of Cl^- channels must be unblocked for Cl^- activation to occur. Low concentrations of Ba^{++} and TEA

substitute only for a sub-threshold number of Ca^{++} on the membrane, and no spike is initiated. Once some stronger binding ion has substituted for Ca^{++} at the blocking sites the threshold is increased. Therefore, no spontaneous spikes are recorded from a cell previously treated with lower concentrations of Ba^{++} or TEA. The prolonged spikes recorded from cells treated with Ba^{++} and TEA are consequence of the fact that these compounds are a less effective substitute for Ca^{++} in terms of the opening of K^+ channels. More time is required to perform the same function as Ca^{++} when Ba^{++} or TEA have substituted for Ca^{++}. Hence, the prolonged spikes.

The effects of chelating agents on the threshold for stimulation has been explained in the beginning of this discussion. The effects on the K^+ activation can be explained in terms of a lack of Ca^{++} on the stimulating sites for K^+ activation. Prolonged spikes are obtained only when a marginal amount of Ca^{++} is available (as for instance with 1 mM EDTA, 10 mM Na Cit.). Ca^{++} must diffuse from other sources, i.e.more time is needed to open the minimum number of K^+ channels to initiate regenerative K^+ activation and hence prolonged spikes are recorded.

Higher concentrations than 1 mM EDTA or 10 mM Na Cit. result in a spike-like depolarization because no Ca^{++} is available to block Cl^- channels, and as Ca^{++} is not available to initiate repolarizing K^+ activation the cell remains in a depolarized state (Chapter II).

The effects of DNP and possibly of NH_4^+ can be explained by the lack of energy required to open the repolarizing K^+ channels. A rate limiting amount of ATP will take longer to open all K^+ channels, resulting in prolonged spikes.

The foregoing hypothesis which was developed entirely from observation of the electrical behavior of internodal cells of Nitella flexilis, has many aspects in common with other hypotheses based entirely on the electrical behavior of animal cells.

As early as 1949 the effect of Ca^{++} on the spike was recognized (Hodgkin 1949). At that time it was postulated that Ca^{++} competes with Na^{+} for a highly negatively charged lipid soluble carrier molecule (Hodgkin 1949). However, the effects of Ca^{++} could not be completely accounted for using Hodgkin's ionic hypothesis (Hodgkin 1949, Monnier 1949).

Duncan (1967) has recently suggested three possible molecular mechanisms for the Na permeability changes that occur during electrical activity of the squid axon and other animal cells, one of which is quoted below.

> "the electrotonic current displaces the calcium ("inhibitory") ions from their strategic, "blocking" sites on the ATPase and that this action alone is sufficient to allow the rapid change of membrane-permeability and entry of sodium ions. However, removal of the inhibitory calcium ions also activates the ATPase system, causes hydrolysis of organic phospates, and accounts for some of the loss of inorganic phosphate from nerve and muscle after activity. Consequent upon this activity is a change of molecular configuration of the enzyme system, causing the observed changes in light-scattering and protein configuration. On this hypothesis, these changes would take

place during the falling phase of the action potential and during the refractory period and recovery processes which follow it." (Duncan 1967)

As can be noted, all basic aspects postulated for the regulation of excitation in Nitella cells are presented. The ions which are regulated are different, squid axon Na^+ and K^+, Nitella Cl^- and K^+, but the mechanisms appear to be very similar if not the same.

Furthermore, an ATP activated protein resembling actomyosin dependent on divalent cations for contraction, has been isolated from Nitella cells (Verobyeva and Poglazov 1963). This protein appears to be directly coupled to excitation and cyclosis (Barry 1968). Protein microfilaments have been observed in Nitella cells and are also thought to be responsible for cyclosis (Nagai and Rebhun 1966). It is probable that similar or even the same contractile proteins are involved in the opening and closing of ion channels as described in Chapter V, similar to the ATPase-ATP system discussed by Duncan (1967).

The erythrocyte membrane has been extensively used as a model for excitable membranes (see Duncan 1967) because of the similar behavior of erythrocyte membranes and cells like squid axons and amoeba.

Recent communications in erythrocyte research indicate that the deformability of these cells is directly related to the amount of ATP present, and is controlled by membrane bound Ca^{++} (Chau-wong and Seeman 1971). Wolf (1972) has shown that Ca^{++} is not required for binding ATP to the ATPase but is essential for breaking the energy rich phosphate bonds. The catalytic centre of the ATPase contains 3 active groups used

during the catalytic reaction. The contractile Ca^{++} dependent ATPase-ATP system of the erythrocyte is analogous if not homologous with the model I proposed for the Ca^{++} dependent ATPase-ATP system regulating the opening and closing of ion channels in nerve and excitable plant cells.

Recent studies on cyclic depsipeptides and peptides show that these molecules are capable of binding alkali metal ions by forming cationic complexes (Andreev et al. 1971) these could act as transporters of cation across hydrophilic zones of biological membranes (Resman et al. 1967, Pressman 1968). The cyclodecapeptide, Antaminida, isolated from the highly poisonous mushroom Aminita phalloides, is an antidote for the toxic peptide, aminitine, synthesized by the same mushroom (Wieland et al. 1968). I have tested crude extracts of an Aminita species on ^{45}Ca loaded Nitella cells and observed release of ^{45}Ca followed by rhythmic spontaneous firing of the cell. These findings indicate that some toxic substance, probably the peptide aminitin present in the mushroom, interfere with the normal ion movement across Nitella cell membranes. The fact that the antidote to aminitin is a cyclodecapeptide, capable of binding alkali metal ions could also indicate that cyclic peptides might have a function in ion transport in Nitella cells. Studies on artificial membranes involving cyclopeptides (Shemyakin et al. 1969) could prove to be valuable in explaining certain ionic phenomena in Nitella cells.

Other studies on artificial membranes have shown that detergent micelles can act as a model for biological membrane pores These micelles bind Ba^{++}, Mg^{++} and Ca^{++}. It was postulated that Ca^{++} has a regulating

function in respect to phospholipid interactions (Buzzel 1970).

It is evident that future studies using artificial membranes could clarify the role of Ca^{++} in activation and inactivation processes.

It might be argued that Nitella is a very specialized cell, however as there is no known effector coupled to excitation, the electrical activity is probably very basic relative to electrically exitable animal cells.

Photosynthesis could also effect the spike, however I have observed no changes in the duration of the spike under different light conditions, changes in membrane potential and effective resistance are observed.

The model of the processes during the spike is, to my knowledge, the simplest although other more complex models could also explain the phenomena observed.

In this thesis some questions have been answered but others have been raised.

With the use of the manipulator and counting arrangement as described in Chapters IV and V, questions concerning ion movement as, for example, during the brief spikes observed after prolonged spikes of Ba^{++} treated cells (Ch. I) or the hyperpolarization of cells treated with TRIS Cl and TRIS prop., (Ch. 2), the mechanism of K^{+} activation under certain drug conditions and other problems could be solved using the appropriate radioisotope.

Other experiments measuring the effect of known metabolic inhibitors on the electrical parameters of the cell could provide additional information about the requirement for and source of energy used during the excitation process of the cell.

Voltage clamp data could clarify the action of some of the drugs used

with respect to their effect on Cl^- and K^+ conductances and current densities but could also provide information whether K^+ activation is regenerative as is the case with Cl^- activation.

Data from these experiments as well as those involving other branches of science such as fluid dynamics, biochemistry, histology, anatomy etc. are essential to explain the excitation process, relative to the other processes occuring in a living cell.

GENERAL CONCLUSIONS

In summary, the conclusions of each Chapter answer the questions posed in the Introduction as follows:

1. The effects of Pharmacological agents that interfere with K^+ activation in nerve and muscle cells have entirely similar effects on Nitella cells.
2. Agents that prolong the spike evidently do so by substitution for Ca^{++} to a rate limiting amount.
3. The repolarizing K^+ activation phase of the spike in Nitella appears to be energy dependent.
4. Evidence for the mode of action of drugs that prolong the spike can be obtained from cells loaded with ^{45}Ca and ^{131}Ba. Ca^{++} is released from the cell on application of the drug before the prolonged response is obtained. Ba^{++} is tightly bound to the cell, the washout curve of Ba from a cell loaded with ^{131}Ba coincides with the time required to reverse some effects of Ba^{++} on the spike.
5. The basic mechanism that regulates depolarizing and repolarizing electrogenesis is similar, if not, the same as in nerve and muscle cells. A simple model was proposed which adequately explained the observation made.

GENERAL DISCUSSION

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A P P E N D I X

Preliminary investigations of the binding of Ba^{++} to cells of *Nitella flexilis*

APPENDIX

INTRODUCTION

In Chapter V it was shown that the effect of Ba^{++} on the duration of the spike was directly related to the removal of cell bound Ca^{++}. Although it was concluded that Ba^{++} substitutes for Ca^{++} on the membrane, no direct evidence to support this was available.

Preliminary experiments were carried out using $^{131}Ba^{++}$ to provide such evidence; they are described in this Chapter.

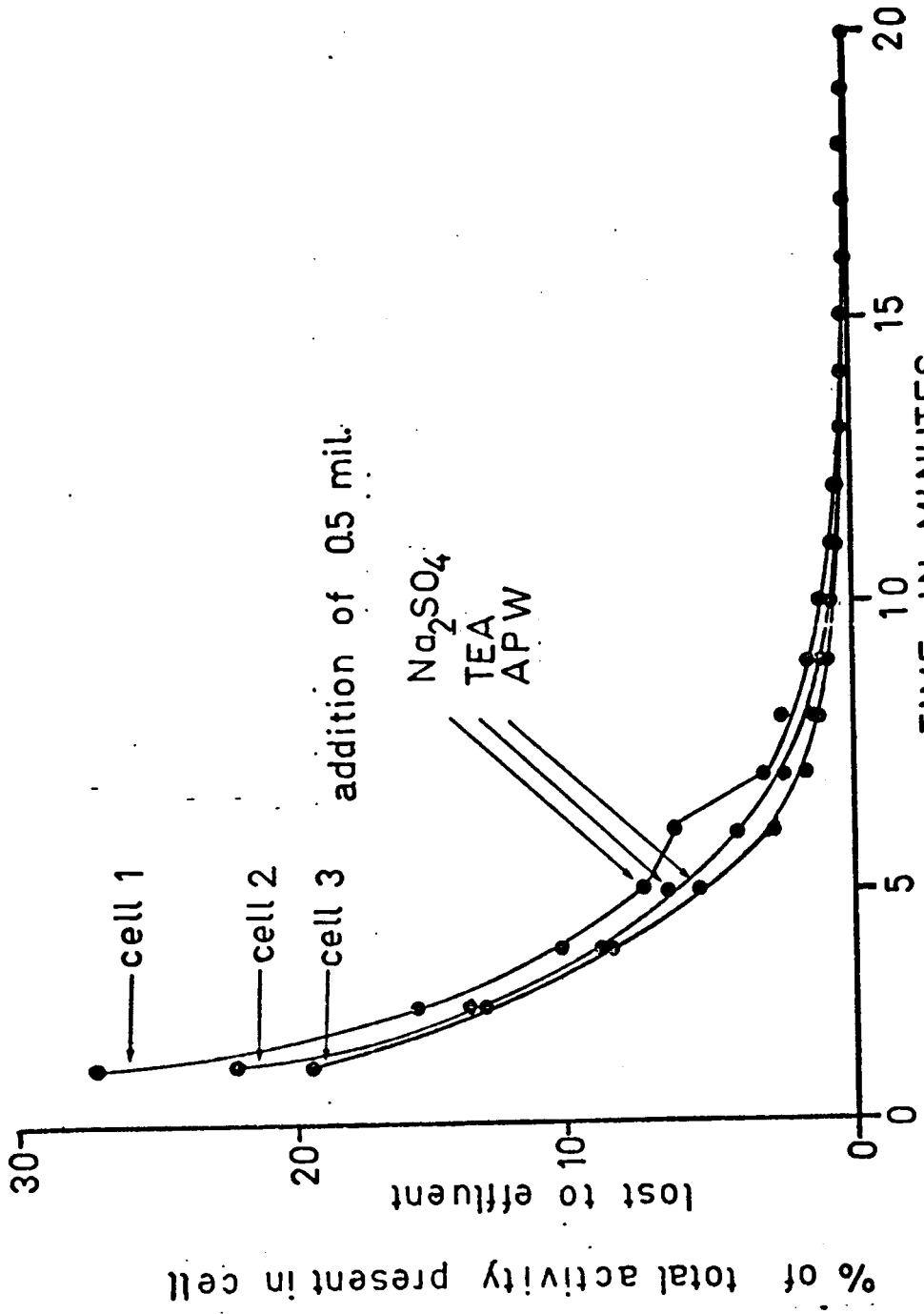
MATERIALS AND METHODS

Internodal cells of Nitella flexilis were placed for 1 minute in a 10 ml solution of 0.4 m ci ^{131}Ba in APW. (New England Nuclear, specific activity 3.6 m ci/mg). At the end of this period the cells were removed, rinsed in APW, and placed individually in a Buchner funnel on top of a filter paper. APW was rinsed over the cell at a constant rate of 1 ml/min. Samples were collected at one minute intervals. At the end of 5 min. a 0.5 ml sample of 100 mM Na_2SO_4, TEA or APW was added to the funnel containing the cell. Samples were collected as before. A new cell was used for every drug. All experiments were repeated three times.

RESULTS

Typical wash out curves are shown in Fig. 1. The percent of the total activity in the cell, lost to the effluent has been plotted

Figure 1 Wash out curves of internodal cells of Nitella after a 1 minute loading period in ^{131}Ba. Cell 1, 0.5 ml of 100 mM Na_2SO_4 added at time 5 minutes. Cell 2, 0.5 ml of 100 mM TEA added at time 5 minutes. Cell 3, 0.5 ml of APW added at time 5 minutes.


% of total activity present in cell
lost to effluent
30
20
10
0
0
5
10
15
20
TIME IN MINUTES
addition of 0.5 mil.
Na_2SO_4
TEA
APW
cell 1
cell 2
cell 3

against time. The activity of cells 1, 2 and 3 before and after a 20 minute washing period with APW is shown below.

Cell number	Treatment	#cnts in cpm before	#cnts in cpm after	% left in cell at the end of experiment
1	.05 ml 100 mM Na_2SO_4	11681	1840	1.4
2	.05 ml 100 mM TEA	143188	2098	1.49
3	.05 ml 100 mM APW	90351	1579	1.75

DISCUSSION

In Chapter I it was observed that the effect of Ba^{++} on the spike duration was not readily reversible by washing with APW. The data in Fig. 1 show that after 20 minutes of washing, with APW, on the average 1.5% of the total activity is still present in the cell. Addition of TEA to the ^{131}Ba loaded cell did not release additional activity, this is also supported by the fact that addition of TEA to a Ba^{++} treated cell does not shorten the time required to reverse the prolongation of the spike. However, I have observed an instantaneous reversal of the prolonged spike recorded from Ba^{++} treated cells on addition of solutions containing $SO_4^{=}$. It is likely that $SO_4^{=}$ reverses the prolongation of the spike brought about by Ba^{++} by combining with it. $BaSO_4$ is evidently released from the cell as shown by the curve for cell 2 in Fig. 1.

CONCLUSION

Ba^{++} are tightly bound to the cell and are not rapidly washed out coinciding with the difficulty of reversing the prolonged spikes of Ba^{++} treated cells with continuous washing. $SO_4^{=}$ can reverse the prolonged spike recorded from Ba^{++} treated cells by causing Ba^{++} to be released from the cell probably as $BaSO_4$.

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