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13. ABSTRACT
Since inception of the study on November 1, 1965, the following objectives have been fulfilled:

Intracellular recording of electrical activity from vascular smooth muscle (superior mesenteric vein of the Guinea pig) has been made a routine experiment, maintaining impalement during spontaneous and drug induced activity. Normal spontaneous activity and the effects of catecholamines, acetylcholine and ions have been studied in terms of electrical and mechanical activity.

Voltage clamp and constant current injection techniques have been used to investigate the membrane properties of vascular and other smooth muscle. Effects of 2-4-dinitrophenol and CN^- have been studied in terms of electrical activity. Attempts have also been made to record electrical activity from the precapillary sphincters of rat mesentry. The above studies indicate that although the electrical properties of the smooth muscle membrane may be somewhat similar to those of nerve and striated muscle, the ionic mechanisms may differ essentially, e.g., the excitation of smooth muscle from the longitudinal layer of the superior mesenteric vein and also taenia coli of the guinea pig is Ca^{++} dependent, Tetrodotoxin (TTX) insensitive and is blocked by transition metals.

The investigation of the so-called "autoregulatory escape" in isolated loops of cat ileum essentially confirms earlier studies by Folkow, et al. and by using TTX we have been able to exclude the involvement of local reflexes (axon reflex) participating in the phenomenon. An additional finding is that TTX treatment of a vascular bed augments the constrictor effects of catecholamines.

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REGULATION OF SMOOTH MUSCLE RESPONSIVENESS

FINAL REPORT

by

LEIF HORN, PH.D.

May 1971

(For the period 1 Nov. 1965 - 1 Nov. 1970)

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"REGULATION OF SMOOTH MUSCLE RESPONSIVENESS"

SUMMARY:

Since inception of the study on November 1, 1965, the following objectives have been fulfilled:

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Voltage clamp and constant current injection techniques have been used to investigate the membrane properties of vascular and other smooth muscle. Effects of 2-4-dinitrophenol and CN^- have been studied in terms of electrical activity. Attempts have also been made to record electrical activity from the precapillary sphincters of rat mesentry. The above studies indicate that although the electrical properties of the smooth muscle membrane may be somewhat similar to those of nerve and striated muscle, the ionic mechanisms may differ essentially, e.g., the excitation of smooth muscle from the longitudinal layer of the superior mesenteric vein and also taenia coli of the guinea pig is Ca^{++} dependent, Tetrodotoxin (TTX) insensitive and is blocked by transition metals.

The investigation of the so-called "autoregulatory escape" in isolated loops of cat ileum essentially confirms earlier studies by Folkow et. al. and by using TTX we have been able to exclude the involvement of local reflexes (axon reflex) participating in the phenomenon. An additional finding is that TTX treatment of a vascular bed augments the constrictor effects of catecholamines.

FOREWORD

Project "Regulation of Smooth Muscle Responsiveness" was started 6 years ago under Contract No. DA 49-193-MD 2843 with the U.S. Army Medical Research and Development Command at the New York Medical College, continued in 1967 at the New Jersey College of Medicine and Dentistry under Contract No. DADA-17-68-C8058, and has for the past three years continued without funding from the Medical Research and Development Command.

The project was initially a pilot study to investigate the feasibility of obtaining electrokinetic data from the smooth muscle of blood vessels by intracellular techniques in order to find out how ionic mechanisms contribute to changes of responsiveness and to the reversal of the blood vessel responses to neurotransmitter in the end stage of shock.

Soon after the project moved into the realm of a definite study, in vivo investigation of the regulation of microcirculation and its derangement in shock was included in the program.

The project demonstrated early the feasibility of using intracellular potential recording techniques to assess changes in vascular response to neurotransmitters. Despite the fact that these extremely delicate techniques had never before been used successfully to study induced changes in responsiveness of blood vessels, several primary objectives were fulfilled, as seen from our two preliminary reports, and in addition, the study yielded a wealth of basic information indispensible for the final evaluation of vascular responses in shock (see enclosures). This comprehensive and detailed final report to the U.S. Army Medical Research and Development Command includes the first phase of a continuing program on the regulation of microcirculation and its derangement in shock, and some interesting but independent aspects suggested by the above project. These other studies have been supported by the New Jersey Heart Association, NSF and a U.S.-Japan International Cooperation grant.

The continuation of our project has been funded partially by our Institution with appropriations to cover some equipment, expendable material, technical help and in part coverage of Salary for Dr. Kumamoto.

It is appropriate to thank the various granting agencies that have made this project possible, and to acknowledge the major contributions by Drs. Nakajima and Kumamoto, to make it a success. Thanks are also due to Drs. Folkow, Wallentin and Niu for valuable discussions and suggestions.

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Prior to the present project the electrical behavior of mammalian vascular smooth muscle was virtually unknown, except for a few reports, notably by Funaki and Bohr (1964), Su and Bevan (1964), Guthbert et al. (1964), Speden (1964) and a series of poor and inconsistent records from our own laboratory. The main problem in obtaining reliable data was to produce microelectrodes of proper diameter ($\sim$0.1 μ), tolerably low resistance ($<$40 megohm) and minimal tip potential ($<$10 mV).

After about 3 months work, starting in November of 1965, testing different types of glass capillaries, studying diffraction patterns under light microscope (tip of electrode being beyond resolution), and, after filling electrodes with 3M KCl, testing the various categories of electrode samples in Taenia coli, we finally ended up with useful micropipettes, suitable for recording from vascular smooth muscle.

Thus, we succeeded in making intracellular recording of electrical activity of vascular smooth muscle a routine experiment, maintaining impalement during spontaneous and induced electrical activity.

For full details of techniques see references 3 and 8. We have studied and described changes in electrical and mechanical activity produced by norepinephrine, epinephrine, isoproterenol, and acetylcholine under normal conditions and under conditions of changed ionic composition of the external bathing solution. The equilibrium potential for acetylcholine was determined. A great deal of effort has gone into the study of the effect of neurotransmitters on maximum rate of rise and maximum rate of fall of the action potential.

All of the significant findings regarding these aspects of our studies have been published, and abstracts and papers are enclosed, see ref. 1-4 and 6-8.

We have also carried out experiments which aim to determine the effects of metabolic poisons (viz., CN- and DNP) on the contractile and electrical activity of vascular smooth muscle. Preliminary results were reported at the International Congress of Microcirculation in Gotenborg, Sweden, in 1968 (6) at the Federation Meeting in 1969 (9) and full details were reported in a paper in 1970 (10), see enclosures.

Our latest experiments have resulted in a very significant observation. Tetrodotoxin (TTX), which specifically blocks the voltage dependent changes in sodium permeability in nerve and skeletal muscle has no effect on the action potential or impulse propagation our preparation, whereas blocking of calcium permeability with manganese abolishes the electrical activity completely and renders the preparation inexcitable. The conclusion drawn from analogous experiments on visceral muscle and on cardiac muscle has been that Ca^{++}, and not Na^{+}, is the current-carrying ion during the upstroke of the action potential, essentially disqualifying the Hodgkin-Huxley hypothesis, (for which they received the Nobel Prize in 1963). We are currently carrying out experiments which involve voltage clamping in effort to assess whether Ca^{++} is directly involved in the excitation and the propagation of impulses in vascular smooth muscle.

Details of techniques and significant findings have been published in several abstracts and a paper (ref. 11-15).

As an adjunct study to the project "Regulation of Smooth Muscle Responsiveness", we carried out in vivo studies in relation to the so-called autoregulatory escape in cats. Unfortunately financial support ceased before the main experiments were started. However, valuable improvements in instrumentation were accomplished.

Thus, we have constructed and tested a new impulse flow meter of the drop counting type (absolute volume) operating an oscillograph (Sanborn 550B or similar). This flow meter is a solid state version of the one used with smoke-drum kymograph in Folkow's laboratory. It offers great stability over a wide range of flows and has minimal drift. Technical reports with performance specifications have been published as the flow meter offers a number of advantages (see ref. 5 and 16). A simple and accurate volume transducer has been constructed utilizing an inexpensive transformer transducer (Linearsyn, Sanborn Div. of Hewlett Packard C9). This volume transducer is used with the plethysmograph. We have alos constructed a new hydraulically operated table with tiltable cat-board and attachment for the variety of equipment used in the experiments. This table

serves as a sturdy base for the microscope and the camera. The assembly is shown with camera-microscope in Fig. 1 and 2.

Enclosed is a report (Ref. 19) by Mr. N.A. Mortillaro delivered at the Federation Meeting this year on preliminary experiments on the autoregulatory escape in ileal loops in the cat. This work (see Abstracts 17,18) is part of his thesis work for the degree of Doctor of Philosophy and was initiated during the last year of the contract (without funding) and has continued with funds granted by N.S.F.

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Reference 1

Reprinted from "THE PHYSIOLOGIST" - Vol. 10(3):205, 1967

ELECTRICAL ACTIVITY OF THE SUPERIOR MESENTERIC VEIN OF THE GUINEA PIG.
L. Horn and A. Nakajima*. New York Medical College, New York.

We have previously reported on intracellular recording of spontaneous and drug-induced electrical changes of smooth muscle from the longitudinal layer of guinea pig superior mesenteric vein (Fed. Proc. 26:330, 1967, Am. J. Physiol. 213:July, 1967). Further data on changes in membrane activity in response to catecholamines and acetylcholine will be reported. Adrenaline and noradrenaline have an excitatory effect brought about by depolarization or increase in spike activity. Adrenaline has an additional positive inotropic effect not associated with detectable change in membrane potential when the muscle has been extensively depolarized by increased external potassium. Isoproterenol abolishes spike activity and hyperpolarizes the membrane. We have previously reported that acetylcholine may briefly hyperpolarize the membrane before the depolarization to about 40 mV associated with the excitatory effects of the drug. The depolarizing effects of acetylcholine reverses when the membrane has been depolarized by KCl to about 30 mV prior to drug application. It is suggested that acetylcholine has an equilibrium potential of about 40 mV in this muscle.
(Supported by Contract DA-49-193-MD-2843 with the U.S. Army R & D Command and a Grant from the American Heart Association).

Reference 2

Reprinted from "FEDERATION PROCEEDINGS" V. 26(1):330, 1967

ELECTRICAL ACTIVITY OF VASCULAR SMOOTH MUSCLE. Akira Nakajima* and Leif Horn, New York Med. Col., New York, N.Y.

Intracellular recording in vitro from the longitudinal muscle layer in guinea pig's superior mesenteric vein showed bursts of action potentials associated with spontaneous contractions. The resting potential between bursts ranged from 41 to 62 mV. Action potentials were accompanied by a slow component with an average amplitude of 25 ± 1.8 mV. The action potentials ranged from 35 to 59 mV and had a fast repolarization phase followed by marked after-hyperpolarization. Overshoot was observed occasionally, but then only a few mV. Adrenaline prolonged the bursts of spike discharges or initiated repetitive firing. High concentrations caused rapid depolarization associated with increasing discharge frequency of action potentials of progressively smaller amplitudes. Acetylcholine excited the muscle but depolarization was usually preceeded by a slight hyperpolarizati[on]. The hyper-polarizing action could not be discerned when the membrane potential was higher than 60 mV at the time of drug application. Asynchronous or partial excitation occurred independently along the muscle. Ionic mechanisms are discussed. (Supported by Contract DA-49-193-MD-2843 with the U.S. Army R & D Command and a grant from the American Heart Association.)

Reference 3

Reprinted from "AMERICAN JOURNAL OF PHYSIOLOGY" Vol. 213, No. 1, July, 1967

"ELECTRICAL ACTIVITY OF SINGLE VASCULAR SMOOTH MUSCLE FIBERS"

Akira Nakajima and Leif Horn

(Reprints enclosed)

Electrical activity of single vascular smooth muscle fibers

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Vol. 213, No. 1, July, 1967
Printed in U.S.A.

Electrical activity of single vascular smooth muscle fibers[1]

AKIRA NAKAJIMA[2] AND LEIF HORN
Department of Physiology, New York Medical College, New York City

NAKAJIMA, AKIRA, AND LEIF HORN. *Electrical activity of single vascular smooth muscle fibers*. Am. J. Physiol. 213(1): 25-30, 1967.—Electrical activity of the longitudinal muscle layer in guinea pig excised superior mesenteric vein was recorded intracellularly. Bursts of action potentials were associated with spontaneous contractions. The resting potential between bursts ranged from 41 to 62 mv. Action potentials were accompanied by a slow component with an average amplitude of 27 ± 1.8 mv. The action potentials ranged from 35 to 59 mv and had a fast repolarization phase followed by marked afterhyperpolarization. Overshoot of a few millivolts was observed occasionally. Adrenaline prolonged the bursts of spike discharges or initiated repetitive firing. High concentrations produced rapid membrane depolarization associated with increasing discharge frequency of action potentials of progressively smaller amplitudes. Acetylcholine had an excitatory effect but the depolarization was usually preceded by a slight hyperpolarization. The hyperpolarizing action of acetylcholine was more prominent when the initial membrane potential was low. Hyperpolarization could not be discerned when it was higher than 60 mv at the time of drug application. Asynchronous or partial excitation occurred independently along the muscle. Ionic mechanisms are discussed.

superior mesenteric vein; vascular muscle, membrane potential; slow potential; adrenaline; action potential; conduction of excitation; acetylcholine; depolarization; contraction; overshoot; hyperpolarization; e-c coupling

IT IS NECESSARY TO KNOW the basic mechanisms influencing contractile activity of vascular smooth muscle to understand the physiological regulation of local blood flow in tissue and the compensatory regional shifts needed for fulfilling the basic economy requirements of a minimal blood volume. Studies of the behavior of vascular smooth muscle proper by means of mechanical methods in vitro and in terms of direct observations through the microscope in vivo, have contributed such knowledge (11, 20). As to the electrophysiological behavior of the smooth muscle membrane, current views are based mainly on data from nonvascular muscle tissues and the validity of transposing such data to vascular muscle has been disputed. It is assumed that catecholamines and acetylcholine play important roles in the regulation of vascular smooth muscle in a manner analogous to their action on other contractile tissues. Excitatory agents decrease the membrane potential of muscle in general to initiate or increase spike frequency, and inhibitory agents increase membrane polarization and decrease or abolish action potentials. Lowering of the membrane potential of vascular muscle under standard in vitro conditions brings about initiation of or increase in spike activity, and this increased frequency is associated with an increase in muscle tension. There is evidence, however, that excitatory agents will increase further the tension of vascular smooth muscle without spike activity and changes in the membrane potential when the latter has been depolarized by high external potassium concentrations (14). It is believed that when muscle tension does not change relative to spike frequency an electromechanical uncoupling has occurred. It has been necessary, therefore to invoke an additional, direct effect of transmitter agents on the contractile activity of muscle independent of action potentials. Nevertheless, the finding that high drug or ion concentrations may stimulate whereas low concentrations may inhibit smooth muscles or vice versa is difficult to explain. Finally, smooth muscle may change (reverse) its contractile responses to stimulation spontaneously or be induced to do so by various means (6, 13).

Unfortunately the electrical events associated with the changes in responsiveness of vascular smooth muscle are for the most part unknown, presumably due to inherent difficulties in the technique of impaling the relatively small fibers. A few reports indicate, however, that vascular smooth muscle may differ somewhat in its electrical properties and behavior from those of other muscle tissue. Thus it has been reported that the membrane potential is less than that of other smooth muscles (3, 10, 14, 17, 18) and only about half that of skeletal muscle. A variety of different patterns of action potentials have been recorded, possibly related to the functional characteristics of the particular muscle fibers. Tomaki

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[1] This work was supported by contract DA-49-193-MD-2844 with the U.S. Army Medical Service Research Development Program and by a grant from the American Heart Association.
[2] Guest Investigator on leave of absence from Kyoto University, Japan.

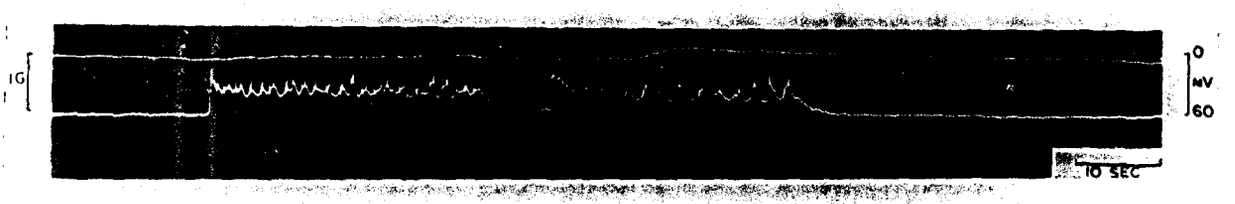

FIG. 1. Spontaneous bursts of spike discharges (conducted responses). Note that maximal tension (upper tracer) developed about 50 sec after the onset of spike activity. This record has been retouched.

(10) obtained two distinctly different types of action potentials from smooth muscle fibers in different parts of the cerebrovascular bed in the frog. The "spike duration" of these action potentials were 60 and 200 msec. Roddie (10) reported "... a wide spectrum of action potentials" in vascular muscle from the turtle. One type resembled a cardiac action potential with an abnormally protracted plateau lasting up to 40 sec. Further, somewhat obscure findings are that acetylcholine has a dual effect on the membrane of vascular muscle giving an initial hyperpolarization followed by a depolarization, both associated with contraction (10). Although similar confusing results have been obtained occasionally in other smooth muscle (5), it would be of interest to know whether these responses are characteristic of vascular muscles and if so how they relate to other parameters.

In the following experiments we have studied the membrane activity of single cells of the superior mesenteric vein of the guinea pig. This vessel exhibits spontaneous contractile activity both in vitro and in vivo. Moreover, it is stimulated by catecholamines as well as acetylcholine and it would be of particular interest to compare the electrical responses to these agents.

METHODS

Longitudinal strips of the vessel, 1 mm wide and 5 mm long, were excised and incubated in a chamber containing warm modified Krebs solution containing (in mM) NaCl 133, $NaHCO_3$ 16.3, NaH_2PO_4 1.38, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 0.105, and dextrose, 7.8. The solution was saturated with gas mixture of 95% O_2 and 5% CO_2. The strip was mounted so that the outer surface of the vessel wall was accessible. Connective tissue covering the longitudinal smooth muscle layer of the wall was removed. One end of the strip was connected to a lever transmitting the contractile tension to a mechanoelectric transducer. Microelectrodes, with resistance of about 50 megohms, were inserted by the floating method described by Woodbury and Brady (19). The tip potential of the electrodes varies between 3 and 10 mv. Calculated amounts of adrenaline or acetylcholine dissolved in standard amounts of Krebs solution were added directly to the bath. It should be noted that in reporting the membrane or action potentials here, impalements giving initial values for the resting potential below 30 mv have been omitted, because such low values are most probably not true

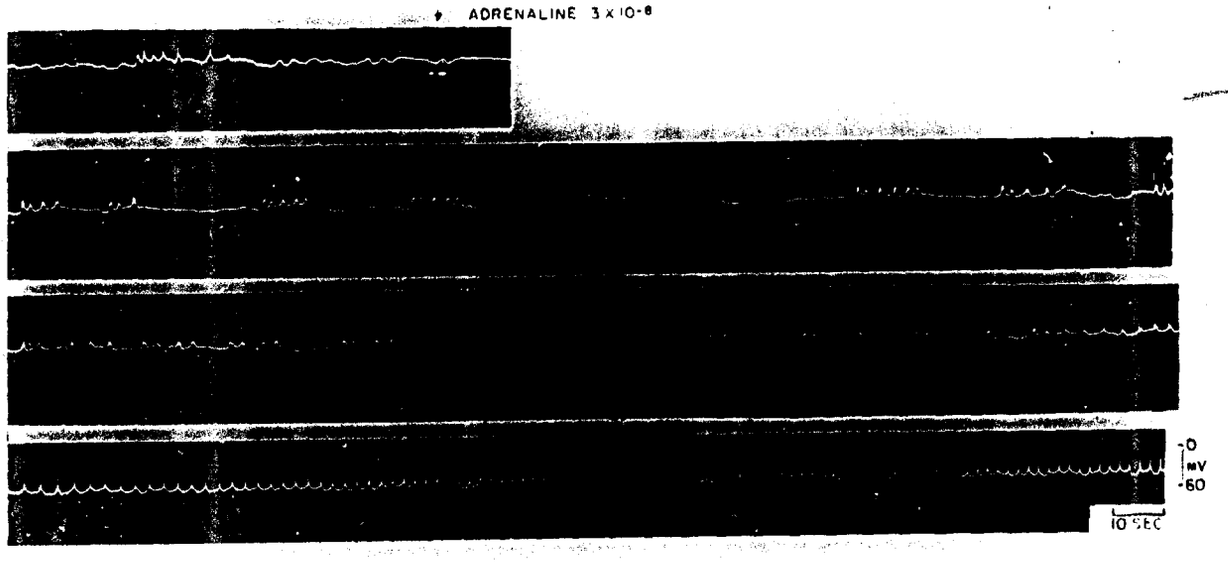

FIG. 2. Effect of low concentration of adrenaline (continuous record). Intervals between spike discharges shorten as the bursts fuse into a pattern of repetitive firing associated with gradual depolarization. This record has been retouched.

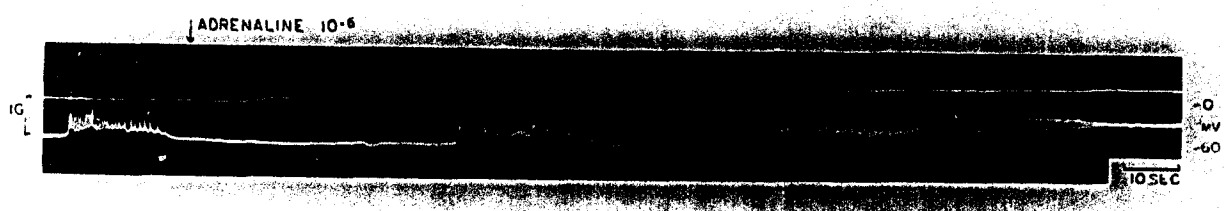

FIG. 3. Repetitive firing and depolarization following 10^{-6} w/v adrenaline applied 2 cm away from the strip. This procedure of drug application, used to avoid dislodging the electrode, produce substantial delay of the response. The slow increase in tension (upper trace) continued after the firing had ceased

intracellular potentials inasmuch as they can be easily obtained by "pressure electrode," as described by Gillespie (12).

RESULTS

The strips of superior mesenteric vein showed spontaneous contractile activity when incubated in warm modified Krebs solution. Bursts of spike discharges associated with contractions were recorded (Fig. 1). The resting membrane potential (± SE) between bursts (range 41–62 mv) was 51±1.4 mv in 27 successful experiments. The spike frequency was always higher in the initial part of the individual trains. The pattern of firing of action potentials varied but the activity could be classified as follows: *1*) long trains of spike discharges, similar to those seen in taenia coli and in uterine muscle (3, 15), *2*) short trains consisting of several spikes, *3*) single spikes or irregular discharges. Occasionally double spikes appeared during the burst of spike discharges. Many of the action potentials rose from slow depolarizations suggesting that the conduction of excitation in this vessel is so poor that the individual cells have marked tendency to exhibit pacemaking activity. Furthermore, the action potentials were usually accompanied by slow potentials of various shapes. This "slow component" associated with action potentials had an amplitude considerably larger (mean 25 ± 1.3 mv) than that of other smooth muscle cells (4) and most frequently, but not always, action potentials appeared to be triggered by these slow potentials. The action potential usually had a fast repolarization phase followed by a marked afterhyperpolarization. Similar results have already been reported by Speden (17) and Trail (18).

The amplitude of the action potential varied, ranging from 35 to 59 mv (mean 45 ± 2.2 mv), and appeared to relate to the initial membrane potential. Occasionally action potentials showed overshoot, but only of several millivolts (max 5 mv).

Effects of adrenaline. Adrenaline had an excitatory effect on the superior mesenteric vein in concentrations ranging from 10^{-8} to 10^{-5} w/v. A total of 54 experiments was carried out. Maintenance of impalement throughout and continuous records were obtained in one-fourth of these. Inhibitory responses were not observed within this concentration range. When the lower concentration of adrenaline was applied, prolonged bursts of spike discharges appeared without detectable change in the membrane potential. When the concentration of adrenaline was increased, prolonged repetitive firing of spike discharges occurred (Fig. 2), and the membrane gradually depolarized. In the highest concentrations of the drug, the membrane depolarized rapidly associated with an increasing discharge frequency of spikes of progressively smaller amplitude. The slow potential initially accompanying action potentials disappeared. Figure 3 shows the effect of 10^{-6} w/v concentration of adrenaline. The membrane potential fell rapidly to a level of about 20 mv as the frequency of spike discharges increased. The amplitude of the spikes decreased and the membrane activity deteriorated into oscillations. After the spikes had disappeared depolarization was maintained. It is noteworthy that the muscle continued to develop tension throughout the process, also after the cessation of spike discharges.

Effects of acetylcholine. Acetylcholine caused contraction of the superior mesenteric vein. Analogous to the excitation by adrenaline, the effect is produced by initiation or increase in spike activity of the muscle membrane. Sixty-six experiments were carried out. Maintenance of impalement throughout and continuous records were obtained in about one-fourth of these. Low concentrations of acetylcholine increased the spike frequency and the duration of bursts without detectable change in the membrane potential. In contrast to adrenaline, acetylcholine initially hyperpolarized the membrane. This effect was most consistent with high concentrations of acetylcholine and appeared to be closely and inversely related to the initial membrane potential. When the initial membrane potential was comparatively low (about 30 mv), a larger degree of hyperpolarization was obtained by applying 10^{-7} and 10^{-6} w/v acetylcholine (see also DISCUSSION).

Figure 4 shows the effect of acetylcholine in a concentration of 10^{-6} w/v. The repetitive firing of spikes appeared soon after the drug reached the muscle as the membrane is being transiently hyperpolarized, about 4 mv. After this initial stage large spikes were elicited repetitively and the membrane potential gradually returned to its initial level. When it was reduced about 60 mv no detectable change in membrane potential could be observed in the initial stage of the drug action. The hyperpolarizing effect of acetylcholine, however, was short lasting even at high concentrations, and after a

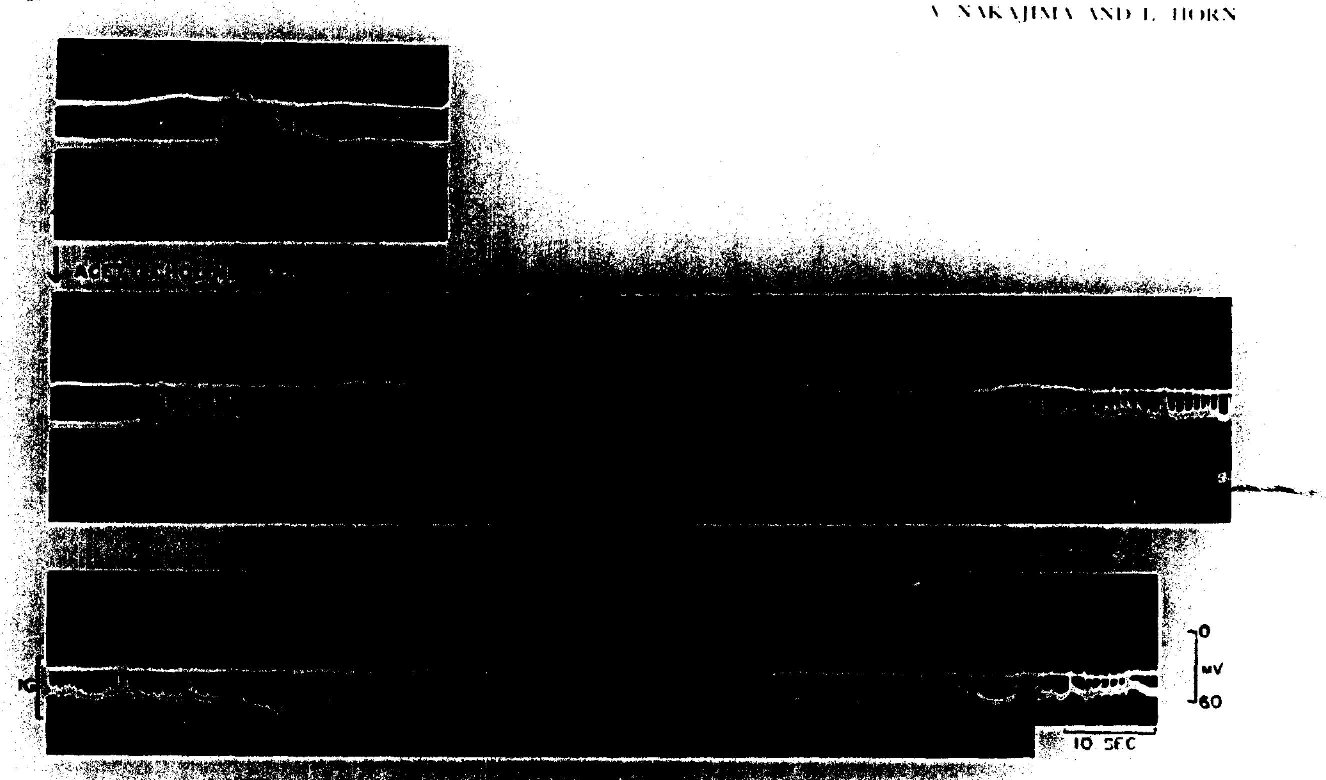

FIG. 4. Effect of acetylcholine (10^{-5} w/v). Resting membrane potential is slightly hyperpolarized (about 4 mv) before the last spontaneous burst preceding the repetitive firing.

brief period the membrane potential shifted to depolarization associated with repetitive firing of spike discharges and decreased to a final level of about 30 mv, as illustrated in Fig. 5. Figure 6 shows an exceptional case of prominent hyperpolarization produced by acetylcholine. Here the initial resting potential was in the range of 42 mv (*part B*) and a very marked hyperpolarization to 63 mv occurred. Some degree of spike inhibition occurred prior to depolarization and regular firing of spike discharges. It is emphasized however, that the typical responses are those illustrated in Figs. 4 and 5. More than two-thirds of our records show that acetylcholine produces a slight hyperpolarization of the membrane before initiation of repetitive firing of action potentials associated with depolarization.

DISCUSSION

The resting membrane potentials of vascular muscle that we encountered were of intermediate value: they were lower than those of visceral smooth muscle (7) but somewhat higher than those found by others also for vascular smooth muscle (17, 18). Several possible explanations come to mind. For example, the vascular smooth muscle cells are so small that it could easily be damaged on impalement with the microelectrode. This [illegible] explain why the recorded membrane [illegible] of this muscle is lower than that of visceral [illegible] cells. It should be noted, however, that when impalement is maintained over long periods of time (about 5 min) no change in the resting potential is recorded.

Overshoots were occasionally recorded but usually of only several millivolts (max 5 mv). Again it is difficult to determine whether the more usual failure to overshoot is an essential feature of this muscle fiber or whether it is due to injury caused by the microelectrode.

It has been reported that the spontaneous contractions of visceral smooth muscle are caused by repetitive firing of action potentials. Each spike discharge is well coordinated with the mechanical event summing up to phasic contraction. Therefore, tetanic contraction is an essential feature of visceral smooth muscle (6). In the present experiments a similar pattern of action potentials was observed in association with spontaneous contractions of the superior mesenteric vein of the guinea pig. The contraction of this vessel may therefore appear to be maintained by essentially similar mechanisms as those of visceral smooth muscle. However, the onset of a burst of spike discharges and increase of tension were not always synchronized, and in extreme cases there was such great disparity that each phenomenon appeared to occur independently. In part this might be accounted for as imperfections in the tension recording system but in the main this shows that asynchronous or partial excitation can occur independently along the muscle strip.

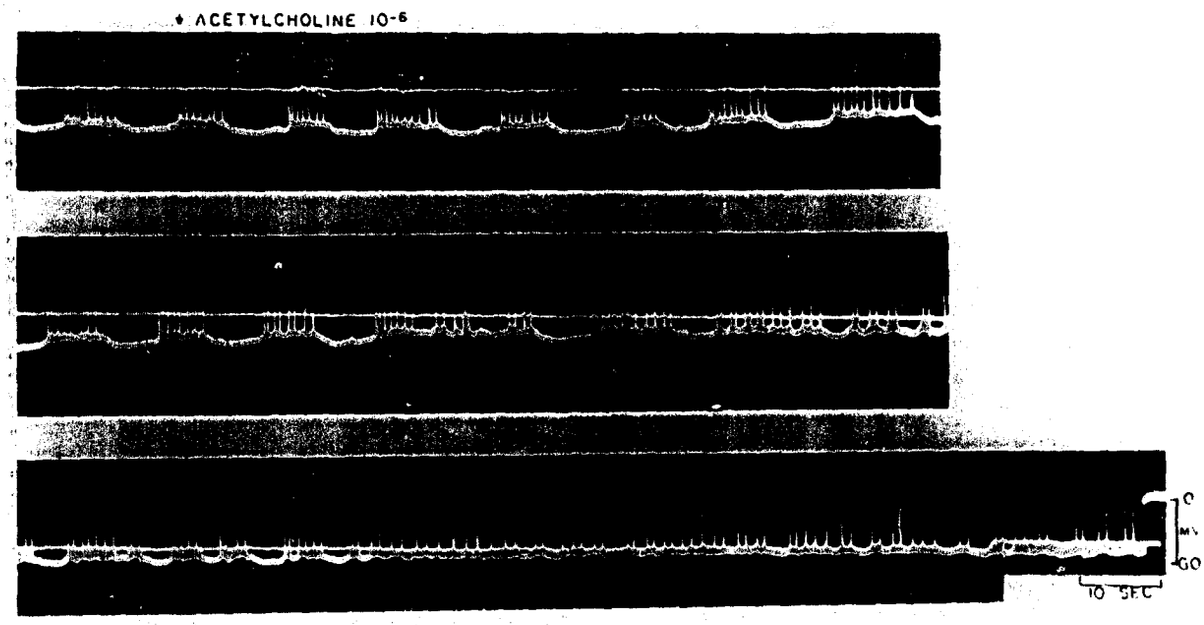

FIG. 5. Acetylcholine (10^{-6} w/v) increased firing frequency and depolarized the membrane from 53 mv to 45 mv after a brief, barely detectable hyperpolarization (3 mv). Disregard upper trace from malfunctioning mechanoelectric transducer.

As mentioned above, the action potentials usually had a slow rising phase of the pacemaker type. This also indicated multiple pacemaker activities in this muscle and, by inference, poor conduction.

It is not possible to assess a definite role to the prominent slow component associated with spike activity. Usually, but not always, it appeared to provide for a generator potential and it may be assumed that it plays a role in the pacemaking activities and thus a part of the mechanism for maintaining some degree of contraction ("tone") in this presumably poorly conducting tissue.

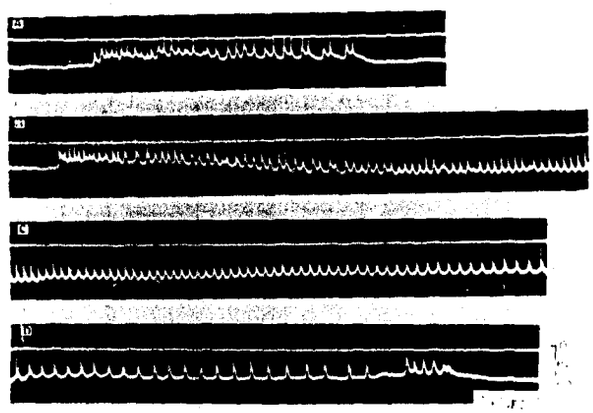

FIG. 6. Effect of acetylcholine (3×10^{-7} w/v). *A* control, *B* 1 min 20 sec after acetylcholine, *C* and *D* continuous records. Tension transducer (upper trace) was defunct.

Adrenaline has a stimulating effect on the superior mesenteric vein that is characterized by membrane depolarization and initiation of, or increase, in spike activity. In contrast to this vascular muscle, most visceral smooth muscles are inhibited by adrenaline. Axelsson and co-workers (1) have suggested that adrenaline has two effects on the membrane of taenia coli. The one "direct" depolarizing effect is masked, however, by another more prominent metabolic effect of the drug which results in hyperpolarization and inhibition. If this is the modus operandi of adrenaline in vascular muscle, the direct effect of the membrane dominates in the superior mesenteric vein at all drug concentrations used in these experiments.

With high concentrations of adrenaline, the membrane depolarized rapidly, the action potentials deteriorated into oscillations, and finally sustained depolarization occurred. However, there was a further increase in muscle tension unaccompanied by spike discharges. Whether or not the further increase in tension can be attributed to some "metabolic" effect of adrenaline maintained after excitation-contraction uncoupling cannot be assessed with certainty. A more plausible explanation would be that the development of contracture is gradual and that activity may remain in other cells for some time after the cessation of the activity of the impaled cell.

The excitatory effects of acetylcholine had some general features in common with those of adrenaline. Nevertheless, the differences are more striking. Even though the hyperpolarizing action is small and short lasting, the dual effect of acetylcholine on this vessel

is in contrast to that of adrenaline and to that reported for acetylcholine on visceral smooth muscles as well, which show only depolarization and increase in spike activity. The acetylcholine effect differed from the adrenaline effect also in that it did not produce a contracture, even at high drug concentrations. This may be due to basic differences of ionic mechanisms by which the two agents act, as has already been demonstrated in taenia coli (6). It should be noted that although the hyperpolarizing effects of acetylcholine usually are small, as reported by Funaki (10) and confirmed by us, they are quite unique, and may be very striking as illustrated in Fig. 6. It would be possible to explain the phenomenon by assuming that acetylcholine increases the potassium permeability earlier than the permeability for other ion species even though the drug might increase the permeabilities of all ions in a general manner, as it appears to do in taenia coli (5, 6). In relation to reversal of contractile manifestations in vascular smooth muscle it would be of interest to know how the effect of a drug on the individual ion permeability varies with the membrane polarization at the time of application.

The authors thank Dr. H. Niu for valuable discussion and advice.

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Reference 4

Reprinted from "FEDERATED PROCEEDINGS" Vol. 27(2):704, 1968

INTRACELLULAR RECORDING OF ELECTRICAL ACTIVITY OF VASCULAR SMOOTH MUSCLE.

L. Horn and M. Kumamoto*, N.J. Col. Med. & Dent., Jersey City, N.J., and A. Nakajima*, Kyoto Univ., Japan.

Spontaneous and drug induced electrical activity of vascular smooth muscle from the longitudinal layer of the superior mesenteric vein of the guinea pig has been recorded with intracellular microelectrode techniques. Adrenaline or noradrenaline caused increase in spike activity; 10^{-6} g/ml adrenaline caused eventually sustained depolarization whereas this concentration of noradrenaline only increased the frequency of action potentials. In contrast isoproterenol first abolished spike activity and then hyperpolarized the muscle membrane. This inhibitory response was followed by repolarization associated with bursts of spike discharges. Solutions containing excessive amounts of potassium depolarized the membrane to about 10 mV. Adrenaline caused further increase in tension. Thus, adrenaline has a direct effect on this muscle not associated with spike activity or changes in the membrane potential. The excitatory effect of Ach was brought about by initiation of or increase of spike activity and depolarization of the membrane. However, the effect on the membrane potential was reversed in the presence of 23.5 mM potassium solutions, hyperpolarization resulted to a steady state of about 40 mV. (Supported by Contract DA-49-193-MD-2843 with the U.S. Army R & D Command and a grant from the American Heart Association.)

Reference 5

Reprinted from THE MICROCIRCULATORY SOCIETY - XVI Annual Conference - 1968

A SOLID STATE IMPULSE FLOW METER. L. Horn and A. Rose. Dept. Physiol. and Electr. Shop, N.Y. Med. Coll., New York, N.Y.

Few methods for recording blood flow utilize direct measurement of volume per unit time. We have constructed an impulse flow meter which in basic principle is a solid state, electronic version of the mechanical ordinate drop flow recorder developed and extensively used by Folkow and associates since 1949. Blood drops, falling through a silicone filled chamber and breaking a light beam focused on a miniature photocell, generate input impulses recorded sequentially by an electromechanical register. Another register, preset to any number from 1 to 999, counts the drops in a backward feshion. When this counter reaches zero it sends out a signal and resets itself. The signal in turn resets a ramp signal that is generated in synchronism with the preset counter. The ramp signal is used to drive an electronic recorder or oscillograph. Self-checking circuits are provided to test the performance of the instrument and for making initial calibrations. Overall accuracy is better than one per cent. Performance data and advantages as used in microcirculatory studies of the cat intestine will be discussed. (Supported by Contract DA-49-193-MD-2843 with the U.S. Army R & D Command.)

Reference 6

Reprinted from "INTERNATIONAL CONGRESS OF MICROCIRCULATION" - 1968

ELECTRICAL AND MECHANICAL ACTIVITY OF BLOOD VESSELS IN RELATION TO METABOLISM. L. Horn and M. Kumamoto. Dept. Physiology, New Jersey College of Medicine and Dentistry, Jersey City, New Jersey, U.S.A.

Local blood flow is controlled through graded changes in the diameter of the peripheral blood vessels and a more or less complete opening or closure of the precapillary sphincters. Little is known about how metabolism affects and regulates the responsiveness of vascular smooth muscle. Utilizing intracellular techniques *in vitro* we have studied spontaneous and drug induced electrical and mechanical activity and some effects thereon of metabolic poisons, in the longitudinal smooth muscle of the guinea pig's superior mesenteric vein. Unlike other smooth muscle, this vascular muscle exhibits no prominent excitatory phase following exposure to 2-4-dinitrophenol (DNP) in concentrations of 10^{-5} to 5 x 10^{-4}M. Only a slight increase in electrical activity was observed and this was not accompanied by increase in tension. Within 40 to 60 seconds the muscle relaxed rapidly to a level of low tension and showed irregular waves of faint contractile activity. These effects of DNP were associated with a change in electrical activity from the characteristic bursts of action potentials to a repetitive firing of single, pacemaker type, action potentials of low, but remarkably constant, frequency. Typical for these action potentials is a large negative after potential or "slow component". This kind of electrical activity continued uninterrupted as long as the preparation was exposed to the drug. Direct microscopic observation revealed considerable, but asynchronous contractile activity, which may account for the low tension and further indicate failing of conduction

under these conditions. Catecholamines (10^{-6}M) elicited their usual, however diminished responses in the presence of DNP in the above concentrations. It is noteworthy that isoproterenol in concentrations of 10^{-5} to 10^{-6}M, which initially abolishes the slow repetitive firing associated with DNP action, subsequently counteracts the effects of the poison and increases tension substantially. The hyperpolarizing effects of DNP, typical in taenia coli and also after prolonged exposure in uterine smooth muscle did not appear in the smooth muscle of the superior mesenteric vein within 30 minutes of observation. These and essentially similar studies with cyanide will be discussed in relation to the responses of vascular smooth muscle to chemical mediators. (Supported by Contract DADA-17-68-C-8058 with the U.S. Army Research and Development Command and a grant from the American Heart Association.)

Reference 7

Reprinted from PROC. OF THE INTERNATIONAL UNION OF PHYSIOLOGICAL SCIENCES - V. VII 200, 1968.

ELECTRICAL PROPERTIES OF VASCULAR SMOOTH MUSCLE. L. Horn and M. Kumamoto*, N.J. Coll. Med. & Dent., Jersey City, N.J. and A. Nakajima*, Kyoto Univ., Kyoto, Japan.

We have investigated the electrical properties of vascular smooth muscle with intracellular techniques. The normal, spontaneous activity of longitudinal fibers of the superior mesenteric vein of the guinea pig is essentially similar to the activity of visceral smooth muscle. Action potentials associated with spontaneous contractions range from 35 to 63 mV and rise abruptly or are generated by slow depolarizations from resting levels of 41 to 67 mV. The marked tendency of pacemaker activity in this muscle may indicate a poor conduction. The spikes appear singly, in pairs, and in bursts of considerable duration. Accompanying action potentials with a half duration of 14 $\pm$ 2.9 msec slow voltage changes of considerable amplitude occurred. This slow component is quite characteristic of this relatively small smooth muscle fiber and will be discussed in terms of conductance changes. Experiments with graded changes in K_o show that the resting potential depends on the Ki/Ko gradient with a maximal slope of 35 mV per ten-fold change in K_o. It is remarkable that linearity extends beyond the range found in other muscle and in nerve. Non-linearity appears in the range of normal K_o, i.e., the apparent decrease in gK occurs at the normal resting potential of this muscle. It was also shown that the membrane is less permeable to $SO_4^=$ than to Cl^-. The slow potential was more prominent in preparations exhibiting low activity and poor conductivity as judged from the preponderance of generated spikes. The results of our studies will be discussed in terms of ion permeabilities and a possible potassium inactivation. (Supported by Contract DADA-17-68-C-8058 with the U.S. Army R & D Command.)

Reference 8

ABSTRACT

Horn, L., M. Kumamoto and A. Nakajima: Electrical Activity Induced by Catecholamines and Acetylcholine in Vascular Smooth Muscle. Am. J. Physiol. --------.

Electrical activity of the longitudinal muscle fibers from the superior mesenteric vein of the guniea pig was studied *in vitro* using intracellular techniques. Adrenaline or noradrenaline increased spike activity and caused a sustained depolarization. Isoproterenol abolished spike activity and subsequently hyperpolarized the membrane. Adrenaline increased tension without causing electrical changes of preparations depolarized to about 10 mV by high concentrations of potassium. Acetylcholine initiated or increased spike activity and depolarized the membrane, but repolarized the membrane when it had been depolarized to about 32 mV by 23.5 mM potassium solution prior to drug application, suggesting that acetylcholine produces an equilibrium potential of about 40 mV in this vascular smooth muscle.

(This work was supported by contract DA-49-193-MD-2843 with the U.S. Army Medical Service Research Development Program and by a grant from the American Heart Association.)

Reference 8

ELECTRICAL ACTIVITY INDUCED BY CATECHOLAMINES AND ACETYLCHOLINE IN VASCULAR MUSCLE. L. Horn, M. Kumamoto and A. Nakajima. Depts. Physiology, N.Y. Med. College, N.Y. and College of Med. & Dent. of N.J. at Newark, Newark, N.J. (Submitted for publication, 1970.) Supported by Contract DA-49-193-MD-2843 with the U.S. Army Medical Service Research Development Program and a grant from the American Heart Association.

Introduction: The effects of catecholamines and acetylcholine on vascular smooth muscle have been studied in considerable detail utilizing a variety of methods (Furchgott, 1955). Electrophysiological recording techniques, i.e., intracellular recording and the sucrose gap method, have only recently been applied to vascular smooth muscle to study the changes in membrane activity produced by transmitter agents. Reports show that the excitatory effects of adrenaline and noradrenaline on blood vessels are brought about by depolarization of the membrane and initiation or increase in spike activity (Roddie, 1962; Funaki, 1964; Keatinge, 1964; Steedmand, 1966; Nakajima and Horn, 1967a,b; Horn and Nakajima, 1967). Inhibitory effects of isoproterenol, although short-lasting even at high concentration, were associated with suppression of spike activity on the portal vein of the rat (Sutter, 1965; Axelssen, Johanson, Jonsen, and Wahlstrom, 1967).

In our present experiments we investigated further the behavior of single cells of the superior mesenteric vein of the guinea pig in response to catecholamines and obtained some additional information including continuous records of the 'direct' action of adrenaline on the contractile elements of the muscle.

Sutter, 1965, reported that in the guinea pig, acetylcholine caused contraction at any dose up to 10^{-5} g/ml of the superior mesenteric vein. The increase in tension was brought about by depolarization of the membrane and increase in spike activity. An initial hyperpolarization preceding the depolarization has also been reported (Funaki and Bohr, 1964; Nakajima and Horn, 1967 a,b).

It is well known that acetylcholine produces an equilibrium potential at the motor end plate, presumably associated with an increase in membrane permeability to all ions (Fatt and Katz, 1951; del Castillo and Katz, 1954, 1955). Acetylcholine also produces an equilibrium potential in taenia coli (Burnstock, 1958b; Bülbring and Kuriyama, 1963). In present experiments we have studied the effects of acetylcholine on the superior mesenteric vein and the results suggest that an equilibrium potential also exists for acetylcholine in this vessel, although the level of this potential was considerably higher than that for taenia coli.

Methods: The superior mesenteric vein of the guinea pig was used for all experiments. Longitudinal strips, 1 x 7 mm, were mounted in a small chamber with modified Krebs solution at 37C, such that the outer surface of the vessel wall was accessible. The standard solution contained: (mM) NaCl 133, $NaHCO_3$ 16.3; NaH_2PO_4 1.38; KCl 4.7; $CaCl_2$ 2.5; $MgCl_2$ 0.105 and dextrose 7.8 (Keatinge, 1964), and was equilibrated with a mixture of 5% CO_2 and 95% O_2. After removing the connective tissue, microelectrodes with a resistance of 20 to 50 megohm were inserted into the longitudinal smooth muscle layer by the floating method (Woodbury and Brady, 1956). The isometric tension of the strip was monitored via a mechanoelectric transducer (RCA 5734) and together with the membrane potential displayed on the oscilloscope. A Grass kymograph camera was used to obtain permanent records suitable for illustrations (slow speed) and for measurement of rates of rise and fall and the duration of the action potentials (high speed). Modified Krebs solutions with high potassium chloride concentrations were prepared by replacing sodium chloride by equimolar amounts of potassium chloride. When excess potassium was required, solid potassium chloride was added to the standard solution. It was technically difficult to maintain impalement during rapid change of the bath medium. Therefore, the microelectrode usually had to be reinserted after the solution had been replaced. Calculated amounts of drugs dissolved in the standard solution were added directly to the bath, 1.5 cm from the recording site.

Results:

Normal Activity: The superior mesenteric vein was spontaneously active. Bursts of spike discharges were usually associated with the contractions, although the two phenomena did not always have the same time course. The burst usually arose abruptly from resting membrane potential, i.e., the burst was triggered by a conducted impulse, also when the membrane appeared to be in a highly excitable condition as determined by degree of spontaneous activity and subsequent responses to drugs. Generated spikes, as judged by a slow depolarizaing phase, appeared sporadically and more frequently when the preparation had a low membrane potential (Fig. 1E). The configuration of the action potentials during a burst varied considerably. For all action potentials the average value (+ SE) of the maximum rate of rise was 4.8 ± 1.7 V/sec., rate of fall was 3.6 ± 0.8 V/sec., and half duration was 14 ± 2.9 msec. Fig. 1A shows a burst consisting of fast action potentials with rapid rates of rise and followed by marked positive after potentials. However, the most common pattern of the burst is shown in Fig. 1B. There can be seen irregular spikes with varying amplitudes intermingled with fast spikes. The maximum rates of rise and fall of the irregular spikes were lower than those of the fast spikes. For example, the averages from three different such bursts were 6.1 V/sec. and 3.6 V/sec. for the fast spikes as compared to 4.7 V/sec. and 3.2 V/sec. for the irregular spikes.

The prominent slow wave during the burst was a characteristic feature of the electrical activity of this vessel. In preparation with low membrane potentials the duration of the burst was short-lasting and the spikes arose with the slow waves (Fig. 1C). After the first spike was triggered by a conducted impulse, the prominent slow wave followed from which a second and third spike generated successively. Not only generated spikes but also conducted ones may be superimposed on the slow wave.

More simplified combinations of spikes and slow waves can be seen in Fig, 1D. After initial complex spike discharges, single spikes followed by prominent slow waves appeared. The slow wave, basically as represented here, but often somewhat modified by abortive spikes, appear to be characteristic for this muscle.

Occasionally single spikes were generated intermittently without slow waves as illustrated in Fig. 1E.

Longer bursts of spike discharges are usually superimposed on a more or less sustained depolarization of the membrane. The amplitude of this depolarization varied, ranging from 0 to 15 mV. No relation could be found between amplitude and initial membrane potential or amplitude and duration of the burst.

Effects of Adrenaline and Noradrenaline: The action of adrenaline on this spontaneous active preparation has been described previously (Nakajima and Horn, 1967a, b). A typical pattern of the drug action is shown in Fig. 2. The changes in membrane potential and spike frequency produced by adrenaline in different concentrations were plotted against time (Fig. 3). Adrenaline in high concentration (10^{-5} or 10^{-6} g/ml) caused rapid depolarization of the membrane associated with increase in spike frequency. The action potentials eventually changed into mere oscillations of declining frequency until finally, a sustained depolarization remained. In lower concentration (10^{-7} g/ml) the sequence of events progressed more slowly. In very low concentrations (10^{-8} or 10^{-9} g/ml), the only effect was a prolongation of the bursts and shortening of the intervals between bursts without detectable depolarization of the resting membrane potential. The final level of the depolarization induced by high concentration of adrenaline was around 20 mV.

Noradrenaline had qualitatively the same, but quantitatively lesser effect than adrenaline. Fig. 4 shows the effect of noradrenaline in a concentration of 10^{-6} g/ml. Spike activity still continued at high frequency four minutes after administration of the drug.

Effects of Adrenaline at High Potassium Concentrations: The effect of adrenaline was examined in muscles completely depolarized by addition of solid potassium chloride, directly to the incubation chamber in order to observe the dissociation between electrical and mechanical activity. The increase in external potassium concentration caused rapid depolarization of the membrane of the superior mesenteric vein accompanied by repetitive discharges of action potentials of high frequency (Fig. 5). After 10 to 20 sec., the spike activity ceased and sustained

depolarization of about 10 mV was obtained. Tension developed rapidly associated with this accelerated membrane activity and depolarization. Adrenaline, when applied after the depolarization, caused a further increase in tension, but without by itself causing detectable change in the membrane potential. It is noteworthy that the onset of this increasing tension usually occurred after some degree of repolarization (in Fig. 5 at a membrane potential of about 20 mV), however, the slight repolarization cannot be attributed to the adrenaline as it usually occurred also in the absence of the drug.

Effects of Isoproterenol: Isoproterenol had an inhibitory effect on the superior mesenteric vein. The effect was brought about by suppression or abolition of spike activity, usually followed by hyperpolarization or repolarization of the membrane. Fig. 6 shows the effect of 10^{-6} g/ml isoproterenol. Two minutes thirty seconds after application of the drug the last train of spike discharges appeared with irregular and abortive spikes. No further spikes were observed even though the impalement was maintained for five minutes.

The membrane hyperpolarized gradually. The maximal degree of the hyperpolarization was only about 6 mV in the case illustrated. The abolition of spike discharges preceded the hyperpolarization.

After the inhibitory effect subsided the membrane returned to its initial resting level or lower and bursts of spike discharges appeared with short intervals.

Similar responses have also been described by Axelsson, 1966 et al., for rat's portal vein. However, the inhibitory period lasted five minutes or more in the guinea pig whereas it was very short-lasting in the rat for the same concentration of the drug.

Effects of Acetylcholine: Acetylcholine depolarized the membrane and initiated or gradually increased the spike activity of the preparation. A typical record is shown in Fig. 7. The maximum rate of rise and rate of fall of the action is shown in Fig. 7. The maximum rate of rise and rate of fall of the action potentials increased after application of acetylcholine. Four series of such measurements were performed. One of these which had the unusually low initial values of 2.5 V/sec

and 2.4 V/sec increased to 4.1 V/sec and 3.4 V/sec five minutes after the application of the drug in a concentration of 10^{-6} g/ml.

Changes in membrane potential produced by acetylcholine have been illustrated graphically in Fig. 8. After a brief time, during which a slight hyperpolarization sometimes occurred as described previously (Nakajima and Horn, 1967b), the membrane potential decreased gradually to the level of 40 to 45 mV. The spike frequency initially increased and firing became repetitive and then returned to control rates before any detectable repolarization occurred. It should be noted that the initial frequency of spike discharge given in Fig. 8 is the frequency within intermittent bursts in the control period and subsequent values for frequency are after firing has become repetitive. The action potentials did not deteriorate into oscillation with drug concentrations ranging from 10^{-8} to 10^{-5} g/ml.

Effects of Acetylcholine with High Potassium Concentrations: The effect of acetylcholine was also tested at high potassium concentrations. When the preparation was exposed to a solution containing 2.5 times normal concentration of potassium, the membrane potential either did not change or it decreased slightly; the spike activity increased and the intervals between the burst shortened or occasionally the firing became repetitive. Under these conditions acetylcholine in concentrations of 10^{-6} to 10^{-5} g/ml depolarized the membrane to 40-45 mV and increased firing frequency further as it did in normal Krebs solution. When exposed to 5 times normal concentration of potassium the preparation depolarized to 28-38 mV and fired repetitive action potentials. Under such conditions acetylcholine would repolarize the membrane slightly (average 6 mV) but nevertheless accelerate the spike activity (Fig. 9). Representative records of these changes in membrane potential have been plotted in Fig. 10 and indicate that the drug action on membrane potential was reversed when the membrane was already depolarized to 40 mV or less. Therefore, acetylcholine shifted the membrane towards a definite potential level.

Discussion:

Intermittent spontaneous bursts of spike discharges are a common feature of the electrical activity of the superior mesenteric vein. The bursts are usually evoked by conducted impulses from active neighbouring cells. However, in preparations with low resting potentials, single spikes with a slow rising phase appear, indicating that the impaled cell is also capable of generating the spikes. Apparently, a usually prominent slow wave (Nakajima and Horn, 1967 a,b) is a most important factor for production of subsequent spikes during a burst. The slow wave or local potential of smooth muscle has been attributed to electrotonic spread from active neighbouring cells (Bülbring, Burnstock and Holman, 1958). It has also been suggested that it may originate at special loci of the cell membrane (Kuriyama and Tomita, 1965; Tomita, 1966). The slow wave observed in our preparation may not be easily attributed to electronic spread because of its long duration. The characteristic (undistorted) pattern of the slow wave is, furthermore, that of a marked negative after-potential as shown in Fig. 1D. When it reaches threshold level, second or third spikes are triggered. Since the amplitude of the slow wave in other smooth muscle has been shown to be strongly influenced by the external sodium concentration (Bülbring et al. 1963; Tamai and Prosser, 1966), changes in the sodium permeability may be important for the production of slow waves. It has been observed, however, that a characteristic feature of the action potential is a rapid rate of repolarization which brings the membrane towards the potassium equilibrium level and produces a marked positive after-potential (Speden, 1964; Nakajima and Horn, 1967 a,b). This may be explained in terms of a rapid and adequate increase in potassium permeability (Steedman, 1966), although the mechanism controlling the pattern of activity could be considered a competitive action between spike generation and repolarization, but where the corresponding increases in potassium permeability vary in duration resulting in slow waves, abortive spikes or membrane oscillations.

When the membrane is highly excitable and the pattern is that of rapid repetitive firing, slow waves do not occur (Fig. 1A). However, when the membrane potential

is low and the preparation fires at a low frequency, activated by the spike, unknown factors may more easily overcome the driving force that tends t bring the membrane back towards the potassium equilibrium potential. The fact that the rate of fall of the spikes with slow waves was much lower than that of the spikes with a marked positive after-potentials may suggest that the sodium inactivation progresses slower, and/or that the potassium permeability is inadequate under these conditions. Other explanations are also possible. The sustained depolarization or plateau during a burst is maintained for varying periods of time. Usually, a subsequent generated full spike causes repolarization. However, it should be emphasized that there are mostly conducted spikes superimposed on the slow wave and on the plateau even though many of the spikes appear to have been generated from both.

As reported previously, adrenaline or noradrenaline have excitatory effects on the superior mesenteric vein, brought about by increase in spike activity and depolarization of the membrane. In addition to the membrane stimulating action, adrenaline appears to have a direct effect on the contractile elements of smooth muscle. This effect has been demonstrated in various smooth muscle more or less fully depolarized by high concentrations of potassium, (Evans, Schild and Thesleff, 1958; Vaugh, 1962; Edman and Schild, 1963). In present experiments, utilizing similar procedures, our preparation depolarized rapidly to the level of about 10 mV as other smooth muscle does (Holman, 1958; Goto and Csapo, 1959; Jung, 1959; Marshall, 1962). Adrenaline caused a further increase in tension without detectable change in membrane potential attributable to the drug. As noted previously, however, the onset of this direct effect does not occur until the membrane has repolarized somewhat. In the illustrated case the tension begins to increase when the membrane potential reaches 15-20 mV, viz., below or about the same level of depolarization where spike activity is abolished by adrenaline in experiments with other-wise untreated smooth muscle from the superior mesenteric vein. It is noteworthy in this regard that tension usually continues to increase also after cessation of membrane activity in the latter type experiments and that this increase most probably must be considered a direct adrenaline effect, rather than one due to remaining electrical

activity in other muscle fibers after the activity has ceased in the impaled one, as suggested by us in a previous report (Nakajima and Horn, 1967b). Edman and Schild (1963) have suggested that an increased permeability to Ca ions may be responsible for the direct effect.

Little is known about the inhibitory effect of isoproterenol on this vessel. The only relevant finding in the present experiments is that the effect was brought about by abolition of spike activity and hyperpolarization of membrane. It is noteworthy that the spike activity ceased before noticeable hyperpolarization. This change in membrane activity is quite similar to that which adrenaline produces in taenia coli (Burnstock, 1958b; Bülbring and Kuriyama, 1963).

Acetylcholine causes increase in spike activity and depolarization of the membrane of the superior mesenteric vein (Funaki and Bohr, 1965; Junaki, 1966; Nakajima and Horn, 1967 a,b; Horn and Nakajima, 1967) analogous to its effect on visceral smooth muscle (Burnstock, Holman and Prosser, 1963). The question arises whether or not the underlying ionic mechanisms are the same as in other smooth muscle. In taenia coli the effect of acetylcholine depended on the initial membrane potential; the higher the initial membrane potential, the greater the depolarization (Bülbring and Kuriyama, 1963), and Burnstock (1958a) has shown that depolarization did not occur or was reversed to a repolarization in a solution containing 40 mM potassium. Similarly, in present experiments, on exposure to 11.7 mM (2.5 times normal) of potassium, no change, or only a slight decrease in membrane potential was observed, as might be expected since this concentration corresponds to the non-linear part of the potassium concentration-depolarization curve (Kumamoto and Niu, 1967). In this solution acetylcholine depolarized the membrane. However, when the membrane was depolarized to about 32 mV on exposure to the solution containing 23.5 mM potassium (5 times normal) acetylcholine had a tendency to repolarize the membrane as was shown in Fig. 10.

Therefore, it is suggested that acetylcholine produces an equilibrium potential of approximately 40 mV in this vascular smooth muscle, as compared to about 20 mV in taenia coli (Burnstock, 1958a).

The ionic mechanisms of this acetylcholine effect in our preparation are unknown. Because of the similarity of the changes in membrane potential it is likely that similar mechanisms may be involved as those described for the motor end plate and for taenia coli.

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LEGENDS

Fig. 1 - Patterns of spontaneous electrical activity. Upper trace is tension. For discussion see text. This record has been retouched.

Fig. 2 - Typical electrical activity in response to adrenaline. Upper trace is tension. Continuous record.

Fig. 3 - Electrical responses to various concentrations of adrenaline plotted against time. The upper graph shows that the membrane depolarizes more rapidly at higher adrenaline concentrations towards a level of about 20 mV. For the lower panel the corresponding spike frequencies have been plotted. It is seen that in the lower concentrations, spike activity was induced and continued throughout the experiment.

Fig. 4 - Typical electrical responses to noradrenaline from a continuous, retouched record. Disregard upper trace.

Fig. 5 - The "direct" effect of adrenaline. See text.

Fig. 6 - The lower trace shows that 10^{-6} g/ml isoproterenol abolishes spike activity before detectable hyperpolarization. The upper trace shows a slight transient decrease in tension.

Fig. 7 - Continuous record of electrical responses to 10^{-5} g/ml acetylcholine. Upper trace is tension. This record has been retouched.

Fig. 8 - Upper graph is a plot of membrane potential against time and shows that acetylcholine depolarizes the membrane to 40-45 mV sometimes after a brief initial period of hyperpolarization. Lower graph shows the effect of acetylcholine on spike frequency. Note that values given at time zero are spike frequencies within bursts of activity and that solid line indicate continuous, repetitive activity.

Fig. 9 - Continuous record of the effects of 10^{-5} g/ml acetylcholine on a preparation which has been depolarized by 5 times normal concentration of potassium to about 35 mV. A slight repolarization (here 6 mV) associated with increased spike activity occurs. Upper trace is a tension record which was repositioned at the time of acetylcholine application. This record has been retouched.

Fig. 10 - Shows a plot of the changes in membrane potential in five representative experiments on the effect of acetylcholine at two concentrations of K_o, suggesting an equilibrium potential of about 40 mV.

Reference 9

EFFECTS OF 2-4-DINITROPHENOL ON THE ELECTRICAL AND MECHANICAL ACTIVITY OF VASCULAR SMOOTH MUSCLE. Minayori Kumamoto* and Leif Horn. N.J. Col. Med. & Dent., Jersey City, N.J. 07304.

With intracellular techniques <u>in vitro</u> we have studied effects of 2-4-dinitrophenol (DNP) (10^{-4}-$5 \cdot 10^{-3}$M) on electrical and mechanical activities of muscle from guinea pigs superior mesenteric vein. Initially phasic contractions decreased in duration and amplitude and increased in frequency in all experiments. Low concentrations of DNP induce depolarization to about -40 mV and repetitive firing of single action potentials with decreased amplitude. At higher concentrations the electrical activity ceases before any detectable change in the resting potential. At the highest concentration of DNP the spike activity ceases more rapidly and after subsequent depolarization to about -40mV the membrane exhibits a characteristic electrokinetic phenomenon resembling repetitive abortive spikes which do not trigger any detectable contractile activity. In all experiments electrical and mechanical changes caused by DNP were reversible. The peculiar bistable flip-flop electrokinetics of the membrane at -40 mV and the cessation of activity prior to depolarization suggest a direct effect of DNP or voltage dependent changes in specific conductances and is presently under investigation with voltage clamp techniques.

(Supported by Contract DADA 17-68-C-8058 with the U.S. Army Research and Development Command.)

Reference 10

REPRINTED FROM MICROVASCULAR RESEARCH 2:182-187, 1970) -

"EFFECTS OF 2:4-DINITROPHENOL ON THE ELECTRICAL AND MECHANICAL ACTIVITY OF VASCULAR SMOOTH MUSCLE" - Leif Horn and Hinayori Kumamoto.

(33 copies enclosed).

MICROVASCULAR RESEARCH 2, 182–187 (1970)

Effects of 2:4-Dinitrophenol on the Electrical and Mechanical Activity of Vascular Smooth Muscle[1, 2]

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Received October 20, 1969

With intracellular techniques *in vitro* we have studied effects of 2-4-dinitrophenol (DNP) (10^{-3} to 5×10^{-3} *M*) on electrical and mechanical activities of muscle from guinea pigs superior mesenteric vein. Initially phasic electrical and mechanical activity decreased in duration and amplitude and increased in frequency in all experiments. Low concentrations of DNP subsequently induce depolarization to about −40 mV and repetitive firing of single action potentials with decreased amplitude. At higher concentrations the electrical activity ceases before any detectable change in the resting potential. At the highest concentration of DNP the spike activity ceases more rapidly, and, after subsequent depolarization to about −40 mV, the membrane exhibits a characteristic electrokinetic phenomenon resembling repetitive abortive spikes which do not trigger any detectable contractile activity. In all experiments electrical and mechanical changes caused by DNP were reversible. The peculiar bistable flip-flop electrokinetics of the membrane at −40 mV and the cessation of activity prior to depolarization suggest a direct effect of DNP on voltage dependent changes in specific conductances.

INTRODUCTION

Control of local blood flow is effected through graded changes in the diameter of the peripheral blood vessels and a more or less complete opening or closure of the precapillary sphincters. Such physiological regulation of local blood flow is believed to be related to ionic and metabolic interrelations of the vascular smooth muscle and parenchymal tissues. Since Funaki first reported intracellular electrical recordings from blood vessels in 1958, a number of papers have dealt with the electrophysiological aspects of vascular smooth muscle (Horn, 1970). The normal spontaneous electrical activity, effects of catecholamines and acetylcholine (Nakajima and Horn 1967; Horn, Kumamoto, and Nakajima, 1970), and the effects of potassium ion (Kumamoto and Niu, 1966) on the smooth muscle of the guinea pig's superior mesenteric vein have been described in detail. However, little is known about how metabolism affects and regulates the responsiveness of vascular smooth muscle although inferences have been made from effects of metabolic poisons on other smooth muscle. In current experiments, utilizing intracellular techniques *in vitro*, we have studied effects of a

[1] A preliminary report on this study was presented at the Annual Meeting of the Federation of American Societies for Experimental Biology in Atlantic City, New Jersey on April 13–18, 1969.

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[3] Dr. Kumamoto's present address is Department of Liberal Arts, Kyoto University, Kyoto, Japan.

commonly used metabolic inhibitor, or uncoupler, 2:4-Dinitrophenol (DNP), on the electrical and the mechanical activities of the longitudinal smooth muscle of the guinea pig's superior mesenteric vein. Our results indicate that DNP has additional physicochemical effects on the vascular smooth muscle membrane that may completely overshadow the metabolic effects.

METHODS

The superior mesenteric vein of the guinea pig was used in all experiments. Isolated longitudinal strips, 5 mm long and 1.5 mm wide, were mounted in an organ bath of 4 ml. Adipose tissues and the adventitia were carefully removed under a dissecting microscope. Microelectrodes with a resistance of 30 to 40 megohms were inserted into the longitudinal smooth muscle layer from the outside of the vein by the floating method (Woodbury and Brady, 1956). The isometric tension of the strip was recorded with a mechanoelectric transducer (RCA 5734).

The modified Krebs solution used in the experiments contained (in mM) NaCl 133; $NaHCO_3$ 16.3; NaH_2PO_4 1.38; KCl 4.7; $CaCl_2$ 2.5; $MgCl_2$ 0.105, and dextrose 7.8 (Keatinge, 1964) and was aerated with 95% O_2 + 5% CO_2. The solution flowed continuously at the rate of 8–10 ml min and at a constant temperature of 37°.

RESULTS

DNP was administered in concentrations of 10^{-4} *M*, 5×10^{-4} *M*, and 5×10^{-3} *M* to spontaneously active preparations with a resting potential of −50 to −60 mV.

The preparations, which were observed under microscope during the experiments, exhibited somewhat arrhythmic, spontaneous contractions in the control period. After the application of DNP, the number of apparent pacemaker sites increased, with smaller clones of fibres contracting regularly but asynchronously at a greater frequency than the intrinsic rate during the control periods.

Typical results obtained are shown in Figs. 1, 2, and 3 and summarized in Fig. 4. Changes in electrical activity and tension occurred within a minute following drug application. The phasic contractions decreased in duration and amplitude and increased in frequency during initial phase of all experiments.

In Fig. 1, with low concentration of DNP, (10^{-4} *M*), complete relaxation is not obtained; the spike bursts associated with phasic concentration increase in frequency but decrease in duration; the spikes decrease in amplitude, and the pattern changes into one of repetitive firing of single action potentials while the membrane is depolarizing to about −40 mV. There is a further tendency for the single action potentials, each accompanied by one phasic contraction, to increase in frequency. The frequency continues to increase for several minutes after drug application. Throughout the repetitive firing phase, the spike amplitude remained at about two-thirds of its normal value.

In Fig. 2, with 5×10^{-4} *M* DNP, the spike activity ceases within 2 min after application of the drug and the membrane depolarizes to about −45 mV. Although slow fluctuations may occur between −45 and −50 mV, there is no marked change in membrane potential within another 10 min. Also at this concentration, before cessation of activity, the spike amplitude declined to about two-thirds of normal value.

FIG. 1. The effect of 10^{-4} *M* DNP. Upper trace shows tension, and lower trace shows electrical activity: (A) control period; (B) 10^{-4} *M* DNP was applied at the end of panel A; (A)–(D) continuous records; (E) after 8 min. 30 sec.

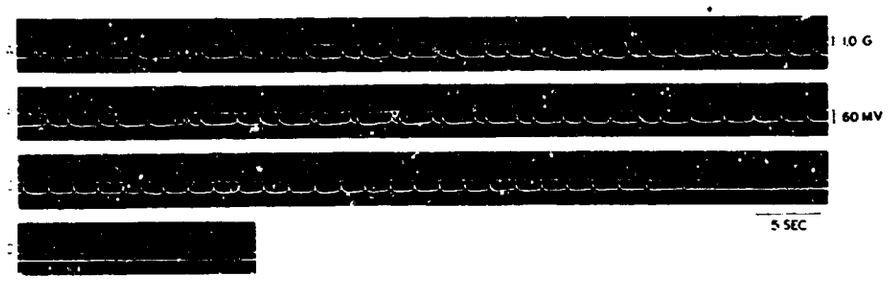

FIG. 2. The effect of $5 \cdot 10^{-4}$ *M* DNP: (A) control period and application of $5 \cdot 10^{-4}$ *M* DNP at the arrow; (A), (B), and (C) continuous records; (D) after 15 min.

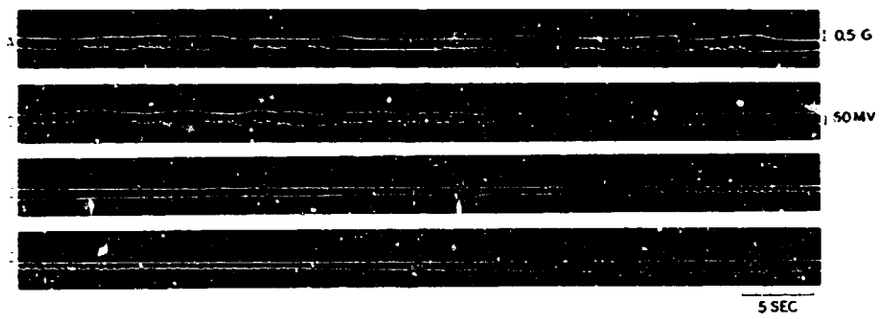

FIG. 3. The effect of $5 \cdot 10^{-3}$ *M* DNP: (A) control period; (B) $5 \cdot 10^{-3}$ *M* DNP was applied at the end of panel A; (A)–(D) continuous records.

In a still higher concentration of DNP ($5 \cdot 10^{-3}$ *M*), the spike activity ceases more rapidly than the case of $5 \cdot 10^{-4}$ *M* DNP. After spike cessation at a membrane potential of approximately −54 mV in the illustrated case, the membrane may remain at the same potential for a considerable length of time, as is shown in Fig. 3, or exhibit slow fluctuations before it depolarizes to about −40 mV and exhibits a characteristic electrokinetic

phenomenon resembling "abortive spikes." These oscillatory electrical changes did not trigger any detectable contractile activity. At this high concentration of DNP in some experiments, the membrane went through a slight depolarization and a repolarization to initial level just after the spike cessation, however, these changes were always followed by a more marked depolarization (to about −40 mV) which led into oscillations without exception.

In all experiments, both electrical and mechanical changes caused by DNP were almost completely reversible.

DISCUSSION

It is well known that, in the smooth muscle of the guinea pig's taenia coli, DNP exerts a diphasic action in terms of an initial excitation and a secondary inhibition (Born and Bülbring, 1955; Bülbring and Lullmann, 1957; Burnstock, 1958). In this vascular muscle, such a remarkable excitatory phase as that occurring in the taenia coli was not observed at any of the employed concentrations of DNP. However, in a concentration of 10^{-4} *M* DNP, after the initial changes in both electrical and mechanical activity, the spike frequency gradually increased as did the frequency of the small, transient increase in muscle tension accompanying each spike. The resting tension did not decline, but the maximal tension decreased for reasons that probably relate to asynchronization (fibrillation) of the preparation and to changes in its electrical properties.

Since the diphasic action of DNP on oxygen consumption (Born and Bülbring, 1955) and on spike discharge (Bülbring and Lullmann, 1957) was associated with an initial fall and then later an increase in the membrane potential, the effects of DNP on the smooth muscle of the guinea pig's taenia coli have been discussed from the point of view that the drug is just a metabolic inhibitor.

In our experiments with low concentrations of DNP (10^{-4} *M*), the initial changes in electrical activity as shown in Fig. 1 are somewhat similar to those reported for the taenia coli. At this low concentration of DNP, the initial changes in both electrical and mechanical activity might be explained by the metabolic effects of the drug, as implicated in the studies of the guinea pig's taenia coli (Born and Bülbring, 1955; Bülbring and Lullmann, 1957; Burnstock, 1958). This could indicate that the DNP reduces the ATP content and the oxygen consumption in the early phase and that the decrease in ATP content suffices to inhibit contractile activity in the taenia coli (Born, 1955).

However, in Fig. 2, with $5 \cdot 10^{-4}$ *M* DNP, the cessation of spike activity is associated with a less marked change in the membrane potential than that at the lower concentrations. Even more striking, in the highest concentration of DNP ($5 \cdot 10^{-3}$ *M*), the spike cessation occurs practically without any change in the membrane potential, and then the membrane potential gradually falls and goes into oscillation as shown in Fig. 3 and further illustrated in Fig. 4. Furthermore, as mentioned above, in some of our experiments with this concentration of DNP a transient repolarization occurred, occasionally almost to the normal resting level but always without the reinitiation of spike activity. Such fluctuations were always followed by the more marked depolarization that led into the peculiar bistable flip-flop electrokinetics of the membrane around −40 mV. Nonetheless, these changes suggest the possibility that the DNP, apart from its metabolic action, has additional effects on the ion permeability of the cell membrane.

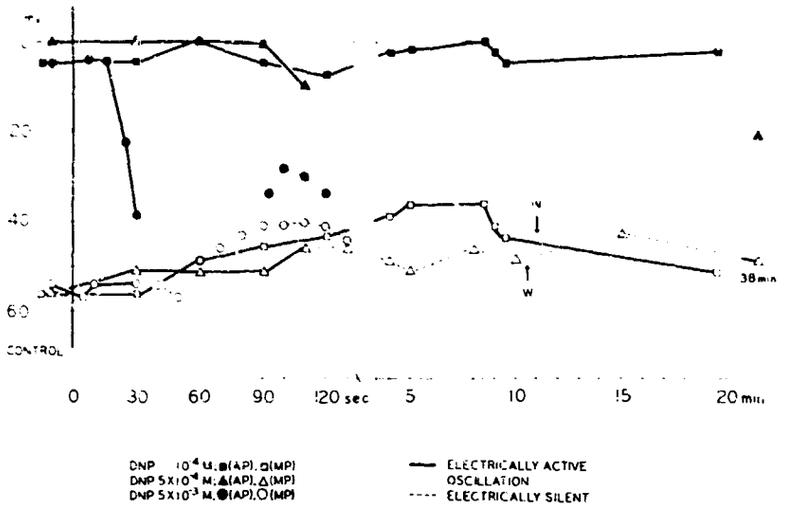

FIG. 4. Changes in electrical activity as a function of time at the three concentrations of DNP. Upper traces with filled in symbols show the peak values of the action potentials and lower traces with open symbols show the resting membrane potentials. Therefore, the distances between these traces represent the amplitude of the action potentials.

In the highest concentrations of DNP $5 \cdot 10^{-3}$ *M* (●-), the spike cessation occurred without any change in the membrane potential. In the concentration of $5 \cdot 10^{-4}$ *M* DNP (▲-), the cessation of spike activity is accompanied by a slight depolarization at a membrane potential of −46 mV which normally would maintain activity. The arrows at W's indicate washing in Krebs solution.

In the frog's skeletal muscle, Koketsu *et al.* (1964) indicated that an initial rapid depolarization caused by DNP was closely related to an estimated increase in the intracellular Cl^--concentration (DNP left potassium permeability essentially unaffected and increased both Na^+ and Cl^- permeability during the first 30 minutes). Hopfer *et al.* (1968), concluded from experiments with artificial phospholipid bilayer membranes that uncoupling agents such as DNP increase the specific conductance of such model membranes by facilitating transport of H^+ or OH^- (or both) across the membrane. Since DNP is lipid soluble, it is conceivable that DNP could alter the physicochemical properties by direct interaction with lipoprotein also in the vascular smooth muscle membrane.

Although the secondary marked depolarization observed at high concentrations of DNP ($5 \cdot 10^{-3}$ *M*) could be caused by the inhibition of the sodium pump and the subsequent rise in the membrane potential might be a consequence of increased metabolic activity, too little is known about the permselectivity of the vascular smooth muscle membrane to warrant any detailed discussion at the present time.

It appears obvious, however, that whatever reasons for the depolarization and subsequent repolarization, the electrokinetic behavior around −40 mV in high concentrations of DNP indicates quite clearly a fundamental change in the membrane properties. The abortive spikes, which have a maximal amplitude of less than 20 mV, the initial fall in spike amplitude, and the apparent conduction impairment indicate a direct effect of DNP on voltage dependent changes in specific conductances.

During the initial increase in electrical activity in vascular muscle at low DNP-concentrations there was no preceding depolarization, indicating that DNP does not here affect the overall resting permeability of the membrane. Rather, DNP affects a conductance, normally subject to slow inactivation and important for the frequency and duration of the burst of spikes, i.e., more specifically the plateau or slow component from which spikes arise.

If one may assume that DNP predominantly exhibits physicochemical effects in the initial phase (spike frequency) and that then gradually the metabolic effects (depolarization-repolarization) manifest themselves in smooth muscle, then the difference between the observed phenomena in the vascular muscle and those in the taenia coli could be attributed to the relative susceptibility to physicochemical and metabolic effects mediated by DNP in the two tissues.

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The authors thank Dr. H. Niu for helpful discussion.

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Reference 11

Reprinted from Proc. of the XVII Annual Meeting of the Microcirc. Soc. 1969.

DOES CALCIUM CARRY THE EARLY TRANSIENT CURRENT OF THE ACTION POTENTIAL IN VASCULAR SMOOTH MUSCLE? Leif Horn and Minayori Kumamoto. N.J. Col. Med. & Dent., Jersey City, N.J. 07304.

The electrokinetics of vascular smooth msucle is similar to that of other spontaneously active smooth muscle and we have assumed that the ionic mechanisms underlying the electrical behavior are those proposed by Hodgkin and Huxley. However, our latest experiments have resulted in a significant observation that tetrodotoxin (TTX) which specifically blocks the voltage dependent changes in sodium permeability in nerve and skeletal muscle, has no effect on the action potential or impulse propagation in our preparation, whereas blocking of the calcium permeability with manganese renders the preparation inexcitable. Analogous experiments with TTX and Mn^{++} on visceral muscle and on cardiac muscle have prompted other investigators to conclude that Ca^{++} and not Na^{+} is the current carrying ion during the upstroke of the action potential, essentially disqualifying the Hodgkin-Huxley hypothesis for those tissues. Voltage clamping experiments in effort to assess whether calcium is directly involved are in progress. (Supported by contract DADA 17-68-C-8058 with the U.S. Army Research and Development Command.)

Reference 12

Reprinted from THE PHYSIOLOGIST Vol. 12, No. 3, August 1969.

VOLTAGE CLAMPING OF SMOOTH MUSCLE. M. Kumamoto* and L. Horn. New Jersey College of Medicine and Dentistry, Jersey City, N.J.

A sucrose-gap voltage clamping technique, modified from Narahashi and Anderson, has been used with smooth muscle preparations from the superior mesenteric vein and taenia coli of the guinea pig. The electrical activities of both preparations are quite unaffected by tetrodotoxin (TTX) in concentrations of 10^{-6} g/ml, which is known to inhibit the voltage dependent increase in specific sodium conductance in the squid giant axon and skeletal muscle. Manganese in concentrations of $5 \cdot 10^{-4}$M has been shown to drastically reduce the calcium permeability and also to render our smooth muscle preparations inexcitable. Our clamping experiments on taenia coli show that Mn^{++} inhibits the early transient current, but has little or no effect on the steady state current, suggesting the possibility that calcium plays a role as a current carrying ion during the early transient phase. Experiments with TTX are underway. The compatibility of our voltage clamp data with the conventional current carrying mechanisms during the action potential will be discussed. (This research was supported by a grant from the New Jersey Heart Association and Contract DADA-17-68-C-8058 with the U.S. Army Medical Research and Development Command.)

Reference 13

Reprinted from MICROVASCULAR RESEARCH 2:188-201, 1970.

VOLTAGE CLAMPING OF SMOOTH MUSCLE FROM TAENIA COLI. Minayori Kumamoto and Leif Horn. N.J. Col. Med. & Dent., Newark, N.J.

(33 copies enclosed)

MICROVASCULAR RESEARCH 2, 188–201 (1970)

Voltage Clamping of Smooth Muscle from Taenia Coli[1,2]

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Received October 20, 1969

INTRODUCTION

In a current paper (Horn, Kumamoto, and Nakajima, 1970), we reviewed experiments on vascular smooth muscle utilizing intracellular recording of electrical activity and alluded to possible differences between the ionic mechanisms of this muscle membrane and those of skeletal muscle and squid giant axon (Hodgkin and Huxley, 1952 a–d). Our reasons for questioning the applicability of the sodium hypothesis to our preparation, the superior mesenteric vein of the guinea pig, are that Tetrodotoxin (TTX) in concentrations of 100 times that required to inhibit excitability in skeletal muscle (Kuriyama, Osa, and Toida, 1966) and nerve (Narahashi, Moore, and Scott, 1964) has no apparent effect on spontaneous or evoked electrical activity (Kumamoto and Niu, 1966; Horn and Kumamoto, 1969; Kumamoto and Horn, 1969); and that Mn^{2+} known for its inhibition of Ca^{2+} permeability (Hagiwara and Nakajima, 1965) renders our muscle preparation inexcitable (Kumamoto and Horn, 1969). Analogous results have been obtained in taenia coli of the guinea pig by Kuriyama, Osa, and Toida (1966), Nonomura, Hotta, and Ohashi (1966), and Hotta and Nonomura (1968), supporting the hypothesis that Ca^{2+} is a current carrying ion during the upstroke of the action potential. Other experiments by the same investigators with graded changes in Na^{+}_{0}, and with Ca^{2+}-free solutions (see also Holman, 1957, 1958; Bülbring and Kuriyama, 1963), essentially corroborate this contention.

For these reasons, we were interested in examining the changes in the transient early current by voltage clamping techniques in the presence of TTX and Mn^{2+} and in the absence of Ca^{2+}.

Our main interest is vascular smooth muscle, but as has been pointed out before (Nakajima and Horn, 1967), the cell-to-cell conduction of strips from the superior mesenteric vein of the guinea pig is relatively poor, and thus the success rate of experiments with this preparation utilizing the double sucrose-gap voltage clamp technique described here is quite low.

Since the essential features of the vascular smooth muscle which we wanted to investigate are the same in taenia coli, practical considerations dictated that we use this latter

[1] A preliminary report of this paper has been given at the Fall meeting of the American Physiological Society, 1969, (Kumamoto and Horn, 1969).

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tissue in our experiments to be presented in this communication. The data obtained on our vascular muscle preparation are practically identical to those from taenia coli.

While this manuscript was in preparation, the first paper on utilization of the double sucrose-gap voltage clamp technique with uterine smooth muscle was published by Anderson (1969). He used estrogen dominated, uterine smooth muscle to facilitate more extensive intercellular coupling and concluded from his experiments with a Na^+-free solution that the changes in the transient current voltage relationship, e.g., shifting the reversal potential to a more negative value, indicate a Na^+-dependent excitation mechanism.

Our voltage clamp experiments show that the inward current carrying mechanism in taenia coli is Ca^{2+} dependent, inhibited by Mn^{2+}, and unaffected by TTX.

METHODS

Preparations and Solutions

The preparations used in these experiments were freshly dissected longitudinal muscle strips from the taenia coli of the guinea pig, about 300 μ wide, and about 1.5 cm long, removed immediately after the animal had been killed by a blow on the head. The muscle strips were mounted in the double sucrose-gap chamber and maintained at approximately 37°, as described below.

The normal Krebs solution used in all experiments is the same as the solution used by Bülbring and Kuriyama (1963) in their taenia coli experiments. It contained (mM) Na^+ 137.4; K^+ 5.9; Mg^{2+} 1.2; Ca^{2+} 2.5; Cl^- 134; $H_2PO_4^-$ 1.2; HCO_3^- 15.5; glucose 11.5 and was aerated with 97% O_2 + 3% CO_2.

Isotonic sucrose solution (10% w/v) was made up with distilled water, and deionized to reduce the conductivity to less than 2 μmho/cm. This conductivity gives a resistance to the sucrose parts of more than 20 MΩ in our chamber in the absence of the muscle strip.

Crystalline TTX (Sankyo Co., Toyko) was dissolved in distilled water to make up a stock solution. This solution was kept under refrigeration and was diluted with Krebs solution to obtain the desired concentration, prior to use. All preparations served as their own controls.

The Double Sucrose-Gap Chamber

The essential part of the chamber used in these experiments is the small piece of Lucite, approximately 12 mm thick, with numerous holes or channels drilled in it, as shown in Fig. 1C. The diameter of the channels, and the distance between them, vary depending on the tissue to be used. This central piece and the two side blocks (Fig. 1A and B) are bolted tightly together.

All inlets are connected to reservoir bottles via finely adjustable needle valves for flow rate control, drop bottles for electrical isolation, and a warming bath, for temperature control. The prepared muscle strip was mounted in the central lumen, as is shown in Fig. 2, which also indicates the flow directions of the solutions as represented by the arrows.

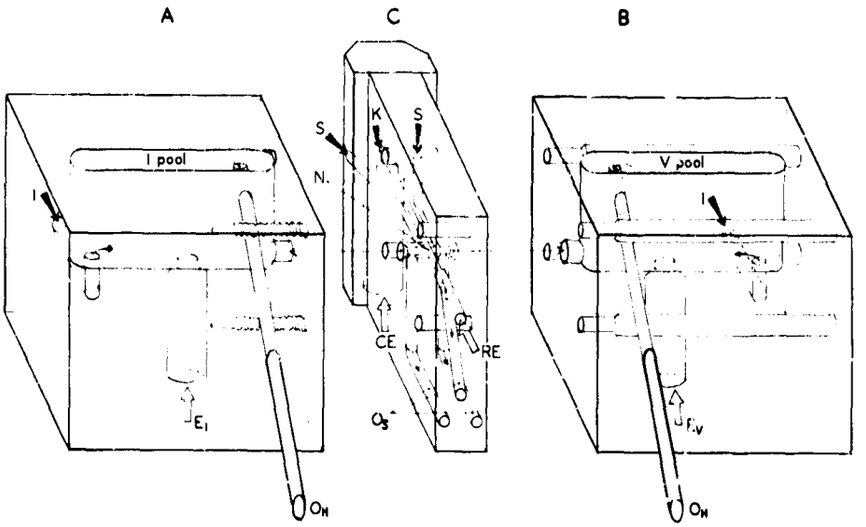

FIG. 1. Schematic diagram of double sucrose gap chamber: A, I pool lucite block, B, V pool lucite block, C, Changeable central piece. (Note: These three blocks are fitted tightly together with four screws.) K, Inlet for Krebs and test solutions. S, Inlets for sucrose solution. I, Inlets for isotonic potassium solution. N, Node (see Fig. 2). E_I, Current injection electrode. E_V, Potential recording electrode. CE, Current recording electrode. RE, Reference electrode. O_S, Outlets for solutions. O_H, Outlets to get constant hydrostatic outflow pressure.

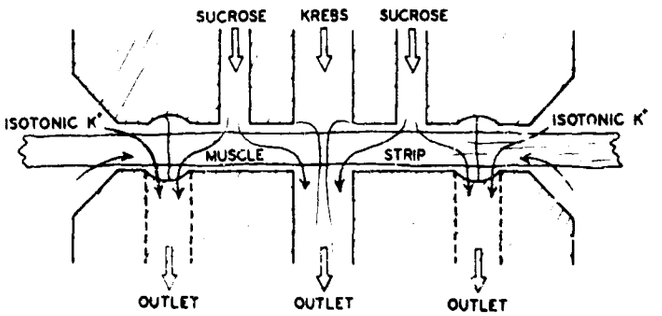

FIG. 2. Flow diagram in the double sucrose gap chamber (see text).

Both ends of the muscle strip were fixed with threads to keep appropriate tension and constant length. The nodal area, formed by the critical surfaces between the Krebs and the sucrose solutions, was controlled by visual inspection through a microscope by adjusting the needle valves of the respective solutions. A typical flow pattern is shown in Fig. 2. When desired, the flow rate could be regulated by raising and lowering the polyethylene tubings connected to the outlets (O_H in Fig. 1), thus changing the outflow pressure. The width of the nodal area is less than 100 μ, and the length of the sucrose parts is about 2.5 mm in the chamber.

To maintain a stable flow pattern, it is necessary to keep the hydrostatic outflow pressure constant. This can be achieved by maintaining a constant head pressure of I and V pools (using O_H in Fig. 1) and by eliminating the effects of the drop separation at the drop bottles and outlets (represented as O_S in Fig. 1), using small pieces of sponge foam or cotton fibers.

This procedure was adequate to permit recordings of normal membrane potentials, full amplitude action potentials, and voltage clamping.

Electric Circuit

The electric circuit is essentially the same as the one used by Moore, Narahashi, and Anderson at Duke University for nerves and myometrium and has only been modified slightly in our laboratory for taenia coli and vascular smooth muscle. It is schematically represented in Fig. 3.

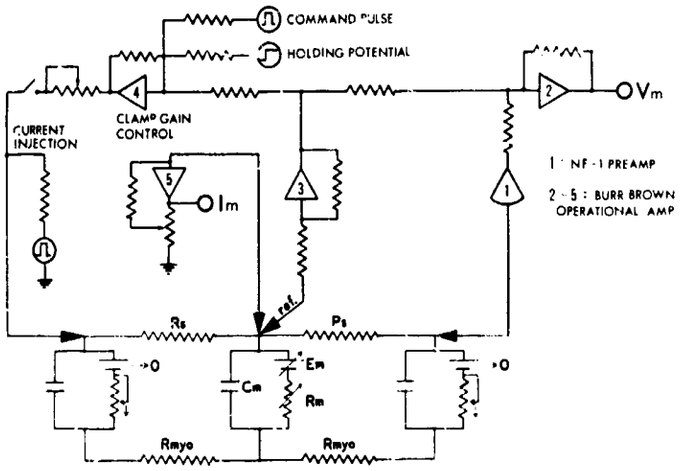

FIG. 3. Equivalent circuit diagram. The circuit schematically shown here is generally the same as one developed by Julian, Moore, and Goldman (1962). The sucrose resistance is labelled R_s and the myoplasmic resistance R_{myo}; the other symbols have their usual meanings. Closed triangles represent electrodes. The current electrode is located in the upstream and a reference electrode in the downstream of the central Krebs solution (see text).

Low resistance, coiled Ag–AgCl electrodes (less than 200 Ω) are inserted from the bottom of each block, and connected to I and V pools and to the upstream of the Krebs solution through Krebs–agar bridges. From the downstream of the Krebs solution, a pointed Ag–AgCl electrode, used as a reference electrode, was inserted as close to the node as possible.

Experimental

The theoretical basis, operation of the clamping circuits and the experimental limitations of voltage clamping performed in a double sucrose-gap chamber have been well described by Julian, Moore, and Goldman (1962) for nerve and by Anderson (1969) for smooth muscle. Because our techniques are essentially similar to those developed by Duke University group, extensive description has been omitted.

Narahashi, Moore, and Scott (1964) used a holding potential 10 to 30 mV hyperpolarized above the resting membrane potential in order to remove possible inactivation of the Na^+-carrying system. Although we alluded to the possibility of a current carrying system different from the Na^+ hypothesis, the holding potential was kept hyperpolarized by 10 to 20 mV above the resting membrane potential in our preparation.

RESULTS

Constant Current Injection

The membrane potential response was recorded in normal preparations after approximately 30 min incubation, during external stimulation, with stepwise hyperpolarizing and depolarizing constant current pulses of 700 msec duration. The resting membrane potential of these preparations varied from 50 to 70 mV.

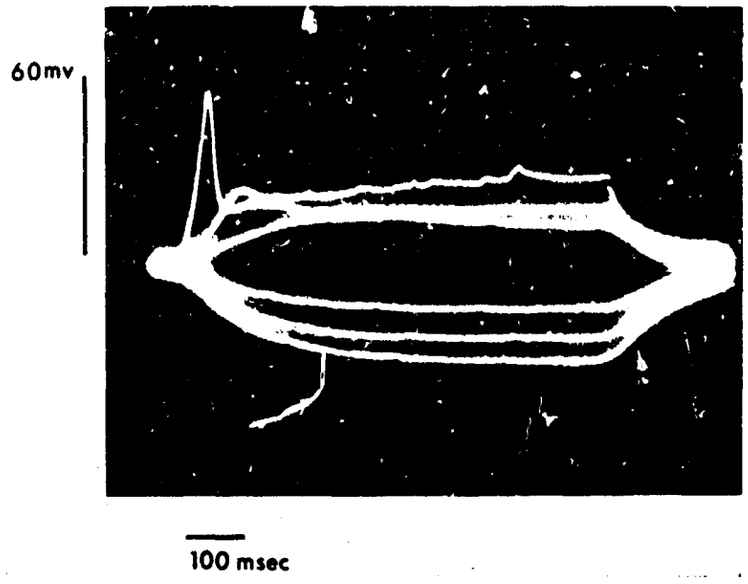

FIG. 4. Upper and lower traces represent the respective responses of the membrane potential to stepwise depolarizing and hyperpolarizing constant current pulses.

Hyperpolarizing pulses give a family of voltage tracings with typical exponential time course, characteristic of electrotonic potentials in these multifiber preparation (Fig. 4). The time constant (τ) varied somewhat from one preparation to another, ranging from 80 to 120 msec. The membrane revealed no anomalous resistance changes in the range of hyperpolarization we studied. The calculated value of the specific membrane resistance ranged from 1 to 10 KΩ cm^2. It should be noted, however, that this multifiber preparation has a very complex membrane structure, and that any calculation of the actual nodal membrane surface area is only approximate and that the effective membrane area of the artificial node is a matter of conjecture. For this reason, current densities are not given but rather the total current across the artificial node membrane.

Depolarizing current pulses caused complex changes in membrane potential. At low currents, the patterns were mirror images of the equivalent hyperpolarizing pulses with identical initial exponential time courses. At higher currents, abortive, delayed action potentials occurred, and as the stimulating current was increased further, full amplitude (60–70 mV) action potentials could be obtained (Fig. 4). These latter action potentials had maximum rates of rise and fall of 4.0 and 2.6 V/sec, respectively and a half duration of about 25 msec.

The current strength required to produce full-size action potentials varied, not only between preparations, but also in consecutive stimulations. Occasionally, abortive action potentials followed a full-size spike within the same current pulse.

Voltage Clamping

Normal Preparations

To assess the adequacy of the experimental conditions, a series of constant current injections was always carried out prior to voltage clamping. Figure 5 shows typical current recordings associated with stepwise voltage changes under clamped conditions. As a routine, three steps of hyperpolarizing pulses were recorded as shown in the panels to the left of Fig. 5. To estimate the leakage current for the preparation, the steady state current produced by these hyperpolarizing pulses were plotted with inverted polarity and the leakage current estimated from the resulting linear function or its extrapolated values.

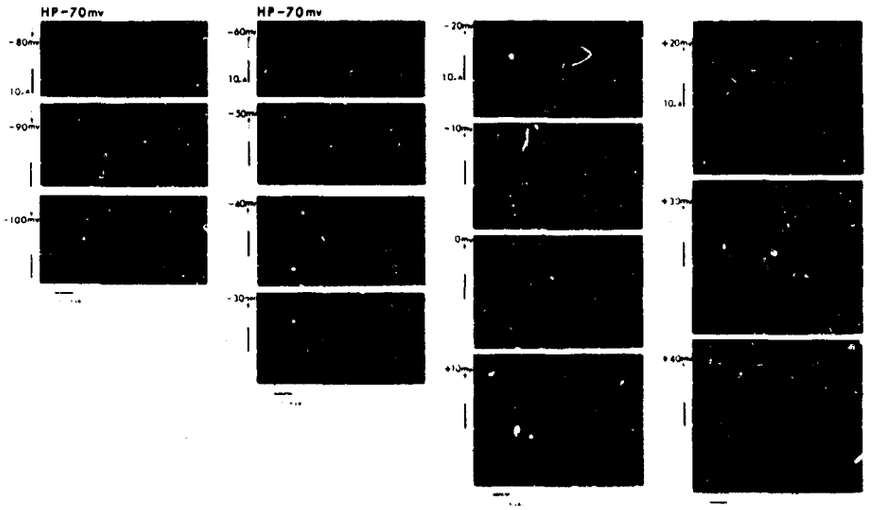

FIG. 5. Typical current recordings under voltage clamp condition. Holding potential −70 mV. The upper trace shows voltage, and the lower trace current. Routinely, three steps of hyperpolarizing command pulses were applied prior to each experiment in order to estimate the initial capacitive and leakage currents.

The reasons for assuming that this is a valid estimate of the actual leakage current are (a) that depolarizing and hyperpolarizing command pulses of low but equal magnitude and opposite polarity produced current curves that were mirror images and symmetrical on both sides of the holding potential and (b) that the voltage–current relationship was linear up to 60 mV hyperpolarization above the holding potential. Beyond this range (actual membrane potential more negative than −130 mV) nonlinear changes occurred that probably can be ascribed to membrane damage.

The current patterns produced by depolarizing command pulses are shown in the remainder of the panels of Fig. 5. The early inward current cannot be easily recognized

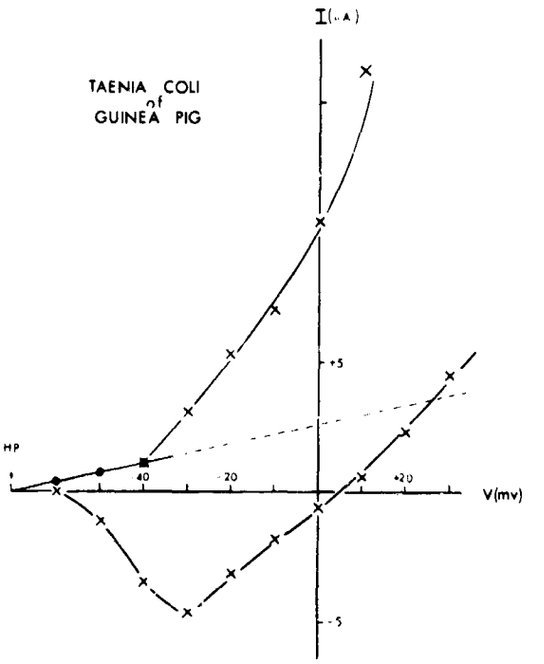

FIG. 6. Voltage–current relation plotted from Fig. 5. The leakage current, represented by the broken line, was extrapolated from the three-steps leakage current measurement (inverted polarity).

as in recordings from squid giant axon. However, when the curves are corrected for capacitive and leakage currents, the early transient and steady state currents appear quite similar to those of squid axon. The peak value of the early transient current and the steady state current are plotted, after correction for leakage current, in Fig. 6, against the clamped potential. The reversal of the transient current from inward to outward (apparent equilibrium potential) was at −5 mV, and the peak inward current occurred about 4 msec following a 40-mV depolarization.

Multiple peak transient currents, as reported by Anderson (1969), were not observed in any of our experiments, but rather a graded, continuous function of the clamping potential.

Effects of TTX

Six experiments with exposure of the muscle strip to TTX in concentrations of 10^{-7} and 10^{-6} g/ml were carried out. A typical record of the current-voltage relationship before and after 10 min exposure to 10^{-6} g/ml TTX has been plotted in Fig. 7. It shows

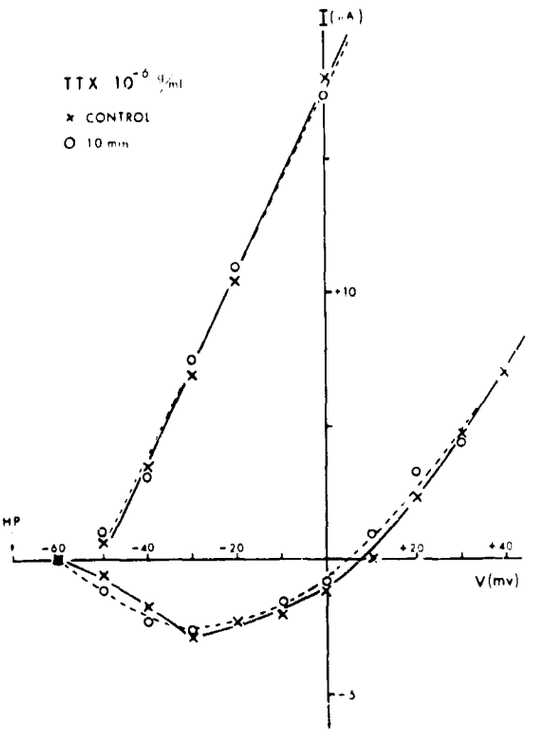

FIG. 7. Effects of 10^{-6} g ml TTX; holding potential −70 mV.

that TTX, in concentrations 100 times that sufficient to completely inhibit the excitability of squid giant axon and skeletal muscle, had no effect on either the early transient current or the steady state current.

Effects of Mn^{2+}

A series of six experiments were carried out with $MnCl_2$ (5×10^{-5} to 5×10^{-4} M) added to the Krebs solutions. All of these experiments show that the early inward current is gradually reduced with increasing duration of the exposure to Mn^{2+}. The apparent equilibrium potential gradually shifted to increasingly more negative values. A typical experiment is illustrated in Fig. 8. The recovery after washing with normal Krebs solution of preparations that had been exposed previously to Mn^{2+} was usually incomplete.

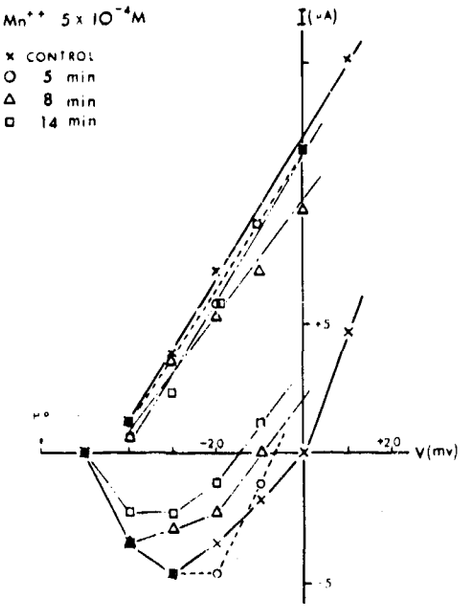

FIG. 8. Effects of 5×10^{-4} *M* Mn^{2+}; holding potential −60 mV.

Effects of Ca^{2+}-Free Solution

In three experiments with the preparations exposed to Ca^{2+}-free Krebs solution, there was a gradual reduction of the early inward current with a concomitant shift of the apparent equilibrium potential toward more negative values. Eventually, the inward current was abolished in all of these experiments. The absence of Ca^{2+} had little or no effect on the steady state current. The experiment illustrated in Fig. 9 is typical.

The current time relationship for this series of experiments at a clamping potential of −20 mV at which the early inward current was maximal, before and after 15 min exposure to Ca^{2+}-free Krebs solution, is illustrated in Fig. 10. The current obtained in Ca^{2+}-free solutions was subtracted from the current in control experiments to obtain the current depending on that ion. The lower panel is a typical record from squid giant axon. It should be noted that the current axis is in arbitrary units for the smooth muscle preparation, and that the time base is ten times that for the squid axon.

In sharp contrast to the current-time relationship of squid giant axon, the activation curve for the inward current carrying system of taenia coli was very slow, relative to the rate of increase in delayed K^+-conductance. The two currents overlap, thus reducing the peak transient current to only 70–80% of the inward ionic current. After correction for K^+-efflux, the current curve in Fig. 6 gives a true reversal potential for inward ion flux of about 25 mV.

DISCUSSION

It appears that the most important factors for successful experimentation are (a) the short circuit factor, i.e., $R_i / R_i + R_{min}$, the ratio of extracellular to extracellular plus

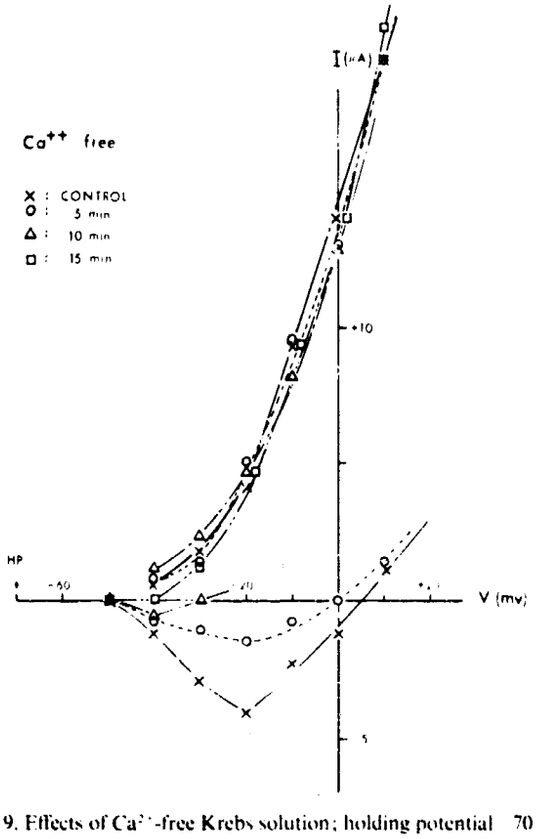

FIG. 9. Effects of Ca^{++}-free Krebs solution; holding potential −70 mV.

myoplasmic resistances in the sucrose parts (Stämpfli, 1954; Julian, Moore, and Goldman, 1962), (b) the degree of synchronization; and (c) the width of the artificial node.

Deionized sucrose solutions with a conductivity of less than 2 μmho cm consistently sufficed for providing enough extracellular insulation to record resting membrane potentials, which, after correction for sucrose hyperpolarization, are in the same range as those obtained with microelectrode techniques. This indicates that the dimensions of our double sucrose-gap chamber, the size of the preparation, and the relation between them are adequate. The maximal amplitude of the action potential was the same as recorded with intracellular techniques and shows that the width of the artificial node (100 μ) is small enough as compared with the space constant for the preparation (approximately 1.5 mm) to allow adequate space clamp.

The half duration of 25 msec and the relatively slow maximal rates of rise and fall of the action potential may indicate somewhat incomplete synchronization of the effective nodal membrane area. It is believed, however, that when the nodal width is less than 100 μ, as measured under the microscope, that at least the superficial fiber layers are excited simultaneously, as the rate of rise of action potentials triggered by supermaximal currents approached that recorded by intracellular electrodes. The half

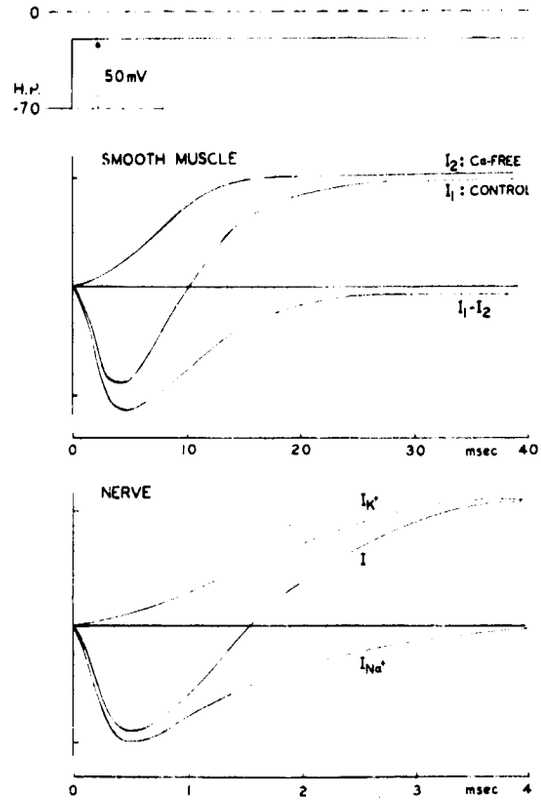

FIG. 10. Typical current-time relationships in smooth muscle and nerve (see text).

duration of the action potential and the rate of fall both indicate that the space clamp, although adequate, may not have been complete. It is not feasible by any direct method to assess the sharpness of demarcation between the sucrose and Krebs solutions in the deeper fiber layers in such a narrow node, and as noted previously, it is also difficult to get an accurate measure of the total membrane surface area in a complex multifiber preparation.

However, the high and scattered values for the specific membrane resistance may indicate, if compared with those calculated by Kuriyama and Tomita (1965), that a variable number of fibers are excited in the current injection experiments.

Although it does appear from the voltage-time changes in response to constant current injections that the simple cable theory (Hodgkin and Rushton, 1946) is applicable to the preparation, as suggested by Abe and Tomita (1968), the considerations above would raise the question of recruitment and a dependence of effective membrane area on the clamping voltage.

The results from the voltage clamping of normal preparations are compatible with the Hodgkin Huxley hypothesis, i.e., a voltage dependent increase in Na^+ conductance, Na^+ inactivation, and a delayed increase in K^+ conductance. We were not able to relate

the amplitude of the evoked action potential (varying from undershoot to several mV overshoot) to the leakage current corrected reversal potential of the early transient current.

It appears that in taenia coli, due to the slow and varying rate of activation of the inward current carrying system, relative to the rate of increase in delayed K^+ conductance, the net inward current varies and thus accounts for the varying amplitude of and rate of change of the membrane potential, i.e., little or no overshoot, abortive spikes, and slow fluctuations of the membrane which are the common features of the electrical activity of this muscle. It is, therefore, important to distinguish between the reversal potential for the net current flow (capacitance and leakage corrected) corresponding to the action potential amplitude measured by intracellular recording techniques, and the *true reversal potential* for the inward ionic current, i.e., the equilibrium potential for inward current carrying system. In squid axon, the time course for activation of the Na^+ conductance and the increase in K^+ conductance are separated sufficiently so that the reversal potential is practically identical to the true equilibrium potential. In taenia coli, the true equilibrium potential is obscured due to the overlap in time of the activation curves for inward and outward ion fluxes, as stated above. The calculated value of 25 mV, based on the experiment illustrated in Figs. 5 and 6, is close to the equilibrium potential for sodium as calculated by Goodford and Hermansen (1961).

As might be predicted from earlier studies with microelectrodes, TTX had no effect on the early transient and the steady state currents. The fact that TTX specifically inhibits the voltage-dependent early transient conductance of membranes, normally employing a Na^+ mechanism for excitation, is well established (Moore and Narahashi, 1967). Therefore, the persistence of unaffected early transient conductance changes of taenia coli in the presence of 100 times the concentration required to completely inhibit excitability in skeletal muscle (Kuriyama, Osa, and Toida, 1966) and nerve (Narahashi, Moore, and Scott, 1964) must be interpreted to mean that the mechanisms are different.

Nonetheless, nerves from pufferfish and newts are resistant to TTX, despite using a sodium mechanism for excitation (Moore and Narahashi, 1967), and this therefore tends to minimize the significance of this series of experiments with the toxin. Although teleologic, it may be noted that most investigators attribute the TTX insensitivity of the above species to the development of self-protective mechanisms against their own toxin, and that there are no reasons why such mechanisms should exist in the guinea pig. Furthermore, in TTX-insensitive, estrogen dominated, uterine smooth muscle, as recently shown by Anderson (1969), the early transient current is affected by the Na^+ gradient during the first 11.5 min in Na^+-free solution. This was interpreted by Anderson to indicate a Na^+ mechanism. The elusiveness of such a conclusion becomes apparent, however, considering the fact that smooth muscle, when transferred from a higher to a lower concentration of sodium, goes through a period of lower or complete inhibition of excitability. The duration of the inexcitable period depends upon the type of smooth muscle used. For uterine smooth muscle and taenia coli the inexcitable period usually has an onset after 2–5 min in Na^+-free solutions and lasts from a few minutes up to 10 min. Thus, Niu, Nakajima, and Kumamoto (1962) were able to record spontaneous electrical activity in the pregnant uterus of the guinea pig after 25 min in Na^+-free

solution and Bülbring and Kuriyama (1963) recorded action potentials with overshoots "for at least 20–30 minutes" in the absence of Na^+. Hotta and Nonomura (1968) recorded action potentials after 2 hr of Na^+ depletion. For other discussion on electrical activity in Na^+-free solutions, see Holman (1958), Daniel and Singh (1958) and Kuriyama (1963).

With regard to the series of experiments with Mn^{2+}, it has been shown conclusively, as mentioned before, that Mn^{2+} inhibits Ca^{2+} permeability in taenia coli. More recently, it has been found that in cardiac fibers (Harrington and Johnson, 1969, personal communication) transition metals will inhibit both the Ca^{2+} permeability and the voltage-dependent Na^+ conductance, thus throwing some doubts as to the specificity of its action in smooth muscle from taenia coli. The rapid and progressive reduction in early transient current in the presence of Mn^{2+} must, for lack of other evidence, at present be attributed to its inhibition of Ca^{2+} permeability. It is surprising that we could demonstrate no effect of the ion on the steady state current, since heavy metals may be expected to cause considerable physicochemical changes of the membrane, and that washing with normal Krebs solution did not bring about complete recovery.

The complete suppression of the early transient current in Ca^{2+}-free Krebs solution must be interpreted to mean that excitation in taenia coli occurs by a Ca^{2+}-dependent mechanism.

What the mode of this mechanism is, and whether Ca^{2+} is actually a current carrying ion during the upstroke of the action potential, can only be speculated. Considering the available evidence only, the normal mechanism of excitation must be obligate Ca^{2+} dependent but nonspecific in terms of current carriers; in other words, the voltage-dependent gates are not specific for Na^+.

ACKNOWLEDGMENTS

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Reference 14

Reprinted from MICROCIRCULATORY SOCIETY, 18th Annual Meeting, Atlantic City, N.J. April 11-12, 1970. Microvascular Res. Vol. 2, No. 2, 1970, p 2.

THE ELECTRICAL BEHAVIOR OF VASCULAR SMOOTH MUSCLE. L. Horn, A. Nakajima and M. Kumamoto. N.J. Col. Med. & Dent., Newark, N.J. and Kyoto Univ., Kyoto, Japan.

The patterns of normal spontaneous and drug-induced electrical activity of the longitudinal muscle fibers of the superior mesenteric vein and smooth muscle from taenia coli of the guinea pig have already been described in considerable detail. (See Horn, Kumamoto, and Nakajima, Microvascular Research, In press.) The present communication attempts to explain the characteristic features seen with intracellular microelectrodes in terms of relative rates of activation of specific conductances, based on analysis of data obtained by double sucrose gap voltage clamping techniques. Essentially the analysis shows that the primary reason for the relatively slow rate of rise of the action potential, usual lack of overshoot, apparent graded responses, and abortive spikes is a slow and variable rate of activation of the inward current carrying system, apparently dependent on the prevailing membrane potential. (Supported by Contract DADA-17-68-C-8058, U.S. Army Medical Research and Development Command, a grant from the New Jersey Heart Association, and NSF General Research Fund.)

Reference 15

Reprinted from XXV International Congress of Physiological Sciences, Munich 1971.

THE ROLE OF CA^{++} IN EXCITATION OF VASCULAR AND OTHER SMOOTH MUSCLE. L. Horn, M. Kumamoto and A. Nakajima. College of Medicine and Dentistry of New Jersey at Newark Newark, New Jersey 07103 and Kyoto University, Kyoto, Japan.

Electrical properties of the longitudinal muscle fibers from the superior mesenteric vein and the taenia coli of the guinea pig has been investigated using intracellular recording, voltage clamp, and constant current injection technqiues. Normal spontaneous electrical activities and responses to catecholamines and acetylcholine (Ach) are compared. The reversal potential of Ach in vascular muscle was found to be substantially more negative than that for skeletal muscle and nerve, and somewhat lower than that of taenia coli. The effects of Tetrodotoxin (TTX), Mn^{++} and graded changes in Ca^{++}-concentration on the two preparations are discussed. It is suggested that the excitation in both preparations is obligate Ca^{++}-dependent, inhibited by transition metals that interfere with Ca^{++}-permeability and that it is unaffected by TTX. (This work has been supported by a general research grant from NSF, a grant from the New Jersey Heart Association and support from U.S.-Japan Cooperative Scientific Program, Office of International Activities, N.S.F.)

Reference 16

Reprinted from MICROVASCULAR RESEARCH 2:268-272, 1970.

A SOLID-STATE IMPULSE FLOWMETER. L. Horn and A. Rose. Dept. Physiology and the Electronic Shop, N.Y. Med. College, N.Y.

(33 copies enclosed)

MICROVASCULAR RESEARCH, 2, 268–272 (1970)

A Solid-State Impulse Flowmeter[1]

L. HORN[2] AND A. TOSI

Department of Physiology and the Electronic Shop, New York Medical College, New York

Received October 20, 1969

This paper describes a solid-state flowmeter that will record blood flows over a wide range (0.1–30 ml/min). The flowmeter is activated by a photoelectric cell in a drop chamber that produces input impulses from the breaking of a light beam by drops of blood falling through the chamber which is filled with silicone fluid. The flowmeter is accurate to ±1% within a range of 3-fold changes in flow and will operate almost any laboratory recorder or oscillograph. Self-checking circuits are provided for making initial calibrations and to test performance during operation. The instrument is also equipped with a register recording total flow over a desired period of time (total number of drops). Performance data and advantages as used in microcirculatory studies have been discussed.

Hemodynamics requires as a prime datum the rate of blood flow through the vessel (Lamport, 1965). Of the various methods available only few actually measure directly volume per unit time. Although the original Volkmann flowmeter (Volkmann, 1850) as modified and improved by Ludwig (Dogiel, 1867) and, lately, Dawes and co-workers (1952) and the Pavlov flowmeter (1887) both are accurate, they are bulky, somewhat inconvenient, and do not provide for continuous recording.

Folkow and co-workers (1949) have over the past two decades used and refined a mechanical ordinate recorder similar to that described by Fleisch (1930). The Folkow flowmeter utilizes a smoke drum kymograph and is activated by a photoelectric drop counter. It gives a reasonably accurate measure of volume flow over a wide range of flows and produces records of the inverse of flow (peripheral resistance) that are most appealing esthetically (Lindgren and Uvnas, 1954).

We have constructed a solid-state impulse flowmeter that in basic principle is similar to the Folkow flowmeter but offers the advantage that it can be used with practically any electronic recorder or oscillograph and will produce both the familiar ordinate records and a single-line graph indicating the peripheral resistance. The signal output may also be stored on magnetic tape for analysis later. The flowmeter has been in routine use for some time and has proved accurate and advantageous in studying microcirculatory changes in segments of cat intestine.

Figure 1 shows the basic circuit elements of the flowmeter. Two major circuits, the ramp generator and the totalizer with its associated amplifier, operate in all modes. In the Units mode, the ramp generator is reset by the occurrence of each input pulse, but in the Preset mode, it is reset only after a predetermined number of input pulses

This work was supported by Contract DADA 17-67-C-8055 and DA-49-193-MD-2813 with the U.S. Army Medical Research and Development Command.

Present address: Department of Physiology, New Jersey College of Medicine and Dentistry, Newark, New Jersey.

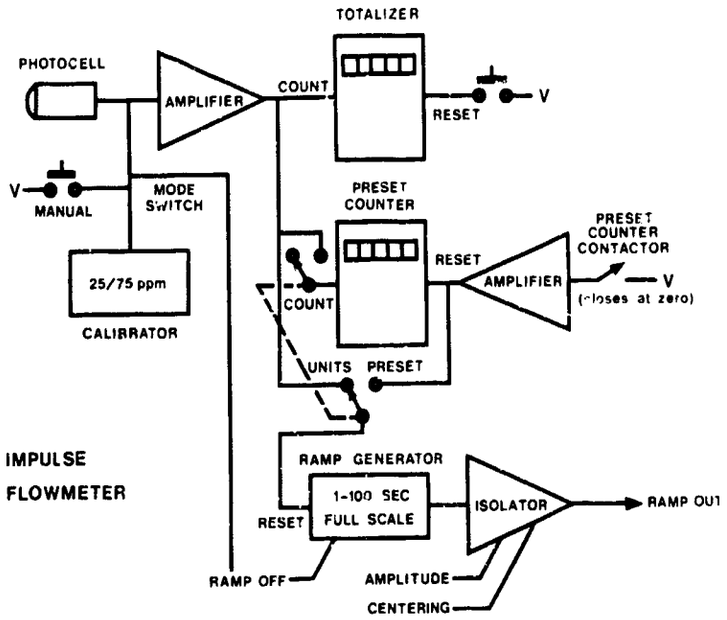

FIG. 1. Basic circuit elements of flowmeter.

FIG. 2. Flowmeter photocell with light beam focused

have occurred. Each input pulse, of course, corresponds to a drop falling through the drop chamber across the path of a light beam focused onto the photocell (see Fig. 2).

The function of the totalizer is invariant – it keeps a running total of the number of drops that have passed through the drop chamber. It may, however, be reset manually at any time.

In the Preset mode, the present counter operates simultaneously with the totalizer, counting backwards until it reaches zero. At this point, contacts in the preset counter close and a reset signal is applied to the preset counter and to the ramp generator. This cycle repeats as long as there is an input to the device.

Unless a reset signal is applied to the ramp generator, it will climb at a predetermined rate until it reaches its maximum amplitude. It will stay at this maximum value until reset and then climb again. Since each drop, or preset number of drops, resets the ramp and allows it to immediately climb toward its maximum amplitude, the amplitude of the ramp at the point where it resets is directly proportional to the time interval between drops – up to the point of maximum ramp amplitude. With strip-chart recorders operating at sufficiently slow speeds, the envelope of the ramp tracings provide a graphic measurement of the rate of drop flow.

The ramp signal may be adjusted for any maximum amplitude between zero and 10V, may be centered about zero to permit use of a recorder that operates in a bipolar fashion with the pen normally at rest in the center of the tracing, and may be adjusted for any time-to-climb from 1 to 100 sec.

It may seem somewhat inconvenient to record time between drops rather than the reciprocal function, rate of flow. However, to record the rate function directly with the same degree of accuracy would entail considerably more electronic circuitry since the input information occurs at a relatively slow rate and is a direct function of time. The reciprocal function could be obtained only by extensive use of computational logic or by the accurately shaped hyperbolic waveform in place of the linear ramp.

In its present form, the flowmeter is trouble-free and readily calibrated over a wide range of flow rates. The base line corresponds to zero time between drops or infinite rate of flow. However, it can be offset effectively so that the area of interest falls within the desired recording area. The maximum pen excursion is set by the ramp amplitude. Adjustments of the ramp time then sets the scale factor and determines the lowest rate of flow that can be recorded.

As an example, the ramp amplitude can be adjusted for a total pen deflection of 4. If the ramp time were set for 4 sec, the range of recording would be from 15 drops/min to infinity. The midscale value, 2, would correspond to 30 drops/min, and the 1 value would correspond to 60 drops/min. Similarly, other settings of ramp time would be set to N times the value used for Units operation, where N is the preset number. Preset operation offers greater convenience in obtaining the average rate of flow.

To aid recorder calibration, two accurate calibration signals have been included in the instrument, 25 and 75 pulse/min. These correspond to 2.4 and 0.8 sec between drops respectively. After the ramp amplitude and centering have been adjusted, the desired calibration signal is switched into the input, and the ramp time is adjusted to obtain a pen deflection to the proper point. A manual input is also provided to permit adjustment without the drop chamber. It is useful in establishing the base line and other calibration procedures.

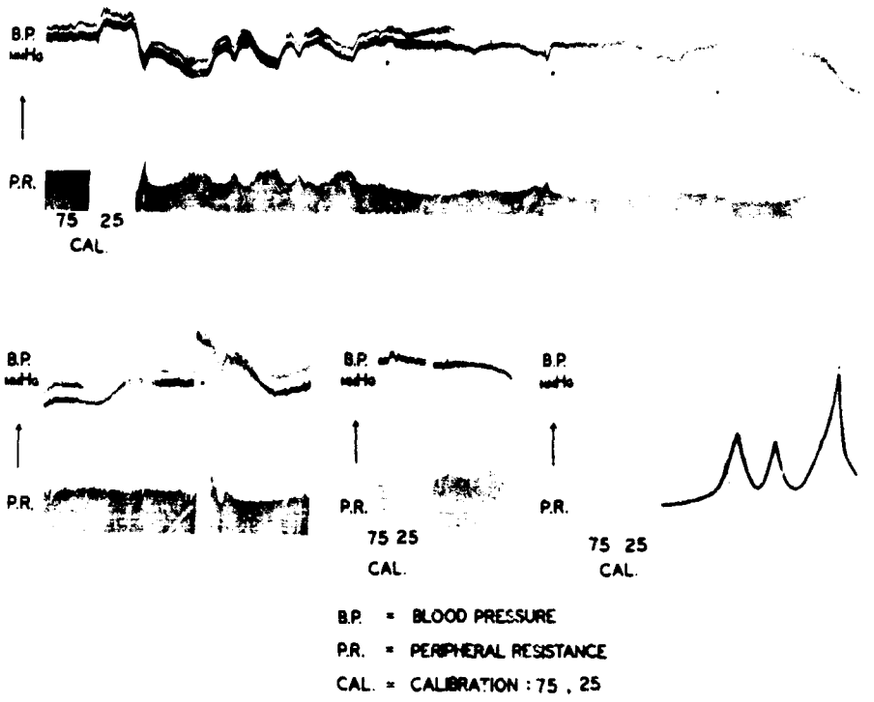

FIG. 3. Records obtained from Electronics-for-Medicine oscillograph with photographic writer adjusted to give "ordinate diagram."

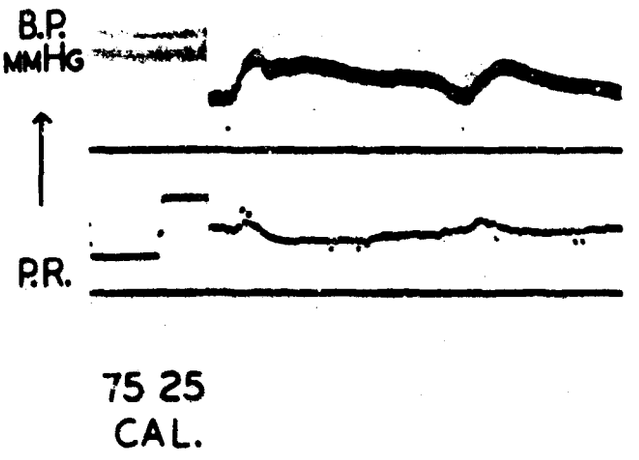

FIG. 4. "Curve diagram" shown when only peak value (ordinate) is registered

Figure 3 shows the flowmeter records as obtained from an Electronics-for-Medicine oscillograph with photographic writer when adjusted to give an "ordinate diagram." Figure 4 shows records where only the peak value of the ordinate is registered, giving a "curve diagram."

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Reference 17

Reprint from the APS Fall Meeting. 1971. (Abstract)

VASCULAR RESPONSE OF THE CAT INTESTINE TO NOREPINEPHRINE FOLLOWING INFUSION OF TETRODOTOXIN (TTX). Nicholas A. Mortillaro* and Leif Horn. College of Medicine and Dentistry of New Jersey at Newark, Newark, N.J.

In cats, eviscerated and adrenalectomized, autoperfused isolated loops of small intestine (ileum) were intra-arterially infused with norepinephrine (1.0-2.5 ug/min) before and following infusion of TTX (10 ug/min). The former taken as the control. In each case the concentration of norepinephrine before and following the infusion of TTX was the same. Total blood flow (ml/min x 100 g) through the intestinal segment was measured utilizing a flowmeter activated by a photoelectric cell in a drop chamber. During infusion of TTX, the TTX containing efflux from the segment was discarded and blood was cross perfused from a donor cat. Both splanchnic nerves were cut and the peripheral ends were mounted on double ring electrodes. Marked inhibition of sympathetic outflow to the TTX perfused segment was indicated by the absence of vascular response during splanchnic nerve stimulation (5-8 imp/sec), i. e., blood flow remained relatively constant during the stimulation period. Intestinal vascular constrictor response to norepinephrine, as indicated by a decrease in blood flow, ranged from 41 to 48% (control) of resting blood flow, whereas following TTX infusion the range was only 13 to 28% of resting blood flow. The results suggest that a condition similar to denervation hypersensitivity results from TTX's inhibition of sympathetic activity, and this condition is seen to develop minutes after the infusion of TTX. The results will be discussed in relation to the "autoregulatory escape". (This research was supported by Contract DADA-17-68-C-8058 with the U.S. Army Medical Research and Development Command and National Science Foundation Grant #FJ5024.)

Reference 18

Abstract of paper presented at the 22nd Annual Session of American Association for Laboratory Animal Science, New York, N.Y., October 11-15, 1971.

INTESTINAL BLOOD FLOW MEASUREMENTS UTILIZING A SOLID STATE DIGITALIZED FLOWMETER.

N. Mortillaro, M.S.E.E.* and L. Horn, Ph.D. College of Medicine and Dentistry of New Jersey at Newark, Newark, N.J.

Ileal segments of cats, anesthetized with pentobarbital, adrenalectomized, atropinized, splanchnic nerve ablated, and with facility for microscopic observation were placed in a plethysmograph. The middle colic artery and the superior mesenteric vein (SMV) were cannulated. Norepinephrine induced changes in blood flow were observed before and after infusion of Tetrodotoxin (TTX). The scope of these experiments was to observe the vascular response of the intestine to norepinephrine before and following intra-arterial infusion of TTX via the middle colic artery. The preparation and experimental setup has proven convenient for studying autoregulation, the autoregulatory escape and the so-called "reactive hyperemia" following stimulation of the peripheral cut end of the splanchnic nerve or the infusion of norepinephrine. The outflow (SMV) of the autoperfused isolated ileal loop was passed through a silicone fluid filled drop chamber with a light source and a photoelectric cell. The output of the photoelectric cell acted as the source for the activation of the flowmeter. Once having passed through the drop chamber the blood was returned to the animal via the right external jugular vein. In the case when TTX was infused the outflow was discarded and an equal amount of blood was transfused from a donor cat. The results suggest a mechanism for the changes in blood flow caused by splanchnic nerve stimulation or norepinephrine infusion at variance with the conventionally accepted hypothesis. (Supported by Contract DADA-17-68-C-8058 with the U.S. Army Med. Res. and Development Command and National Science Foundation Grant #FJ5024.)

Reference 19

Preliminary Report on the Autoregulatory Escape in the Cat Ileum

by

Nicholas A. Mortillaro

August, 1971

In 1964, it was reported that the vascular response of the cat intestine to splanchnic nerve stimulation and intra-arterial infusion of norepinephrine underwent three distinct phases. An initial increase in vascular resistance, followed by a decrease in resistance with continued stimulation, and sequentially, upon cessation of the stimulation a further decrease in resistance (hyperemic phase). The second phase was termed the "autoregulatory escape" implying a release from the constrictor fiber influenced by local mechanisms.

We have been investigating the "autoregulatory escape" phenomenon in the cat intestine utilizing pharmacological denervation by means of Tetrodotoxin, a poison extracted from the Japanese fugu fish, more commonly known as the Puffer fish. It has been amply demonstrated that the action of Tetrodotoxin is to render mammalian nerves inexcitable by blocking the increase in specific sodium conductance, that is, the early inward current, and thereby rendering the nerve inexcitable. Evidence from other experiments in our laboratory indicates that Tetrodotoxin has no such effect upon smooth muscle.

In our preparation, the animals were adrenalectomized, splenectomized and evicerated except for a small loop of autoperfused ileum, weighing 10-20 grams. In some cases, when intestinal volume recording were made, and in order to accommodate a plethysmograph, a total gastroectomy was performed.

The ileal loop was then subjected to splanchnic nerve stimulation and intra-arterial infusions of norepinephrine and Tetrodotoxin. The infusions were carried out via a cannula inserted into the middle colic artery, the other end of the cannula was connected to a continuous [illegible] pump, thereby insuring that the

sions were initially localized to the isolated small intestine.

Figure 1 illustrates the method of measuring the physiological parameters of interest. Recordings of arterial blood pressure were made from the femoral artery.

To measure blood flow through the intestinal segment, the superior mesenteric vein was cannulated and connected to a silicone fluid filled drop chamber onto which was mounted a photoelectric cell. The signal from the cell activated a solid state digitilized flowmeter. After having passed through the drop chamber, the animal's blood was returned to the circulation via the external jugular vein, thereby creating an extracorporeal loop.

Venous pressure was measured from a side branch of the superior mesenteric vein cannula.

In some experiments, the intestine was enclosed into a plethysmograph, and changes in tissue volume were recorded simultaneously with intestinal blood flow, arterial blood pressure and venous blood pressure.

However, in the results to be presented now, volume recordings were not made.

Figure 2 gives the results obtained as a consequence of splanchnic nerve stimulation and intra-arterial infusion of norepinephrine and Tetrodotoxin.

The upper trace is arterial blood pressure, the center trace is venous blood pressure and the bottom trace is the intestinal segment's blood flow normalized to 100 grams of tissue weight. The rectangles at the bottom indicate the on-off time of the applied stimulus. Recording speed was 0.5 cm/min.

In the first panel splanchnic nerve stimulation, in the form of square waves was applied for 4.5 minutes at a frequency of 6 Hz, with an amplitude of 7v, and a duration of 3 msec. The vascular response seen is characterized by the three phases previously discussed. That is, an initial constriction, then with continued stimulation we see the "autoregulatory escape", and finally, with the removal of the stimulus - a hyperemic period.

In the second panel is shown the vascular response to intra-arterial infusion of norepinephrine, in this case at a dose rate of 1.25 µg/min for 4.5 minutes. Again as in the nerve stimulation the pattern of vascular response is the same.

FIGURE 1

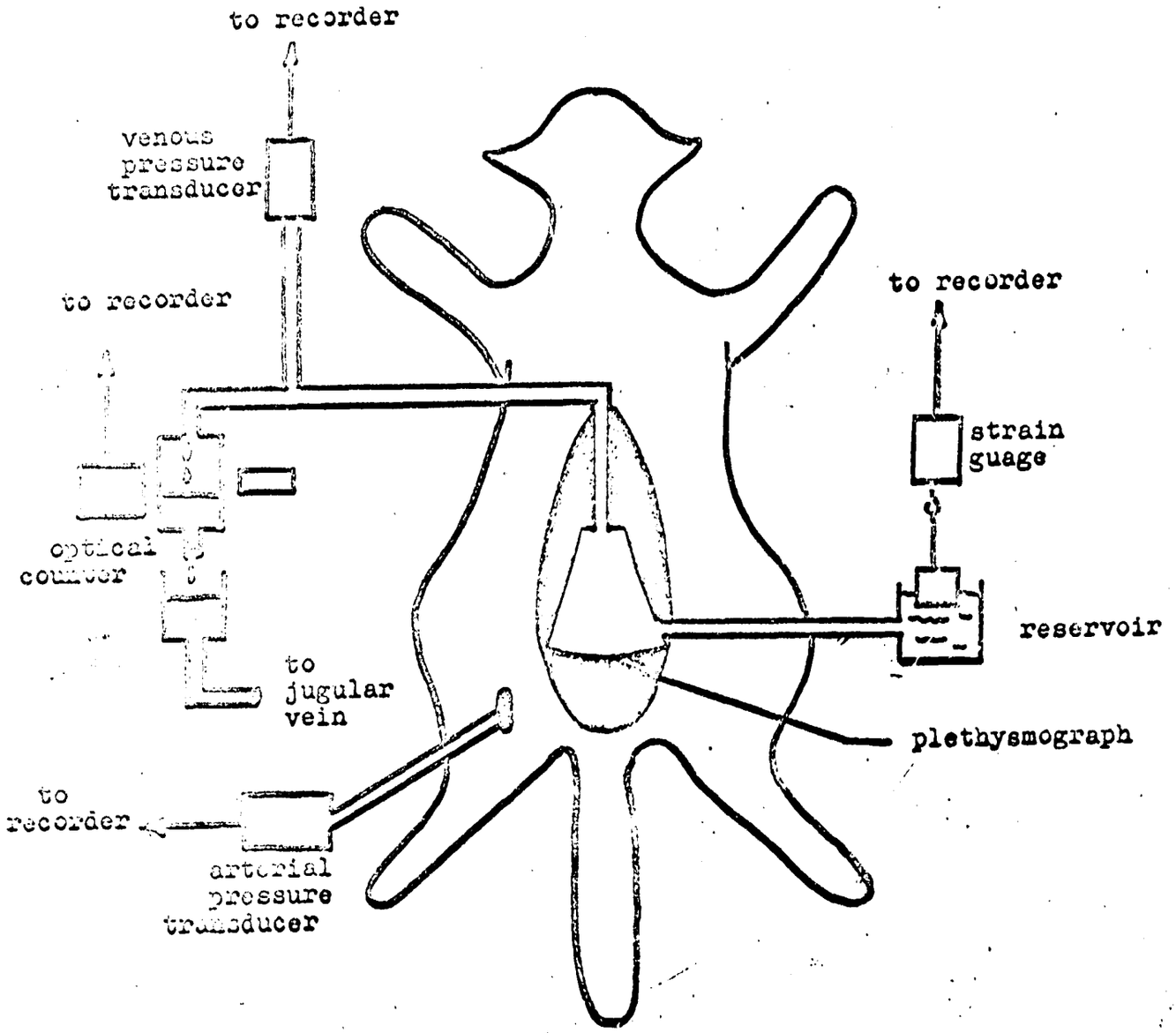
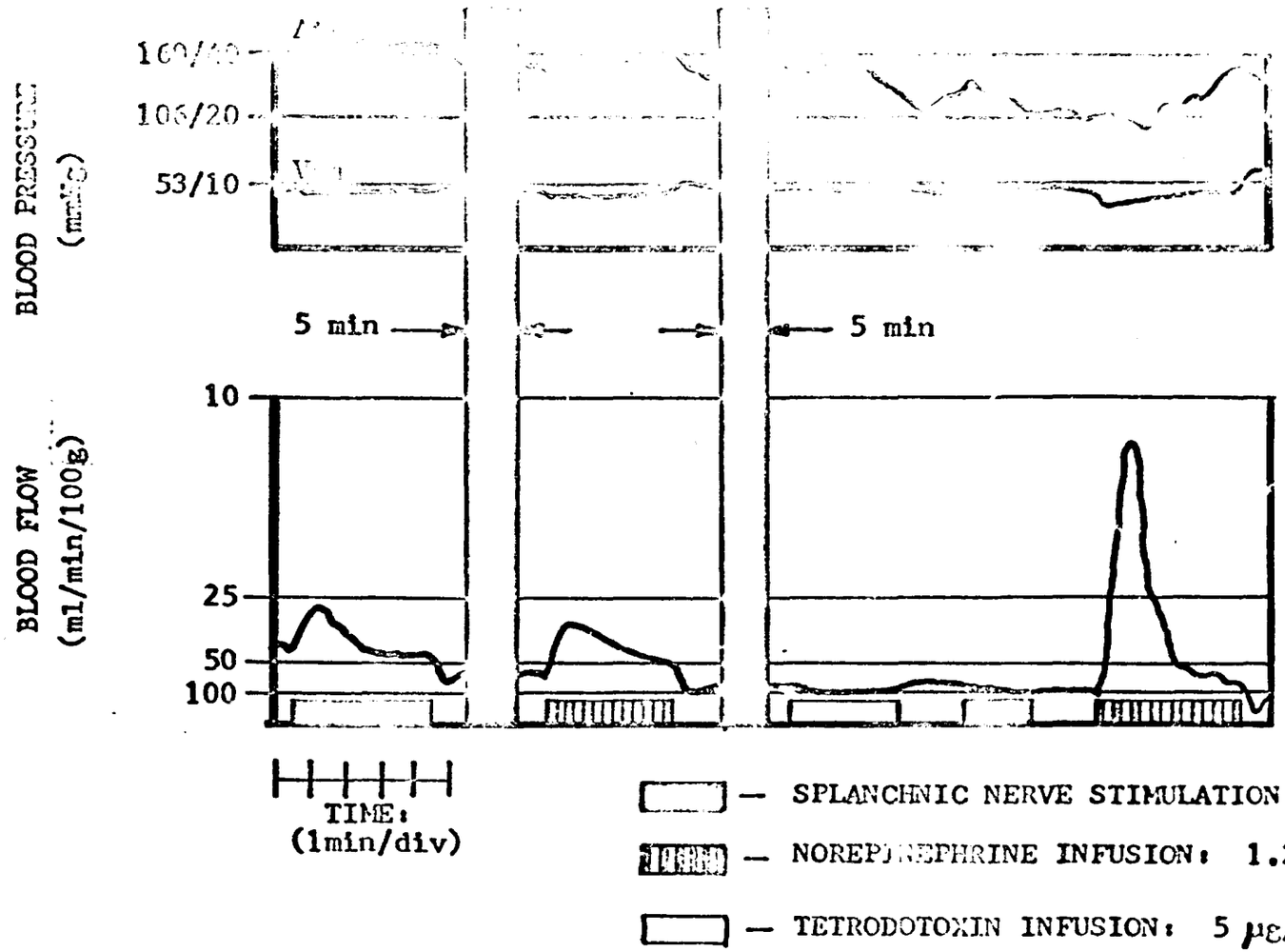

to recorder
venous pressure transducer
to recorder
optical coupler
to jugular vein
to recorder
arterial pressure transducer
to recorder
strain guage
reservoir
plethysmograph

FIGURE 2


BLOOD PRESSURE
(mmHg)
106/20
53/10
5 min
5 min
BLOOD FLOW
(ml/min/100g)
10
25
50
100
TIME:
(1min/div)
— SPLANCHNIC NERVE STIMULATION: 8Hz, 7v, 3msec
— NOREPINEPHRINE INFUSION: 1.25 μg/min
— TETRODOTOXIN INFUSION: 5 μg/min

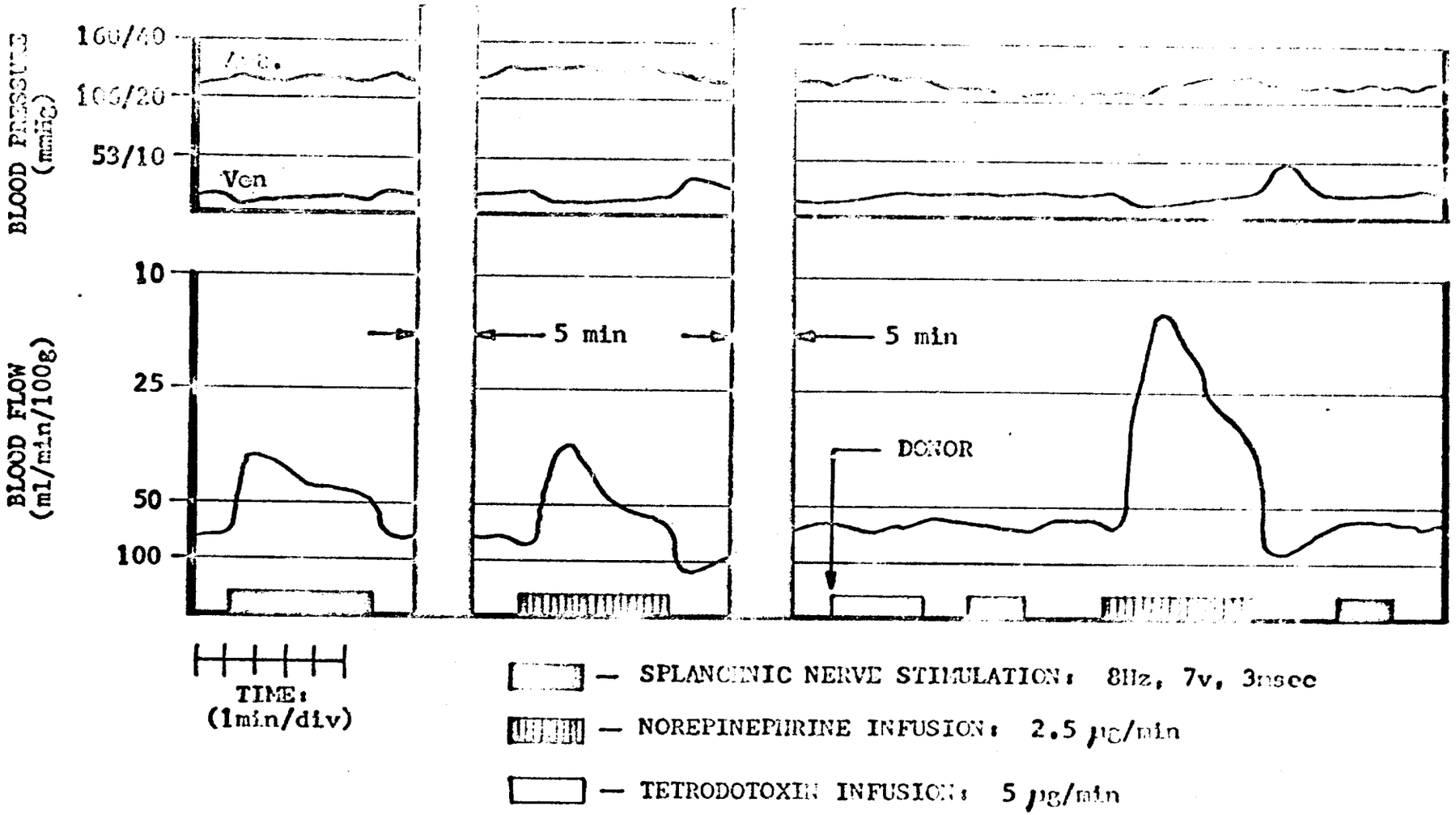
In the third panel is shown the vascular response to norepinephrine following the intra-arterial infusion of Tetrodotoxin. Here, Tetrodotoxin was infused at 5 μg/min. for 3 minutes, then after a brief pause of approximately 2 minutes, the splanchnic nerve was stimulated for an additional 2 minutes. The lack of vascular response to nerve stimulation, as compared to the response in the first panel, indicates that a conduction block was achieved. Norepinephrine was then intra-arterially infused for 4.5 minutes in the same concentration as in the pre-Tetrodotoxin period. Once again the characteristic response, that is, constriction, escape and hyperemia. However, comparing this response to that in the second panel, we see that the increase in resistance is more pronounced indicating a seemingly potentiated response to norepinephrine in the presence of Tetrodotoxin.

But, in this particular experiment, there was a large precipitous drop in the arterial blood pressure during the infusion of Tetrodotoxin. (149 → 112). As discussed above, the blood passing out of the intestine and through the drop chamber was returned to the animals general circulation. In this case then, that meant Tetrodotoxin contaminated blood. The present literature indicates that Tetrodotoxin does have a direct depressor effect upon cardiac function, and the drop in blood pressure may be a result of such an effect. This was of concern to us, since it is known that smooth muscle may become more sensitive to catecholamines during the early stages of hypotension.

To eliminate this possibility, we resorted to a donor cat technique. During the infusion of Tetrodotoxin and thereafter, the TTX containing blood from the intestinal segment, once having passed through the drop chamber was discarded, and an equal amount of blood was transfused from a donor cat.

Thus, in figure 3 which is the record of such an experiment, no large precipitous drop in arterial blood pressure occurs during the infusion of Tetrodotoxin (133 → 123). However, the potentiated response to norepinephrine in the presence of Tetrodotoxin is still present, as well as the typical vascular response pattern.

FIGURE 3


BLOOD PRESSURE (mmHg)
160/40
100/20
53/10
Art.
Ven
BLOOD FLOW (ml/min/100g)
10
25
50
100
5 min
5 min
DONOR
TIME: (1min/div)
— SPLANCHNIC NERVE STIMULATION: 8Hz, 7v, 3msec
— NOREPINEPHRINE INFUSION: 2.5 μg/min
— TETRODOTOXIN INFUSION: 5 μg/min

From the experiments performed on 16 animals we have computed the mean peripheral resistance at rest and at peak constriction for both cases, that is, prior to and following the infusion of Tetrodotoxin, illustrated in figure 4.

The open circles represent the rest to maximum constriction response to norepinephrine in the absence of Tetrodotoxin. A change from 2.3 to 6.2 mean peripheral resistance units/100 gram of tissue. An overall increase in mean peripheral resistance of two and a half fold above the resting state.

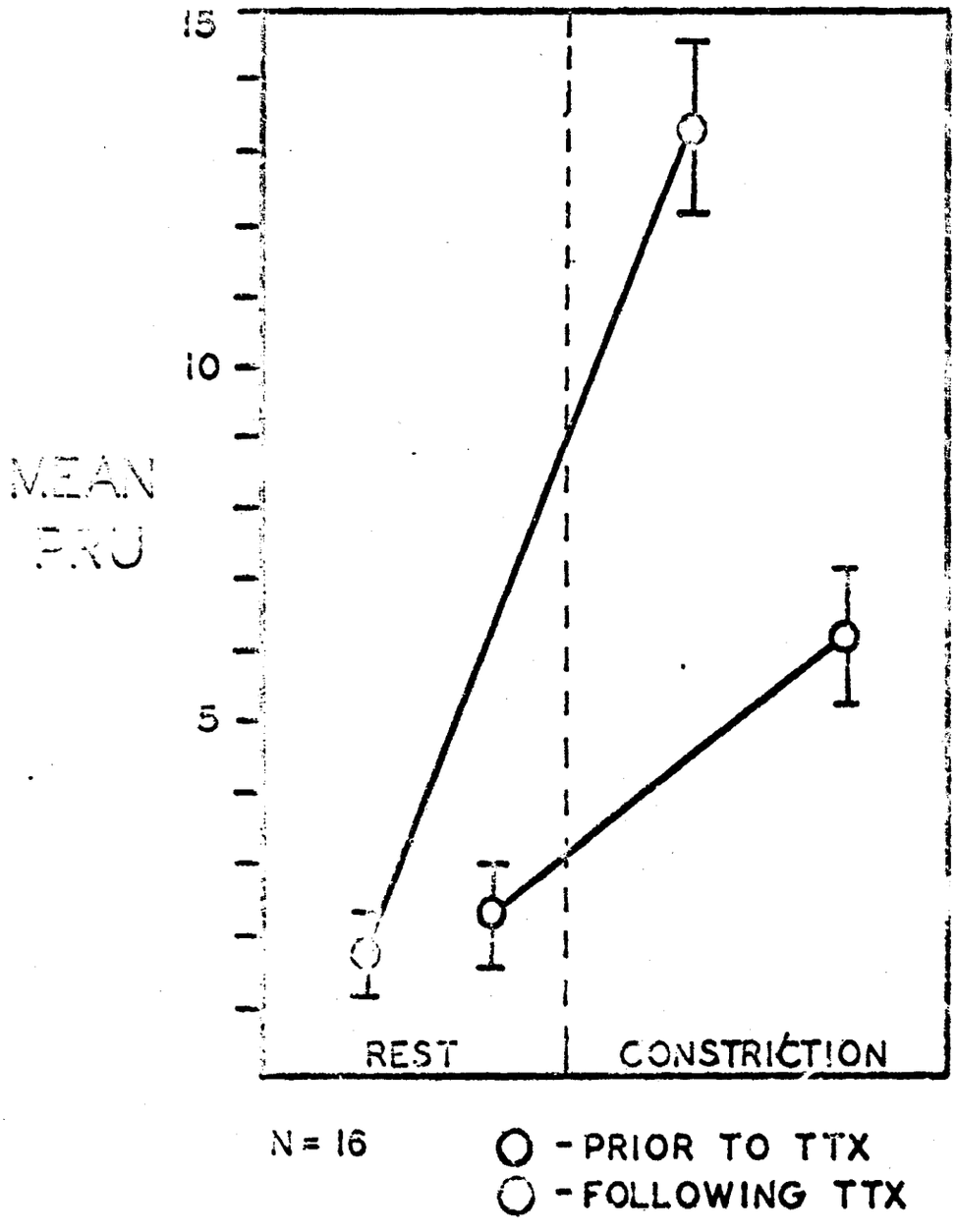
The closed circles show the response to norepinephrine in the presence of Tetrodotoxin. A change from 1.75 to 13.3 mean peripheral resistance units/100 gram of tissue. An increase of seven and a half fold above the resting state. A very significant increase when compared to the pre-Tetrodotoxin values.

Thus, we have demonstrated that in the Tetrodotoxin denervated intestine of the cat the pattern of vascular response to infusion of norepinephrine remains unaltered, that is, an initial constriction, and with continued stimulation an autoregulatory escape, followed by a hyperemic period after the cessation of the stimulation. The implication being that local reflexes such as the axon reflex do not participate in the autoregulatory escape.

Also we have shown that the peak constrictor vascular response of the small intestine to intra-arterial infusion of norepinephrine appears to be potentiated by Tetrodotoxin.

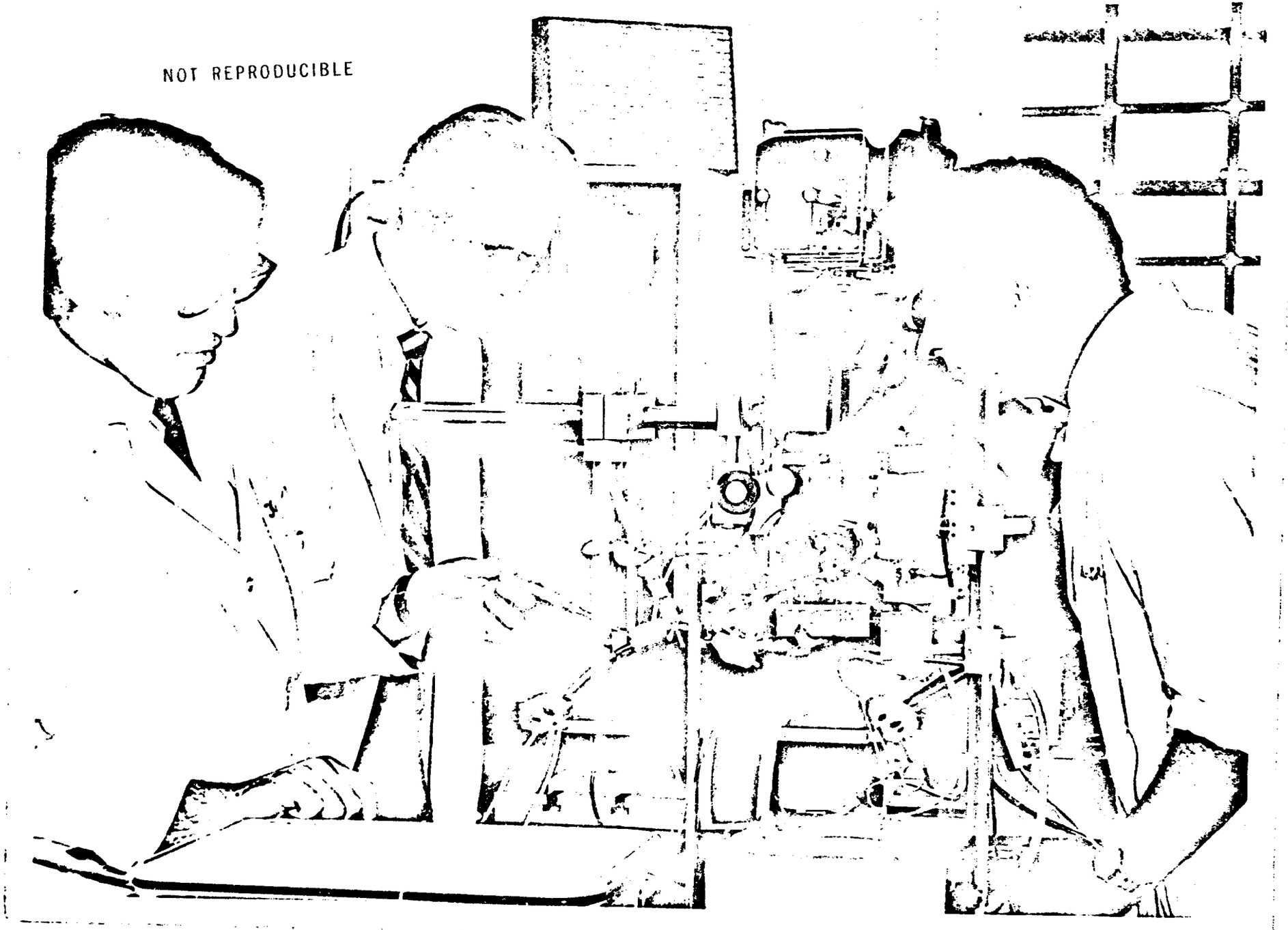
Thus, in this respect, and only in this respect, the vascular response is similar to denervation hypersensitivity, that is, in the presence of Tetrodotoxin it takes less norepinephrine to elicite the same degree of response. (This research was supported by Contract DADA-17-68-C-8058 with the U.S. Army Medical Research and Development Command and the National Science Foundation Grant #FJ5024.)

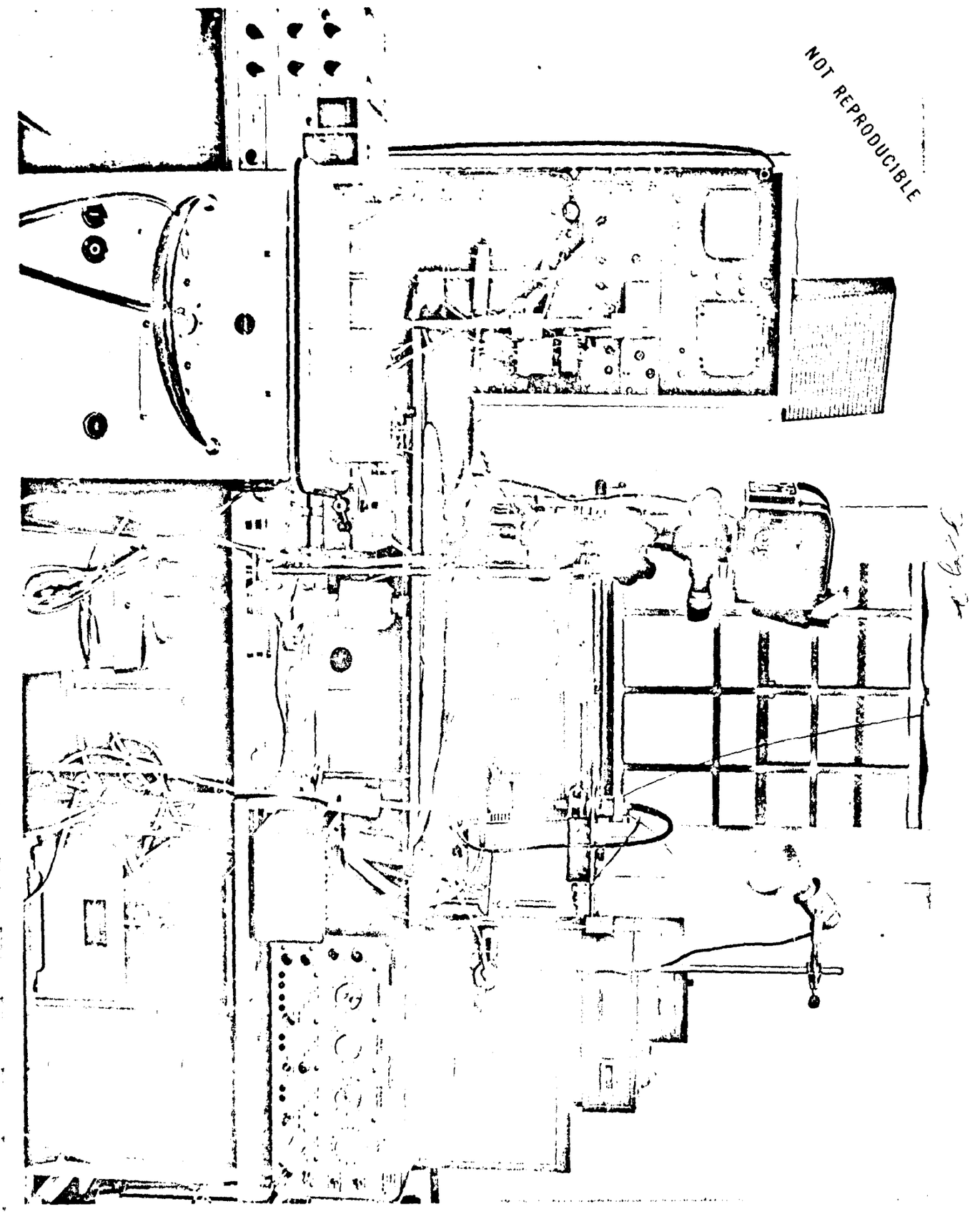
FIGURE 4


15
10
5
MEAN
PRO
REST
CONSTRICTION
N = 16
- PRIOR TO TTX
- FOLLOWING TTX

APPENDIX A

Figures 1 and 2 cited on page 3.





APPENDIX B

Figures 1 - 10 - Cited In Reference 8.

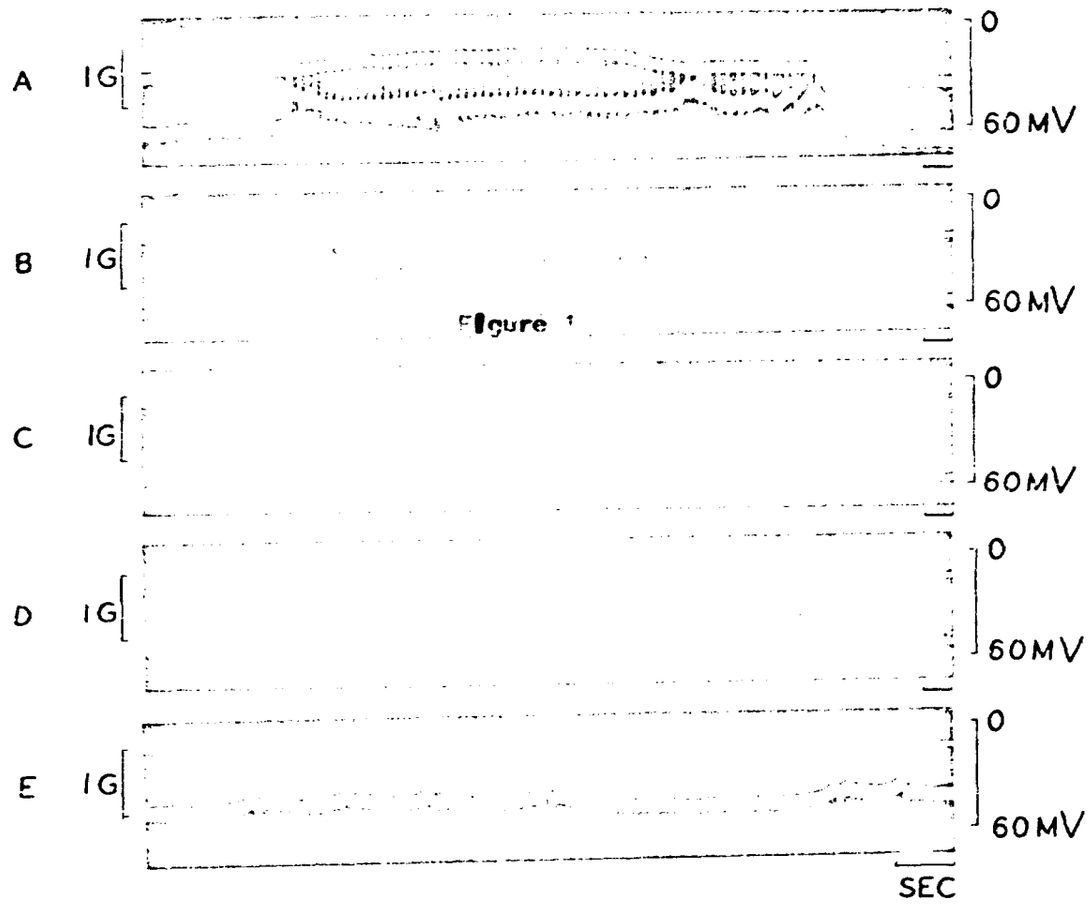


Figure 1

Figure 7

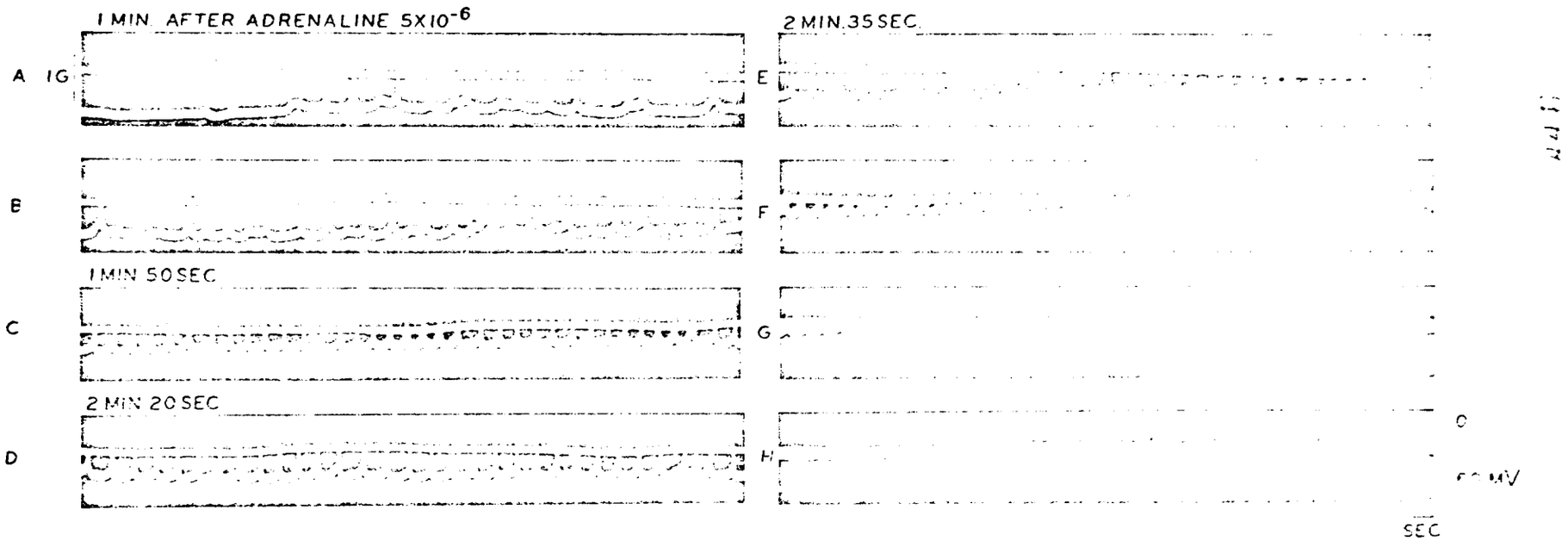


Figure 3

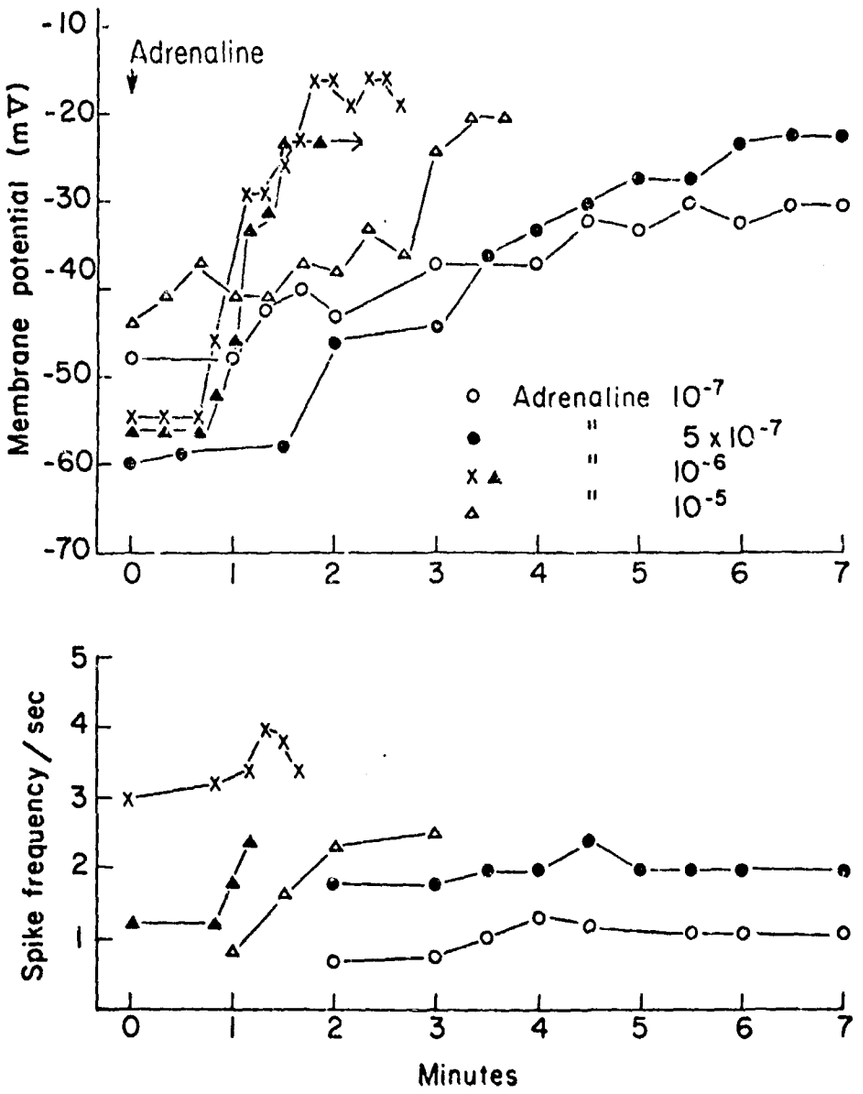

Adrenaline
Membrane potential (mV)
-10
-20
-30
-40
-50
-60
-70
0
1
2
3
4
5
6
7
Adrenaline 10-7
" 5 x 10-7
" 10-6
" 10-5
Spike frequency / sec
Minutes

Figure 1

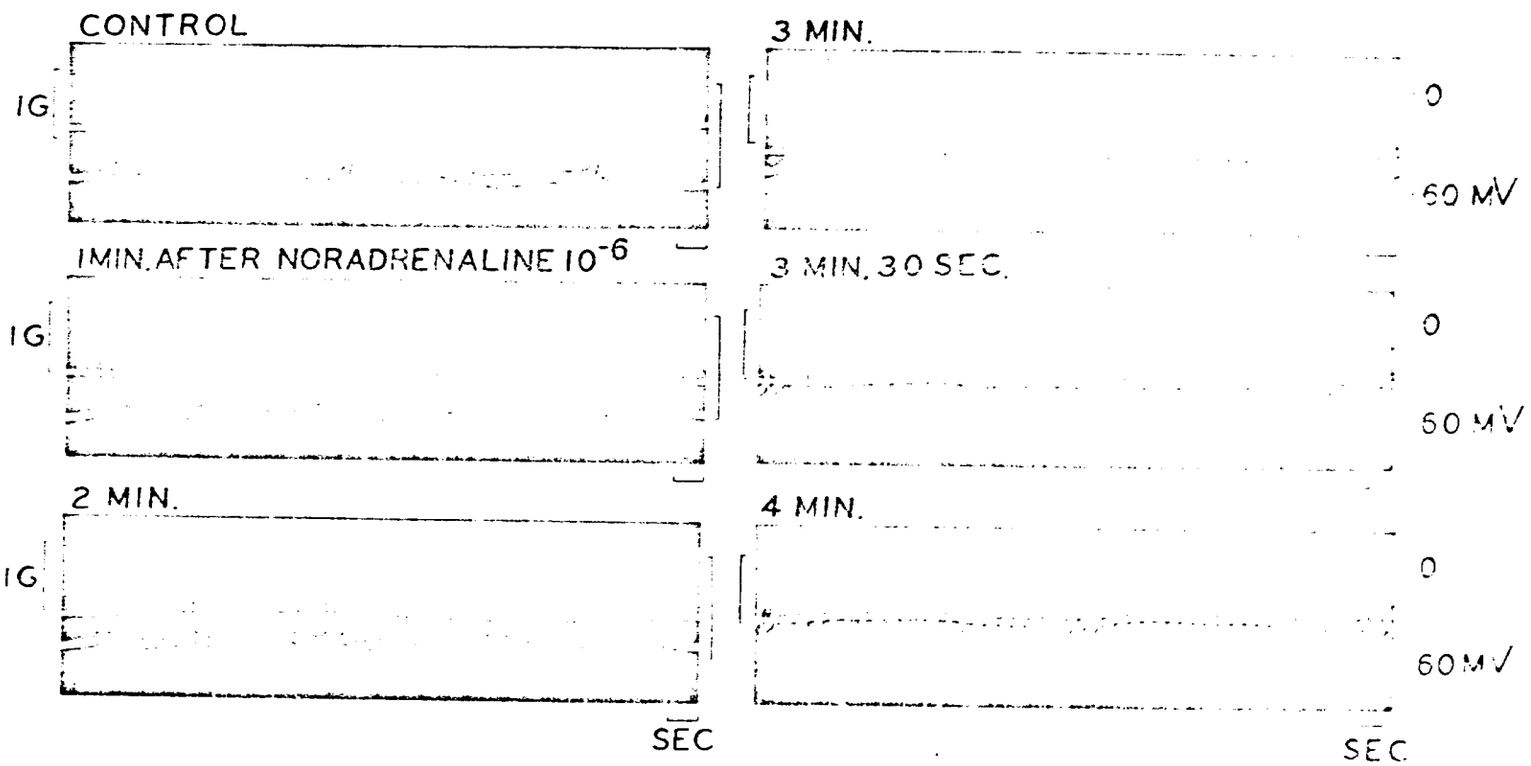


Figure 5

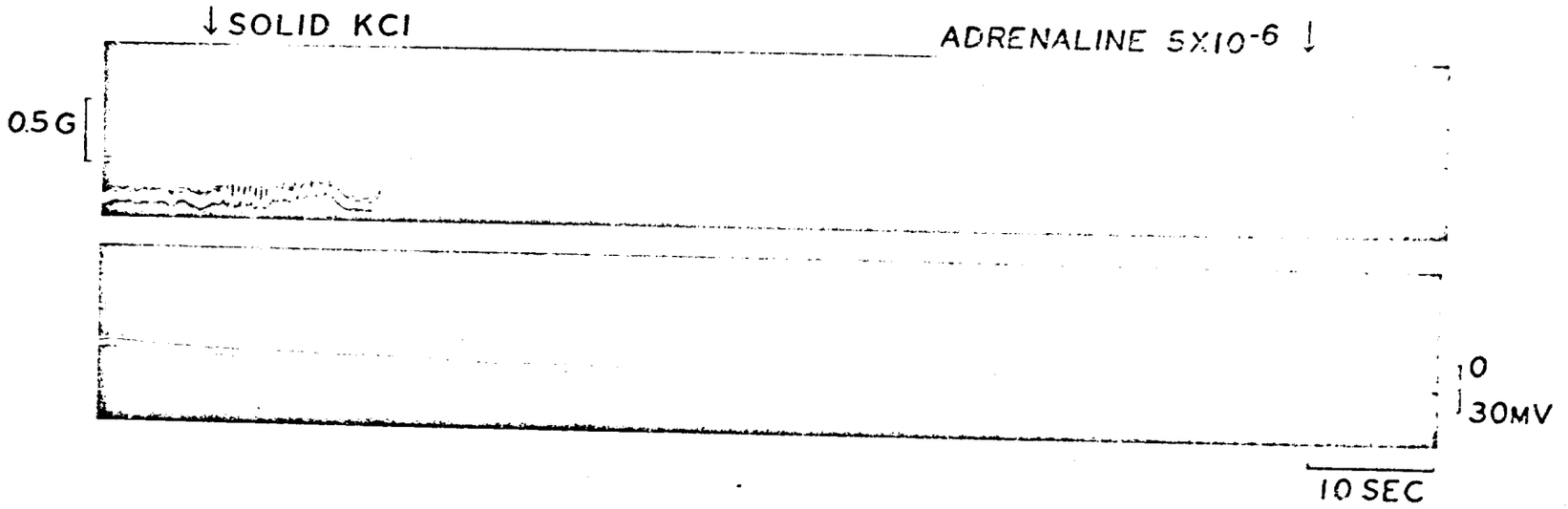
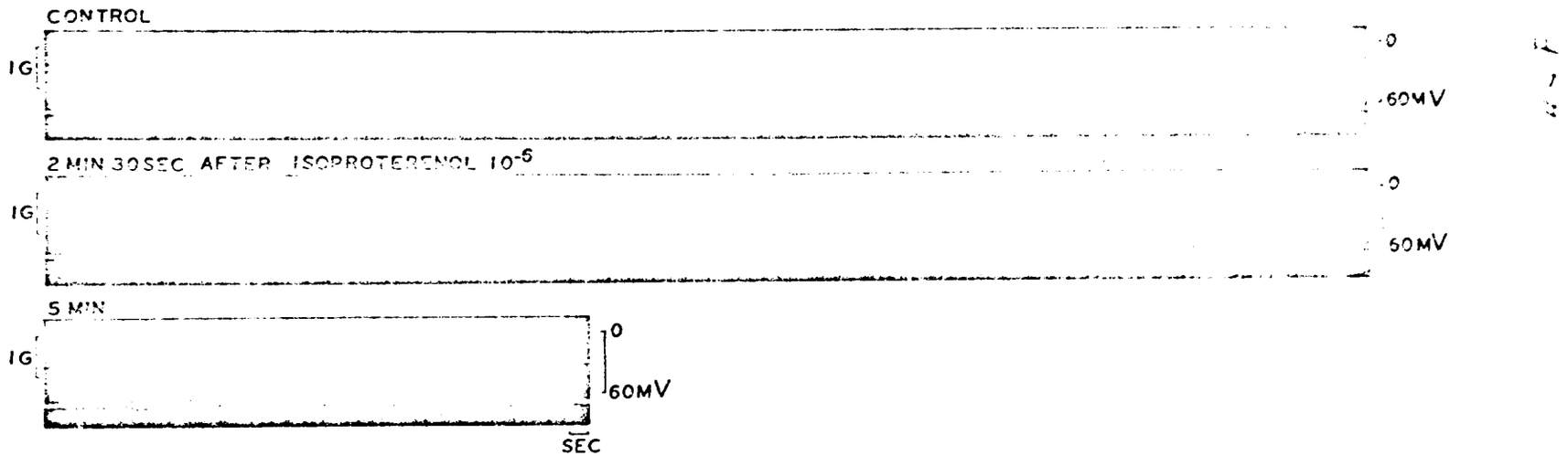


Figure 6



NOT REPRODUCIBLE

Figure 7

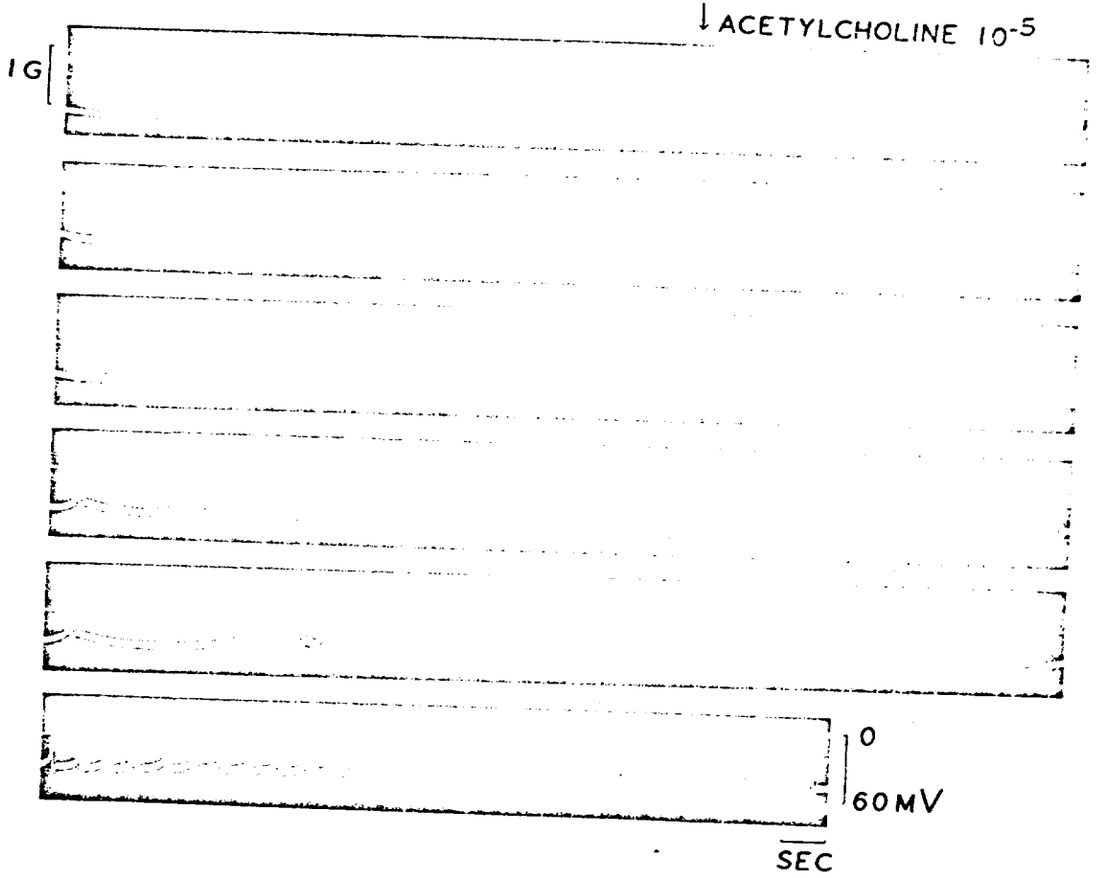


Figure 5

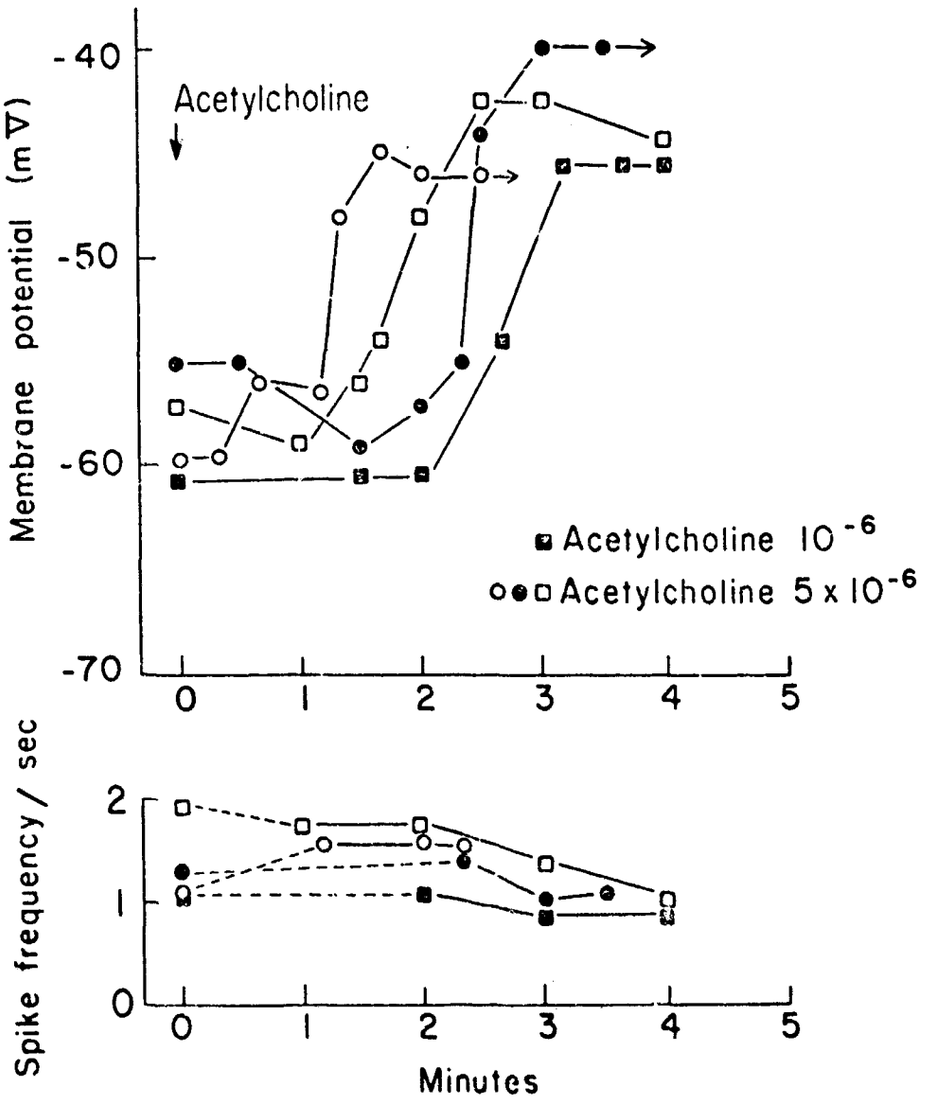

-40
-50
-60
-70
Membrane potential (m∇)
Acetylcholine
Acetylcholine 10 -6
Acetylcholine 5 x 10-6
0
1
2
3
4
5
Spike frequency / sec
2
1
0
Minutes

Figure 9

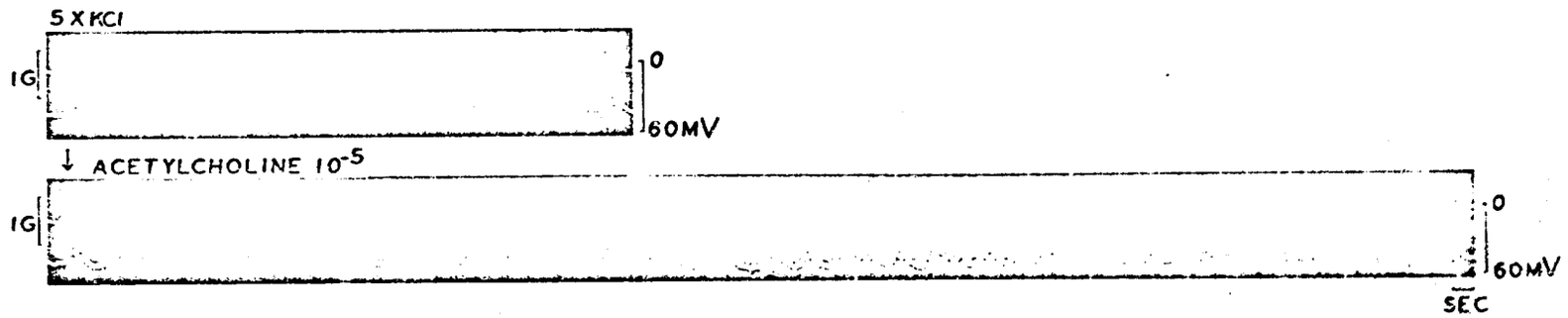
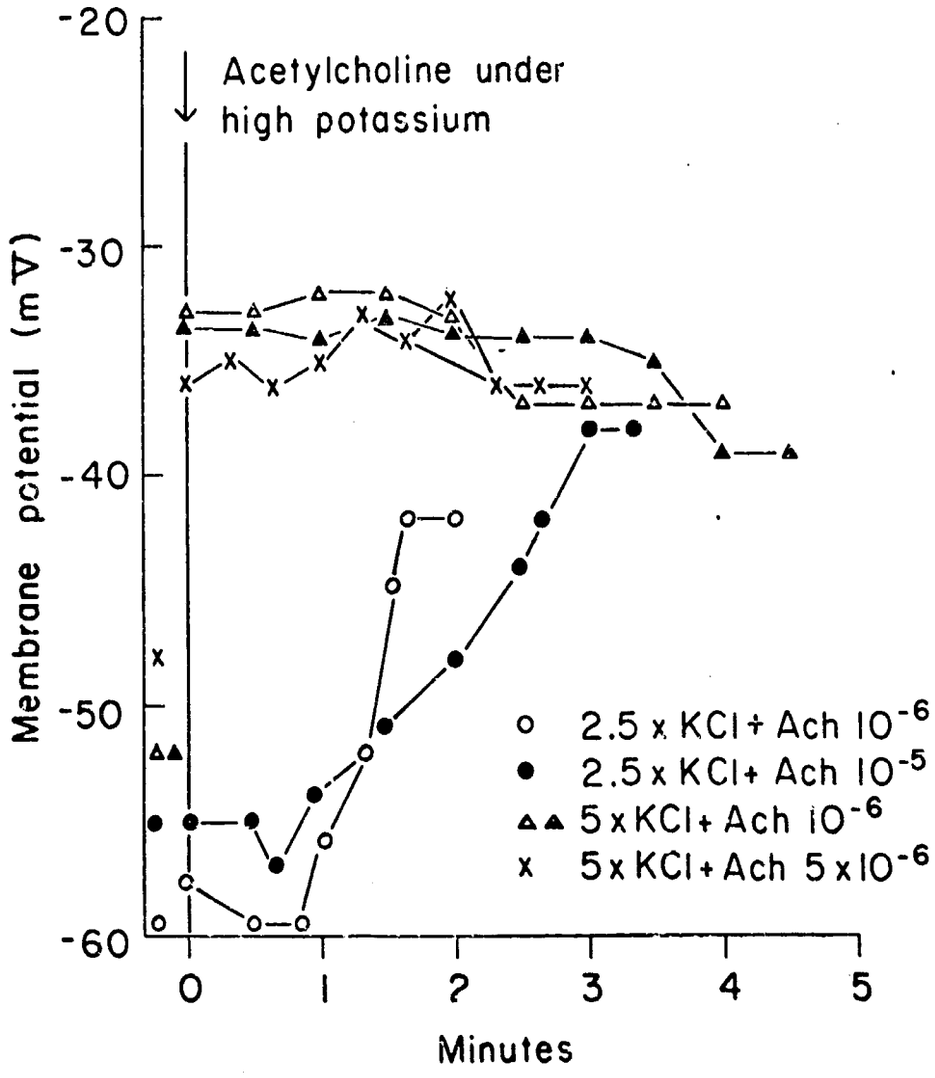


Figure 10


-20
-30
-40
-50
-60
Membrane potential (mV)
Acetylcholine under
high potassium
0
1
2
3
4
5
Minutes
2.5 x KCl + Ach 10-6
2.5 x KCl + Ach 10-5
5 x KCl + Ach 10-6
5 x KCl + Ach 5 x 10-6

APPENDIX C

FINAL REPORT

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