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COLCHICINE REVERSIBLY INHIBITS ELECTRICAL
ACTIVITY IN ARTHROPOD
MECHANORECEPTORS

THESIS

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Paul D. Reagan B. S.

Denton, Texas

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Dendrites of cockroach tibial spine mechanoreceptors contain hundreds of free microtubules, which may have some relation to the generation of electrical activity. Deflection of a spine produces a train of action potentials. Continuous perfusion over a period of 4 hours results in no response decrement. Perfusion with 10mM colchicine reversibly inhibits the response within 5-7 minutes. Irreversible inhibition is produced by perfusion with 1mM vinblastine sulfate in perfusion solution containing 1% dimethyl sulfoxide. Deuterium oxide does not inhibit at concentrations less than 50%, nor does it counteract inhibition by 10mM colchicine.

Colchicine may be affecting (1) intracellular microtubules, (2) membraneous tubulin, (3) other membrane components, or (4) axoplasmic transport of essential materials to the sensory dendrites.

TABLE OF CONTENTS

	Page
LIST OF TABLES	iv
LIST OF ILLUSTRATIONS.	v
 Chapter	
I. INTRODUCTION	1
Morphology Biochemistry Distribution Function Assembly Sensory Neurons Disruptive Agents Colchicine Campaniform Organ Purpose	
II. MATERIALS AND METHODS.	12
Animals Stimulation Recording Perfusion Summary	
III. RESULTS.	18
Train Duration as a Measurement Necessity for Constant Perfusion Longevity of Preparation Rate of Stimulation Effects of Microtubule-Active Agents	
IV. DISCUSSION	65
Adapting Properties of the Mechanoreceptor Colchicine and Vinblastine Inhibition Effects of Deuterium Oxide Effects of Tetrodotoxin and 2,4-Dinitrophenol Related Experiments Where Do Colchicine and Vinblastine Act? Summary	
REFERENCES	73

LIST OF TABLES

Table	Page
I. Summary.	64

LIST OF ILLUSTRATIONS

Figure	Page
1. Instrumentation and Preparation.	13
2. Effect of Stopping Perfusion	25
3. Continuous Perfusion	27
4. Effect of Varying Stimulus Rate.	29
5. 1mM Colchicine	31
6. 4mM " 	33
7. 5mM " 	35
8. 6mM " 	37
9. 7mM " 	39
10. 8mM " 	41
11. 9mM " 	43
12. 10mM " 	45
13. 1mM Vinblastine.	47
14. 1×10^{-5} M Tetrodotoxin	49
15. 0.1mM 2,4-Dinitrophenol.	51
16. 10% and 20% Deuterium Oxide.	53
17. 30% Deuterium Oxide.	55
18. 40% " " 	57
19. 50% " " 	59
20. 60% " " 	61
21. 70% " " 	63

CHAPTER I

INTRODUCTION

Morphology

Microtubules (MT) are hollow-appearing filaments of protein which have become the subject of extensive research since the introduction of gluteraldehyde fixation for electron microscopy. Prior to the 1960's, intracellular microtubules were often confused with microfilaments. A clear distinction has now emerged between these two major intracellular fibrous proteins. Microfilaments are actin-like polymers which share no chemical similarity with microtubules. A third type of fibrous protein, neurofilaments, are found only in nerve cells, and differ from microfilaments. They are much larger in diameter and are not made up of actin-like subunits (Yamada et al., 1970; Fine and Bray, 1971).

Microtubules have the most elaborate structure among the three types of neuronal fibrous proteins. Microtubules appear to be hollow cylinders 24 ± 2 nm in diameter with an electron lucent center 15 nm in diameter (Olmsted and Borisy, 1973) and 5 nm thick walls. The wall is made up of eleven to thirteen protofilaments, which can be seen in cross section (Ambrose and Easty, 1970; Guttman, 1971; Erickson, 1975).

Behnke and Forer (1967) proposed four subclasses of MT based on structural and chemical stability.

1. Cytoplasmic or free MT, the most sensitive, are labile to colchicine and low temperature (2-4°C).

2. The central pair of MT found in the 9 + 2 complex of motile cellular extensions, cilia and flagella, are sensitive to high colchicine concentrations and prolonged low temperature (0°C).

3. The peripheral doublets of the 9 + 2 complex fall into two groups. The "B tubules" are insensitive to colchicine and decreased temperature, but disrupted by pepsin and high temperature (50°C).

4. The "A tubules" of ciliary and flagellar doublets are the most stable. They are insensitive to colchicine and low temperature, and only slightly sensitive to high temperature.

Olmsted and Borisy (1973) recognize the four classes of Behnke and Forer, but suggest that degrees of stability are probably due to "slight modifications of subunits or the association of other materials with tubules, such as inter-microtubular links...." Thus in the opinion of these later investigators there maybe no difference between the four classes of tubules.

Biochemistry

Microtubules are composed of 13 protofilaments arranged to form a cylinder. Each protofilament is composed of dimers, and each dimer composed of one alpha and one beta tubulin monomer. The molecular weight of the dimer is approximately 110,000 daltons, with each of the tubulins contributing 55,000 daltons (Rosenbaum, 1975; Erickson, 1975).

Distribution

Although MT may have different classifications, they are remarkably similar structurally and chemically. Such similarities are found in all eukaryotic cells from protozoa to multicellular organisms (Bryan, 1973). Microtubules have also been reported in at least two species of bacteria (Iterson et al., 1967; Pope and Jurtshuk, 1967; Vela et al., 1970).

Microtubules are found throughout the cell and cellular appendages. Motile cilia and flagella have a 9 + 2 doublet organization, while nonmotile cilia and flagella, centrioles, and basal bodies usually have a 9 + 0 doublet or triplet organization. Other cytoplasmic MT occur singly and are generally referred to as "free microtubules." They are often associated with other cellular organelles such as the nucleus, golgi apparatus, and mitochondria (Burnside, 1975). In addition, the use of fluorescent antibodies and radioisotopically labeled colchicine has disclosed the presence of microtubule protein or colchicine-binding activity associated with

cytoplasmic membranes (Feit and Barondes, 1970; Wilson, 1970; Dahl et al., 1970; Lagnado et al., 1971; Stadler and Franke, 1972, 1974).

Function

Microtubules function in a variety of cellular processes including motility (cilia and flagella), maintenance of cell shape, orientation of cellular structures, outgrowth of axons and dendrites in cultured neurons (Burnside, 1975; Olmsted and Borisy, 1973; De Robertis et al., 1971). Microtubules have tensile properties which play an important role in the arthropod connective tissue (Burnside, 1975). Arthropods contain no fibrocytes or collagen fiber producing cells, but have cells packed with MT which provide structural strength. Of interest in the present study is the fact that arthropod glial cells contain just as many MT as do neurons (Smith, 1968). Microtubules also function in intracellular transport, including the movement of organelles, metabolites, and biosynthetic precursors to various areas of the cell (Burnside, 1975). An important neuronal function is the transfer of neurotransmitter precursors to synapses (De Robertis et al., 1971; Olmsted and Borisy, 1973; Rosenbaum et al., 1975).

Sensory transduction has been suggested as a function of MT. According to Atema (1973, 1975), MT activate a generator potential in olfactory cells by initiating depolarization of the neuron and, if large enough, activate propagated

action potentials. Spondelin (1968) has suggested that the hair cells within the cochlea of the ear are activated via MT found within each cell. Callatay (1969) and Hejnowicz (1970) has suggested that MT play an active role in producing generator and action potentials within the neuron.

Assembly

Assembly of MT involves several steps and accounts for the differences between stable and labile MT noted above. Stability seems to be related to the chemical bonding pattern between the subunits (Behnke and Forer, 1967; Wittman, 1975; Inoue' and Sato, 1967). The extensive pattern of cross bridges between ciliary and flagellar microtubules suggests that cross-binding confers stability, i.e., low turnover and resistance to disrupting agents.

The formation of tubules takes place by addition of monomers or dimers at one end of the MT. Sensitivity to various disruptive agents is due to the constant turnover of subunits within the tubules (Behnke and Forer, 1967; Wittman, 1975; Rosenbaum et al., 1975). A "dynamic equilibrium" (Inoue' and Sato, 1967) is maintained between formed MT and free tubulin pools. Treatment with disruptive agents, which bind to the soluble subunits, shifts the equilibrium toward the dissociated state. Stabilizing agents shift the equilibrium toward the formed MT.

Microtubules are formed or lengthen by two methods from the soluble tubulin pools. The tubule lengthens when the

dynamic equilibrium shifts toward the associated state, in which alpha and beta tubulin attach to the preformed microtubule. The second method involves alternating attachment of the alpha and beta tubulins, 55,000 daltons each (Burnside, 1975; Rosenbaum et al., 1975; Erickson, 1975), forming a coiled filament, a protofilament. The protofilaments attach to one another side by side, as they uncoil forming a sheet of filaments. Due to some biophysical requirement, 13 adjacent protofilaments (Erickson, 1975) form a tubule. The uncoiling protofilaments acquire tubulin monomers from the free tubulin pools (Erickson, 1975). This causes lengthening of the tubule to its functional length. The equilibrium between the tubulin pools and formed MT maintains this length.

A specific example of cytoplasmic MT in a state of dynamic equilibrium occurs in neuronal axons and dendrites (Chapman, 1965; Daniels, 1975; Moran and Varela, 1971). These "neurotubules" are assembled in the same fashion as other MT and increase in length by addition of tubulin to the distal ends. Tubulin is synthesized in the cell body and transported via preexisting MT to the distal portion of the extensions and added to the MT (Daniels, 1975).

Sensory Neurons

Neurotubules are found in all nerve cells: motor, sensory, and interneurons. In all neurons investigated, MT serve to transport the neurotransmitters, metabolic precursors,

proteins, lipids, and many other cellular constituents to distal areas. Dendritic neurotubules are most abundant in sensory receptors, including thermal receptors, mechanoreceptors, chemoreceptors, and photoreceptors (Dyson, 1974; Ham, 1974; De Robertis et al., 1970).

Mechanoreceptors, the subject of this investigation, are widely distributed. Mechanoreceptive modalities include touch, hearing, balance, vibration, and proprioception. Most mechanoreceptors have dendrites which are ciliary outgrowths. The dendrites are often further specialized (e.g., laminated nerve endings) (Ham, 1974). Mechanoreceptors are activated by deformation of the dendritic membrane of the neuron, eliciting a generator potential (Kuffler and Nicholls, 1976). Mechanoreceptor generator potentials are generally produced by increased sodium membrane conductance. Based on one experiment, and the circumstantial presence of large numbers of MT in mechanoreceptor dendrites, Moran and Varela (1971) have proposed a transducing role for MT in arthropod mechanoreceptors.

Disruptive Agents

Alteration in cellular function often follows MT disruption by antimitotic agents. For example, axonal transport is interruptible by colchicine, colcemid, and the vinca alkaloids (Margulis, 1973).

Colchicine binds to tubulin in the soluble pool within the cell. Tubulin with colchicine bound to it is incapable

of recombining with the formed MT, thus shifting the dynamic equilibrium toward the dissociated state (Wilson et al., 1974). Colchicine specifically binds to tubulin (Borisy and Taylor, 1967), but an analog, lumicolchicine, does not (Wilson et al., 1974) and can be used as a control for non-specific effects of colchicine.

Podophyllotoxin also binds to tubulin, shifting the equilibrium toward the dissociated state. Similarity to colchicine is due to attachment at the same binding site (Wilson, 1975; Margulis, 1973). The inactive isomer, picropodophyllotoxin provides verification of the specific action on MT and on tubulin.

The vinca alkaloids, vinblastine and vincristine, like colchicine, bind to soluble tubulin. Unlike colchicine, the vinca alkaloid-tubulin complex aggregates to form highly organized crystals. Vinblastine binds to the tubulin at a different site from the colchicine binding site. There are two types of binding sites, the high affinity binding site, which displaces guanosine triphosphate, and the low affinity site, characterized by the displacement of calcium when vinblastine attaches (Olmsted and Borisy, 1973). Griseofulvin affects functions ascribed primarily to MT, but treatment with this agent produces no obvious alteration of the MT structure or distribution when examined with the electron microscope (Wilson, 1975; Hoebeke, 1975).

Stabilizing Agents

Stabilizing agents provide an added chemical tool which allows greater precision in evaluating MT function. Such agents are rapidly becoming necessary tools to evaluate MT function. Heavy water, deuterium oxide, has been demonstrated to stabilize MT (Malaisse et al., 1975; Salmon, 1975; Daniels, 1975; Inoue' and Sato, 1967). Presumably deuterium oxide (D_2O) displaces ordered water on the tubulin molecules to produce a stabilization (Inoue' and Sato, 1967). Binding to MT shifts the tubulin-MT equilibrium toward the associated state. Deuterium oxide overstabilizes MT altering their function (Salmon, 1975). This observation seems to indicate that the dynamic equilibrium is necessary for proper functioning of MT. Further biochemical studies need to be done for D_2O and another agent, dimethyl sulfoxide (DMSO), in order to determine their specific actions on tubulin.

Colchicine

Alteration of cell form, cellular movement, secretory processes, neurotransmission, and presently, alteration of mechano-sensory activity in the nervous system have been shown to be colchicine sensitive. In the last two decades colchicine has been shown to disrupt MT and has become the chemical tool of choice with which to investigate MT.

Colchicine is an alkaloid extracted from the plant Colchicum autumnale. It is sensitive to ultraviolet light (UV) at about 355 nm wavelength, and exposure to UV converts

colchicine to lumicolchicine (Wilson and Friedkin, 1966). Colchicine and lumicolchicine have been reported to alter the cellular uptake of the nucleosides, thymine, uracil, guanine, and adenine (Margulis, 1973). Being equally active as inhibitors of nucleoside uptake, an effect on MT can be ruled out as causing such inhibition, because lumicolchicine is inactive as a MT disrupter (Wilson and Meza, 1973).

The polymerized tubulin in MT is insensitive to colchicine, but it binds to all soluble tubulin that becomes available, thus shifting the dynamic equilibrium toward the dissociated state. Wilson and Meza (1973) have shown that the tubulin dimer has one colchicine binding site, which is inaccessible in the assembled MT. Colchicine-tubulin bonding has been demonstrated not to be covalent or electrostatic, but probably involves hydrogen bonding (Wilson et al., 1974).

Campaniform Organ

The investigation of the possible involvement of MT in the process of sensory transduction should be on a simple system, in which as many variables as possible are eliminated. The insect campaniform organ is a single unit mechanoreceptor containing one sensory neuron and numerous MT in the sensory (dendritic) portion of the neuron. Pringle (1936) suggested that the tactile spines on the cockroach leg contained a single mechanoreceptive neuron, and Pumphrey (1936) illustrated the use of tactile spines as a preparation with which to study mechanoreceptors.

The tactile spine campaniform organ is a derivative of (and attached to) the cuticle. Movement of the spine causes the deformation of the cuticular dome covering the sensory receptor. Distortion of the dome somehow results in electrical excitation of the receptor. Underlying the dome is a receptive dendrite which contains several hundred to a thousand MT (Moran and Varela, 1971). The MT extend from a connecting cilium, such as that found in other sensory cells. The receptor's cell body is found in the periphery, near the dendrite, and has an axon which feeds into the leg nerve, and carries impulses to the thoracic ganglia.

Moran and Varela (1971) proposed that the dendritic MT may play a role in mechanotransduction and showed that the use of the MT disruptors, colchicine and vinblastine, would inhibit electrical activity in the receptors. They suggest that the MT in some fashion activate a generator potential in the dendrite which is transformed into a train of action potentials for conduction down the afferent nerve.

Purpose

The purpose of this investigation is to repeat and expand Moran and Varela's (1971) experiments on colchicine inhibition of the electrical activity in the campaniform organ. The effects of another MT disruptive agent, vinblastine, will also be evaluated. Tetrodotoxin and 2,4-dinitrophenol have specific effects on neuronal activity, and their relationship to colchicine effects will be evaluated.

CHAPTER II

MATERIALS AND METHODS

Animals

Male and female cockroaches, Periplaneta americana L., were used for all experiments. Colonies were maintained on Purina Lab Chow, apples, and water. The metathoracic leg was severed at the coxa and mounted on modeling clay pressed on to a Plexiglas block (Chapman and Duckrow, 1975; Moran and Varela, 1971; Chapman, 1965). Experiments were done at ambient room temperature, 23-26°C.

Stimulation

Tibial tactile spines were stimulated by flexing them proximally (Pumphrey, 1936) with a tungsten wire loop attached to a toothpick. The toothpick, attached to a small speaker cone, was driven by square wave pulses from a Grass Model S88 Stimulator. The onset of the square wave pulled the spine proximally and at cessation of the pulse, the spine was returned to its resting position. The degree of spine movement back and forth depended on the magnitude of stimulator output voltage, which varied from 10-20 volts. In all experiments the stimulus amplitude (voltage) was adjusted to elicit a train of action potentials 300 msec long. This typically required a spine deflection of approximately 15°.

FIGURE 1

Instrumentation and preparation

Recording

Figure 1. illustrates the stimulating and recording setup. Action potentials were detected using extracellular electrodes (Chapman, 1965; Moran and Varela, 1971). A perfusion syringe needle inserted into the tarso-tibial joint served as the indifferent electrode. The femur was impaled with the active electrode, another modified syringe needle. Signals picked up by the electrodes were amplified by a WPI differential preamplifier (model DAM 5A). The bandpass filter of the preamplifier was set at 10Hz (lower -3dB cutoff frequency) and 10KHz (upper -3dB cutoff frequency). The preamplified signal was fed to a Tektronics high gain preamplifier (Model AM 502, low frequency cutoff at 100Hz and high frequency cutoff at 3KHz). The second preamplifier permitted greater amplification with a narrower band width. The signal was then displayed on a master oscilloscope (Tektronics 5103 model D11) which controlled a slave oscilloscope (Tektronics 5103 model D10). The slave oscilloscope display was photographed by a Grass C4R Kymograph Camera to provide a permanent experimental record. The film, Kodak Plus-X Pan, was developed in D76 or D19 developer depending on availability.

Perfusion

Many investigators have determined the ionic concentrations of Periplaneta americana hemolymph and serum (Tobias, 1948; Asperen and Esch, 1956; Lockwood, 1961; Treherne and

Beament, 1965; Edney, 1966; Guthrie and Tindall, 1968).

Hemolymph ionic concentrations are differentiated from serum concentrations, in that serum is defined as the extracellular fluid only, while hemolymph includes the blood cells (hemocytes). All the documented concentrations closely approximate one another, with that of Tobias (1948) being typical. A physiological solution was developed to closely approximate the serum concentrations of ions, determined by Tobias.

Normal Perfusion Fluid:

<u>Compound</u>	<u>Concentration (mM)</u>
NaCl	117.4
KCl	15.0
CaCl ₂	4.0
MgCl ₂	1.8
NaHCO ₃	10.0
Sucrose	110.0
Trehalose	10.0

The osmotic concentration of the perfusion fluid was maintained at 410 mOsm/liter in all solutions (Edney, 1966; Prosser, 1973; Shaw and Stobbart, 1963). Sucrose, physiologically inert in cockroaches, was varied in concentration with the addition of test compounds in order to maintain 410 mOsm/liter.

Experimental Solutions:

1. Normal perfusion fluid
2. Perfusion fluid with 1-10mM colchicine
3. " " with 1mM vinblastine sulfate in
 1% dimethyl sulfoxide (DMSO)
4. Perfusion fluid with 1×10^{-5} M tetrodotoxin (TTX)
5. " " with .1mM 2,4-dinitrophenol (DNP)
6. " " with 10-70% deuterium oxide (D_2O
 or heavy water)
 - a. Without 10mM colchicine
 - b. With 10mM colchicine

The leg was perfused via gravity flow through a 27 gauge syringe needle inserted into the tarso-tibial joint. The rate of perfusion varied among legs, but the average rate was approximately 1.5 ml/hour. The perfusion fluid flowed through the tibia and femur, draining at the coxal end of the femur.

Summary

The leg was removed and mounted on the perfusion needle and supported with modeling clay. Perfusion was started immediately and the femur impaled with the active electrode. The tungsten loop, attached to the stimulating probe was positioned on a spine, and the stimulus intensity adjusted to give a 300 msec spike train every five seconds. Results were monitored visually every five seconds, and by the kymograph

camera, which was triggered every 60 seconds. A Bioelectric Reflexor camera mount (model RCM-3) counted the time in seconds and allowed photographing the time with each frame of film taken.

CHAPTER III

RESULTS

Train Duration as a Measurement

The duration of the spike train was used as an indicator of treatment effects. Responses greater than 250 msec were normal and less than 250 msec were abnormal.

Necessity for Constant Perfusion

Constant perfusion of the cockroach leg was necessary to maintain responses. Spike train duration declined rapidly when continuous flow of the normal perfusion fluid was interrupted. Typically, the response duration declined to almost zero four to five minutes after cessation of flow. When flow was reestablished, the response returned to greater than 250 msec (Figure 2).

Longevity of Preparation

The effects of constant perfusion over time were investigated, since the experiments ran from a few minutes to a few hours. In several experiments, train duration was maintained at the maximal level for four hours during continuous perfusion and stimulating every five seconds. No experiment lasted more than four hours. The manual deflection of other tactile spines on the same leg showed no difference with the spine stimulated continuously (Figure 3).

Rate of Stimulation

The rate of stimulation was varied to determine the adapting properties of the preparation using a 300 msec repetitive stimulus. Response duration decreased when the interstimulus interval was less than 3-4 sec (Figure 4). A five second interval was adopted for subsequent experiments since it is near the threshold for adaptation. The effects of drug treatments might become observable through changes in spike train length, caused by changes in the adapting threshold. Experimental parameters used throughout further experimentation were continuous perfusion, 5 sec interstimulus interval, 300 msec stimulus duration, and limiting the time of each experiment to less than four hours.

Effects of Microtubule-active Agents

Colchicine

Colchicine, a known MT disruptor, did not affect the response through the concentration range of 1-4mM (Figures 5 & 6). At 5mM concentration, colchicine decreased the response duration and induced erratic shifts in duration from one stimulation to the next after 15 minutes of colchicine perfusion. This effect was not well defined even after 45 minutes of perfusion (Figure 7). At 6mM, a decrease in train length appeared at 15 minutes, followed by a further decline to a level slightly above 225 msec by 30 minutes (Figure 8). At 7mM, the response duration began to decline

at 20 minutes, reaching 112 msec by 45 minutes (Figure 9). At 8-9mM, the response duration dropped rapidly to about 120 msec (Figure 10-11). In each case, return of the response duration to a maximal level upon reperfusing with normal solution followed approximately the same time course as the decline. At 10mM, inhibition was seen within 5 minutes and the response duration reached 60 msec within 30 minutes (Figure 10). Both decline rate and recovery rate appear to be concentration dependent. Inward and outward diffusion of colchicine to its site of action would be concentration dependent. Reperfusing with normal solution gradually dilutes the colchicine to an ineffective concentration.

Vinblastine

Vinblastine sulfate, another MT disrupter, was investigated to determine its effect on train duration. It was necessary to dissolve the vinblastine in perfusion solution containing 1% dimethyl sulfoxide (DMSO), because it is relatively insoluble in water. Control perfusion with 1% DMSO produced no inhibition, but vinblastine definitely caused a decline in response duration within 10 minutes, and was irreversible (Figure 13). The response failed to return upon reperfusion with 1% DMSO in normal perfusion solution.

Tetrodotoxin

Microtubule protein seems to be closely associated with the membranes of some cells (Stadler and Franke, 1974). Since

the possibility existed that colchicine's inhibitory effect resulted from an interaction with membrane-associated tubulin, it seemed wise to test the effects of an agent known to affect membrane ion fluxes. Therefore, tetrodotoxin (TTX), a compound known to affect the excitability of the neuronal membrane, was used to evaluate the similarity of effect on the duration (Figure 14). Perfusion with $1 \times 10^{-5}M$ tetrodotoxin produced an inhibition whose form and time course was similar to that produced by 10mM colchicine. A decline in the average amplitude of individual action potentials was also produced (a factor not seen with colchicine inhibition). Tetrodotoxin also produced an irreversible inhibition similar to vinblastine's effect. The time course of the decline of train duration paralleled the colchicine data.

2,4-Dinitrophenol

Dynamic equilibrium of the MT with soluble tubulin requires an energy source in the form of ATP or GTP. Lack of the energy source inhibits mitotic spindle formation and several other MT-related processes (Rebhun et al., 1975). Inhibition of action potentials by 2,4-dinitrophenol (DNP), an inhibitor of oxidative phosphorylation, is well known, but the relationship to MT in the present preparation has yet to be investigated. Treatment with 0.1mM DNP produced a decline in train duration paralleling the effects of colchicine (Figure 15). A decrease in train duration is seen at 10 minutes and continues to decline to 10^4 sec

within 35 minutes. The response returns rapidly with normal fluid perfusion in a pattern similar to recovery from colchicine inhibition.

Deuterium Oxide

The leg was perfused with deuterium oxide (D_2O), a MT "stabilizer," to evaluate its effect on the train duration. Deuterium oxide was applied both with and without 10mM colchicine. It was hypothesized that D_2O might counteract the destabilizing effect of colchicine.

Ten to forty percent D_2O was ineffective in blocking colchicine inhibition (Figures 16-18). Deuterium oxide alone at those concentrations had no effect. At 50% and 60% D_2O the response duration began to decline without colchicine (Figures 19 & 20). Reperfusion with normal solution brought the response back to the starting level. Upon perfusion with D_2O plus 10mM colchicine, the response declined parallel to inhibition by 10mM colchicine alone. Seventy percent D_2O showed a rapid decline in response and, with 10mM colchicine, the decreased response became irreversible (Figure 21).

Other Microtubule Agents

Dimethyl sulfoxide, a reported MT stabilizer, produced erratic responses in concentrations of 3-10%. Spontaneous activity, of an amplitude exceeding elicited response activity, obscured the results of any possible stabilizing effects.

Use of the colchicine analog, lumicolchicine, was abandoned because of insufficient quantities of the compound. Preliminary efforts were made to obtain lumicolchicine by ultraviolet irradiation of colchicine, but at the rate of production achieved, it would have taken six months to a year to obtain enough for one experiment.

Fig. 2. Effect of Stopping Perfusion. Cross-hatched bar at top indicates perfusion with normal perfusion fluid. Interrupting the flow of perfusion fluid (blank bar) inhibits the response within four minutes. Resumption of flow is followed by recovery of the response to its initial level.

n = 30

Time/division = 20 seconds

Fig. 3. Continuous Perfusion. Perfusion of an excised leg over a period of nearly four hours has no effect on the response duration.

n = 20

Time/division = 5 minutes

FIGURE 3

Fig. 4. Effect of Varying Stimulus Rate. Stimuli were given at different rates: 1 stimulus per 10 seconds; 1 per 9 sec; 1 per 8 sec; etc. At a rate of 1 stimulus per 3 seconds and higher, the train duration decreased. When the interval between stimuli was increased, the response recovered, but not until the rate reached 1 stimulus per 5 seconds.

$n = 5$

Time/division = 5 minutes

Fig. 5. 1mM Colchicine. Perfusion with 1mM colchicine (black bar) does not affect response duration.

n = 5

Time/division = 2.5 minutes

Fig. 6. 4mM Colchicine. Perfusion with 4mM colchicine (black bar) does not affect response duration.

n = 3

Time/division = 2.5 minutes

Fig. 7. 5mM Colchicine. Perfusion with 5mM colchicine (black bar) produces some decline in response duration and an increase in variability between stimuli. The inhibition is reversed by perfusion with normal perfusion fluid (cross-hatched bar).

n = 4

Time/division = 2.5 minutes

FIGURE 7

Fig. 8. 6mM Colchicine. Perfusion with 6mM colchicine (black bar) produces some inhibition.

n = 3

Time/division = 2.5 minutes

FIGURE 8

Fig. 9. 7mM Colchicine. Perfusion with 7mM Colchicine (black bar) inhibits the response by 50% within 30 minutes.

n = 3

Time/division = $3.\overline{33}$ minutes

FIGURE 9

Fig. 10. 8mM Colchicine. Perfusion with 8mM colchicine (black bar) inhibits the response by 50% within 10 minutes.

n = 3

Time/division = $3.\overline{33}$ minutes

FIGURE 10

Fig. 11. 9mM Colchicine. Perfusion with 9mM colchicine (black bar) inhibits the response by 50% within 10 minutes. The response declines in an asymptotic manner to a new level which is reached about 30 minutes after the beginning of colchicine perfusion. The response recovers when the leg is reperfused with normal perfusion fluid.

n = 3

Time/division = $3.\overline{33}$ minutes

FIGURE 11

Fig. 12. 10mM Colchicine. Perfusion with 10mM colchicine (black bar) inhibits the response by 50% within 7 minutes. The response declines in an asymptotic manner to a new level within 30 minutes after the beginning of colchicine perfusion. The response recovers with a comparable time course when the leg is reperfused with normal perfusion fluid. The normal variation in response from stimulation to stimulation is not abolished by colchicine inhibition (cf. fig.3).

n = 10

Time/division = 2.5 minutes

Fig. 13. Vinblastine Inhibition. Perfusion with 1mM vinblastine sulfate in 1% dimethyl sulfoxide (DMSO, black bar) inhibits the response. Perfusion with 1% DMSO (vertically-hatched bar) prior to the vinblastine treatment has no effect. The response cannot be revived by reperfusing with 1% DMSO or normal perfusion fluid (not shown).

n = 4

Time/division = $3.\overline{33}$ minutes

FIGURE 13

Fig. 14. Tetrodotoxin Block of Sodium Channels. Perfusion with tetrodotoxin (10^{-5} M, black bar) inhibits the response fully within 45 minutes. Tetrodotoxin inhibition cannot be reversed by reperfusion with normal perfusion fluid. Coupled with the decrease in response duration is a decline in spike amplitude (not shown).

n = 3

Time/division = 3.33 minutes

FIGURE 14

Fig. 15. 2,4-Dinitrophenol. Perfusion with .1mM 2,4-dinitrophenol (black bar) results in a reversible inhibition of response duration.

n = 4

Time/division = 5 minutes

FIGURE 15

Fig. 16. Effect of 10 and 20% Deuterium Oxide.
Perfusion with perfusion fluid containing 10% D₂O
(vertically-hatched bar) has no effect. Perfusion with
10mM colchicine plus 10% D₂O (first black bar) is inhi-
bitory. Perfusion with 10mM colchicine plus 20% D₂O
(second black bar) is also inhibitory. Neither 10% nor
20% D₂O block the inhibitory effect of colchicine.
n = 3
Time/division = 5 minutes

Fig. 17. Effect of 30% Deuterium Oxide. Perfusion with perfusion fluid containing 30% D₂O (vertically-hatched bar) has no effect. Perfusion with 10mM colchicine plus 30% D₂O (black bar) is inhibitory. The inhibition can be reversed by reperfusion with 30% D₂O.

n = 3

Time/division = 5 minutes

Fig. 18. Effect of 40% Deuterium Oxide. Perfusion with perfusion fluid containing 40% D₂O (vertically-hatched bar) has no effect. Perfusion with 10mM colchicine plus 40% D₂O (black bar) is inhibitory. The inhibition can be reversed by reperfusion with 40% D₂O.

n = 5

Time/division = 2.5 minutes

Fig. 19. Effect of 50% Deuterium Oxide. Perfusion with perfusion fluid containing 50% D₂O alone (first vertically-hatched bar) is slightly inhibitory. Perfusion with 10mM colchicine plus 50% D₂O (first black bar) is strongly inhibitory, but can be reversed with 50% D₂O (second vertically-hatched bar). Full recovery is achieved by reperfusion with normal perfusion fluid.

n = 3

Time/division = 5 minutes

Fig. 20. Effect of 60% Deuterium Oxide. Perfusion with perfusion fluid containing 60% D₂O alone (first vertically-hatched bar) is inhibitory as is perfusion with 10mM colchicine plus 60% D₂O (black bar). Sixty percent D₂O does not block colchicine inhibition.

n = 3

Time/division = 5 minutes

FIGURE 20

Fig. 21. Effect of 70% Deuterium Oxide. Perfusion with perfusion fluid containing 70% D₂O alone (vertically-hatched bar) is initially inhibitory, but the response recovers rapidly. Perfusion with 10mM colchicine plus 70% D₂O (black bar) is strongly inhibitory. This inhibition cannot be reversed by perfusion with normal perfusion fluid (third cross-hatched bar).

n = 3

Time/division = 5 minutes

FIGURE 21

TABLE I

SUMMARY

Treatment	Inhibition	Reversibility
Colchicine, 5-10mM	+	+
Vinblastine + 1% DMSO	+	-
Four Hour Perfusion	-	
Interstimulus Interval		
10-5 sec.	-	
4-1 sec.	+	-
Perfusion Pause (Hypoxia)	+	+
2,4-Dinitrophenol, .1mM	+	+
Tetrodotoxin, $1 \times 10^{-5}M$	+	-
D ₂ O only, 50-70%	+	+
D ₂ O + Colchicine, 10mM		
10-60%	+	+
70%	+	-

+ = yes

- = no

CHAPTER IV

DISCUSSION

Sensory transduction is a mysterious process which has not been explained for any sensory system, including the much researched visual system. The presence of microtubules in a wide variety of sensory dendrites fostered the belief that they might have something to do with the initiation of electrical responses in receptors (Atema, 1973, 1975; Hejnowicz, 1972; Callatay, 1969; Palay et al., 1968; Spondelin, 1968). The only test of this hypothesis is the experiment by Moran and Varela (1971), who were able to inhibit mechano-sensory responses in the cockroach leg using colchicine. The purpose of this research was to (1) repeat and verify Moran and Varela's (1971) experiment, and, if successful, (2) extend their work using other microtubule-active agents under more controlled conditions.

Adapting Properties of the Mechanoreceptor

The duration of the train of action potentials produced by a single stimulus in the cockroach campaniform organ is inversely proportional to the rate of stimulation. In effect, an interval of at least five seconds is required between stimuli to obtain a maximal response duration with a 300 msec stimulus. This requirement is presumably related to the

adapting properties of the receptor. However, like sensory transduction, the physiological basis for adaptation is as yet unexplained.

Colchicine and Vinblastine Inhibition

Colchicine reversibly inhibits responses of the cockroach leg mechanoreceptor, while vinblastine produces irreversible inhibition. This action correlates with the known biochemical action of colchicine, whose binding to microtubules is reversible, and vinblastine, whose effect is to produce an irreversible condensation of microtubule protein into crystalline structures within the cell. Colchicine and vinblastine inhibition suggest microtubules or microtubule protein are a part of the excitatory mechanisms within the neuron.

High concentrations of colchicine and vinblastine are required to produce inhibition (5-10mM and 1mM, respectively). This, however, may be explained by the fact that any perfusing agent used in this system must traverse several cell layers between the hemocoel and the neuron. In addition, high concentrations of colchicine and vinblastine may be required if their effect is intracellular and the agents have to penetrate the cells in sufficient quantity to produce an electrical effect which is evident within minutes. Cell biologists and biochemists typically do experiments on cell growth and metabolism with much lower concentrations of colchicine and vinblastine, but their experiments last hours to days. The

time period under investigation here is seconds to minutes, and the object of investigation is electrical excitability, not metabolism.

Effects of Deuterium Oxide

Deuterium oxide has no detectable effect on colchicine inhibition. Since D_2O has been reported to be a microtubule stabilizer (Inoue' and Sato, 1967), it might have the ability to block the colchicine inhibition, but it does not. In fact, perfusion with 50-70% D_2O inhibits electrical activity in the absence of colchicine. Based on the data at hand, there is no way to conclude whether or not microtubules are affected by the D_2O treatment. Deuterium oxide has other biological effects (Thompson, 1963), including the creation of pH and viscosity changes, and altering all processes which involve hydrogen bonds between water molecules and cellular constituents.

Effects of Tetrodotoxin and 2,4-Dinitrophenol

Tetrodotoxin (TTX) is known to specifically inhibit increases in sodium conductance associated with the action potential (Tasaki, 1968). Tetrodotoxin was therefore tested on this preparation as an example of an agent which is known to interfere with the excitatory process by affecting the cell membrane. Its effect on the cockroach mechanoreceptor is similar, in one respect, to that of colchicine and vinblastine, i.e., it causes a decrease in response duration

and frequency of spiking within the train of action potential. However, it also causes a decrease in spike amplitude, an effect which is not observed with colchicine. The action of TTX is to limit the amount of sodium diffusing through the membrane during the action potential. Since no decrease in spike height occurred with colchicine or vinblastine, we may conclude that these agents are not directly interfering with sodium conductance changes in the same manner as TTX. The observed effects with colchicine and vinblastine would be the effect expected if the treatment decreased the amplitude of the generator potential in the dendrite or cell body. When the magnitude of the generator potential decreases in any sensory neuron, there is a reduction in train duration and spiking frequency within the response (Kuffler and Nicholls, 1976). Tetrodotoxin does not affect the generator potential, but colchicine and vinblastine may affect it.

2,4-Dinitrophenol (DNP) is an inhibitor of oxidative phosphorylation; i.e., it deprives cells of adenosine triphosphate (ATP). It would also eliminate guanosine triphosphate (GTP); this is of importance, since GTP seems to be necessary for microtubule assembly. Neurons deprived of ATP show a gradual decrease in the ability to produce generator potentials and conduct propagated action potentials. The effect of DNP on the cockroach mechanoreceptor mimics the effects of colchicine and vinblastine. Dinitrophenol reduces duration and spiking frequency, but not spike amplitude.

Stopping perfusion, and consequently depriving the preparation of oxygen, has the same effect as DNP. We may therefore conclude that colchicine's effect may be primary (cutting off ATP and GTP production), or secondary (blocking transport of ATP, GTP, or other necessary metabolites to some part of the excitatory system, probably the dendrite). Colchicine and vinblastine are known inhibitors of axoplasmic transport. In this system they may be cutting off axoplasmic flow of some necessary metabolite to the sensory dendrites.

Related Experiments

Colchicine does not alter the propagation of action potentials down axons in the rabbit vagus nerve (Hinkley and Green, 1971). Okafu (unpublished) has incubated desheathed crab leg nerves and frog sciatic nerves overnight in 10mM colchicine with no effect on the compound action potential. Therefore, axonal microtubules are either unaffected or they have no functional relationship to propagation of the nerve impulse in axons. Since it seems likely that overnight treatment with 10mM colchicine would obliterate all microtubules, free microtubules in axons must have no role in impulse conduction. Additionally, Okafu's experiments indicate that axonal membranes, which mediate impulse propagation, are not poisoned by colchicine. By analogy, it is likely that impulse conduction in cockroach leg nerves is unaffected by colchicine, and that the effects observed in these experiments are due to changes in the dendrite or cell body.

Where Do Colchicine and Vinblastine Act?

The colchicine and vinblastine effect on cockroach mechanoreceptors is probably an effect at the site of the sensory dendrites or soma. Colchicine and vinblastine may be affecting (1) intracellular microtubules, (2) membrane associated tubulin, (3) other membrane components by binding to them in a nonspecific fashion, or (4) axoplasmic transport of essential materials to the sensory dendrites. The possibility of a nonspecific effect of colchicine could be tested by using a colchicine derivative, lumicolchicine, which does not bind to tubulin. However, an insufficient supply of lumicolchicine has so far prevented this important test.

These experiments could be carried further by employing a variety of other agents which are known to disrupt microtubule function: podophyllotoxin, melatonin, griseofulvin, nocodazole, etc. The insolubility of podophyllotoxin in water prevented its use in the present study, but it is still a candidate for future use. Another agent, cytochalasin B, may prove to be a valuable control, since it is supposed to affect microfilaments, not microtubules, and should not affect a microtubule-mediated process.

It may be wisest to follow up these experiments on another preparation, rather than continue experiments with the cockroach leg whose limitations have been reached in these experiments. For example, mechanoreceptors exist in the crab leg which have similar response characteristics to the

cockroach mechanoreceptors (Wyse, 1967, 1971). The advantage of the crab leg is that impulse conduction in its long nerve can be studied independently of the generation of receptor potentials in mechanoreceptor dendrites. Another preparation with potentially more usefulness is the crayfish stretch receptor. Intracellular recordings of both receptor and action potentials are possible in the crayfish stretch receptor and microtubule-active agents can be injected into the cell or applied to the outer membrane by microiontophoresis. Thus, the intracellular versus extracellular effects of these agents could be directly tested, and the receptor and action potentials clearly separated. Fluorescent antibodies and radioisotope labeled colchicine could also be used to demonstrate the exact sites microtubule protein and colchicine binding sites. Finally, a number of vertebrate preparations may offer similar advantages, e.g., muscle spindle, cat Paccinian corpuscle, etc.

At present, the generality of these observations is unknown. Colchicine inhibition of electrical activity may be limited to insects or arthropods, or it may be present at all phylogenetic levels. We are also ignorant of whether these observations apply only to mechanoreception, or are representative of a broader phenomenon which is represented in other sensory modalities. If these observations are found to extend either across species lines or across sensory modalities, they may be important clues to the workings of a fundamental process in excitable cells.

SUMMARY

The conclusions derived from this investigation may be summarized as follows.

1. The decline in response duration during perfusion with colchicine and vinblastine suggest that protein may be involved in the excitatory process.

2. Stopping continuous perfusion through the leg inhibits the response duration, indicating the need for oxygenated fluid to maintain neuronal activity.

3. 2,4-Dinitrophenol inhibition, which parallels colchicine inhibition, suggests a metabolic effect by colchicine.

4. Tetrodotoxin inhibition of the action potential train parallels colchicine inhibition, but the decline in action potential amplitude is dissimilar. This data indicates that colchicine does not alter sodium ion conductance during the action potential.

6. Deuterium oxide does not counteract the colchicine inhibition, indicating that a hypothesized D_2O stabilization of MT is ineffective in this preparation.

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