THE EFFECTS OF TETRODOTOXIN ON THE REACTIVITY OF SMOOTH MUSCLE

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MICHIGAN STATE UNIVERSITY
THOMAS D. BURNS
1973

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ABSTRACT

THE EFFECTS OF TETRODOTOXIN ON
THE REACTIVITY OF SMOOTH MUSCLE

BY

Thomas Dudley Burns

Tetrodotoxin (TTX), also called tarichatoxin, the toxic extract from the poisonous puffer fish and California newt affects excitable tissues by selectively blocking sodium conductance. In the present study in situ canine preparations of the intestine and gracilis muscle as well as isolated arterial (femoral) strips were employed to study the effect of TTX on the responses of vascular and visceral smooth muscle. The study also focused on the effects of TTX on the vascular responses to other vasoactive agents (epinephrine, norepinephrine, acetylcholine, ganglionic stimulants, KCl and changes in osmolality). TTX in doses (0.05-10 μg/min producing 10^{-6}-10^{-9} g/ml of blood) sufficient to depress or abolish 1) nerve-induced motor responses of gracili muscles, 2) vagal-induced intestinal motility reponses and 3) vascular responses to carotid occlusion produced significant increases in blood flow or decreases in vascular resistance of innervated gracili but failed to effect denervated gracili or innervated intestinal preparations. In cross-perfused innervated gracili muscles TTX produced a maximum vasodilation at doses which

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abolished the resistance responses to carotid occlusion. TTX induced vasodilation correlated with the level of initial resistance in innervated gracili ($r = 0.92$) but showed no correlation ($r = 0.27$) in denervated preparations. In innervated intestinal preparations, locally infused TTX generally produced no vasodilator response. However, occasionally TTX increased resistance which occurred concomitantly with increases in motility. TTX had no effect on the vasodilation produced by locally infused KCl into gracili muscles but frequently converted KCl-induced vasodilation to vasoconstriction in the intestine. TTX altered the motility responses to KCl from a pattern showing little phasic activity to one exhibiting large phasic responses. Intra-arterial or luminal placement of hyperosmotic NaCl produced vasodilation followed by vasoconstriction. TTX abolished the motility responses occurring during the vasoconstrictor phase; thus allowing hyperosmotic NaCl to produce only vasodilation. In isolated arterial strips the responses to all agents studied were augmented as pre-load was increased (1, 2, 4 and 6 gms). TTX (10^{-9}-10^{-6} g/ml) had no effect on strip before or after 24 hrs of cold storage. TTX depressed the increases in tension to nicotine or DMPP (5×10^{-6} g/ml) but failed to effect the responses to norepinephrine, acetylcholine, epinephrine, tyramine, KCl or hyper and hypo-osmolality. Increasing extracellular K^+ (+1.5, 4.5, 7.5, and 15.0 mEq/l) above control (4.7 mEq/l) produced increases in tension of vascular strips which were greater at higher pre-loads. When the strips were actively contracted with $BaCl_2$ (1 mM), $[K^+] < 12.2$ mEq/l produced relaxation while $[K^+] > 12.2$ mEq/l produced contractions. Increasing

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bath osmolality (50 mOsm/Kg) above control (300 mOsm/Kg) produced contractions of passively loaded strips but relaxation following $BaCl_2$. Hyposmolality (- 30-50 mOsm/Kg) produced increases in tension before and after $BaCl_2$. The responses to change in bath K^+ or osmolality were not affected by TTX, adrenergic or cholinergic blockade. These data strongly support the contention that: 1) TTX in neural blocking doses (10^{-9}-10^{-6} g/ml) has no direct action on vascular smooth muscle but produces vasodilation through neural mechanisms; 2) TTX may stimulate molility of visceral muscle by removal of intrinsic inhibitory nerves; 3) in the intestine extracellular K^+ and changes in osmolality may produce neurogenic motility responses which frequently alters the direct vascular action, and 4) the responses of isolated arterial strips may differ quantitatively and qualitatively depending on equilibration pre-load and the degree of active tension.

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THE EFFECTS OF TETRODOTOXIN ON THE REACTIVITY OF SMOOTH MUSCLE

By

Thomas D. Burns

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

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1973

To my family and fri

happiness, and

To Caroll and Tommy

heaven.

DEDICATION

To my family and friends who have shown me there are many ways to happiness, and

To Caroll and Tommy who I hope will receive some rewards before heaven.

The author would
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for his desire to mak

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T.M. Brody, R.M. Daug
Scott for their valua
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The author owes
Mrs. J. Johnston, Mi
assistance in the pr

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The author would like to thank Dr. J.M. Dabney not only for his encouragement, financial support and guidance but especially for his desire to make learning enjoyable.

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INTRODUCTION

Tetrodotoxin (TTX) the toxic extract from the poisonous puffer fish (Fugu, Spheroides rubripes) and California newt (Taricha torosa) has been shown to affect excitable tissues in animals and man (73). Among the physiological effects of TTX are paralysis of nerves (61, 95, 97) and systemic hypotension (33, 73). Nerve paralysis results from depression of the propagated action potential without depolarization (29), reportedly due to a selective inhibition of the sodium conductance (54, 95, 97) normally occurring during depolarization. Feinstein and Paimre (33) maintain that a blockade of conduction occurring in peripheral sympathetics and nerves innervating the adrenals is responsible for the profound hypotensive action of TTX. However, Kao et al. (74, 77, 88) subsequently reported that TTX-induced hypotension results from a direct relaxant action on vascular smooth muscle. Although the hypotension produced by systemic administration of TTX has been ascribed to numerous factors (33, 61, 74, 76, 77, 78, 82, 87, 88) the mechanism producing vasodilation remains unclear.

The responses of nonvascular smooth muscle to TTX are reported to be limited to neural mechanisms (39, 86, 100). Wood (130) cautioned against Gershon's (39) contention that TTX specifically blocked enteric neurons without affecting smooth muscle and reported that TTX altered both the electrical and mechanical activity of isolated cat jejunum but concluded that the stimulation occurs upon removal of an inhibitory neural mechanism. The stimulation of rat jejunum by TTX was reported to be similar to the stimulation resulting from whole body X-irradiation, hexamethonium and physostigmine (71). Kuriyama

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et al. (86) and Gershon (39) presented electrophysiological and functional evidence from guinea-pig taenia coli, mouse stomach, guinea-pig stomach, rabbit jejunum, and guinea-pig ileum favoring a non-direct action of TTX. Based on the strong evidence that TTX affects nonvascular smooth muscle primarily via extrinsic and/or intrinsic neural pathways, many investigators have used this neurotoxin in numerous preparations to separate neurogenic and non-neurogenic mechanisms (6, 39, 71, 73, 100 and 105).

Intrinsic nerves have been ascribed important roles in regulation of gastrointestinal secretion and motility (83), however, the role of intrinsic nerves in local regulation of intestinal blood flow remains questionable. Although, little evidence exists relating intrinsic nerves with local regulation of intestinal blood flow, a few studies indicate that local neural mechanisms may be involved directly or indirectly in the responses of intestinal vascular and/or visceral smooth muscle to certain vasoactive agents (23, 26, 112, 113, 114 and 127). Paton and Zar (102) studying innervated and denervated longitudinal muscle of guinea-pig ileum, reported that $BaCl_2$ and KCl (thought to have primarily direct effects) produce part of their action on visceral smooth muscle by neural mechanisms and part via direct effects. Gershon (39), by pharmacologically denervating longitudinal strips with tetrodotoxin, confirmed Paton and Zar's findings. Although the data reported by these investigators suggest only visceral smooth muscle effects, evidence suggesting that intrinsic neural elements may be involved in the intestinal vascular responses to luminal placement of

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Visceral smooth muscle activity can have a profound passive effect on the intestinal vasculature (24, 27, 112 and 114). This interaction between visceral and vascular smooth muscle reactivity has been described and emphasized by Haddy et al. (49). According to these authors the net vascular response of any vasoactive agent is determined by three interrelated effects: 1) the direct action on vascular smooth muscle, 2) the effect on tissue metabolism, and 3) any mechanical effect resulting from the activity of nonvascular parenchyma. Therefore, any attempt to study the role of intrinsic neural elements in local blood flow regulation by use of a pharmacological agent such as TTX requires that the action of TTX on vascular as well as visceral smooth muscle must first be defined.

The following review briefly presents a historical background regarding TTX, previous investigations directed at the cardiovascular effects of TTX, the action of TTX on nonvascular smooth muscle.

LITERATURE REVIEW

Historical Background of Tetrodotoxin, TTX - Descriptions of the toxic puffer fish (tetrodons, the first recognized source of tetrodotoxin) can be found in ancient Chinese literature over 2000 years ago (73). Although tetrodon eggs were used as a therapeutic agent for

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centuries, a detailed description of the tetrodon fish and its therapeutic relevance didn't appear until the pharmacopea Pen-t'so Kang Mu (the Great Herbal) was published around 1600 A.D. Tetrodon poisoning and the toxic puffer fish first became known to Europeans in the 18th century (70) when many cases of poisoning as well as clear descriptions of tetrodon fish were reported by early visitors to the Orient (73). G. Foster, Cook's naturalist, described the illness as producing numbness of the limbs, flushing and generalized muscular weakness (34).

The first scientific study on tetrodon poisoning was published by Remy in 1884 (104). However, the first extensive study of tetrodon poisoning was completed by Takahashi in 1890 (cited by Kao, 73). Interested in all the species of toxic tetrodon, Takahashi studied the relative toxicity of various organs, their physiological effects in mammals, and began work to chemically extract the toxic agent. Tahara, working in Japan, completed extraction of the toxin in 1894 and coined the term "tetrodotoxin" (121). The extract from spheroides eggs, given a molecular formula of $C_{16}H_{13}NO_{16}$, and was used experimentally and clinically until 1950 when crystallization of the toxic agent from Spheroides rubripes, called "spheroidin", was reported by Yokoo (135).

Interest in tarichatoxin, the poison from California newt (Taricha torosa), developed following transplantation studies by V. Twitty in 1927 (126). Twitty, an experimental embryologist at Stanford, reported that transplantation of eye vesicles from local salamander embryos into embryos of eastern salamanders (Amblystoma) produced prolonged paralysis of the host (126). Subsequent

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experiments showed that the taricha embryos contained a toxin which selectively paralyzed nerves. The growing interest by other investigators resulted in extraction and purification of a "tarichatoxin" preparation available for study in 1940 (59). Continuing efforts to purify tarichatoxin led to the development in 1962 of a crystalline preparation 10 times more potent (7000 mouse units/mg) than the previously reported extract (14); and further studies on tetrodotoxin indicated that the toxic extract from the eggs of spheroides rubripes, "spheroidin", (135) which was shown in 1956 to be identical to the toxic principle extracted from the ovaries by Tsuda and Kawamura (125) was pharmacologically and chemically similar to tarichatoxin (14). Buchwald et al. (15) elucidated the chemical structure of tetrodotoxin and reported in 1964 that tarichatoxin and the puffer fish poison were in fact chemically identical amino perhydraquinazoline compounds with a molecular formula $C_{11}H_{17}N_3O_8$ (mol. wt. = 319.28) (132). The toxin, known to be present in many species of puffer fish, is readily absorbed by most routes of administration, distributes rapidly throughout the body and exhibits a half-life of 30 minutes to 4 hours (73).

Crystalline TTX, having a LD_{50} in mice of 8 μg/kg, is one of the most potent non-protein neurotoxins known being 160,000 times more effective than cocaine in blocking axonal conduction (73, 76). This powerful neural blocking ability of TTX was first reported by Ishihara (61) in 1918. More recently Hamada (55) and Ogura (99) using isolated smooth muscle preparations designed as bioassays reported that TTX in doses of 1-25 ng/ml was effective in depressing nicotine

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induced contractions of the guinea pig ileum (55) and contractions of the rat stomach elicited by vagal stimulation (99), without altering the responses to acetylcholine, histamine, serotonin, substance P, or other polypeptides. According to Kao (73), the neural inhibitory properties can be demonstrated in both afferent and efferent nerves as well as skeletal muscle with sensory nerve fibers exhibiting more sensitivity to TTX than efferent or somatic nerves (31). Watanabe (128), using direct oscillographic recordings, reported that TTX doses of 0.5-3 μg/kg i.v. were effective in reducing or abolishing afferent impulses in the superficial peroneal, saphenous and mesenteric nerves of cats. Although the neuromuscular effect occurs on the motor axons and muscle membrane, the dose of TTX and time required to affect skeletal muscle directly is greater than that necessary to produce neural blockade (61).

Blockade of the propagated action potential reportedly occurs only in the treated segment of isolated axons (61), and results from a selective inhibition of the rapid sodium current accompanying excitation (97). Electrophysiological studies have shown that TTX as well as saxitoxin, the poison extracted from the Alaska butter clam (Saxidomas giganteus), affect only the excitatory phenomenon generating spike potentials but fails to affect delayed or resting membrance currents (74, 97). Voltage-clamp studies on giant squid or lobster axons also show that, unlike the local anesthetics procaine or cocaine, TTX decreases the early sodium current while the late inward potassium current remains unchanged (97). Therefore, nerve paralysis induced by TTX is manifest by ablation of the propagated action

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potential in potential studies and inhibition of net sodium movement in voltage-clamp studies. It is now generally agreed (75) that the sole cellular action of TTX is to prevent the increase in early sodium permeability, thus producing a conduction blockade without depolarization (73). Because TTX specifically affects sodium permeability of nerve membranes at low TTX concentrations without affecting other membrane phenomena or tissue, it is now used widely as a tool in neurophysiological and neuropharmacological studies (74, 96).

The specific mechanism by which TTX affects excitable tissue appears to be by affecting the sodium sites extracellularly. Intracellular perfusion of giant squid axons with TTX in concentrations 1000 times (1 μM) that effective extracellularly fails to alter either inward or outward sodium currents and concentrations 10,000 times higher (10 μM) has no effect on the propagated action potential (61, 73). This highly selective action of TTX has encouraged numerous investigators to study structure-activity relationship in an attempt to identify the sodium canal (4, 25, 28, 81, 96, 116). It is now believed that TTX, an amino perhydraquinazoline molecule consisting of numerous oxygen and hydroxyl groups as well as a guanidine moiety, is positioned steriochemically within the canal to block Na^+ movement (4, 116). Smythies et al. (116) has postulated that separation of a nucleotide-protein complex within the membrane may form the sodium channel, and TTX or saxitoxin (STX) plugs this canal by filling the space while batrachotoxin (BTX, from the deadly Colombian frog, Phyllobates aurotaenia) allows continous

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sodium movement by holding the canal open. Further, speculation by Smythes et al. (116) with molecular models indicates that the sodium gate may in fact be composed on uracil and cytosine nucleotides, and that TTX, STX and BTX exert their action by binding differently to these molecules. More recently, investigators attempting to quantify the number of sodium channels responsible for the TTX-sensitive sodium and calcium currents have assumed that a single TTX molecule blocks a single sodium canal (4, 25, 81). Keyne et al. (81) determined the number of sodium sites in non-myelinated rabbit vagus, crab leg nerve and lobster leg nerve to be 75/μm^2, 49/μm^2 and 36/μm^2 of nerve membrane respectively. However, Colquhaun et al. (4) using tritium-labelled TTX calculated no more than 27 binding sites/μm^2 in the desheathed vagus of the rabbit. Therefore, current investigators generally agree that although the number of TTX-sensitive sites may range between 13 and 75/μm^2 of nerve membrane, the relative number of sodium channels per μm^2 of nerve membrane is small.

The Cardiovascular Effects of TTX - Systemic administration of TTX produces a profound decrease in arterial blood pressure (33, 73, 74, 77, 88). This hypotensive action of TTX occurs in 2 to 3 minutes following intravenous administration of 2 µg/Kg and may last for more than 30 minutes (33). Although it was thought for sometime that the hypotensive action of TTX was due to depression of the central nervous system (87, 92), Kao (33) reported that cross-perfusion studies on cats and dogs indicated that the medullary vasomotor centers were relatively unimportant in TTX induced hypotension. Intravenous administration of TTX in concentrations sufficient to produce

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hypotension in the donor animal or i.a. infusion of TTX into the vascularly isolated head of the recipient animal never produced a decrease in systemic blood pressure of the recipient animal. Kao's studies (73, 88) differed from those of previous investigators in that the TTX doses were reduced to concentrations comparable to those which produce hypotension when given intravenously. Where as, Murtha et al. (92) and Li (87) injected into the carotid or vertebral arteries of cats and rats a crystalline TTX solution at concentrations equal to those given i.v.; thus resulting in TTX concentrations to the brain 50 to 100 times higher than that generally seen during i.v. administration of 2 μg/Kg.

Higher doses of TTX (20 μg/Kg) have also been shown to produce bradycardia, abnormal A-V conduction and arrhythmia (61, 73, 76); yet, during hypotension induced by TTX cardiac rhythm and function appears to be unaffected except for slight alterations of the T-wave and A-V interval often seen with reflex release of epinephrine from the adrenals (61, 73, 76). Cardiac effects appear not to be important in tetrodon poisoning for Kao (73) states that, even in animals dying from TTX, cardiac rate is usually normal. Although high doses of TTX may affect cardiac function via depression of the conduction system (73), in vitro and in situ studies show that in hypotensive doses TTX (1 - 5 μg/Kg) has no significant affect on the excitability of the CNS (73), myocardium (73, 75) or vascular smooth muscle (73, 80). Therefore, it is now generally agreed that TTX produces hypotension not via either a CNS or cardiac action but rather by a peripheral effect. Although, in his review in 1966 Kao (73,

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76) stated that there is no unequivocal proof that the hypotensive action of TTX is due to block of vasomotor nerves nor interference with spinal vasomotor control mechanisms, he postulated that hypotension resulted from release of vasomotor tone.

More recently, Kao and collegues (74, 77, 88) have reported that the peripheral vasodilation produced by low doses of TTX (0.5 - 2.0 μg/Kg) is due primarily to a direct relaxant action on vascular smooth muscle. In their first study Lipsius et al. (88) used several canine preparations (cross-perfused head-body, cross-perfused isolated gracilis muscle, and pump-perfused gracilis) to study the peripheral vascular action of TTX and procaine. They reported that: 1) TTX (0.5 - 0.8 μg/Kg) produced vasodilation but failed to block the reflex vasomotor responses to decreased systemic blood pressure, 2) electrical stimulation of the sympathetic chain at L_2 - L_4 with supra-threshold current produced an increase in hindlimb perfusion pressure before and after TTX, 3) the vasodilation produced by TTX was not blocked by phentolamine (1.5 mg/Kg), phenoxybenzamine (15 mg/Kg), bretylium (5 mg/Kg) nor reserpine pretreatment (1 mg/Kg, 24 hrs) and 4) unlike Li's (87) observation the antihistamine, diphenylhydramine (2 mg/Kg) had no effect on the systemic response to TTX. These data reported by Lipsius et al. (88) are in disagreement with those reported in 1968 by Feinstein and Paimre (33), which suggested that vasodilation to TTX was not due to a direct vascular smooth muscle effect but occurred primarily as a result of conduction blockade in the peripheral sympathetic nerves and fibers innervating the adrenals.

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Feinstein and Paimre (33) studied the cardiovascular effects of TTX in cats anesthetized with α-chloralose (40 mg/Kg) and pretreated with ether or pentobarbital (35 mg/Kg). TTX (1 to 10 µg/Kg, i.v.) decreased blood pressure (systolic and diastolic), pulse pressure, heart rate, myocardial strength, cardiac output and peripheral resistance. The sinus bradycardia occurred simultaneously with a fall in blood pressure and was blocked by acute sympathetic denervation. Similarly in isolated atrium preparations, TTX (1 to 2 x 10^{-8} g/ml) abolished the positive inotropic and chronotropic responses to electrical stimulation of the sympathetic nerves, but failed to affect spontaneous contractile activity or that induced by tyramine (10^{-6} g/ml). They also reported that responses to DMPP (1, 1-dimethyl-4-phenylpiperazinium, 5-20 µg/Kg) and stimulation of the celiac ganglia or splanchnic innervation to the adrenal medulla were all attenuated by TTX. Also, in the pump-perfused hindlimb pretreated with d-tubocurarine (0.3-1.0 mg/Kg, i.v.), TTX produced a profound vasodilation and attenuated or blocked the pressor responses to electrical stimulation of the pre-ganglionic and post-ganglionic vasomotor nerves. Neither TTX nor stimulation of the sympathetic fibers to the hindlimb were effective following alpha-blockade by phenoxybenzamine (10 mg); yet, histamine (4-10 µg), acetylcholine (4-40 µg), isoproterenol (10 µg) and epinephrine (4 µg) still decreased resistance.

More recently, Kao et al. (74, 77, 98), re-examining the cardiovascular actions of TTX and saxitoxin (STX) in cats, confirmed their previous reports that low doses of TTX (2.0 µg/Kg) or STX

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(1.5 µg/Kg) produce vasodilation via a direct non-neurogenic vascular action and at higher doses demonstrated an indirect neural action. In these studies Kao et al. again report that reports that TTX induced vasodilation was unaffected by an alpha or beta adrenergic blockade but showed in their data that TTX (1.0 µg/Kg) in a dose 2.5 times the control (0.4 µg/Kg) decreased perfusion pressure of the hindlimb following propranolol (5 mg/Kg i.v.), but failed to affect pressure following pronethalol (5 mg/Kg) even when the TTX dose was increased 50 times (20 µg/Kg) more than in the control. These data differ from their previous study on dogs (88), where they clearly showed that 0.1 µg/Kg of TTX was effective in decreasing resistance of the pump-perfused gracilis following pronethalol (5 mg/Kg) combined with reserpine pretreatment (1 mg/Kg). Therefore, the recent work by Kao et al. (74, 77, 88, 93) and Feinstein and Paimre (33) fails to clearly define the peripheral vascular action of TTX.

Effects of TTX on Nonvascular Smooth Muscle - The action of TTX on nonvascular smooth muscle appears to be limited to responses mediated by neural mechanisms (39, 71, 73, 86, 130). The concensus that TTX acts primarily via neural tissue is supported by studies showing that TTX blocks numerous smooth muscle responses induced by nerve stimulation (piloerection, secretory activity of salivary and sweat glands, pupillary responses and contractions of the cat nictitating membrane, mouse stomach, rabbit jejunum and guinea-pig iteum) but has no effect on the responses to epinephrine, pilocarpine, acetylcholine, or carbamylcholine (39, 61, 73, 75). Thus, it is generally agreed that TTX and saxitoxin have little effect on

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smooth muscle except at doses much higher than the neural blocking levels (39, 73, 75).

Although, TTX has little effect on denervated smooth muscle, it can stimulate motility of innervated intestine (71, 86, 130). This stimulation of motility which has been shown to occur at TTX ($<10^{-5}$ g/ml) doses much less than that needed to produce any direct action on smooth muscle. Kuriyama et al. (86) used microelectrodes to study the electrophysiological responses of guinea-pig intestinal, cardiac and diaphragm muscle. They reported that TTX occasionally increased spike frequency of the isolated taenia coli, but it failed to alter membrance potential (45-58 mV), spike amplitude (40-65 mV) or rates of rise and fall of the action potential, even at concentrations 100 times (5×10^{-7} g/ml) that which blocks spike generation in nerve, heart and skeletal muscle. TTX did not affect the spontaneous activity of cardiac pacemaker cells, changes in spike amplitude of smooth muscle induced with hyperpolarizing or depolarizing currents, membrane responses (hyperpolarization) to increased extracellular calcium, or responses of smooth muscle (slight depolarization, 7 mV) induced with aconitine (10^{-8}-10^{-6} g/ml).

Unlike Kuriyama et al. (86), Gershon reported that TTX (10^{-7} g/ml) never stimulated motility of the isolated rabbit jejunum nor affected the spontaneous contractions (regularity, frequency or magnitude). However, TTX (10^{-7} g/ml) abolished the motility responses of innervated smooth muscle (mouse and guinea-pig stomachs, rabbit jejunum and guinea-pig ileum) to nerve stimualtion (vagus

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and perivascular) and ganglionic stimulants (nicotine and DMPP) but failed to abolish the responses to noradrenaline (10^{-8} - 10^{-6} g/ml), histamine (10^{-8} g/ml), 5-HT (10^{-9} g/ml) acetylcholine (10^{-6} g/ml, BaCl, KCl or bradykinin (2.5-5.0 x 10^{-8} g/ml).

Recent studies indicate that TTX and other pharmacological agents may stimulate intestinal smooth muscle by removing a neural inhibitory mechanism (71, 130). Kagnoff and Kivy-Rosenberg (71) reported that TTX (0.1-8.3 µg/ml) converted the spontaneous contractions of isolated rat jejunum from an irregular pattern into a regular spindling response. The stimulation induced by TTX was like that produced by hexamethonium (240 µg/ml), physostigmine (0.5 µg/ml), and whole-body X-irradiation (1,500 R, 2-3 days previously). Similarly, Wood (130) who studied the electrical and mechanical activity of cat jejunum, reported that TTX (5 x 10^{-8} - 7.5 x 10^{-7} g/ml), atropine (5 x 10^{-5} - 7.5 x 10^{-4} g/ml), procaine (5 x 10^{-5} - 10^{-4} g/ml) and xylocaine (2.5 x 10^{-6} - 10^{-4} g/ml) produced action potentials, thus, augmenting contractions of circular muscle from 350 $\pm$ 56% of control at a TTX dose of 3.3 x 10^{-7} g/ml to 470 $\pm$ 69% at 1.3 x 10^{-6} g/ml of TTX. These authors suggest that the mechanical and electrical stimulation of intestinal motility by neural blocking agents indicates that intestinal smooth muscle is primarily influenced by an intrinsic mechanism.

Although TTX may increase intestinal activity, stimulation of smooth muscle is seldom mentioned by investigators using TTX (6, 17, 105, 122). Bennett *et al.* (6) employing TTX (0.2 µg/ml) to study the action of prostaglandins E_1 and E_2 on the intrinsic

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nerves of human, guinea-pig and rat small intestine, failed to mention any stimulation induced by TTX. Likewise, Burnstock et al. (17) used TTX (1.5×10^{-7} g/ml) to study the role of ATP as a transmitter of non-adrenergic inhibitory nerves; Rikimaru and Susuki (105) further studied the intrinsic inhibitory nerves by pharmacologically denervating guinea-pig taenia coli with TTX (10^{-9} - 5×10^{-8} g/ml); and Taira et al. (122) studied the effect of TTX (1-10 µg, i.a.) on nerve-induced responses of canine urinary bladder; however, none of these investigators reported stimulation of smooth muscle. Thus, from previous work it may be concluded that TTX is highly effective in abolishing the responses of smooth muscle induced by nerves. In some preparations an increase in spontaneous motility may follow this action on nerves. Furthermore there is little data to support the contention that tetrodotoxin has a direct action on smooth muscle at dosages which effect nerves.

Statement of Problem

Intrinsic nerves have been shown to play an important role in regulating secretory and motor activity of the gastrointestinal tract (83, 105); however, regulation of local blood flow by intrinsic neural elements has not been well studied. Although, it is well documented that intrinsic nerves may indirectly affect intestinal blood flow via visceral smooth muscle activity (112, 113, 114), little is known of the role, if any, that the intrinsic intestinal nerves play in local blood flow regulation. Recent work by Dabney et al. (23, 26) suggests that the intrinsic nerves may be

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Dabney et al. (23, 26) studied intestinal vascular responses before and after luminal placement of local anesthetics (dibucaine and piperocaine) which are known to affect permeability (Na^+ and K^+) and conduction in many tissues including smooth muscle (42). Therefore in order to more thoroughly study the role intrinsic nerves play in regulation of intestinal blood flow, vascular responses should be re-examined before and after locally denervating the intestine by a method having minimal direct effect on both intestinal and vascular muscle.

A technique that selectively and permanently destroys the intrinsic nerves of the small intestine has been reported by Hukahara et al. (60). However, this technique requires long periods of local ischemia, thereby resulting in degradation of the preparation. A more desirable technique for selectively denervating the intestine appears to be the pharmacological agent tetrodotoxin (poison from the puffer fish) which has been shown to selectively block nerve propagated action potentials with little direct effect on visceral smooth muscle (39, 73, 122). Although tetrodotoxin had been reported to affect the cardiovascular system only through neural mechanisms (33, 73), Kao et al. (74, 77, 88) recently reported that TTX directly relaxes vascular smooth muscle. Therefore the action of TTX on vascular smooth muscle must first be established before it can be used as a tool in studying neurogenic responses of the intestinal vasculature.

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Thus the objectives of this study were:

1) To define the action of TTX on vascular smooth muscle by using established techniques currently employed in cardiovascular studies [intestinal (23, 26, 27, 108), skeletal muscle (94, 111), and in vitro (1, 21, 36, 80)].

2) To establish doses of TTX necessary to locally block nerves without appreciably affecting vascular or nonvascular smooth muscle.

3) To determine the effect of TTX on the responses of vascular and nonvascular smooth muscle of the intestine induced by drugs and vasoactive agents known to affect these muscles.

4) To compare the intestinal vascular responses induced by various vasoactive agents to those of the skeletal muscle vasculature focusing on the role of neural elements in local blood flow regulation.

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EXPERIMENTAL METHODS

The action of various agents on vascular and visceral smooth muscle were investigated using both *in situ* and *in vitro* preparations. Established techniques as well as preparations modified for experimental improvement were employed in the studies. The basic intestinal (23, 24, 26, 27, 108), skeletal (94, 111), and isolated (1, 36) muscle preparations have previously been described. Smooth muscle responses were studied with natural or pump-perfused blood flow in the jejunum or ileum, natural or pump-perfused gracilis muscle, and isolated arterial (femoral) strips.

Preparation of the Animal

Mongrel dogs of either sex weighing between 16 and 23 Kg were anesthetized with sodium pentobarbital[1] (30 mg/Kg, i.v.) and placed on a positive pressure respirator[2]. The ventral regions of the neck and abdomen or medial aspects of the hindlegs were shaved and wiped clean with a damp sponge. Following the surgical procedures heparin sodium[3] (5 mg/Kg, i.v.) was administered to prevent coagulation. All pressures mentioned were continuously monitored by low volume displacement pressure transducers[4] which served as inputs into a

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[1]American Pharmeceutical Company, Bronx, New York

[2]Harvard Apparatus Co., model 607, Dover, Mass.

[3]National Biochemicals Corp., Cleveland, Ohio

[4]Statham Laboratories, model P23Gb, Hato Rey, Puerto Rico

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direct writing oscillograph[5]. During the experiments additional anesthetic or heparin was given as needed intravenously or into the collecting reservoir. In dogs to be used for intestinal preparations, food was withheld 24 hours prior to the experiment. All gross surgery was performed by cauterization[6] and blunt dissection. The preparations were maintained moist and near 37°C by covering the preparations with a plastic film[7], and using a heat lamp.

Experimental Preparations

Skeletal Muscle Preparations

1. Naturally-perfused gracili muscle (innervated and denervated)

(Figure 1) - In this preparation both gracili muscles were exposed and freed from connective tissue. All blood vessels communicating with the gracili except the major artery and vein were ligated. Heavy occlusive cord-ligatures were placed at each end of the muscles to eliminate collateral flow. A short section (5-8 cm) of each gracilis nerve was carefully freed from investing fascia and one muscle was denervated by cutting its isolated nerve trunk.

A small arterial branch from the gracilis artery was cannulated[8] (PE 50) for subsequent intra-arterial infusions. The gracilis vein

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[5]Sanborn Co., model 60-1300, Boston, Mass.

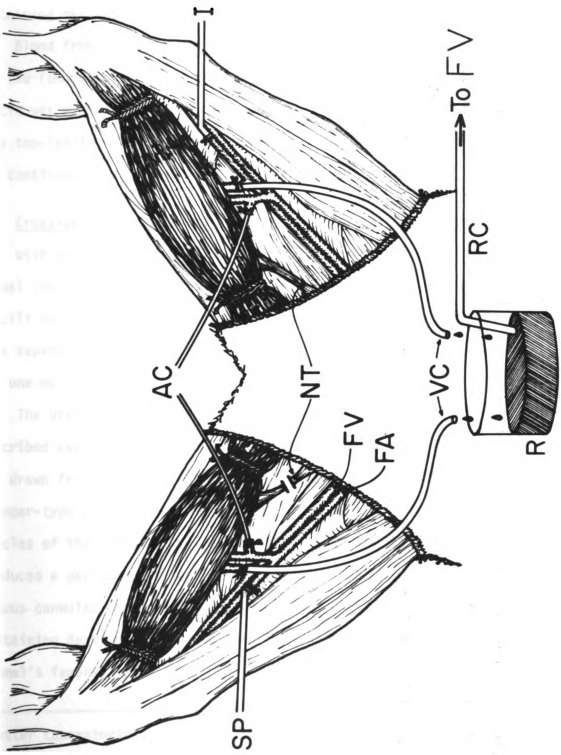
[6]Statham Instruments, Inc., National Prod., Oxnard, California.

[7]Dow Chemical Co., Handi-Wrap, Midland, Michigan

[8]Intramedic[R], Clay Adams Div., Becton, Dickinson & Co., Parsippany, N.J.

Figure 1

Schematic drawing (ventral view) of the naturally perfused gracilis muscle preparation. One muscle was denervated by cutting the nerve trunk (NT). Local infusions were made through intra-arterial cannulas (AC). Venous cannulas (VC) directed the outflow into a collecting reservior (R) from which it was returned through a return cannula (RC) to the animal. Systemic pressure (SP) was monitored from the femoral artery (FA) downstream to the gracilis artery. Systemic injections (I) were made through the femoral vein (FV).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was cannulated (PE 240) and its flow diverted into a glass reservoir containing dextran[9].

Blood from the reservoir was continuously returned to the animal via the femoral vein by means of a pump[10]. Blood flow through the muscle was periodically determined by weighing timed venous collections on a top-loading semi-analytical balance[11]. Abdominal aortic pressure was continuously recorded via a femoral arterial cannula.

2. Cross-perfused gracili muscles (innervated and denervated) with constant flow (Figures 2 and 3) - In this preparation one animal (donor) served as the blood source for perfusion of the gracili muscles of another animal (recipient). Neurogenic responses were separated from direct vascular responses by surgically denervating one muscle.

The gracili muscles of the recipient were prepared as previously described except the muscles were perfused at a constant rate. Blood was drawn from a femoral arterial cannula in the donor and pumped (finger-type pump)[10] into the arterial supply of the isolated gracili muscles of the recipient. The pump flows were set at a rate that produced a perfusion pressure approximately equal to aortic pressure. Venous cannulas (PE 240) directed the blood into a glass beaker containing dextran[9]. The blood was continuously returned to the donor animal's femoral vein.

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[9]Cutter Laboratories, Berkeley, California

[10]Sigma Motor Inc., model T-6SH, Middleport, N.Y.

[11]Mettler Co., Model P 1200, Princeton, N.J.

Figure 2

Schematic drawing of the cross-perfusion preparation in which femoral arterial (FA) blood was drawn from one animal (donor) and pumped at a constant rate into the arterial supply of the isolated gracili muscles (one innervated, one denervated) of another animal (recipient). Venous cannulas directed the outflow into a reservoir from which it was returned by a pump (RP) to the femoral vein (FV) of the donor. Pressures (P_1 and P_2) were monitored downstream to the perfusion pumps (PP). An infusion pump (IP) delivered the test agents upstream to the perfusion pumps (PP). The carotid arteries (CA) were isolated for occlusion and systemic pressure(SP) monitored in both animals from the femoral arteries.

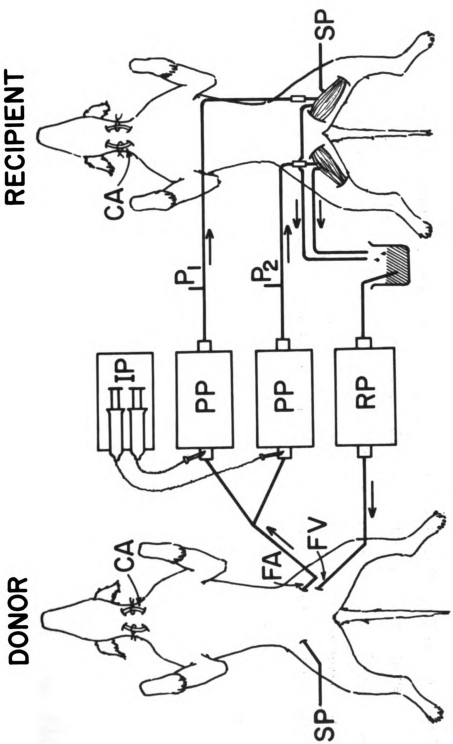
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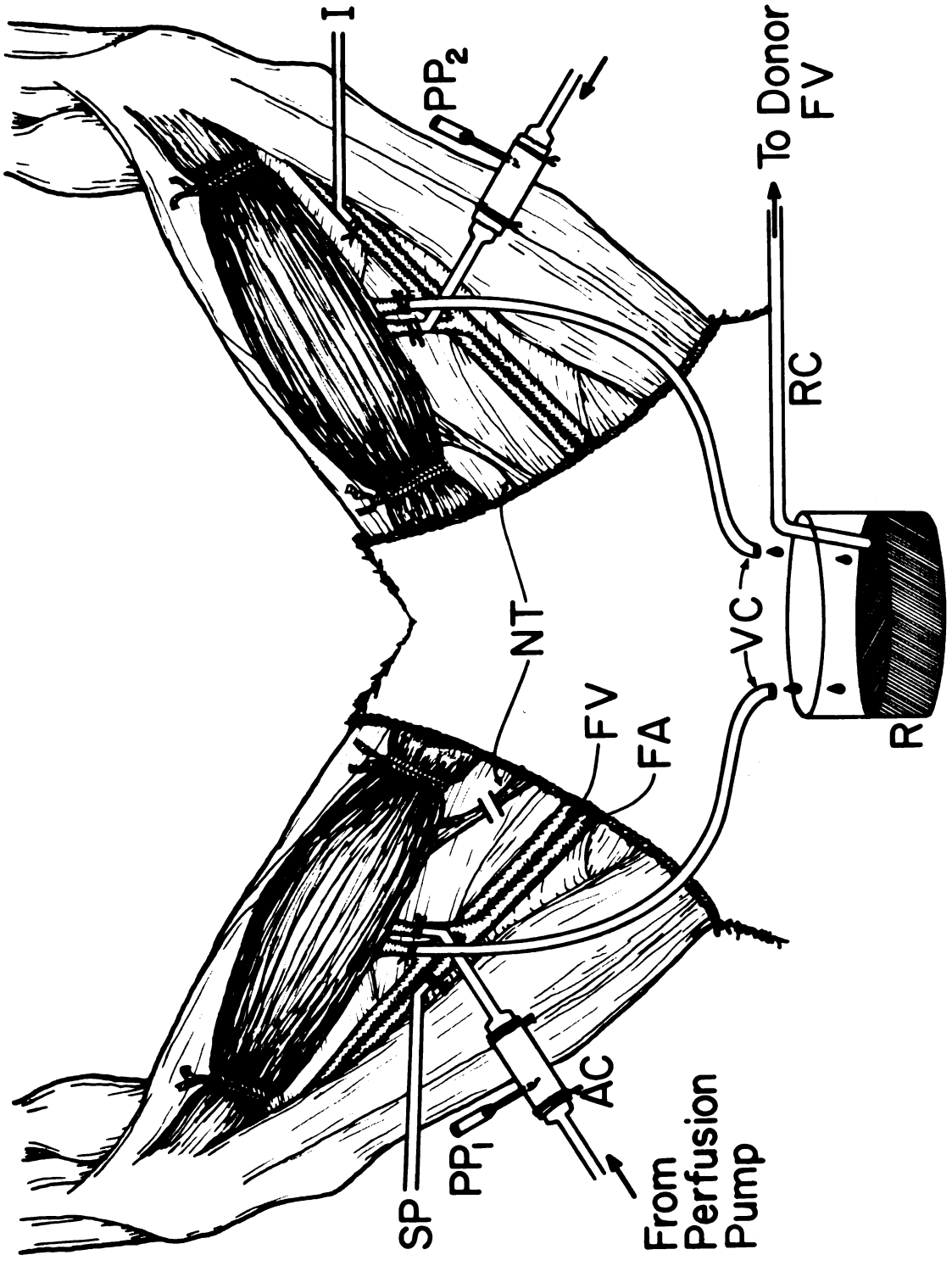
Figure 3

Schematic drawing (ventral view) of the pump perfused gracilis muscle preparation. One muscle was denervated by cutting the nerve trunk (NT). Arterial blood from a donor animal was pumped through arterial cannulas (AC) placed in the artery supplying each muscle. Perfusion pressures (PP_1 and PP_2) were monitored from points near the AC. Venous cannulas (VC) directed the outflow into a reservoir (R). The blood was returned to the donor through a return cannula (RC). Systemic injections (I) were made into the femoral vein (FV) and systemic pressure monitored from the femoral artery (FA).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The perfusion pressures were monitored from small hypodermic needles (21 gauge) inserted through the rubber tubing connected to the arterial cannulas (15 gauge, blunt-end hypodermic needles)[12]. All test agents were infused[13] or injected behind the pumps perfusing the muscles. Systemic pressures were continuously monitored in both animals from femoral arteries. A cannula was inserted into the femoral vein of the recipient for subsequent systemic intravenous injections. Prior to heparinization the carotid arteries and vagi in both animals were isolated for subsequent occlusion or electrical stimulation[14].

Intestinal Preparations

3. Naturally-perfused double-segments with intra-arterial (i.a.) infusion of test agents (Figure 4) - A loop of jejunum (20 cm, aboral to the ligament of Treitz) or ileum was exteriorized through an abdominal incision and divided into two adjacent segments (10 cm), then placed on a supporting platform constructed with towels and gauze.

The carotid arteries, cervical vagal trunks, the single vein draining each intestinal segment and small side-branch arteries (upstream to segmental supply) were isolated at least 15 minutes prior to heparinization. Venous (PE 240, ID = 1.67 mm; OD = 2.42 mm) and arterial (PE 50, ID = 0.58 mm; OD = 0.96 mm) polyethylene

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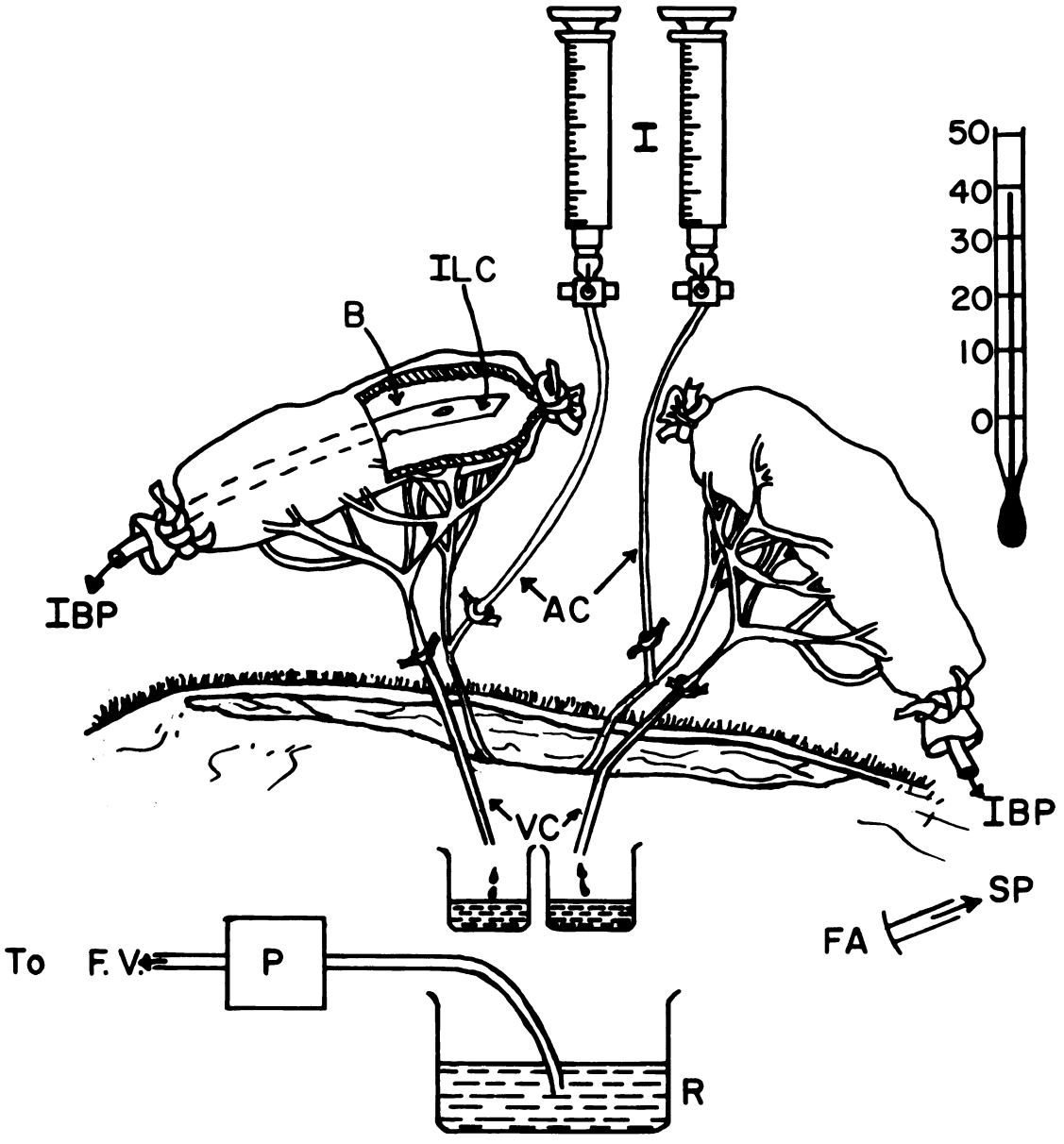
[12]Arista Surgical Co., New York, N.Y.

[13]Harvard Apparatus Co., Model 600-910/920, Dover, Mass.

[14]Grass Instrument, Square-Wave Stimulator, model 55, Quincey, Mass.

Figure 4

Schematic drawing of the naturally perfused intestinal preparation (double-segment) showing venous (VC) and arterial (AC) cannulas. Test agents were infused (I) intra-arterially and the venous outflow returned from the reservoir (R) to the femoral vein (FV) by a pump (P). Motility, recorded as intra-balloon pressure (IBP), was monitored by balloons (B) with intraluminal cannulas (ILC). Systemic pressure (SP) recorded from the femoral artery (FA). The preparation was maintained at 37°C.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cannulas[8] were inserted into the vessels and secured by ligatures (4-0). The venous cannulas directed the outflow into a collecting reservoir initially containing dextran (6% in saline)[9]. The blood was pumped[10] back into the dog continuously via a femoral vein cannula (PE 320, ID = 2.69 mm; OD = 3.50 mm)[8]. Timed venous outflows were collected in clean glass beakers and measured by weighing on a top-loading semi-analytical balance[11].

Rubber tubes[15] (ID = 2.5 mm; OD = 5.3 mm) to which balloons (condoms) had been tied were inserted into the intestinal lumen and secured by tying both ends of the segments. Fluid (10 ml, H_2O) was added to the balloons and intraballoon pressure (IBP) which served as an indication of intestinal motility was monitored and recorded. To prevent collateral circulation the attached mesentery was separated by cauterization. A cannula[8] (PE 280, ID = 2.5 mm; OD = 3.25 mm) was inserted through the femoral artery and positioned in the lower aorta for monitoring systemic pressure. Test solutions, agents, and drugs were delivered through the arterial cannulas by a constant rate infusion pump[13].

4. Naturally perfused double-segments with luminal placement of test agents (Figure 4) - This preparation was similar to the one previously described. However, after washing the intestinal lumen with saline, rubber tubes[15] (ID = 2.5 mm; OD = 5.3 mm) were inserted directly into the lumen for introducing and withdrawing the test agents or saline. The tubes were connected to pressure

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[15]Davol Inc., Levin Type No. 16, Providence, R.I.

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transducers[4] for direct monitoring of intraluminal pressure (ILP). In those preparations designed to employ intra-arterial (i.a.) infusion, i.a. cannulas (PE 50) were also inserted. As previously described, blood flow was measured by timed collections which were then weighed[11].

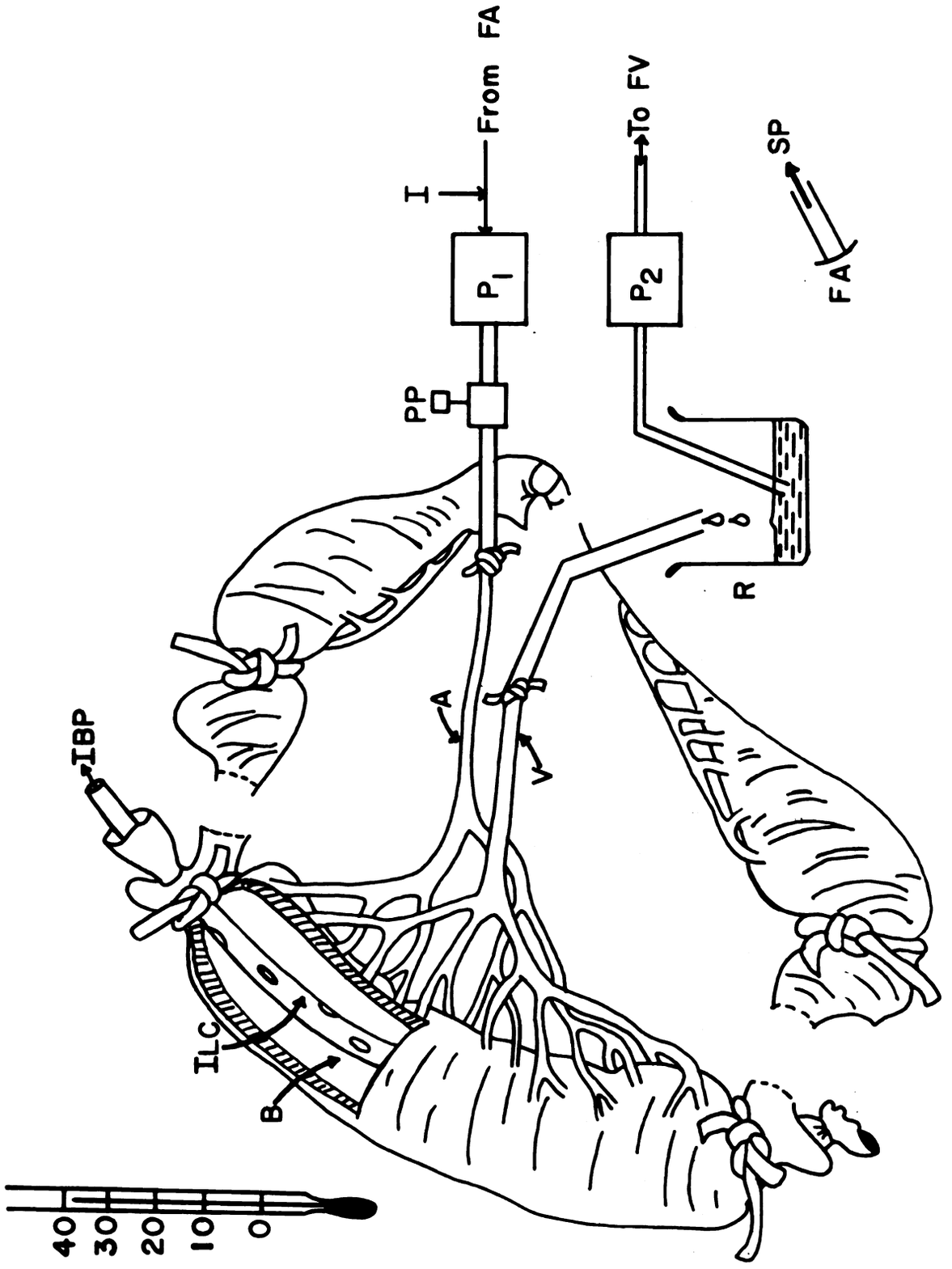
5. Pump-perfused single segment with intra-arterial infusion of test agents (Figure 5) - A loop of jejunum (20 cm, aboral to the ligament of Treitz), exteriorized through an abdominal incision, was placed on a supporting platform constructed with saline dampened towels and gauze. Fifteen minutes prior to heparinization the carotid arteries, cervical vagi, single artery supplying and vein draining the segments were isolated such that the paravascular nerves remained intact. A blunt hypodermic needle[12] (15 gauge), serving as an arterial cannula, was inserted into the artery and secured with ligatures (00). The segment was perfused with a constant volume pump[10] interposed between the arterial cannula (15 gauge)[12] and a femoral arterial cannula (PE 280). Perfusion pressure was monitored from a small hypodermic needle[12] (21 gauge) inserted into the rubber tubing just upstream to the cannula.

A polyethylene cannula (PE 240) inserted into the vein draining the segment directed the venous blood into a collecting reservoir containing 200 ml dextran[9]. The blood was returned to the animal through a femoral venous cannula (PE 320).

A rubber tube[15] to which a condom had been tied was inserted into the intestinal lumen and secured by tying both ends of the segment. Fluid (10 ml, H_2O) was added to the balloon and intraballoon pressure (IBP) served as an indicator by cauterization to further prevent

Figure 5

Schematic drawing showing the exposed single-segment intestinal preparation. Blood from the femoral artery (FA) was pumped (P_1) at a constant rate into the arterial supply (A) of the isolated segment. Perfusion pressure (PP) was monitored from a site downstream and test agents injected (I) upstream to the pump (P_1). A cannula placed into the vein (V) directed the outflow into a reservoir (R) from which a pump (P_2) returned it to the femoral vein (FV). Systemic pressure (SP) was monitored from the femoral artery (FA) Motility was monitored as intra-balloon pressure (IBP) by a balloon (B) with an intraluminal cannula (ILC). The preparation was maintained at 37°C.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collateral circulation. Test agents were infused[13] or injected upstream to the pump perfusing the segment.

6. Vascular Strips in vitro - Isolated femoral arterial smooth muscle strips were studied under different preload tensions.

A) - In vitro baths (Figure 6) -

Four glass baths[16] (200 ml capacity) were employed in the study. The baths were constructed with a sealed outer and open inner chamber. A pump[17] continuously circulated water through the outer chambers of the four baths which were connected in series by rubber tubing (ID = 1/8 in.; OD = 1/4 in.)[18]. A large temperature controlled reservoir[19] was interposed into the circulation to maintain the baths at near 37°C. A mixture of 95% O_2 and 5% CO_2 was continuously bubbled through the porous filters in the bases of the baths. The inner chambers of the baths could be drained and rinsed through the appropriate outlets.

Four displacement transducers[20] were held by adjustable isometrics tension clamps[21] which allowed the preload tensions to be continuously

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[16]Hand-made, Department of Chemistry, Michigan State University, East Lansing, Michigan.

[17]Universal Electric Co., model AA2M 108N, Owosso, Michigan

[18]Tygon^R, Norton, Plastics and Synthetics, Akron, Ohio

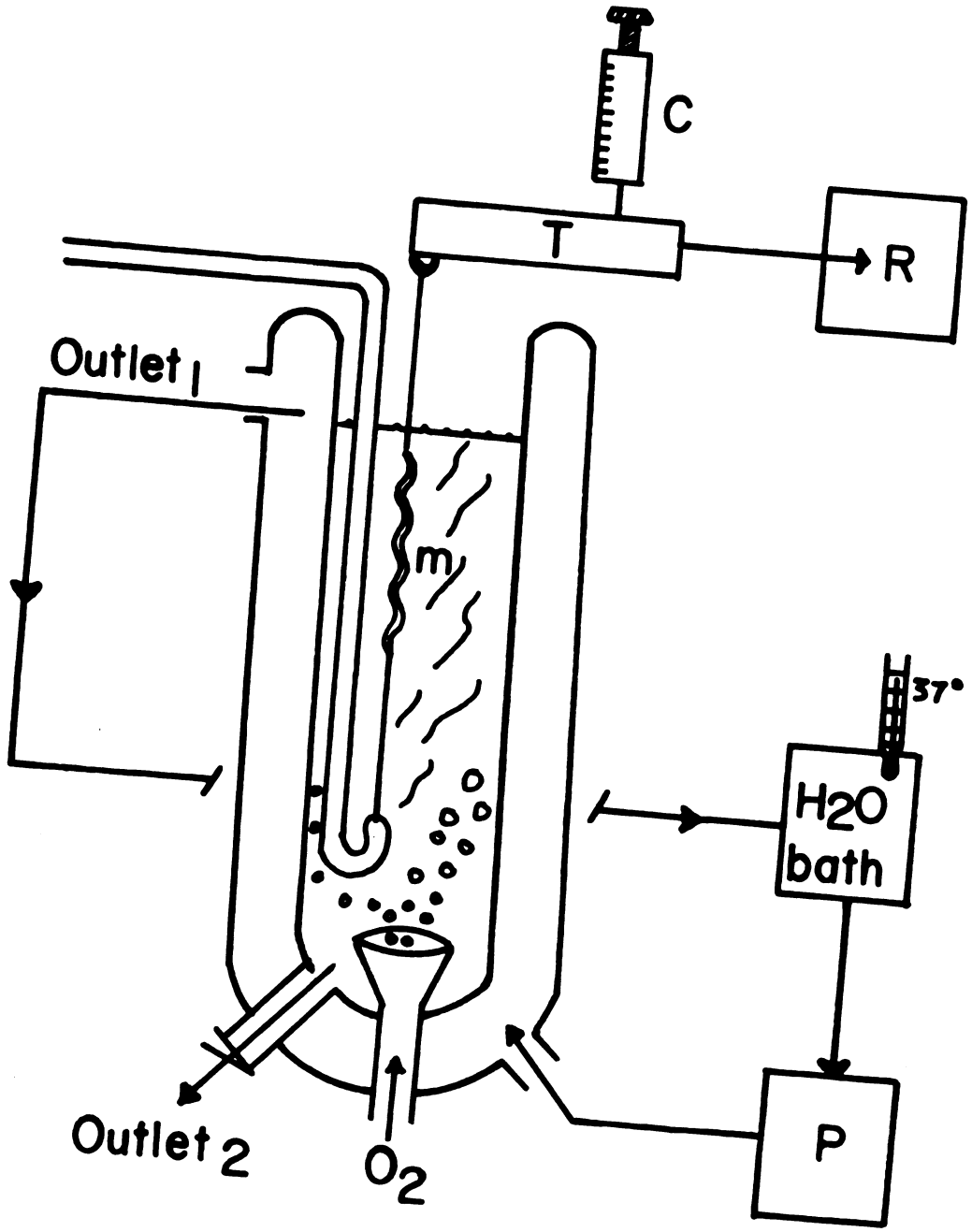
[19]P.M. Tamson, Model T29/100, Holland

[20]Grass Instruments, model FT-03, Quincey, Mass.

[21]Harvard Apparatus Co., Isometric Tension Clamp 214, Millis, Mass.

Figure 6

Schematic drawing of the isolated strip preparation. Muscle strips (M) were anchored to a glass rod and suspended in a bath containing Krebs-Ringer solution. Contractile tension was re-recorded (R) from a force transducer (T). The muscle was subjected to various levles of preload tension by an adjustable clamp (C) used to hold the tranducer. The preparation was maintained at 37°C by pumping (P) water from a constant temperature reservoir (H_2O bath) through the outer chamber of the in vitro bath. Test agents placed directly into the inner chamber were drained and rinsed through outlet 2. The medium was oxygenated by bubbling O_2 through the porous filter.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controlled. The transducers served as inputs to a curvilinear oscillograph (Dynograph,Type R)[22].

B) - Preparation of the strips -

Both femoral arteries or a section of jejunum were rapidly excised from anesthetized mongrel dogs (5-10 Kg), and placed in room temperature Krebs-Ringer (pH = 7.35 - 7.42, 300 ± 5 mOsm/Kg) solutions. The arterial strips were cut by hand in a helical fashion similar to that described by Furchgott (36). During the cutting phase an attempt was made to keep the amount of stretching at a minimum. Each helically cut artery was divided into four strips ($\bar{X}$ = 18.2 x 3.9 mm, 118.45 mg). One set of strips was placed in a stopper-sealed flask containing 200 ml of Krebs-Ringer and placed in cold storage (<10°C) for 24-48 hrs. The fresh strips were attached to glass rods (dia = 3 mm) by small hooks. Light suture (6-0) was inserted through the free ends with a needle and secured for connection to the transducers. The glass rods and muscle strips were suspended in the inner chambers of the baths and connected to the previously calibrated transducers[20]. The four clamps[21] were then adjusted to give the desired tension as indicated by the oscillograph.

The strips were allowed to equilibrate for 2 hrs with frequent adjustment of the tension to the desired preload. All test agents were administered directly into the bath.

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[22]Spinco Division, Beckman Instruments, Lincolnwood, Illinois.

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C) Formulation of the solutions -

Isosmotic, hyperosmotic and hyposmotic solutions were used in this study.

Isosmotic Krebs-Ringer solution - Formulation of the Krebs-Ringer solution was similar to that reported by Altura and Altura (1). The solution which contained NaCl (118 mM), KCl (3.5 mM), $CaCl_2$ (2.5 mM), KH_2PO_4 (1.2 mM), $MgSO_4 \cdot 7\ H_2O$ (1.2 mM), $NaHCO_3$ (25.0 mM) and glucose (10.0 mM) was made up as a stock solution (10 liter) without the $NaHCO_3$. Thus, each stock solution (10 l) contained NaCl (69.55 g), KCl (2.61 g), CaCl (2.77 g), KH_2PO_4 (1.63 g), $MgSO_4 \cdot 7\ H_2O$ (2.96 g) and glucose (18.00 g). In order to prevent calcium precipitation, the $NaHCO_3$ was added daily to 2 liter aliquots. Osmolality (300 ± 5) and pH (7.2-7.4) of the solutions were checked daily.

Hyperosmotic solutions - Osmolality of the solution was increased either by increasing all ion concentrations 3 times that of the stock solution or by adding d-mannitol such that the osmolality was increased in both cases to 900-1000 mOsm/Kg.

Hyposmotic solutions - Two hyposmotic solutions were used. In one case bath osmolality and ion concentrations were decreased by adding distilled water (0 mOsm/Kg). The second means of decreasing bath osmolality was by adding a hyposmotic solution (30 ± 10 mOsm/Kg) from which NaCl was deleted, thereby reducing only NaCl concentration.

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Experiemental Protocols

1. The effects of tetrodotoxin (TTX) on the vasculature of cross-perfused innervated and denervated gracilis muscle -

In this study the constant flow (Figure 3) cross-perfused gracili muscles (Figure 2) allowed the separation of a neural influence from non-neural influences. The primary questions studied were: 1) Does TTX have a vascular action independent of neural mechanisms?; and 2) What effect does initial resistance have on the vasodilator action of TTX?

In this preparation, changes in perfusion pressures directly reflect changes in vascular resistance. The effects of TTX (0.05-1.0 μg/min) on vascular resistance and the carotid reflex response were studied. The study focused on the responses to TTX when vascular resistance was varied by either neurogenic or non-neurogenic mechanisms. The effect of TTX given i.a. on vascular resistance in the gracilis was compared to the response when resistance was lowered reflexly (in the innervated gracilis) by systemic intra-arterial infusion of norepinephrine into the neurally intact animal (recipient). The effect of TTX was then tested when resistance was increased reflexly in the innervated gracilis by hemorrhaging (arterial pressure = 40-60 mmHg) the recipient animal, and by local i.a. infusion of norepinephrine (1.0 μg/min) into the denervated gracilis.

Reactive-dilation and venous-arteriolar responses were also studied before and during TTX infusion. Reactive dilation was produced by stopping the perfusion pump (1-3 min), and the degree of

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dilation determined by comparing the perfusion pressures before and after the period of ischemia. The venous-arteriolar response was elicited, when venous pressure was increased, by raising the venous outflow cannulas 10 cm. Perfusion pressure and venous pressure (monitored from a small side-branch vein or the proximal end of the cannula) were used to determine the pressure gradient. TTX was also studied before and during alpha-adrenergic (phentolamine), beta-adrenergic (propranolol), or cholinergic (atropine) blockade.

2. Effects of tetrodotoxin (TTX) on the responses of intestinal and vascular smooth muscle to KCl - The naturally-perfused intestine (Figure 4) and skeletal muscle (Figure 1) preparations were utilized in this series of studies, to study the following questions: 1) What effect does TTX have on vascular smooth muscle and is there any non-neurogenic action? 2) Do low doses of TTX have any different direct vascular actions than high doses? 3) What effect does TTX have on vascular and visceral muscle of the intestine and are local intrinsic nerves involved in intestinal blood flow regulation?; and 4) What effect does TTX have on the vascular action of K^+ in skeletal and intestinal muscle beds and are local nerves involved in the vascular responses to K^+?

TTX in saline (0.5 or 5.0 μg/ml, 1.6×10^{-9} - 10^{-8}M), isosmotic KCl (110-115 mEq/l) and TTX (0.5 or 5.0 μg/ml) in KCl were infused intraarterially. Dose-response curves were obtained by progressively increasing the rate of infusion (0.1, 0.2, 0.5, 1.0, 2.0 ml/min). Venous outflows were determined by taking one minute collections with one minute intervals beginning 10 sec. after changing the infusion

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rate. All vascular responses were compared to the effect of an isosmotic saline control.

During the infusion of TTX the presense of neural blockage was indicated by recording the motility response to vagal stimulation (electrical, 20 v, 6 msec, and 10 cps) in the intestinal preparations, and vascular responses to carotid occlusion in the gracilis preparations. In some experiments venous blood containing TTX was allowed to return to the animal; however, in most of the experiments venous outflow was discarded during the TTX infusion period and replaced with dextran (6% in saline).

In experiments using the intestine one segment served as a control receiving only solutions (saline, KCl) not containing TTX. However, in the study of skeletal muscle vasculature the effects on innervated and denervated preparations were compared by infusing simultaneously the same solutions (controls or test) into both muscles.

3. The action of tetrodotoxin (TTX) and hexamethonium (C_6) on the responses of vascular and visceral muscle to stimulation by potassium, sodium and drugs action on ganglia - The jujunum was perfused at constant blood flow (Figure 5) to study the role of local nerves in intestinal vascular and motility responses with the study primarily directed at obtaining information helpful in answering the following questions: 1) Does TTX affect the intrinsic nerves?; 2) What effect does TTX and ganglionic blockade have on the intestinal vascular and visceral responses to local stimuli?; and 3) Does K^+ and/or Na^+ produce their vascular or visceral smooth muscle responses via the intrinsic nerves?

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Vascular resistance and intestinal motility responses were studied during intra-arterial infusion of isosmotic NaCl (0.1-2.0 ml/min) (300 mOsm/Kg), hyperosmotic NaCl (1500 mOsm/Kg), isosmotic KCl (300 mOsm/kg) and ganglionic stimulation with nicotine (50 µg, bolus).

In order to determine the influence of local nerves on the responses to the previously mentioned agents, the agents were tested before and after ganglionic blockade by hexamethonium (C_6, 0.5 mg/min) and non-selective neural blockade by TTX (1.0 µg/min). As an index of the responsiveness to agents which directly affect smooth muscle, acetylcholine (3 µg, bolus) and epinephrine (3 µg, bolus) were injected before and after infusion of the blocking drugs. Following a steady control period, the test solutions (NaCl and KCl) were infused (i.a., upstream to the pump) for 1 minute test periods, progressively increasing the rate (0.1, 0.2, 0.5, 1.0 and 2.0 ml/min). When the perfusion pressure and motility returned to near control levels a different agent was infused. Bolus injections of acetylcholine, epinephrine, nicotine, and saline never exceeded 0.5 ml. All test agents were infused before and during the infusion of C_6 or TTX.

In this series of experiments the reactive-dilation and venous-arteriolar responses were tested before and after ganglionic or non-selective neural blockade. As previously described, reactive-dilation was induced by stopping flow (pump off) and the venous-arteriolar response by raising the venous cannulas 10 cm.

The degree of neural blockade was evaluated by its effectiveness in blocking the vagally induced motility responses (electrical

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stimulation) and vascular resistance responses to carotid occlusions.

Venous blood containing C_6 or TTX was usually discarded and replaced with dextran; however, in some experiments the blood was returned to the animal.

4. Effects of TTX on the responses of vascular and visceral muscle to luminal placement of hyperosmotic solutions in the jejunum - Using the naturally perfused double-segment preparation (Figure 4) four series of experiments were performed to study the local intestinal vascular and motility effects of hyperosmotic glucose, KCl, and NaCl. The study focused on the following questions: 1) What effect does hyperosmotic solutions of glucose, K^+, or Na^+ have on the vascular and visceral muscle of the intestine?; and 2) Are local neural elements located in the mucosa involved in the regulation of intestinal blood flow, motility, and absorption?

1st Series - Venous outflows and motility from the two segments were compared while the segments contained either glucose solutions of different osmolality or glucose and polyethylene glycol (PEG; mol. wt. 4000) solutions of the same osmolality. The solutions to be tested were paired into the following four combinations: 1) hyposmotic glucose (2.5% = 150 mOsm/Kg) and isosmotic glucose (5.4% = 300 mOsm/Kg); 2) 20% and 50% glucose, 3) 20% glucose and 34% PED, and 4) 50% glucose and 85% PEG. The osmolality of 20% glucose and 34% PEG was about 1200 mOsm/Kg and 50% glucose and 85% PEG about 3000 mOsm/Kg. Ten milliliters of one of the paired solutions (e.g. 2.5%

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glucose) was introduced into the lumen of one segment and 10 ml of the other solution (e.g., 5.4% glucose) introduced into the lumen of the remaining segment. Since both segments were at any given time subjected to the same systemic influences (e.g., blood pressure, blood constituents and systemic nerve activity), differences in the effects of the test solutions could be reasonably attributed to differences in their local actions. To gain information relative to the mechanisms causing changes in blood flow, venous samples were collected and the osmolality and glucose or ion (Na^+ and K^+) concentration measured.

In this series of experiments, 10 ml of the control or test solutions were placed in the lumen of the segments for 11 minutes. During this period, venous outflow was collected in 3 minute samples with 1 minute intervals between collections. The blood was weighed on a direct-reading balance and expressed as grams per minute, and then poured into the reservoir. After the 11 min test period, the solution in the lumen was withdrawn and its volume measured with a graduated cylinder. The lumen was washed with normal saline before introducing the next solution. An isosmotic solution of PEG (8.5%) served as control and was introduced into the lumen before and after any test solution.

2nd Series - In this series of experiments, the possibility that local hyperglycemia or local plasma hyperosmolarity might contribute to increased flow was studied by infusing various glucose solutions into the segment via side-branch arterial cannulas. The solutions (2.5%, 5.4% and 16.2% glucose) were infused in random sequence at

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increasing rates (0.1, 0.2, 0.5, 1.0 and 2.0 ml/min). Each infusion rate was maintained for 3 min and venous outflow was measured during the last 2 min. As a control, normal saline was infused intra-arterially into the segment before and after each test soluation. As previously mentioned the osmolality and glucose concentration of the venous outflows were measured.

3rd Series - In this and the following series the possible participation of local nerves in the responses (vascular and visceral) to hyperosmotic solutions were examined by comparing the responses before and after subjecting the segment to a neural blocking agent.

Hyperosmotic glucose (50%) was placed in the lumen before and after exposing the lumen to a local anesthetic (dibucaine, 0.4% in saline). Dibucaine was placed in the lumen for 20 minutes. The glucose was then placed in the lumen immediately following withdrawal of the dibucaine solution, omitting the usual saline rinse.

4th Series - This series was performed in a manner similar to series 3, however, in this series TTX rather than dibucaine was used to attain neural blockade. Luminal placement of hyperosmotic KCl (1500 mOsm/Kg) and NaCl (1500 mOsm/Kg) were tested before intra-arterial infusion of TTX. TTX was then infused at a dose sufficient to block motility responses to vagal stimulation. After neural blockade with TTX, hyperosmotic KCl, NaCl, and glucose (50%) were tested. All responses were compared to those when isosmotic PEG was in the lumen.

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5. The effects of TTX, K^+, osmolality, and pharmacological agents on arterial and intestinal muscle, in vitro - In this series of experiments the contractile responses of vascular and visceral muscle strips were studies in vitro (Figure 6). The following questions were studied: 1) Does pre-load tension alter the in vitro responses of vascular and visceral smooth muscle?; 2) What effect does TTX have on vascular and visceral smooth muscle?; and 3) What effect does TTX have on the responses of vascular and visceral smooth muscle to stimuli which act directly on the smooth muscle as well as stimuli acting via neural elements?

In order to simulate in vivo conditions of varying degrees of vascular resistance, the reactivity (contractile response) of arterial (femoral) muscle was studied under different levels of pre-load tension (1, 2, 4 and 6 gm). The strips were equilibrated under tension in isosmotic (300 ± 5 mOsm/Kg) buffered Krebs-Ringer (pH = 7.35 - 7.42) solution. Following the equilibration period the test agents were added directly into the baths were drained and then rinsed before adding fresh Krebs-Ringer solution (100 ml). The strips were again allowed to equilibrate for 15-45 minutes before any subsequent procedures.

Cold storage of smooth muscle strips (21, 84) has been shown to be effective in rendering neural tissue non-functional, and reserpinization (42) is effective in depleting catecholamines from nerve terminals. In this study we used fresh, cold stored, and reserpinized strips of vascular smooth muscle. The degree of the neural inhibition was tested by using the ganglionic stimulants nicotine and DMPP and the catecholamine releasing agent tyramine.

Potassium ion was studied by adding (1, 3, 5 and 10 ml) of an isosmotic solution of KCl (150 mEq/l) to the bath medium containing a K^+ concentration of 4.7 mEq/l. K^+ concentration was altered such that the bath K^+ concentration progressively increased 1.5, 4.5, 7.5, and 15.0 mEq/l.

Osmolality was increased from control (300 ± 5 mOsm/Kg) to near 400 mOsm/Kg, by adding either a hyperosmotic (800 - 1000 mOsm/Kg) Krebs-Ringer solution in which all ions were increased, or one in which osmolality was increased by d-mannitol[40]. Bath osmolality was decreased (from 300-200 mOsm/Kg) by adding either distilled water, decreasing all ion concentrations, or by adding a Krebs-Ringer solution in which only the sodium concentrations was decreased.

Although the primary objective of this study was to investigate the action of tetrodotoxin[23], a number of test agents were included. This preparation allowed one to add and maintain the concentration of one or more agents in the bath for any desired length of time. Those agents employed in this study were acetylcholine[24],

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[23]Crystalline 3X, Sankyo Company, Ltd., Tokyo, Japan

[24]Acetylcholine chloride: Calbiochem, Los Angeles, Calif.

norepinephrine[25], epinephrine [26], potassium[27], tetrodotoxin[23], reserpine[28], atropine[29], propranolol[30], phentolamine[31], nicotine[32], dimethphenyl piperazinim (DMPP)[33], barium [34], methysergide[35], isoproterenol[36], tyramine[37], ouabain[38], hexamethonium[39], and d-mannitol[40].

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[25]Levarterenol bitartrate, Winthrop Labs, New York, N.Y.

[26]Epinephrine hydrochloride, Wolins Pharm. Corp., Farmingdale, N.Y.

[27]Potassium iodide: Mallinckrodt Chem. Works, St. Louis, Mo.

[28]Aldrich Chem. Co., Inc., Milwaukee, Wisc.

[29]Atropine, puriss: Aldrich Chem. Co., Inc., Milwaukee, Wisc.

[30]D-Propranolol: Ayerst Labs. Inc., New York, N.Y.

[31]Regitine dry powder: CIBA Pharm. Co., Summit, N.J.

[32]Nicotine Salicylate: R.S.A. Corp., Ardsley, New York.

[33]1, 1-Dimethyl-4-phenylpiperazinium iodide: Aldrich Chem. Co. Inc., Milwaukee, Wisc.

[34]Barium chloride: J.T. Baker Chem. Co., Phillipsburg, N.J.

[35]Methysergide: Sandoz Pharm., Hanover, N.J.

[36]D-isoproterenol bitartrate: Sterling Winthrop Research Inst., Sterling Drug Inc., Rensselaer, N.Y.

[37]Tyramine hydrochloride, B grade: Calbiochem, Los Angeles, Calif.

[38]Ouabain: Nutritional Biochem Corp., Cleveland, Ohio.

[39]Hexamethonium chloride dihydrate (99%): Mann Research Labs., Becton: Dickinson & Co.

[40]Sigma Chem. Co., St. Louis, Mo.

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Analysis of Venous Samples and Statistical Evaluation of Data

All determinations of pH were made with an expanded scale microelectrode pH meter[41]. Potassium and sodium ion concentrations were determined by flame photometry[42] and osmolality by freezing point depression method[43]. Plasma glucose levels were determined colorimetrically by a hexokinase assay system[44].

The statistical analysis of the data utilized tests described by Sokal and Rohlf (117). Student's *t* test for paired and non-paired replicates, correlation coefficients, and regression analysis were employed in evaluating the data.

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[41]Radiometer Inc., model 22, Copenhagen, Denmark.

[42]Beckman Inc., model 105, Fullerton, Calif.

[43]Advanced Instruments, model 31L, Watertown, Mass.

[44]General Diagnostics, Gluco Strate, Morris Plains, N.J.

RESULTS

Vascular Action Of TTX In Naturally Perfused Gracili Muscles

1) Effect of TTX on venous outflow of innervated and denervated gracili - Local intra-arterial (i.a.) administration of tetrodotoxin (TTX) produced a profound increase in blood flow of innervated gracilis muscle but had little effect on blood flow through the acutely denervated muscle (Figures 7, 8, and 9).

Figure 7 shows the change in venous outflow from naturally perfused gracili muscles (prep #1, Figure 1) before and after denervating the muscle by cutting the nerve trunk. Venous outflow immediately increased following denervation, gradually decreasing to stabilize at a blood flow significantly higher ($\bar{x}$ = 14.79 ml/min/100 g) than control flow ($\bar{x}$ = 13.87 ml/min/100 g). Local i.a. administration of TTX (5 µg/ml, 0.1-2.0 ml/min, 0.5 µg/min) increased venous outflow significantly more (225%) in the innervated muscles as compared to the denervated (125%) gracilis. The increase in venous outflow to TTX in the denervated muscle was not different from that to normal saline in either the innervated or denervated gracilis.

To study the possibility that the doses previously mentioned were too high to show any direct effect, another series of experiments were performed using a TTX dose range (0.05-1.0 µg/min) similar to that reported by Kao (74, 77). Figure 8 shows that the lower doses of TTX as compared to the saline control, significantly increased venous outflow from innervated gracili. Although following

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denervation TTX appears to increase flow over the lower end (0.1 and 0.2 ml/min; 0.05-0.1 µg/min) of the dose response curve; this increase failed to differ significantly from the controls. Venous outflow from denervated muscles were the same during saline or TTX infusion (0.1-1.0 µg/min).

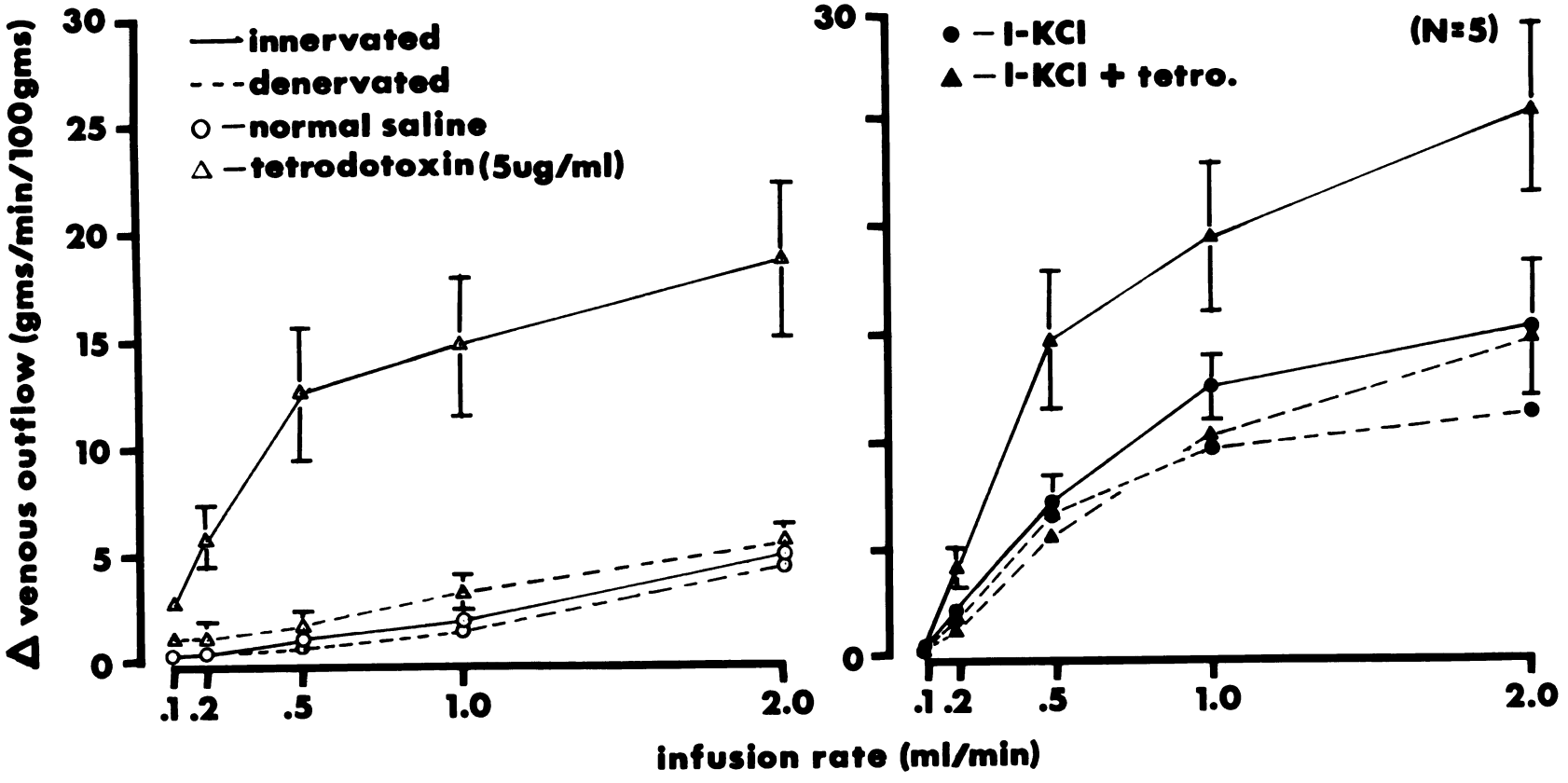
When TTX of high (5.0 µg/ml) and low (0.5 µg/ml) concentrations were administered in the same preparations, one finds that blood flow responses differ in the innervated muscles but were the same in the denervated muscles (Figure 9). Figure 9 shows the venous outflow, minus the saline dilutional effects, during i.a. infusion of TTX. Notice that in the denervated gracili TTX at 1.0 or 10.0 µg/min failed to change blood flow as compared to the pre-infusion value. Although, there is some indication of an increase in blood flow over the lower end of the dose response curve, this non-significant change was the same at a concentration of TTX of either 0.5 or 5.0 g/ml. TTX of 5.0 µg/ml concentration increased venous outflow from innervated muscles to a greater extent than did 0.5 µg/ml. However, the curves were not significantly different at 1.0 ml/min (0.5 and 5 µg/ml) and appeared to reach a maximum at 2.0 ml/min.

2) <u>Effects of TTX on the vascular responses to KCl in innervated and denervated gracili</u> - Local infusion of isosmotic KCl into the naturally perfused gracilis muscle (prep #1, Figure 1) produced an increase in venous outflow from both innervated and denervated muscles (Figure 7). This change in venous outflow progressively increased as the influsion rate was increased

Figure 7

Effects of tetrodotoxin adn potassium chloride (KCl) on blood flow of the innervated (solid lines) and denervated (broken lines) naturally perfused gracilis muscle (prep #1, Figure 1). Change in venous outflow is shown as indicated on the ordinates in gms/min/100 gms of tissue. Each test solution (normal saline, O; isosmotic KCl; tetrodotoxin, 5 µg/ml; Δ; and isosmotic KCl + tetrodotoxin, ▲) was simultaneously infused into the innervated (mean blood flow = 13.87 ml/min/100 gms) and denervated (mean blood flow = 14.79 ml/min/100 gms) gracili muscles at progressively increasing rates as indicated on the abscissa in ml/min.

In the graph on the left, innervated were compared to denervated muscles while on the right the test solutions were compared against each other (i.e. KCl vs. KCl + TTX). Mean values (N = 5) are given with standard errors denoting those points significantly different from each other ($p < 0.05$) as indicated by student-t analysis for paired comparisons.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reaching a maximum at 1.0 or 2.0 ml/min. A decrease (data not shown in Figure 7) in venous outflow in response to KCl occurred in about 25% of the animals but only at an infusion rate of 2.0 ml/min.

When TTX (5 μg/ml) was added to the KCl solution the increase in blood flow was significantly ($p < .05$) augmented in the innervated muscle. The addition of TTX failed to alter the response of denervated gracili to KCl. The dose response curves for KCl or KCl + TTX in denervated muscles were not significantly different from that for KCl infused into the innervated muscles. A maximum change in flow of about 200% occurred in the denervated muscles in response to KCl and KCl + TTX, or in the innervated gracili to KCl alone. In the innervated muscle, simultaneously receiving KCl and TTX, a 300% increase in blood flow occurred at an infusion rate of 2.0 ml/min.

3) Effect of TTX on the vascular responses of gracili to neural stimulation - The efficacy of TTX in locally blocking neural mechanisms was studied in the naturally perfused gracilis (prep #1, Figure 1) by evaluating the effect of carotid occlusion on local blood flow and the effect of electrical stimulation of the nerve trunk on motor activity.

Occlusion of both carotid arteries increased systemic pressure an average of about 25 mmHg, thus increasing venous outflow before and after denervation. When TTX (5.0 μg/ml) was infused locally at 0.2 ml/min (1.0 μg/min), the motor responses to electrical stimulation were gradually depressed and completely blocked after 15 to 20 min. However, the amount of change in

blood flow during carotid occlusion was greater 10 minutes after influsing TTX. Infusing TTX solution at 1.0 μg/min changed the calculated vascular resistance during carotid occlusion from vasoconstriction to vasodilation. In the series of experiments using a TTX concentration of 0.5 μg/m; near complete inhibition of the responses to carotid occlusion or stimulation of the motore nerve occurred in 15 to 25 min following infusion of TTX at 0.25 μg/min. This indicates that time of action in addition to the rate of administration of TTX is important in local paralysis of neural elements with TTX.

When TTX was allowed to return to the systemic circulation a gradual decrease in systemic arterial pressure occurred after 25-40 min and accompanied a decrease in the cardiac response (bradycardia) to electrical stimulation of the decentralized vagi.

Vascular Action Of TTX On Cross-Perfused Gracili Muscles

1) Effect of TTX on vascular resistance and the vascular responses of innervated and denervated gracili to carotid occlusion - Local intra-arterial administration of TTX produced a decrease in the calculated vascular resistance of cross-perfused (prep #2, Figures 2 and 3) innervated gracili muscles but failed to alter the vascular resistance of denervated gracili (Figure 10). This vasodilator action, which occurred only in the innervated muscles, was accompanied by a local neural blocking action as shown by inhibition of the increase in resistance during carotid occlusion (Figure 11).

Figure 8

Effects of low dose tetrodotoxin (TTX) on blood flow of the innervated (solid lines) and denervated (broken lines) naturally perfused gracili muscles (prep #1, Figure 1). Venous outflows are shown as indicated on the ordinate in gm/min/100 gm tissue. NaCl (300 mOsm/l) and TTX (0.5 μg/ml, ●) were infused locally at progressively increasing rates as shown on the abscissa in ml/min.

Mean value (N = 8) for each point are shown with their standard errors.

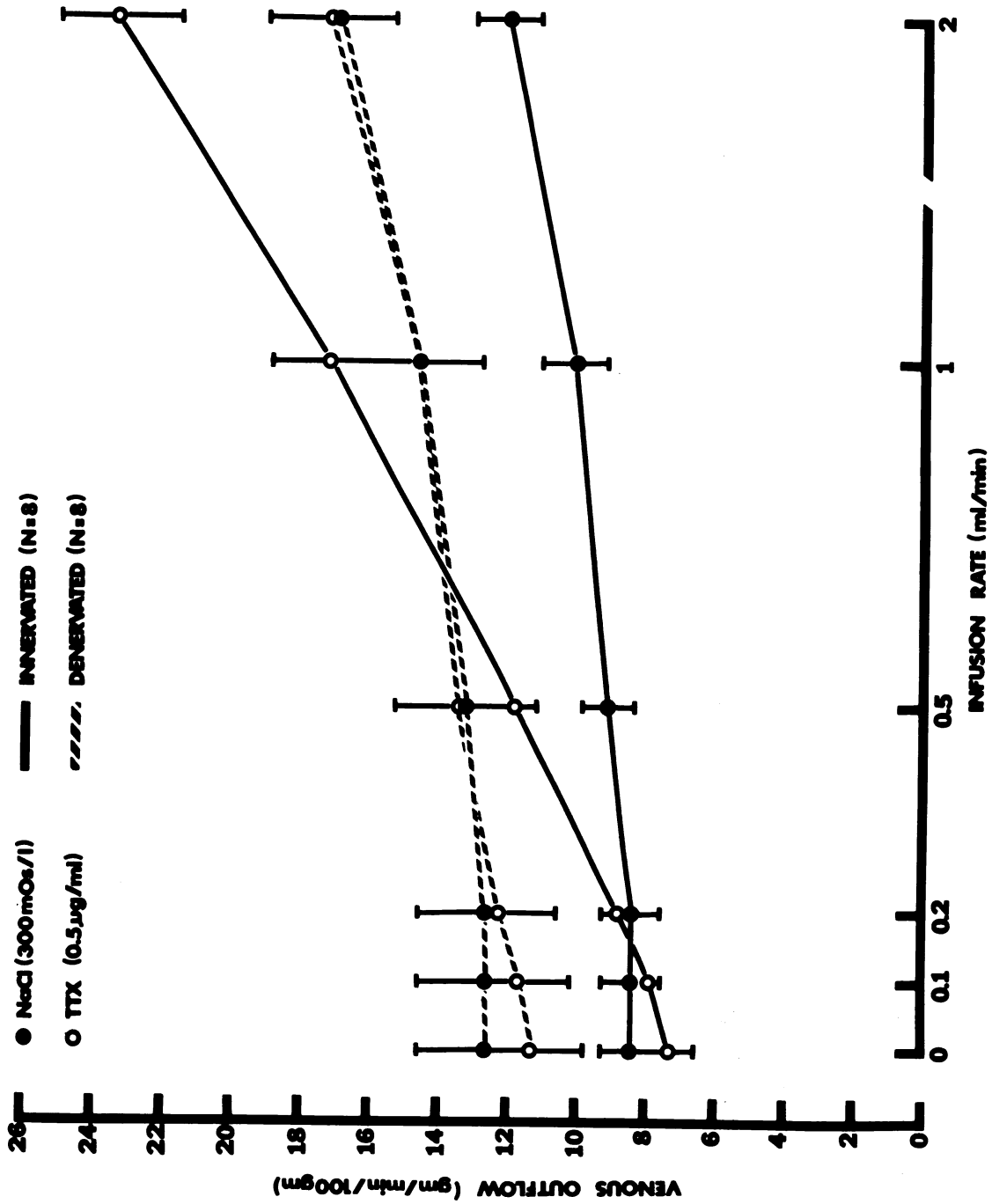
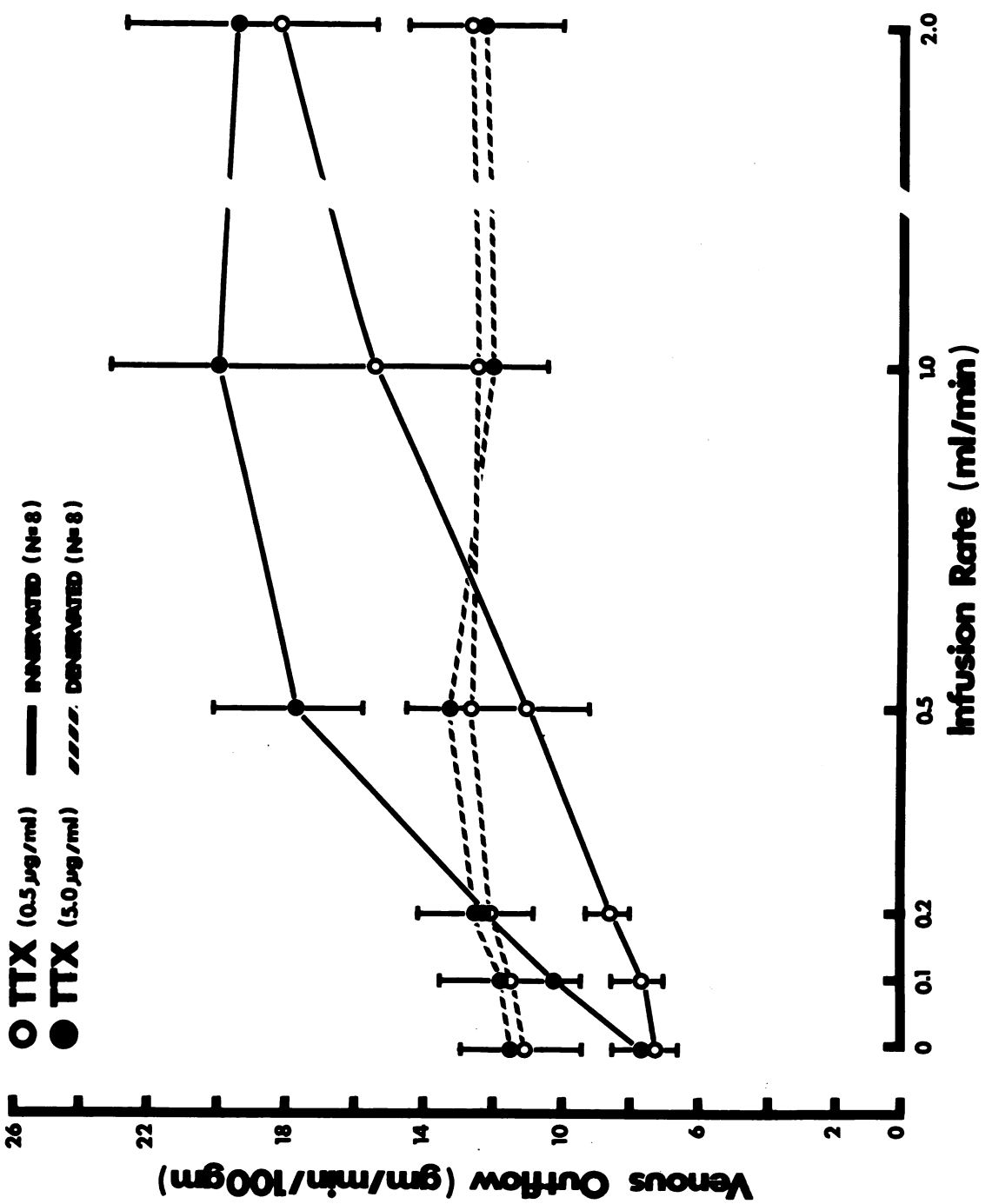

● NaCl (300mOs/l)
○ TTX (0.5μg/ml)
INNERVATED (N=8)
DENERVATED (N=8)
VENOUS OUTFLOW (gm/min/100gm)
INFUSION RATE (ml/min)
0
0.1
0.2
0.5
1
2
0
2
4
6
8
10
12
14
16
18
20
22
24
26

Figure 9

Effect of high (0.5 - 10.0 µg/ml) and low (0.05 - 1.0 µg/min) doses of tetrodotoxin (TTX) on blood flow of naturally perfused innervated (solid lines) and denervated (broken lines) gracili muscles (prep #1, Figure 1). Venous outflows (gm/min/100 gm) minus the saline effects are shown as mean values (N = 8) ± standard errors. High and low dose responses curves were obtained by infusing TTX solutions of two different concentrations (5.0 and 0.5 µg/ml) at progressively increasing rates (ml/min) as shown on the abscissa. Controls are denoted by 0 ml/min.


TTX (0.5 µg/ml)
TTX (5.0 µg/ml)
INNERVATED (N=8)
DENERVATED (N=8)
Venous Outflow (gm/min/100gm)
Infusion Rate (ml/min)
0
2
6
10
14
18
22
26
0
0.1
0.2
0.5
1.0
2.0

Int
increas
pressur
However
denerv
fusion
shows
produ
(p <
that
was
valu
Figu
when
were
caro
(inne
muscl
recip
press
the r
pressu
while
failed
the ci
Cuttin

Intra-arterial infusion of a TTX solution (0.5 µg/ml) at increasing rates (0.1-2.0 ml/min) progressively decreased perfusion pressure of both innervated and denervated muscles (Figure 11). However, after subtracting the effect of the saline diluent the denervated muscles showed no change in resistance during TTX infusion at any dose from 0.05 to 1.0 µg/min (Figure 10). Figure 10 shows that progressively increasing the dose of TTX every 3 minutes produced a decrease in resistance that differed significantly ($p < 0.05$) from the pre-infusion value at 0.5 µg/min. Notice that the average control resistance of the innervated muscles was higher than that of the denervated muscles and the resistance values were not different following a TTX dose of 0.5 µg/min. Figure 11 shows that at an infusion rate of 2.0 ml/min (1.0 µg/min), when vascular resistance of the innervated and denervated muscles were the same (Figure 10) the response of perfusion pressure to carotid occlusion was totally abolished and the perfusion pressures (innervated = 55 mmHg; denervated = 65 mmHg) were similar in both muscles. In these cross-perfused muscles carotid occlusion of the recipient animal produced an increase (10-40 mmHg) in perfusion pressure of the neurally intact muscles. Bilateral occlusion of the recipient animal's common carotid arteries increased systemic pressure (10 to 60 mmHg) in all of the animals (N = 8) studied, while bilateral occlusion of the donor animal for 3 to 6 minutes failed to alter perfusion pressure of either muscle even though the circuit lag-time was always less than 60 seconds (Figure 11). Cutting the nerve trunk innervating one gracilis produced,

simultaneously; 1) a decrease in perfusion pressure of the denervated muscle, 2) an increase in perfusion pressure of the neurally intact muscle and 3) an increase in systemic pressure of the recipient animal.

Following denervation, perfusion pressure gradually returned to near control levels in 15-30 minutes and denervation was substantiated by carotid occlusion. TTX (0.5 μg/ml) infused at progressively increasing rates (every 3 minutes) yielded a dose range of 0.05-1.0 μg/min. TTX progressively decreased the change in local resistance to carotid occlusion (Figure 11) with a significant inhibition occurring at a dose of 0.1 μg/min. Although a decrease in vascular resistance occurred before the carotid reflex response was completely abolished, when it was abolished the effect of TTX was the same in both innervated and denervated muscles. The magnitude of the responses to carotid occlusion returned to control levels 30 to 45 minutes after cessation of TTX infusion.

Notice that during the infusion period TTX had no systemic hypotensive effect on either the recipient or donor animal even though the venous outflow containing TTX was returned to the donor animal. The total TTX dose returned to the donor animal was generally less than 10.0 μg with only 5.70 μg infused during the normal test period of 15 minutes. In about 50% of the animals the donors systemic pressure gradually decreased to less than 75% of the control values 45-60 minutes after the first test sequence. In all animals a significant hypotensive (< 75 mmHg) effect appeared

Figure 10

Effect of tetrodotoxin (TTX) on vascular resistance, minus the saline dilutional effect, of the constantly perfused innervated (solid lines) and denervated (broken lines) gracilis muscles. (prep #2, Figures 2 and 3) Resistance in mmHg/ml/min/100 gm is shown on the ordinate as a function of TTX infusion rate as shown on the abscissa in µg/min with the control values denoted by 0 µg/min.

Each datum point is the mean value ± S.E. for eight experiments. Statistical analysis by student's t test for paired comparisons indicated that only the innervated muscles differed significantly ($p < 0.05$) from their controls only at doses of 0.5 and 1.0 µg/min.

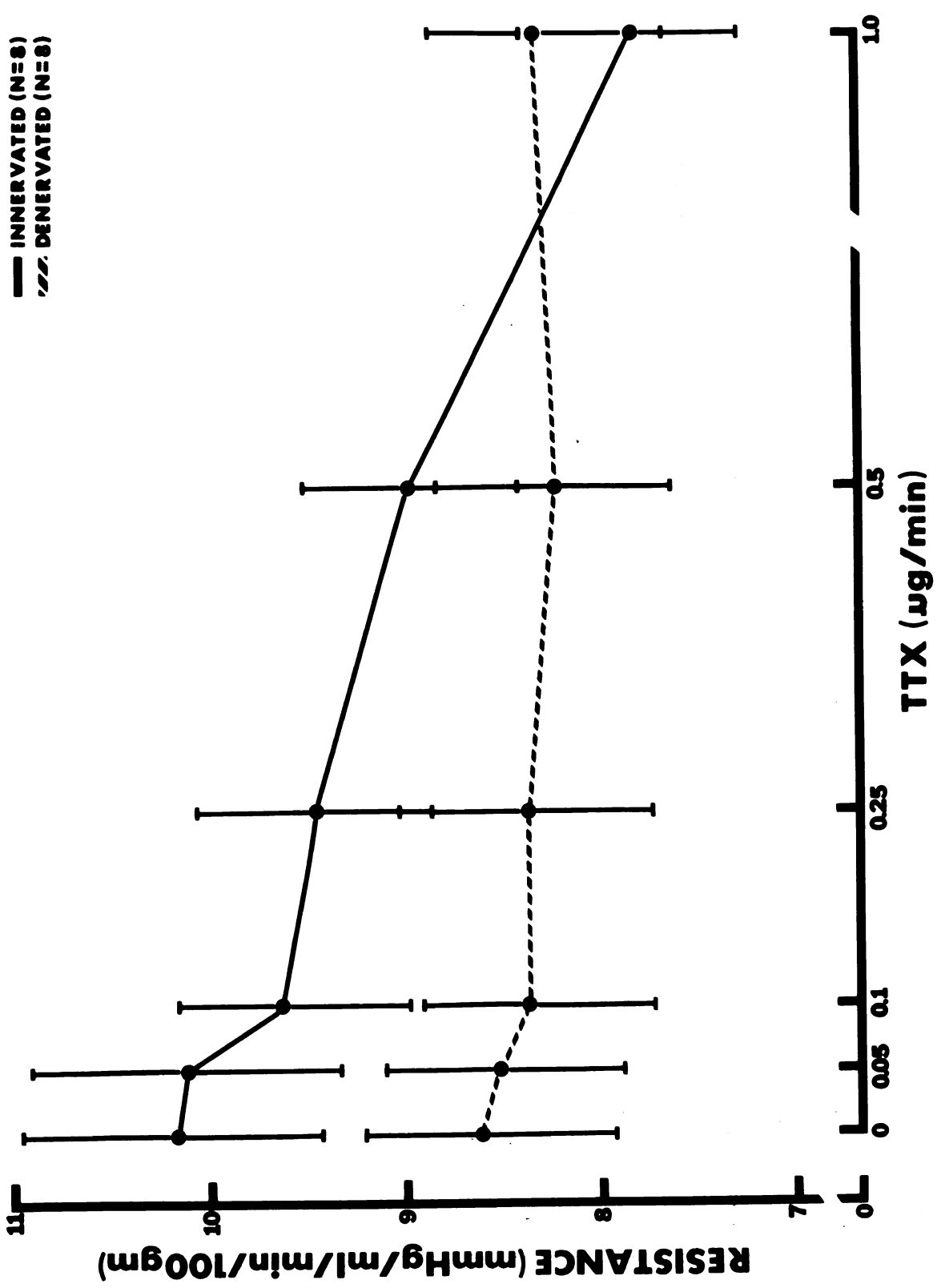
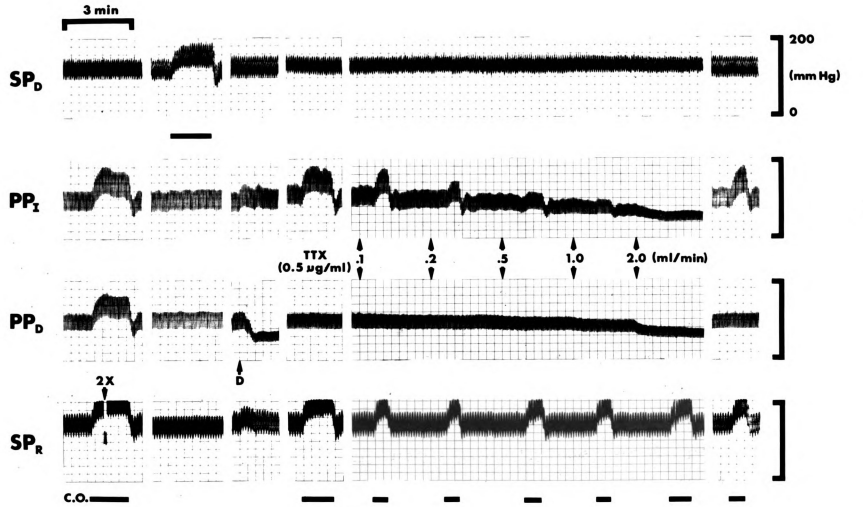

INNERVATED (N=8)
DENERVATED (N=8)
RESISTANCE (mmHg/ml/min/100gm)
11
10
9
8
7
0
0.05
0.1
0.25
0.5
1.0
TTX (µg/min)

Figure 11

Effect of tetrodotoxin (TTX, 0.5 μg/ml) on vascular resistance and the local response to carotid occlusion in cross-perfused gracili muscles (prep #2, Figures 2 and 3). Tracings from a single animal are denoted as SP_D (systemic pressure of donor animal), PP_I (perfusion pressure of innervated gracilis), PP_D (perfusion pressure of the gracilis denervated at point D), and SP_R (systemic pressure of the recipient animal). Carotid occlusion (C.O.) of the recipient or donor animal are indicated by the thick dark horizontal bars below the systemic pressure tracings. Following pre and post denervation control responses, TTX was infused locally at progressively increasing rates (ml/min). Calibration bars (200 mmHg) are shown on the right. Breaks in the tracings represent 15 - 45 min.


3 min
SP$_D$
200
(mm Hg)
0
PP$_I$
TTX
(0.5 µg/ml)
.1
.2
.5
1.0
2.0 (ml/min)
PP$_D$
D
2X
SP$_R$
C.O.

in the donor animals after the second or third TTX infusion sequence 60 to 90 minutes following the initial administration of TTX.

2) Effect of initial resistance on the vascular action of TTX - Increasing initial resistance by neural mechanisms potentiated the vasodilator action of TTX in the neurally intact gracilis, while in the denervated muscles increasing resistance by direct means failed to alter the effect of TTX (Figures 12, 13, 14).

In the cross-perfused gracili (prep #2, Figure 2) TTX (0.1 and 0.25 µg/min) infused at a volume (0.1 ml/min) insufficient to show a dilutional effect produced a decrease in perfusion pressure of only the innervated muscles. This vasodilator action of TTX was dramatically decreased or abolished when resistance of the innervated muscle was reflexly decreased (Figure 12, 13). Intravenous infusion of norepinephrine (NE, 10 µg/min) into the recipient animal increased systemic pressure 15 to 50 mmHg, simultaneously decreasing perfusion pressure of the innervated muscle 10-40 mmHg; thus, decreasing or abolishing the vasodilator effect of TTX (0.1 and 0.25 µg/min).

Hemorrhaging the recipient animal until 25 to 50% of the calculated blood volume (8% of body wt = 100%) was removed immediately increased resistance of the innervated gracilis, concommitantly augmenting the response to TTX (Figures 12, 13). However,TTX failed to produce a resistance change in the denervated muscles where initial resistance was simultaneously increased by local i.a. infusion of NE (0.1 µg/min). Notice in Figure 13 that in the controls which were taken before initial resistance was

Figure 12

Effect of tetrodotoxin (TTX) on vascular resistance when initial resistance was varied (prep #2, Figure 2 and 3). Tracings from a single animal are shown for the donor animals systemic pressure (SP_D), perfusion pressure of the denervated (PP_D) and innervated (PP_I) muscles, and systemic pressure of the recipient animal (SP_R). TTX (0.25 μg/min) was simultaneously infused (2 min) into both muscles starting as indicated at the arrows. Control responses are shown on the left, initial vascular resistance was then reflexly decreased in only the innervated muscle by intravenous infusion into the recipient animal of norepinephrine (NE, 10 μg/min). Initial vascular resistance of the innervated muscle was reflexly increased by hemorrhaging the recipient animal and resistance of the denervated muscle simultaneously increased by local i.a. infusion of NE (0.1 μg/min).

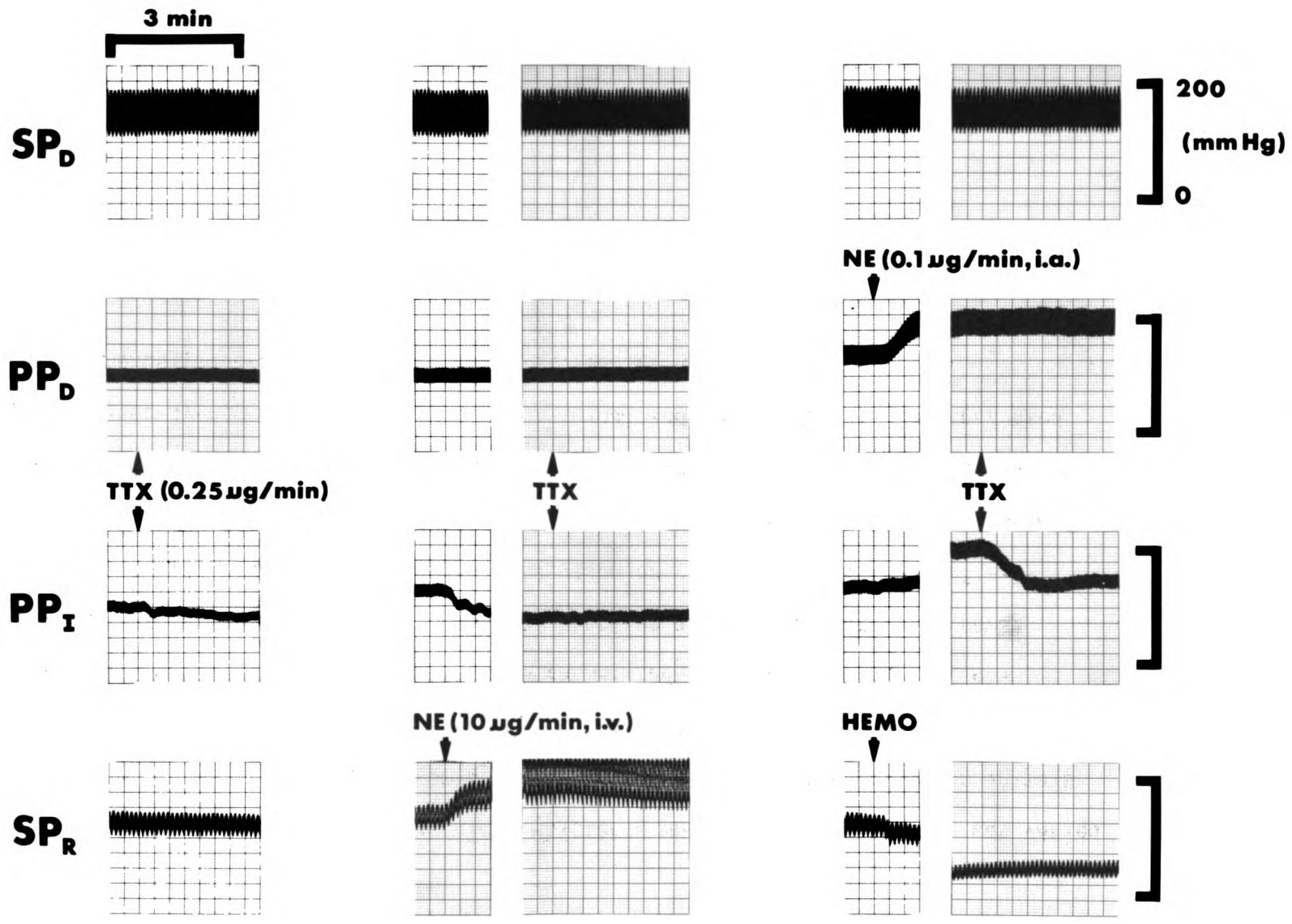

3 min
SP_D
200
(mm Hg)
0
NE (0.1 µg/min, i.a.)
PP_D
TTX (0.25 µg/min)
TTX
TTX
PP_I
NE (10 µg/min, i.v.)
HEMO
SP_R

Figure 13

Mean vascular resistance responses (N = 6) of cross-perfused innervated (solid lines) and denervated (broken lines) gracili muscles (prep #2, Figures 2 and 3) to local i.a. infusion of TTX (0.1 and 0.25 μg/min; on abscissa) when initial resistance was varied. Control responses are those obtained before either raising or lowering initial resistance. Initial resistance was lowered in the innervated muscle by i.v. infusion of norepinephrine (10 μg/min) into the recipient animal; and initial resistance increased in both muscles by hemorrhaging the recipient animal and simultaneously infusing norepinephrine (0.1 μg/min) into the denervated muscle.

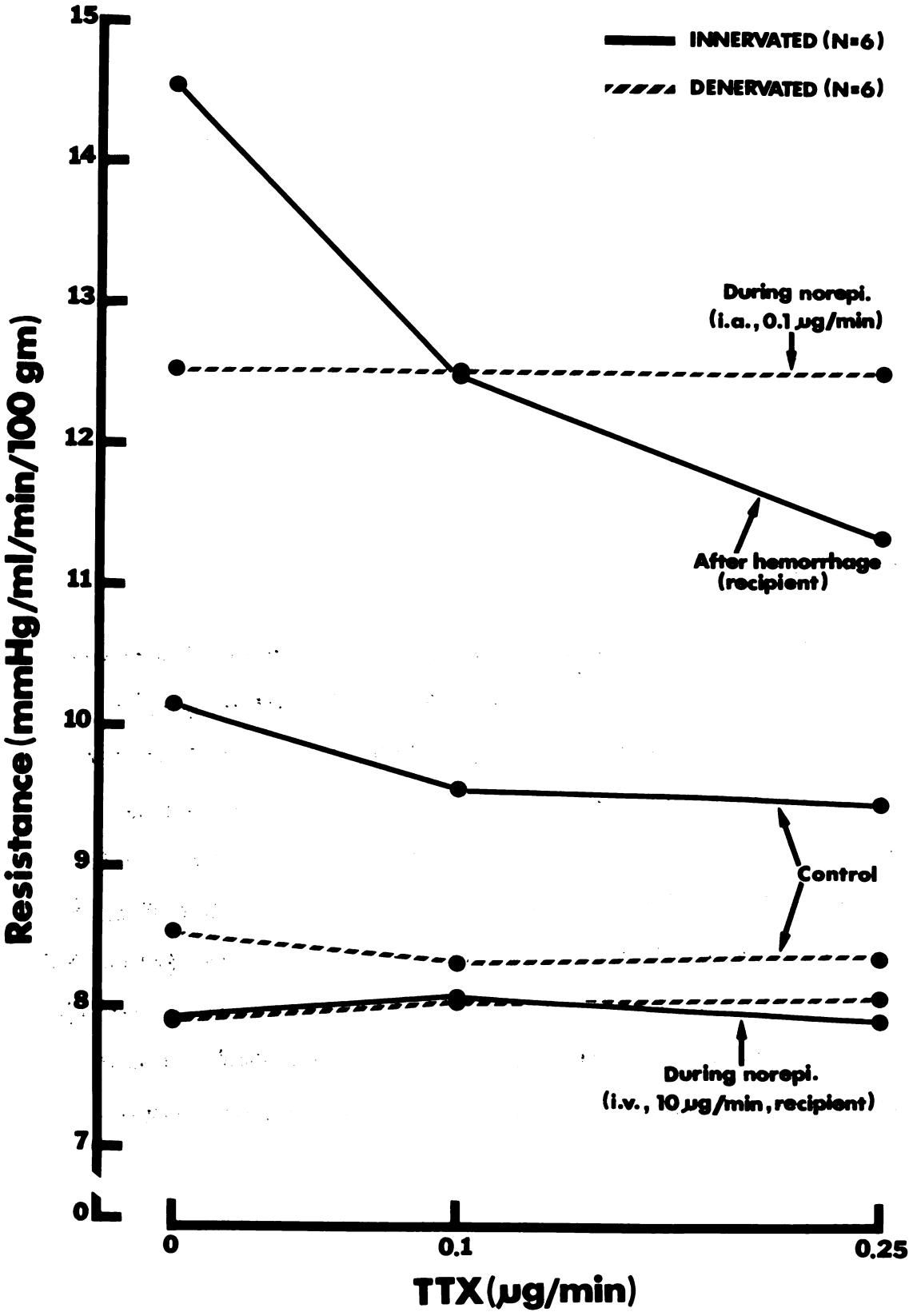
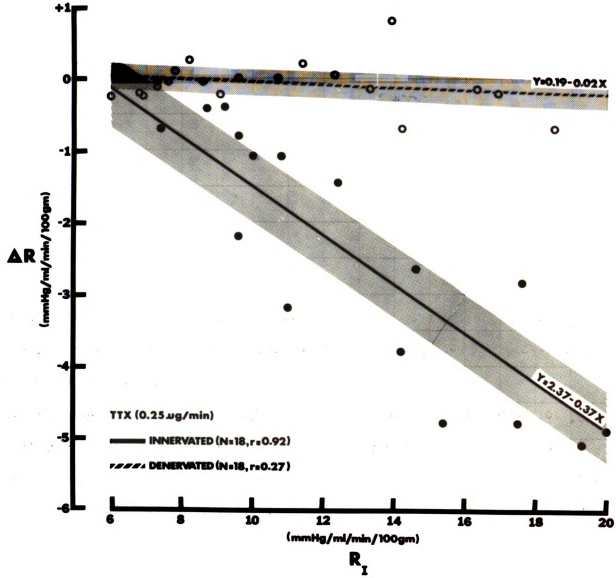

INNERVATED (N=6)
DENERVATED (N=6)
During norepi.
(i.a., 0.1 µg/min)
After hemorrhage
(recipient)
Control
During norepi.
(i.v., 10 µg/min, recipient)
Resistance (mmHg/ml/min/100 gm)
15
14
13
12
11
10
9
8
7
0
0
0.1
0.25
TTX (µg/min)

Figure 14

Effect of initial resistance (R_I, abscissa) on the change in resistance (ΔR, ordinate) to local i.a. infusion of tetrodotoxin (TTX, 0.25 μg/min) into innervated (solid dots and lines) and denervated (open circles and broken lines) gracili muscles (prep #2, Figure 2 and 3). Regression lines with their regression equations are shown for 18 resistance values taken from 6 animals.

Variance of the regression lines are denoted by the shaded area. Correlation coefficients are given for both the innervated ($r = 0.92$) and denervated ($r = 0.27$) muscles.


+1
0
-1
-2
-3
-4
-5
-6
ΔR
(mmHg/ml/min/100gm)
Y=0.19-0.02X
Y=2.37-0.37X
TTX (0.25 μg/min)
INNERVATED (N=18, r=0.92)
DENERVATED (N=18, r=0.27)
6
8
10
12
14
16
18
20
(mmHg/ml/min/100gm)
R_I

changed by any neurogenic or direct means, resistance of the innervated muscles was greater than that of the denervated gracili. In the innervated muscles the dose response curves during TTX infusion got progressively steeper as initial resistance was increased. When neural activity was essentially eliminated by i.v. infusion of norepinephrine into the recipient animal TTX failed to produce a change in resistance.

Figure 14 shows 18 accumulated initial resistances from 6 animals with the corresponding vasodilator response (decrease in resistance) to TTX (0.25 µg/min). The change in resistance of the innervated muscles to TTX positively correlated ($r = 0.92$) with the level of initial resistance. However, in the denervated muscles initial resistance appeared to have no relationship ($r = 0.27$) to the vasodilator action of TTX. Regression analysis of these data showed the responses from neurally intact gracili to have a slope of -0.37 with a y-intercept of 2.37 while those from the denervated preparations show a slope of -0.02 and a y-intercept of 0.19.

A period (1 or 2 minutes) of ischemia produced by turning the perfusion pump off resulted in a lower perfusion pressure when the pump was turned on, thus a decrease in resistance (reactive-dilation). However, when venous pressure was increased 12 to 18 mmHg by elevating the venous cannula (20 cm) vascular resistance was elevated (venous-arteriolar response). TTX in neural blocking doses (0.5 - 10.0 µg/min for 5 to 15 minutes) had no effect on the venous-arteriolar response or reactive dilation of innervated or denervated gracili muscles. In four perfused denervated gracili muscles when

perfusion pressure (80 ±10, 102 ± 20 mmHg) thus vascular resistance (8.0, 9.5, and 10.7 mmHg/min/ml/100 gms) was varied while keeping flow constant ($\bar{X}$ = 15 ml/min/100 gms) turning the pump off for one minute produced a subsequent fall in perfusion pressure to the same absolute level (40-55 mmHg = 2.8-3.7 mmHg/min/ml/100 gms). In the same animals elevating venous pressure ($\bar{X}$ = 14 mmHg) produced similar increases in perfusion pressure ($\bar{X}$ = 28 mmHg) even when initial resistances differed ($\bar{X}$s = 5.0, 6.5, and 9.5 mmHg/min/ml/100 gms) thus producing mean increases in vascular resistance of 1.0, 1.0 and 0.7 mmHg/min/ml/100 gms. TTX in a dose (0.25 μg/min, i.a.) sufficient to block vascular resistance changes in 5 minutes had no effect on reactive dilation or the venous arteriolar response.

Vascular Action Of TTX On Naturally Perfused Ileum And Jejunum

1) Effect of TTX on venous outflow of the ileum - TTX (5 μg/ml) given locally into the naturally perfused neurally intact ileum (prep #3, Figure 4) produced an increase in venous outflow which progressively became greater as the infusion rate was increased from 0.1 to 2.0 ml/min. However, at no point in the dose range of 0.5 to 10.0 μg/min did the change in venous outflow during TTX infusion differ statistically from that of the saline controls even though TTX frequently stimulated motility of the intestinal segment (Figure 15).

Electrical stimulation of the decentralized vagi and carotid occlusion produced an increase in motility and venous outflow respectively from both intestinal segments. Following the infusion

of TTX into one of the segments ($\bar{x}$ wt. = 25.9 g; $\bar{x}$ blood flow = 16.4 ml/min) vagal stimulation failed to affect motility of that segment and carotid occlusion produced a greater increase in blood flow as compared to the pre-infusion control. Thus, indicating that at some point in the TTX dose response curve local blockade of the neural elements occurred. In two animals (not included in Figure 15) where the neural blocking action of TTX was studied at each dose, 2.5 µg/min (0.5 ml/min) infused for 5 minutes was sufficient to significantly ($p < 0.05$) depress but not abolish the vagally induced motility response.

2) Effect of TTX on vascular responses to KCl - Figure 15 shows that infusion of isosmotic KCl into the intestinal segments, not previously receiving TTX, produced an increase and then a decrease in venous outflow. However, segments which had previously received TTX and were then given KCl with TTX (5 µg/ml) showed only a decrease in venous outflow. The increase in venous outflow reached a maximum at 0.5 ml/min. Infusion of KCl at rates of 1.0 and 2.0 ml/min always produced an increase in intestinal motility which accompanied the decrease in venous outflow. KCl in combination with TTX often increased motor activity at a lower infusion rate (0.5 ml/min). Furthermore, in this series of experiments the vasodilator response to KCl alone was abolished when TTX was present.

In all of the animals studied, i.a. infusion of KCl increased systemic arterial pressure. This increase was not abolished by local infusion of TTX. However, when the blood containing TTX was allowed to return to the animal systemic pressure began to gradually

decrease 30 to 45 minutes after completion of the first TTX test sequence and the systemic pressure response to local infusion of KCl was then depressed or abolished. The total dose of TTX given before a decrease in systemic pressure was observed was always greater than 20 μg and in 5 of the 7 dogs was greater than 40 μg.

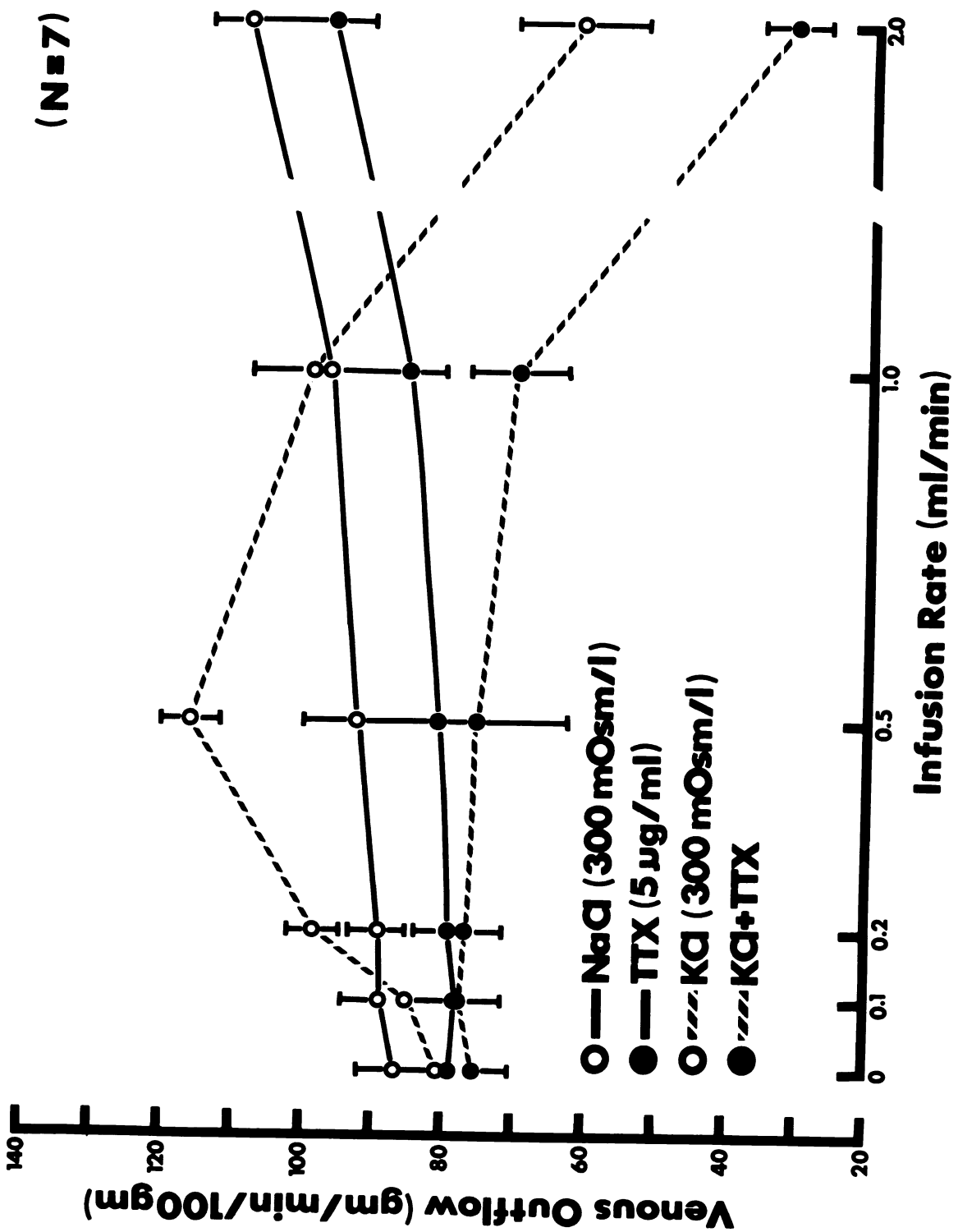
3) Effect of TTX on the vascular responses to luminal placement of hyperosmotic NaCl, hyperosmotic KCl, and glucose - Hyperosmotic NaCl (1500 mOsm/Kg), hyperosmotic KCl (1500 mOsm/Kg), and glucose (2.5, 5.4, 20 and 50%) were placed in the lumen of naturally perfused jejunum (prep #4, Figure 4) to study the local vascular responses, motility and fluid movement before and after neural blockade by TTX or dibucaine. The vascular responses to intraluminally placed and i.a. glucose have been reported (Chou et al., 22).

NaCl - Luminal placement of hyperosmotic NaCl (1500 mOsm/Kg) increased venous outflow in the first 3 minutes. This increase in venous outflow was significantly greater ($p < 0.01$) than that during luminal placement of isosmotic polyethylene glycol solution and was not affected by an i.a. TTX dose (0.5 - 2.0 μg/min) sufficient to block the vagally induced motility responses (Table 1). Systemic arterial pressure gradually decreased during the course of the experiments showing a significant hypotensive effect of TTX about 30 minutes after TTX was returned to the animal (Table 1). Table 1 shows that the calculated vascular resistance of the segments decreased during the first 3 minutes and increased slightly over the next 8 minutes but remained significantly ($p < 0.01$) lower than

Figure 15

Effects of tetrodotoxin (TTX) and potassium chloride (KCl) on blood flow of the naturally perfused ileum (prep #3, Figure 4). Venous outflow responses are shown (gm/min/100 gms of tissue, as given on the ordinate) during intra-arterial infusion of NaCl (300 mOsm/l; O———O), TTX (5 g/ml; ●———●), KCl (300 mOsm/l; O----O), and KCL (300 mOsm/l) + TTX (5 µg/ml) (●----●) at increasing infusion rates as indicated on the abscissa in ml/min.

Mean values (N = 7) and their standard errors are shown, with control flows given at infusion rate of 0 ml/min. The two broken line (-----) values and two solid line (_____) values were compared to each other at each infusion rate; and only KCl and KCL + TTX at 0.5 ml/min were significantly different from each other as indicated by student's t analysis for paired comparisons ($p < 0.05$).


(N=7)
Venous Outflow (gm/min/100gm)
140
120
100
80
60
40
20
0
0.1
0.2
0.5
1.0
2.0
Infusion Rate (ml/min)
NaCl (300 mOsm/l)
TTX (5 µg/ml)
KCl (300 mOsm/l)
KCl+TTX

the control resistances. However, during TTX infusion hyperosmotic NaCl produced a decrease in resistance which continued to fall over the entire test period (Table 1, Figure 16). This slight increase in resistance over the last 8 minutes to hyperosmotic NaCl was accompanied by an increase in motility which was abolished by TTX.

Hyperosmotic NaCl placed in the lumen significantly altered venous blood osmolality, Na^+ and K^+ concentrations and the volume of fluid recovered from the lumen. Osmolality of the venous effluent was significantly ($p < 0.01$) increased in 3 minutes, increasing more after 11 minutes, and was unchanged by TTX (Table 1, Figure 16). Na^+ concentration of the venous blood significantly increased before and after TTX. However, potassium concentration of the venous blood increased during luminal placement of hyperosmotic NaCl only before TTX infusion (Table 1). Notice though, that the K^+ values before and after TTX were significantly different only during the control. Recovery of the fluid in the lumen at the end of the test period showed a significant increase which was attenuated by local TTX infusion (Table 1).

KCl - Luminal placement of hyperosmotic KCl (1500 mOsm/Kg) increased venous outflow before TTX but decreased flow after TTX (Table 2, Figure 16). During luminal placement of KCl before TTX, vascular resistance of the segments significantly decreased in 3 minutes then increased over the next 8 minutes remaining below the control values. However as compared to the controls, after TTX resistance was unchanged during the first 3 minutes but profoundly increased over the remainder of the test period. KCl increased motor activity of the segment before and after TTX.

Table 1 - Effects of tetrodotoxin (TTX) on the intestinal vascular responses in naturally perfused jejunum to luminal placement (10 ml) of isosmotic polyethylene glycol (Control, PEG) and hyperosmotic NaCl (1500 mOsm/Kg). The venous responses (flow, osmolality, Na^+, and K^+ concentrations), systemic arterial pressure, calculated vascular resistance, and recovered volume (from the lumen) are shown before (B) and during (A) intra-arterial infusion of TTX (0.5-2.0 μg/min). The numbered columns indicate the 1st, 2nd, or 3rd collection (3 min) of the test period (11 min). The mean values and standard errors are shown. Values statistically different from their appropriate control (i.e., B vs. B) are denoted by asterisks. In the lower chart all values were compared to those obtained from the 3rd collection of the control.

Luminal Contents		Flow (gm/min/100 gm)			Pressure (mm Hg)			Resistance (mm Hg/gm/min/100 gm)		
		1	2	3	1	2	3	1	2	3
Control (PEG) (N=16)	B	42.4±3	40.3±3	40.2±2	115±3	116±2	116±3	2.88±.3	3.00±.3	3.03±.3
NaCl (N=16)	B	54.1±3**	50.0±3**	50.1±3**	111±3*	111±3*	112±2*	2.16±.1**	2.37±.1**	2.34±.1**
—TTX—										
Control	A	36.7±2	35.0±2	35.1±2	100±6	100±6	94±6	2.85±.3	2.94±.3	2.76±.3
NaCl	A	41.3±3	40.4±2**	40.4±2**	89±6**	85±5**	82±4**	2.25±.1**	2.13±.1**	2.07±.1**

		Osmolality (mOsm/Kg)		Na^+ Conc. (mEq/L)		K^+ Conc. (mEq/L)		Volume (ml)
		1	3	1	3	1	3	3
Control (N=14)	B		300±3		150± .9		2.8±.1	9.4±.4
NaCl (N=14)	B	313±4**	323±4**	160±1.8**	162±1.6**	3.4±.2*	3.6±.1*	15.0±.4**
—TTX—								
Control	A		299±3		155± .9		3.3±.2	10.0±.4
NaCl	A	316±4**	322±3**	160±1.8**	165±2.4**	3.4±.1	3.5±.2	13.7±.4**

$\bar{x}$ ± S.E.
* = P <.05
** = P <.01

Table 2 - Effect of tetrodotoxin (TTX) on the intestinal vascular responses in naturally perfused jejunum to luminal placement (10 ml) of isosmotic polyethylene glycol (Control, PEG) and hyperosmotic KCl (1500 mOsm/Kg). The venous responses (flow, osmolality, Na^+ and K^+ concentrations), systemic arterial pressure, calculated vascular resistance and recovered volume (from the lumen) are shown before (B) and during (A) intra-arterial infusion of TTX (0.5 - 2.0 µg/min). The numbered columns indicate the 1st, 2nd, or 3rd collection (3 min) of the test period (11 min). The mean values and standard errors are shown. Those values statistically different from their appropriate control (i.e., B vs. B) are denoted by asterisks. In the lower chart all values were compared to those obtained from the 3rd collection of the control.

Luminal Contents		Flow (gm/min/100 gm)			Pressure (mm Hg)			Resistance (mmHg/gm/min/100 gm)		
		1	2	3	1	2	3	1	2	3
Control (PEG) (N=8)	B	72.3±12	66.4±11	67.4±12	123±5	123±5	121±6	2.13±.3	2.28±.3	2.25±.3
KCl (N=8)	B	108.7±20**	93.7±17**	85.6±17*	114±6**	114±6**	116±5*	1.35±.3**	1.65±.3**	1.92±.3*
—TTX—										
Control	A	43.4±12	43.9± 9	40.5± 9	104±5	104±5	100±6	3.27±.6	3.18±.6	3.03±.6
KCl	A	43.2± 9	32.1± 8*	29.6± 8*	94±7*	101±7	106±4*	2.70±.3	4.14±.6**	4.77±.6**

		Osmolality		Na^+ Conc.		K^+ Conc.		Volume
		1	3	1	3	1	3	3
Control (N=14)	B		300±3		150± .9		2.8± .1	9.4± .4
KCl (N=6)	B	301±.8*	304± .6**	153±1.7	152±1.7	4.3±.2**	4.5± .1**	14.9±1.2**
—TTX—								
Control	A		299±3		155± .9		3.3± .2	10.0± .4
KCl	A	304±.7*	311±1.4**	153±2.0	151±2.1	5.2±.4**	9.2±1.0**	13.2± .8**

$\bar{x}$ ± S.E.
* = $P < .05$
** = $P < .01$

Intraluminal placement of hyperosmotic KCl increased venous blood osmolality before and during TTX. However during infusion of TTX this increase in osmolality was significantly greater than control 3 minutes after placement of KCl in the lumen and was further augmented during the last 3 minutes of the test period (Table 2, Figure 16). Sodium concentration of the venous blood was unchanged by hyperosmotic KCl before or during TTX (Table 2). However, K^+ concentration of the venous outflow was significantly ($p < 0.01$) increased at 3 and 11 minutes of the test period and was significantly ($p < 0.05$) greater after TTX than before (Figure 16). Again the volume of fluid recovered from the lumen was greater after KCl as compared to the polyethylene glycol controls and the increase was attenuated by TTX.

Glucose - The data collected during luminal placement or i.a. administration of glucose (2.5, 5.4, 20 and 50%) and polyethylene glycol solutions of similar osmolalities were obtained from a series of experiments which have been published (22). Therefore, these data will be briefly described along with the series utilizing TTX.

Luminal placement of 2.5, 5.4, 20 and 50% glucose and 85% PEG solutions all significantly increased blood flow as compared to flow values measured in the preceding period during which the lumen contained isosmotic PEG solution. The 2.5 and 5.4 percent glucose solutions caused small and similar increases in flow and venous glucose concentration, but did not alter venous osmolality. Lumen volume decreased with the 2.5 percent glucose solution but

Table 3 - Vascular responses in naturally perfused jejunum to luminal placement (10 ml) of isosmotic polyethylene glycol (Control, PEG) and hyperosmotic Glucose (3000 mOsm/Kg) during intra-arterial infusion of tetrodotoxin (TTX, 0.5-2.0 µg/min). The venous responses (flow, osmolality, and glucose concentration), systemic arterial pressure, calulated vascular resistance and recovered volume (from the lumen are shown for the 1st, 2nd, and 3rd collections (3 min) of the test period (11 min). The mean values and standard errors are shown. Those values statistically different from the controls are denoted by asterisks.

Luminal Contents	Flow (gm/min/100 gms)			Pressure (mm Hg)			Resistance (mm Hg/gm/min/100 gm)		
	1	2	3	1	2	3	1	2	3
Control (N=16) (PEG)	33.3±4	28.3±3	28.0±3	90±3	92±4	91±4	3.45±6	4.05±.6	3.96±.6
Glucose (N=16)	36.6±4**	36.2±4**	38.8±4**	85±3*	81±3**	77±3**	2.94±.3**	2.64±.3**	2.34±.3**

	Osmolality (mOsm/Kg)		Glucose (mg/100 ml)		Volume (ml)
	1	3	1	3	3
Control (N=6)		299±3		95± 9	10.0±.4
Glucose (N=6)	309±2**	314±1**	378±51**	446±67**	13.4±.4**

$\bar{x}$ ± S.E.
* = P <.05
** = P <.01

did not change with the 5.4 percent glucose solution. Fifty percent glucose caused a greater increment in flow, venous osmolality, glucose concentration, and lumen volume than did 20% glucose. Although 20% glucose and 34% PEG solutions have the same osmolality, and both solutions raised venous osmolality and lumen volume to the same extent, the 20% glucose solution raised venous outflow, yet 34% PEG solution did not. Similarly, the osmolality of 50% glucose and 85% PEG are the same, and both solutions raised venous osmolality and lumen volume to the same extent; but the rise in flow caused by 50% glucose was significantly greater than that caused by 85% PEG.

Fifty percent glucose significantly increased venous outflow and motility before the jejunal mucosa was exposed to dibucaine, but after dibucaine administration, 50% glucose did not significantly alter flow or motility. For example, when 50% glucose was placed in one segment there was an increase in flow and motility but the other segment which contained isosmotic PEG showed no change in flow or motility. However, after exposure to dibucaine there was no response to 50% glucose, yet the other segment responded to 50% glucose with increases in flow and motility. Thus, the effects of 50% glucose and dibucaine appear to be local since they affected only the segment exposed. The possibility that dibucaine might have paralyzed the vascular smooth muscle making it unresponsive was examined by intra-arterial infusions of vasodilators (isoproterenol, isosmotic KCl, or hyperosmotic NaCl solution) before and after luminal placement of dibucaine. The increased flow

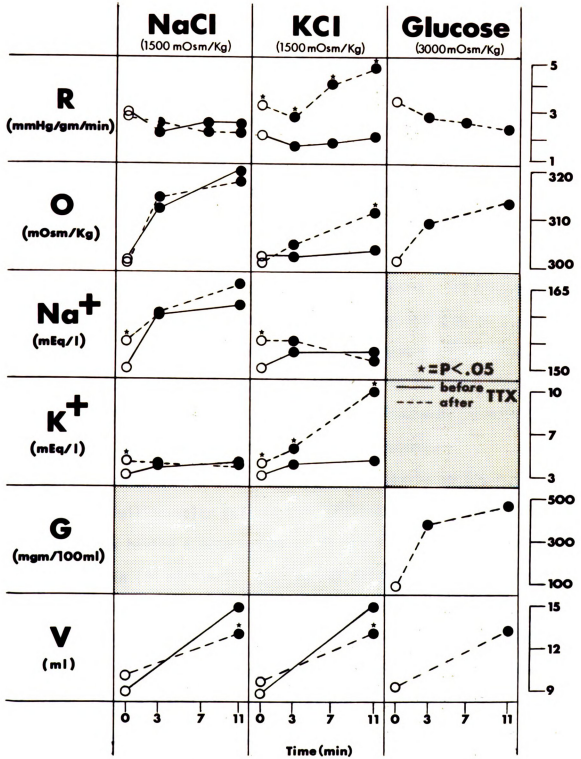
caused by intra-arterial infusion of the vasodilators was not altered by luminal placement of dibucaine. The increases in venous osmolarity, glucose concentration, and lumen volume caused by luminal placement of 50% glucose were the same before and after dibucaine. Dibucaine per se did not alter blood flow or lumen volume but did increase motility. This increase in motility, however, waned and disappeared shortly after the removal of dibucaine from the lumen and its replacement with isosmotic PEG.

Hyperosmotic glucose (50%, 3000 mOsm/Kg) solution placed in the lumen increases venous outflow and decreases vascular resistance before (22) and after i.a. infusion of TTX (Table 3, Figure 16). In this series of experiments the glucose solution was introduced into segments previously receiving NaCl or KCl and TTX. Glucose decreased resistance. This vasodilation progressively increased throughout the test period (Table 3). Venous osmolality, glucose concentration, and volume recovered from the lumen all increased after TTX. Before neural blockade by TTX, 50% glucose increases motility of the isolated segment; however, after TTX motility was unchanged during luminal placement of hyperosmotic glucose.

Intra-arterial infusion of 2.5, 5.4, and 16.4 percent glucose solutions all increased glucose concentration in the venous plasma but only 16.4 percent glucose at or above infusion rates of 0.5 ml per minute significantly increased blood flow as compared to normal saline. Venous plasma osmolality was increased by the 16.4 percent glucose solution and lowered by 2.5 percent glucose.

Figure 16

Intestinal vascular responses of naturally perfused jejunum (prep #4, Figure 4) to lumenal placement (10 ml) of hyperosmotic NaCl (N = 14), hyperosmotic KCl (N = 6) and 50% glucose (N = 6) before (solid lines) and during (dashed lines) intra-arterial infusion of TTX (0.5 - 2.0 μg/min). Calculated vascular resistance (R), venous blood osmolality (O), venous blood sodium concentration (Na^+), venous blood potassium concentration (K^+), venous blood glucose (G) and the volume (V) recovered from the lumen are shown during luminal placement of the control solution (isosmotic polyethylene glycol) denoted as open circles at time zero and then 3, 7, and 11 minutes after introducing the test solutions (denoted as solid dots). Calibration bars given on the right represent values in those units listed on the left. Stars (*) denote those values during TTX infusion significantly different ($p < 0.05$) from those before TTX.


NaCl
(1500 mOsm/Kg)
KCl
(1500 mOsm/Kg)
Glucose
(3000 mOsm/Kg)
R
(mmHg/gm/min)
O
(mOsm/Kg)
Na+
(mEq/l)
K+
(mEq/l)
G
(mgm/100ml)
V
(ml)
5
3
1
320
310
300
165
150
10
7
3
500
300
100
15
12
9
*=P<.05
before TTX
after
0 3 7 11
Time (min)

Vascular Action Of TTX On Pump-Perfused Jejunum

1) Action of C_6 and TTX on the vasoactivity of isosmotic KCl and hyperosmotic NaCl (H-NaCl) - Local i.a. infusion of isosmotic KCl produced a biphasic vascular response in the pump-perfused jejunum (prep #5, Figure 5), dilation at low infusion rates and constriction at high infusion rates. Hexamethonium (C_6) or TTX stimulated motility of the segment and concomitantly increased initial resistance, but failed to alter the biphasic response of resistance (Figure 17). As with isosmotic KCl local infusion of hyperosmotic NaCl produced a biphasic change in vascular resistance. The increase in vascular resistance occurred concomitant with an increase in motility. However, during infusion of TTX, hyperosmotic NaCl produced only dilation which progressively increased as the infusion rate was increased (Figure 17 and 18). Furthermore no increase in motility was seen during infusion of H-NaCl when TTX was present.

Figure 17 shows the calculated vascular resistance during i.a. infusion of 300 milliosmolar KCl and 1500 milliosmolar NaCl into jejunal segments having a mean weight of 31 grams with a mean blood flow of 16 ml/min (54 ml/min/100 g). KCl at infusion rates of 0.5 ml/min or less, (increasing blood $[K^+]$ 4.7 mEq/l) significantly decreased resistance while infusion rates of 1 or 2 ml/min (increasing $[K^+]$ 9.4 and 18.8 mEq/l) increased resistance. In all cases infusion of C_6 (0.5 mg/min = 2.5×10^{-5} g/ml of blood) or TTX (1.0 µg/min = 5×10^{-8} g/ml of blood, 1.5×10^{-10} M) increased initial resistance of the segment and simultaneously increased motility. However, the biphasic response to KCl was unchanged by C_6 or TTX. Infusing 0.5

Figure 17

Effect of hexamethonium (C_6, 0.5 mg/min) and tetrodotoxin (TTX, 1.0 μg/min on vascular resistance responses to local intra-arterial infusion of isosmotic KCl (300 mOsm/l) and hyperosmotic NaCl (1500 mOsm/l) into the innervated pump-perfused jejunum. The test solutions were infused (1 to 2 min) before (●), and during C_6 (O) or TTX (Δ), at progressively increasing rates as shown on the abscissa. Data points represent mean values (N = 9) with the asterisks denoting those points significantly different ($p < 0.05$) from their control (0 ml/min).

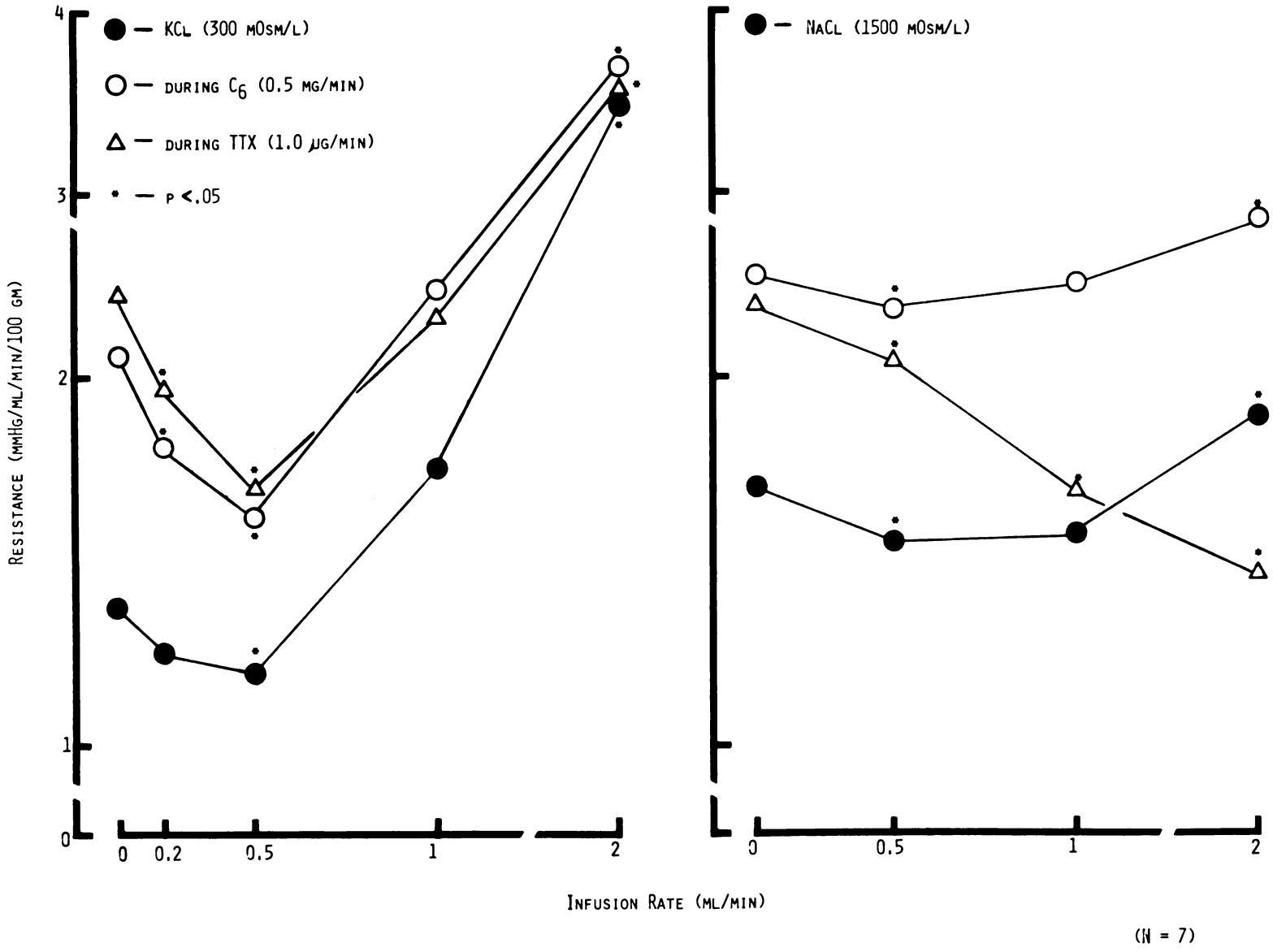
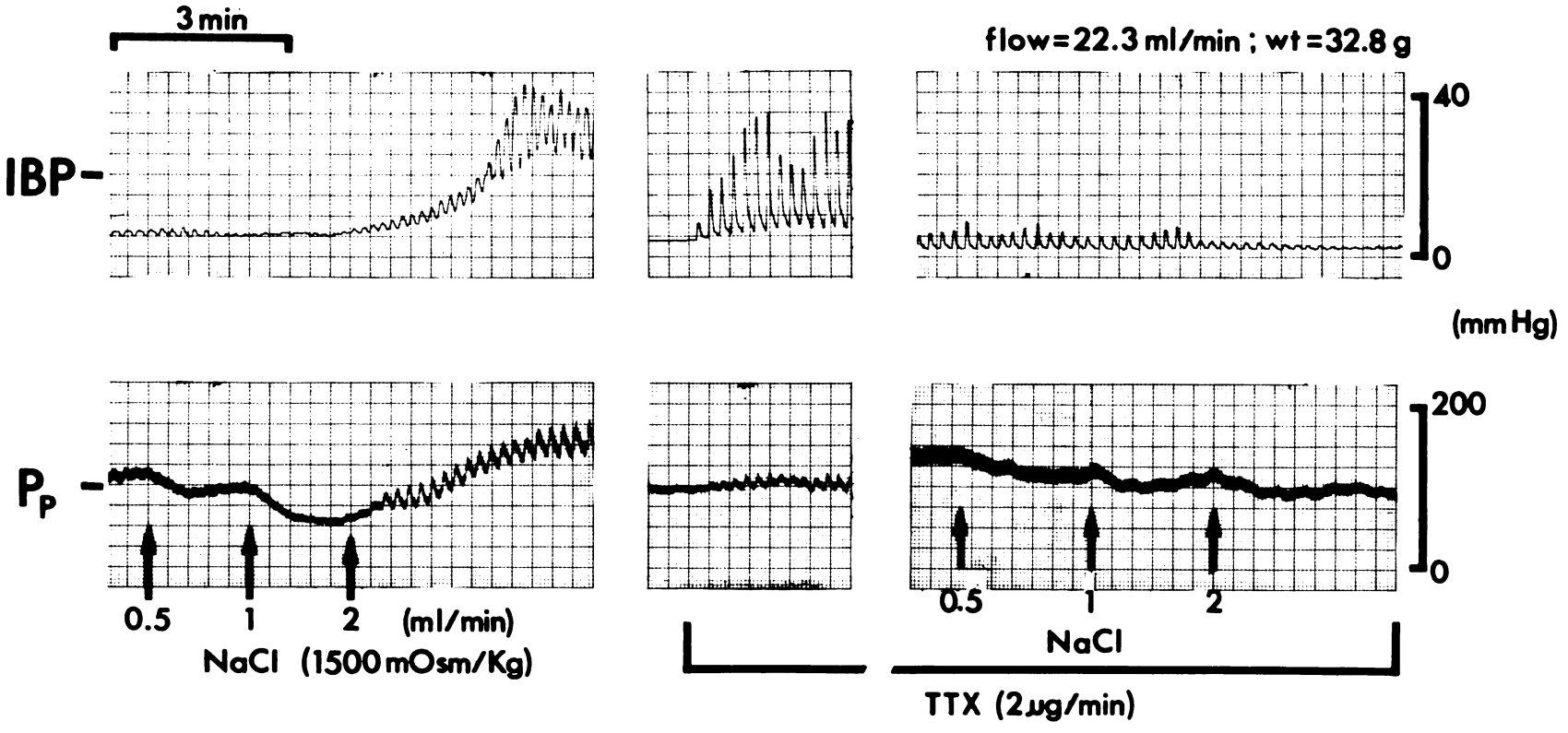

KCL (300 mOsm/L)
DURING C6 (0.5 mg/min)
DURING TTX (1.0 µg/min)
* — P <.05
NaCL (1500 mOsm/L)
RESISTANCE (mmHg/ml/min/100 gm)
INFUSION RATE (ml/min)
0
1
2
3
4
0.2
0.5
(N = 7)

Figure 18

Representative intraballoon pressure (IBP) and perfusion pressure (P_p) tracings to local intra-arterial (i.a.) infusion of hyperosmotic NaCl (1500 mOsm/Kg) before and during i.a. infusion of tetrodotoxin (TTX, 2 µg/min). NaCl was infused at progressively increasing rates into a constantly perfused jejunum (prep #5, Figure 5) weighing 32.8 gm and receiving 22.3 ml of blood per min. The breaks in the tracings represent 15 min.


3 min
flow=22.3 ml/min ; wt=32.8 g
IBP
40
0
(mm Hg)
P_P
200
0
0.5
1
2
(ml/min)
NaCl (1500 mOsm/Kg)
0.5
1
2
NaCl
TTX (2 µg/min)

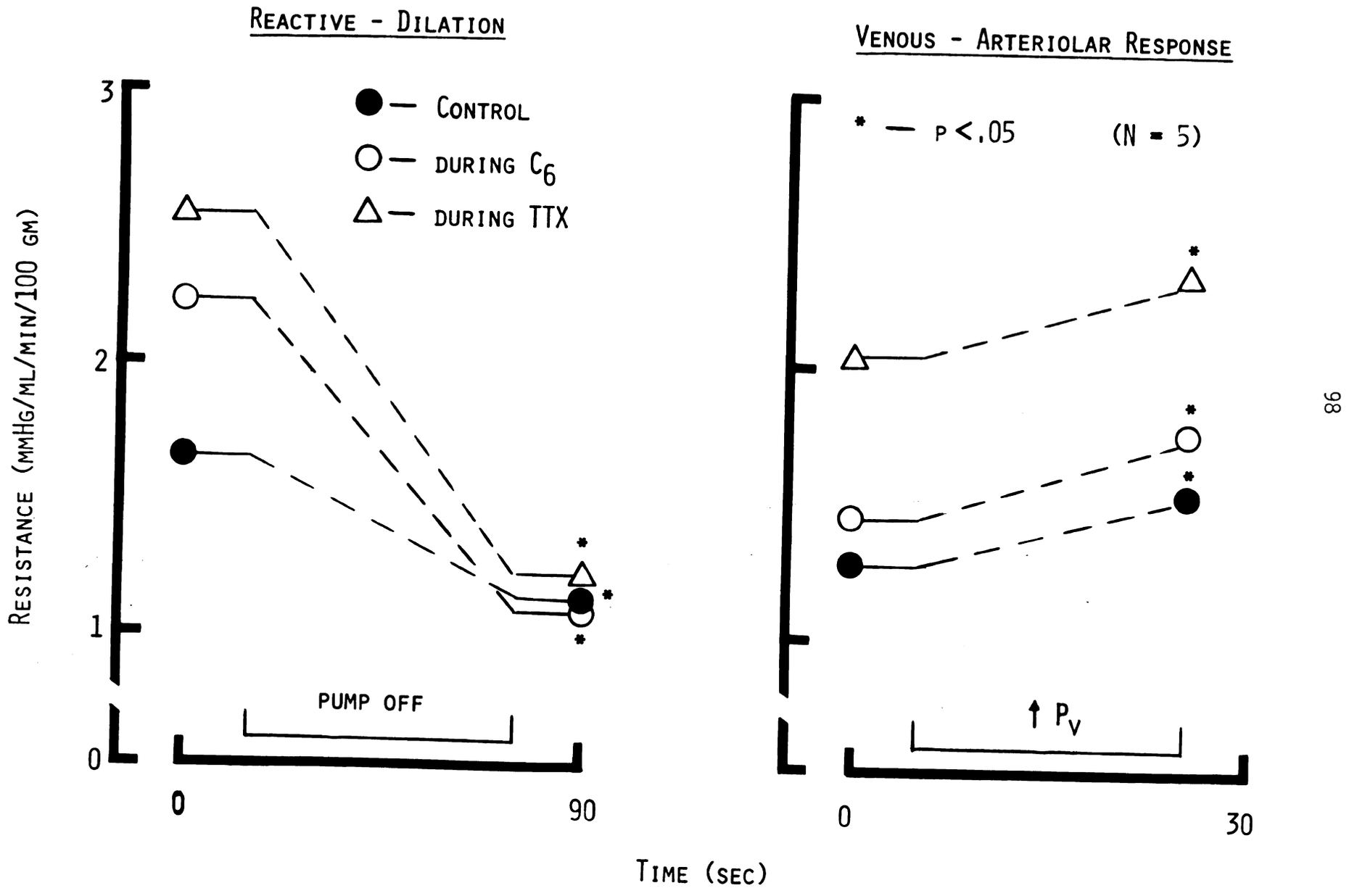
ml/min of hyperosmotic NaCl (increasing blood osmolality 37 mOsm/Kg) produced a significant decrease in resistance; however, as the infusion rate was increased vascular resistance and motility were also increased. C_6 failed to alter either the motility or resistance responses to NaCl but TTX depressed or abolished the changes in motility and converted the constrictor phase to dilation.

Figure 18 shows intraballoon and perfusion pressure tracings from a single jejunal segment receiving a blood flow of 22.3 ml/min. Infusing NaCl at 0.5 and 1.0 ml/min, thus increasing blood osmolality (34 and 68 mOsm/Kg), produced a decrease in perfusion pressure. Intraballoon pressure began to increase slightly after a 1 minute infusion of 1 ml/min; but, when the infusion rate was increased to 2 ml/min (+ 136 mOsm/Kg) intraballoon pressure and perfusion pressure dramatically increased. Infusion of TTX i.a. (2 µg/min, 9×10^{-8} g/ml of blood, 2.7×10^{-10} M) stimulated motility of the segment. This increase in motor activity, which wained with time, was reflected in the perfusion pressure. In the presence of TTX the motility response to NaCl was abolished and only a decrease in vascular resistance was seen.

2) <u>Effect of TTX on the venous-arteriolar response, reactive dilation and the vascular responses to nicotine, acetylcholine and epinephrine</u> - A period of ischemia produced a fall in the subsequent perfusion pressure, thus a decrease in vascular resistance (reactive-dilation). However, when venous pressure was increased vascular resistance of the segment was also increased (venous-arteriolar response). Neither the venous-arteriolar response nor

Figure 19

Effect of hexamethonium (C_6, 0.5 mg/min) and tetrodotoxin (TTX, 1.0 µg/min) on reactive dilation and the venous-arteriolar response of the constantly perfused jejunum (prep #5, Figure 5). Vascular resistance (mmHg/ml/min/100 gm) is shown on the ordinates and time in sec. on the abscissa. The graph on the left shows vascular resistance 15 sec before the perfusion pump was turned off (for 1 min) and 15 sec after it was turned on. On the right is shown the calculated resistance 5 sec before and 20 sec after venous pressure was increased (P_v) by raising the venous cannula 10 cm. Each datum point represents the mean value (N = 5) before (●), and during C_6 (0) or TTX (Δ), with asterisks (*) denoting those values significantly different from their pre-response value ($p < 0.05$) as indicated by student's t test for paired comparisons.


REACTIVE - DILATION
VENOUS - ARTERIOLAR RESPONSE
CONTROL
DURING C_6
DURING TTX
* — P<.05
(N = 5)
RESISTANCE (MMHG/ML/MIN/100 GM)
3
2
1
0
PUMP OFF
↑ P_V
0
90
0
30
TIME (SEC)

reactive dilation was affected by local infusion of C_6 or TTX (Figure 19). Nicotine increased motility and vascular resistance before infusion of C_6 and decreased motility and resistance during C_6. TTX depressed or abolished the changes both in motility and vascular resistance produced by nicotine (Figure 20). Neither C_6 nor TTX affected the vasodilatory response of acetylcholine or the vasoconstrictor action of epinephrine (Figure 20).

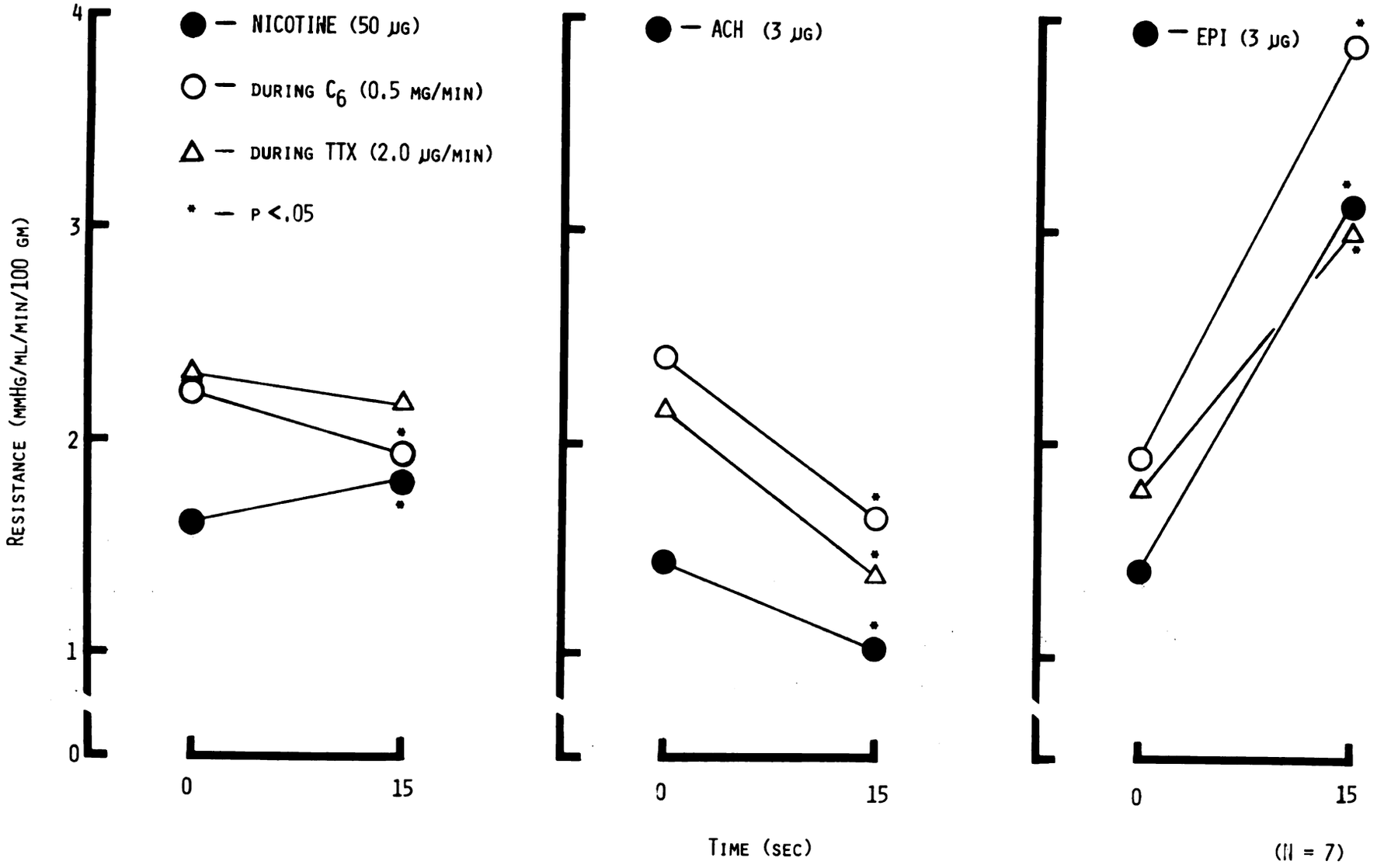
Figure 19 shows the vascular resistance 15 sec before the perfusion pump was turned off for 1 minute and 15 sec after it was turned on. Notice that C_6 (0.5 mg/min, 2.5×10^{-5} g/ml of blood) and TTX (1.0 µg/min, 5×10^{-8} g/ml of blood) increased resistance. This occurred simultaneously with an increase in motility. But even though the control resistances differed, a one minute period of ischemia significantly decreased resistance to the same absolute level before and during C_6 or TTX.

The venous-arteriolar response was induced by raising (10 to 20 cm) the venous cannula, (Figure 19). Figure 19 shows that the increase in resistance in response to an increase in venous pressure was unaltered by C_6 or TTX. The magnitude of this venous-arteriolar response appeared to be unrelated to initial vascular resistance.

Figure 20 shows the vascular resistance before and 15 sec after bolus injections of nicotine (50 µg), acetylcholine (3 µg) and epinephrine (3 µg). Nicotine produced a vasoconstriction before and a vasodilation during C_6 infusion (2.5×10^{-5} g/ml of blood). Occurring concomitantly with the changes in resistance were similar visceral muscle responses; i.e., stimulation of motility before and

Figure 20

Effect of nicotine (50 μg), acetylcholine (ACH, 3 μg) and epinephrine (EPI, 3 μg) on vascular resistance of the constantly perfused jejunum (prep #5, Figure 5) before (●), and during i.a. infusion of hexamethonium (O, C_6, 0.5 mg/min) or tetrodotoxin (Δ, TTX, 2.0 μg/min). Data points show mean (N = 7) values before and 15 sec after bolus injections of the test agents. Asterisks denote those points significantly different ($p < 0.05$) from their pre-injection controls.


NICOTINE (50 µG)
DURING C_6 (0.5 MG/MIN)
DURING TTX (2.0 µG/MIN)
* — P <.05
ACH (3 µG)
EPI (3 µG)
RESISTANCE (MMHG/ML/MIN/100 GM)
0
1
2
3
4
0
15
TIME (SEC)
(N = 7)

inhibition during C_6. TTX (5×10^{-8} g/ml blood, 1.5×10^{-10} M) significantly depressed or abolished both the vascular and visceral muscle responses to nicotine. Neither the decrease in resistance following a bolus injection of acetylcholine (3 g) nor the increase in resistance following epinephrine were affected by C_6 or TTX.

Responses of Femoral Arterial Strips In Vitro

1) Effect of preload on the contractile responses of arterial strips to TTX and other vasoactive agents - In an attempt to simulate in vitro conditions of varying initial vascular resistances, femoral arterial strips were equilibrated (1 1/2 to 2 hours) under preloaded tensions of 1, 2, 4 and 6 grams.

The vasoconstrictor, norepinephrine (0.1-1.0 µg/ml) increased tension in all strips studies with the magnitude of the response positively related to the dose and preload tension, while the vasodilator, acetylcholine (0.1-1.0 µg/ml) decreased tension of the strips (Figure 21 and 22). The relaxation elicited by acetylcholine (0.2 µg/ml) was most evident in those strips equilibrated at high preload (4 and 6 gm) and seldom seen in strips with low preloads (1 gm). When the strips were actively contracted with $BaCl_2$, acetylcholine (0.2 µg/ml) produced a greater decrease in tension as compared to the response before barium. The magnitude of the response to barium as well as the relaxation to acetylcholine was augmented at the greater preload (Figure 21). Occasionally acetylcholine increased the tension of strips equilibrated at low preloads

(1 gm); however, it never produced a contraction in strips preloaded with 2 or more grams.

The vasoconstrictors, barium chloride (1.0 mM), norepinephrine (0.1 µg/ml) nicotine (50 µg/ml), and dimethylphenylpiperizinium (DMPP, 10 µg/ml) all produced an increase in tension of arterial strips. This increase in tension was greater at the higher preload tensions (Figure 22). Phentolamine (50 µg/ml) abolished the response to norepinephrine but only attenuated the responses to nicotine or DMPP. Increasing the barium chloride concentration to 5.0 mM frequently produced rhythmic contractions oscillating every 1-3 minutes. Therefore only concentrations of 1.0 mM or less of $BaCl_2$ were used to actively contract the strips.

TTX (1.0 µg/ml, 3.1×10^{-9} M) generally failed to affect the tension of vascular strips equilibrated at low or high preloads. In four strips (preloaded with 6 gm) exposed to TTX concentrations of 10 µg/ml a decrease in tension occurred in two strips, an increase in one, and no change in the remaining strip. However, in all these strips acetylcholine produced relaxation. Acetylcholine (0.2 µg/ml) always produced a decrease in tension of strips preloaded with 2 or more grams. This relaxation was augmented at higher preloads (Figure 22) or after actively contracting the strips with $BaCl_2$ (Figure 21).

2) Effect of TTX and cold-storage on the reactivity of arterial strips - TTX in doses less than 1.0 µg/ml (3.1×10^{-9} M) failed to produce a change in tension of arterial strips. Yet, acetylcholine (0.2 µg/ml) still relaxed the strips and epinephrine (0.1 µg/ml)

Figure 21

The effect of pre-load tension on the in vitro (prep #6, Figure 6) responses of freshly prepared femoral arterial strips to norepinephrine (NE, 1 μg/ml) and acetylcholine (Ach, 1 μg/ml) before and after tension was increased by barium choloride ($BaCl_2$, 0.5 mM). Four different strips were equilibrated under four levels of pre-load tension (1, 2, 4 and 6 gm). The base-line pre-load tension is shown on the left as horizontal dashes. Calibration bars on the right show 2 gms change in tension. The agents were given at the arrows. Following the test period the baths were drained and washed (w) with Krebs-Ringer solution.

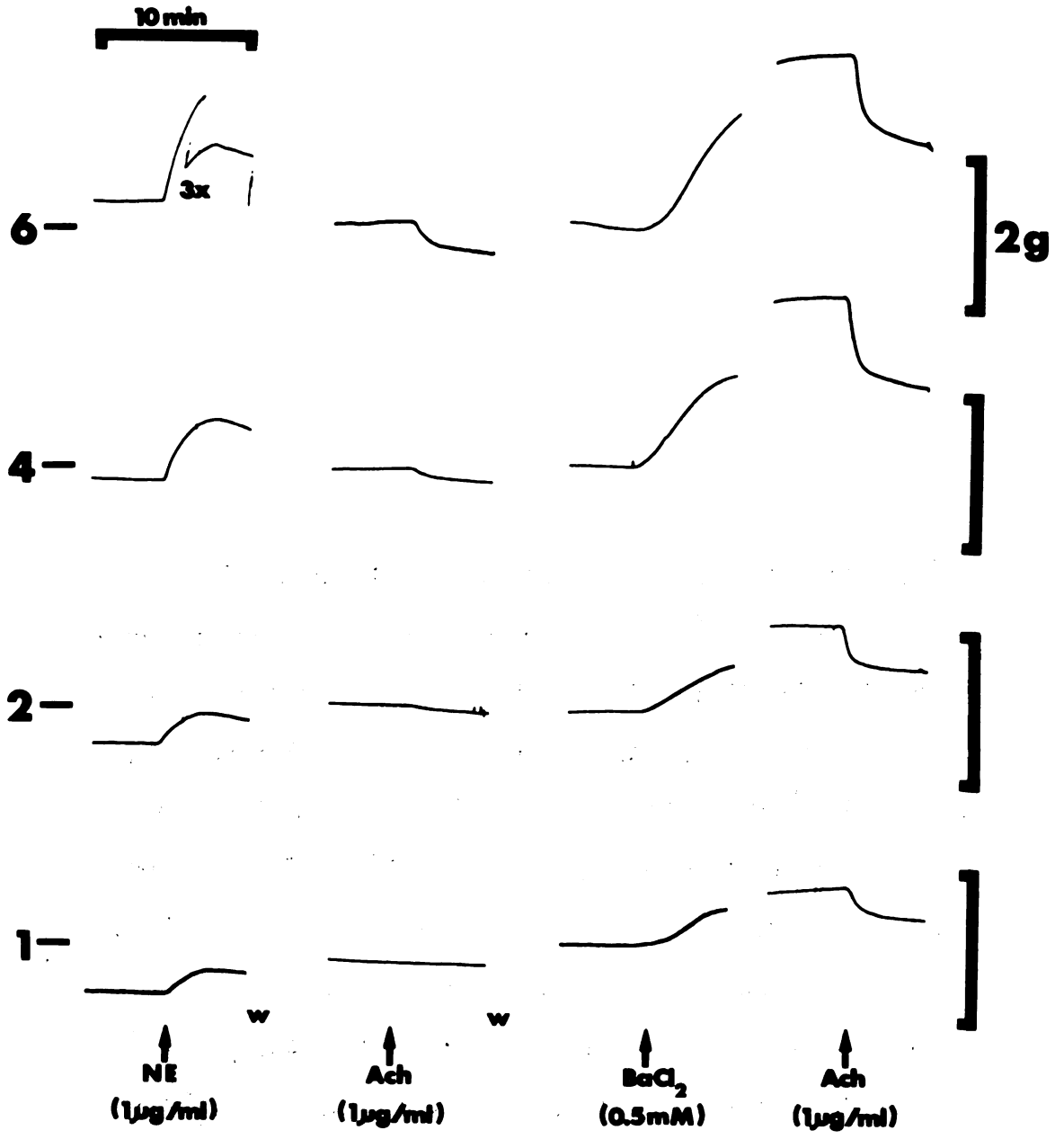

10 min
3x
6
4
2
1
2g
W
W
NE
(1µg/ml)
Ach
(1µg/ml)
BaCl2
(0.5mM)
Ach
(1µg/ml)

Figure 22

Effect of pre-load tension on the tension responses (Δ tension) of arterial (femoral) strips (prep #6, Figure 6) to $BaCl_2$, norepinephrine, nicotine, DMPP, tetrodotoxin (TTX), and acetylcholine (Ach). Change in tension is shown on the ordinate as a function of four equilibration pre-load tensions shown on the abscissa in grams. Asterisks denote those points statistically different ($p < 0.05$) from their controls (0) as evaluated by student's t analysis for paired comparisons. Standard errors of the responses and pre-loads are shown.

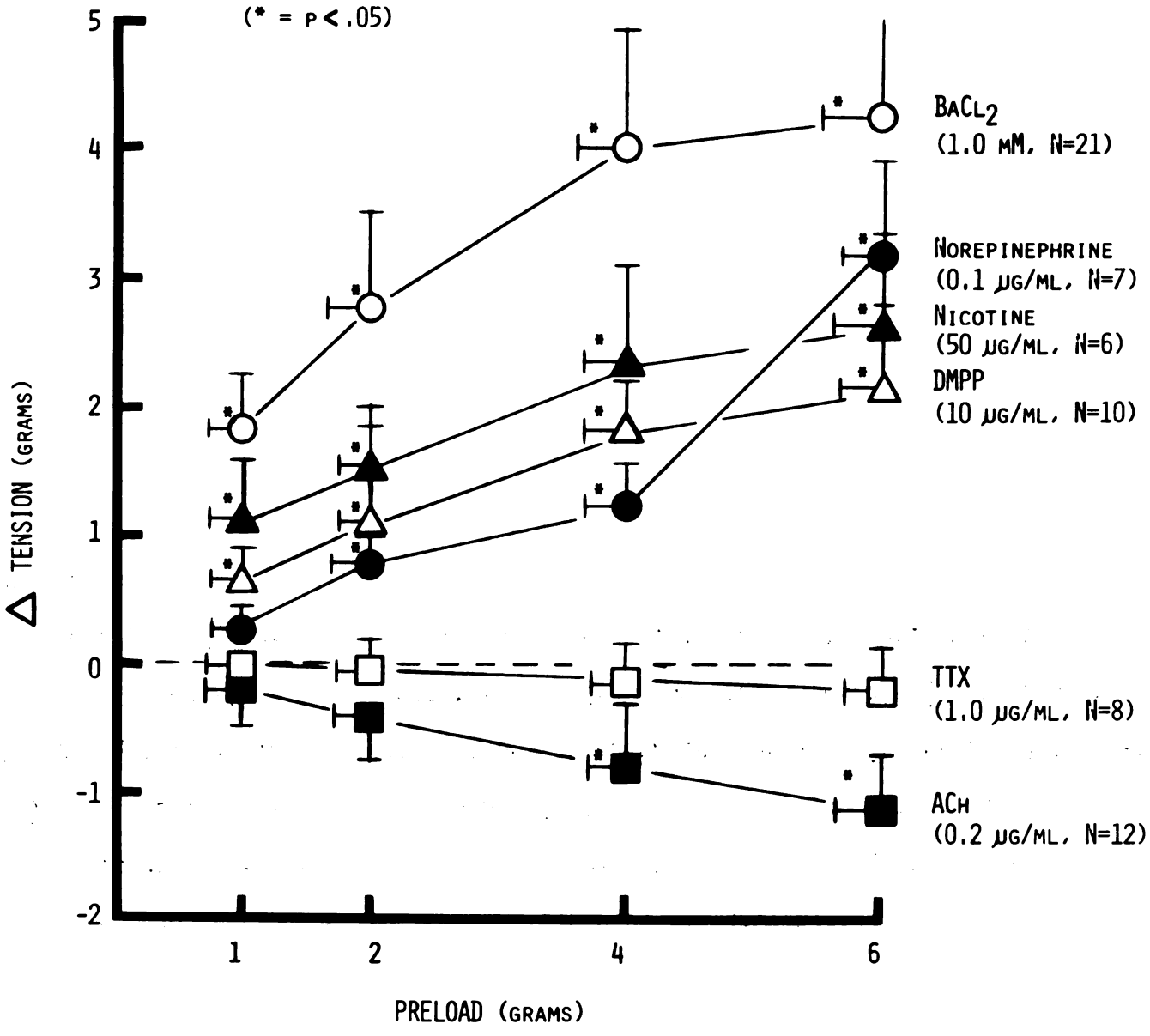
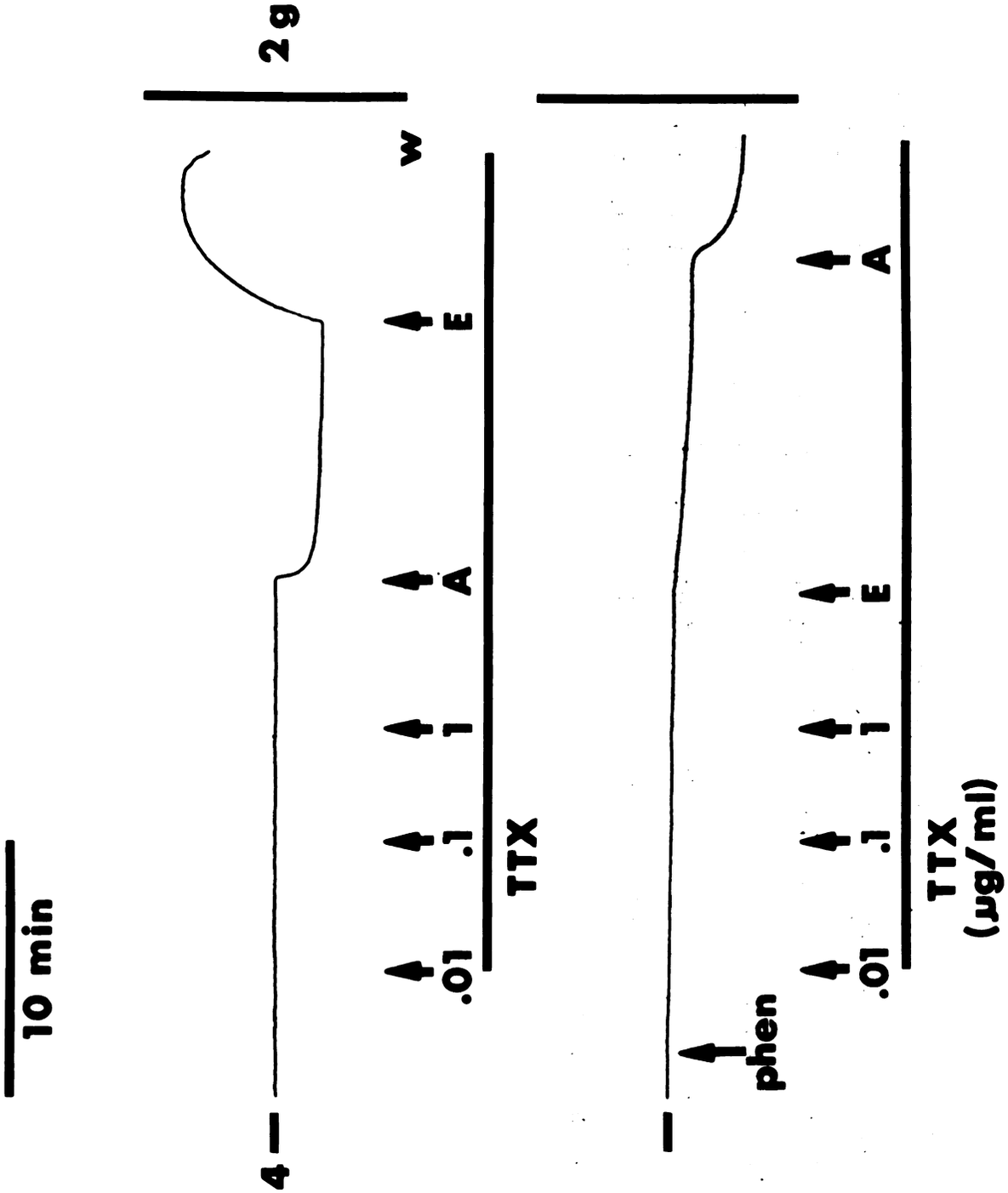

(* = P < .05)
5
4
3
2
1
0
-1
-2
Δ TENSION (GRAMS)
1
2
4
6
PRELOAD (GRAMS)
BaCl2
(1.0 mM, N=21)
Norepinephrine
(0.1 µg/ml, N=7)
Nicotine
(50 µg/ml, N=6)
DMPP
(10 µg/ml, N=10)
TTX
(1.0 µg/ml, N=8)
ACh
(0.2 µg/ml, N=12)

Figure 23

Tracings showing the effect of tetrodotoxin (TTX; 0.01, 0.1, 1.0 μg/ml), acetylcholine (A; 0.2 μg/ml) and epinephrine (E; 0.1 μg/ml) on the tension of a freshly prepared arterial strip in vitro (prep #6, Figure 6) before and after phentolamine (phen; 50 mg/ml). The strip was equilibrated under a 4 gram preload (dash at left of tracings). TTX concentration was progressively increased as denoted by the arrows and maintained at 1 μg/ml during the administration of A and E. The bath was drained and washed at (w). 2 gram calibration bars are shown on the right.


10 min
2g
4
w
.01
.1
1
A
E
TTX
phen
TTX
(µg/ml)

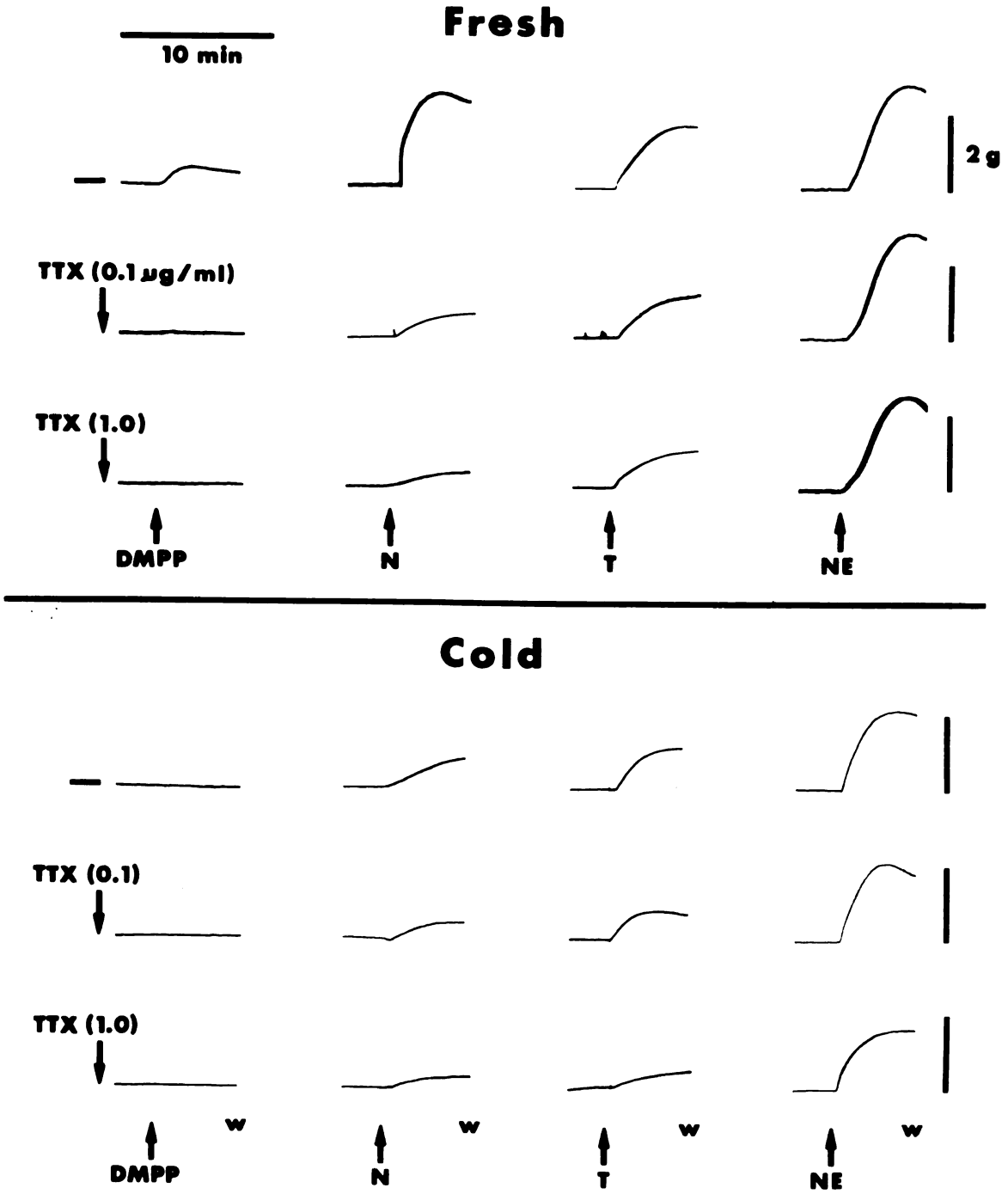
produced contractions (Figure 23). In the presence of phentolamine (50 µg/ml), TTX again had no effect on the vascular strips; however, both epinephrine and acetylcholine produced decreases in tension.

Following a period of 24 hours in cold (5-10°C) storage under anoxic conditions, the sensitivity of arterial strips to all vasoactive agents was decreased to less than 60% of that of the corresponding fresh strips. Increasing the period of storage to 48 hrs decreased the sensitivity of the strips to norepinephrine, acetylcholine and $BaCl_2$ to less than 40% of that of fresh strips and abolished the responses to 5 µg/ml of either DMPP or nicotine. Figure 24 shows the effect of DMPP (5 µg/ml), nicotine (50 µg/ml), tyramine (50 µg/ml) and norepinephrine (0.1 µg/ml) in freshly prepared arterial strips and strips following 24 hrs of cold-storage. DMPP (5 µg/ml) produced an increase in tension which was abolished by TTX (0.1 or 1.0 µg/ml) or by cold-storage (24 hrs). The increase in tension produced by 50 µg/ml of nicotine was attenuated but not abolished by TTX and/or cold storage. Increasing the dose of DMPP produced responses similar to higher doses of nicotine, and lowering the dose of nicotine yielded responses similar to lower doses of DMPP. TTX had little effect on the responses to tyramine (50 µg/ml) or norepinephrine in fresh strips as well as strips exposed to cold-storage.

Thus these data indicate that: 1) Low doses of DMPP or nicotine produced their vascular responses <u>via</u> neural mechanisms which are inhibited by TTX or cold-storage; 2) High doses of DMPP or nicotine affect both neural and non-neural mechanisms;

Figure 24

Effects of dimethylphenylpiperazinium (DMPP, 5 µg/ml); nicotine (N, 50 µg/ml); tyramine (T, 50 µg/ml); and norephinephrine (NE, 0.1 µg/ml) before and after tetrodotoxin (TTX, 0.1 and 1.0 µg/ml) in fresh and cold stored (24 hrs at 5°C) arterial strips in vitro (prep #6, Figure 6). The strips were equilibrated under a 4 gm preload tension denoted by a small dash to the left of the control responses (upper rows). The TTX concentrations are indicated at the arrows to the left of each row of tracings. Following each test response the baths were drained, washed (w) and refilled with Krebs-Ringer solution. 2 gm calibration bars are shown on the right.


Fresh
10 min
2 g
TTX (0.1 µg/ml)
TTX (1.0)
DMPP
N
T
NE
Cold
TTX (0.1)
TTX (1.0)
W
DMPP
N
T
NE

3) Tyramine produces vasoconstriction by neural and/or non-neural mechanisms which are only slightly sensitive to either TTX or anoxia, and 4) TTX appears to have no affect on the sensitivity of vascular smooth muscle in doses sufficient to block neurogenic responses; however, cold-storage under anoxic conditions decreases the sensitivity of vascular strips to drugs acting via both neural as well as direct mechanisms. TTX in doses of 0.1 or 1.0 μg/ml failed to affect the responses of acetylcholine, norepinephrine, epinephrine, isoproternol, or 5-hydroxytryptamine; however, following cold-storage the responses to these agents were greatly attenuated.

3) Effect of K^+ on arterial strips before and after $BaCl_2$ - Increasing K^+ concentration of the bath produced only an increase in tension of both fresh or cold-stored strips not exposed to $BaCl_2$. However, following an increase in tension due to $BaCl_2$, increasing the concentration of K^+ in the bath had a biphasic effect, relaxation at low concentrations and contraction at high concentrations. The magnitude of the relaxation was increased at the higher preloads and appeared to be related to the level of tension developed by $BaCl_2$ (Figure 26).

Figure 25 shows a set of four strips which produced rhythmic contractions as the concentration of K^+ in the bath was increased over the constrictor range. Although rhythmical contractions to K^+ were not generally seen, strips which responded to $BaCl_2$ by producing cyclic contractions often showed rhythmical responses to high K^+ concentrations.

Figure 25

The effect of K^+ on arterial strips equilibrated at different preloads (1, 3, 5 and 7 gm), before and after increasing tension with $BaCl_2$ (1.0 mM). The K^+ concentration was simultaneously and progressively increased in all four strips from a control of 4.7 to 19.7 mEq/l as indicated at the arrows. The baths were drained and washed as indicated by the (w). All calibration bars to the right of the tracings represent 2 grams tension.

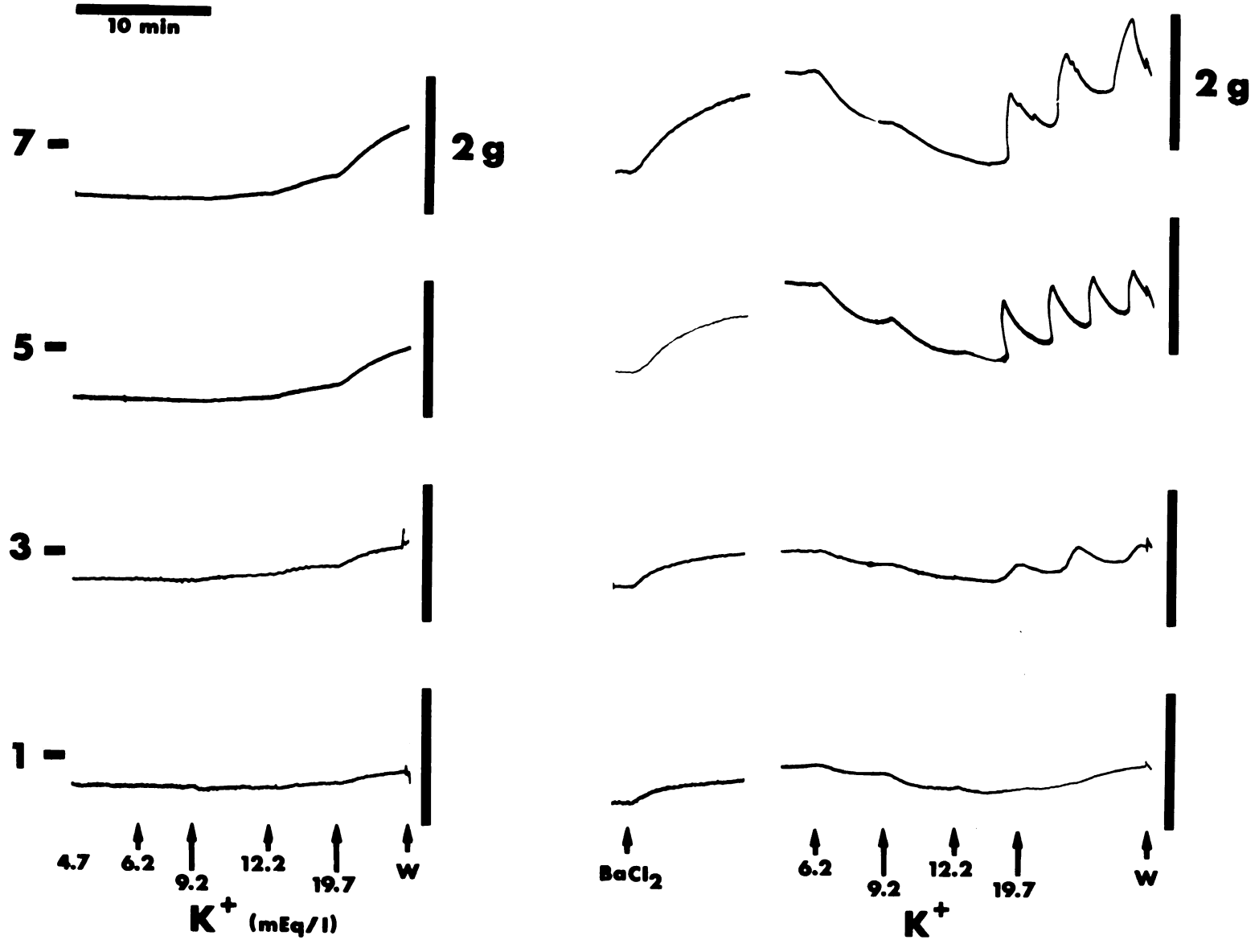

10 min
7 -
5 -
3 -
1 -
2 g
2 g
4.7
6.2
9.2
12.2
19.7
W
K+ (mEq/l)
BaCl2
6.2
9.2
12.2
19.7
W
K+

Figure 26

The effects of increasing bath K^+ on arterial strips in vitro before (solid lines) and after (dashed lines) increasing tension with $BaCl_2$ (1.0 mM). Change in tension (Δ tension) from control (denoted as 0) is shown on the ordinate (grams) as a function of bath K^+ concentration. To the right of each curve is given the average preload before and preload plus the active tension after $BaCl_2$. Mean values (N = 10) and their standard errors are shown, with asterisks denoting those points significantly ($p < 0.05$) different from their control (C, 4.7 mEq/l).

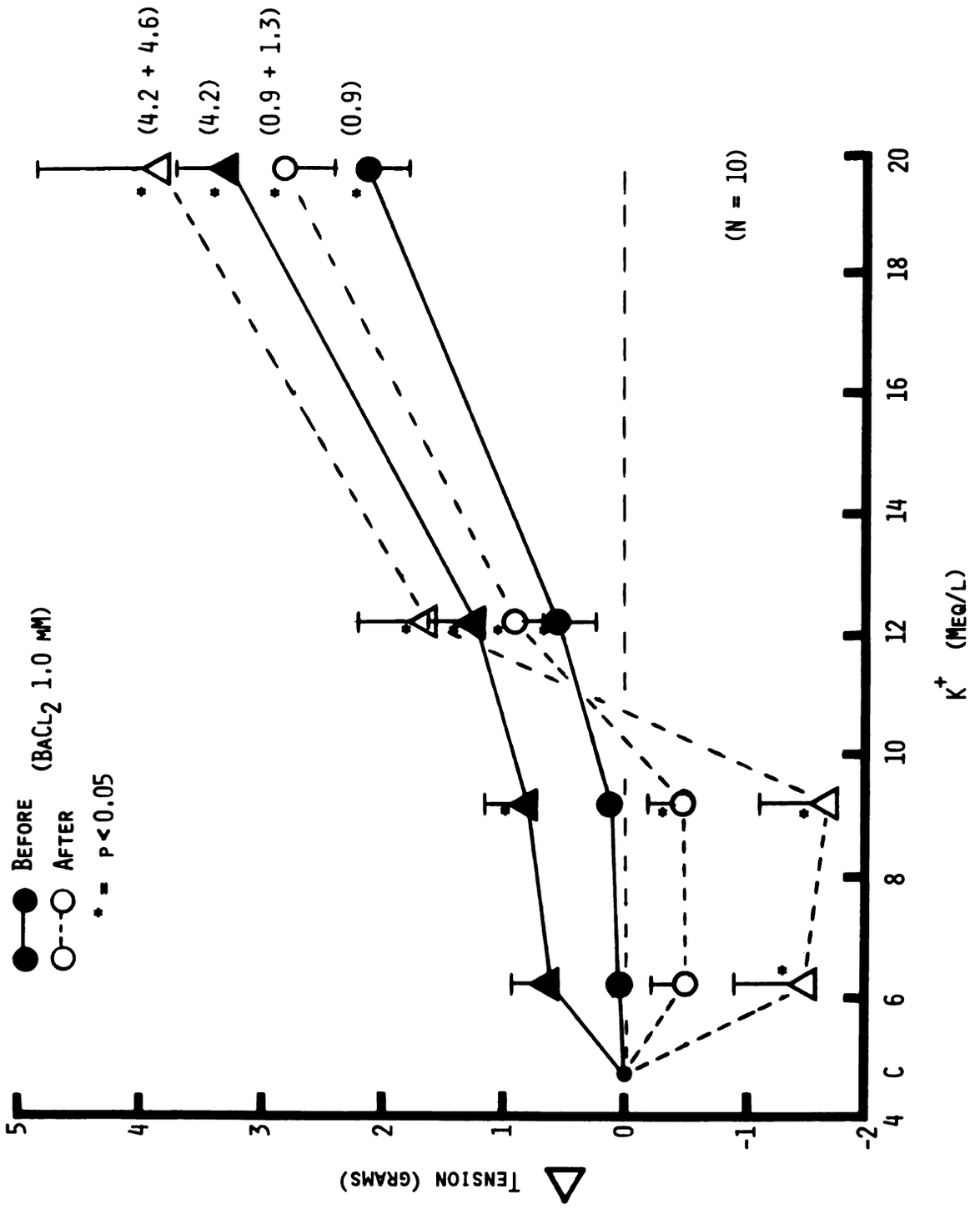

(4.2 + 4.6)
(4.2)
(0.9 + 1.3)
(0.9)
(N = 10)
BEFORE (BACL2 1.0 MM)
AFTER
* = P<0.05
TENSION (GRAMS)
K+ (MEQ/L)
C
4
6
8
10
12
14
16
18
20
-2
-1
0
1
2
3
4
5

Figure 27

Tracings of arterial (fresh) strips <u>in vitro</u> (prep #6, Figure 6) showing the tension responses to increasing bath concentrations of K^+, before (A) and after $BaCl_2$ (B - G) (1.0 mM); and in the presence of tetrodotoxin (TTX, 0.1 µg/ml) (C), phentolamine (phen, 10 - 50 µg/ml) (D); propranolol (prop, 1 - 5 µg/ml) (E); atropine (atr, 1 - 5 µg/ml) (F), and ouabain (oua, 10^{-5} m) (G). The K^+ concentration was progressively increased from the control of 4.7 mEq/l to those values given at the arrows below the tracings. The tracings on the left (A, B and C) are of the same arterial strip (equilibrated at 4 gm preload) with the K^+ concentration changed in all three as indicated by the arrows, while on the right are responses (D, E, F and G) to four different strips also equilibrated at a preload of 4 gm, with the K^+ concentration progressively increased as shown by arrows for each tracing. Numerals to the left of each tracing give the absolute tension level at the dashes. 2 gm calibration bars are shown to the right of each tracing.

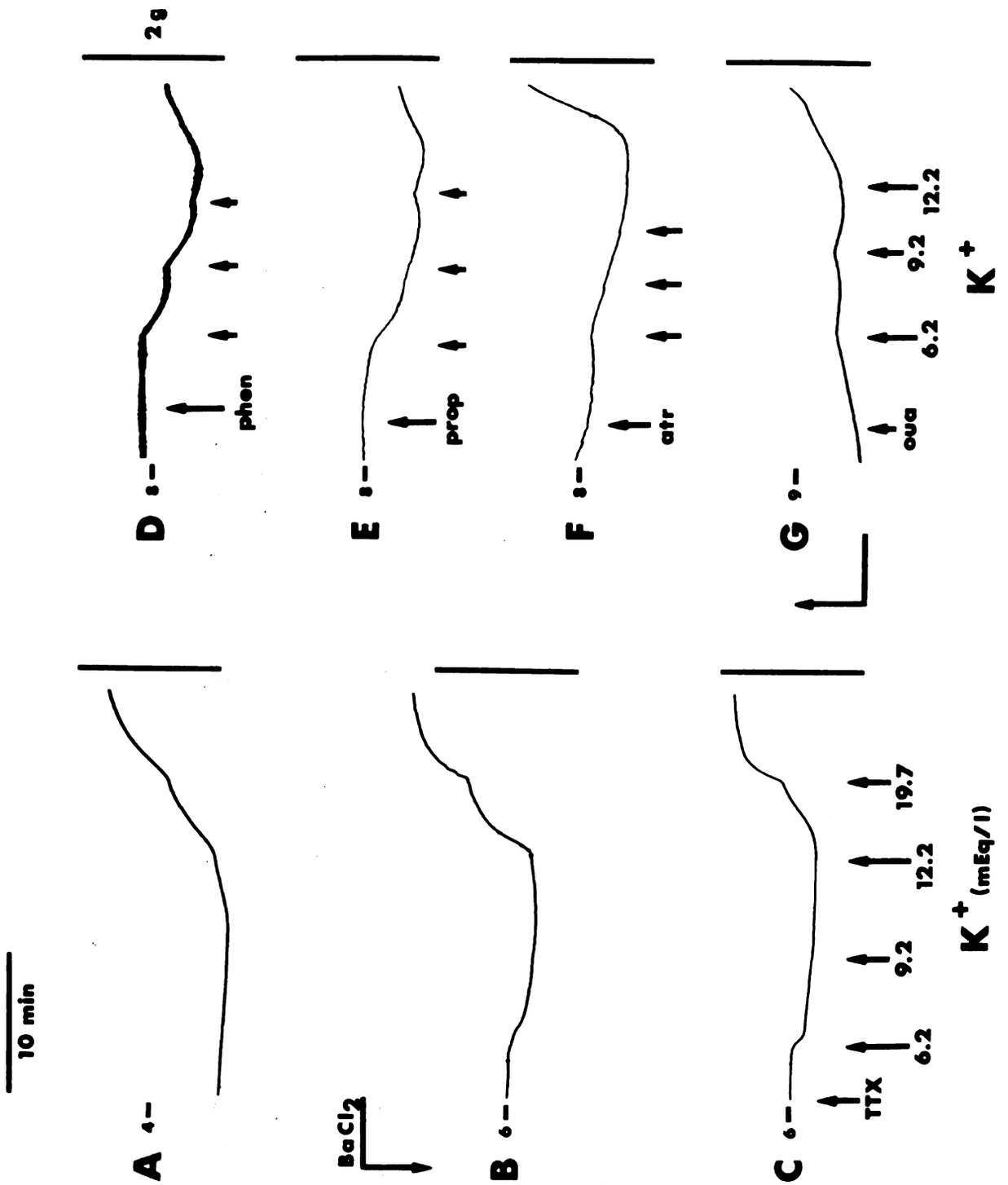

10 min
A
BaCl2
B
TTX
6.2
9.2
12.2
19.7
K+ (mEq/l)
C
2g
D
phen
E
prop
F
atr
G
oua
6.2
9.2
12.2
K+

Figure 26 shows that before actively contracting the strips, increasing K^+ from 4.7 to 19.2 mEq/l produced only an increase in tension which was greatest in the strips at higher preload levels. After $BaCl_2$, increasing K^+ in the bath over the range of 4.7 to 9.2 mEq/l produced only relaxation; however, increasing K^+ to levels above 10 mEq/l produced an increase in tension not different from that seen before $BaCl_2$.

Figure 27 shows that this dilator action of K^+ in the presence of active tension was not blocked by TTX (1.0 µg/ml), phentolamine (50 µg/ml), propranolol (5-10 µg/ml) or atropine (1-5 µg/ml); but appeared to be greatly attenuated by ouabain (10^{-5} M).

These data show that in the presence of active tension increasing extra-cellular K^+ in vitro produces responses which closely duplicate those seen in vivo.

4) Effect of hyper- and hyposmolality on arterial strips before and after $BaCl_2$ - Increasing osmolality decreased the tension of arterial strips which had been actively contracted by $BaCl_2$. However, in the absence of active tension, hyperosmolality frequently increased tension of strips equilibrated at high preloads (4 or 6 gm) (Figure 28 and 29). Hyposmolality produced an increase in tension before and after contracting the strips with $BaCl_2$. The responses of vascular strips were quantitatively and qualitatively different depending upon the manner in which osmolality was altered.

Figure 28 shows that before $BaCl_2$, increasing bath osmolality to 345 mOsm/Kg by the addition of a concentrated Krebs-Ringer

solution, thus increasing bath concentration of all ions, produced a significant increase in tension. Notice that the increment of increase for any given change in osmolality was greater in the strip receiving a concentrated Krebs-Ringer solution than the change in tension produced by a hyperosmotic solution in which osmolality was increased by d-mannitol. After actively contracting the strips with $BaCl_2$, increasing osmolality to as much as 390 mOsm/Kg produced only a relaxation. This decrease in tension was greater when osmolality was increased by d-mannitol as compared to the concentrated Krebs-Ringer solution (Figure 28). Although the increase in tension induced by hyperosmolality was potentiated at higher preloads, a small decrease in tension was frequently seen when osmolality was increased 10 to 40 mOsm/Kg in strips equilibrated at 4 or 6 gm (Figure 29). Hyperosmolality generally failed to increase tension until osmolality exceeded 350 mOsm/Kg.

The relaxation produced by hyperosmolality following $BaCl_2$ increased in magnitude as preload was increased and appeared to be related to the level of active tension, this relaxation was not sensitive to phentolamine (10-50 µg/ml), propranolol (1-5 µg/ml), atropine (1-5 µg/ml), or TTX (0.1-1.0 µg/ml) and closely duplicated the vascular responses to hyperosmolality which have been observed in situ (111).

Decreasing osmolality by the addition of distilled water generally failed to change the tension of strips equilibrated at high or low preloads; however, distilled water occasionally decreased tension in strips preloaded with 6 gm. Following $BaCl_2$ the addition of

distilled water produced an initial decrease in tension followed by a gradual increase, thus showing little net change in tension when bath osmolality was sequentially decreased to 255 mOsm/Kg. However, when osmolality was lowered by decreasing only the Na^+ concentration, an increase in tension developed before and after actively contracting the strips with $BaCl_2$ (Figure 28). This increase in tension induced by hyposmolality was unaffected by phentolamine (10-50 µg/ml), propranolol (1-5 µg/ml), atropine (1-5 µg/ml) or TTX (0.1-1.0 µg/ml).

Action of TTX on Visceral Smooth Muscle in Naturally Or Pump-Perfused Intestine

1) Effect of TTX on spontaneous motility of the ileum and the motility during vagal stimulation - Local intra-arterial infusion of TTX produced either a decrease or increase in spontaneous activity of the naturally perfused ileum (Figure 31).

In intestinal segments exhibiting spontaneous motor activity, infusion of TTX in low doses of less than 1 µg/min occasionally depressed the ongoing motility. The decrease in motility occurred within one minute after starting TTX, and generally remained depressed during the infusion period. Spontaneous activity of the segment failed to return to pre-infusion levels within 20 minutes after stopping TTX.

In quiesent segments showing little or no spontaneous motor activity TTX in doses of 1.0 µg/min or more generally stimulated motility (Figures 18, 31, 32). The increase in motility induced by TTX wained with time and generally returned to pre-infusion

Figure 28

Tracings showing the effect on fresh arterial strips (prep #6, Figure 6) of increasing osmolality (A and B) and decreasing osmolality (C and D) before and after tension was increased by $BaCl_2$ (1.0 mM) given as indicated at the arrow. Osmolality of the baths were increased or decreased from the controll of 300 mOsm/Kg to the osmotic values (mOsm/Kg) given at the arrows by adding (A) hyperosmotic Krebs-Ringer solution (1000 mOsm/Kg) in which all ions were increased (B) hyperosmotic Krebs-Ringer solution (1000 mOsm/Kg) in which osmolality was increased by d-mannitol, (C) distilled H_2O and (D) hyposmotic Krebs-Ringers solution depleted of NaCl. The dashes to the left of each tracing represents 4 grams tension. All the baths were drained and washed as denoted by w. Calibration bars to the right represent 2 grams.

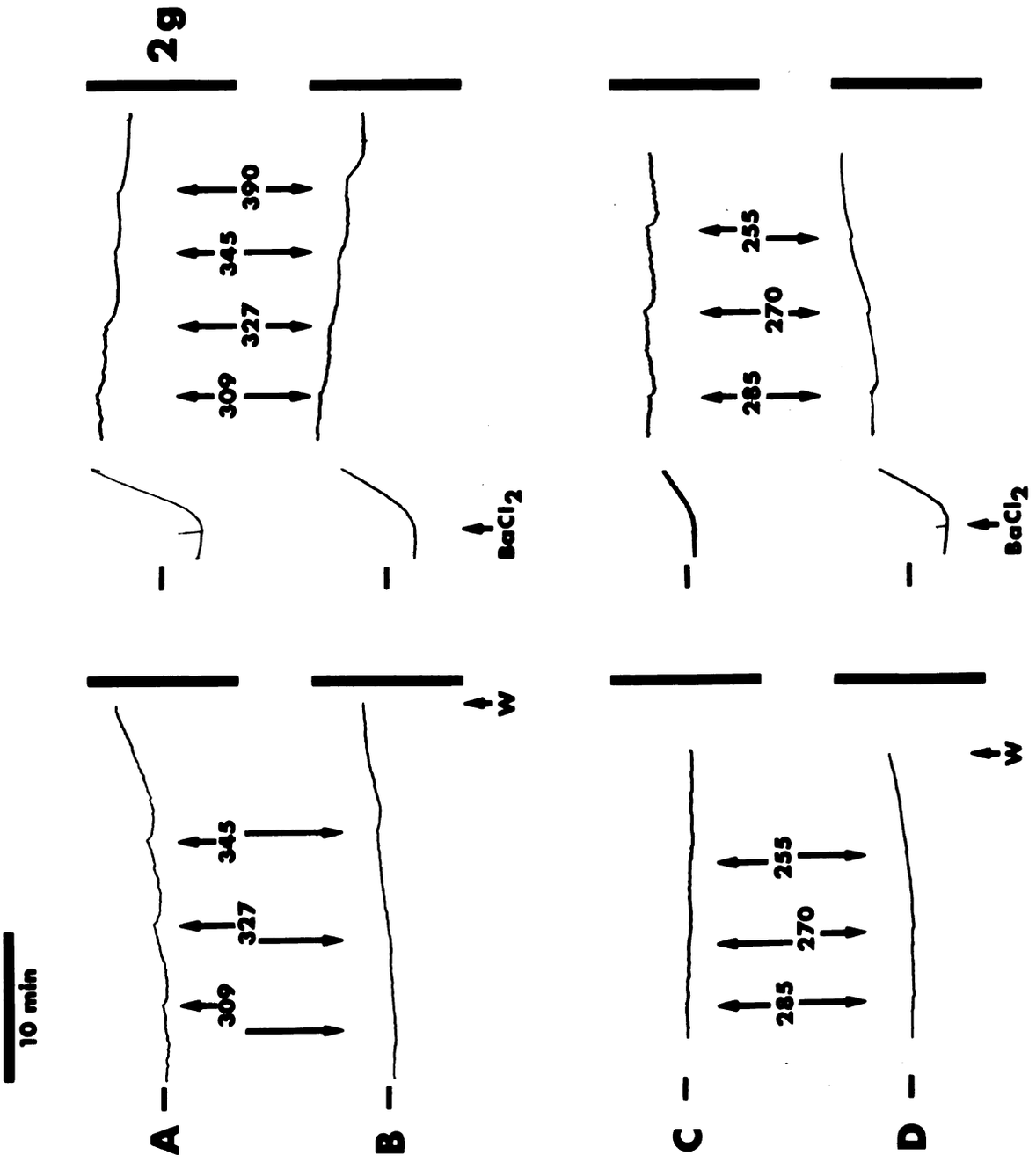
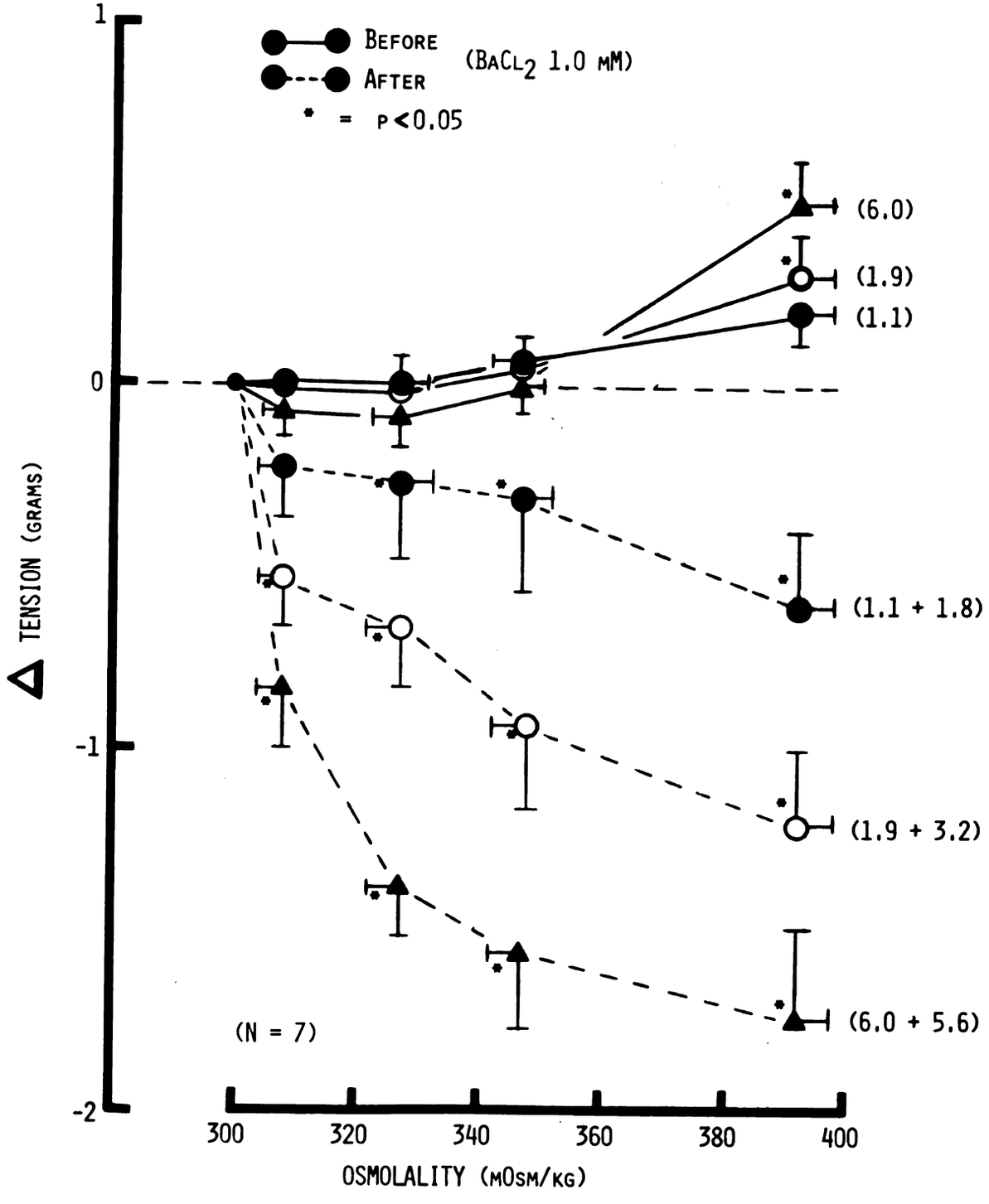

2g
10 min
A
B
C
D
309
327
345
390
285
270
255
W
BaCl2

Figure 29

Effect of three different preloads and increasing osmolality on the tension of fresh arterial strips in vitro (prep #6, Figure 6), before (solid lines) and after (dashed lines) actively contracting the strips with 1.0 mM $BaCl_2$. To the right of each curve is given the average preload tension before and the corresponding preload plus active tension after $BaCl_2$. Osmolality was increased as shown on the abscissa by adding to the bath a Krebs-Ringer solution in which osmolality was increased by d-mannitol. Mean values (N = 7) and their standard errors are shown, with the asterisks denoting those values significantly different ($p < 0.05$) from their controls (300 mOsm/Kg).


BEFORE
AFTER
($BaCl_2$ 1.0 mM)
* = P<0.05
(6.0)
(1.9)
(1.1)
(1.1 + 1.8)
(1.9 + 3.2)
(6.0 + 5.6)
(N = 7)
Δ TENSION (GRAMS)
1
0
-1
-2
300
320
340
360
380
400
OSMOLALITY (MOSM/KG)

values in 10-20 minutes. The magnitude of increase in motility varied from slightly detectable increases to large rhythmical contractions producing increases of 10 to 30 mmHg in luminal pressure.

The local neural blocking action of TTX was studied by evaluating its efficacy in abolishing the motility responses to electrical stimulation of the decentralized vagi (6v, 6 cps, and 16 msec). TTX in doses as low as 0.1 μg/min was effective in depressing the motility response to vagal stimulation. However, TTX doses of less than 2 μg/min generally required prolonged infusion periods of more than 15 minutes to produce an effective block. If the TTX dose was increased to 2.5 or 5.0 μg/ml complete block to vagal stimulation was obtained in less than 10 minutes and could be maintained by a TTX dose of 1/10 to 1/4 that of the initial blocking dose. Figure 30 shows that TTX gradually depressed the motility response to vagal stimulation, and completely abolished the response at a TTX dose of 2.5 μg/min. Placing TTX (10 μg/ml) in the intestinal lumen attenuated the motility response to vagal stimulation only after a 15 minute exposure period. Increasing the period of exposure to 30 minutes failed to abolish the response.

When TTX decreased spontaneous motor activity, vagal stimulation still produced an increase in motility; but when TTX stimulated motility, vagal stimulation generally had little effect. When the motility responses to vagal stimulation were effectively depressed or abolished the changes in resistance to carotid occlusion were also abolished but the increase in systemic pressure to local infusion of KCl was only attenuated.

Figure 30

Intraluminal pressure (ILP) and systemic arterial pressure (SAP) responses in mmHg to electrical stimulation (thick horizontal bars) of the cut vagi during local intra-arterial (i.a.) infusion (ml/min) of tetrodotoxin (T, 5 μg/ml), normal saline (NS), and acetylcholine (Ach, 0.5 μg/bolus) into two adjacent, naturally perfused ileal segments (A and B) (prep #3) ——— Control responses (C) are shown on the left. Breaks in the tracings represent periods of 5 - 20 min with the thin horizontal bars below each tracing representing the periods of i.a. infusion.

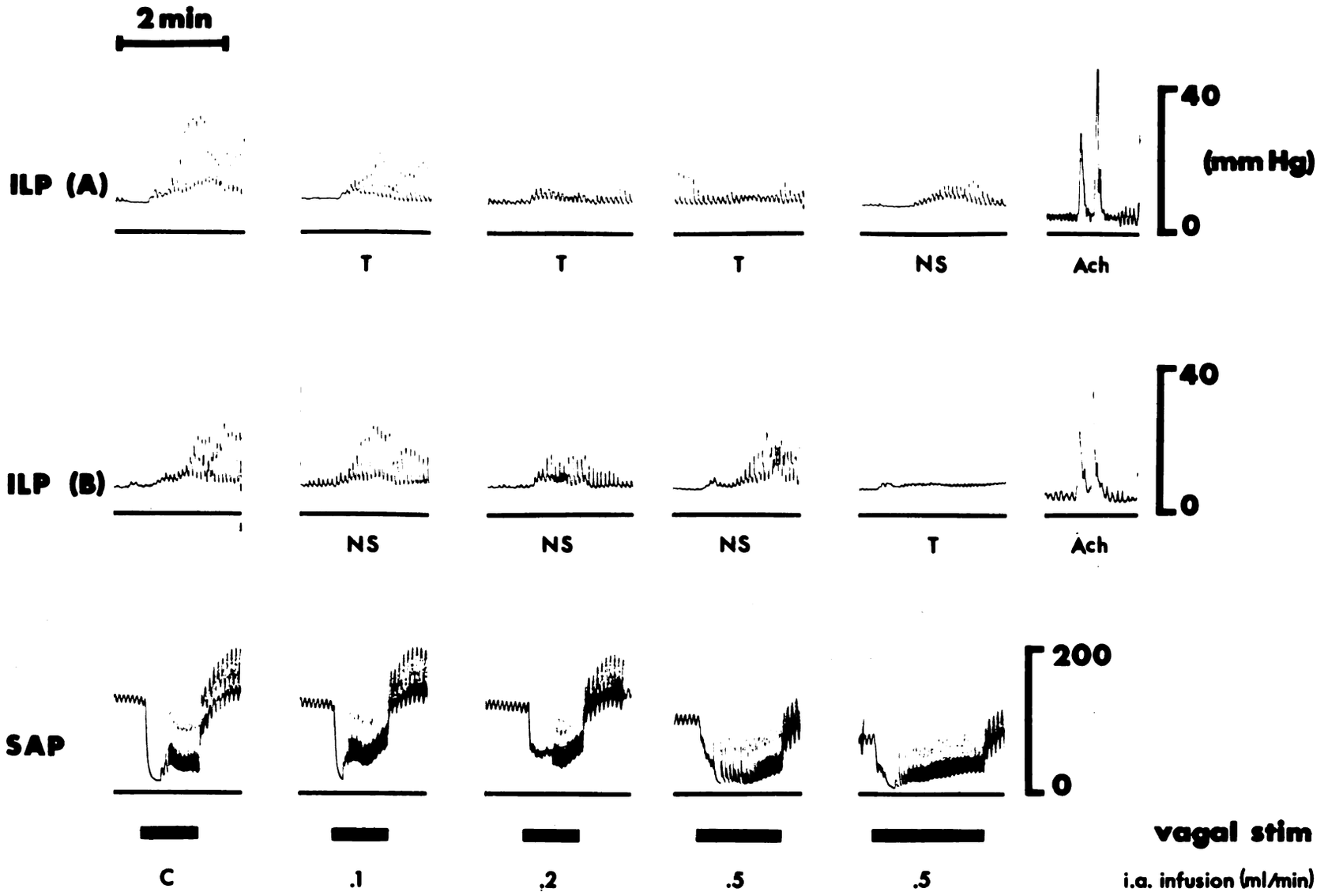
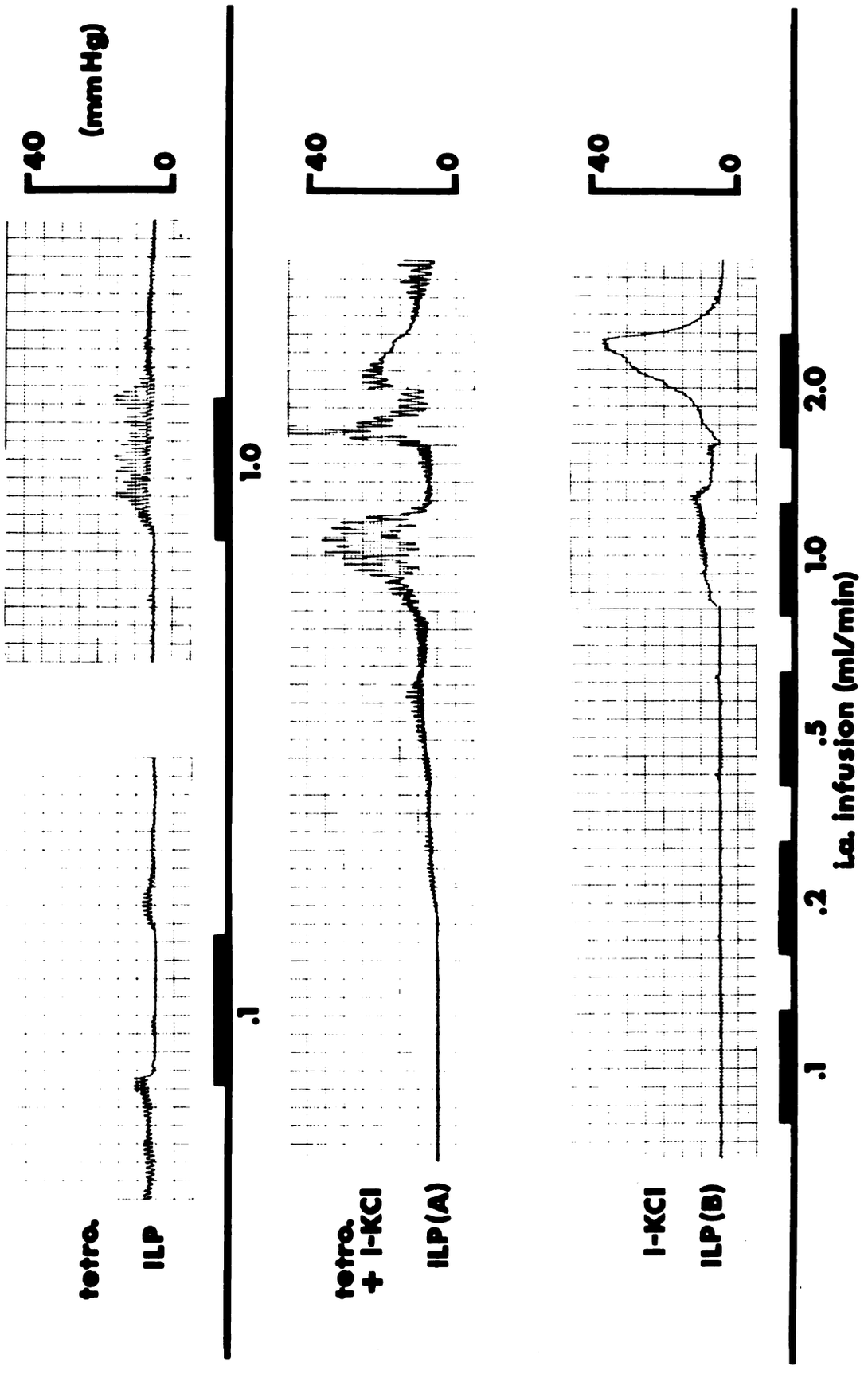

2 min
ILP (A)
T
T
T
NS
Ach
40
(mm Hg)
0
ILP (B)
NS
NS
NS
T
Ach
40
0
SAP
200
0
vagal stim
C
.1
.2
.5
.5
i.a. infusion (ml/min)

Figure 31

Intestinal (ileum) motility responses to intra-arterial (i.a.) infusion of tetrodotoxin (5 µg/ml) and isosmotic potassium choloride (I-KCl; 300 mOsm/Kg). Intraluminal pressure responses (ILP) are shown in mmHg as indicated by the calibration bars on the right. The periods and rates of infusion (ml/min) are indicated by the thick horizontal bars and numerals respectively, below each set of tracings. The upper set of tracings shows the responses of a single naturally perfused ileal segment (prep #3, Figure 4) to tetrodotoxin infused (2 min 20 sec) at two different rates (0.1 and 1.0 ml/min). The lower set of tracings shows the responses of two adjacent segments (A and B) to infusions (2 min) of KCl alone (B) and KCl + tetrodotoxin (5 µg/ml) (A).


40
0
(mm Hg)
tetra.
ILP
.1
1.0
tetra.
+ I-KCl
ILP(A)
I-KCl
ILP(B)
.1
.2
.5
1.0
2.0
La. infusion (ml/min)

Notice in Figure 30 that systemic pressure gradually decreased when TTX was allowed to enter the systemic circulation. This hypotensive action was generally seen if 30 to 50 µg of TTX had been returned to the animal. As systemic hypotension developed the cardiac response (bradycardia) to vagal stimulation progressively decreased.

2) Effect of TTX and C_6 on the motility responses to i.a. KCl, hyperosmotic NaCl, nicotine, acetylcholine, and epinephrine - Intra-arterial infusion of isosmotic KCl, hyperosmotic NaCl, nicotine and acetylcholine all stimulated intestinal motility; while epinephrine exhibited an inhibitory action (Figures 31, 32, 33 and 34). Hexamethonium (C_6) (0.5 µg/min) and TTX (1.0 µg/min) also stimulated motility; however, C_6 converted the response to nicotine from stimulation to inhibition and TTX depressed or abolished the response to nicotine and hyperosmotic NaCl (Figure 32). Neither C_6 nor TTX in the doses studies changed the response to acetylcholine or epinephrine.

KCl - Local infusion of isosmotic KCl stimulated motility of both natural ($\bar{x}$ weight = 19.8 gm, $\bar{x}$ flow = 10.5 ml/min) and pump-perfused ($\bar{x}$ weight = 31 gm, $\bar{x}$ flow = 16 ml/min) intestine (Figures 31, 33, and 34).

Figure 31 shows that when isosmotic KCl was infused locally into a neurally intact, naturally perfused ileal segment intraluminal pressure (ILP) increased. Although numerous rhythmical contractions appeared to occur at an infusion rate of 0.5 ml/min the responses were generally too small to be recorded. Increasing the rate of infusion to 1.0 ml/min produced a generalized

constrictor response exhibiting little evidence of rhythmical phasic contractions. Occurring concomitantly with the increase in ILP was a decrease in venous outflow. KCl at 2 ml/min produced a rapid increase in ILP along with a complete blanching of the segment. Isosmotic KCl in the presence of TTX (5 μg/ml) produced a stimulatory response exhibiting large rhythmical contractions. During infusion of TTX, the stimulatory effect of KCl occasionally appeared at lower infusion rates (0.2 and 0.5 ml/min) but was most evident at 1 and 2 ml/min.

The effects of KCl on motility of the constantly perfused jejunum were similar to those of the naturally perfused intestine (Figures 33 and 34). KCl alone produced an increase in motility exhibiting large changes in baseline pressure with little evidence of phasic rhythmical responses. C_6 had little effect on the motility responses to KCl but TTX attenuated the increases in baseline pressure (Figure 33) and augmented the phasic pressure responses (Figure 34).

Hyperosmotic NaCl - Local infusion of a hyperosmotic (1500 mOsm/Kg) NaCl solution produced both an increase in baseline pressure and phasic contractions (Figures 33 and 34). The stimulatory effect of NaCl (1500 mOsm/Kg) was often evident at an infusion rate of 0.2 ml/min but was not significantly altered until infusion rate was increased to 0.5 ml/min. C_6 (o.5 μg/ml) did not change the increase in pressure produced by NaCl (1500 mOsm/Kg) and only slightly decreased the phasic responses at the highest infusion rate of 2 ml/min. However, TTX (1.0 μg/min) completely abolished both the increases in baseline pressure and the phasic responses.

Nicotine, acetylcholine, and epinephrine - Figure 32 shows representative tracings of the intestinal motility responses to 1 ml intra-arterial injections of nicotine (50 μg), acetylcholine (3 μg) and epinephrine (3 μg) before and during infusion of Hexamethonium (C_6) (0.5 mg/min) or tetrodotoxin (TTX) (1.0 μg/min).

Infusion of C_6 or TTX stimulated motility which decreased in magnitude with time. Bolus injections of nicotine (50 μg) into the relatively quiesant gut stimulated motility before C_6 but during C_6 infusion nicotine inhibited motility. TTX abolished both the stimulation and inhibition produced by nicotine. Injections of acetylcholine (3 μg) stimulated and epinephrine (3 μg) inhibited motility before and during C_6 or TTX.

3) Effect of TTX on the intestinal motility produced by luminal placement of hyperosmotic NaCl, KCl and glucose - Luminal placement of hyperosmotic NaCl (1500 mOsm/Kg), Kcl (1500 mOsm/Kg), glucose (1000 to 3000 mOsm/Kg) and polyethylene glycol (1000 to 3000 mOsm/Kg) all increased baseline luminal pressure and elicited rhythmical contractions of the jejunum. TTX abolished the responses to NaCl, glucose and polyethylene glycol but only attenuated the responses to KCl.

Hyperosmotic NaCl - Placing 10 ml of a hyperosmotic NaCl solution (1500 mOsm/Kg) in the lumen of naturally perfused jejunum ($\bar{x}$ wt = 19.8 gm, $\bar{x}$ flow - 10.5 ml/min) stimulated motility. An increase in both baseline pressure and phasic pressure changes frequently appeared within the first 3 minute collection period. Generally the increase in intra-luminal pressure exceeded 30 mmHg

Figure 32

Representative tracings of intestinal motility responses to intra-arterial (i.a.) injections (1 ml) of nicotine (N, 50 μg), acetylcholine (Ach, 3 μg) and epinephrine (Epi, 3 μg) before and during local i.a. infusion of hexamethonium (C_6; 0.5 mg/min) or tetrodotoxin (TTX, 1.0 μg/min) into the constantly perfused jejunum (prep #5, Figure 5). Calibration bars on the right indicate the absolute intraballoon pressure in mmHg. Control responses (C) for all test conditions (pre-drug, during C_6, and during TTX) are shown on the left. Administration of the drugs and test agents are denoted by the arrows.

INTESTINAL MOTILITY

I.A. BOLUS INJECTIONS OF NICOTINE(N), ACETYLCHOLINE AND EPINEPHRINE

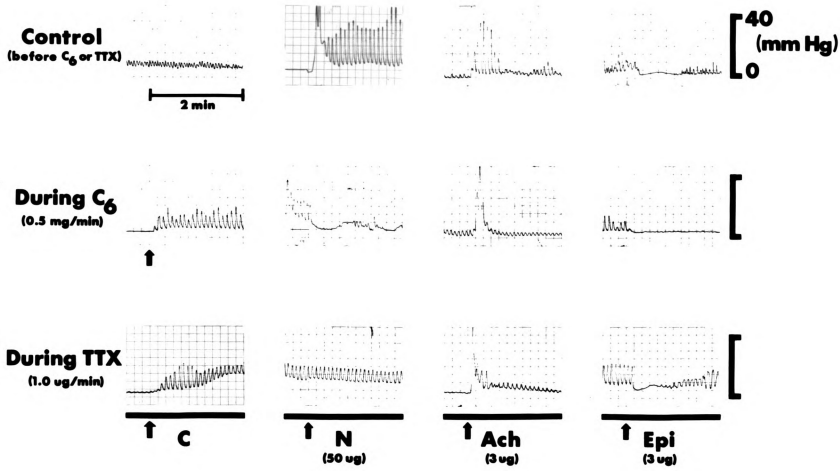


Figure 33

Effects of tetrodotoxin (TTX, 1.0 μg/min) and hexamethonium (C_6, 0.5 mg/min) on the baseline tension of the constantly perfused jejunum (prep #5, Figure 5), to local intra-arterial (i.a.) infusion of isosmotic NaCl (300 mOsm/l), hyperosmotic NaCl (1500 mOsm/l) and KCl (300 mOsm/l). Each datum point represents the mean intraballoon pressure response (ILP) (N = 9) in mmHg to i.a. infusion (ml/min) of the test agents alone (●), during C_6 (○) or during TTX (Δ)

BASELINE INTRABALLOON PRESSURE

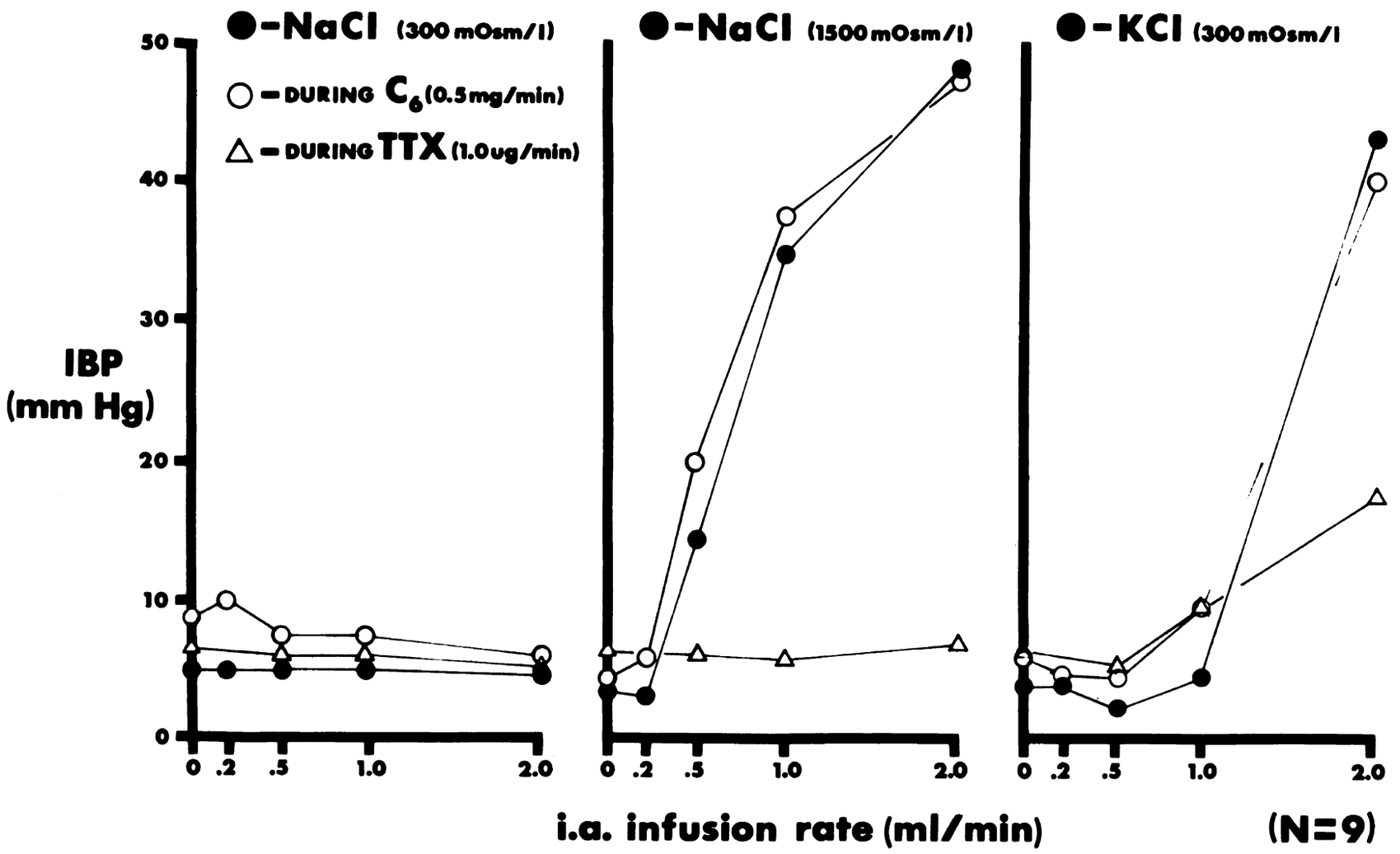
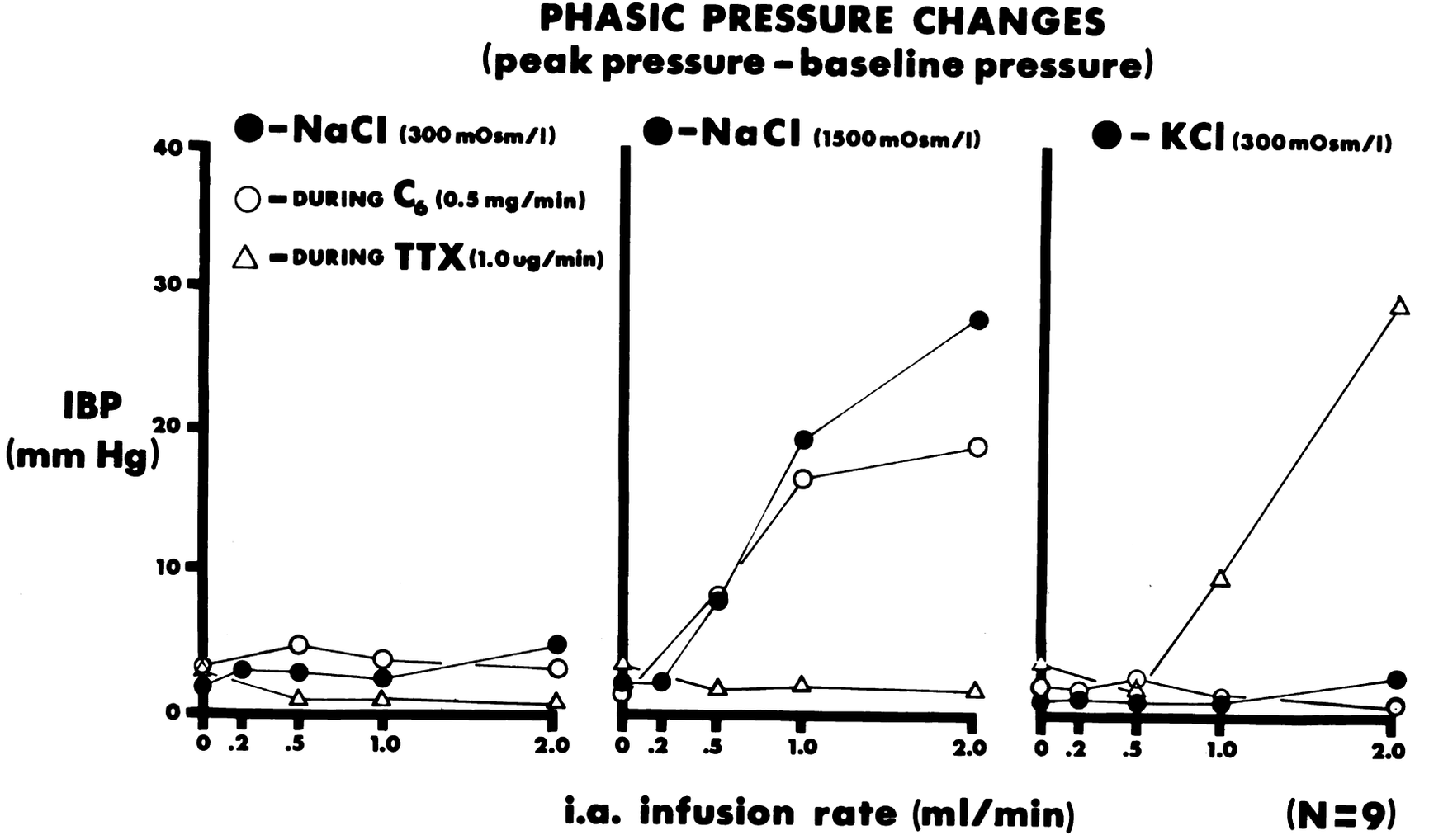


Figure 34

Effect of hexamethonium (C_6) and tetrodotoxin (TTX) on phasic motility of the constantly perfused jejunum (prep #5, Figure 5) to local intra-arterial (i.a.) infusion of isosmotic NaCl (300 mOsm/l), hyperosmotic NaCl (1500 mOsm/l) and KCl (300 mOsm/l). Each datum point represents the mean (N = 9) intraballoon pressure (IBP) in mmHg as shown on ordinate. Each test solution was infused before (●), and then during C_6 (0, 0.5 µg/min), or TTX (Δ, 1.0 µg/min) at progressively increasing rates as indicated on the abscissa (ml/min).


PHASIC PRESSURE CHANGES
(peak pressure - baseline pressure)
● - NaCl (300 mOsm/l)
○ - DURING C6 (0.5 mg/min)
△ - DURING TTX (1.0 ug/min)
● - NaCl (1500 mOsm/l)
● - KCl (300 mOsm/l)
IBP
(mm Hg)
40
30
20
10
0
0 .2 .5 1.0 2.0
i.a. infusion rate (ml/min)
(N=9)

7-9 minutes following introduction of the NaCl solution into the lumen. TTX at a dose (0.5-2.0 μg/min) sufficient to block the increases in motility to vagal stimulation also abolished the increase in motility during luminal placement of hyperosmotic NaCl.

Hyperosmotic KCl - Luminal placement of hyperosmotic KCl (1500 mOsm/Kg) produced an increase in luminal pressure usually within the first minute following introduction of the solution. The response was characterized primarily by an increase in baseline pressure, frequently exceeding 50 mmHg, yet phasic contractions were generally superimposed on the increased baseline pressure. Intra-arterial infusion of TTX (0.5-2.0 μg/min) significantly depressed or abolished the vagally induced increases in motility and attenuated but did not abolish the responses to luminally placed hyperosmotic KCl. Although TTX increased the phasic responses to KCl, this increase was significantly less than that seen during i.a. administration of KCl.

Glucose - Luminal placement of 50 percent glucose and 85 percent PEG solutions increased base luminal pressure and caused rhythmic contractions of the segment in all animals studied. However, 2.5 percent and 5.4 percent clucose or 8.5 percent PEG solutions failed to alter luminal pressure, while a 20 percent glucose or 34 percent PEG solutions caused minimal or no changes in pressure. Fifty percent glucose produced a small change in motility during the first three minutes in the lumen. A further increase in motility which was apparent during the second and third flow periods was not accompanied by a significant change in blood flow.

Introduction of dibucaine (0.4% in saline) into the lumen for 20 minutes abolished both the vascular and motility responses to 50% glucose, however, TTX (0.5 to 2.0 µg/min) abolished only the increase in motility. Following either dibucaine or TTX the vasculature responded to intra-arterial infusions of isoproterenol, isosmotic KCl, and hyperosmotic NaCl. Dibucaine per se increased motility which wained with time but disappeared only after the dibucaine was removed from the lumen and replaced with isosmotic PEG.

Intra-arterial infusion of only 16.4 percent glucose increased jejunal lumen pressure and caused rhythmic contractions at and above infusion rates of 1.0 ml/min.

DISCUSSION

Tetrodotoxin (TTX) blocks propagated action potentials in nerves (61, 73, 96, 97, 100) but has been reported to have little effect on smooth muscle (33, 39, 73). If TTX depresses only neurogenic responses it should prove beneficial in studying neural regulation of vascular and/or visceral smooth muscle. However, due to recent claims that TTX has a direct smooth muscle action (74, 77, 88), and before using this poison to study local neural regulation of intestinal blood flow it is necessary to establish more clearly the effects of TTX on vascular and visceral smooth muscle. Therefore, the reactivity of smooth muscle to TTX and other vasoactive agents were studied both *in situ* and *in vitro*.

The Action of TTX on Vascular Smooth Muscle

Skeletal muscle vascular responses - Local infusion (i.a.) of TTX increased blood flow and decreased vascular resistance of innervated gracili muscle but failed to demonstrate any vascular effect in denervated preparations (Figures 7, 8, 9, 10, 14). These data which agree with earlier work by Ishihara (61) and Feinstein and Paimre (33) suggest that the vascular action of TTX was primarily via neural mechanisms. In view of the fact that acutely denervated preparations were seldom affected by TTX in doses (0.05-10.0 µg/min, i.a.) adequate to depress both the carotid reflex responses (Figure 11, 12, 13) and the skeletal muscle responses to motor nerve stimulation it appears that any direct vasodilator property of TTX, as reported by Kao *et al.* (74, 77, 88), is negligible.

The direct vasodilator action of TTX is claimed to occur at concentrations (0.5-2.0 μg/Kg) much lower than those necessary to abolish nerve mediated responses (74, 77, 88). In the present study the vasodilation induced by TTX appeared to increase concomitantly with neural blockade and maximum vasodilation was reached when nerve mediated responses were completely abolished (Figures 11, 12, 13). Although it is true in this present study and previous studies by others that TTX was not administered via the same route, the doses are comparable. Assuming complete vascular distribution, the blood concentrations of TTX in this study (4×10^{-9} g/ml) as well as those reported by Feinstein and Paimre (10^{-8}-10^{-7} g/ml, based on a blood volume of 8% body weight) were in part within the range of those reported (4×10^{-9}-2.5×10^{-8} g/ml, 0.2-2.0 μg/Kg) to exhibit direct vasodilator actions, yet neither this study nor Feinstein and Paimre's (33) demonstrated vasodilation independent of neural blockade. This, suggests that statistically significant vasodilation results via neural mechanisms at both low and high TTX concentrations.

Although this vasodilator action of TTX was first implied to occur only at low doses (0.3-0.8 μg/Kg), TTX-induced vasodilation by high doses (2.0 μg/Kg) has been subsequently reported to result from both a direct smooth muscle action as well as release of vasomotor tone (77, 88). Therefore, high doses of TTX should also produce vasodilation when neural influences are eliminated. Yet inthis present study TTX in high (5×10^{-8}-1×10^{-6} g/ml blood) or low concentrations (5×10^{-9}-1×10^{-7} g/ml blood) failed to show

any significant increase in venous outflow (Figure 8, 9) or decrease in vascular resistance (Figure 10) of denervated gracili muscles. The slight but insignificant increase in venous outflow of acutely denervated muscle (Figure 8, 9) to TTX may indicate a variable degree of neural activity elicited by injury potentials. Of importance is that these changes were not significant and were quantitatively similar for both high and low doses of TTX.

The failure in these studies to show a systemic hypotension until late in the experiment has been of concern to some investigators including Kao and co-workers (72, 74). It is generally agreed that 2 μg/Kg of TTX given systemically produces an immediate and profound systemic hypotension (33, 73, 88). But in the present studies the venous outflow containing TTX was either discarded and the volume replaced with dextran or if returned to the animal the total volume in which it was contained included a reservoir (500 ml). Furthermore, the absolute amount of TTX given (2-20 μg) over a 10-15 minute test period (dogs weighing 17-20 Kg) was well below the hypotensive dose (2.0 μg/Kg). In view of the dilutional effect of the reservoir, the relative low total doses and intra-arterial administration, it is not surprising (32) that hypotension was seldom seen until late in the experiment (45-60 min) or after subsequent TTX infusions (Figure 30 and Table 1, 2, 3).

The claim (74, 77, 88) that TTX produces vasodilation <u>via</u> a direct effect on smooth muscle is based on the following observations: 1) TTX (i.v. or i.a.) produced vasodilation in dogs and cats but failed to block the reflex vascular responses of the pump perfused

gracilis, hindlimb or head, 2) TTX in vasodilating doses often failed to block the vascular responses to electrical stimulation of the sympathetic chain (L_1-L_4), and 3) TTX-induced vasodilation was not blocked by alpha or beta adrenergic blocking drugs, antihistamines, or adrenergic depleting agents. However, relevant to the question and damaging to his contention is the work by Kao himself (77) that showed a progressive decrease in magnitude of the vascular reflex responses accompanied by an increase in vasodilation as the TTX dose was increased. They also show that in the cat the beta-blocking agent, pronethalol (5 mg/Kg), which in the dog failed to block the response to 0.1 μg/Kg of TTX, abolished the response to 20.0 μg/Kg of TTX, yet the response was unaffected by propranolol (5 mg/Kg). In claiming that alpha-adrenergic blockade has no effect on TTX induced vasodilation, Lipsius et al. (88) fail to show either a control response to TTX (1.0 μg/Kg) or a saline dilution response before simultaneous administration of phenoxybenzamine (15 mg/Kg), phentolamine (1.5 mg/Kg) and bretylium (5 mg/Kg). Also in a subsequent study Kao et al. (77) reported that alpha-adrenergic blocking agents have no effect on vasodilation induced by TTX but never show data confirming their previous work. Lastly, Kao (74) shows that systemic administration of TTX (0.8-1.6 μg/Kg) into the recipient animal simultaneously produced a decrease in systemic pressure and a reflex increase in resistance of the cross-perfused gracilis. However, the tracings from two different cats do not allow one to compare the responses quantitatively. Therefore, the findings of Kao et al. (74, 77, 88) may in fact be somewhat similar to those of other investigators.

It is well known that TTX in concentrations of 10^{-9} g/ml is effective in depressing or abolishing nerve conduction (20, 73, 76, 95). The toxin in concentrations of less than 10^{-5} g/ml has also been reported to have no effect on the tension of isolated arterial strips (80, 118), electrical activity of arterial (80), venous (58) or cardiac (33) smooth muscle, or resistance of microperfused vessels (11). Therefore, it seems unlikely that the responses reported by Kao et al. (77, 88) are independent of neural mechanisms. The dissimilarity between Kao's work and those of other investigators may suggest important differences in the experimental techniques and/or interpretation of the data. Species differences does not appear to be an important factor in that Kao and co-workers (77, 88) report vasodilation via direct actions in both dogs and cats.

Further support of the selective action of TTX on neural tissue is its failure to affect either the venous-arteriolar response or reactive dilation in skeletal muscle (present study) or intestine (Figure 19). Both of these vascular phenomena involve only non-neural mechanisms (48, 50, 65, 134). The increase in vascular resistance accompanying an increase in venous pressure (variously named veni-vasomotor reflex, 134; venous-arteriolar reflex, 48, 50; or venous-arteriolar response, 65) apparently results from a direct stimulation of vascular smooth muscle upon stretching (myogenic hypotheses, Bayliss response, 62, 65); while the decrease in vascular resistance following a period of ischemia (reactive dilation) is the result of accummulation of vasoactive metabolites (chemical hypotheses, 48, 51, 52). Therefore, either or both of these

phenomena should be altered if TTX directly affects vascular smooth muscle. Failure of TTX to depress or inhibit either of these responses strongly suggests that TTX, in concentrations of $< 10^{-5}$ g/ml has minimal if any direct vascular action.

In agreement with Nagle et al. (94), elevation of venous pressure in this present work by partial venous obstruction or elevation of the outflow cannula always raised vascular resistance of both innervated and denervated canine gracilis muscle preparations. Nagle et al. (94) showed that the rise in total vascular resistance was accompanied by a fall in venous resistance thus they concluded that pre-capillary resistances must have increased. However, in Nagle's studies all post-capillary vessels were assumed to respond similar to the studied large vessel segments. Yet, the present study and Nagle's findings are in agreement with Johnson (65) who first studied the venous-arteriolar responses by indirect determination of capillary pressures using an isogravimetric method and reported that blood flow in the intestine is adjusted to meet tissue demands by an intrinsic response not susceptible to neural blocking agents. However, changes in post-capillary resistance during alterations in perfusion pressure are susceptable to neural blocking agents, thus indicating the involvement of neural elements in the arterio-venous reflex (56, 66). Of interest in the present study was that when the venous cannula was raised 20 cm the increase in venous pressure averaged 16 mmHg and perfusion pressure always increased more than 20 mmHg ($\bar{x}$ = 24 mmHg). This indicates that in these constantly perfused preparations precapillary resistances changed in a manner which tended

to maintain the hydrostatic pressure gradient across the capillaries. Also the present study showed that, in both gracili muscle and intestinal preparations, the absolute change in precapillary resistance is dependent upon the level of change in the venous pressure and independent of initial resistance.

The increase in blood flow (reactive hyperemia) or decrease in vascular resistance (reactive dilation) following a period of partial or complete ischemia has been studied in many different tissues (48, 51, 52, 94). These local regulatory phenomena have been attributed to numerous vasoactive agents (hydrogen, potassium, acetate, citrate, pyruvate, osmolality, adenosine, AMP, ADP, ATP, O_2 and CO_2) (48, 51, 52, 94, 111). Although, all of these agents have been shown to have potent vasodilating properties it is generally agreed that reactive hyperemia or dilation is probably due to a multiplicity of these agents with possibly different agents predominating at different periods during the response (52). In the present study when blood flow to the gracilis muscles or intestine was stopped for 1 or 2 minute periods there was a significantly lower perfusion pressure upon return of blood flow. This apparent vasodilation occurred in both innervated and denervated gracili as well as the intestine (Figure 19) and was unaltered by intra-arterially infused TTX (0.5 - 10 µg/min) into muscles having blood flows ranging between 5 and 12 ml/min. The level of perfusion pressure upon restarting flow following a 1 minute period of ischemia in any given experiment was to the same absolute level even when initial resistance differed. Therefore, these data from gracili showed that, as in

the intestine (Figure 19), a 1 minute period of ischemia is sufficient to produce maximum dilation by this mechanism with the absolute change (i.e. pre-post ischemia pressures) dependent upon initial resistance.

TTX had little effect on the vascular responses to KCl (Figure 7, 8). K^+ which has been implicated as having important roles in various local regulatory phenomena is known to have a direct vascular action (9, 12, 20, 109). The biphasic response to K^+ (vasodilation followed by vasoconstriction) has been demonstrated in many tissues including heart, hindlimb, intestine, stomach, kidney and gracilis muscle (49, 52, 53). It is well known that elevating serum potassium over the range of 4 to 12 mEq/l produces vasodilation (53, 109). Recent work by Chen et al. (20), has shown that in canine gracilis muscle preparations the vasodilator action of K^+ as well as vasoconstriction produced by hypokalemia during dialysis are sensitive to ouabain (2.5 µg/min, i.a.). Gulati and Jones (47) reported that ouabain in concentrations greater then 10^{-9} M depressed or abolished the uptake of K^+ by isolated canine carotid arteries. The fact that ouabain is a known inhibitor of the Na^+ - K^+ sensitive ATPase (40, 115) allowed Chen et al. to hypothesize that K^+-induced vasodilation is via stimulation of the ATPase mediated Na^+ - K^+ pump. Although the effect of TTX on Na^+ - K^+ activated ATPase has not been studied, Kao (73, 76) reported that 25 µM concentrations of TTX intra or extracellularly failed to affect the active transport of sodium across frog skin. The present study shows (Figure 7) that if Chen et al. (20) are correct, TTX in doses of 0.5 to 10 µg/min (calculated blood concentrations = 3×10^{-8}-7×10^{-7} µg/ml)

had no affect on the responses regulated by Na^+ - K^+ ATPase during periods of increased extracellular potassium. Figure 7 shows that when KCl was infused locally over the range of 0.1 to 2.0 ml/min (calculated blood concentration elevated 1 to 8 mEq/l) venous outflow increased to about 200% of control in both innervated and denervated gracili muscles. However, TTX produced a further increase in innervated muscles. Thus, these data indicate: 1) That vasodilation induced in gracili by K^+ is via a mechanism insensitive to TTX and 2) TTX in doses 0.5 to 10 µg/min given locally affects vascular muscle only through neural elements.

Figure 11 shows the responses of innervated and denervated gracili constantly perfused with blood from a donor animal. Similar to Lipsius et al. (88) and Kao et al. (74, 77) reflex responses were demonstrated even after vascular resistance appeared to decrease. However, the magnitude of the increase in resistance to carotid occlusion of the recipient animal progressively decreased as vasodilation became greater, and at 2.0 ml/min (1.0 µg/min) to TTX both the innervated and denervated muscles appear to have similar resistances. These data may indicate that in these preparations, TTX first blocks vascular nerve fibers on the periphery of the nerve trunk and later those in the interior of the trunk. It is also conceivable that TTX may depress ongoing activity of vascular nerves yet the nerves continue to respond to high levels of stimulation as carotid occlusion or lumbar root stimulation with supramaximal stimuli as shown by Kao (74, 77, 88). Further support for the gradual loss of functional nerve fibers is the fact that maximum

vasodilation to TTX was reached at the same time that reflex activity was completely abolished. Failure of the innervated muscle to show a clearly greater dilation in Figure 11 is probably due to the high systemic pressure of the recipient animal reflexly decreasing the level of vasomotor neural activity.

If TTX possessed any direct vasoactive properties one might reason that such action should be augmented at higher resistances when the capacity for relaxation is increased. However, Figures 12, 13 and 14 show that when resistance was increased reflexly by hemorrhaging the recipient animal or increased by local infusion of norepinephrine (0.1 μg/min, i.a.) TTX produced a greater decrease in resistance only in the innervated muscles. Regression analysis of arbitrarily selected responses from innervated ($r = 0.92$) and denervated ($r = 0.27$) muscles shows that the vasodilator action of TTX (0.25 μg/min) is related to initial resistance in innervated preparations (Figure 14), with denervated muscles failing to show any response to intra-arterial infusion of TTX over the range 0.05-1.0 μg/min.

Intestinal vascular responses - The intestinal vasculature demonstrated variable responses to TTX in doses between 0.5 to 10.0 μg/min. Generally, there was no change in venous outflow (Figure 15) but occasionally an increase in vascular resistance occurred concomitant with increased motor activity (Figure 17 and 18).

Figure 15 shows that the increase in venous outflow was not statistically different from that of isotonic saline; thus suggesting

that vasomotor tone in the intestinal vasculature was minimal or absent in these preparations. Yet, these preparations responsed to carotid occlusion with increases in resistance and these increases were depressed by TTX. Both double-segment and single segment preparations appeared to be neurally intact by this test.

Haddy et al. (49) reviewing the intestinal vascular effects of vasoactive agents points out that vasoactive agents may produce vascular changes, at least in part, by their effect on visceral muscle. Kao (73) and Gershon (39) have shown that visceral smooth muscle is relatively insensitive to TTX at concentrations less than 10^{-5} g/ml. Yet, Kagnoff and E. Kivy-Rosenberg (71) have shown that TTX (0.1-8.3 μg/ml) stimulated motility of the isolated rat jejunum. The present study also showed that increases in motility may occur during TTX infusion thereby altering vascular resistance (Figure 18). The responses of the vasculature in skeletal muscle to TTX to indicate that TTX has no direct action on intestinal or vascular muscle. Assuming this true, the use of TTX in studying the role of neural elements in the regulation of local blood flow in the intestine seems profitable.

Although the gastrointestinal circulation has been studied well (62), very little physiological evidence exists which relates intrinsic nerve activity with regulation of intestinal blood flow. However, recent work by Dabney et al. (23, 26) suggests that local neural elements may be significantly involved in the intestinal vascular responses to certain vasoactive agents. Sharma and Nasset (113) also have reported that luminal perfusion of cat and dog intestinal

loops with various solutions (glucose, amino acids, dextrose, and salts) altered mesenteric nerve activity. The intestinal vasculature freely anastomoses (arterial and arteriolar) without endarteries, forming vascular plexuses which appear to be located in those areas possessing the vast intramural neural plexuses. Schofield (106, 107) reports that the axon reflex phenomena described by prior investigators (35) may be mediated via afferent fibers with collateral branches in enteric ganglia, and that the most extensively studied components of the myenteric plexus lie adjacent to the small intestinal vessels. Therefore, it is conceivable that vasoactive agents present in the intestinal lumen or vasculature may stimulate local neural elements, thus producing local vascular changes either via neural pathways or indirectly via neural regulation of motility which, in turn, could influence local blood flow (24, 27, 108, 112, 114).

In the present study TTX, which is known to depress neural activity by blocking sodium conductance (29, 73, 95, 97), abolished the increase in venous outflow from the ileum elicited by i.a. infusion of KCl (Figure 15), but failed to block K^+-induced vasodilation of the pump-perfused jejunum (Figure 17). This disparity between the two series is, at present, unexplainable. However, it was noticed that the abolishment of the vasodilation occurred concomitantly with a great increase in motility. When hyperosmotic KCl (1500 mOsm/Kg) was placed in the lumen, i.a. TTX (0.5-2.0 µg/min): 1) Converted vasodilation to vasoconstriction, 2) Produced greater increases in venous osmolality and K^+ concentrations and 3) Decreased

the volume of fluid recovered from the lumen. Therefore, these data show that intra-arterial infusion or luminal placement of KCl can produce vasodilation, as shown in many tissues by others, at serum concentrations of less than 12 mEq/l. The increase in venous osmolality is also known to produce vasodilation. Since, low concentrations of K^+ and increased osmolality favor a direct vasodilatory effect on vascular muscle while the same factors may affect intestinal motility by hyperpolarizing intrinsic nerves thus removing inhibitory neural mechanisms, it is conceivable that TTX may alter the intestinal vascular responses to KCl by an action on both visceral and vascular smooth muscle. Therefore, the vascular action of TTX in the intestine may be via neural mechanisms affecting motility and/or altering smooth muscle by altering cell permeability to K^+.

Table 2 and Figure 16 show that after TTX, venous potassium approached the vasoconstrictor range. It is conceivable that visceral smooth muscle is stimulated at lower K^+ concentrations than vascular muscle. It should also be remembered that the interstitial concentrations may be greater than those obtained in venous outflow. These data may indicate that TTX in the intestine alters K^+ conductance thus differing from other tissues reported to have no changein potassium conductance in the presence of TTX (1, 2, 3). It may also indicate that local neural elements directly or indirectly participate in intestinal and vascular muscle responses to potassium by either altering K^+ permeability or by releasing intracellular K^+ during increases in visceral muscle activity. Thus, increases in vascular resistance may be produced by elevating

extracellular potassium. The fact that K^+ produced vasoconstriction during TTX in face of two vasodilating factors (hyperosmolality and increased potassium) strongly suggests that vascular resistance is influenced by changes in visceral muscle.

Sodium has been shown not to be vasoactive (52, 109); however, in the present study intra-arterial or luminal placement of hyperosmotic sodium chloride produced responses directionally similar to KCl although much smaller (Table 1, Figures 16, 17, 18). Figures 16 and 17 show that these responses, were significantly altered by TTX (0.5-2.0 µg/min). Figures 17 and 18 show that TTX blocked the increase in motility and produced dilation over the entire infusion range (0.2-2.0 ml/min) of NaCl (1500 mOsm/Kg). Intraluminally placed hyperosmotic NaCl produced significant decreases in resistance before and after TTX. However, before TTX resistance, although below control levels, was gradually increasing during the 4 and 8 minutes collection periods, whereas after TTX, resistance decreased even more during these same periods (Table 1). These data and Figure 18 indicate that the vasodilation to hyperosmotic NaCl which is probably the result of increased osmolality (+ 13-23 mOsm/Kg), (89, 111), is masked by passive vasoconstriction due to stimulation of visceral smooth muscle. The fact that the known ganglionic blocker, hexamethonium, failed to affect the response indicated that NaCl or hyperosmolality affect either neural pathways not involving ganglia or affect visceral muscle directly (Figure 18). However, hyperosmolality has been reported to produce hyperpolarization (63, 69, 89), therefore, stimulation of motility could only occur by depression of

inhibitory neural activity known to predominate in the intrinsic plexuses (7, 8, 17, 85, 105). It is possible that TTX abolished this neural mechanism thus allowing the direct vascular action of hyperosmolality to remain unaltered (Figure 17, 18).

The effect of hyperosmolar glucose has been shown by Chou et al. (22) to produce greater changes in motility (increased) and blood flow (increases) than equal osmolar (3000 mOsm/Kg) polyethylene glycol solutions. Both of these changes were blocked by dibucaine (0.4%); however, in the present study TTX (0.5-2.0 µg/min) blocked only the change in motility produced by hyperosmotic glucose; thus indicating 1) that the increase in motility to hyperosmotic glucose is via neural mechanism and 2) the increase in blood flow is via a direct vascular action of hyperosmolality and glucose. The fact that dibucaine blocks the vascular response may suggest that this local anesthetic actually has a paralyzing affect on vascular smooth muscle. All of these data are in agreement with Scott and Dabney (108), Sidky and Bean (114), and Boatman and Brody (10) who have reported that intestinal tonus and motor activity may substantially alter the vascular responses by extravascular means. This is further supported by the fact that nicotine (50 µg, i.a.) a known ganglionic stimulant produced increases in motility and vascular resistance before hexamethonium (C_6), decreases in motility and vascular resistance after C_6 (0.5 mg/min) and no change in vascular resistance or motility after TTX (2.0 µg/min) (Figure 20).

Even though TTX altered the intestinal vascular responses to intra-arterial and luminal placement of vasoactive agents its indirect action is supported by the fact that it failed to affect either reactive dilation, or venous-arteriolar response. Also the decreases in resistance to locally infused acetylcholine (3 µg, i.a.) or increases in resistance to epinephrine (3 µg, i.a.) were unaffected by TTX (Figure 19 and 20). Therefore these data show that local infusion of TTX over the range of 0.5 to 10.0 µg/min will produce local nerve paralysis with minimal affect on vascular muscle.

Vascular Smooth Muscle Responses In Vitro

Vasoconstrictor drugs (norepinephrine, nicotine, dimethylphenylpiperizinium, epinephrine, tyramine and $BaCl_2$) increased the tension of isolated arterial (femoral) strips and the vasodilator, acetylcholine, produced relaxation while TTX had no effect on strips before or after cold storage (Figures 22, 23, 24). Equilibration at higher pre-loads augmented the responses to both vasoconstricting and vasodilating agents (Figure 23). Increasing extracellular K^+ concentration (9.2 mEq/l) before constricting the strips with $BaCl_2$ (1.0 mM) produced primarily increases in tension; however, after $BaCl_2$, KCl produced decreases in tension at low concentrations (< 12.2 mEq/l) and increases in tension at higher concentrations (> 12.2 mEq/l) (Figures 25, 26). Increasing extracellular osmolality (> 50 mOsm/Kg) before increasing strip tension with $BaCl_2$ (1.0 mM) produced small increases in strip tension: however after $BaCl_2$ hyperosmolality (+10-90 mOsm/Kg) produced only relaxation (Figure 28, 29) while hyposmolality (-15-45 mOsm/Kg)

produced increases before and after $BaCl_2$ (Figure 28). Cold storage (24 hrs at 5°C) attenuated the responses to all vasoactive agents tested.

The responsiveness of isolated vascular muscle has been reported by Bohr (12) to be greatly attenuated immediately following removal of the tissue from the animal. Burnstock et al. (18) reported that this decreased sensitivity following dissection results from intracellular loss of K^+ and gain of Na^+ ions. The increase in sensitivity of isolated strips which reaches maximum in 3 to 5 hours (2, 37) appears to be related to cellular gain of K^+ (12, 18, 41). The sensitivity, cellular gain of K^+ and loss of Na^+ has been reported to be accelerated by increasing extracellular K^+ concentration or equilibrating the strips under tension (41, 133). Therefore, in the present study arterial strips were equilibrated for at least 1 1/2 hours at preloads equal to and greater than those used by Furchgott (36) and Altura (2) for similar sized strips. An attempt to simulate the conditions of various degrees of initial resistance in vivo was accomplished by equilibrating isolated strips from the same femoral arterial segment under four different preloads (1, 2, 4, 6 gm). Various vasoactive agents were then studied before and after contracting the strips with $BaCl_2$ which has been reported to act directly on smooth muscle by decreasing K^+ conductance (57, 123).

Figure 21 shows that the constrictor response to norepinephrine (1×10^{-6} g/ml) were progressively higher at higher pre-loads, thus suggesting that 1) intracellular K^+ accumulation was higher at higher

pre-loads thereby increasing the sensitivity of the strips and/or 2) equilibrating the strips under greater degrees of stretch increased the area or sensitivity of the cell membrane receptor sites. Figure 21 also shows that when the capacity to relax was increased as at higher pre-loads or following $BaCl_2$, the relaxation produced by a given dose of acetylcholine was augmented. Acetylcholine also exhibited a constrictor action shown by other investigators (2) however this occurred only at the lowest pre-load, possibly due to a low or inability to relax.

Drugs acting directly on smooth muscle or via neural stimulation produced progressively greater responses as pre-load was increased. Yet, TTX over the range of 10^{-6} g/ml failed to produce any significant change in tension (Figure 22) and cholinergic as well as alpha and beta-adrenergic responses were demonstrated in the presence of TTX (10^{-6} g/ml). Neither TTX (10^{-7}-10^{-6} g/ml) nor 24 hours of cold storage, known to render neural elements nonfunctional (21, 84), blocked the responses to norepinephrine (10^{-6} g/ml), tyramine (5×10^{-5} g/ml), or high concentrations of nicotine or dimethylphenylpiperazinium (5×10^{-5} g/ml) but both TTX and cold storage blocked the responses to low levels (5×10^{-6} g/ml) of the ganglionic stimulants (Figure 24). These data indicate that: 1) Equilibration of vascular strips under increased pre-loads increases the sensitivity of the strips to both vasoconstrictor and vasodilator agents, 2) TTX in concentrations over the range of 10^{-8}-10^{-6} g/ml has no effect on vascular strips either fresh or following cold storage; 3) Responses to tyramine as previously

shown by Bell (5) to be independent of Na± conducted action potentials, are unaffected by TTX or cold storage; and 4) The ganglionic stimulants, nicotine and dimethylphenylpiperizinium, in low concentrations produce release of adrenergic mediators via Na^+ related action potentials but in higher concentrations appear to possess a direct action.

Increasing extracellular K^+ concentrations in additive increments (+ 1.5, 4.5, 7.5 and 15.0 mEq/l) above control (4.7 mEq/l) generally produced only contraction of isolated arterial strips before actively constricting them with $BaCl_2$ (1 mM) (Figure 25, 26). However, after increasing the tension with $BaCl_2$ the strips demonstrated biphasic responses with relaxation at low K^+ concentrations (< 12.2 mEq/l) and constriction at high concentrations (12.2 and 19.7 mEq/l). The magnitude of both contractions (increases in tension) and relaxations (decreases in tension) were augmented at higher pre-loads; however, the magnitude of the relaxation appeared to be related to the level of active tension present following $BaCl_2$ (Figure 26). It is well known that Ba^{++} depolarizes nerves (98) and muscle cells (30, 44) and alters membrane phenomena by specifically decreasing K^+ conductance of excitable and nonexcitable tissue (57, 123). This divalent ion also increases cardiac pacemaker activity (45, 103, 123) which can be altered by different experimental conditions including reduction of intracellular K^+ (19, 91). Therefore the excitatory effect of Ba^{++} demonstrated in the present study which results from its affect on K^+ conductance apparently is counteracted by the ATPase stimulating effect of elevated extracellular concentrations

of K^+ (20). Neither in the present study on vascular strips nor in that reported by Hermsmeyer and Sperelakis (57) on frog ventricular muscle did TTX alter responses produced by Ba^{++} or K^+.

Figure 27 shows that the relaxing affect of K^+ at low concentrations (< 12.2 mEq/l) appears not be be sensitive to TTX, phentolamine, propranalol or atropine, however, ouabain significantly inhibited the relaxation phase and augmented the constrictor phase. Figure 27 clearly shows that ouabain (10^{-5} M) failed to abolish the response. Although, the control responses before subjecting the strips to the blocking drugs are not shown, the strips clearly exhibit relaxations which were not significantly different from their controls except following ouabain. The presence of K^+ in the control solution may account for ouabain's failure to abolish the relaxation, as it is known to decrease the efficacy of ouabain binding to cell membranes (43). These data appear to indicate that: 1) Similar to <u>in vivo</u> responses (48, 51, 52) increasing extracellular K^+ concentrations over the range of 4-12 mEq/l produced relaxation of isolated vascular strips only if the strips were first actively contracted; 2) The magnitude of relaxation was related to the level of active tension present; 3) The K^+ - induced relaxation phase was not significantly altered by TTX, cholinergic, or alpha and beta-adrenergic blockade; and 4) Ba^{++} and K^+ - induced responses were significantly augmented in strips equilibrated at high pre-loads.

Increasing extracellular osmolality always produced relaxation of isolated arterial strips which were actively contracted with $BaCl_2$ (Figures 28, 29). Decreasing osmolality generally

produced increases in contractile tension before and after contracting arterial strips. Both contraction and relaxation were greater in strips equilibrated at the higher pre-loads. Increasing blood osmolality has been reported to produce vasodilation in many tissues and has been implicated in many local regulatory phenomena (38, 53, 39, 110, 111). In vitro studies have clearly shown that isolated guinea-pig taenia coli (124), frog sartorius muscle (46) and rat portal vein (64) respond to hyperosmolality by hyperpolarization, decreased mechanical activity and reduced cell volume. Jonsson (67, 68, 69) has postulated that altering extracellular osmolality may produce responses by altering intra and extracellular ion concentrations as well as permeability of the cell membrane. In the present study hyperosmolality was studied over the range of 300 (control) to 390 ± 10 mOsm/Kg by adding concentrated Krebs-Ringer solution (3 x normal), thus increasing all extracellular ions, or by increasing osmolality with d-mannitol which is known to not cross cell membranes.

Figure 28 shows that before the strips were contracted with $BaCl_2$, increasing extracellular osmolality and ion concentrations produced increases in tension. These increases, which became significant when osmolality was increased more than 20 mOsm/Kg, were greater at higher pre-loads and substantially higher than those seen when d-mannitol was used. When osmolality was increased by using d-mannitol significant increases did not occur until osmolality was increased more than 50 mOsm/Kg. However, both solutions produced relaxation of the strips stimulated by $BaCl_2$. A greater relaxation was produced by the d-mannitol solution. These data

seem to indicate that increasing extracellular ion concentrations and osmolality activates two opposing responses with the predominating response dependent upon the capacity to relax or contract. Figure 29 clearly shows that when the capacity to relax is elevated, as indicated by the degree of active tension, the response to any given change in osmolality is augmented. These data may suggest that, *in vivo*, blood vessels may show greater vasodilator responses when initial vascular resistance is increased.

These results are compatable with both *in vitro* (38, 89, 110) and *in vivo* (63, 69, 124) studies reported by other investigators and are explainable by altered ratios of intracellular to extracellular ions concentrations. When extracellular ions are increased H_2O tends to move from the cell thus increasing intracellular ion concentrations, predominantly K^+, thereby favoring hyper-polarization by increasing $[K^+]_I/[K^+]_0$ ratio. However, occurring concomitantly would be movement of Na^+ into the cell due to the large extracellular pool thus favoring depolarization. Increasing the pre-load tension may lead to increased permeability thus aiding Na^+ movement, augmenting depolarization and producing contractions as seen in the present study. Following $BaCl_2$ when the cells are presumably depolarized the intracellular concentration of Na^+ is probably elevated thus decreasing the $[Na^+]_0/[Na^+]_I$ gradient thereby decreasing the effects of sodium movement and augmenting the hyperpolarizing effects of elevated intracellular K^+ on the membrane.

Decreasing osmolality would have somewhat different effects. Decreasing extracellular ion concentrations and osmolality may

decrease intracellular concentrations by loss of ions to extracellular fluid as well as by increasing intracellular volume (68, 73). Decrease in $[K^+]_I/[K^+]_0$ ratio would favor depolarization. However diluting other extracellular ions appears to favor hyperpolarization as shown by the strips subjected to distilled H_2O (Figure 28) which demonstrated no significant increase in tension. Yet when only extracellular NaCl was decreased the strips responded with an increased tension even after actively contracting the strips. This seems to indicate that hypo-osmolality favors depolarization by decreasing the intracellular K^+ concentration. This occurs when H_2O moves into the cell thus increasing cellular volume (68, 73). Increasing cell volume may also alter Na^+ movement by increasing membrane permeability. All responses to change in extracellular osmolality were augmented at higher pre-loads, thus suggesting that permeability changes occurring in strips under stretch facilitate both ion and H_2O movement. These changes in strip tension during either hyper or hyposmotic conditions appear to result from direct membrane phenomena not sensitive to TTX, adrenergic or cholinergic blockade.

The Action of TTX on the Reactivity of Visceral Smooth Muscle

Local intra-arterial infusion of TTX occasionally depressed spontaneous motility (Figure 31) but generally exhibited a stimulatory effect (Figure 18, 31 and 32). KCl increased motility which following TTX was converted from contractile responses showing little phasic activity to responses showing an active phasic pattern (Figure 31, 33 and 34). However, the increases in motility

to hyperosmotic NaCl, hyperosmotic glucose, nicotine and dimethylphenylpeperizinium were abolished by TTX (Figures 18, 32, 33 and 34).

Depression of spontaneous motility by TTX occurred at low concentrations (0.1 µg/min, Figure 31) insufficient to block vagal induced increases in motility. Figure 30 shows that TTX infusions of 0.5 µg/min (0.1 ml/min) depressed vagally-induced motility changes and only after the infusion was increased to 2.5 µg/min were the responses completely abolished. In this series, using ileal segments TTX infusion was started 15 sec before vagal stimulation. In subsequent series TTX, in concentrations as low as 0.5 and 1.0 µg/min, was effective in inhibiting motility changes induced by vagal stimulation or ganglionic stimulation by nicotine (5-50 µg) and DMPP (5-50 µg). TTX concentrations of less than 2 µg/min generally required prolonged infusion periods of 5-15 minutes before they were effective in abolishing vagally induced contractions. During periods when TTX showed depression of spontaneous activity vagal stimulation still produced significant increases in motility but when TTX stimulated motility, vagally induced contractions were blocked. Therefore, these data may indicate that when excitatory neural activity is present that small doses of TTX may affect it by blocking more susceptible excitatory fibers, however, when intrinsic inhibitory mechanisms are removed by TTX motility is stimulated. This stimulation wains with time but never returns to control values. Therefore it appears as though the removal of local inhibitory neural mechanisms is overcome either by adaptation of the excitatory neural components or alteration in visceral

smooth muscle activity. The later seems more logical in that excitatory as well as inhibitory nerves are susceptible to TTX blockade (16, 17, 85, 105).

The ability of TTX to stimulate motility has been demonstrated in rat (71), and cat jejunum (130); yet Gershon (39), Rigimaru and Susuki (105) and Taira et al. (122) fail to mention any stimulatory affect on their isolated smooth muscle preparations. However Kagnoff and E. Kivy-Rosenberg (71) studying the affect of whole body X-irradiation reported that isolated rat jejunal strips of irradiated animals produced similar motility patterns as non-irradiated strips subjected to TTX (0.1-8.3 x 10^{-6} g/ml). They showed that the motility pattern produced by TTX was of regular, rhythmical contraction with primarily augmentation in magnitude. These authors hypothesized that both X-irradiation and TTX produce increased motility by removal of intrinsic neural mechanisms, which have been well studied (16, 17, 85, 105). This concept of intrinsic inhibitory neural control is further supported by the studies of Wood (130) on isolated cat jejunum. This author reported that atropine (5.0 x 10^{-5} - 5.0 x 10^{-4} g/ml), procaine (5.0 x 10^{-5} - 5.0 x 10^{-4} g/ml), TTX (5.0 x 10^{-8} - 7.5 x 10^{-7} g/ml) and lidocaine (2.5 x 10^{-6} - 2.5 x 10^{-4} g/ml) produced action potentials and large increases in amplitude of circular muscle contractions.

Similar increases in frequency and amplitude of phasic contractions elicited by TTX have been reported by Kao (73) to occur in rabbit intestine by an unknown mechanism. Yet Wood (130) has hypothesized a two neuron system within the enteric network,

theoretically agreeable with the experimental findings. A spontaneously active neuron which receives no synaptic input drives a non-spontaneous neuron by a cholinergic synapse. The driven neuron releases a nonadrenergic inhibitory transmitter substance (16, 17) at muscarinic sites on the circular muscle layer. The existence of this type of an intrinsic neural pathway is supported by: 1) direct electrical recordings from enteric nerve cells indicating that one neuron drives another (101); 2) acetylcholine affects the electrical activity of only 21% of the intrinsic neurons (129); 3) atropine affects only a fraction (21%) of the intrinsic neural activity (101); 4) procaine, lidocaine and TTX augments the circular muscle activity to electrical stimulation of intrinsic neurons (131); and 5) transmural electrical stimulation inhibits mechanical and electrical activity produced by atropine, and is sensitive to TTX (130).

Atropine appears to produce excitation by removing the driving influence on inhibitory nerves and TTX produces excitation by removing all neural influence thus allowing spontaneous myogenic activity to occur. The ability of TTX to inhibit ongoing spontaneous activity as was seen in the present study may be related to the cholinergic synapse between the driver neuron and the non-adrenergic inhibitory neuron. If TTX, which has been shown to inhibit acetylcholinesterase at low concentrations (136), prolonged or augmented the effect of the transmitter at the cholinergic synapse spontaneous motility may be inhibited by producing a more efficacious inhibitory influence.

Intra-arterial or intra-lumenally administered KCl stimulated motility of both natural and pump-perfused intestinal segments (ileum and jejunum). Like vascular smooth muscle (12, 20, 119) KCl has been reported to have similar direct effects on visceral smooth muscle (18, 26, 49). However, the fact that K^+ permeability may differ between these two muscles may be important in the visceral muscle action and thus the indirect vascular responses. In the present study KCl infused locally (elevating calculated serum concentrations 1-30 mEq/l) or placed intralumenally (elevating serum K^+ 1.5-10.0 mEq/l) always increased motility as infusion rate was increased (> 1.0 ml/min) or mucosal exposure to a hyperosmotic solution (1500 mOsm/Kg) exceeded 3-4 minutes. These data are in agreement with Dabney et al. (23, 26, 27). However, when KCl was placed in the lumen, venous K^+ concentration was well below the vasoconstrictor levels as shown in other tissues, yet increases in motility and vascular resistance occurred (Figure 16 and Table 2). The fact that TTX changes the K^+ - induced motility from a nonrhythmical pattern to highly phasic responses may indicate that KCl has a neural as well as non-neural action and TTX removes the neural effect thus demonstrating only myogenic responses synchronous with the basic electrical rhythm of the tissue. However, these data do not rule out the fact that TTX may alter smooth muscle permeability to K^+. One must agree that TTX alters the venous concentration of K^+. This is explainable by the decreased blood flow but may also indicate either altered K^+ flux or cellular loss occurring during increased muscle activity (111).

Intra-arterially or luminally placed NaCl or glucose in hyperosmolar concentrations (1500 mOsm/Kg) frequently elicited increases in motility (Figure 18, 33 and 34). These increases were characterized by both elevations of baseline pressures as well as phasic activity. Although ganglionic blockade with hexamethonium (0.5 mg/min) failed to alter the H-NaCl induced contractions the responses appear to be neurally mediated by their susceptability to TTX (1.0 μg/min). Hyperosmolality has been shown to hyperpolarize different tissues and sodium movement into cells is known to cause loss of membrane potential thus producing action potentials. Therefore hyperosmotic NaCl could possibly affect intestinal muscle by: 1) Hyperpolarizing the inhibitory nerves thus removing the previously mentioned inhibitory components, 2) Stimulating excitatory neural components by a different mechanism (i.e. movement of Na^+ into the neuron) and 3) Directly stimulating smooth muscle following extracellular to intracellular movement of Na^+. If any or all of these mechanisms were true, TTX must block the motility by: 1) Removing the inhibitory neural elements thus eliminating the effect of hyperosmolality, 2) Block depolarization of excitatory nerves by its known effect on Na^+ conductance in neural tissue (97), or 3) Blocking sodium conductance thus depolarization at the muscle cell membrane. Evidence strongly rules against any action of TTX directly on the smooth muscle membrane (5, 39, 80, 86) therefore hyperosmotic NaCl and glucose appears to influence visceral smooth muscle activity through excitatory and/or inhibitory neural mechanisms.

Acetylcholine and epinephrine are known to act directly on the smooth muscle membrane. In the present study neither hexamethonium (C_6) nor TTX significantly altered the motility responses to these agents (Figure 32). However, ganglionic stimulation with nicotine (5-50 μg, i.a.) (Figure 32) or DMPP produced excitation before C_6 and inhibition after C_6 (0.5 mg/min). Both of these responses to ganglionic stimulation were blocked by TTX. These data may indicate that the hypothesized cholinergic synapse driving the inhibitory neuron is insensitive to hexamethonium blockade. Thus, if nicotine and DMPP stimulate both excitatory and inhibitory nerves and hexamethonium only blocked excitatory post-synaptic receptors the inhibitory elements would remain functional. Both of these neural pathways would then be susceptible to TTX thus eliminating both excitatory and inhibitory responses to nicotine or DMPP.

SUMMARY AND CONCLUSIONS

In the present work in situ canine preparations of the intestine and gracilis muscle as well as isolated arterial strips were used to study: 1) What effects the neurotoxin tetrodotoxin (TTX) has on vascular smooth muscle, 2) What effects TTX has on intestinal muscle, 3) What doses of TTX block neurogenic responses, 4) The effects of TTX, in neural blocking doses, on the vascular responses to other vasoactive agents (epinephrine, norepinephrine, acetylcholine, ganglionic stimulants, KCl and changes in osmolality).

Local infusion of TTX in doses of 0.05-10 µg/min (producing calculated blood concentrations of 10^{-6}-10^{-9} g/ml) produced significant increases in blood flow or decreases in vascular resistance of innervated gracilis muscles but has no significant effect on denervated gracili. The neural blocking action of TTX was evaluated by its ability to depress and abolish the motor responses to electrical stimulation of the cut nerve trunk and the increases in vascular resistance following carotid occlusion. Doses of TTX as low as 0.1 µg/min i.a. (for 5-15 min) were shown to be effective in depressing neurogenic responses, however, the efficacy of TTX was increased as the dose was elevated. In these neuro-blocking doses, TTX has no effect on the vasodilator action of KCl in acutely denervated gracili muscles, but augmented the response in innervated muscles. In cross perfused innervated gracili muscles the vasodilator action of TTX reached a maximum at those doses which abolished vasoconstriction subsequent to carotid occlusion. The vasodilation induced by TTX correlated with the level of initial

resistance in innervated preparations (r = 0.92) but showed no correlation (r = 0.27) in denervated preparations.

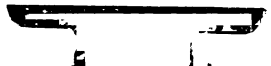
In innervated intestinal preparations local infusion of TTX never produced increases in venous outflow or decreases in vascular resistance. However, occasionally TTX produced increases in vascular resistance which occurred concomitantly with the TTX-induced increases in motility. In doses (0.05-10.0 μg/min, i.a.; 5-15 min) sufficient to block vagally induced increases in motility or vascular reflex responses to carotid occlusion, TTX abolished the vasodilator action of locally infused KCl in natural-flow experiments but not in constant-flow intestinal preparations. Luminal placement of KCl significantly decreased vascular resistance and increased venous osmolality and K^+ concentration, and volume recovered from the lumen. TTX augmented the changes in venous osmolality and potassium but converted vasodilation to vasoconstriction. TTX altered the responses in motility induced by KCl from a pattern showing little phasic activity to one exhibiting large phasic responses. Intra-arterial or luminal placement of hyperosmotic NaCl produced vasodilation followed by vasoconstriction which was accompanied by increased motility. Following TTX, motility responses were abolished with only vasodilation occurring.

Isolated arterial strips responded to vasoconstricting agents by an increase in contractile tension and vasodilator agents by a decrease in tension. Responses to all agents studied, were progressively augmented as pre-load was increased (1, 2, 4 and 6 gms). TTX (10^{-9}-10^{-6} g/ml) had no significant effect on strips before or

after 24 hrs of cold storage. TTX blocked the increases in tension to low doses of nicotine or DMPP (5×10^{-6} g/ml) but had no effect on the responses to norepinephrine, acetylcholine, epinephrine, tyramine, KCl or hyper and hypo-osmotic solutions. Increasing extracellular K^+ (+ 1.5, 4.5, 7.5 and 15.0 mEq/l) above control (4.7 mEq/l) generally produced only increases in tension in vascular strips subjected to different pre-load tensions; however, in the presence of active tension, produced by $BaCl_2$, increasing bath K^+ to levels below 12.2 mEq/l produced relaxation while concentrations greater than 12.2 mEq/l produced contractions. Increasing bath osmolality (50 mOsm/Kg) above control (300 mOsm/Kg) frequently produced contractions of passively loaded strips. After actively contracting the strips with $BaCl_2$ hyperosmolality (310-390 ± 10 mOsm/Kg) always produced relaxation; however, decreasing osmolality frequently produced increases in tension before or after $BaCl_2$. The responses to increasing extracellular K^+, or changing bath osmolality were all augmented in strips equilibrated at higher pre-loads with relaxation appearing to be related to the level of active tension produced by $BaCl_2$. Neither the responses to change in bath K^+ or osmolality were altered by TTX, adrenergic or cholinergic blockade.

Based on the present study the author makes the following conclusions:

1) Local infusion of TTX in doses producing blood concentrations of 10^{-9} g/ml has no vascular action in denervated gracilis muscles but demonstrates a vasodilator effect in innervated gracili



with the level of dilation directly related (r = 0.92) to initial resistance.

2) TTX in doses as low as 0.1 μg/min (10^{-9} g/ml of blood) for 5-15 min exhibits neural blocking action in skeletal and intestinal muscle with the efficacy of neural blockade increased at higher doses.

3) TTX in neural blocking doses (10^{-9}-10^{-6} g/ml, 5-15 min) has no effect on the vascular action of vasoconstrictor or vasodilator agents which act directly on vascular muscle.

4) TTX in bath concentrations of 10^{-9}-10^{-6} g/ml has no effect on the tension of fresh arterial strips or following cold storage.

5) TTX (10^{-7}-10^{-6} g/ml) and cold storage (24 hrs) abolishes the responses of isolated arterial strips to neural stimulating agents which work through Na^+ related action potentials.

6) Equilibrating isolated arterial strips under tension augments the responses to vasoconstrictor and vasodilator agents.

7) In isolated arterial strips, actively contracted with $BaCl_2$, elevating extracellular K^+ produces responses similar to those seen <u>in vivo</u> with relaxations at $[K^+]_0$ < 12 mEq/l and contractions at $[K^+]_0$ > 12 mEq/l. Ouabain (10^{-5} g/ml) significantly attenuates the relaxing action of K^+.

8) In the presense of active tension hyperosmolality (+10-100 mOsm/Kg) produces a decrease in tension of arterial strips and hyposmolality (-10-50 mOsm/Kg) produces increases in tension.

9) TTX, adrenergic and cholinergic blocking agents have no effect on responses produced by K^+ or hyperosmolality or hyposmolality.

10) Local infusion of TTX may inhibit spontaneous intestinal activity in low doses but generally stimulates motility in neural blocking doses.

11) TTX frequently alters the intestinal vasodilator action of i.a. or luminally placed KCl. Vasoconstriction is augmented concomitantly with increases in motility. This indicates that local vascular responses to K^+ in the intestine may be regulated in part either via intrinsic nerves or by altering the motility of visceral smooth muscle.

12) TTX abolishes the motility responses and vasoconstrictor responses to i.a. (NaCl, 1500 mOsm/Kg) or luminally (NaCl, 1500 mOsm/Kg, glucose and polyethylene glycol, 3000 mOsm/Kg) administered hyperosmotic solutions, allowing only vasodilation. This indicates that hyperosmolality has a direct vasodilator action in the intestine which may be masked by the motility responses activated by neural mechanisms.

13) As shown by its failure to affect either reactive dilation or the venous arteriolar response, TTX in neural blocking doses appears to have no direct vascular action in innervated or denervated skeletal muscle or intestinal preparations.

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