NALOXONE POTENTIATION OF EPINEPHRINE INDUCED VASOCONSTRICTION IN CANINE SKELETAL MUSCLE ARTERIES

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

BY

Scott Thomas Stoll, B.S., D.O.
Denton, Texas
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Naloxone (NX) potentiated epinephrine (EPI) induced submaximal vasoconstriction in canine renal and skeletal muscle arterial segments, yet had no vasoconstrictor action alone. Developed tension generated *in-vitro* by 4 x 1mm. O.D. rings from 1st degree branches of canine femoral arteries was expressed as % of KCl induced maximum response. NX (10^{-5} M) potentiated EPI induced submaximal contractions (34.2%) significantly more than contractions induced by norepinephrine, phenylephrine, lofexidine, ADH, KCl and serotonin (13.8, 13.4, 4.7, 13.5, 14.4 and 11.4% respectively). The NX response was unaffected by beta-adrenergic blockade and NX did not reverse an isoproterenol mediated vasodilation. Alpha-adrenergic blockade with phentolamine completely eliminated EPI plus NX induced vasoconstriction. After washout, vessels exposed to EPI plus NX relaxed by 50% significantly faster than vessels exposed to EPI alone (18.5 and 27.9 min respectively). EPI induced vasoconstrictions were potentiated by 10^{-5} M corticosterone (49.0%) which inhibits extraneuronal catecholamine uptake, but not by 10^{-7} M desipramine (1.1%) which inhibits neuronal uptake. EPI induced vasoconstrictions were also potentiated by 10^{-4} M pyrogallol (33.0%) which inhibits catechol-o-methyl transferase activity, but not by 10^{-5} M pargyline (-1.1%) which inhibits monoamine oxidase activity. The NX effect was endothelium independent. The dose-response of various opioid receptor agonists and antagonists were compared to the NX response. A specific opioid receptor subclass could not be identified as the mediator

of the NX effect. The ED_{50}s for NX ($3.7x^{-6}$ M) and (+)NX ($8.1x^{-7}$ M) indicated a significant stereoselectivity for the (+)enantiomer. A variety of sigma receptor ligands, steroids and steroid metabolites were tested for the ability to augment EPI vasoconstrictions. Several of the opioid, sigma and steroid ligands, all with polycyclic structures, induced responses similar to those of NX. NX exerted its effect independent of traditional opiate receptors and may have influenced the cellular uptake or degradation of EPI. Endogenous compounds with sigma or steroid activity may modulate these processes *in-vivo*.

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The persistence, dedication and effort invested by Dr. James L. Caffrey, to ensure academic excellence, is greatly appreciated. The contributions of all other committee members is appreciated as well.

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CHAPTER I

INTRODUCTION

The control mechanisms involved in the regulation of blood pressure are very complex and involve a wide variety of organ systems. The autonomic nervous system (ANS) occupies a central position in the maintenance of minute to minute cardiovascular homeostasis. The ANS conveys information between the peripheral organs which sense changes in perfusion pressure, the central nervous system (CNS) which coordinates responses and the effector organs (heart and vasculature) which raise or lower blood pressure. The ANS enables blood pressure to be accurately monitored and regulated.

The cardiovascular effects of exogenous opiates (morphine) have been evident to the user (or abuser) for centuries in the form of orthostatic hypotension. Early research into the cardiovascular consequences of morphine revealed localized ANS effects. Morphine and morphinomimetic substances increase parasympathetic and decrease sympathetic tone (27, 30, 31, 39). Little research was directed toward these cardiovascular effects because relatively high doses of morphine were required compared to the doses required for analgesia or euphoria. The discovery of endogenous opiates (55) stimulated research in the field of opioid/cardiovascular interactions.

Opioids

Morphine was the first compound to be isolated from the opium poppy and therefore labeled an opiate. Compounds with similar alkaloid structures that

mediated similar pharmacologic effects were designated as opiates as well. The receptors which mediate physiologic responses to the opiates are termed opiate receptors. Peptides produced by the body and active at opiate receptors are termed endogenous opioids. These peptides are called opioids because they are opiate-like. This nomenclature is widely used in endocrine literature.

Morphine and other exogenous opiates have their effects by mimicking the actions of endogenous opioid peptides. Three broad categories of endogenous opioid peptides have been characterized: endorphins, enkephalins and dynorphins. Each category is derived from a distinct protein precursor molecule. Figure 1 diagrams the opioid precursor molecules and the major active peptide products resulting from enzymatic cleavage and processing (38).

Pro-opiomelanocortin is the precursor for alpha-lipotropin, beta-lipotropin and beta-endorphin. These form the core of the endogenous endorphin family. Proenkephalin is the precursor for methionyl-enkephalin (met-enk), leucyl-enkephalin (leu-enk), methionyl-enkephalyl-argeninyl-glycyl-leucine (met-enk arg-gly-leu), and methionyl-enkephalyl-argenyl-phenylalanine (met-enk arg-phe). These are the primary active endogenous enkephalins. Prodynorphin is the precursor for alpha-neoendorphin, beta-neoendorphin, dynorphin A, and dynorphin B. These are the endogenous dynorphins.

The anatomic location of the endogenous opioids reveals a close correlation between sites of opioid production and release and areas of known autonomic significance. Immunohistochemical examination of the CNS reveals enkephalin-like immunoreactivity concentrated in the dorsal ventricular nucleus (DVN), nucleus ambiguous (NA), and nucleus tractus solitarius (NTS) (24, 53, 56, 99). These specific brain centers are crucial for autonomic cardiovascular regulation (37, 67).

ENDOGENOUS OPIOID PRECURSERS AND PRODUCTS

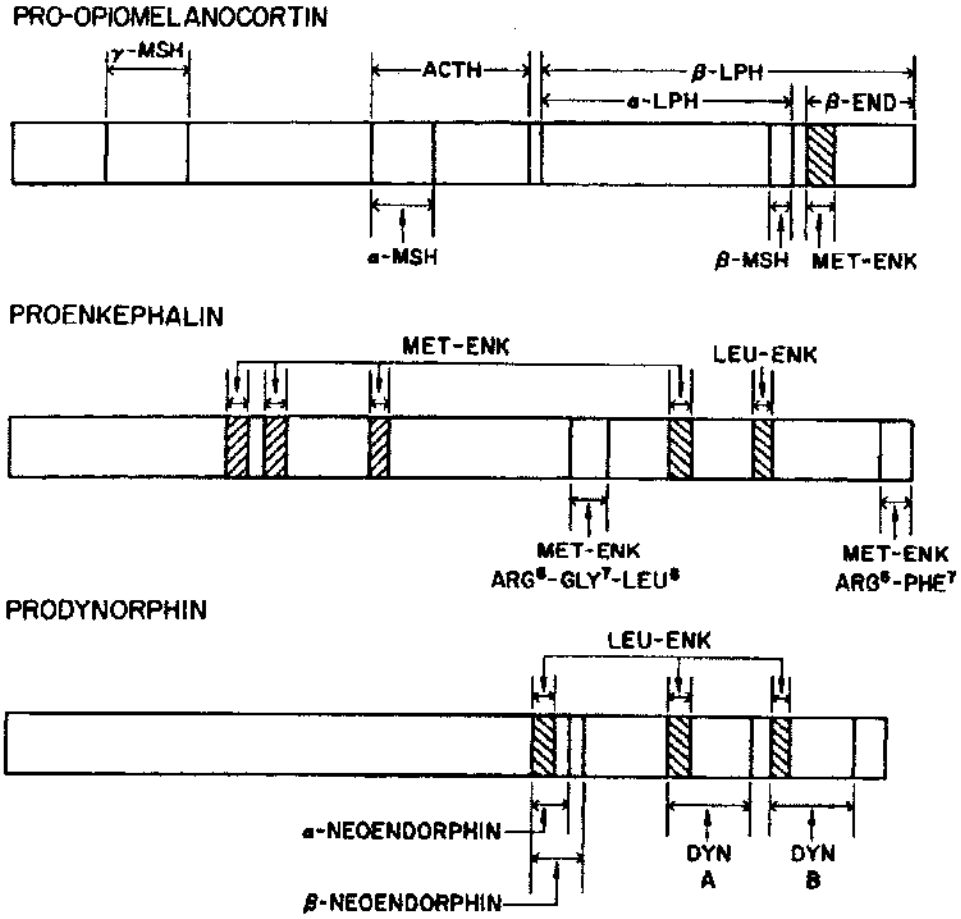


Figure 1. Schematic representation of the protein precurser molecules of the three endogenous opioid peptide families: endorphins, enkephalins and dynorphins. Abbreviations: END = endorphin; ENK = enkephalins; DYN = dynorphin; MSH = melanocyte stimulating hormone; ACTH = adrenocorticotropic hormone; LPH = lipotropin; MET = methionine; LEU = leucine; ARG = arginine; GLY = glycine; PHE = phenylalanine. (Reproduced with permission, from Goodman and Gillman's Pharmacologic Basis of Therapeutics, 1990.)

Beta-endorphin is localized to cell bodies in the hypothalamus with axonal projections to a variety of brain regions with significant contributions to cardiovascular control (117). Enkephalin-like immunoreactivity has been localized to the superior cervical, and the superior and inferior mesenteric sympathetic ganglia (20, 22, 98) and in sympathetic nerve axons and terminals (120).

Opioids are stored and released along with a variety of stress related hormones (82, 118). The pituitary gland stores beta-endorphin and adrenocorticotropin (ACTH) together in secretory vesicles and releases them concomitantly in response to stress (42, 91). Epinephrine and enkephalin are stored in and released from the adrenal medulla together (9, 45, 111, 112, 120). Opioid peptides have been isolated in most peripheral tissues including the heart (54, 56, 69) and vasculature (29).

Opiate Receptors

The discovery and characterization of the various opioid peptide families soon revealed the heterogeneity inherent in the opiate receptor itself. The variability in rank order of potency of opioids in standard opioid bioassays (guinea pig ileum and mouse vas deferens) indicated the existence of multiple opiate receptor subtypes (77, 121). There are now three clearly characterized opiate receptor subtypes: mu, delta and kappa (84). The mu-opiate receptor modulates nociceptive neural traffic and mediates the analgesic response to morphine. Delta-opiate receptors inhibit presynaptic norepinephrine release from sympathetic nerve terminals (35, 57-60, 102, 113). Kappa-opiate receptors modulate spinal transmission of nociceptive information. There is considerable cross-reactivity between receptor subtypes, and little is known about the physiologic effects opiate receptor subtype activation.

Sigma receptors were postulated by Martin et. al. in 1976 (78) to explain the actions of the racemic benzomorphans like (+/-)SKF10,047. (+/-)SKF had unique psychoactive properties. (-)SKF binds to mu- and kappa-opiate receptors while (+)SKF binds to phencyclidine (PCP) receptors and a unique site which is still called a sigma receptor. Sigma receptors bind compounds from several distinct classes. Sigma ligands include compounds such as: a) dextrorotary benzomorphans such as (+)pentazocine; b) analogs of U50,488; c) PCP analogs; d) analogs of di-o-tolylguanidine (DTG); e) analogs of (+)-1-propyl-3-(3-hydroxyphenyl)piperidine [(+)ppp]; f) steroids; and g) butyrophenones like haloperidol (115).

There remains considerable debate as to whether the sigma receptor is to be considered an opiate receptor subtype (61, 105, 115). As the field of opioid pharmacology has expanded, so has the number of selective opiate receptor agonists and antagonists. These ligands greatly facilitate endogenous opioid research.

Opiate receptors are widely distributed (36, 77) throughout the central and peripheral nervous systems, as well as the central and peripheral cardiovascular systems. Just as there is a strong correlation between opioid peptides and catecholamines, there appears to be a correlation between opiate receptors and adrenergic targets. Opiate receptors, as defined by binding studies or functional responses, are found in the brain (7, 86, 100, 107), spinal cord (6), sympathetic ganglia (20, 65, 83, 112) as well as on presynaptic sympathetic nerve terminals (35, 57-60, 66, 101, 102, 113, 120). Opiate receptors are found in the heart (13, 14, 73, 74, 101) and on vascular smooth muscle (4, 15, 25, 46, 48, 116).

Opioid/Catecholamine Interaction

The anatomic locations of endogenous opioids and opiate receptors enable this system to modulate the adrenergic regulation of cardiovascular hemodynamics. The first evidence of opioid modulation of cardiovascular hemodynamics came from an in-vivo rat model of endotoxic shock (49). Holaday and Faden discovered that the pretreatment with the opiate receptor antagonist naloxone prevented the hypotension and bradycardia associated with an injection of endotoxin. Naloxone was found to reverse rat endotoxic shock (49), hemorrhagic shock (28) and spinal shock (50); mouse anaphylactic shock (5); rabbit hemorrhagic shock (97); dog hypovolemic shock (43, 44, 73-76, 110) and endotoxic shock (89); cat hemorrhagic shock (17, 19) and splanchnic artery occlusion shock (18); pig hypovolemic shock (93); and monkey hemorrhagic shock (79).

Naloxone appeared to reverse shock states only in the presence of circulating catecholamines. Adrenalectomy, associated with sympathectomy, prevented the naloxone induced reversal of the cardiovascular consequences of shock (1, 75, 85). Intravenous naloxone also potentiated the cardiovascular effects of exogenous catecholamines in the dog (14, 40, 75) and the rat (32). Naloxone in these studies appears to augment the effects of catecholamines at the receptor site and not through an increase in sympathetic output or catecholamine release (76).

The in-vivo shock models demonstrate the cardiovascular significance of opioid blockade with naloxone. Many studies followed to determine the site of action of naloxone. All major systems involved in cardiovascular hemodynamics were investigated to determine at what level naloxone potentiates adrenergic control of cardiovascular hemodynamics. The ANS was examined including its baroreflex control system, CNS autonomics, autonomic ganglia and autonomic nerve termi-

nals. The effector organs (the heart and peripheral vasculature) were studied also as potential mediators of the naloxone response.

Opioid peptides inhibit and naloxone augments adrenergic effects in all cardiovascular structures listed above. Baroreflex sensitivity is augmented in the cat by naloxone pretreatment (68, 71). Naloxone acts at opiate receptors in the CNS to reverse shock hypotension (50) and morphomimetic agents have profound cardiovascular depressor effects in the CNS (7, 21,70-72) which are reversed by opiate antagonism. Activation of ganglionic opiate receptors with met-enk causes a naloxone-reversible peripheral vasodilation by interupting ganglionic transmission (16). Exogenous opioid peptides, acting on prejunctional opiate receptors, inhibit electrically stimulated release of norepinephrine from sympathetic nerve terminals in vascular smooth muscle in a naloxone-reversable manner (35, 57-60, 102, 113). The above data demonstrate that opioids tend to inhibit, and blockade with naloxone enhances, sympathetically mediated cardiovascular control at all levels of the ANS.

Naloxone also acts locally in the heart. Intracoronary dynorphin depresses and naloxone enhances cardiac function (13). Intracoronary naloxone acts to potentiate the effects of catecholamines in the canine intact (73) and isolated heart (14) in dosages ineffective when given intravenously. Despite evidence suggesting that naloxone may augment cardiac contractile responses to catecholamines by inhibiting extraneuronal catecholamine uptake, Gu et. al. (40) were unable to demonstrate a naloxone mediated change in uptake. Intracoronary naloxone failed to alter cardiac uptake of epinephrine in the canine isolated heart. Intracoronary dynorphin depresses nerve stimulation induced cardiac norepinephrine overflow and myocardial performance in a naloxone reversable fashion, yet dynorphin has no effect on exogenously administered norepinephrine. (+)Naloxone, the less active

enatiomer of naloxone, is as effective as (-)naloxone in facilitating the contractile response to epinephrine in the isolated canine heart (41). Naloxone may have dual effects in augmenting cardiac function, partially by blocking endogenous opioids at the presynaptic sympathetic nerve terminal and partially by a post junctional, non-opiate receptor mediated mechanism.

There is substantial evidence supporting the existence of opiate receptors in vascular smooth muscle. Opioid peptides infused into the peripheral circulation cause vasodilation in a naloxone-reversable fashion (26, 80, 119). A series of experiments has demonstrated the presence of delta- and kappa-receptors in the rabbit ear artery (35, 57-60, 66, 90). The receptors are on the postganglionic, presynaptic sympathetic nerve terminals. When stimulated, these receptors inhibit norepinephrine release from the sympathetic nerve terminals which reduces field stimulation induced vasoconstriction. The opioid induced inhibition of norepinephrine release is blocked by naloxone. These observations have also been made in the branch of the ileocolic artery (113), the jejunal artery (87), the smaller mesenteric artery (58), and the pulmonary artery (102) of the rabbit. The rabbit pulmonary artery has predominantly kappa-receptors. At high doses (10 μM), morphine and other morphomimetic agents inhibit electrically stimulated norepinephrine release and subsequent constriction of in-vitro canine saphenous veins in a naloxone insensitive manner (81). Naloxone does not augment vasoconstriction in these preparations when given alone which argues against tonic enkephalinergic inhibition *in-vitro*. Perhaps these neurovascular opiate receptors respond to circulating opioids.

Several vascular beds have been examined using tissue bath preparations in order to determine the effects of opioids and naloxone in the peripheral vasculature, isolated from neural or hemodynamic influences. Sharkawy et. al. (25) tested

a variety of opiate receptor agonists and antagonists on rings of rat aorta precontracted with norepinephrine, prostaglandin F_2alpha or KCl. They concluded that opioids do have a direct action on vascular smooth muscle, but endogenous opioid activity is unlikely because of the extremely high doses required. Ruth et. al. (92) found a significant, naloxone-reversable relaxation in norepinephrine precontracted spiral strips of rat aorta with low dose (0.1 nM) leu-enk. Naloxone alone had little effect, again arguing against tonic endogenous opioid activity. Various researchers often find seemingly conflicting results due to tissue and species variability in opiate receptor populations.

Cerebral arteries seem to respond to opioids and naloxone differently than other vascular beds. In canine cerebral arteries, naloxone inhibits norepinephrine induced vasoconstriction and has no effect on KCl or serotonin induced contractions. Morphine actually augmented the naloxone response in these vessels (95). Altura et. al. have discovered that in-vitro canine cerebral arteries relax in response to kappa-opiate receptor agonists (ketocyclazocine, ethylketocyclazocine and bremazocine) and contract in response to sigma-receptor ligands (MR1,452 and U50,488) in a naloxone insensitive manner (2, 3, 4). Morphine (46-48) and met- and leu-enkephalin (46, 47) were shown to induce naloxone-reversable relaxation in cat middle cerebral arteries. These studies indicate naloxone reverses enkephalin mediated vasodilation and reverses catecholamine mediated vasoconstriction. The effect of naloxone given alone in the cerebral circulation is as yet undescribed.

Naloxone potentiates the effects of catecholamines in the peripheral vasculature, although the mechanism by which it does so is unclear. Sasaki et. al. (94) found that naloxone (0.3 - 30 μM) alone caused no vasoconstriction. Naloxone augments epinephrine and norepinephrine induced vasoconstriction in a dose

dependent fashion, yet fails to affect contractions induced by phenylephrine (94). The effect of naloxone was abolished by pretreatment with an extraneuronal catecholamine uptake antagonist (normetanephrine) and unaffected by neuronal catecholamine uptake blockade. Canine renal interlobar (15) and skeletal muscle (103) arteries react similarly. Naloxone augments epinephrine induced vasoconstriction more so than norepinephrine or phenylephrine induced vasoconstriction and has no vasoactive effect alone. The ED_{50} for naloxone is higher than necessary for activity at known opiate receptors. Mu, delta and kappa opiate receptor agonists do not shift the naloxone dose response curve. Additionally, the opiate receptor inactive stereoisomer, (+)naloxone, actually has greater efficacy in augmenting epinephrine induced contractile responses (15). These data suggest that naloxone augments catecholamine induced vasoconstriction in an epinephrine selective, non-opiate receptor mediated manner. In all regards, the effects of naloxone appear similar to those of corticosterone (an extraneuronal catecholamine uptake blocker) which suggests a role for naloxone as a catecholamine uptake inhibitor (15, 94).

Caffrey also noted that canine renal vessels, contracted with epinephrine and naloxone, relaxed more rapidly than vessels contracted without naloxone once the epinephrine was removed (15). This observation supports the hypothesis that naloxone potentiates catecholamine induced vasoconstriction by blocking extraneuronal catecholamine uptake. Inhibition of extraneuronal catecholamine uptake could augment an existing catecholamine contraction by allowing a higher concentration of catecholamine to accumulate at the receptor sites. This mechanism could also allow for more rapid relaxation since less epinephrine accumulates intracellularly while uptake is blocked. Once the bath is cleared of all catecholamines, there is less epinephrine to diffuse out of the tissue, past the adrenergic receptors, thereby shortening the relaxation time.

Naloxone was originally described as a pure opiate receptor antagonist when it was first utilized to reverse various types of shock (49-52). Evidence now suggests that naloxone has cardiovascular effects unrelated to its action as an opiate receptor antagonist (96). Endogenous opioids and opiate receptors are strategically located to mediate cardiovascular responses. Opiate receptors function in a naloxone reversable manner to modulate autonomic regulation of the cardiovascular system. This is accomplished through action in the baroreflexes; the brain; the spinal cord; and the autonomic nerves, ganglia and effector terminals. Opiate receptors modulate autonomic regulation of the cardiovascular system via direct actions on the heart and peripheral vasculature as well. Naloxone appears to augment catecholamine influences on the heart and peripheral vasculature by a dual mechanism. Naloxone clearly reverses opioid peptide induced inhibition of norepinephrine release from postganglionic, presynaptic sympathetic nerve terminals. In addition, it appears that naloxone selectively potentiates epinephrine induced cardiovascular responses by a nontraditional opiate receptor mechanism or by way of a non opiate receptor mechanism.

Relatively high doses of naloxone are required to augment catecholamine induced responses in the heart (13, 14, 40, 41, 73) or peripheral vasculature (15, 25, 46-48, 81, 92, 94). (+)Naloxone is less active at traditional opiate receptors yet has equal or greater efficacy than (-)naloxone in the isolated canine heart (41) and *in-vitro* renal arteries (15). These data suggest the effect of naloxone is not mediated via mu-, kappa- or delta-receptors. Perhaps naloxone is binding to a nontraditional opiate receptor at which naloxone has much less affinity and therefore requires a very high concentration for receptor blockade. (+)Naloxone may have equal efficacy to (-)naloxone because the enantiomers may be equally poor ligands at the receptor in question.

The sigma receptor may be the site of action of naloxone in these studies. Sigma receptors seem to subserve vasoconstriction in cerebral vasculature (2-4). Naloxone has relatively poor affinity for the sigma receptors (61, 115). Sigma receptors also have the unique characteristic of binding with greater affinity to the (+) enantiomer of many known sigma ligands such as (+)SKF 10,047 (N-allyl-normetazocine), (+)pentazocine, (+)cyclazocine and (+)3-PPP [1-propyl-3-(3-hydroxyphenyl) piperidine] (61, 115). (+)Naloxone may have greater efficacy than (-)naloxone because it is acting at a sigma receptor.

Corticosteroids augment the cardiovascular effects of catecholamines *in-vivo* (40, 88) and *in-vitro* (15, 34, 62-64, 108). Corticosterone exactly mimicked the effects of naloxone in the canine renal interlobar artery (15). Perhaps naloxone is not only an opiate receptor antagonist at low doses, but also a corticosteroid substitute at high doses.

Naloxone may potentiate adrenergic cardiovascular effects both by mimicking endogenous corticosteroids and acting as a sigma ligand. Certain steroid hormones have demonstrated high affinity as sigma-receptor ligands (104, 115). Testosterone, progesterone and deoxycorticosterone were among the steroids with the highest sigma affinity. There is not sufficient data to determine whether naloxone augments adrenergic induced vasoconstrictor tone by mimicking the action of corticosteroids via extraneuronal catecholamine uptake blockade, by action as a sigma-receptor ligand or by another unrelated mechanism.

Summary and Experimental Inquiry

The effect of naloxone must be examined at all levels of the cardiovascular system in order to fully understand the mechanism by which naloxone reverses

cardiovascular decline in a variety of shock states. Naloxone clearly functions as an opiate receptor blocker in the central and peripheral nervous system, but evidence suggests a different mechanism of action in the heart and peripheral vasculature. Naloxone reverses opioid peptide mediated inhibition of norepinephrine release from sympathetic nerve terminals in the heart and peripheral vasculature. But, naloxone also demonstrates a high dose, non-stereoselective augmentation of exogenous catecholamine responses in the heart and vasculature isolated from neural influences. It is unclear which mechanism of action of naloxone contributes most significantly to the reversal of cardiovascular decline in shock. This work examines the mechanism by which naloxone potentiates adrenergic vasoconstriction in canine skeletal muscle arteries isolated from neural or humoral influences.

This laboratory has investigated the effects of opioid peptides and naloxone in the *in-vivo* and isolated canine heart, the isolated canine renal interlobar artery, the sympathetic ganglion regulating hindlimb vascular resistance, and the *in-vivo* and *in-vitro* hind limb vasculature (10-16, 40, 41, 103). This work continues to use canine arteries so that the results obtained may be integrated with other hemodynamic data collected in this lab. Skeletal muscle arteries were selected because the skeletal muscle vasculature comprises the greatest single vascular bed in the body. If naloxone reverses the hemodynamic decline in shock predominantly via a direct action on the peripheral vascular smooth muscle, the skeletal muscle vasculature must surely participate. It is unlikely that naloxone could reverse shock hypotension by constricting vascular beds without a significant vasoconstrictive contribution from the skeletal muscle vasculature.

Isolated canine skeletal muscle vasculature has not been examined for its response to opioid peptides or naloxone. Therefore, this work initially focuses on the

reproduction of observations in other vascular beds. The following questions therefore had to be answered:

1. What is the optimal resting tension?
2. Does naloxone induce vasoconstriction alone?
3. Does naloxone affect adrenergic vasoconstriction?
4. Does naloxone differentially affect various adrenergic agonists?
5. Does naloxone affect non-adrenergic vasoconstrictors?

If skeletal muscle arteries respond like other non-cerebral vascular beds, then naloxone is expected to selectively potentiate epinephrine induced vasoconstriction and have no vasoconstrictive activity alone. Given these results, several questions addressing the mechanism of action of naloxone must be answered.

6. Does naloxone affect alpha-adrenergic receptor activity?
7. Does naloxone affect beta-adrenergic receptor activity?
8. Does naloxone alter catecholamine disposal mechanisms?
9. Is the naloxone effect endothelium dependent?
10. How does the response to naloxone compare with that of various opiate receptor agonists and antagonists?
11. Is the response to naloxone stereoselective?
12. How does the response to naloxone compare with that of various sigma-receptor ligands?
13. How does the response to naloxone compare with that of various steroid hormones?

The answers to the above questions will give information that is both descriptive and mechanistic. The untried *in-vitro* skeletal muscle vasculature must be proven to be an adequate model to confirm past observations. Once the effect of naloxone is qualitatively and quantitatively known, the mechanism can accurately be pursued.

Elevated peripheral vascular resistance can significantly elevate arterial blood pressure. A new method of regulating peripheral vascular resistance may be unveiled once the mechanism by which naloxone augments vascular smooth muscle responsiveness to catecholamines is discovered. Knowledge of the local vascular effects of naloxone may support its use in clinical treatment of various shock states. By expanding knowledge in the field of cardiovascular opiate/catecholamine interactions, a new tool may be gained to control hypertension.

CHAPTER II

MATERIALS AND METHODS

Materials

A) Apparatus

All experiments described within this dissertation utilize the vessel bath apparatus illustrated in Figure 2. The basic materials utilized to conduct these experiments include:

1. A heated water reservoir tank with a circulating pump.
2. Jacketed test tubes with inflow and outflow ports to the jacket and an oxygen bubbling port, a drainage port, and small inverted glass hooks mounted near the bottom of the inner tube. Radnoti tissue-organ bath size, 10 milliliter, catalog No. 158410.
3. Strain gauge transducers sensitive in the range from 0.002 to 50 grams with accuracy of ± 1%. Grass model number FT03.
4. Grass Polygraph recorders models 7D (eight channel) and 79D (four channel).
5. Compressed gas cylinders (size G) filled with 95% oxygen and 5% carbon dioxide (± 1%) and pressure regulator valves.
6. Dissecting microscope. Olympus model MTX.
7. pH meter. Fisher Accumet model 325.
8. Vernier scales.

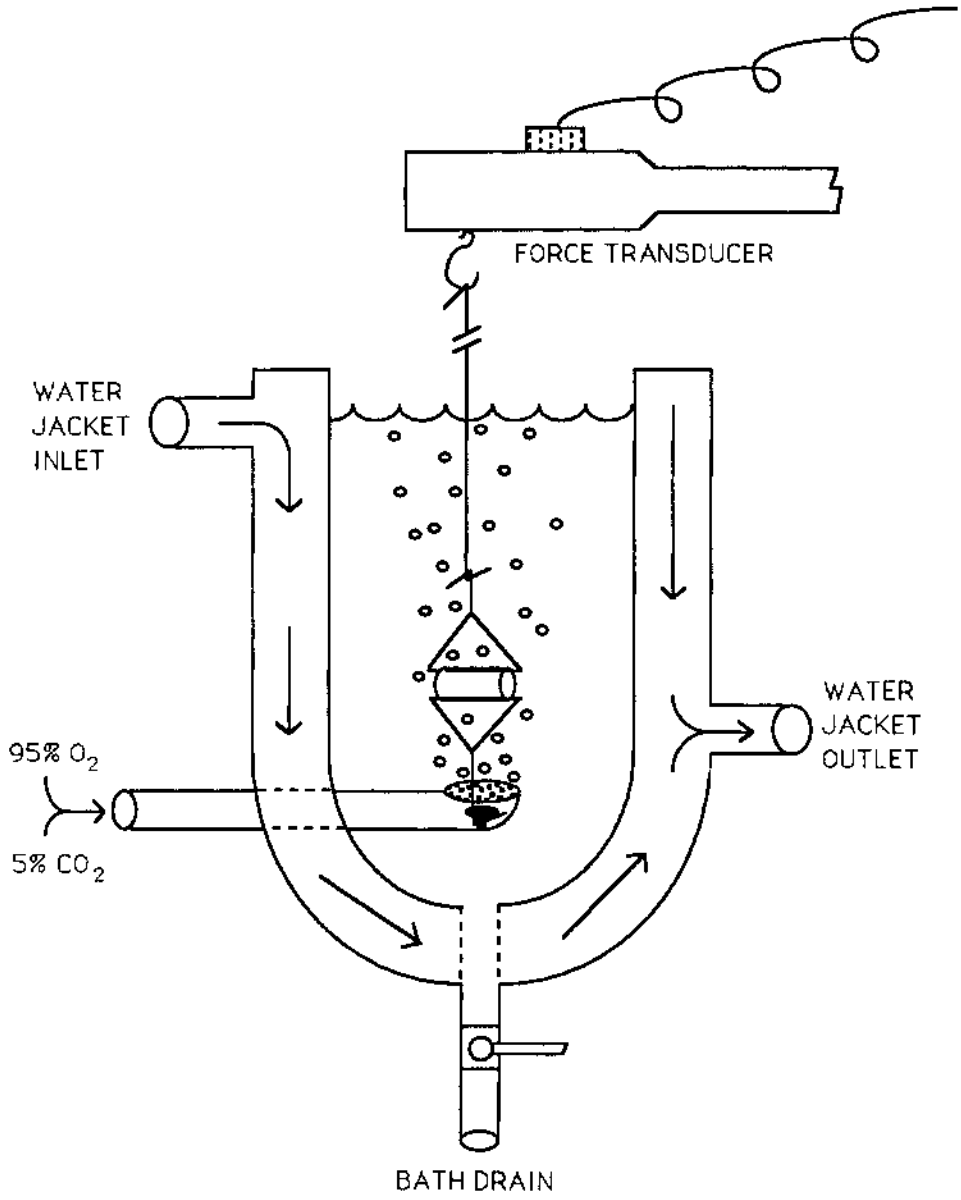


Figure 2. Illustration of in-vitro vessel bath apparatus.

9. Gas flow control valves (Cole-Palmer, model #6393-60).
10. Cornwall continuous pipetters (model #3054).
11. Modified Krebs Ringers bicarbonate solution (KRB) (pH: 7.4 ± 0.1)
    a. Sodium chloride (NaCl).....118.2 mM.
    b. Potassium chloride (KCl).....4.6 mM.
    c. Potassium phosphate (KH_2PO_4).....1.2 mM.
    d. Magnesium sulfate ($MgSO_4 \cdot 7H_2O$).....1.2 mM.
    e. Sodium bicarbonate ($NaHCO_3$).....23.8 mM.
    f. Dextrose ($C_6H_{12}O_6$).....9.1 mM.
    g. Ethylenediaminetetraacetic acid (EDTA).....0.03 mM.
    h. Calcium chloride dihydrate ($CaCl_2$).....2.5 mM.

B) Pharmacologic agents

As follows is a listing of all pharmacologic agents employed, their pharmacologic action and their corresponding commercial or private supplier.

1. epinephrine (endogenous, nonselective adrenergic agonist) Sigma
2. norepinephrine (endogenous, nonselective adrenergic agonist) Sigma
3. phenylephrine ($alpha_1$-adrenergic agonist) Sigma
4. lofexidine ($alpha_2$-adrenergic agonist) Merell Dow
5. vasopressin (nonadrenergic vasoconstrictor) Sigma
6. serotonin (nonadrenergic vasoconstrictor) Sigma
7. phentolamine (nonselective alpha-adrenergic antagonist) Sigma
8. timolol (nonselective beta-adrenergic antagonist) Sigma
9. propanolol (nonselective beta-adrenergic antagonist) Sigma
10. acetylcholine (endogenous, nonselective cholinergic agonist) Sigma

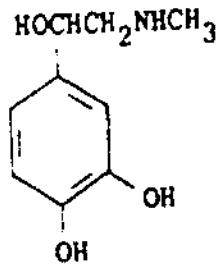
11. imipramine (neuronal catecholamine uptake inhibitor) Sigma
12. desipramine (neuronal catecholamine uptake inhibitor) Sigma
13. pargyline (monoamine oxidase inhibitor) Sigma
14. pyrogallol (catechol-O-methyl transferase inhibitor) Sigma
15. naloxone (alkaloid, mu-opiate receptor antagonist) DuPont
16. naltrexone (alkaloid, mu-opiate receptor antagonist) DuPont
17. morphine (alkaloid, mu-opiate receptor agonist) Sigma
18. mophiceptin (peptide, mu-opiate receptor agonist) Vega Biotech
19. naltrindolol (alkaloid, delta-opiate receptor antagonist) Sigma
20. leucine-enkephalin (peptide, endogenous delta-opiate receptor agonist) C.R.B.
21. methionine-enkephalin (peptide, endogenous delta-opiate receptor agonist) C.R.B.
22. MR1,452 (alkaloid, kappa-opiate receptor antagonist) Boehringer Ingelheim
23. dynorphin 1-8 (peptide, endogenous kappa-opiate receptor agonist) Peninsula
24. dynorphin 1-9 (peptide, endogenous kappa-opiate receptor agonist) Peninsula
25. U50,488 (alkaloid, kappa-opiate receptor agonist) Upjohn
26. diprenorphine (alkaloid, nonselective opiate receptor antagonist) N.I.D.A.
27. (+)-1-propyl-3-(3-hydroxy-phenyl)piperidine [(+)-3-PPP] (phenylpiperidine, sigma opiate receptor ligand) R.P.I.
28. (-)-1-propyl-3-(3-hydroxy-phenyl)piperidine [(-)-3-PPP] (phenylpiperidine, sigma receptor ligand) R.P.I.
29. haloperidol (butyrophenone, sigma receptor ligand) R.P.I.
30. rimcazole (atypical antipsychotic, sigma receptor ligand) R.P.I.

31. (+)pentazocine (benzomorphan, sigma receptor ligand) Sigma
32. (-)pentazocine (benzomorphan, sigma receptor ligand) Sigma
33. di-o-tolylguanidine (DTG) (N,N'-diaryl substituted guanidine, sigma receptor ligand) R.P.I.
34. corticosterone (endogenous active steroid hormone) Sigma
35. hydrocorticone(endogenous active steroid hormone) Sigma
36. progesterone (endogenous active steroid hormone) Sigma
37. tetrahydrocorticosterone (inactive corticosterone metabolite) Sigma
38. tetrahydrocortisol (inactive hydrocortisone metabolite) Sigma
39. pregnanalone (inactive progesterone metabolite) Sigma
40. 11-dehydrocorticosterone (corticosterone metabolite) Sigma
41. 11-deoxycorticosterone (corticosterone metabolite) Sigma

Most structures for the above compounds are on the following pages.

Subjects

Mongrel dogs were procured by the Texas College of Osteopathic Medicine (TCOM) Animal Care Facility under strict adherence to the official guidelines regulating collection, care and housing of research animals. The TCOM Animal Care Facility met or exceeded all official standards as set by the National Institutes of Health (NIH), United States Department of Agriculture (USDA), United States Public Health Service (USPHS) and the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Use of all animals was approved by the TCOM Animal Care and Use Committee. The NIH, USDA, USPHS and AAALAC guidelines for humane care and treatment of research animals was always carefully observed in this laboratory. Great care was taken to ensure these animals did not suffer.



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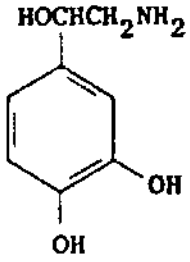

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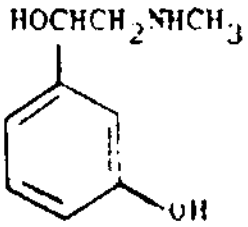
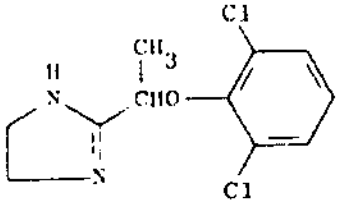


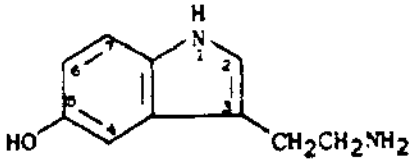
PHENYLEPHRINE



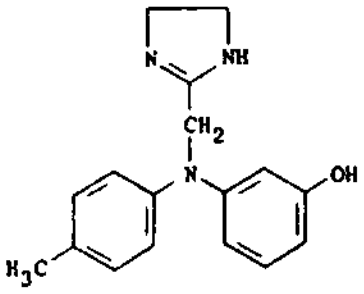
LOFEXIDINE

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH2

VASOPRESSIN



SEROTONIN



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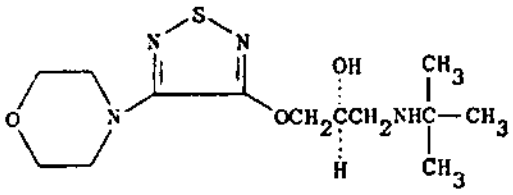

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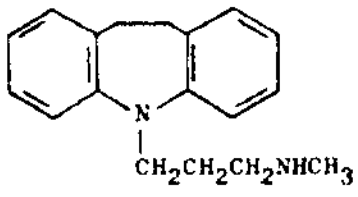

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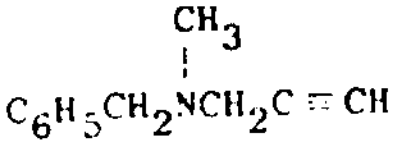

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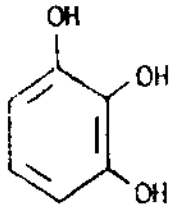
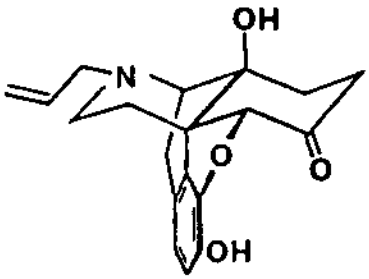


PYROGALLOL



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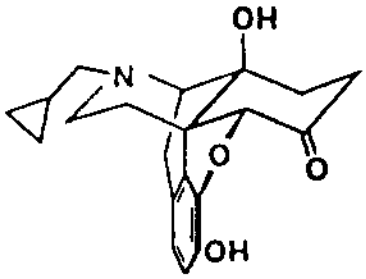

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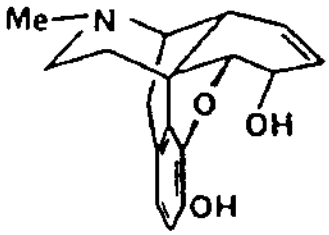

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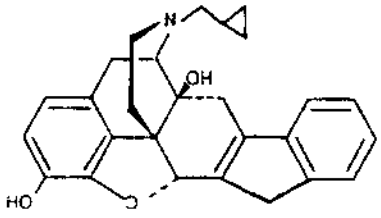

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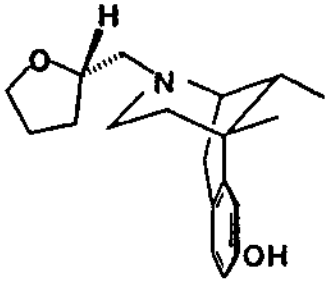

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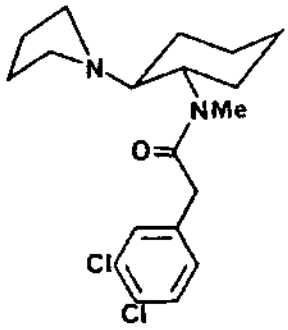

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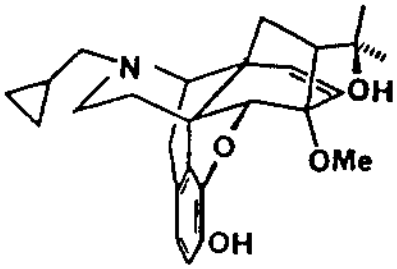

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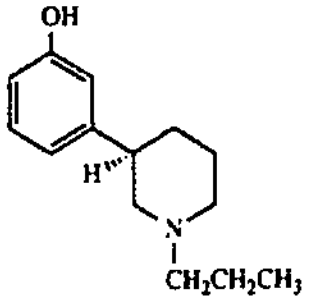
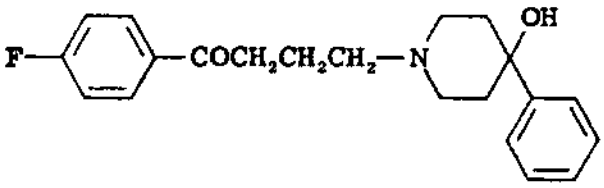


(+)PPP



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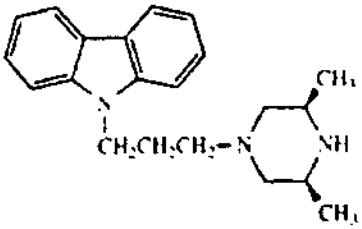

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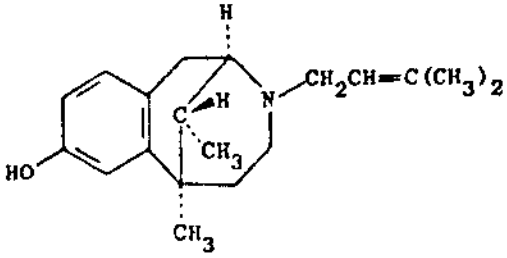

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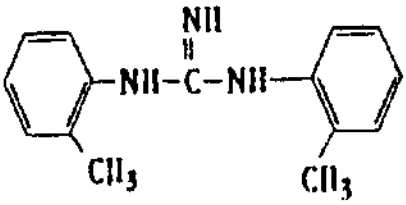

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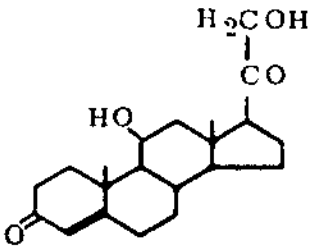

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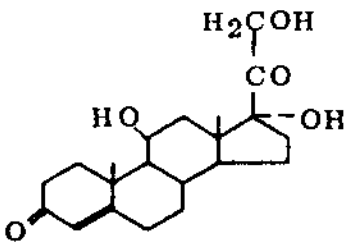
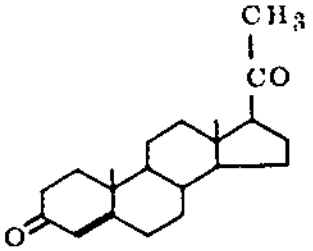


HYDROCORTISONE



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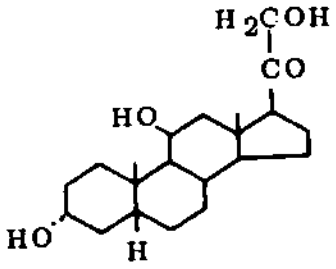
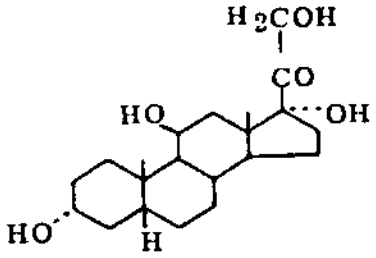


TETRAHYDROCORTICOSTERONE



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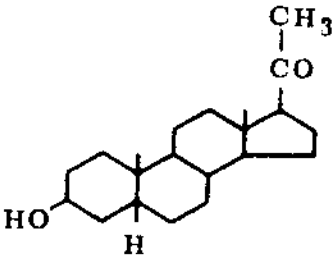
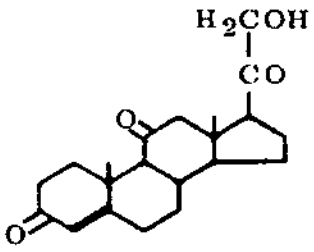
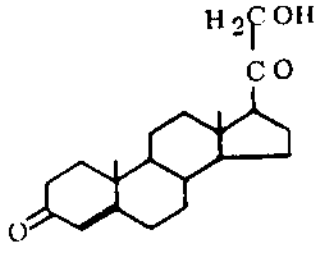


PREGNANALONE



11-DEHYDROCORTICOSTERONE



11-DEOXYCORTICOSTERONE

All experiments are performed with arterial segments harvested from mongrel dogs. A representative sample of 12 dogs had a mean weight of 15.7 ± 1.4 kilograms. A representative sample of 74 arterial segments from 8 dogs had a mean length of 3.8 ± 0.07 millimeters and a mean outer diameter of 1.5 ± 0.04 millimeters.

Procedures

Arteries were suspended in a physiologic tissue bath so that changes in wall tension could be measured with the strain gauge and recorded via the chart recorder. A variety of pharmacologic probes were employed to investigate the mechanism by which naloxone potentiates epinephrine induced vasoconstriction. Tissue was collected from the animals within thirty minutes after euthanasia under anesthesia. The skin overlying the medial thigh, from the knee to the inguinal canal was dissected away. Skeletal muscle surrounding the femoral artery from the knee to the inguinal canal including several centimeters of tissue medial and lateral to the artery was removed. This single, large piece of femoral muscle tissue was immediately submerged in iced, oxygenated, Krebs Ringers bicarbonate solution (KRB).

A dissecting microscope was employed to prepare the arterial segments for suspension in the baths. The muscle tissue was transferred to a shallow dissection pan set in ice and filled with KRB. The dissection followed the femoral artery to the saphenous artery which is a branch immediately off the main artery. The saphenous artery can usually be seen, once the skin is removed, traversing the distal medial aspect of the thigh and bifurcating at the knee. The saphenous artery was carefully dissected free leaving little visible adventitia present on the vessel wall. The artery was sectioned into four millimeter long cylinders, each individually hung on two opposing stainless steel triangles fashioned from 4-0 surgical steel suture. Figure

3 depicts the method for hanging a vessel onto a folded wire. These vessels threaded onto triangular frames were refrigerated in KRB and used within thirty-six hours.

The transducers, amplifiers, preamplifiers and chart recorders were calibrated daily. The jacketed tissue baths were filled with 6 ml of KRB and were maintained at 37° C. The KRB was made fresh daily and also maintained at 37° C. in a heated reservoir. The humidified 95% O_2 and 5% CO_2 gas mixture was bubbled steadily through the KRB solution in the reservoir flask and each of the jacketed tubes. The pH of the KRB was continually monitored and maintained at 7.4 ± 0.05.

Figure 2 illustrates how vessels appeared suspended in the bath. The vessels were suspended such that the hook on one triangle caught the inverted hook at the bottom of the baths. The hook on the other triangle was linked via a connecting piece of 3-0 surgical steel suture to the force transducers mounted above. The force transducers are mounted on vernier scales that allow for minute adjustment of the vessel wall tension.

Once suspended in the KRB at 37°C, the vessels underwent a two hour equilibration period. During equilibration, vessels were washed (the bath is drained and refilled with fresh KRB) every fifteen minutes. Continuous pipetters allowed for rapid and accurate refilling of the tissue baths with KRB once emptied by momentary opening of the drain valves. Figure 4 illustrates the method by which vessels were raised to resting tension. The equilibrium period began with thirty minutes of no tension on the vessels. Tension was then increased stepwise by carefully raising the force transducers with the vernier scales over a thirty minute period. Tension was increased one to two grams, the vessels relaxed for several minutes until equilibrated to a new resting tension. This process was repeated until the equilibrated

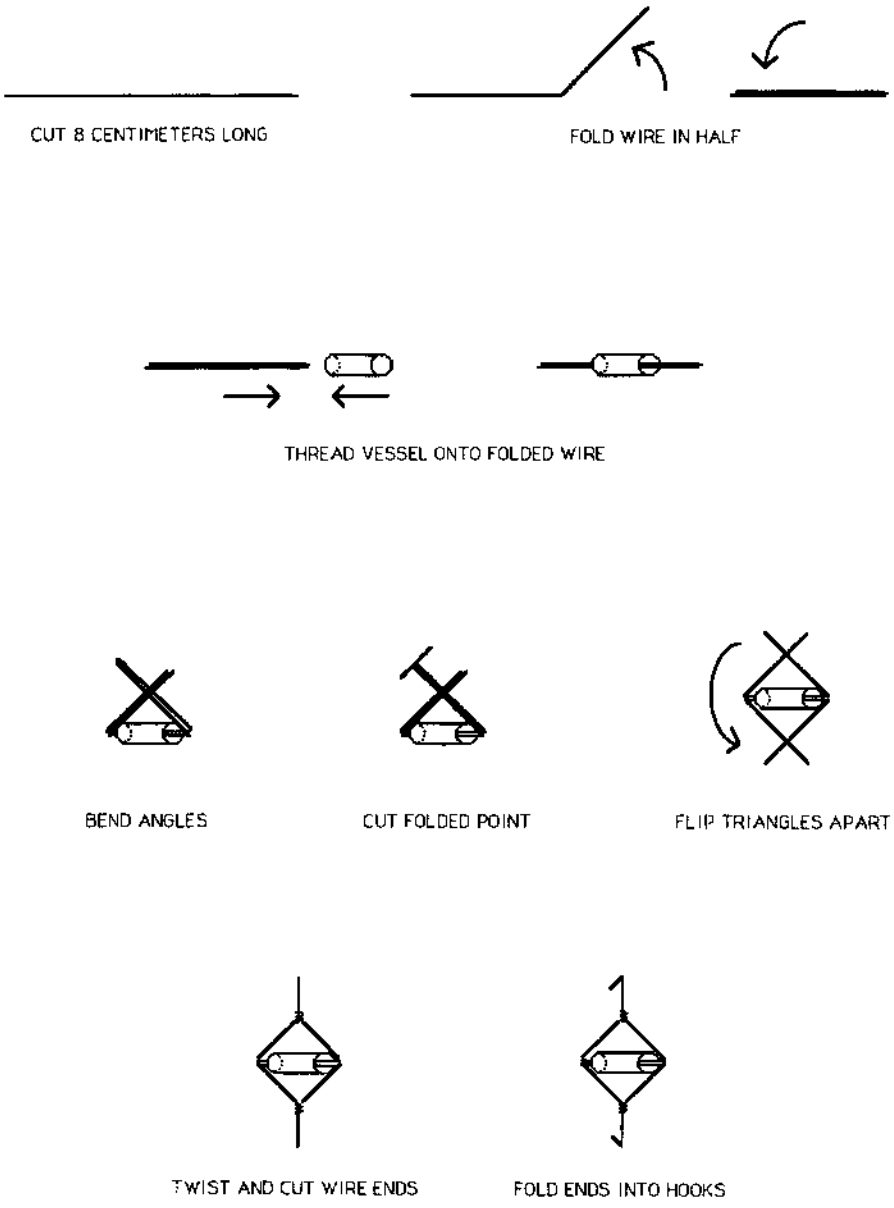


Figure 3. Illustration depicting method of hanging vessel segments on apposing stainless steel wire triangles.

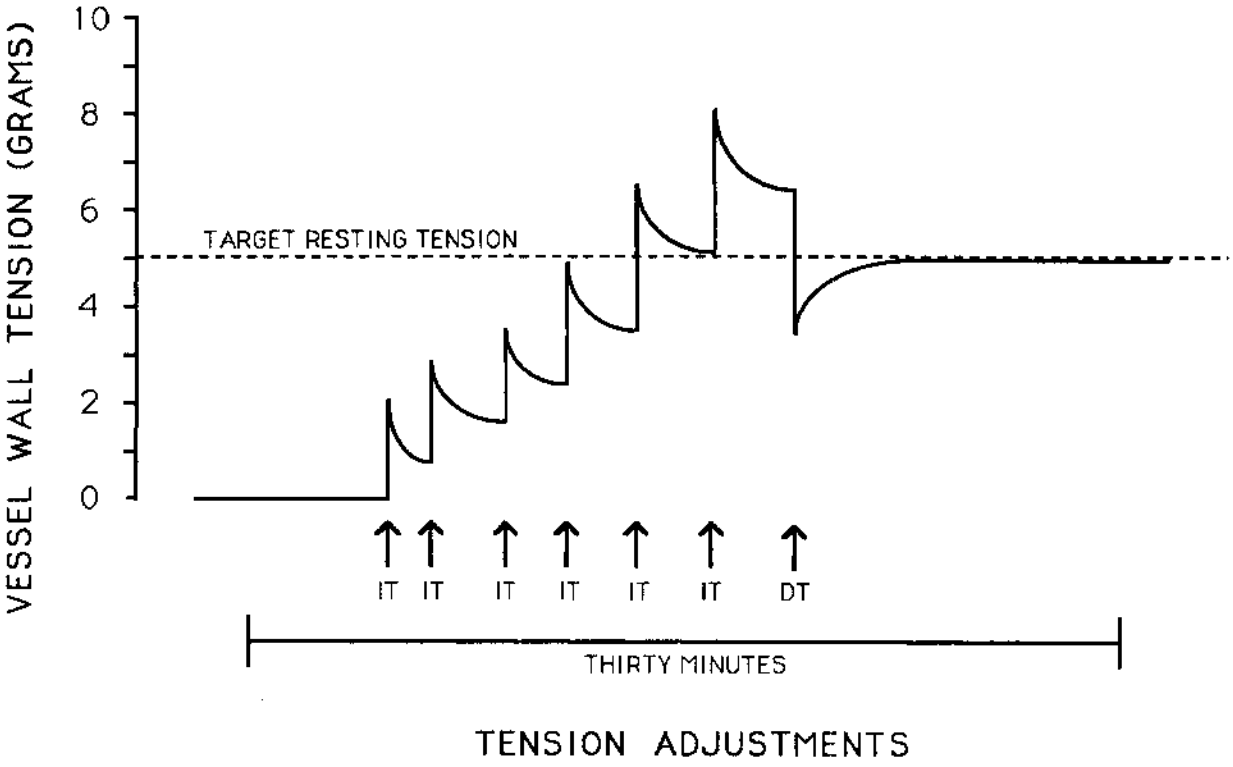


Figure 4. Illustration of how vessels were gradually taken from zero tension to five grams of resting tension. Tension was increased incrimentally. After each increase in tension (IT), the vessel underwent reflex relaxation. Once vessel tension was maintained above the target resting tension, the tension was decreased (DT) and the vessel underwent reflex contraction back up to the target resting tension.

tension was one to two grams above the target resting tension. The tension was then decreased such that after a small reflex contraction the wall tension was at the desired resting tension.

Vessel smooth muscle was depolarized when potassium chloride (KCl) was added to the KRB in the baths to a final concentration of 80 mM, which caused the vessels to undergo KCl induced maximal contraction. The vessels were washed three times and allowed to return to resting tension. This process was repeated once again, two times total, for all vessels prior to experimentation.

All vessels also underwent a test for the presence of intact endothelium. Once vessels returned to resting tension following the second KCl induced contraction, norepinephrine (0.1 - 1.0 μM) was added to the bath. The dose of norepinephrine was adjusted to stimulate a 2 - 4 gram contraction. Acetylcholine (1.0 μM) was then added to the bath. Vessels were judged to have intact endothelium, and therefore acceptable for experimentation, if they relaxed by at least 50% within five minutes. The vessels were washed three times in succession once endothelial viability was established. Vessel wall tension was adjusted as described above until the appropriate resting tension (five grams) was reestablished.

Experimentation began once the two hour equilibration process was complete, vessel baths had been thoroughly washed with fresh KRB and resting tension was maintained for 15 minutes. Great care was taken to ensure that all vessels were treated identically before the onset of experimentation. Experimental procedures and protocols specific to the various experiments performed are described in the results section.

Statistical Analyses

Due to the wide variety of experimental protocols employed, a variety of statistical analyses were applied as appropriate. An unpaired student t-test was used to compare results from two groups of vessels each undergoing a different experimental condition. A paired student t-test was used to compare results from a single group of vessels undergoing two different experimental conditions in sequence. A factorial ANOVA was used to make comparisons between results from several groups of vessels each undergoing a different experimental condition. A repeated measures ANOVA was used to make comparisons between results from a single group of vessels undergoing a variety of experimental conditions in sequence. The unpaired and paired t-tests and the major effects of the ANOVA analyses were all considered significant at $p < 0.05$. Post-hoc ANOVA Scheffe analyses were employed to determine the significance of minor effects. In order to ensure the validity of examining the ANOVA minor effects, post-hoc Scheffe analyses were only considered significant at $p < 0.01$. If ANOVA minor effects were reported as significant, the major effects were significant as well.

Statistical analyses on dose-response data were applied to the log dose data. The statistical analyses employed were valid given an assumption of a normally distributed data set. The log dose data more closely approximated a normal distribution than does the raw data (33).

CHAPTER III

RESULTS

A large variety of experimental results are presented below. These are the results obtained in the course of answering the questions posed in the introduction of this dissertation. The presentation of these results is sequenced to parallel the sequence of questions posed as well as the general chronology of the studies. Statistical tools are identified as they are employed. All drug concentrations are expressed as final bath concentrations. The number of vessels in each study is expressed as the total number of vessels from the total number of dogs [# of vessels (# of dogs)]. Results are presented as mean ± standard error of the mean. Data detailing the magnitude of arterial vasoconstriction is given as either absolute grams tension generated or tension change as a percent of KCl maximum contraction. Presentation of data as percent of maximum KCl induced vasoconstriction normalized data and reduced inter-vessel variation caused by differences in vessel wall smooth muscle content.

Optimal Resting Tension

The resting tension is the vessel wall tension at the onset of all experiments. The resting tension is a passive tension induced by the applied external force of stretching. Active tension is tension that is generated internally by vasoconstriction. Active tension is determined by subtracting resting tension from total tension generated after administration of a vasoconstrictor. The optimum resting tension

(T_{max}) is that which allows for the maximum possible active tension. T_{max} was determined experimentally, in this work, using two studies. First, norepinephrine (10 μM) was used to maximally contract vessels at a variety of resting tensions (1, 2, 3, 4 and 5 grams). Figure 5 illustrates the results as each vessel undergoes maximal contraction with 10 μM norepinephrine at each resting tension. Active tension varied significantly between the following resting tensions: 1 vs. 4 and 5 grams; and 2 vs. 5 grams. There was a direct relationship of increasing maximum active tension with increasing resting tension without an evident plateau in the curve in Figure 5.

A second, similar study was completed for two reasons. Resting tensions had to be tested which were great enough to demonstrate declining active tension with increasing resting tension to ensure that the optimum or plateau resting tension had been tested. The norepinephrine study failed to demonstrate this decline in active tension. Norepinephrine contractions relaxed more slowly than KCl and there was concern that vessels did not reequilibrate completely between each resting tension trial. In Figure 6, vessels were maximally contracted by 80 mM KCl from 2, 5, 8, 11 and 14 grams resting tension. Maximum active tensions varied significantly between all resting tensions except for 2 vs. 5 and 8 grams and 5 vs. 8 grams. The data indicated that 2, 5, and 8 grams allowed for statistically equivalent active tensions with an apparent maximum at 5 grams resting tension (T_{max}). In addition, there was a progressive decline in active tension as resting tension increased from 8 to 11 to 14 grams. This declining portion of the curve demonstrates that Tmax had been surpassed. The portion of the curve in Figure 6 between 2 and 8 grams resting tension defined a relative plateau. It was important to use a resting tension which corresponded to this plateau because it reduced inter-vessel variability and ensured maximum vessel reactivity. Five grams was chosen as the optimal resting tension and all experiments began from this resting tension.

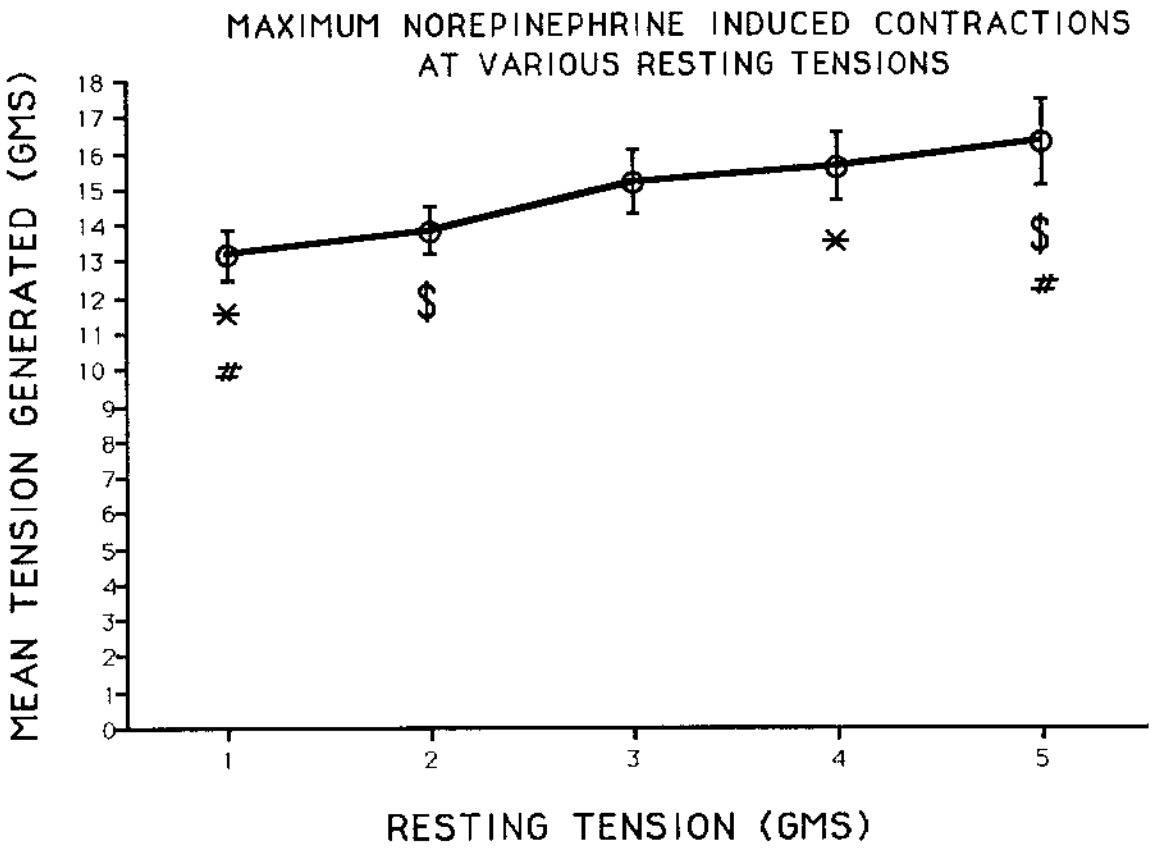


Figure 5. Vessels were maximally contracted with norepinephrine (10 μM) at each of the given resting tensions. A post-hoc ANOVA Scheffe analysis revealed that the maximum tensions generated marked with paired symbols were different at $p < 0.01$. Values are mean ± SEM. N = 15(2).

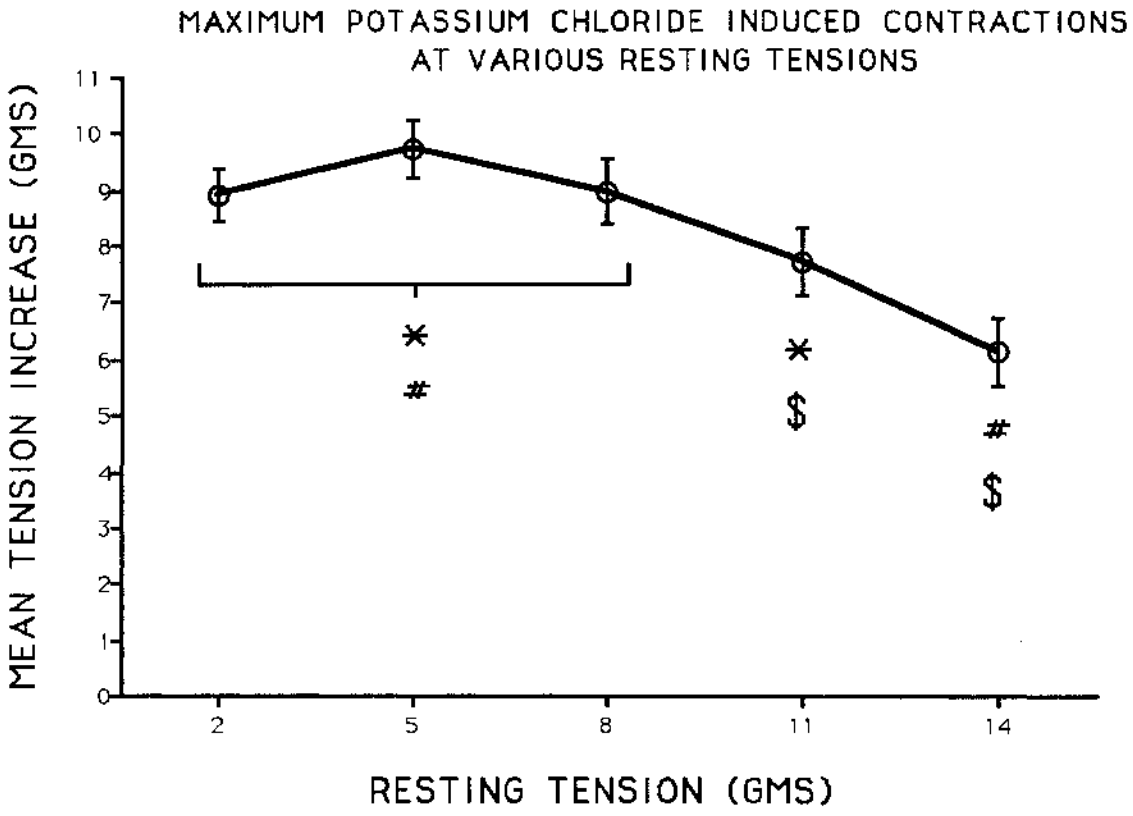


Figure 6. Vessels were maximally contracted with KCl (80 mM) at each of the given resting tensions. A post-hoc ANOVA Scheffe analysis revealed that the maximum tensions generated marked with paired symbols were different at $p < 0.01$. The maximum tensions generated marked with the bracket were not different from each other. Values are mean ± SEM. N = 46(5).

Naloxone Effect

Naloxone was used initially to help study opiate/catecholamine interactions in the peripheral vasculature. Preliminary studies shifted the focus of this dissertation to the elucidation of the mechanism by which naloxone potentiates epinephrine induced vasoconstriction in canine skeletal muscle arteries. This effect of naloxone to potentiate epinephrine induced vasoconstriction (naloxone effect) was most apparent when naloxone was applied to a vessel with a submaximal epinephrine induced contraction. Naloxone (10 μM) applied to vessels precontracted by 15 - 20% with epinephrine, stimulated a 205.1 ± 35.7% increase in vessel wall tension. Naloxone applied alone without precontraction had no effect. The naloxone effect is illustrated in Figure 7. The epinephrine induced contraction was augmented 2 - 3 fold with naloxone. The vessel was washed and returned to resting tension. Naloxone reapplied in the absence of epinephrine had no effect, yet when the naloxone was followed by a repeat of the original epinephrine dose, the full augmented response recurred.

This specific naloxone effect observed in isolated skeletal muscle arteries was characterized in three types of studies. In the first, single, near maximum doses of naloxone were added to vessels precontracted with epinephrine. In the second, dose-response relationships for epinephrine were examined in the presence and absence of naloxone. Finally, a dose-response relationship was determined for naloxone in the presence of an epinephrine induced precontraction.

A. Single Dose Naloxone

Many of the following experiments used a single dose of naloxone to study its effects. Ten μM was selected as an adequate dose of naloxone to elicit a substantial,

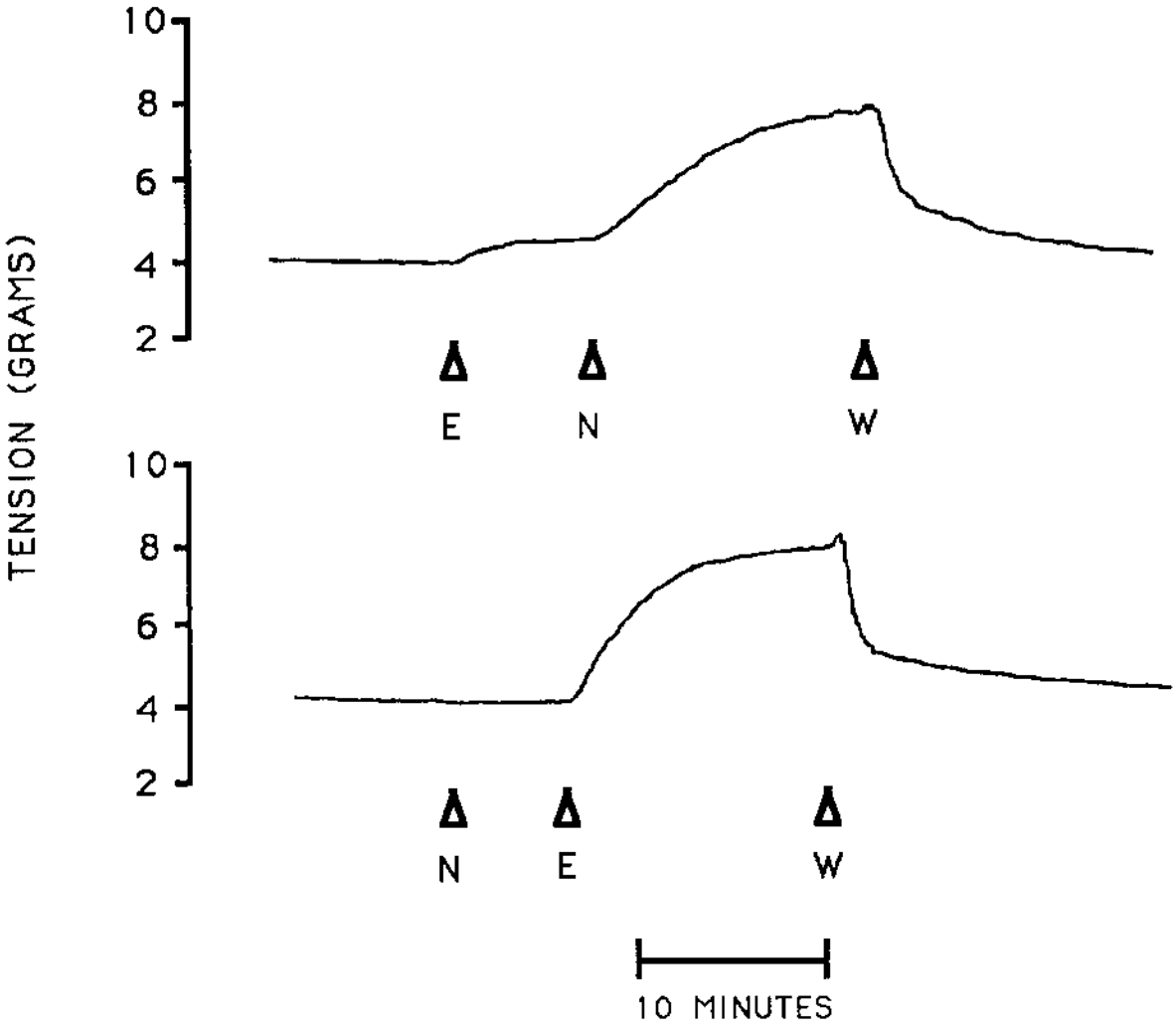


Figure 7. The above tracings are from the same vessel. Naloxone given after a submaximal epinephrine contraction caused marked vasoconstriction (upper tracing). Naloxone given alone had no vasoconstrictor action. Epinephrine given after naloxone stimulated a vasoconstriction greater than that of epinephrine alone and equal to the contraction of epinephrine with naloxone (lower tracing). "E" is epinephrine at a submaximal dose. "N" is naloxone (10^{-5} M). "W" is wash of the bath solution.

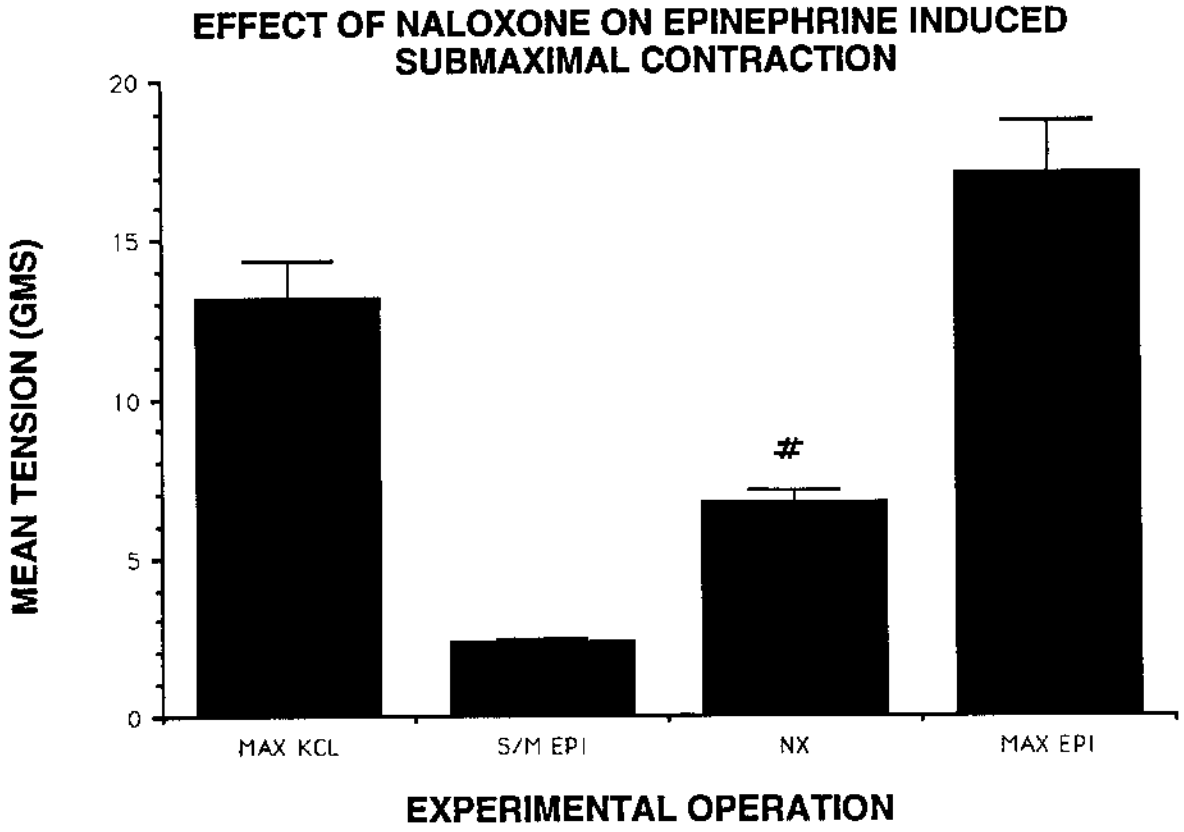


Figure 8. MAX KCL and MAX EPI are the mean maximum tension induced by KCl (80 mM) and epinephrine (10 μM). S/M EPI is the mean submaximal tension induced by epinephrine. NX is mean tension induced by epinephrine with naloxone (10 μM). S/M EPI was 20.0 ± 2.2% of MAX KCL and 15.6 ± 1.8% of MAX EPI. (*) indicates different from S/M EPI at $p < 0.05$. Values are mean ± SEM. N = 15(6) vessels.

reproducible response when added to an epinephrine precontraction. Subsequent data illustrated in Figure 13 indicated that this dose elicited a near maximum naloxone response.

Naloxone (10 μM) was added to vessels precontracted with epinephrine. Figure 8 illustrates the sequential course of the experiment from left to right as well as the vascular responses in absolute terms. The first bar represents the maximum KCl induced contraction. Vessels were washed and allowed to reequilibrate. Vessels were then precontracted with epinephrine to the level indicated by the second bar. Naloxone (10 μM) was added while vessels were precontracted and the resultant tension is indicated by the third bar. Finally, epinephrine (100 μM) was added to induce maximal contraction (fourth bar). Naloxone increased the epinephrine precontraction by 194.3 ± 19.7%. This was equivalent to an increase of 34.2 ± 2.2% of KCl maximum contraction.

B. Epinephrine Dose-Response

Vessels were divided into two groups, A and B. Two sequential epinephrine dose-responses (10^{-9} to 10^{-4} M) were constructed in each group. See Figures 9 and 10. Group A was exposed to epinephrine alone for the first run, then epinephrine in the presence of naloxone for the second run. Group B was treated identically, but in reverse order. All vessels in each group were thoroughly washed and allowed to relax and reequilibrate for over two hours between the first and second runs. The ED_{50} for epinephrine in the presence and absence of naloxone was calculated from a best fit line which included two doses above and below the point of half maximum contraction. The ED_{50}s for Group A first and second runs are listed in Table 1, below. Naloxone caused a significant leftward shift of both the first and second run

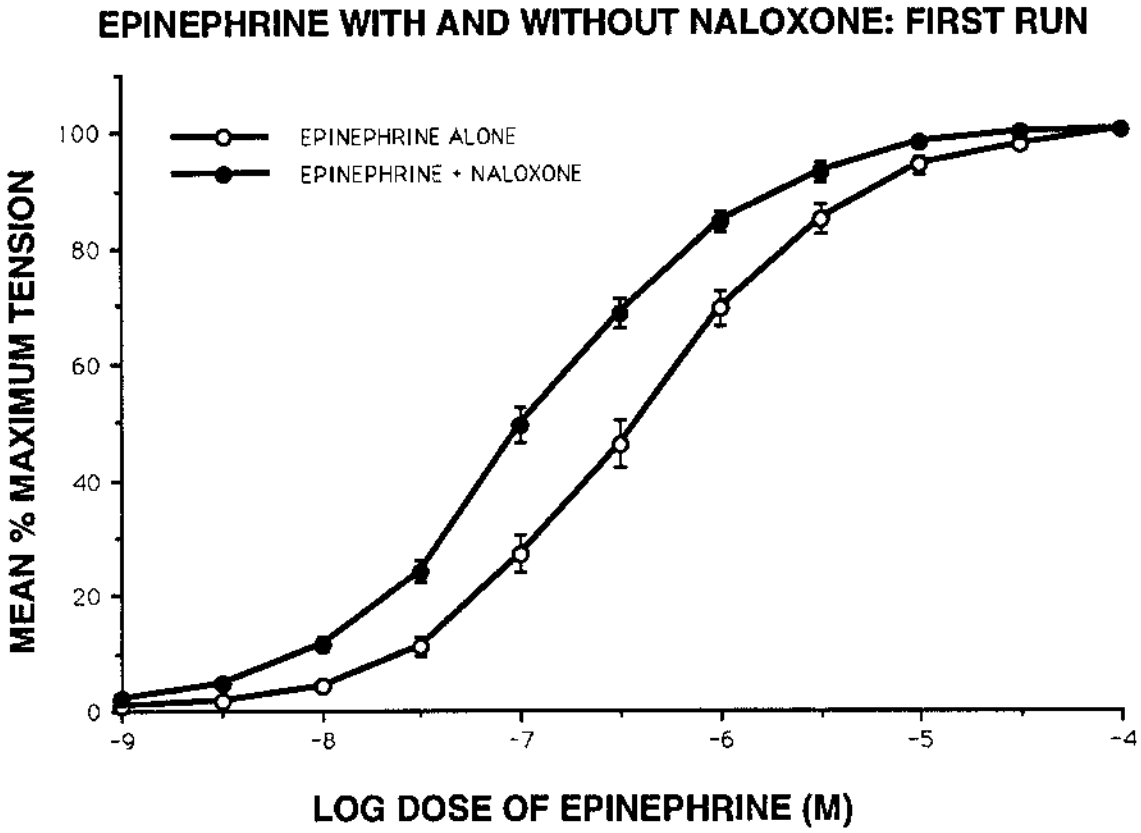


Figure 9. Each vessel underwent an epinephrine dose-response in the presence and absence of naloxone (10 μM). Group A vessels first underwent epinephrine alone, then epinephrine with naloxone second. Group B vessels underwent the reverse order. This figure depicts the results of the first run dose-response of group A vs. group B. The ED_{50}s were 4.8×10^{-7} and 1.3×10^{-7} M for epinephrine alone and epinephrine with naloxone first run curves respectively. A factorial ANOVA post-hoc Scheffe analysis of the log ED_{50}s for groups A and B, first and second runs, revealed the above curves were different at $p < 0.01$. Values are mean ± SEM. N = 22(5) vessels for both epinephrine alone and epinephrine with naloxone first run curves.

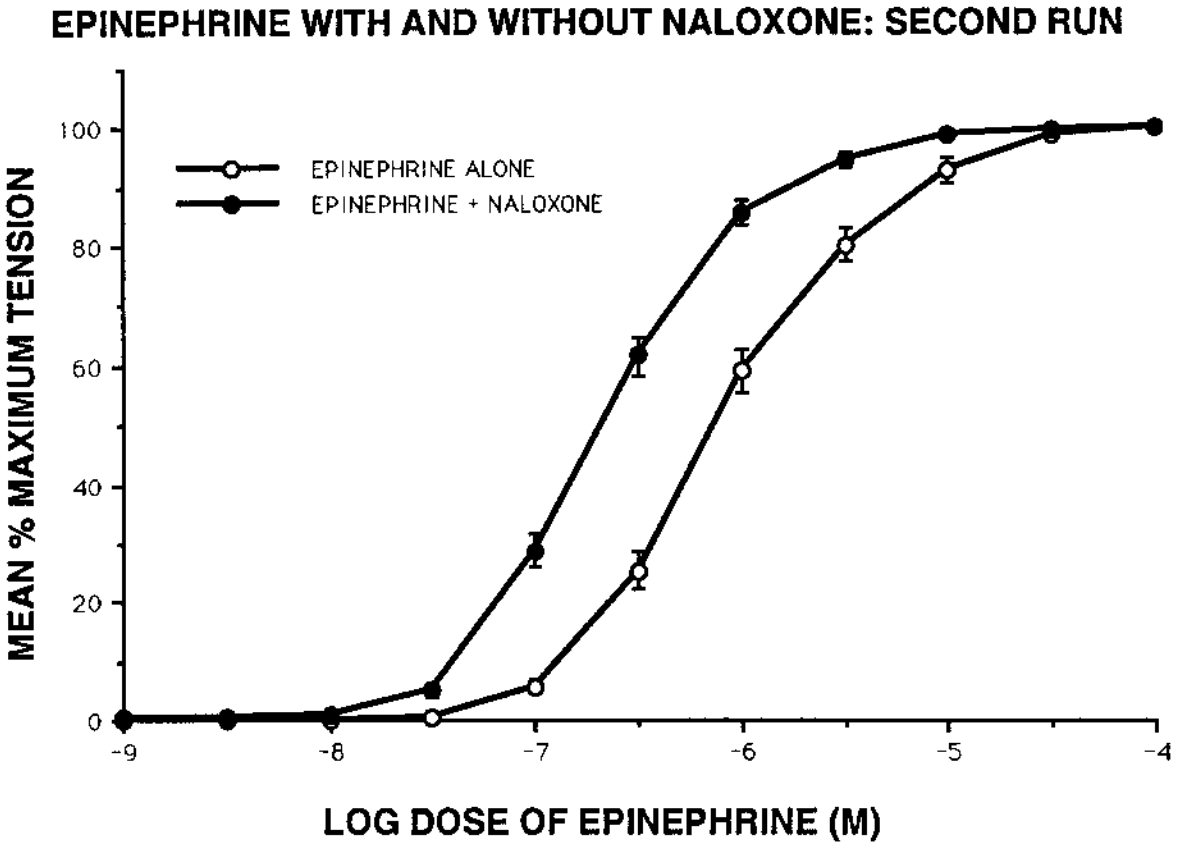


Figure 10. Each vessel underwent an epinephrine dose-response in the presence and absence of naloxone (10 μM). Group A vessels first underwent epinephrine alone, then epinephrine with naloxone second. Group B vessels underwent the reverse order. This figure depicts the results of the second run dose-response of group A vs. group B. The ED_{50}s were 9.7×10^{-7} and 2.5×10^{-7} M for epinephrine alone and epinephrine with naloxone second run curves respectively. A factorial ANOVA post-hoc Scheffe analysis of the ED_{50}s for groups A and B, first and second runs, revealed the above curves were different at $p < 0.01$. Values are mean ± SEM. N = 22(5) and 20(5) vessels for epinephrine alone and epinephrine with naloxone second run curves respectively.

epinephrine dose-response curves and significantly reduced the epinephrine ED_{50}s. This was most evident when comparing within the first run and within the second run between Groups A and B.

ED_{50} S FROM DOSE-RESPONSE DATA		
	EPINEPHRINE ALONE	WITH NALOXONE
FIRST RUN	4.8×10^{-7} M	1.3×10^{-7} M
SECOND RUN	9.7×10^{-7} M	2.5×10^{-7} M

TABLE 1

Statistical analysis also indicated a major effect of increasing epinephrine ED_{50} from the first to the second run. See Figures 11 and 12. The analysis did not detect a significant minor effect of increased ED_{50}S in second run vessels when tested individually either in the presence or absence of naloxone. A significant decrease in vascular responsiveness was apparent between the first run and second run when groups A and B were viewed collectively as suggested by the increase in ED_{50}S and the overall run effect. The rightward shift of the dose-response curves between first and second runs for vessels undergoing similar experimental conditions is illustrated in Figures 11 and 12.

This crossover design was employed to ensure the reliability of the results. Vessels that defined a leftward shifted dose-response curve in the first run, defined a curve to the shifted to the right in the second run. This ensured the results were not due to random vessel variability or a sequence dependent change in responsiveness. The overall shift of the dose-response curves to the right in the second run indicated a partial desensitization of the tissue to epinephrine subsequent to the first run.

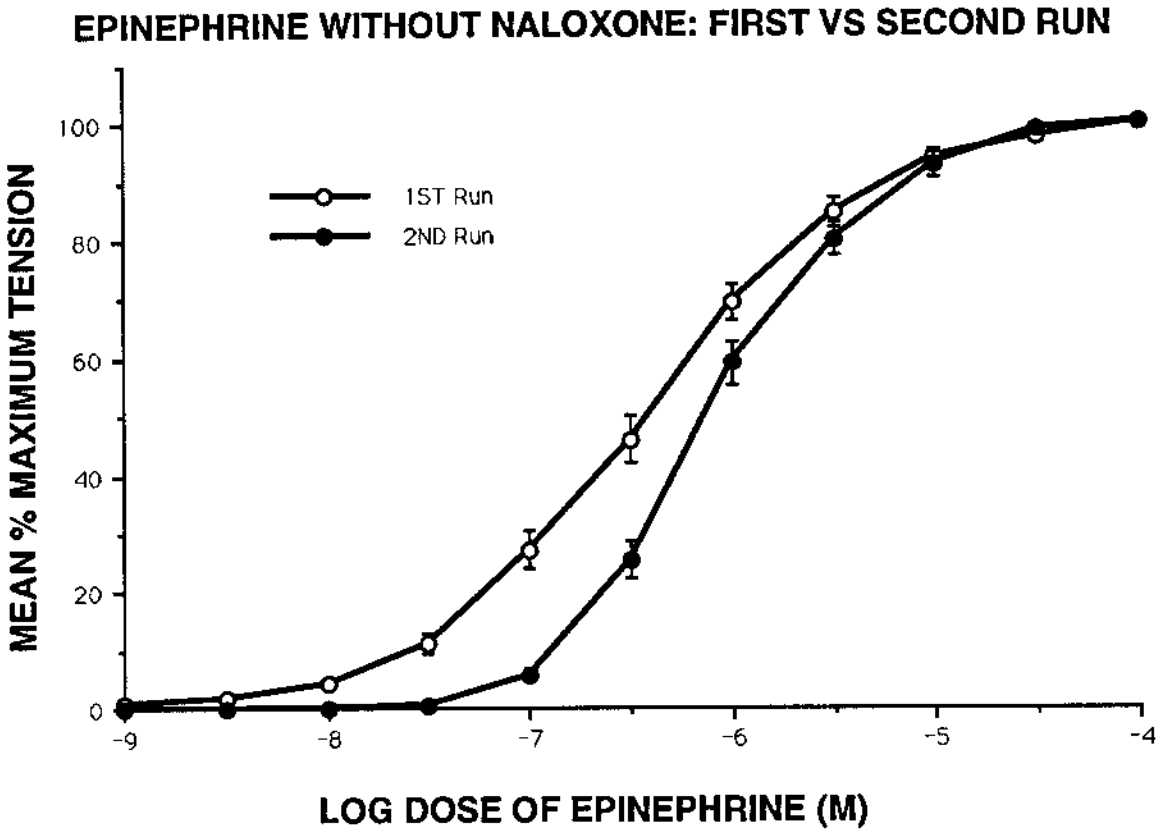


Figure 11. Each vessel underwent an epinephrine dose-response in the presence and absence of naloxone (10 μM). Group A vessels first underwent epinephrine alone, then epinephrine with naloxone second. Group B vessels underwent the reverse order. This figure depicts the results of the epinephrine alone dose-response for the first vs. second run. The ED_{50}s were 4.8×10^{-7} and 9.7×10^{-7} M for epinephrine alone first and second run curves respectively. A factorial post-hoc ANOVA Scheffe analysis of the ED_{50}s for groups A and B, first and second runs, revealed the above curves were not different at $p < 0.01$, yet there was an ANOVA major effect of increasing ED_{50}s from first to second runs at $p < 0.05$. Values are mean ± SEM. N = 22(5) vessels for both epinephrine alone first and second run curves.

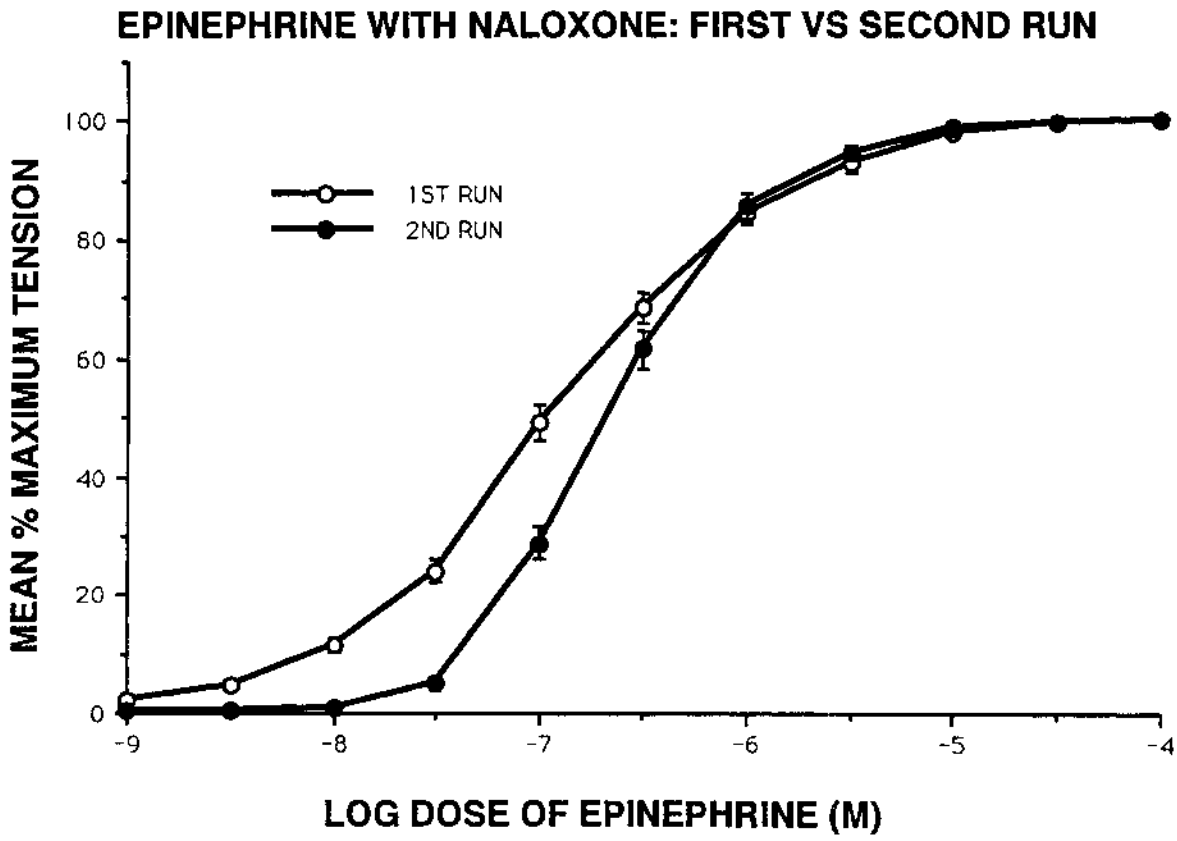


Figure 12. Each vessel underwent an epinephrine dose-response in the presence and absence of naloxone (10 μM). Group A vessels first underwent epinephrine alone, then epinephrine with naloxone second. Group B vessels underwent the reverse order. This figure depicts the results of the epinephrine with naloxone dose-response for the first vs. second run. The ED_{50}s were 1.3×10^{-7} and 2.5×10^{-7} M for epinephrine with naloxone first and second run curves respectively. A factorial post-hoc ANOVA Scheffe analysis of the ED_{50}s for groups A and B, first and second runs, revealed the above curves were not different at $p < 0.01$, yet there was an ANOVA major effect of increasing ED_{50}s from first to second runs at $p < 0.05$. Values are mean ± SEM. N = 22(5) and 20(5) vessels for epinephrine with naloxone first and second run curves respectively.

C. Naloxone Dose-Response

Vessels were precontracted 15 - 20% with epinephrine and naloxone (10^{-8} - 10^{-4} M) was added in increments to construct a naloxone dose-response curve. Figure 13 illustrates the resultant relationship. The ED_{50} for naloxone is 3.7×10^{-6} M. This ED_{50} is relatively high for the known affinity of opiates for their receptors.

Adrenergic Specificity

Several types of experiments were conducted to determine the role of the adrenergic receptors in the naloxone effect. Several experiments examined the effect of naloxone (10 μM) on a variety of adrenergic and non-adrenergic vasoconstrictors. Several other experiments examined the contribution of alpha- and beta-adrenoreceptors respectively.

A. Adrenergic vs Non-Adrenergic

Naloxone was applied to vessels submaximally contracted with the adrenergic vasoconstrictors epinephrine, norepinephrine, phenylephrine and lofexidine. Figure 8 illustrates an example of the sequence of the experimental protocol from left to right and the absolute magnitude of the naloxone effect relative to KCl and epinephrine induced maximum contractions. Figure 14 summarizes the data, collected in the same manner, from the adrenergic vasoconstrictors . Naloxone augmented the submaximal contractions induced by all of the adrenergic vasoconstrictors. Naloxone augmented epinephrine induced submaximal contractions the most and augmented the norepinephrine, phenylephrine and lofexidine contractions to an equal, but much lower degree.

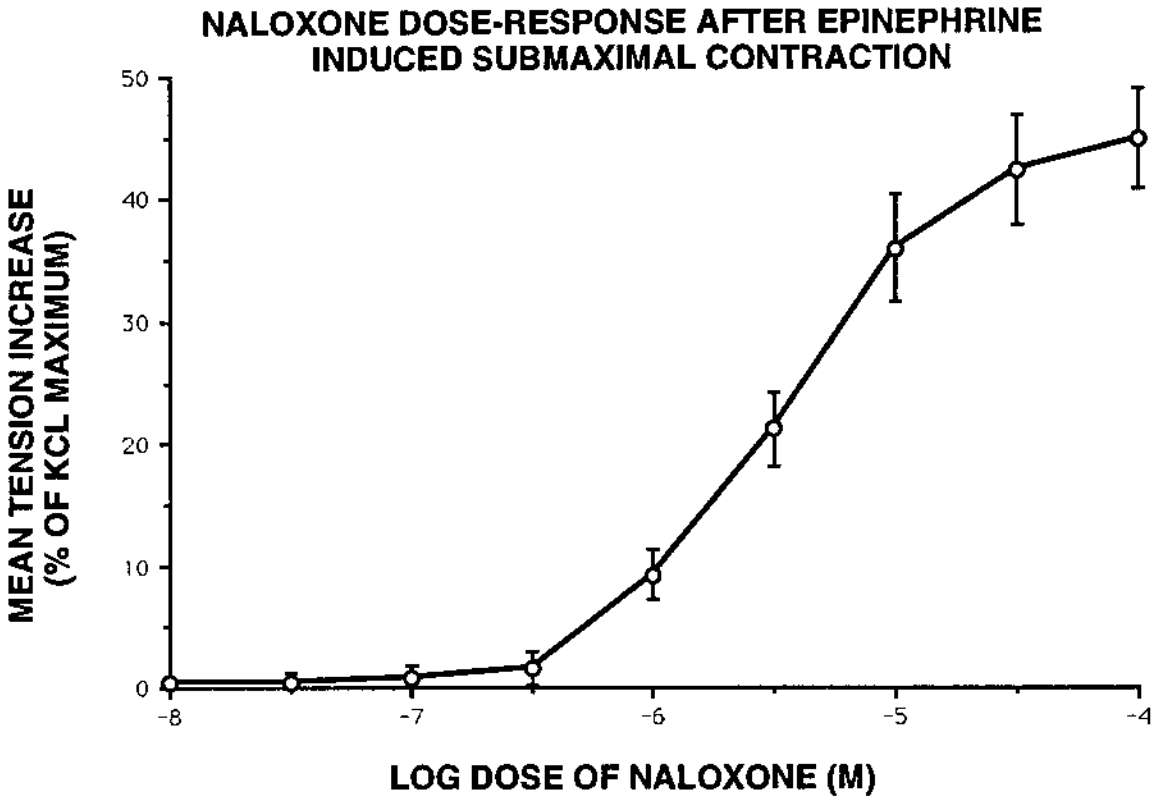


Figure 13. The ED_{50} for naloxone was $3.7x10^{-6}$ M. The epinephrine induced precontraction was 25.9% and 16.3% of maximum KCL and epinephrine contractions respectively. Values are mean ± SEM. N = 18(5).

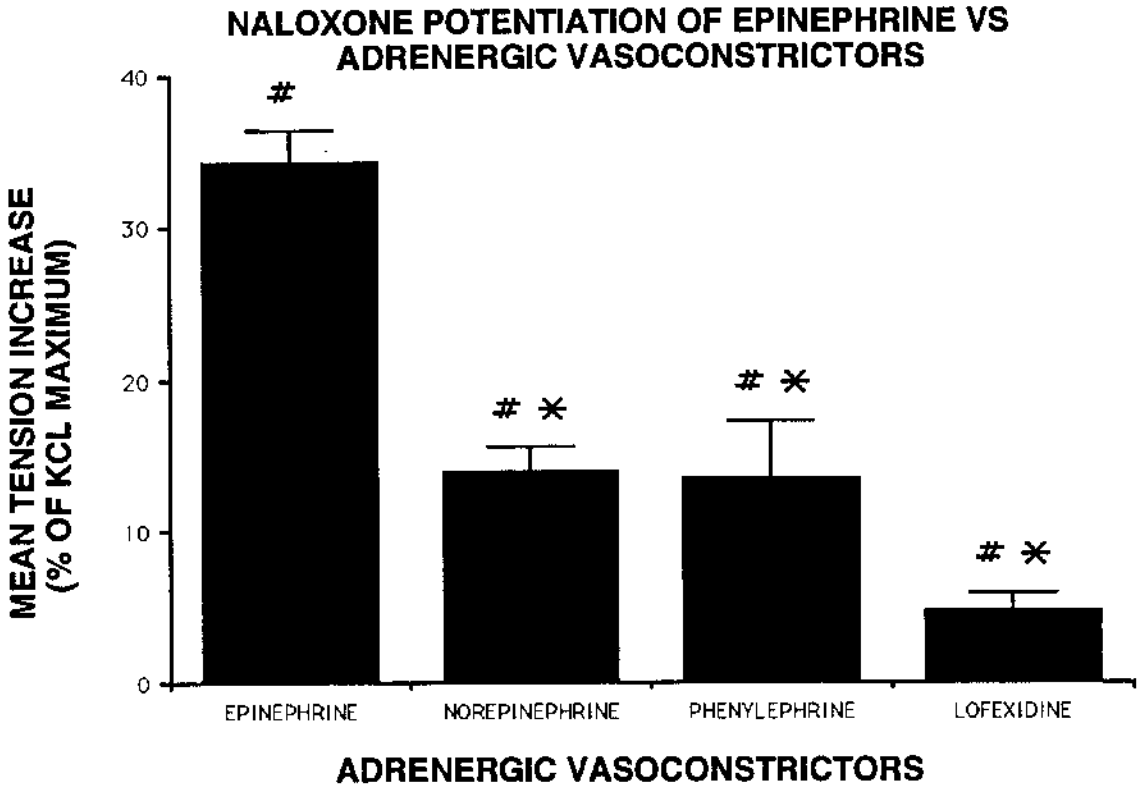


Figure 14. Naloxone (10 μM) was added to vessels precontracted with the given adrenergic vasoconstrictor. (*) indicates different from epinephrine plus naloxone via an ANOVA post-hoc Scheffe analysis, $p < 0.01$. A paired student t-test between the submaximum tension and the naloxone induced tension increase for each vasoconstrictor revealed that all vasoconstrictors were augmented by naloxone $p < 0.05$ (#). The submaximal contractions of epinephrine, norepinephrine, phenylephrine and lofexidine were 20.0, 19.4, 56.5 and 31.3% of maximum KCL and 15.6, 14.4, 24.1 and 32.6% of maximum vasoconstrictor contractions respectively. Values are mean ± SEM. From left to right, N = 15(5), 15(5), 20(5), and 22(6) vessels(dogs).

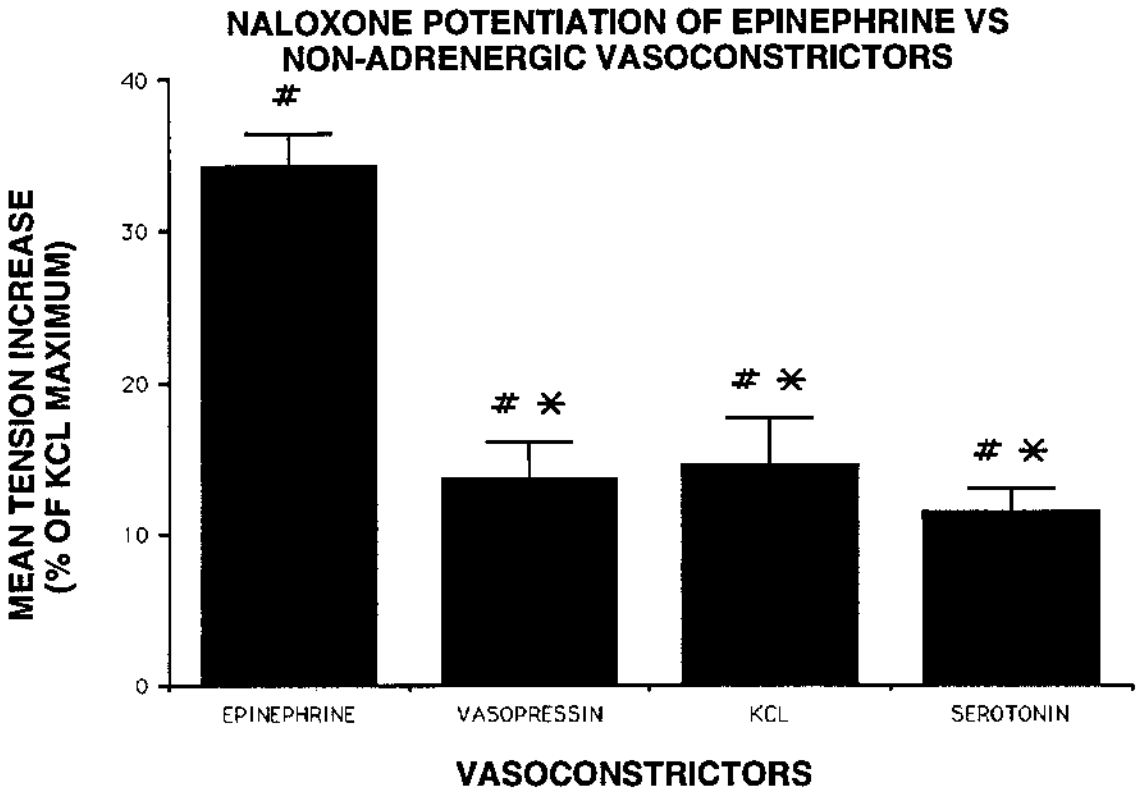


Figure 15. Naloxone (10 μM) was added to vessels precontracted with the given non-adrenergic vasoconstrictor. (*) indicates different from epinephrine plus naloxone via an ANOVA post-hoc Scheffe analysis, $p < 0.01$. A paired student t-test between the submaximum tension and the naloxone induced tension increase for each vasoconstrictor revealed that all vasoconstrictors were augmented by naloxone $p < 0.05$ (#). The submaximal contractions of epinephrine, vasopressin, KCL and serotonin were 20.0, 59.7, 56.5 and 29.3% of maximum KCL and 15.6, 31.4, 24.1 and 27.1% of maximum vasoconstrictor contractions. Values are mean ± SEM. From left to right, N = 15(5), 15(6), 18(6), and 15(4) vessels(dogs).

Naloxone also was applied to vessels submaximally contracted with various non-adrenergic vasoconstrictors. Figure 15 summarizes this data. These non-adrenergic agents included vasopressin, KCl and serotonin. Naloxone augmented the submaximal contractions induced by all of the nonadrenergic vasoconstrictors. Naloxone augmented vasopressin, KCl and serotonin induced contractions equally, but augmented epinephrine induced submaximal contractions significantly more.

Naloxone augmented all vasoconstrictors tested, but selectively augmented epinephrine vasoconstrictions to the greatest extent.

ED_{50}S FROM DOSE-RESPONSE DATA		
	AGONIST ALONE	WITH NALOXONE
NOREPINEPHRINE	1.2×10^{-6} M	1.2×10^{-6} M
PHENYLEPHRINE	8.4×10^{-7} M	7.2×10^{-7} M

TABLE 2

B. Norepinephrine and Phenylephrine Dose-Responses

Dose-response curves were constructed for norepinephrine and phenylephrine which are similar to the first run epinephrine dose-response curves described above. Figures 16 and 17 illustrate the results. The ED_{50}s for norepinephrine and phenylephrine in the presence and absence of naloxone are listed in Table 2. Neither norepinephrine nor phenylephrine demonstrated a significant change in their log ED_{50}S subsequent to the presence of naloxone. The figures demonstrate no shift of the curves to the right or left as a result of the naloxone exposure. Attempts at constructing dose-response relationships for clonidine or lofexidine ($alpha_2$-adrenergic agonists) were unsuccessful due to the extraordinarily long time required for

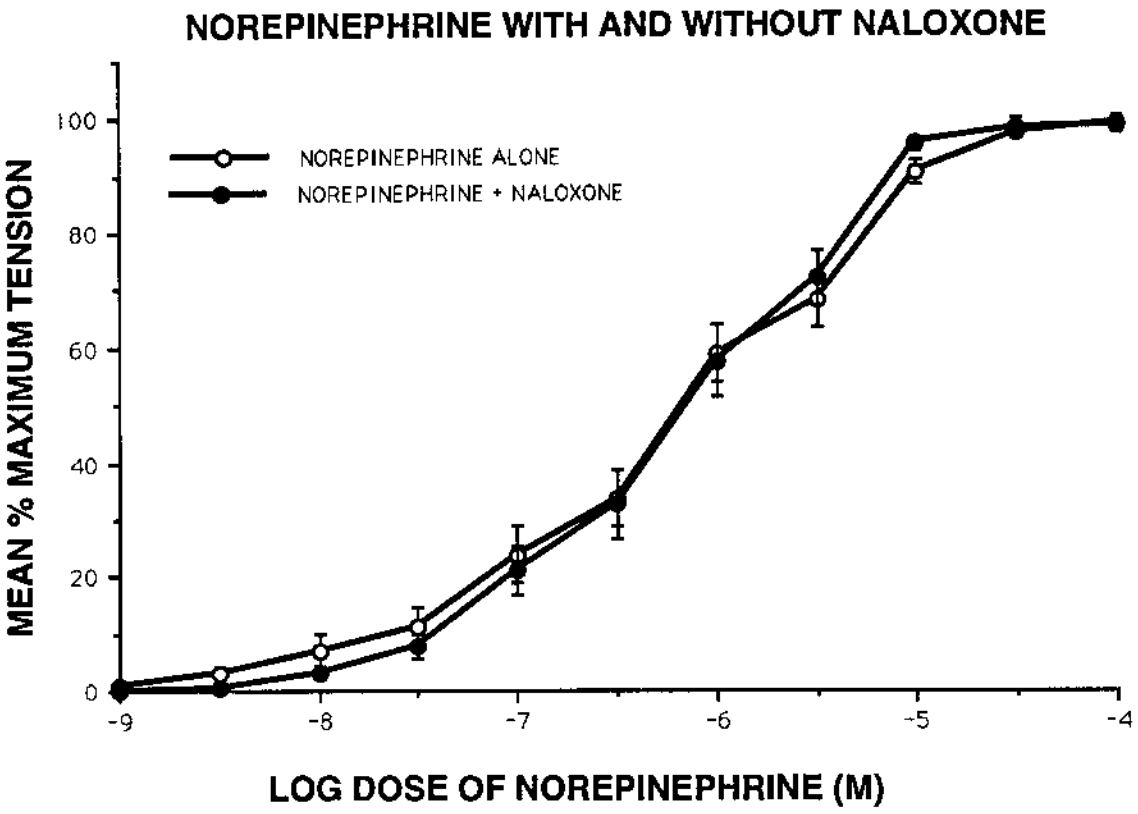


Figure 16. Vessels underwent a norepinephrine dose-response in the presence and absence of naloxone (10 μM). The ED_{50}s were $1.2x10^{-6}$ and $1.2x10^{-6}$ M for norepinephrine alone and norepinephrine with naloxone dose-response curves respectively. Unpaired t-test analysis of the ED_{50}s for norepinephrine alone and norepinephrine with naloxone dose-response curves revealed the above curves were not different at $p < 0.05$. Values are mean ± SEM. N = 13(4) vessels in each group.

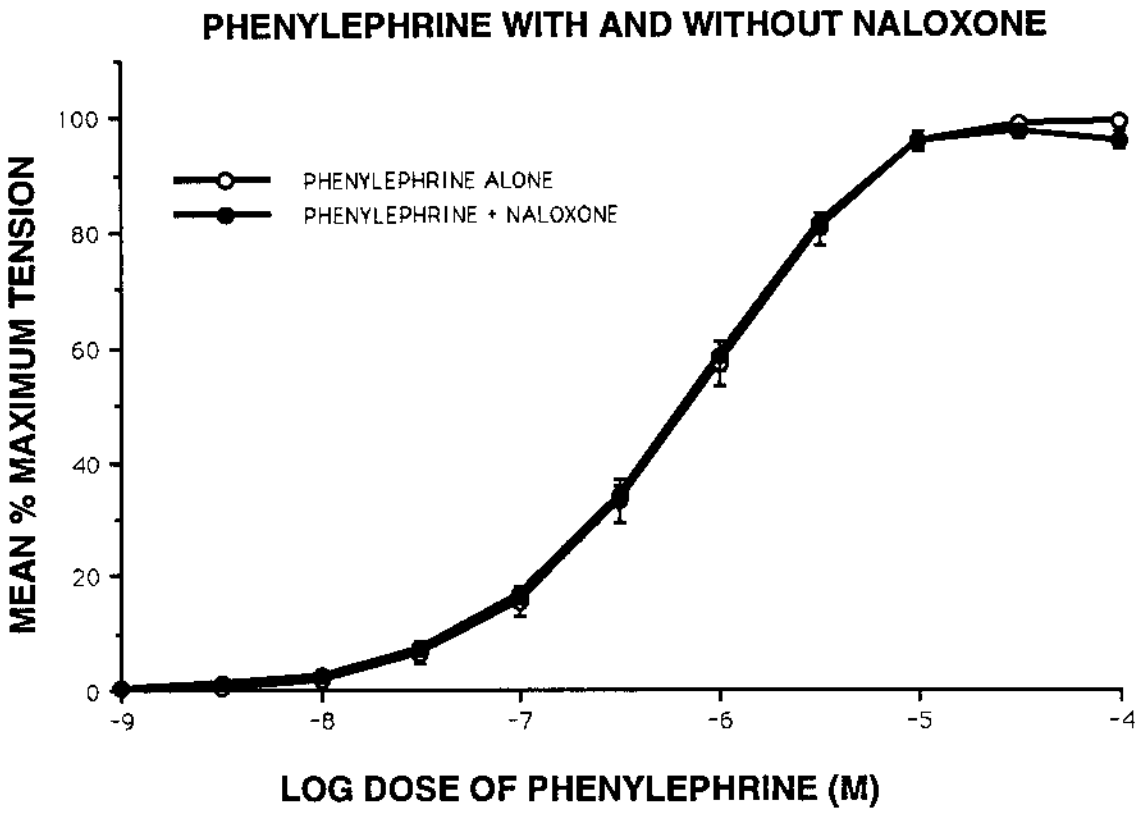


Figure 17. Vessels underwent a phenylephrine dose-response in the presence and absence of naloxone (10 μM). The ED_{50}s were 7.2×10^{-7} and 8.4×10^{-7} M for thephenylephrine alone and phenylephrine with naloxone dose-response curves respectively. Unpaired t-test analysis of the ED_{50}s for phenylephrine alone and phenylephrine with naloxone dose-response curves reveal the above curves were different at $p < 0.05$. Values are mean ± SEM. N = 13(4) and 14(4) vessels in groups phenylephrine alone and phenylephrine with naloxone respectively.

alpha$_2$-adrenoreceptor induced vasoconstriction to plateau. Epinephrine induced vasoconstriction by the same mechanism as norepinephrine and phenylephrine. These data confirmed the selectivity with which naloxone augmented epinephrine induced vasoconstriction.

C. Alpha-Adrenergic Contribution

Alpha-adrenergic receptor involvement was assessed with the use of the nonselective, reversible alpha-adrenergic receptor antagonist, phentolamine. Figure 18 is a tracing of a representative vessel that undergoes phentolamine blockade. Phentolamine rapidly induced a total reversal of the naloxone augmented epinephrine vasoconstriction. Figure 19 illustrates the results from a group of vessels that were precontracted with epinephrine and then treated with naloxone. Phentolamine (1 μM) caused a rapid relaxation of 106.1 ± 3.4 percent of the pre-phentolamine tension. Phentolamine completely eliminated the contraction induced by the combination of epinephrine and naloxone. This indicates that the contractions induced by both epinephrine and naloxone were alpha-adrenergic receptor mediated.

D. Beta-Adrenergic Contribution

Epinephrine is active at both alpha- and beta-adrenergic receptors. Activation of alpha receptors induces vasoconstriction and activation of beta receptors induces vasodilation. Vasoconstriction induced by epinephrine is a combination of alpha receptor mediated vasoconstriction and beta receptor mediated vasodilation. It is possible that naloxone may selectively potentiate epinephrine induced vasoconstriction by acting as a beta-adrenergic receptor antagonist.

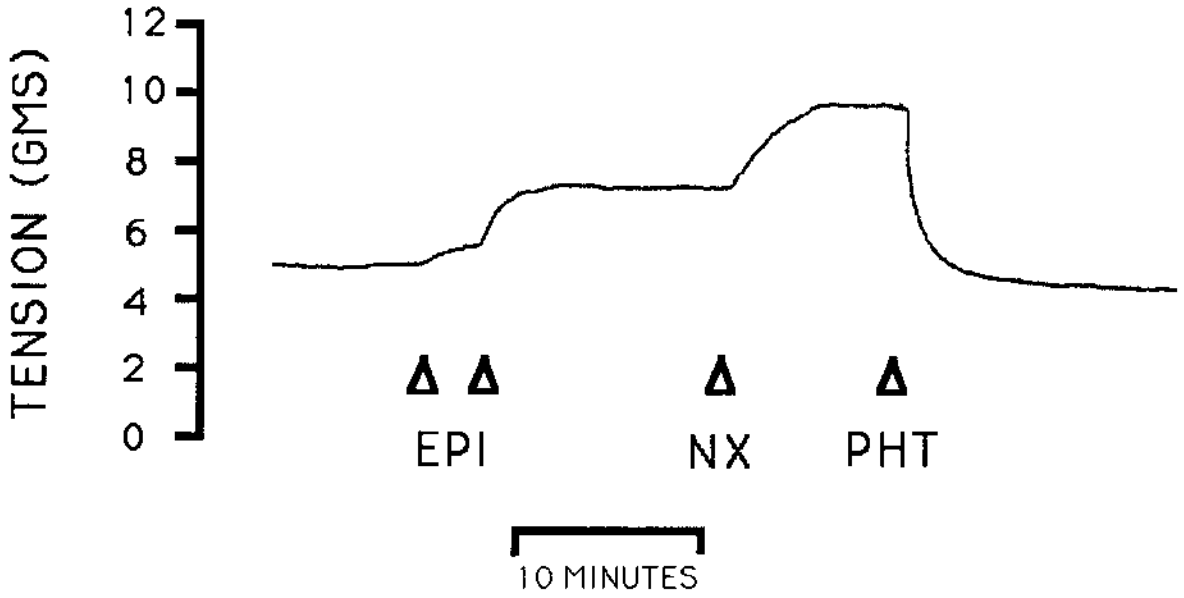


Figure 18. The above tracing is representative of vessels that underwent alpha-adrenergic receptor blockade with phentolamine (PHT, 10 μM) after precontraction with epinephrine (EPI, 0.03 - 0.1 μM) and naloxone (NX, 10 μM). Note the immediate reversal of both the epinephrine and the naloxone induced vasoconstrictions. This suggests that the naloxone induced epinephrine augmentation was all alpha-adrenoreceptor mediated.

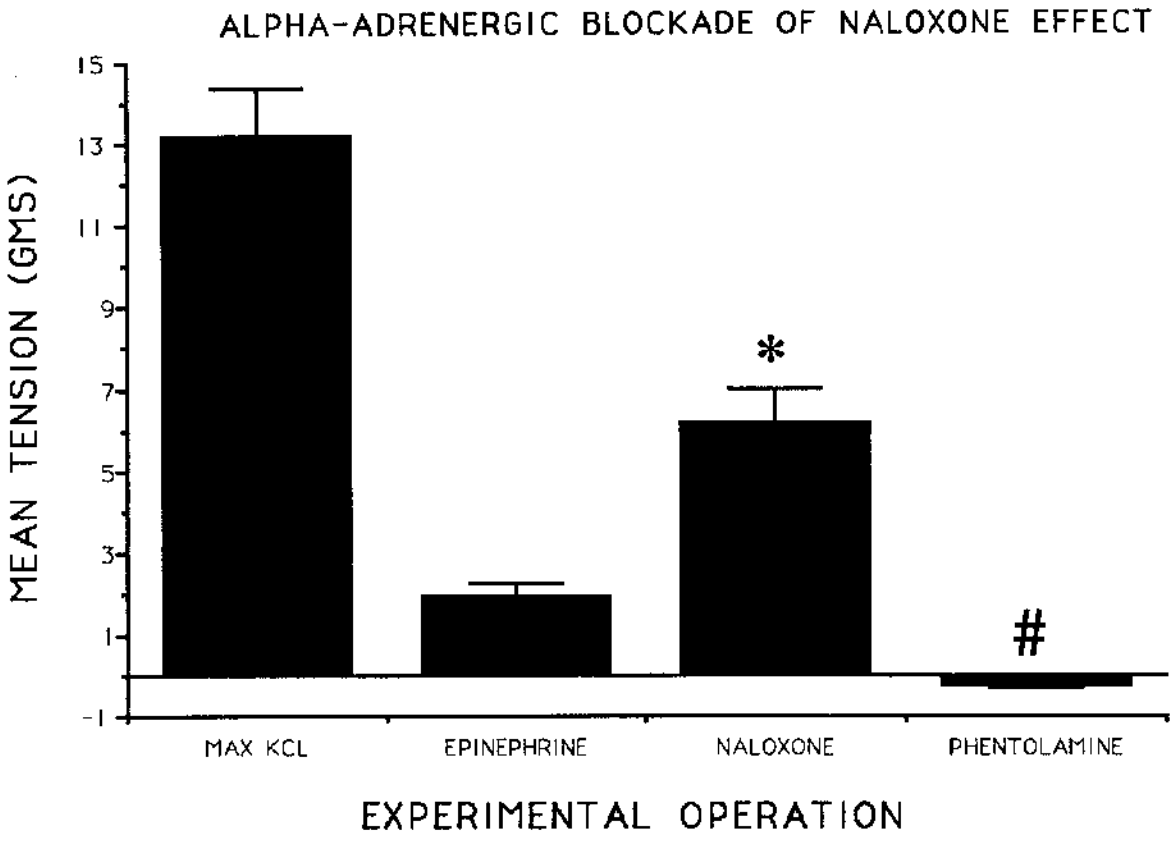


Figure 19. MAX KCL is the maximum tension induced by KCl (80 mM). EPINEPHRINE is the submaximal tension induced by epinephrine. NALOXONE is tension induced by epinephrine with naloxone (10 μM). PHENTOLAMINE is the tension resulting from the addition of phentolamine (1.0 μM) to vessels precontracted with epinephrine and naloxone. Naloxone augmented the epinephrine induced submaximal contraction and phentolamine rapidly returned the vessel to resting tension. EPINEPHRINE was 15.6% of MAX KCL. (*) indicates different from EPINEPHRINE at $p < 0.05$. (#) indicates different from NALOXONE at $p < 0.05$. Values are mean ± SEM. N = 9(4) vessels(dogs).

Two types of experiment were used to assess beta-adrenergic receptor involvement in the naloxone effect. Figures 20 and 21 illustrate the first type of experiment wherein beta receptors were blocked both before and after applying naloxone to a submaximal epinephrine vasoconstriction. Timolol (nonselective beta receptor antagonist) was employed at 0.1 μM to ensure adequate beta receptor blockade. Higher concentrations of timolol initiated vascular relaxation. Vessels were divided into two groups. All vessels first underwent a submaximal epinephrine contraction. In one group, epinephrine was followed by timolol and then naloxone (Figure 20). In the other group, the epinephrine was followed by naloxone and then timolol (Figure 21). In neither group did beta-adrenergic blockade alter the naloxone effect. Figure 22 compares the effects of naloxone and timolol added after epinephrine precontractions in separate vessels. Naloxone augmented and timolol had no effect on an epinephrine precontraction. These data suggest that these vessels had no epinephrine induced beta-adrenergic mediated vasodilation competing with the epinephrine induced alpha-adrenergic vasoconstriction. The above results also indicate that naloxone does not augment epinephrine induced vasoconstriction in these vessels via beta-adrenergic receptor blockade.

The second type of experiment used to assess the role of beta-adrenergic receptors in the naloxone effect was more complex. This experiment was designed to examine the effect of naloxone on a clearly beta-adrenergic mediated vascular response in the absence of epinephrine. The previous experiments demonstrated that beta receptor blockade did not alter the naloxone effect. This experiment was designed to clearly show that naloxone did not function as a beta-adrenergic receptor antagonist. See Figure 23. A group of vessels was precontracted with phenylephrine to 41.0 ± 3.6% of maximum. These vessels were then stimulated to

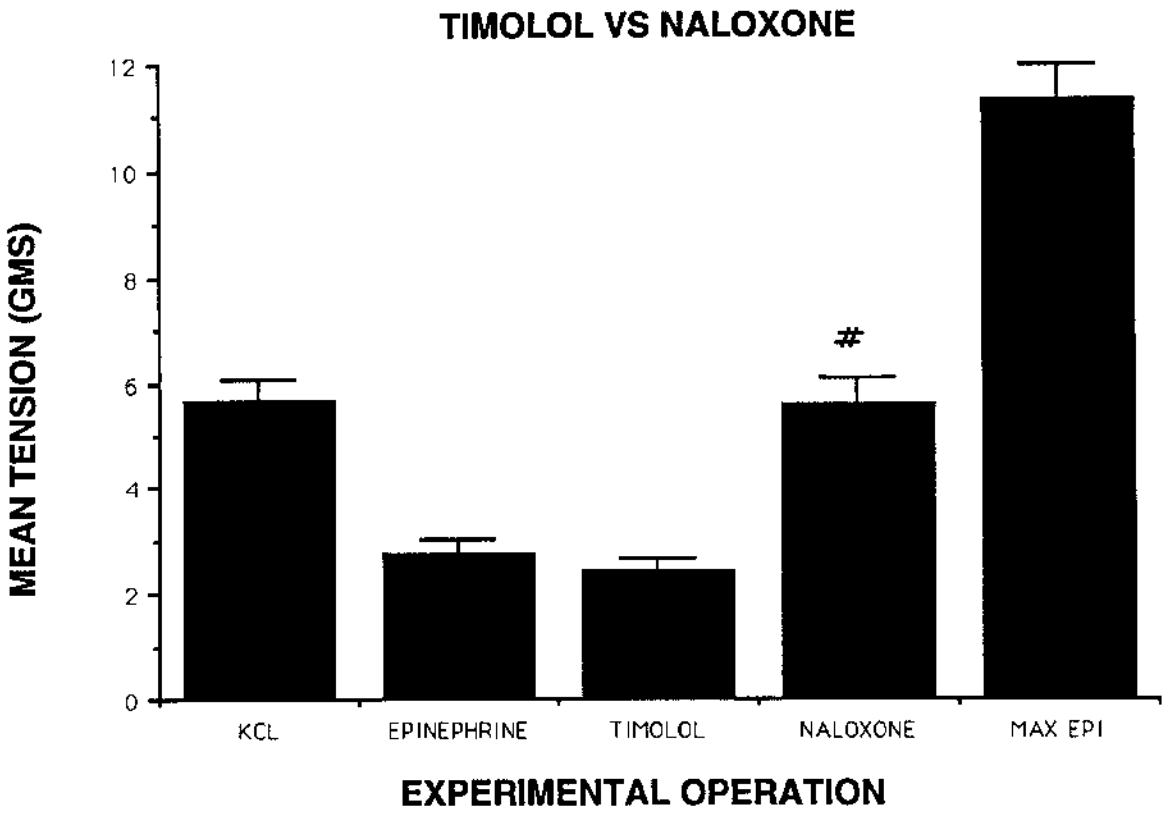


Figure 20. KCL and MAX EPI are the maximum tension induced by KCl (80 mM) and epinephrine (10 μM). EPINEPHRINE is the submaximal tension induced by epinephrine. TIMOLOL is the mean tension induced by timolol (0.1 μM) and epinephrine. NALOXONE is mean tension induced by epinephrine, beta blockade and naloxone (10 μM). EPINEPHRINE was 48.2% of MAX KCL and 24.1% of MAX EPI. A repeated measures ANOVA post-hoc Scheffe analysis revealed that naloxone augmented the epinephrine induced contraction, but beta blockade did not at $p < 0.01$, indicated by the (#). Values are mean ± SEM. N = 12(5) vessels(dogs).

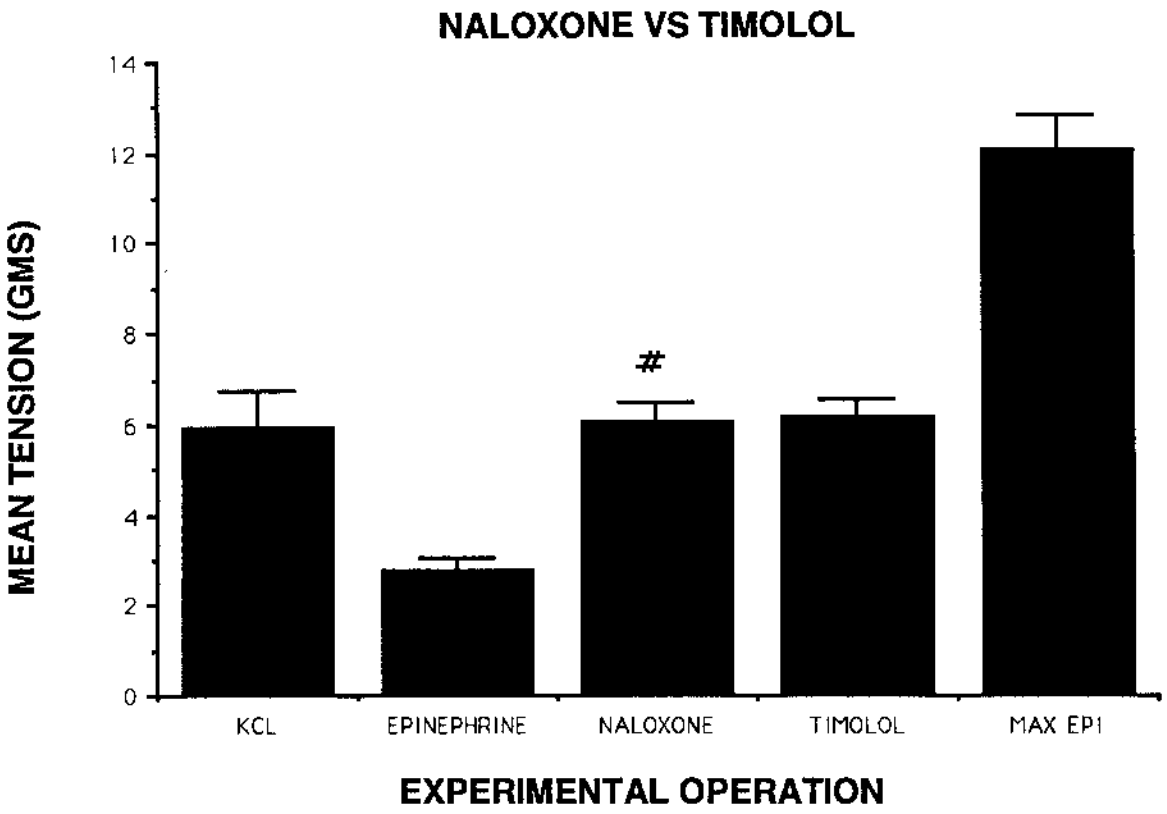


Figure 21. KCL and MAX EPI are the maximum tension induced by KCl (80 mM) and epinephrine (10 μM). EPINEPHRINE is the submaximal tension induced by epinephrine. NALOXONE is tension induced by epinephrine and naloxone (10 μM). TIMOLOL is then tension induced by epinephrine, naloxone and timolol (0.1 μM). EPINEPHRINE was 46.4% of KCL and 22.9% of MAX EPI. A post-hoc ANOVA Scheffe analysis revealed that naloxone augmented the epinephrine induced contraction, indicated by the (#), but timolol did not induce any further increase at $p < 0.01$. Values are mean ± SEM. N = 12(5) vessels(dogs).

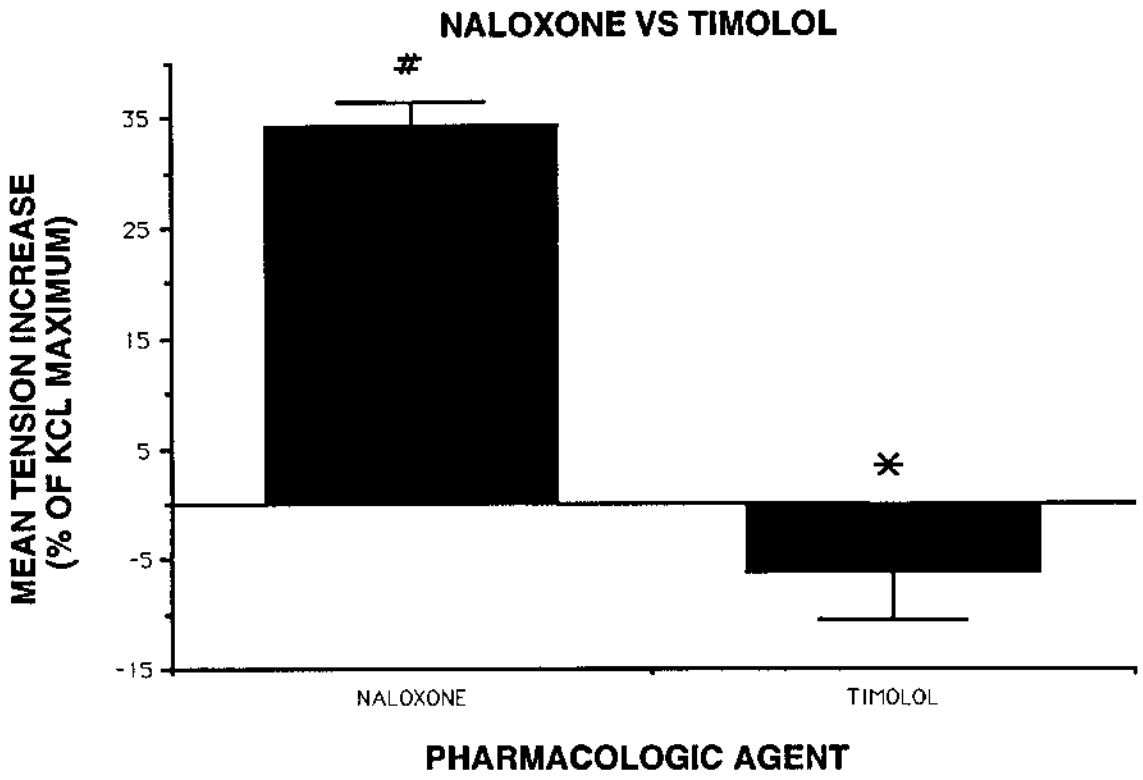


Figure 22. Timolol (0.1 μM) or naloxone (10 μM) was added to vessels precontracted with epinephrine. Paired t-tests revealed that naloxone induced a change from epinephrine precontraction and timolol did not at $p < 0.05$ (#). An unpaired t-test between the percent increase in tension induced by timolol and naloxone revealed a difference at $p < 0.05$ (*). Values are mean ± SEM. N = 12(5) vessels(dogs).

relax by employing the non-selective, beta-adrenergic agonist isoproterenol (0.3 μM). Naloxone (10 μM) was added, and then timolol (0.1 μM). Naloxone had no significant effect on the beta-adrenergic induced vasodilation and the timolol quickly reversed it. Beta blockade with timolol stimulated a greater reversal of the beta-adrenergic induced relaxation than did naloxone at 84.5 ± 18.7 and -1.0 ± 6.1% respectively. Vessel tension after naloxone in this experiment was no different than the tension before naloxone. Naloxone did not reverse a beta-adrenergic induced vasodilation. The naloxone effect was not the result of beta-adrenergic receptor antagonism.

The above experiment did not indicate whether naloxone augmented beta-adrenergic mediated vasodilation. The isoproterenol induced vasodilation was maximum and therefore further beta-adrenergic mediated vasodilation was not possible. A submaximal isoproterenol induced vasodilation could not be reliably obtained and verified. Naloxone augmented an epinephrine induced alpha-adrenergic mediated vasoconstriction and it remains unclear whether naloxone augmented the effect of epinephrine at the beta-adrenergic receptor as well.

Relaxation Time

Vessels contracted with epinephrine in the presence of naloxone relax more rapidly once washed than do vessels contracted with epinephrine alone. The vessels in groups A and B described above which underwent epinephrine dose-responses in the presence and absence of naloxone were washed and allowed to relax after both the first and second runs. Rate of relaxation was described as the time required for a vessel to relax to 50% of its pre-washout tension ($T_{1/2}$). Table 3 displays the relaxation $T_{1/2}$s for vessels contracted with epinephrine alone and

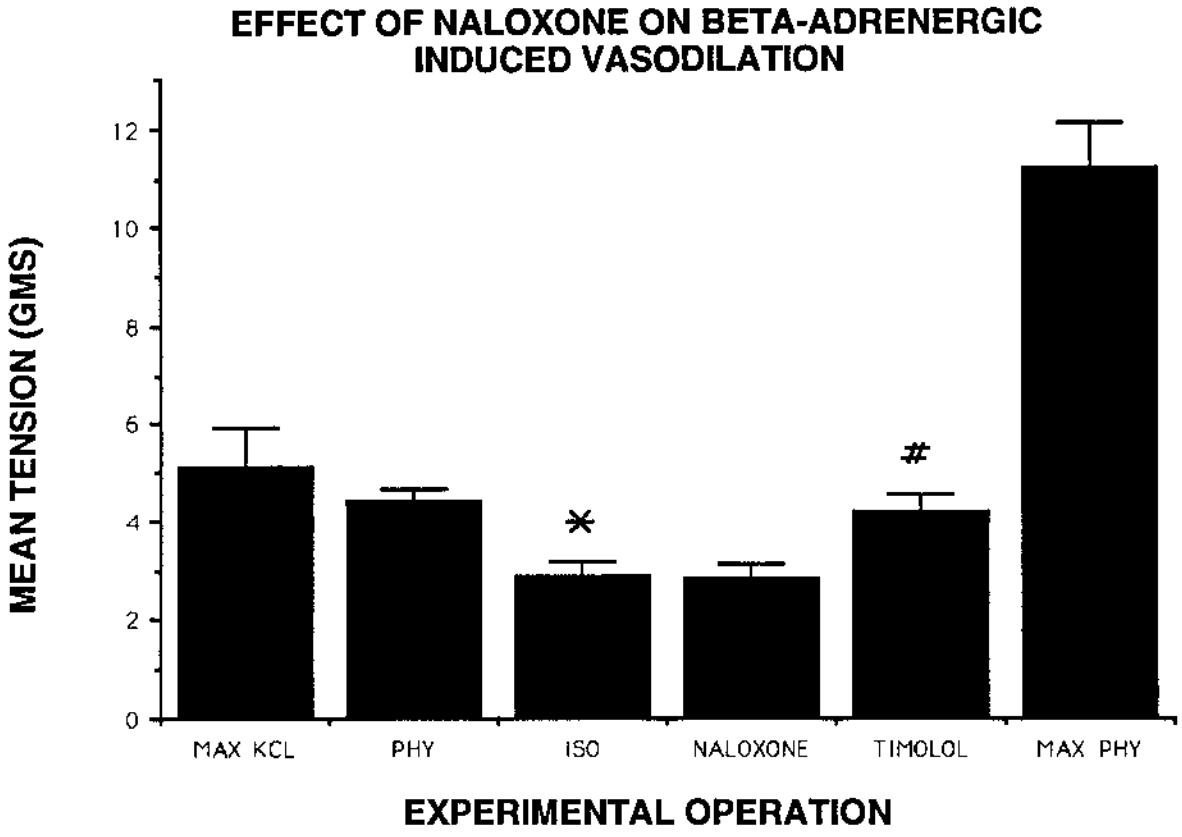


Figure 23. MAX KCL and MAX PHY are the maximum tension induced by KCl (80 mM) and phenylephrine (10 μM). PHY is the submaximal tension induced by phenylephrine. ISO is the vessel tension after isoproterenol (0.3 μM) is added to the submaximal phenylephrine induced contraction. NALOXONE is tension induced by phenylephrine, isoproterenol and naloxone (10 μM). TIMOLOL is the tension induced by phenylephrine, isoproterenol, naloxone and timolol (0.1 μM). PHY was 86.5% of MAX KCL and 39.4% of MAX PHY. A paired t-test revealed that isoproterenol decreased the phenylephrine induced contraction at $p < 0.05$ (*). A repeated measures ANOVA post-hoc Scheffe analysis revealed that naloxone had no effect and timolol reversed the isoproterenol induced attenuation of the phenylephrine vasoconstriction at $p < 0.05$ (#). Values are mean ± SEM. N = 20(5) vessels(dogs).

epinephrine with naloxone after the first and second runs. Relaxation $T_{1/2}$s were significantly different between the presence and absence of naloxone during the first run, but not the second run. There was a tendency for naloxone to reduce relaxation $T_{1/2}$ in the second run, but it was not statistically significant. Naloxone reduced the relaxation $T_{1/2}$ for vessels contracted maximally with epinephrine.

RELAXATION $T_{1/2}$S (MINUTES)		
	EPINEPHRINE ALONE	WITH NALOXONE
FIRST RUN	27.9 ± 1.1	18.5 ± 0.8
SECOND RUN	20.6 ± 1.0	17.3 ± 0.8

TABLE 3

RELAXATION $T_{1/2}$S (MINUTES)		
	AGONIST ALONE	WITH NALOXONE
NOREPINEPHRINE	9.4 ± 1.7	11.7 ±1.8
PHENYLEPHRINE	7.8 ± 0.8	8.7 ±1.8

TABLE 4

The vessels which underwent dose-response contractions with norepinephrine and phenylephrine in the presence and absence of naloxone were also washed and allowed to relax. These relaxation $T_{1/2}$s are listed in Table 4. There was no difference in relaxation $T_{1/2}$s for vessels contracted in the presence or absence of naloxone for norepinephrine nor phenylephrine. The effect of naloxone to shorten relaxation $T_{1/2}$s was specific for epinephrine.

Comparison of the data in Tables 3 and 4 shows that the relaxation $T_{1/2}$s for epinephrine were significantly longer than for norepinephrine or phenylephrine in either the presence or absence of naloxone. The longer $T_{1/2}$s for epinephrine indicate that epinephrine was handled in a different manner than was norepinephrine or phenylephrine even in the absence of naloxone. It appears that the tissue reversed catecholamine activation of adrenergic receptors in a manner that was different between the endogenous catecholamines, epinephrine and norepinephrine.

RELAXATION $T_{1/2}$S (MINUTES)		
	EPINEPHRINE ALONE	WITH NALOXONE
3 MINUTES	14.1 ± 1.0	10.9 ± 1.0
10 MINUTES	22.2 ± 0.7	18.8 ± 0.8

TABLE 5

The tendency for naloxone to shorten relaxation time was closely examined in an experiment where exposure time of vessels to epinephrine was more closely monitored and controlled. The results are presented in Table 5. Vessels were exposed to epinephrine (100 μM) for either three or ten minutes in the presence and absence of naloxone (10 μM). These groups included from 12 - 15(5 - 8) vessels(dogs) each. The vessels were thoroughly washed, allowed to relax and the relaxation $T_{1/2}$ calculated. The presence of naloxone reduced the relaxation $T_{1/2}$ for the 10 minute incubation, but not the 3 minute incubation. There was a trend toward shortened relaxation $T_{1/2}$ after the three minute epinephrine exposure, but it was not statistically significant. The longer incubation time prolonged relaxation $T_{1/2}$s in the presence and absence of naloxone.

RELAXATION $T_{1/2}$S (MINUTES)		
	EPINEPHRINE ALONE	WITH PHENTOLAMINE
3 MINUTES	14.1 ± 1.0	5.0 ± 0.8
10 MINUTES	22.2 ± 0.7	4.4 ± 0.8

TABLE 6

Naloxone reduced relaxation $T_{1/2}$S for vessels precontracted with epinephrine. It was unclear why vessels exhibited such a gradual relaxation after epinephrine had been removed from the tissue bath. Was the persistent contraction due to 'post-receptor' mechanisms which remained active despite the discontinued adrenergic receptor activation, or did the contraction persist because of continued adrenergic receptor occupancy? Phentolamine was administered immediately after washout to vessels exposed to epinephrine (100 μM) for three [N = 7(4)] and ten minutes [N = 10(5)] and relaxation $T_{1/2}$s were recorded. The results are listed in Table 6 along with the mean relaxation $T_{1/2}$ of vessels exposed to epinephrine (100 μM) for three and ten minutes, but relaxed without phentolamine. Phentolamine induced an abrupt reduction in relaxation $T_{1/2}$. Phentolamine is a competitive antagonist and has no vasodilatory properties by itself. Phentolamine can accelerate relaxation only by displacing epinephrine molecules from alpha-adrenergic receptors. The acceleration of relaxation by phentolamine indicated that the persistent contraction and relatively slow relaxation of vessels after washout was a result of continued epinephrine occupancy of alpha receptors. Therefore epinephrine either diffused away from the receptor site very slowly, or there was a significant eflux of epinephrine from uptake storage that had continued interaction with the receptors as it left the tissue.

Catecholamine Uptake and Metabolism

The naloxone effect could be a result of inhibition of epinephrine disposal mechanisms. Catecholamines are transported to their site of action (adrenergic receptors) by two pathways. Norepinephrine is released from sympathetic nerve terminals directly onto the receptors and epinephrine is released from the adrenal medulla and is transported via the circulation. There are two predominant pathways of catecholamine disposal once they are in the proximity of the adrenergic receptors. There are active mechanisms to take up catecholamines into the nerve terminals and the smooth muscle and reduce the concentration of catecholamine at the receptor site. There are also enzymes available to degrade the catecholamines into molecules which no longer activate the adrenergic receptors.

Catecholamine uptake is often classified as either neuronal ($uptake_1$) or extraneuronal ($uptake_2$). Neuronal uptake selectively takes up norepinephrine better than epinephrine and extraneuronal uptake selectively takes up epinephrine better than norepinephrine. There are two predominant pathways of enzymatic catecholamine degradation. These enzymes, catechol-o-methyl transferase (COMT) and monoamine oxidase (MAO), degrade epinephrine and norepinephrine equally well. COMT is associated predominantly with the extraneuronal uptake process and MAO is associated predominantly with the neuronal uptake process. As a result of these enzyme/uptake associations, COMT degrades predominantly epinephrine and MAO degrades predominantly norepinephrine. Because of the selective elimination of epinephrine by extraneuronal uptake and COMT, it is possible that naloxone selectively augmented epinephrine induced vasoconstriction by inhibiting one of these processes.

A variety of studies were employed to examine this relationship. First, the effect of naloxone was compared to that of an extraneuronal uptake inhibitor (corticosterone) and a neuronal uptake inhibitor (desipramine). Next, the effect of naloxone was compared to that of a COMT inhibitor (pyrogallol) and a MAO inhibitor (pargyline). Compounds which closely mimic the effect of naloxone provided evidence as to the mechanism of the naloxone effect.

A. Catecholamine Uptake Inhibition

Figures 24 and 25 illustrate the results of the studies on catecholamine uptake inhibition. Vessels underwent a submaximal epinephrine induced vasoconstriction followed by desipramine (0.1 μM) or corticosterone (10 μM). Naloxone (10 μM) was given after both desipramine and corticosterone. The tension change induced by desipramine was not different from epinephrine alone and the tension generated by naloxone was different from both epinephrine alone and epinephrine with desipramine. The tension generated by corticosterone and naloxone were different than epinephrine alone, but not different from each other. Naloxone, desipramine and corticosterone induced an increase in epinephrine induced precontraction of 34.2 ± 2.2, 1.1 ± 1.3 and 49.0 ± 6.0% of KCl maximum (See Figure 26). The percent tension increase for naloxone vs desipramine vs corticosterone demonstrated that corticosterone and naloxone caused contractions similar to each other, yet both stimulated contractions different than desipramine. It is evident from inspection of these data (Figures 24 - 26) that desipramine had no effect and that the effect of corticosterone was indistinguishable from that of naloxone. Corticosterone was also administered to vessels without epinephrine induced precontractions and caused no significant change in resting tension. The fact that the responses to naloxone and corticoste-

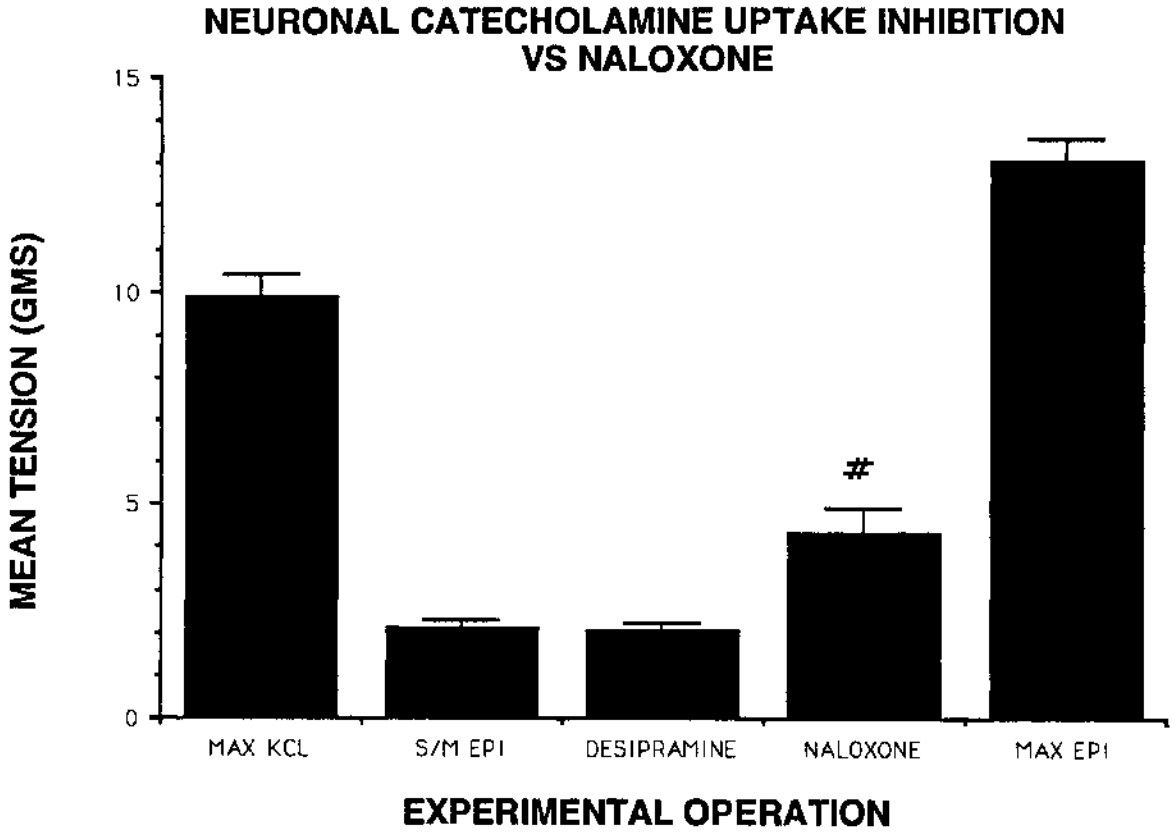


Figure 24. MAX KCL and MAX EPI are the maximum tension generated by KCL (80 mM) and epinephrine (10 μM). S/M EPI is the submaximal tension induced by epinephrine. DESIPRAMINE is the tension generated by desipramine (0.1 μM) and epinephrine. NX is the tension induced by epinephrine, desipramine and naloxone (10 μM). A repeated measures ANOVA post-hoc Scheffe analysis revealed that desipramine had no effect and naloxone augmented the epinephrine precontraction (#) $p < 0.01$. S/M EPI was 21.8% of MAX KCL and 16.1% of MAX EPI. Values are mean ± SEM. N = 16(5) vessels(dogs).

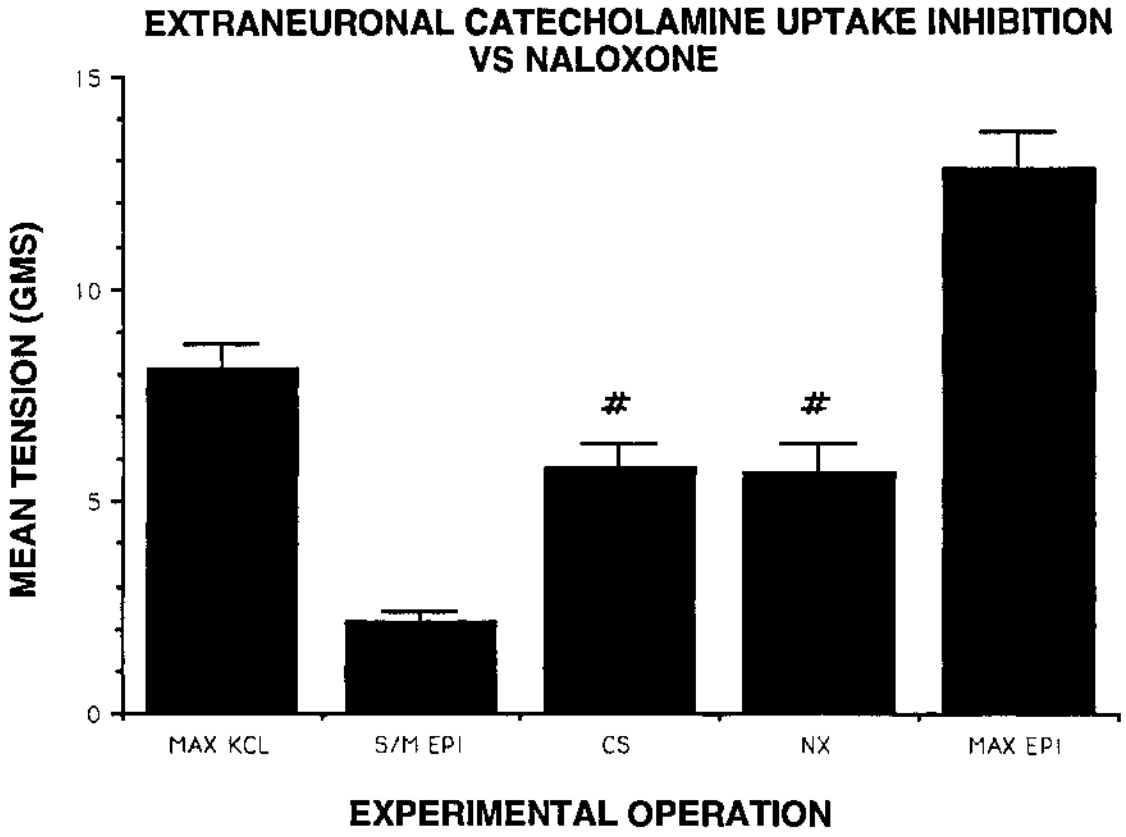


Figure 25. MAX KCL and MAX EPI are the maximum tension generated by KCL (80 mM) and epinephrine (10 μM). S/M EPI is the submaximal tension induced by epinephrine. CS is the tension generated by corticosterone (10 μM) and epinephrine. NX is the tension induced by epinephrine, corticosterone and naloxone (10 μM). A repeated measures ANOVA post-hoc Scheffe analysis revealed that both naloxone and corticosterone augmented the epinephrine precontraction (#) to an equivalent degree $p < 0.01$. S/M EPI was 28.1% of MAX KCL and 17.7% of MAX EPI. Values are mean ± SEM. N = 15(5) vessels(dogs).

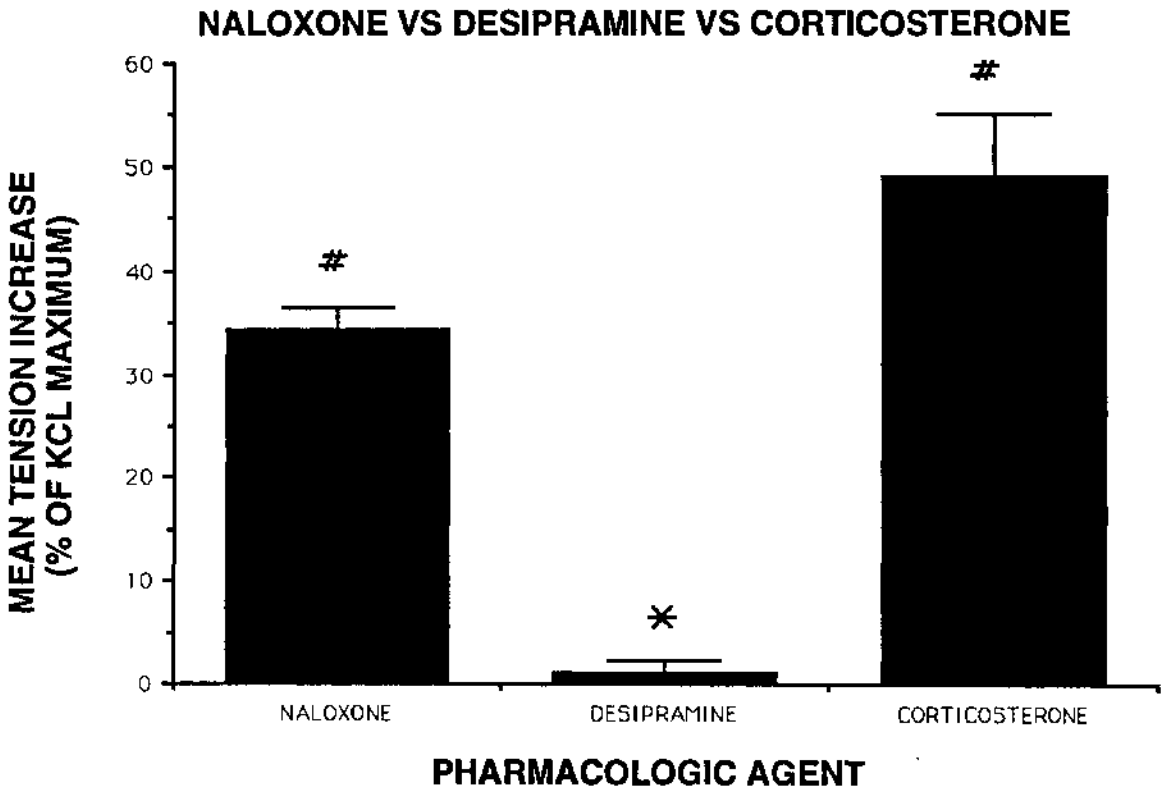


Figure 26. Naloxone (10 µM), desipramine (0.1 µM) and corticosterone (10 µM) were applied to vessels precontracted with epinephrine. Paired t-tests comparing the vessel tension before and after the administration of these drugs revealed that desipramine had no effect and that corticosterone and naloxone caused a significant increase in tension at $p < 0.05$ (#). An ANOVA post-hoc Scheffe analysis revealed that the change in the baseline submaximum epinephrine contraction induced by desipramine was different from that induced by corticosterone and naloxone at $p < 0.01$ (*). Values are mean ±SEM. N = 15(5), 15(5) and 15(6) vessels(dogs) respectively.

rone were indistinguishable suggests that naloxone may have augmented epinephrine induced vasoconstriction by inhibiting extraneuronal catecholamine uptake.

B. Catecholamine Metabolism Inhibition

Figures 27, 28 and 29 illustrate the results of the studies on catecholamine enzymatic degradation. Preliminary studies suggested that pyrogallol closely mimicked the effect of naloxone and pargyline had no effect. Therefore, the effect of pargyline was evaluated only on vessels precontracted with epinephrine, whereas pyrogallol was tested for a possible differential effect on epinephrine and norepinephrine precontractions. Figures 27 and 28 demonstrate the sequential experimental procedures as well as the absolute magnitude of the results. Vessels were precontracted with epinephrine and then exposed to pargyline (10 μM). Other vessels were precontracted with norepinephrine and then exposed to pyrogallol (100 μM). These vessels given pyrogallol and norepinephrine were washed, quickly relaxed, allowed time to recover, and then precontracted with epinephrine followed by pyrogallol (100 μM). After the pargyline and the last pyrogallol dose, naloxone (10 μM) was added as a paired control.

Figure 29 summarizes the results and compares the effects of pargyline and pyrogallol to the naloxone effect. Naloxone and pyrogallol augmented both epinephrine and norepinephrine induced precontractions and pargyline had no effect on an epinephrine precontraction. The effect of naloxone and pyrogallol on epinephrine and norepinephrine precontractions are indistiguishable, and pargyline had no effect. Both pargyline and pyrogallol were introduced to vessels in the absence of any precontraction and they produced no vasoconstrictions alone. Pyrogallol selectively augmented epinephrine precontractions over norepinephrine induced

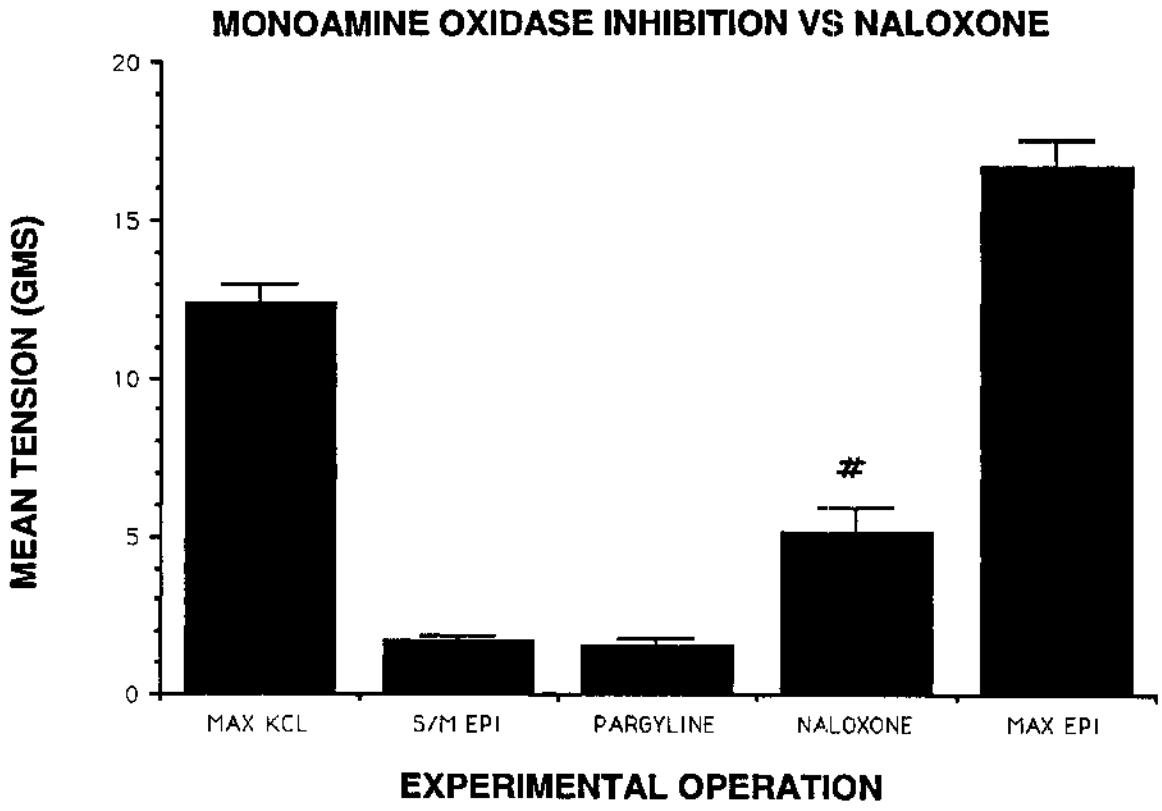


Figure 27. MAX KCL and MAX EPI are the maximum tension generated by KCL (80 mM) and epinephrine (10 μM). S/M EPI is the submaximal tension induced by epinephrine. PARGYLINE is the tension generated by pargyline (10 μM) and epinephrine. NALOXONE is the tension induced by epinephrine, pargyline and naloxone (10 μM). A repeated measures ANOVA post-hoc Scheffe analysis revealed that pargyline had no effect and naloxone augmented the epinephrine precontraction (#) $p < 0.01$. S/M EPI was 14.1% of MAX KCL and 10.4% of MAX EPI. Values are mean ± SEM. N = 10(5) vessels(dogs).

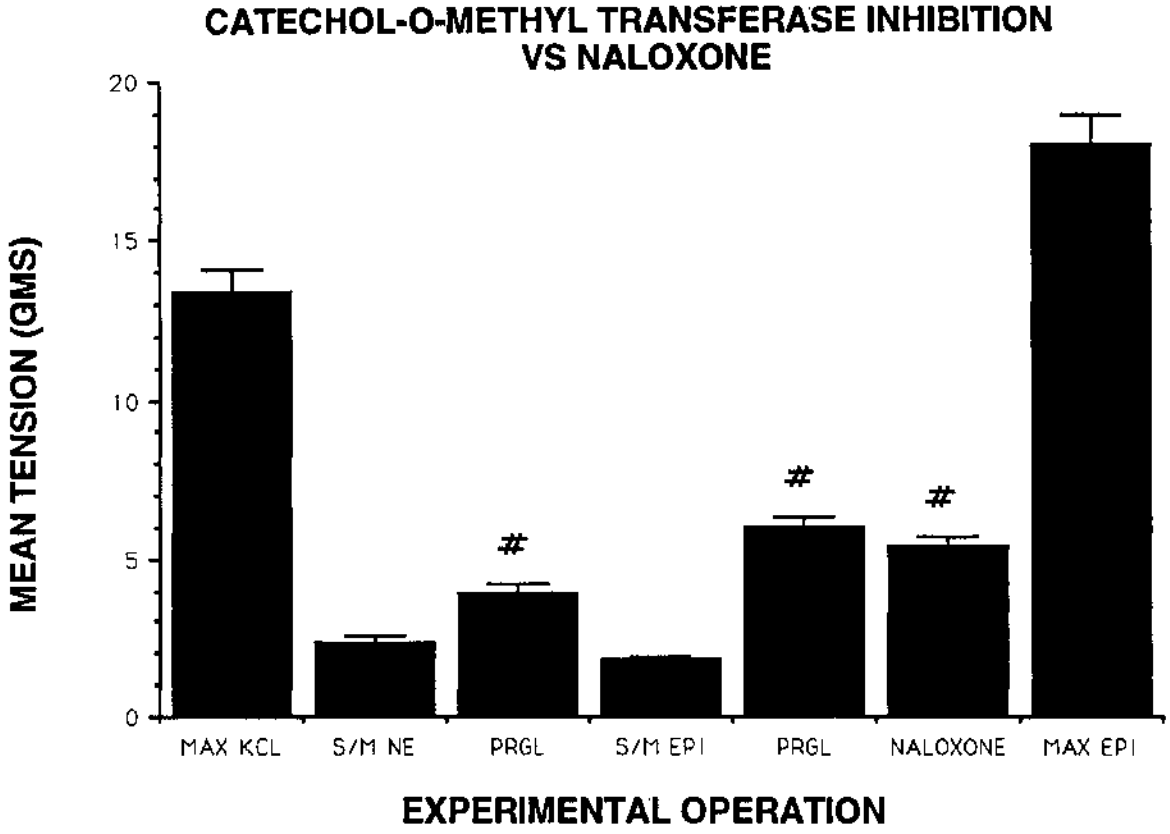


Figure 28. MAX KCL and MAX EPI are the maximum tension generated by KCL (80 mM) and epinephrine (10 μM). S/M EPI is the submaximal tension induced by epinephrine. S/M NE is the submaximal tension induced by norepinephrine. Both PRGLs are the tension generated by pyrogallol (100 μM) added to the preceding vasoconstrictor (epinephrine or norepinephrine). NALOXONE is the tension induced by epinephrine, pyrogallol and naloxone (10 μM). Vessels were washed and allowed to recover between the first PRGL and the S/M EPI. Repeated measures ANOVA post-hoc Scheffe analyses revealed that pyrogallol augmented both epinephrine and norepinephrine precontractions and was indistiguishable from naloxone at $p < 0.01$ (#). S/M NE was 17.7% of MAX KCL and 13.7% of MAX EPI. S/M EPI was 13.5% of MAX KCL and 10.1% of MAX EPI. Values are mean ± SEM. N = 12(5) vessels(dogs).

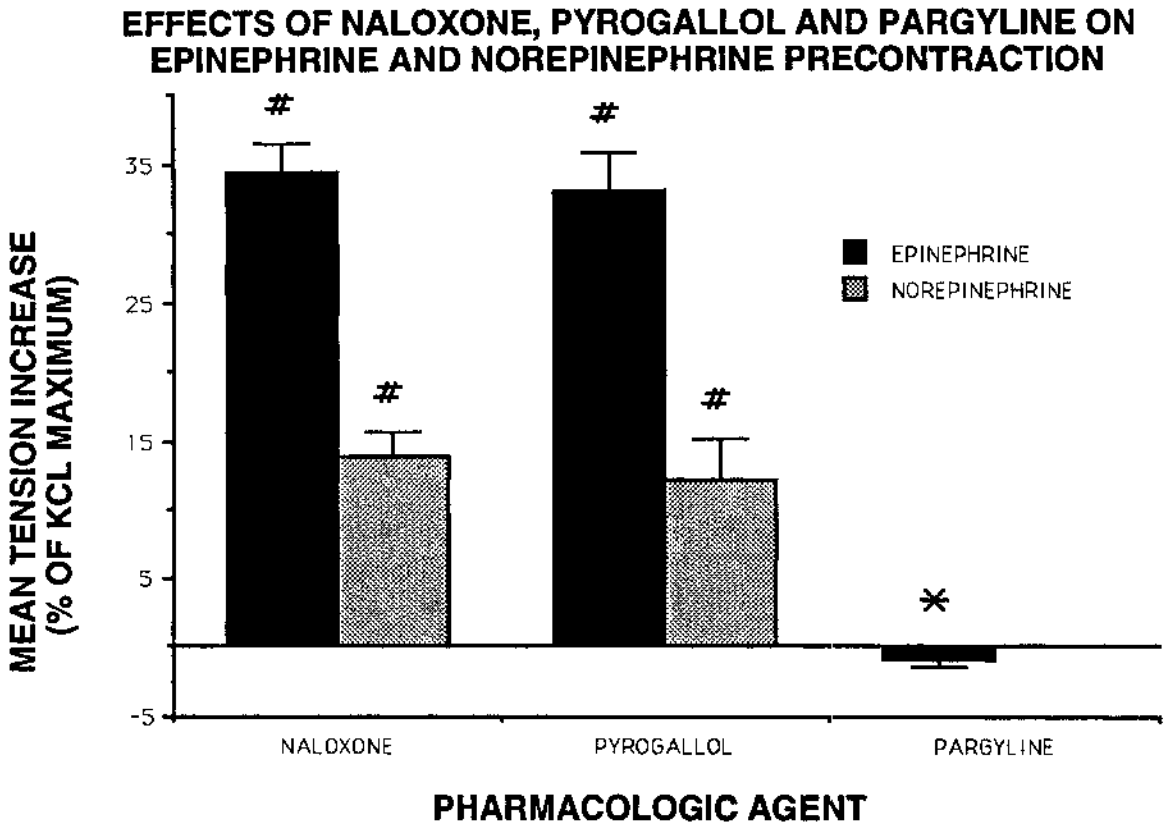


Figure 29. Vessels were precontracted with either epinephrine or norepinephrine and then exposed to naloxone (10 μM), pyrogallol (100 μM) or pargyline (10 μM). Statistics revealed that naloxone and pyrogallol augmented epinephrine and norepinephrine precontractions and pargyline caused no change in an epinephrine precontraction (#). The effects of naloxone and pyrogallol were indistinguishable from each other and different than pargyline (*). Values are mean ± SEM. N = 15(5), 12(5) and 10(5) for naloxone, pyrogallol and pargyline respectively.

precontractions in a manner that was indistinguishable from naloxone. This suggests that naloxone may have augmented epinephrine induced vasoconstrictions via COMT inhibition.

Endothelial Dependency

The endothelium is an important target, mediator and source of many vasoactive compounds. Vessels were tested to determine if the endothelium has a role in the naloxone effect. See Figure 30. Endothelium was intentionally stripped from a group of vessels and confirmed ablated by lack of endothelial dependent acetylcholine induced vasodilation. These vessels were precontracted with epinephrine and then given naloxone (10 μM). There was no difference in the effect of naloxone on submaximal epinephrine induced vasoconstrictions between vessels with endothelium intact vs absent. The vascular endothelium was not involved in naloxone mediated augmentation of epinephrine induced vasoconstriction.

Opioid Receptor Interactions

The ED_{50} for naloxone augmentation of epinephrine induced vasoconstriction was in the micromolar range which is very high relative to the affinity of naloxone for the mu-opiate receptor. Naloxone is best known as a mu-opiate receptor antagonist, yet also exhibits significant binding to all opiate receptor subtypes. The high ED_{50} for the naloxone effect suggests that naloxone may have mediated this response via an opiate receptor subtype to which it had relatively poor affinity. A study of the mechanism of action of naloxone would be incomplete without a thorough investigation of all opiate receptor subtypes. A wide variety of opiate receptor agonists and antagonists were tested for a tendency to augment epinephrine precontractions over

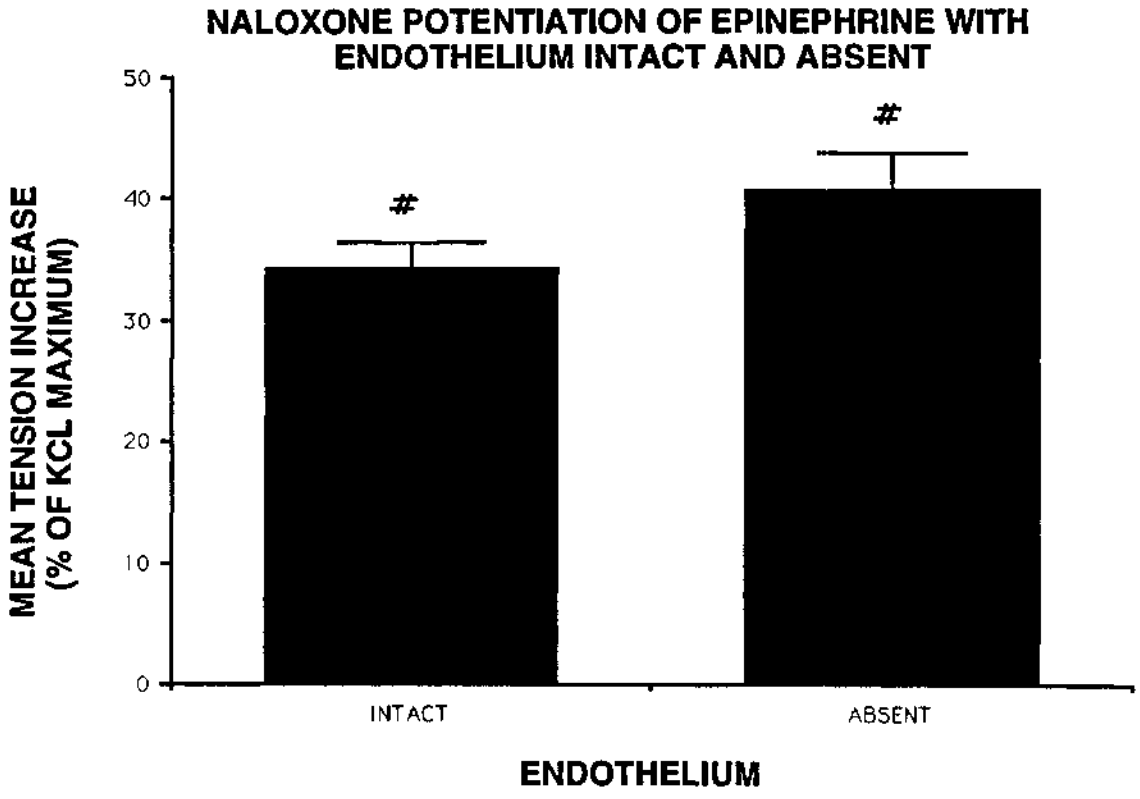


Figure 30. Naloxone (10 μM) was added to vessels precontracted with epinephrine with the endothelium intact and absent. An unpaired t-test revealed no difference at $p < 0.05$. Paired t-tests revealed that naloxone augmented the epinephrine precontraction in both groups at $p < 0.05$ (#). Values are mean ± SEM. N = 15(5) and 21(7) vessels(dogs) for the groups with endothelium intact and absent respectively.

a wide dose range. If the naloxone effect was mediated by a specific opiate receptor subtype, agonists or antagonists to that receptor subtype would induce a response similar to naloxone at a much lower dose. Dose-response relationships for naloxone and its opiate receptor inactive stereoisomer, (+)naloxone, were also compared as a further test of opiate receptor participation in the naloxone effect. If the naloxone effect was mediated via an opiate receptor, (+)naloxone would only augment epinephrine induced vasoconstrictions at a much higher dose than naloxone, if at all.

A. Opioid Dose-Responses

The effects of a series of opioids were compared to that of naloxone. Agents were chosen to include selective agonists and antagonists to the mu-, delta- and kappa-opiate receptors as well as a non selective agonist. This selection of opioids included both alkaloid and peptide ligands to each receptor subtype. Dose-response relationships were determined for each opioid in the presence of a submaximal epinephrine induced vasoconstriction. Doses ranged from 10^{-9} to 10^{-5} molar and the peak dose was followed by naloxone (10^{-5} molar) for comparison. Figure 31 depicts naltrexone as an example to demonstrate the experimental sequence from left to right and gives the absolute vascular tension generated by each experimental operation. The highest dose of opioids used was 10 μM. Higher doses were not employed because of problems including solubility, availability, cost and physiologic relevance.

Figures 32 - 38 depict comparisons between dose-responses from selected groups of opioids vs naloxone. The opioids were grouped with respect to activity (agonist vs antagonist), selectivity (mu vs kappa vs delta vs sigma ligands) and structure (alkaloid vs peptide). Detailed examination of these dose-response curves

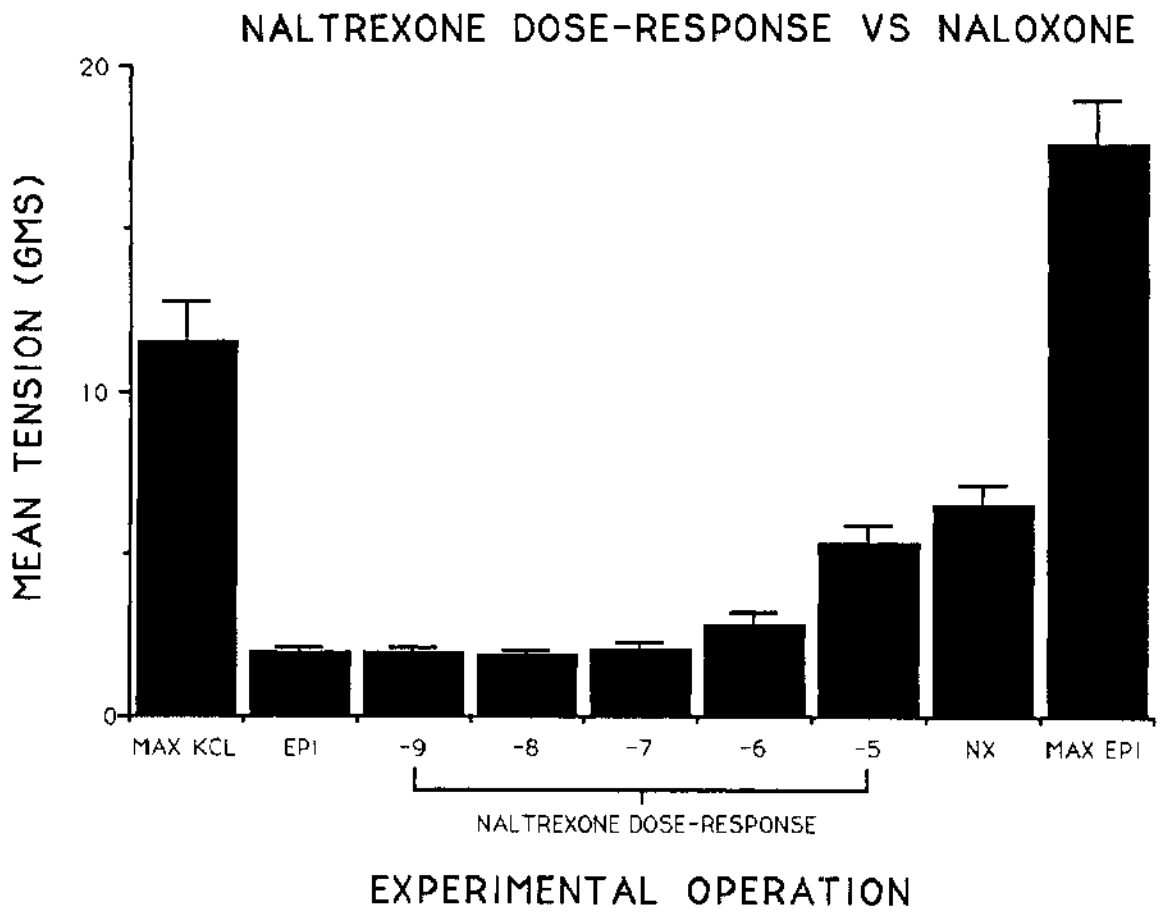


Figure 31. MAX KCL and MAX EPI are the maximum tension generated by KCl (80 mM) and epinephrine (100 μM). EPI is the tension generated by epinephrine (0.03-0.1 μM) and was 19.5% of MAX KCL and 11.6% of MAX EPI. The naltrexone dose-response (10^{-9} - 10^{-5} M) was performed on vessels precontracted with epinephrine. NX is the tension generated by the addition of naloxone (10 μM) to vessels after the peak dose of naltrexone. Values are mean ± SEM. N = 10(5) vessels(dogs).

provided substantial information. Across the various receptor subtypes, there was no clear correlation between agonist nor antagonist activity and the naloxone effect. Various agonists and antagonists within the same class augmented epinephrine vasoconstriction whereas others did not. It is, however, notable that the mu antagonists (naloxone and naltrexone) augmented epinephrine induced submaximal vasoconstrictions wherein the mu-opiate receptor agonists (morphine and morphiceptin) are inactive. None of the ligands to a single opiate receptor subtype demonstrated a consistent tendency to augment epinephrine precontractions. The only consistent finding was that none of the peptide structures and almost all of the alkaloid structures augmented epinephrine precontractions.

Figure 39 summarizes the change in epinephrine precontraction induced by the highest dose of each opioid. Statistical comparisons were based on the tension change in epinephrine precontraction as a percent of KCl maximum induced at a 10 μM dose. ED_{50}s could not be calculated because the opioid dose-response curves did not reach a maximum response even at this high dose and there was insufficient data to estimate maximum responses via a double reciprocal plot. Naltrexone; naltrindolol; MR1,452; dynorphin 1-9; U50,488 and diprenorphine all stimulated a change in the initial epinephrine contraction, yet only naltrexone; MR1,452; U50,488 and diprenorphine induced responses which were statistically equivalent to naloxone.

B. Opioids Alone

Any agent which augmented epinephrine induced vasoconstriction might have been a vasoconstrictor itself. Naloxone augmented epinephrine precontractions, yet had no effect alone. Agents which induced responses identical to those of naloxone might have had a mechanism of action in common with nalxone. All agents

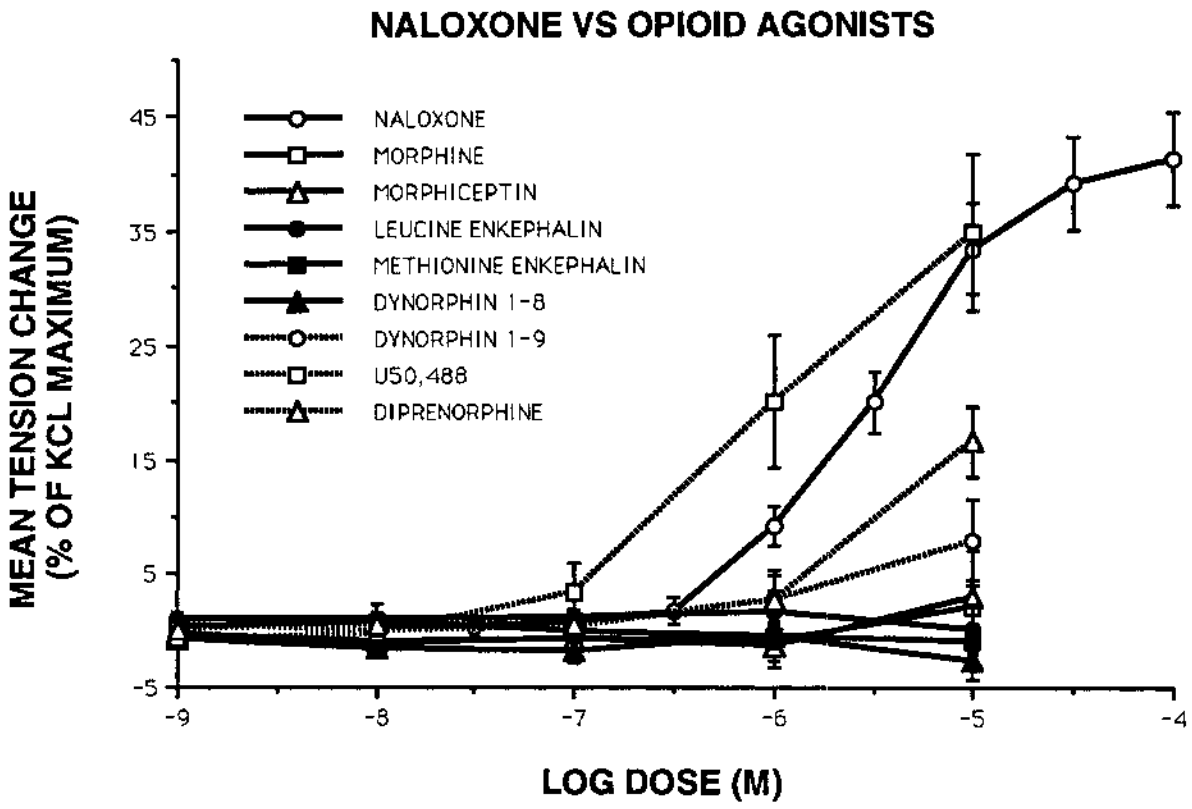


Figure 32. The listed opioids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

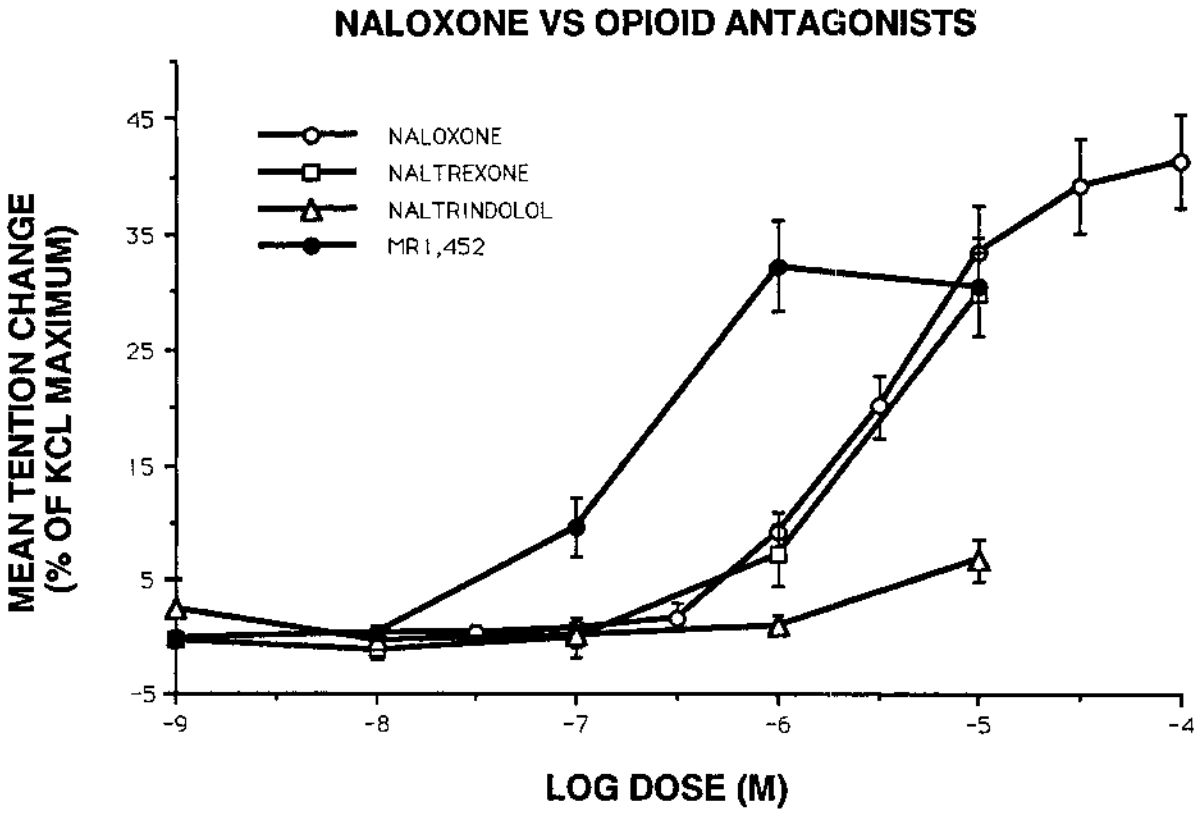


Figure 33. The listed opioids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

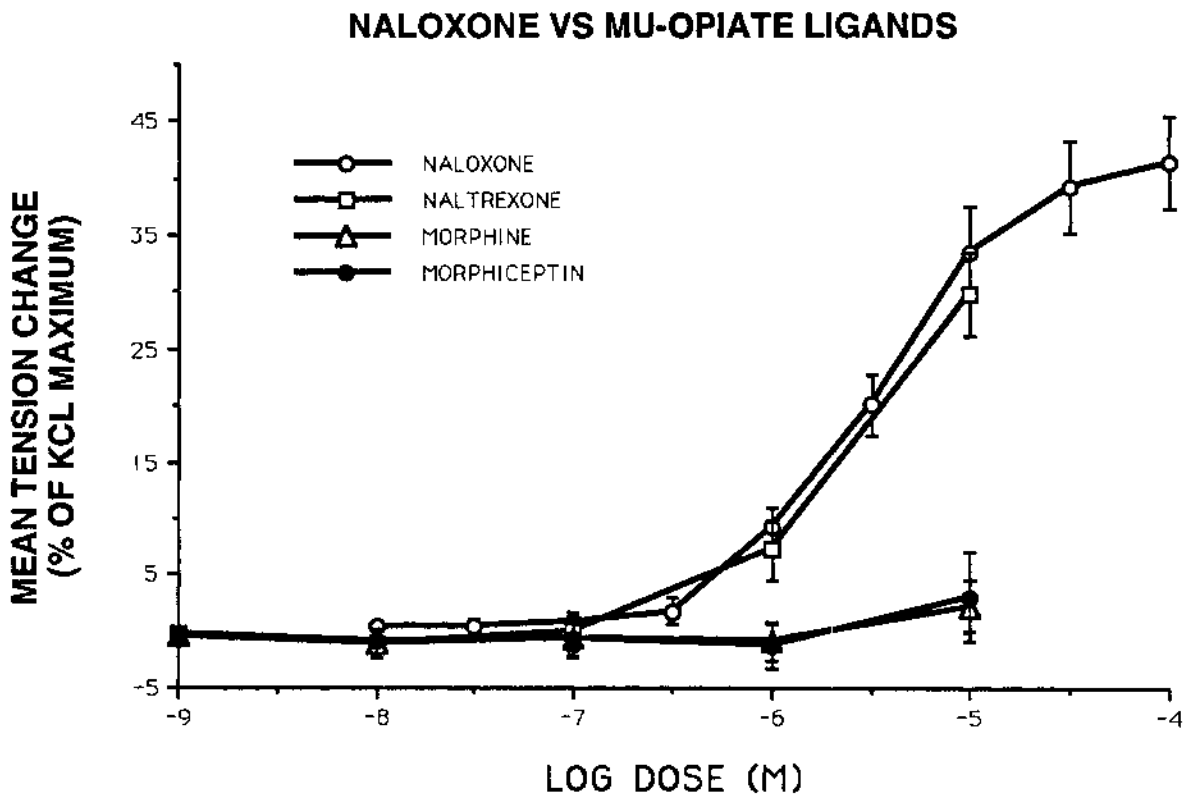


Figure 34. The listed opioids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

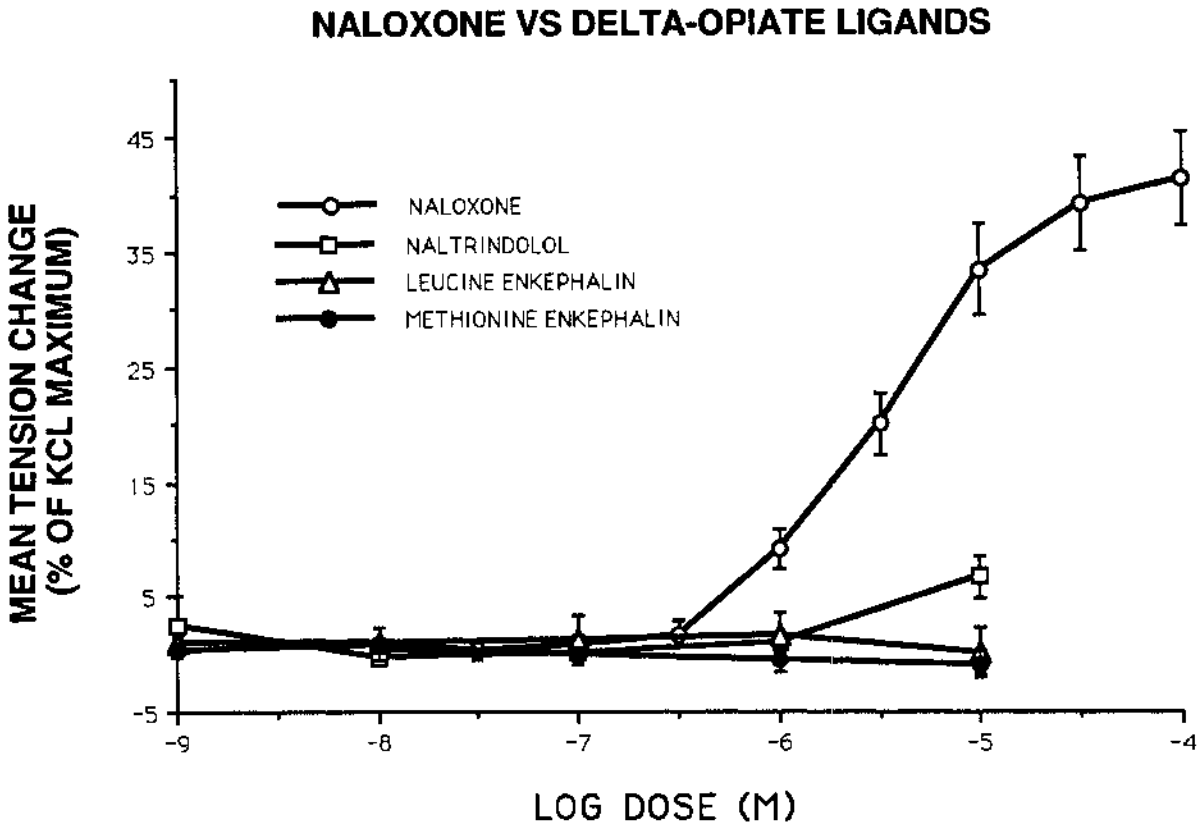


Figure 35. The listed opioids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

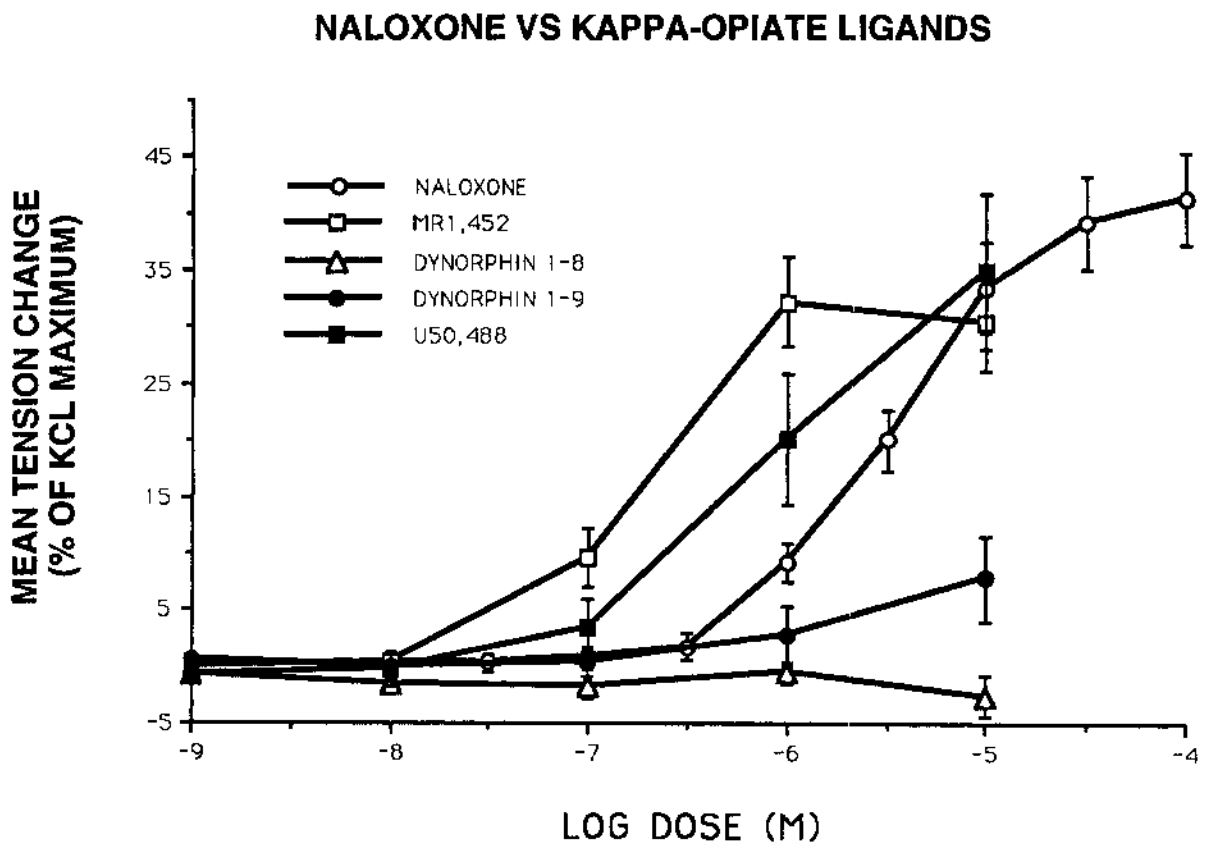


Figure 36. The listed opioids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

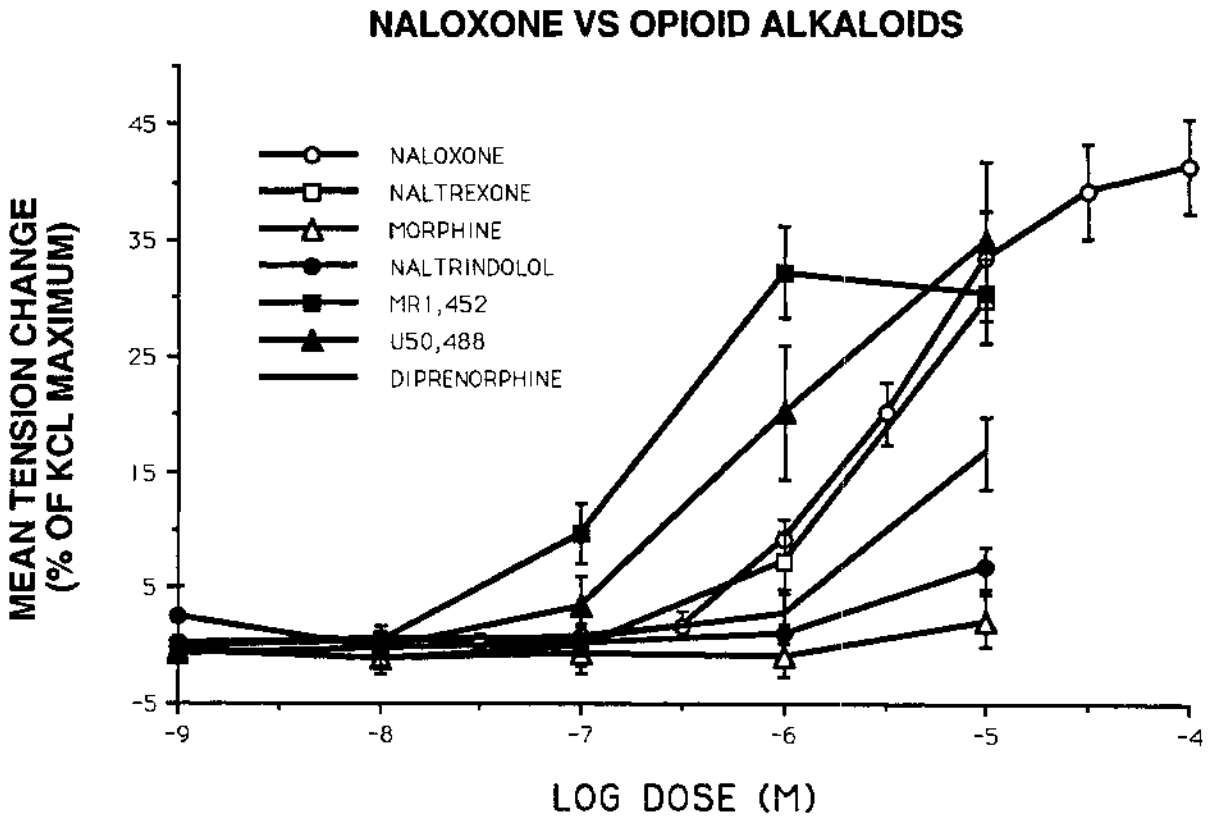


Figure 37. The listed opioids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

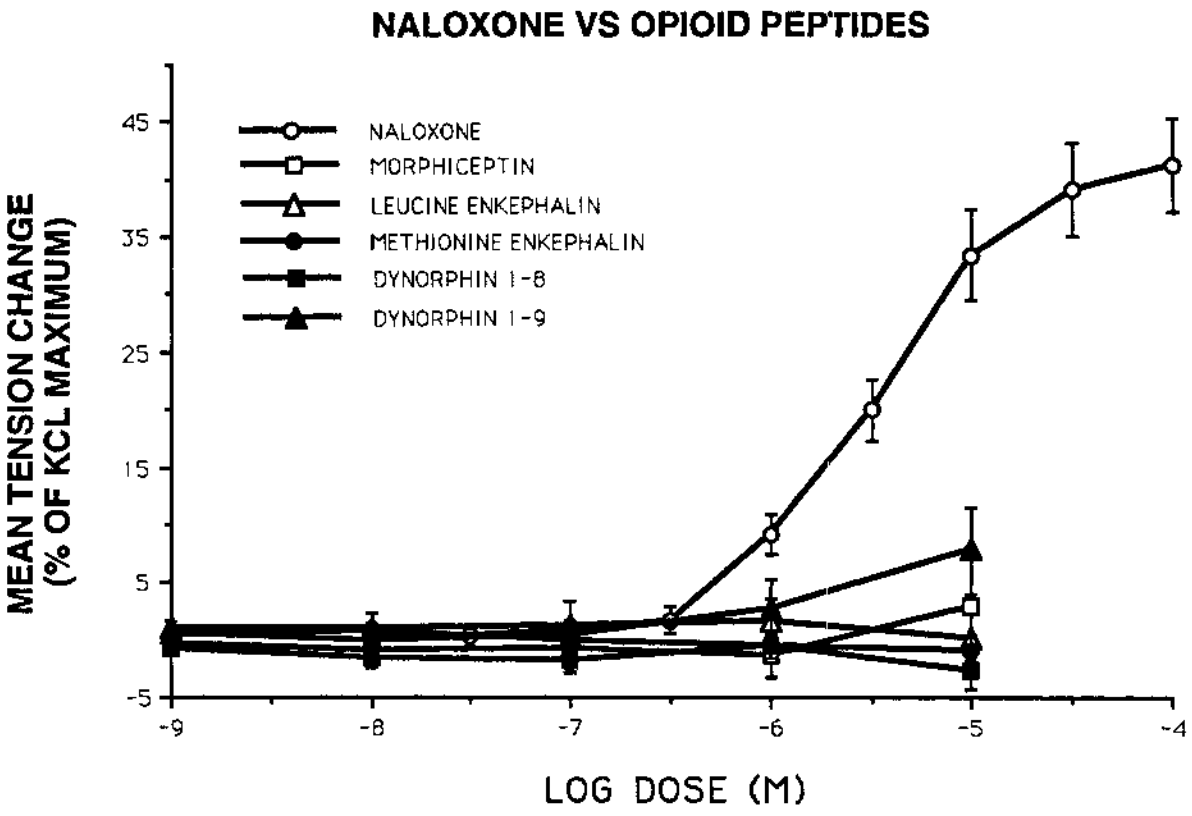


Figure 38. The listed opioids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

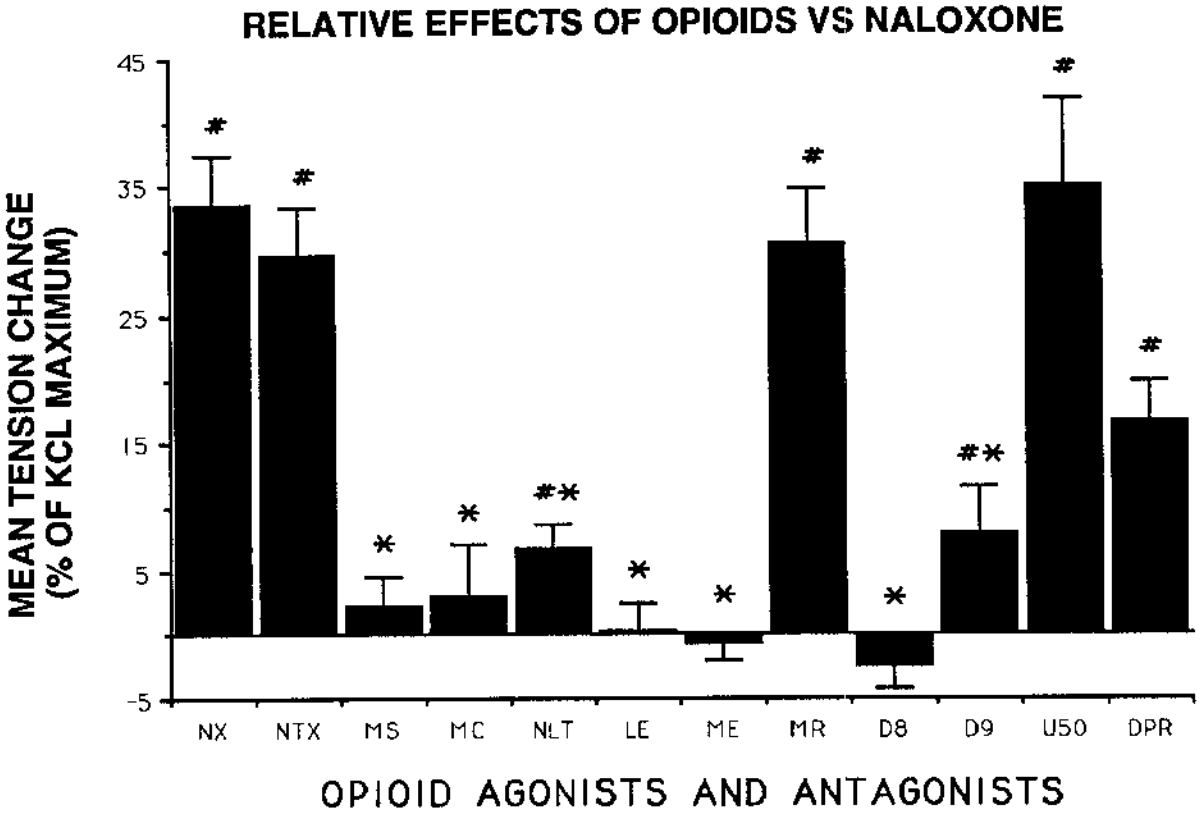


Figure 39. Vessels precontracted with submaximum epinephrine (0.03-0.1 μM) were exposed to a variety of opiate receptor agonists and antagonists (10 μM). NX = naloxone, NTX = naltrexone, MS = morphine sulfate, MC = morphiceptin, NLT = naltrindolol, LE = leucine enkephalin, ME = methionine enkephalin, MR = MR1,452, D8 = dynorphin 1-8, D9 = dynorphin 1-9, U50 = U50,488, and DPR = diprenorphine. (#) indicates opiate effect was different than submaximum epinephrine contraction and (*) indicates naloxone effect was significantly different from opioid effect. The mean submaximum epinephrine contractions for the above opioids were 25.9, 19.5, 21.8, 20.7, 15.6, 22.7, 16.8, 21.1, 22.0, 20.8, 22.8, and 18.8% of maximum KCL contractions respectively. Values are mean ± SEM. N = 15(4), 10(5), 11(5), 10(5), 10(5), 10(5), 11(5), 11(5), 10(5), 10(5), 10(5) and 12(6) vessels(dogs) respectively.

which augmented epinephrine vasoconstriction were also tested for vasoconstrictive action alone. Naltrexone; MR1,452 and U50,488 all had effects similar to that of naloxone. These agents were applied to vessels at 10 μM without precontraction with epinephrine. None of these agents demonstrated vasoconstrictive action when given alone. Therefore, these agents may have had mechanisms of action similar to that of naloxone.

C. Naloxone Stereoisomers

The stereoselectivity of the naloxone effect was examined by comparing the effects of the opiate antagonist, (-)naloxone, with its less active enantiomer, (+)naloxone. See Figure 40. Vessels were divided into two groups and dose-response curves are constructed for (-) and (+)naloxone in the presence of a submaximal epinephrine induced vasoconstriction. Both stereoisomers were tested between 10^{-8} to 10^{-4} M. The ED_{50}s for each enantiomer were determined by computer assisted graphical analysis. The ED_{50}s for (-) and (+)naloxone are $3.7x10^{-6}$ and $8.1x10^{-7}$ M respectively. The ED_{50} for (+)naloxone was less than the ED_{50} for (-)naloxone. (-)Naloxone exhibits greater affinity for opiate receptors than (+)naloxone, yet (+)naloxone exhibited a greater potency than (-)naloxone to augment an epinephrine induced submaximal vasoconstriction. This suggests that the naloxone effect was not mediated via an opiate receptor mechanism.

Sigma Receptor Ligands

Evidence from the study of opioids and naloxone stereoisomers suggested possible involvement of sigma receptors. Sigma receptors demonstrate a higher affinity for the (+)stereoisomers of various benzomorphan opiate receptor ligands.

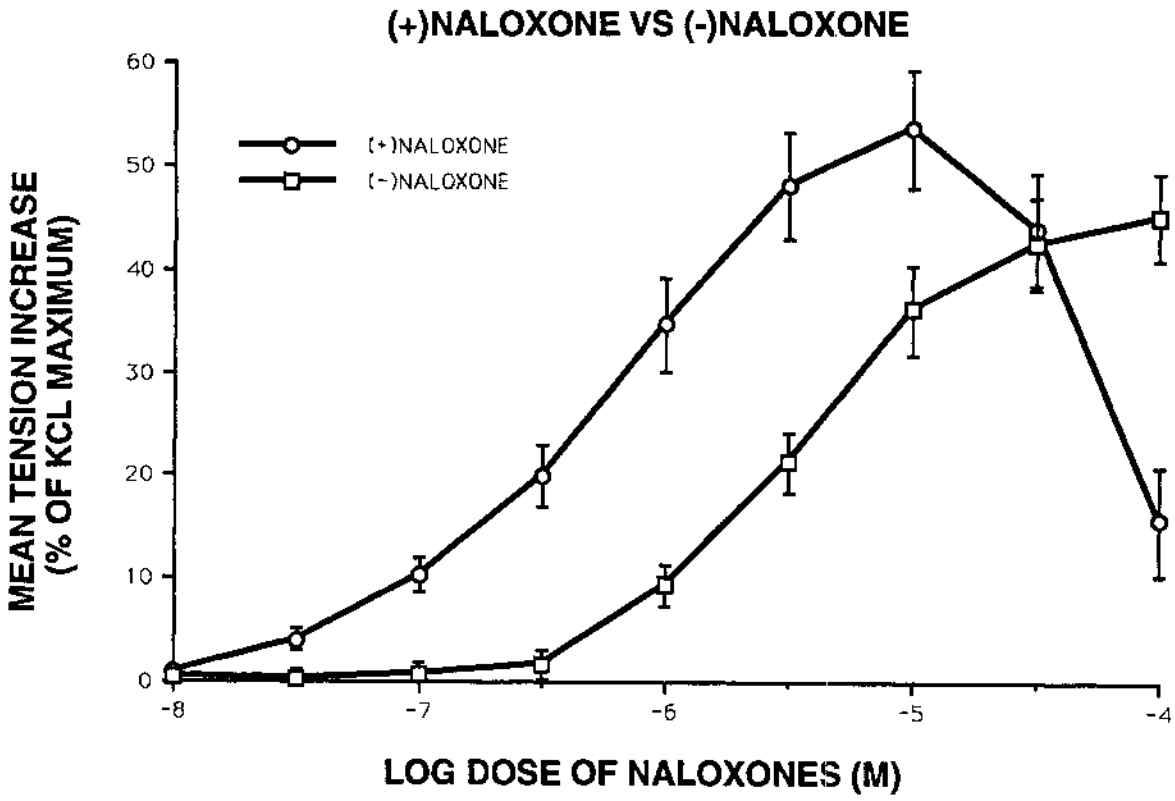


Figure 40. Vessel underwent a (+) or (-)naloxone dose-response in the presenceof a submaximum epinephrine contraction (0.03-0.1 μM). The ED_{50}s were $8.1x10^{-7}$ and $3.7x10^{-6}$ M for the (+) and (-)naloxone groups respectively. Submaximal epinephrine contractions for (+) and (-)naloxone groups were 25.9% and 25.9% of a maximum KCl contraction and 17.3% and 16.3% of a maximum epinephrine contraction respectively. An unpaired t-test comparing log doses of the naloxones demonstrates that the ED_{50} for (+)naloxone was less than the ED_{50} for (-)naloxone at $p < 0.05$. Values are mean ± SEM. N = 14(4) and 15(4) vessels(dogs) for the (+) and (-)naloxone groups respectively.

Since the (+)naloxone stereoisomer had greater efficacy than (-)naloxone to augment epinephrine vasoconstriction, one might propose sigma receptor involvement. Certain kappa-opiate receptor ligands have demonstrated cross reactivity with sigma receptors. Both U50,488 and MR1,452 are compounds with a significant degree of sigma receptor cross reactivity. U50,488 and MR1,452 demonstrated the greatest ability to potentiate epinephrine induced vasoconstriction and had no vasoconstrictive action alone. MR1,452 appears to have had even greater potency than naloxone in this regard. Corticosterone also mimicked the action of naloxone. Several steroids are potent sigma ligands (105, 115). It is possible that corticosterone augmented epinephrine induced vasoconstriction via a sigma receptor mediated mechanism. The above considerations indicate that the naloxone effect could be mediated via a sigma receptor.

A variety of sigma ligands were tested for their ability to alter an epinephrine precontraction over a wide range of doses. Some sigma ligands augmented epinephrine contractions, others had no effect, while others reversed the epinephrine vasoconstrictions. Sigma ligands which augmented epinephrine precontractions were tested alone (without epinephrine precontraction). Sigma ligands which reversed epinephrine induced precontraction were tested in the presence of a precontraction induced by a vasoconstrictor for which naloxone was not selective. Sigma ligands which induced vasodilation after epinephrine precontraction may have acted via the same mechanism as naloxone only in the opposite direction. These inhibitory sigma ligands may also simply have been vasodilators. Inhibitory sigma ligands were not tested for vasodilatory action in the absence of epinephrine unless a different vasoconstrictor was used to induce a precontraction. Some vasodilators may be effective only in the presence of an active vasoconstriction.

Therefore, inhibitory sigma ligands were applied to vessels precontracted with phenylephrine. Since naloxone selectively potentiated epinephrine over phenylephrine, reversal of phenylephrine vasoconstrictions indicated a mechanism of action distinct from naloxone.

A. Sigma Ligand Dose-Response

Sigma ligands were selected from a variety of different molecular conformations with reported sigma receptor affinity. The sigma ligands selected include di-o-tolylguanidine (DTG), (+)-1-propyl-3-(3-hydroxy-phenyl)piperidine [(+)PPP], (-)PPP, haloperidol, rimcazole, (+)pentazocine and (-)pentazocine. Each sigma ligand (10^{-9} - 10^{-5} M) was incrementally introduced to a group of vessels precontracted with epinephrine. This series of experiments followed the identical protocol as is depicted in the example in Figure 31. Figures 41 and 42 illustrate the resultant dose-response relations obtained. Figure 41 compares the naloxone effect to that of the sigma ligands which augment epinephrine precontractions. Figure 41 illustrates that (-)pentazocine had greater potency than naloxone and (+)PPP had equal potency to naloxone. Figure 42 illustrates the dose-response relation of the sigma ligands which actually reversed the epinephrine precontraction. Note that in figure 42, the percent inhibition instead of percent augmentation is plotted.

Figure 43 summarizes the change in the epinephrine precontracton induced by the highest dose of sigma ligand (10 μM) as a percent of KCl maximum contraction. All of the sigma ligands induced a significant change in vascular tone. Only (+)PPP and (-)pentazocine were statistically equivalent to naloxone at the 10 μM dose.

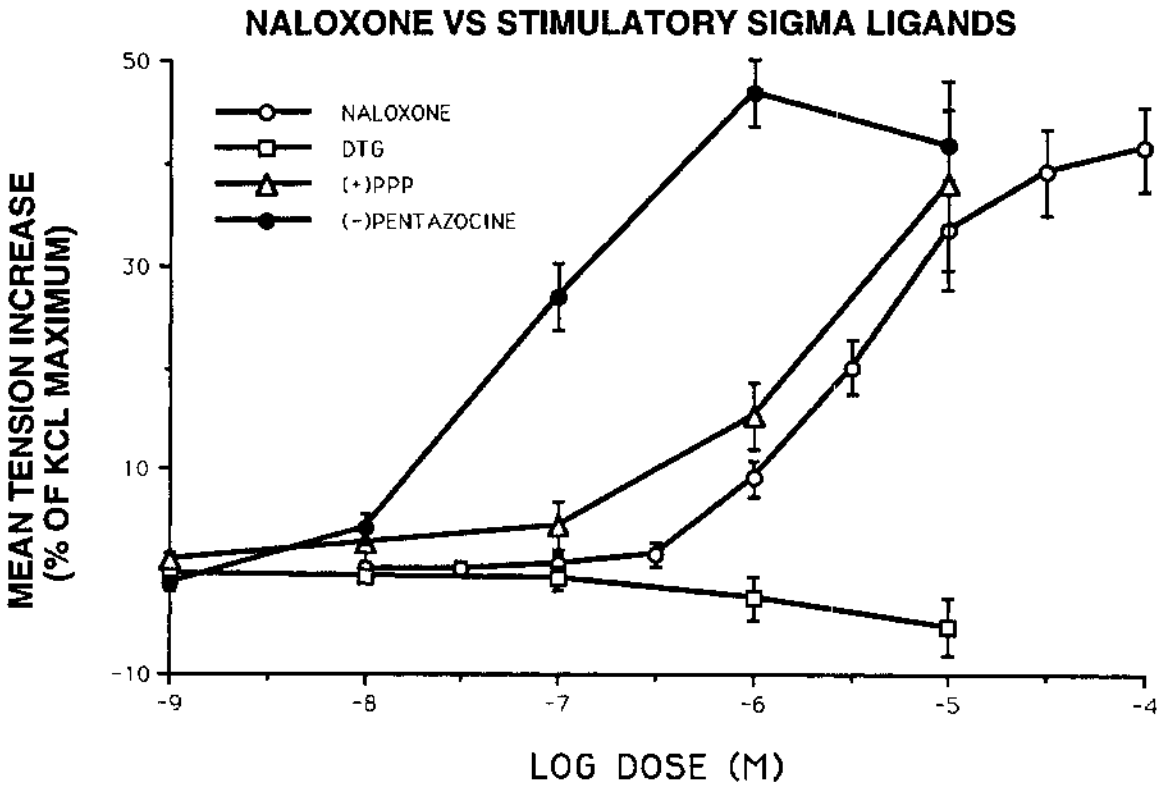


Figure 41. The listed sigma ligands and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

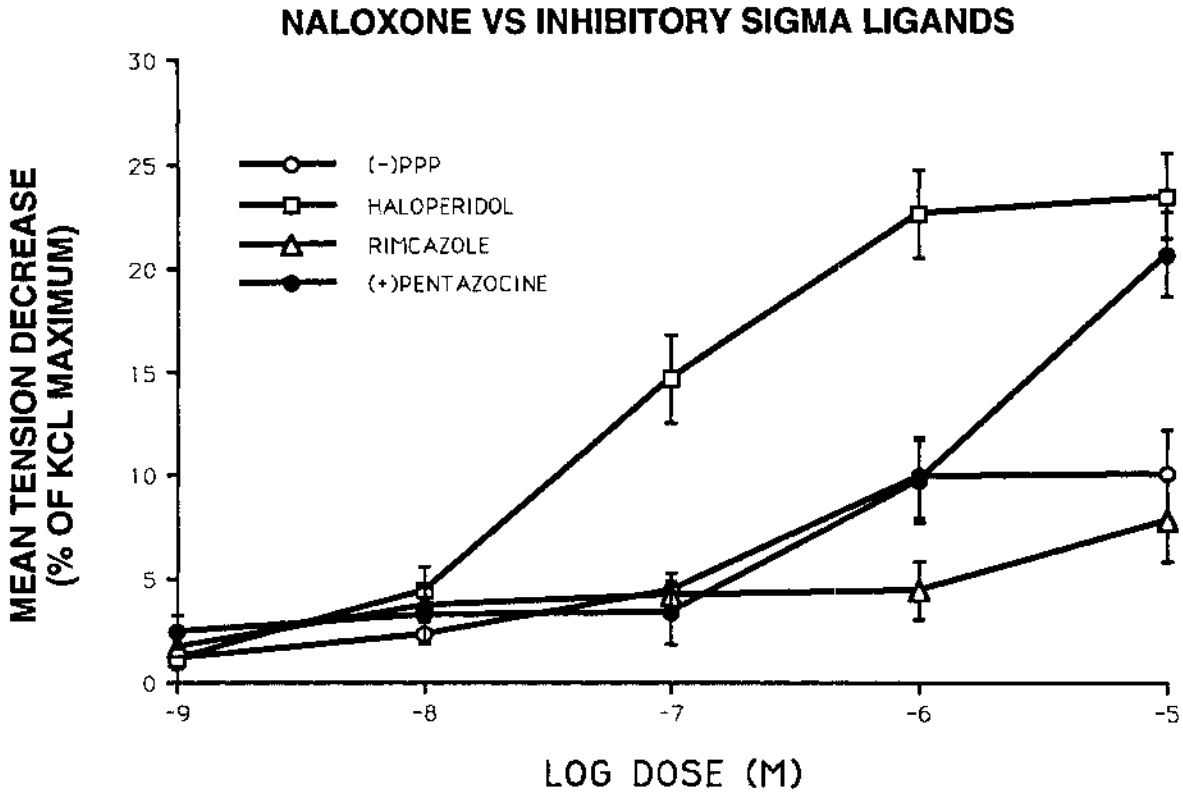


Figure 42. The listed opioids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

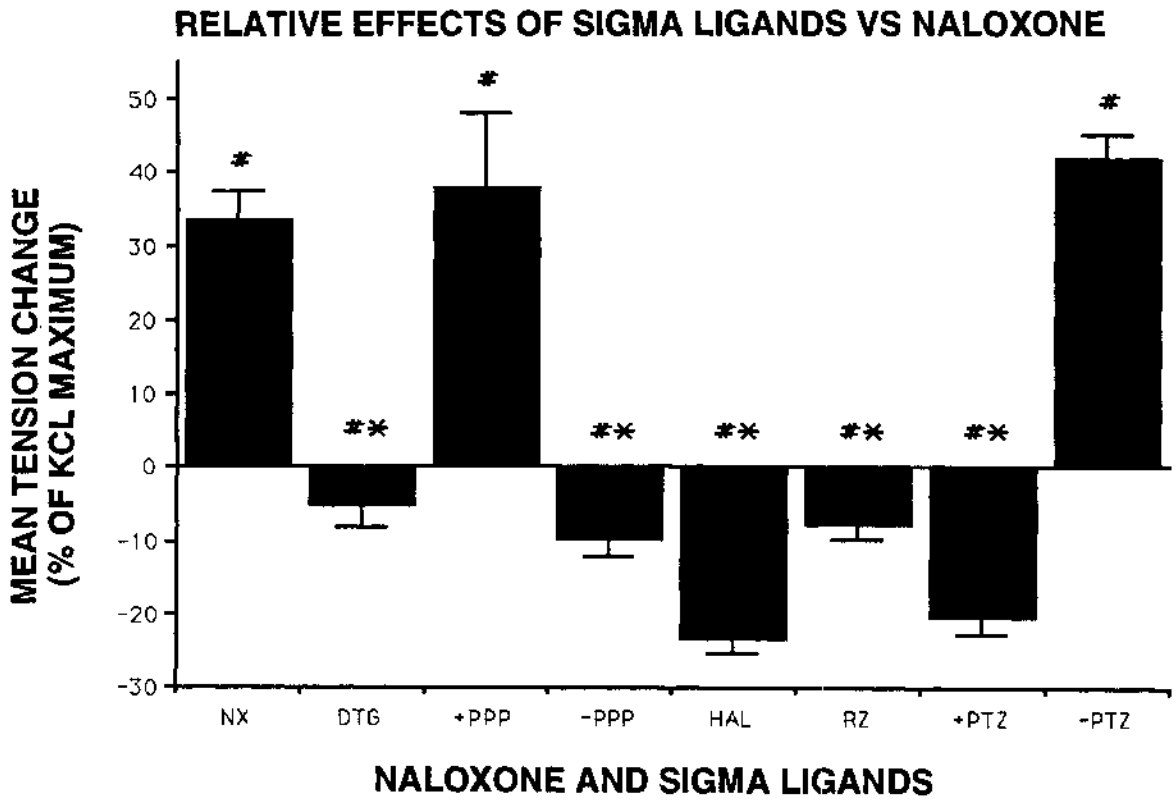


Figure 43. Vessels precontracted with submaximum epinephrine (0.03 - 0.1μM) were exposed to a variety of sigma receptor ligands (10 μM). NX = naloxone, DTG = di-o-tolylguanidine, +PPP = (+)PPP, -PPP = (-)PPP, HAL = haloperidol, RZ = rimcazole, +PTZ = (+)pentazocine, -PTZ = (-)pentazocine. (#) indicates sigma effect was different than submaximum epinephrine contraction and (*) indicates naloxone effect was significantly different from sigma effect. The mean submaximum epinephrine contractions for the above agents were 25.9, 20.1, 15.6, 19.0, 19.8, 19.5, 22.7, and 25.6% of maximum KCL contractions respectively. Values are mean ± SEM. N = 15(4), 10(5), 10(5), 10(4), 10(5), 11(5), 10(5) and 10(5) vessels(dogs) respectively.

B. Sigma Ligands Without Epinephrine

(+)PPP and (-)pentazocine were sigma ligands which had the greatest similarity to naloxone in their vascular effects. These two ligands (10 μM) were applied to vessels without precontraction with epinephrine. Neither (+)PPP nor (-)pentazocine induced a change in vascular tone in the absence of epinephrine.

(-)PPP, haloperidol and (+)pentazocine all substantially reversed an epinephrine induced precontraction. These agents were tested for vasodilator action in the absence of an epinephrine precontraction. Preliminary data demonstrated that vessels had to first be partially contracted in order to reveal vasodilator action. Figure 44 illustrates the percent inhibition of epinephrine and phenylephrine precontraction by the three most inhibitory sigma ligands. Inhibitory sigma ligands all nonselectively affected epinephrine and phenylephrine induced vasoconstrictions, whereas naloxone selectively affected epinephrine over phenylephrine induced vasoconstrictions. These sigma ligands probably inhibited epinephrine and phenylephrine induced vasoconstriction via a mechanism unrelated to the naloxone effect.

Steroids and Steroid Metabolites

A variety of data indicated that naloxone may have mimicked the effects of steroids. Corticosterone had vascular effects indistinguishable from those of naloxone. In the study on extraneuronal catecholamine uptake, corticosterone augmented epinephrine precontractions in a manner similar to naloxone. Data from the survey of the various opiate receptor subtypes suggested that the effect of naloxone was not opiate receptor specific and that alkaloid structures (like steroids) most closely mimicked the naloxone effect. Finally, several sigma receptor ligands had effects similar to naloxone and several steroids are now known to be potent sigma ligands (104, 115).

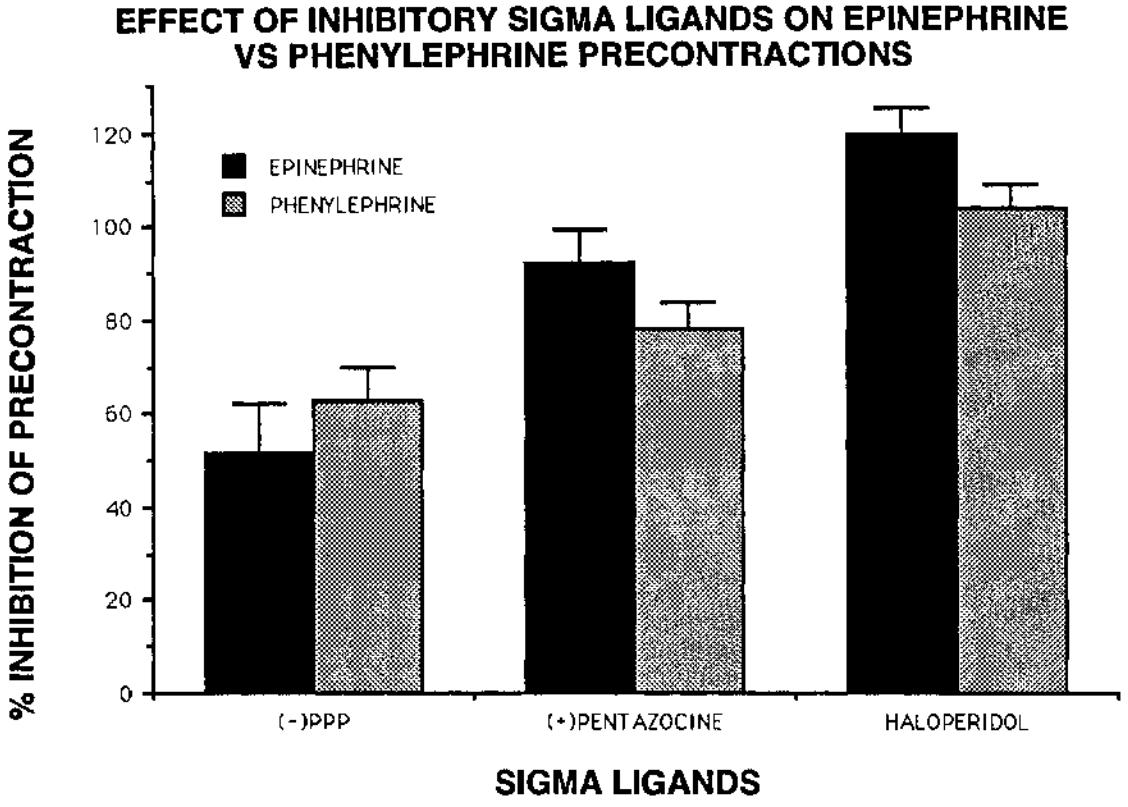


Figure 44. Vessels were precontracted with phenylephrine or epinephrine and then exposed to the inhibitory sigma ligands at 10 μM. Paired student t-tests indicate that each listed sigma ligand reduced precontractions by epinephrine and phenylephrine. Unpaired t-tests indicate that each listed sigma ligand affected epinephrine and phenylephrine precontractions alike. There was a trend for increasing inhibition of precontractions between sigma ligands from left to right in the figure.
Values are mean ± SEM.

To investigate the relationship between steroids and naloxone, dose-response relationships were determined for a variety of steroids and steroid metabolites. Biologically active steroids were compared to inactive steroid metabolites. Comparisons were made between types of biologic activity of steroids (glucocorticoid vs mineralocorticoid vs progestin). Structure activity relationships were examined as well. A selection of biologically active steroids were tested for vasoconstrictive action in the absence of epinephrine precontraction to ensure lack of independent vasoconstrictor activity.

A. Steroid Dose-Responses

Vessels were precontracted with epinephrine and then exposed to incrementally increasing doses (10^{-9} - 10^{-5} molar) of a variety of steroids and steroid metabolites. This series of experiments followed the identical protocol as is depicted in the example in figure 31. Figures 45 - 47 compare the dose-response relationships between selected groups of steroids and naloxone. Again, the absence of maximums on these curves prevented measurement of ED_{50}s for comparison. All the biologically active steroids depicted in Figure 45 significantly augmented epinephrine induced vasoconstriction. Progesterone had the least effect. Figure 46 depicts the effects of steroids rendered biologically inactive by A-ring saturation. Tetrahydrocorticosterone and tetrahydrocortisol had much less activity than their biologically active molecular precursors. Progesterone and pregnanalone augmented epinephrine precontractions equally. Biologic activity appears to have been important in the mediation of steroid induced augmentation of epinephrine contractions.

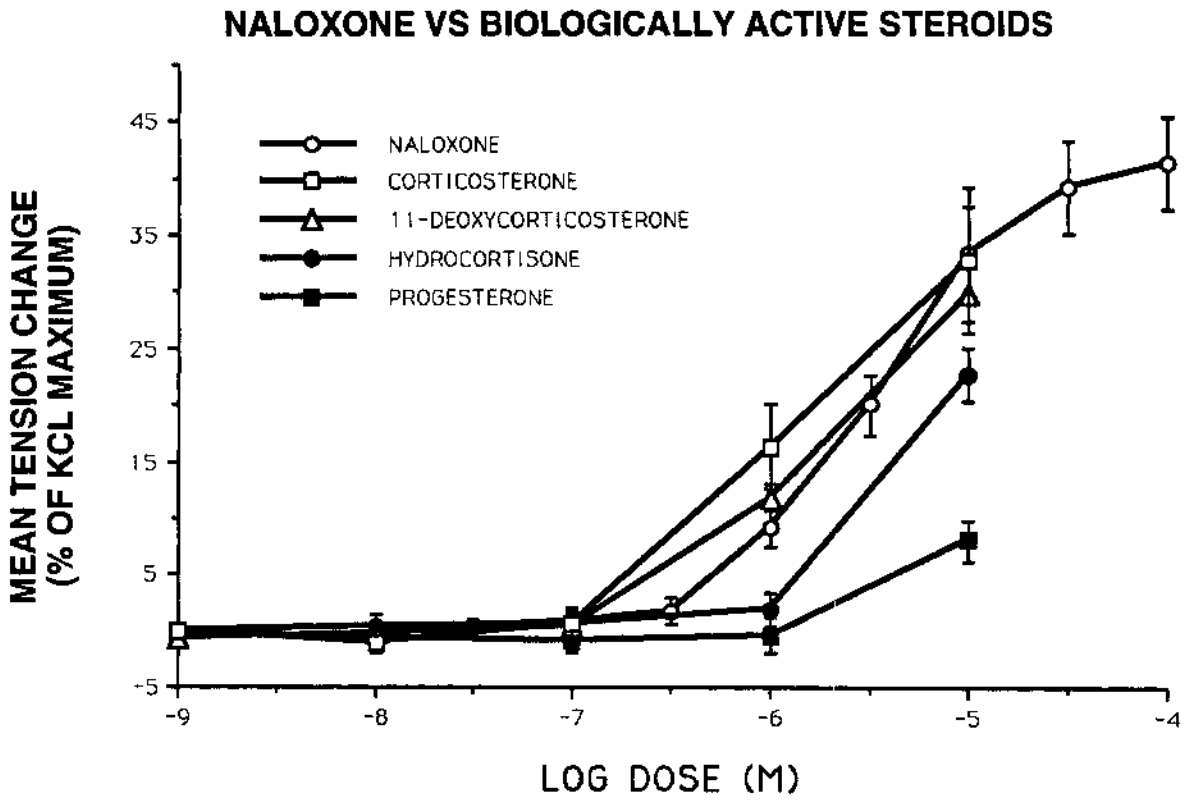


Figure 45. The listed steroids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

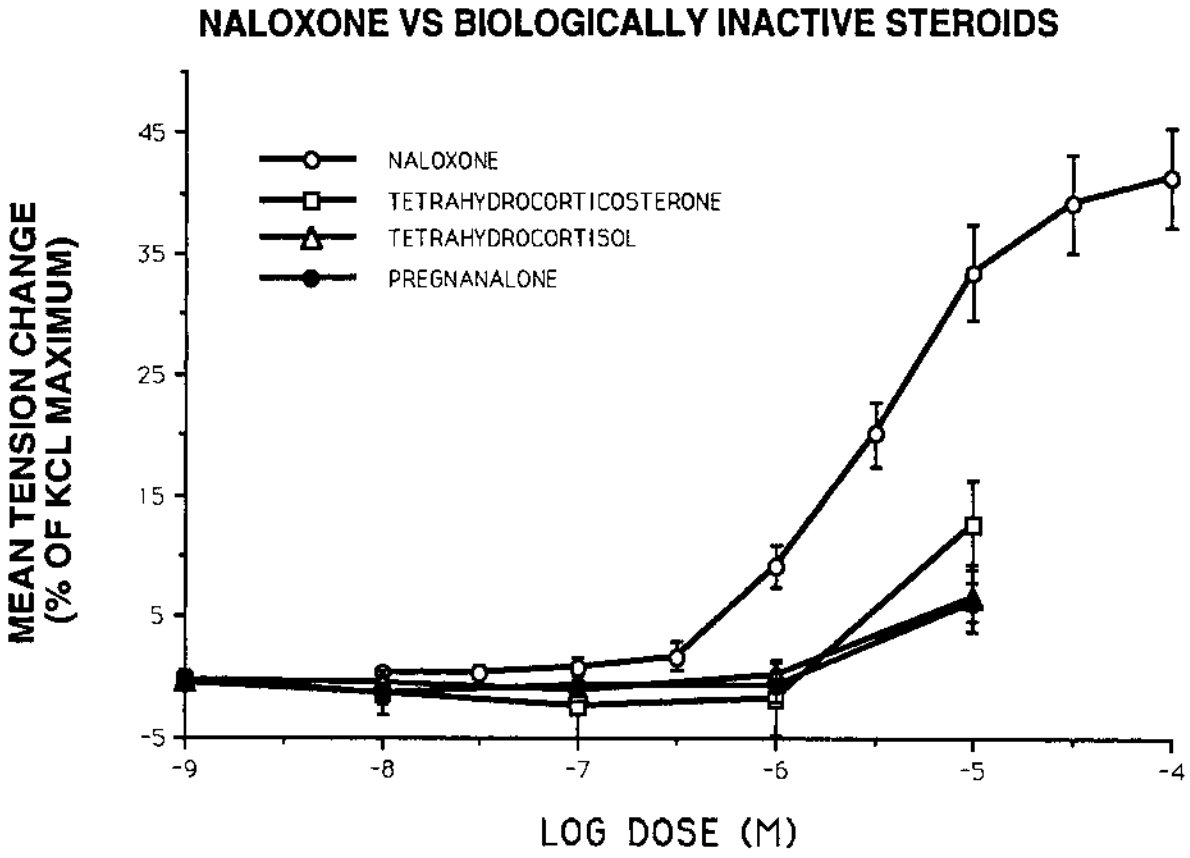


Figure 46. The listed steroids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

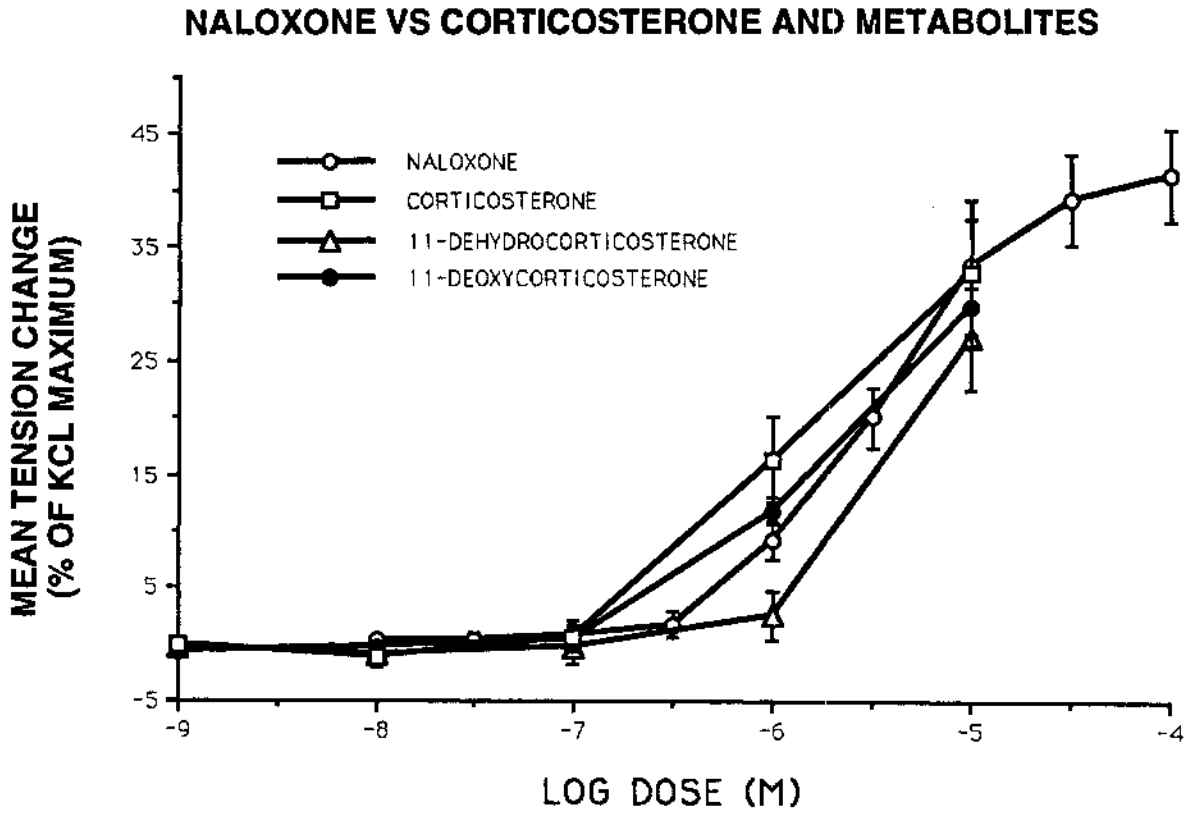


Figure 47. The listed steroids and naloxone were applied to vessels precontracted with epinephrine. Values are mean ± SEM.

Corticosterone and hydrocortisone have mixed mineralocorticoid and glucocorticoid activity. 11-Deoxycorticosterone has only mineralocorticoid activity. Progesterone has predominant effect as a progestin. Figure 45 compares the effects of these various classes of steroids on epinephrine vasoconstriction. Compounds with either glucocorticoid or mineralocorticoid activity appear to have augmented epinephrine precontractions the best. Progestins seem to have had little effect. In fact the biologically inactive metabolite of progesterone (pregnanalone) was as effective as progesterone itself.

Figure 47 illustrates the effect of altering the structure of corticosterone about the 11-hydroxyl. Conversion of the 11-hydroxyl to a carbonyl group or elimination of the 11-hydroxyl altogether had no effect on the resultant structure's ability to augment epinephrine induced vasoconstriction. In contrast, A-ring saturation greatly reduced the steroids' capacity to augment epinephrine vasoconstrictions.

Figure 48 summarizes the augmentation of the epinephrine induced precontraction as a percent of KCl maximum for each steroid analog at 10 μM. All steroids potentiated epinephrine induced vasoconstriction. Corticosterone, hydrocortisone, tetrahydrocorticosterone, 11-DHCS and 11-DOCS induced responses indistinguishable from naloxone at $p < 0.01$. Tetrahydrocorticosterone was different from naloxone at $p < 0.05$. None of the steroids appear to have had greater efficacy than naloxone to potentiate an epinephrine induced vasoconstriction. These results support the conclusions drawn above from visual inspection of the dose-response relationships. Steroids with glucocorticoid and mineralocorticoid activity augmented epinephrine induced vasoconstrictions better than steroids with progestin activity or steroids which lack biologic activity. Structural manipulations about the 11-hydroxyl group of corticosterone did not change the effect of corticosterone on epinephrine

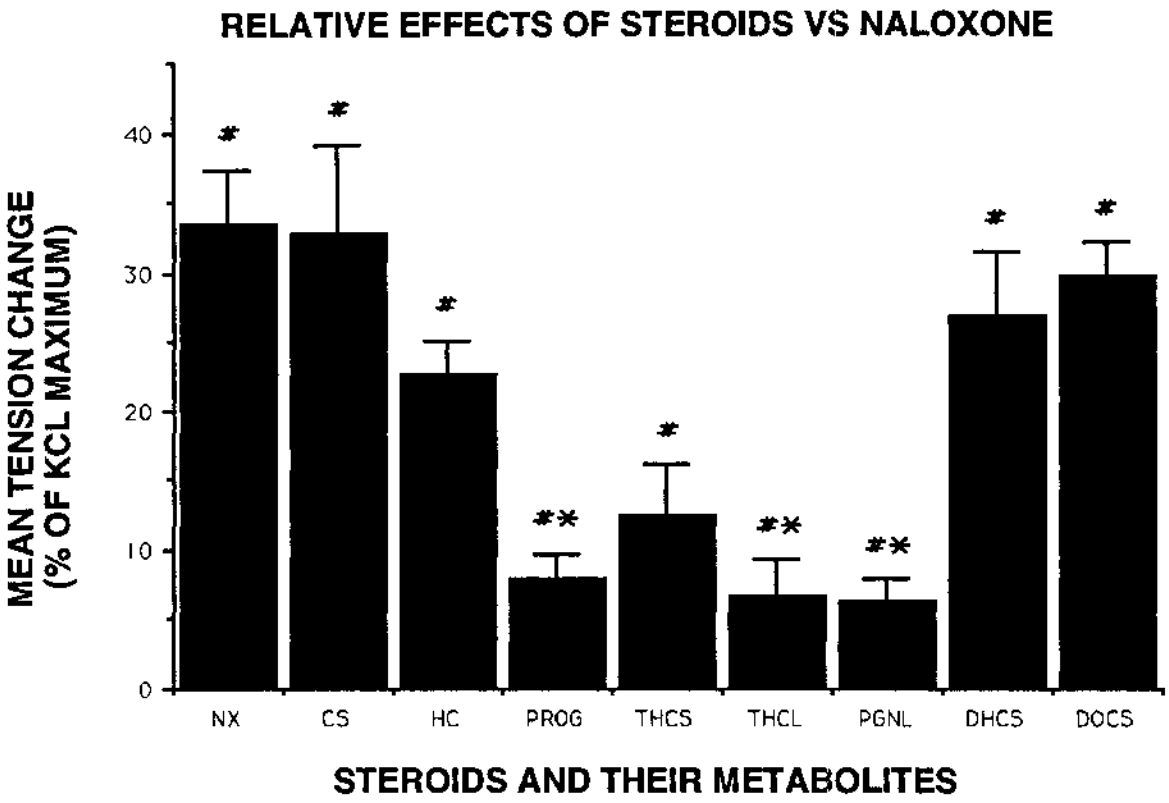


Figure 48. Vessels precontracted with submaximum epinephrine (0.03 - 0.1 μM) were exposed to a variety of steroids (10 μM). NX = naloxone, CS = corticosterone, HC = hydrocortisone, PROG = progesterone, THCS = tetrahydrocorticosterone, THCL = tetrahydrocortisol, PGNL = pregnanalone, DHCS = 11-hydroxycorticosterone, DOCS = 11-deoxycorticosterone. (#) indicates steroid effect was different than submaximum epinephrine contraction and (*) indicates naloxone effect was significantly different from steroid effect. The mean submaximum epinephrine contractions for the above agents were 25.9, 16.4, 18.0, 18.6, 18.4, 14.3, 16.8, 19.3 and 18.9% of maximum KCL contractions respectively. Values are mean ± SEM. N = 15(4), 9(5), 9(5), 10(5), 9(5), 8(4), 7(4), 8(4) and 8(4) vessels(dogs) respectively.

precontractions. None of the steroids augmented epinephrine vasoconstriction better than naloxone, but many appeared to be equally efficacious.

B. Steroids Without Epinephrine

Corticosterone, hydrocortisone and progesterone were tested for vasoconstrictor activity independent of an epinephrine induced precontraction. None of these steroids induced a vasoconstriction in the absence of epinephrine. Naloxone augmented epinephrine induced vasoconstrictions, but had no independent vasoconstrictive activity. Drugs which exhibit these characteristics may have augmented epinephrine precontractions by a similar mechanism. Certain steroids may have enhanced contractions induced by epinephrine by a similar mechanism to that of naloxone. It is unclear whether steroids mediated this effect via extraneuronal catecholamine uptake, via sigma receptors, via steroid receptors or by a totally unrelated mechanism.

CHAPTER IV

DISCUSSION

The autonomic nervous system and the endogenous opiate system interact extensively. Opioids modulate adrenergic control of the cardiovascular system in the central and peripheral nervous systems as well as in the heart and peripheral vasculature. The opiate receptor antagonist naloxone greatly augmented catecholamine induced vascular tone. This project began with a study of opioid modulation of adrenergic vasoconstriction in canine skeletal muscle vasculature using naloxone as a pharmacologic tool. Early studies indicated, however, that traditional opiate receptors did not mediate the naloxone response. This discovery redirected the investigation. The goal of this project became the characterization of the effect of naloxone to augment catecholamine induced vasoconstriction and the determination of the mechanism by which naloxone operated.

Naloxone is best known as a reversable opiate receptor antagonist. Naloxone has long been employed as a tool in the study of opioids and opiate receptor systems. In fact, investigators assumed that naloxone induced changes indicated 'by definition' that opiate receptors were involved. For example, when Holaday and Faden found that naloxone reversed the hypotension associated with rat endotoxic shock, they concluded that opiate receptor activation must exacerbate shock hypotension (49). Their discovery was followed by a rapid succession of experiments which revealed that intravenous naloxone reversed a variety of shock states (endotoxic, spinal, splanchnic occlusion, anaphylactic and hypovolumic) in a variety of animal models (dog, cat, mouse, pig, rabbit and rat) (5, 18, 19, 28, 43, 44, 49, 73-76, 89, 93,

97, 110). Naloxone was next found to reverse shock hypotension only in the presence of circulating catecholamines (1, 32, 75, 76, 85).

Endogenous opiates and catecholamines are synthesized and released together at a variety of functional sites (9, 42, 45, 82, 91, 111, 112, 118, 120). Several lines of evidence suggest that naloxone reverses shock hypotension via an opiate receptor mechanism. Endogenous opiates and catecholamines are cosecreted in times of stress and the reversal of shock by naloxone is dependent on circulating catecholamines. The action of naloxone as an opiate receptor antagonist is well documented. The above evidence indicates that the antihypotensive effect of naloxone is probably a direct result of opiate receptor blockade.

Naloxone augments sympathetic adrenergic stimulation of the cardiovascular system in the baroreflex (68, 71), the brain (7, 21, 70-72), the sympathetic ganglia (16) and sympathetic neurons (35, 57-60, 102, 113) as well as via direct action on the heart (13, 14, 40, 41, 73) and peripheral vasculature (15, 25, 92, 94, 103) -- seemingly everywhere.

The involvement of opiate receptors in the naloxone response is undeniable in many tissues. For example, numerous studies have proven the existence of opiate receptors on presynaptic sympathetic nerve terminals which, when stimulated, inhibit norepinephrine release in a naloxone sensitive manner (35, 57-60, 81, 87, 90, 102, 113). Other studies suggest naloxone potentiates cardiovascular responses to adrenergic stimulation via a non-opiate receptor mediated mechanism (15, 41, 103).

Naloxone has been extensively studied in the central and peripheral nervous system and in the heart. Insufficient data has been collected in the peripheral vasculature. It is unclear whether naloxone potentiation of peripheral adrenergic vasoconstriction is sufficient to contribute significantly to the naloxone induced

reversal of shock hypotension. Further, the mechanism by which naloxone potentiates adrenergic vasoconstriction *in-vitro* is unclear.

These studies were originally intended to examine the relationship between the endogenous opiates and the sympathetic nervous system. Early results refocused the goals, which became two fold: to characterize the effect of naloxone on adrenergic vasoconstriction in canine skeletal muscle vasculature and to investigate the mechanism by which naloxone had its effect. Canine skeletal muscle vasculature was chosen for two reasons. First, this lab has studied opiate/ catecholamine interactions in the canine intact and isolated heart, sympathetic ganglia and renal circulation and intended to correlate the data using a consistent animal model. Secondly, if naloxone significantly contributes to reversal of shock hypotension via peripheral vasoconstriction, it must involve the skeletal muscle vasculature which comprises the largest portion of the peripheral vasculature.

This discussion follows a similar pattern as the proposed questions in the introduction and the presentation of results. First, the canine skeletal muscle arteries themselves were studied to determine optimal resting tension. The effect of naloxone to potentiate adrenergic vasoconstriction was examined in detail. The roles of opiate receptors and sigma receptors were examined. Finally, a survey of steroids and steroid metabolites was completed in search of an endogenous ligand which subserved the naloxone effect.

Resting Tension

The optimum resting tension (T_{max}) is that resting tension (passive tension) which allows for the greatest possible generated contractile tension (active tension). For any given muscular contraction, the force generated is directly dependent on the

muscle fiber length immediately preceeding the contraction. There is a typical relationship wherein an optimal resting muscle length (L_{max}) allows for the greatest possible active tension. Near this optimal resting length maximum active tension is stable, but below and above this optimal resting length maximum active tension declines. This experimental design accurately recorded vessel wall tension instead of muscle fiber length. The muscle fiber length is directly proportional to the tension applied to it. Therefore, the resting tension which allows for the maximum active tension is the T_{max}.

Two similar experiments were performed to determine T_{max} for this tissue. In the first experiment (Figure 5), norepinephrine was the vasoconstrictor used and in the second experiment (Figure 6), KCL was the vasoconstrictor. The norepinephrine experiment failed to demonstrate a plateau in maximum active tension with increasing resting tension. The aximum active tension continues to rise slightly with increasing resting tension. Therefore, it was unclear that T_{max} was reached.

The second experiment was designed to ensure that resting tension was sufficiently high to demonstrate a clear plateau in maximum active tension with increasing resting tension. KCL was used as the vasoconstrictor because it washed out of the tissue readily and allowed vessels to relax more quickly and completely. Maximum active tension reached a plateau between two and eight grams resting tension and declined at higher resting tensions. Five grams resting tension was T_{max} according to these two studies and was employed as such throughout this series of experiments.

A few early experiments were completed using two grams resting tension. Two grams resting tension allowed for similar results as five grams. Data collected at two grams resting tension was never compared with data collected at five grams

resting tension. All statistical comparisons were made only between data collected at a consistent resting tension in order to maintain statistical integrity.

Naloxone Effect

The original observation which prompted this work involved the effect of naloxone on an epinephrine induced vasoconstriction in isolated canine renal interlobar arteries (15). Caffrey (15) determined that naloxone enhanced an epinephrine precontraction by over 150% and the presence of naloxone shifted an epinephrine dose-response curve leftward. That work was replicated here in canine skeletal muscle arteries. Examination of Figure 7 will familiarize the reader with the essence of what will be herein referred to as the 'naloxone effect'. Note that naloxone induced no vasoconstriction alone, yet greatly enhanced an epinephrine induced vasoconstriction.

The naloxone effect was characterized in three manners. The first method of characterizing the naloxone effect consisted of adding a single dose of naloxone (10 μM) to vessels precontracted with a submaximal (< 20% of KCL maximum) epinephrine induced vasoconstriction. Figures 7 and 8 demonstrate the potential magnitude of the naloxone effect. Naloxone enhanced an epinephrine precontraction by over 200%. It is this effect that subsequent experiments attempted to describe and explain.

Naloxone was found to shift the epinephrine dose-response curve leftward. The results of this experiment are depicted in Figures 9 - 12. The study had a crossover design to ensure the reliability of the results. Figures 9 and 10 illustrate that naloxone induced a leftward shift of the epinephrine dose-response curves for the first run and again during the subsequent crossover study. Figures 11 - 12

illustrate that there was a tendency for the epinephrine dose-response curves to shift rightward from the first to second run indicating a slight desensitization of the preparation over time.

Finally, a dose-response relationship was determined for naloxone in the presence of a submaximum epinephrine precontraction (Figure 13). The ED_{50} for naloxone was $3.7x10^{-6}$ M. The high (μM) ED_{50} suggests that if naloxone was operating via a receptor mechanism, it must have a relatively low affinity for that receptor. Because of the high dose of naloxone required, any of the opiate receptors might be involved despite the relative specificity of naloxone as a mu-receptor antagonist. High dose naloxone has even demonstrated opiate receptor agonist activity (96) and therefore its high ED_{50} in these experiments suggests activity as an opiate agonist. Naloxone may also be a weak agonist or antagonist at a non-opiate receptor site for which the endogenous ligand has yet to be discovered.

Adrenergic Specificity

This series of experiments demonstrated that the naloxone effect was specific to epinephrine precontractions and was directly dependent on alpha-adrenergic receptor stimulation.

Figures 14 and 15 compare the augmentation by naloxone of a series of adrenergic and nonadrenergic vasoconstrictors. Naloxone (10 μM) augmented precontractions by all adrenergic and nonadrenergic vasoconstrictors equally, except for epinephrine. Epinephrine precontractions were augmented to a much greater degree. Figures 16 and 17 demonstrate that naloxone did not shift the norepinephrine and the phenylephrine dose-response curves leftward as it did to the epinephrine dose-response curves. Figures 18 and 19 depict the rapid and total

reversal of epinephrine plus naloxone vasoconstrictions by phentolamine (alpha-adrenergic receptor antagonist).

The above data has clear and important implications. Norepinephrine, phenylephrine ($alpha_1$-adrenergic receptor agonist) and lofexidine ($alpha_2$-adrenergic receptor agonist) precontractions were all augmented by naloxone, yet epinephrine was selectively augmented the most. Reversal of the nalxone induced vasoconstriction by phentolamine implied the naloxone effect was mediated via the alpha-adrenergic receptor. Epinephrine, norepinephrine and phenylephrine have near equal efficacy at the $alpha_1$-adrenergic receptor, yet the precontractions induced by these alpha-adrenergic agonists were augmented differentially by naloxone. Once an alpha-adrenergic agonist activates an alpha-adrenergic receptor, it matters not which agonist supplied the activation, the subsequent intracellular cascade of events should be identical. Therefore, if naloxone acted at a point in the 'post-receptor' cascade of events to augment vasoconstriction, it should have augmented all alpha-adrenergic receptor agonists similarly. For naloxone to have selectively potentiated epinephrine induced alpha-adrenergic mediated vasoconstriction, naloxone must have selectively altered the epinephrine to receptor binding relationship. This binding relationship can be changed by changing the affinity or number of the receptors for epinephrine or by changing the concentration of epinephrine at the receptor (biophase). Biophase epinephrine concentration can be affected if naloxone selectively alters the uptake or degradation of epinephrine. This possibility was addressed by comparing the naloxone effect with that of known catecholamine uptake and degradation inhibitors. The actual affinity of the receptor for epinephrine cannot be measured with the tools applied here.

Norepinephrine, phenylephrine, lofexidine, vasopressin, KCL and serotonin precontractions were all augmented by naloxone, yet epinephrine was selectively

augmented to a greater extent. Following the same reasoning as above, naloxone must have augmented the precontractions of this diverse group of vasoconstrictors via a common 'post-receptor' pathway. Although this group employs a wide variety of mechanisms to initiate the cascade to vasoconstriction, there are numerous mechanistic commonalities. In order to have augmented this diverse group of vasoconstrictors equally, naloxone must have affected mechanistic pathways common to all. Naloxone, therefore, had two distinct effects. It selectively potentiated epinephrine induced vasoconstriction via a 'pre-receptor' mechanism and nonselectively potentiated vasoconstriction induced by norepinephrine, phenylephrine, lofexidine, vasopressin, KCL and serotonin to a lesser degree via a 'post-receptor' mechanism. These studies focused on characterization of the much larger selective augmentation of epinephrine induced vasoconstrictions.

Another possible explanation for the selective naloxone effect on epinephrine vasoconstriction is that naloxone may function as a beta-adrenergic antagonist. $Beta_2$-adrenergic receptors on vascular smooth muscle subserve vasodilation. Epinephrine stimulates alpha-adrenergic mediated vasoconstriction and beta-adrenergic mediated vasodilation simultaneously. Blockade of beta-adrenergic receptors would remove any vasodilatory effect and allow further vasoconstriction. Since epinephrine has greater efficacy at beta-adrenergic receptors than any of the other vasoconstrictors tested, antagonism at this receptor would have selectively augmented epinephrine induced vasoconstriction.

Figures 20 - 23 demonstrate that naloxone did not augment epinephrine induced precontractions by acting as a beta-adrenergic receptor antagonist. Figure 23 clearly demonstrates that naloxone did not act as a beta-adrenergic receptor antagonist, but fails to demonstrate whether naloxone potentiated beta-adrenergic

mediated vasodilation. The isoproterenol (beta-adrenergic receptor agonist) induced vasodilation was at maximum and therefore could not be potentiated. The magnitude of beta-adrenergic mediated vasodilation was small enough that a reliable submaximal vasodilation could not be obtained and confirmed.

Caffrey et. al. found that naloxone potentiated the beta-adrenergic effects of isoproterenol in the canine isolated heart (14). Naloxone did not augment epinephrine induced vasoconstriction via beta-adrenergic receptor blockade. If naloxone augmented the effects of epinephrine by either increasing the biophase epinephrine concentration, increasing the number of receptors or increasing adrenergic receptor affinity for epinephrine, beta-adrenergic receptor effects would probably be augmented along with the alpha-adrenergic receptor effects. Unfortunately, the effect of vascular beta-adrenergic receptor activation in this preparation was too small to obtain definitive results.

Relaxation Time

Kalsner (64) utilized relaxation times extensively to study catecholamine uptake and metabolism. Kalsner determined that catecholamine uptake and metabolism are intimately related and that the time required for a vessel to relax after vasoconstriction is a function of catecholamine disposal mechanisms. The time required for vessels to relax to 50% of their maximum contractile tension after washout (relaxation $T_{1/2}$) was determined for epinephrine, norepinephrine and phenylephrine after the completion of dose-response curves. See Tables 3 and 4. Naloxone selectively reduced relaxation $T_{1/2}$ for vessels contracted with epinephrine and had no effect on the relaxation $T_{1/2}$ for vessels contracted with norepinephrine or phenylephrine.

Relaxation $T_{1/2}$ for vessels contracted maximally with epinephrine were significantly longer than for norepinephrine or phenylephrine. See Table 3 and 4. The differential rates of relaxation after washout between epinephrine and norepinephrine induced vasoconstriction suggest that these vasoconstrictors were somehow taken up or metabolized differently by the tissue.

Table 5 demonstrates that naloxone also reduced the relaxation times for vessels contracted maximally for ten minutes with a single dose of epinephrine (100 μM). In this second experiment, exposure times were more rigidly controlled and the results remained the same. The relaxation $T_{1/2}$ for the epinephrine contracted vessels were similar between the vessels exposed to a dose-response (Table 3) and for those exposed to 100 μM epinephrine for ten minutes (Table 5). This consistency of relaxation $T_{1/2}$s increases confidence in the validity of these data. Both Tables 3 and 5 demonstrate the effect of naloxone to reduce relaxation $T_{1/2}$. It is significant that although naloxone augmented epinephrine induced vasoconstriction, naloxone also allowed vessels to relax more rapidly once washed free of the epinephrine. Both naloxone mediated augmentation of vasoconstriction and reduction of relaxation $T_{1/2}$ were specific for epinephrine which implied a common mechanism of action.

Table 5 demonstrates that as exposure time was increased from three to ten minutes, the relaxation $T_{1/2}$ was prolonged. The effect of naloxone to change relaxation $T_{1/2}$ increased with time as well. Naloxone failed to induce a significant reduction in $T_{1/2}$ at three minutes, but did reduce relaxation $T_{1/2}$ at ten minutes of epinephrine exposure. These trends indicate that a time dependent system existed which was the determinant of relaxation rate and that naloxone initiated a reduction in relaxation $T_{1/2}$ by a mechanism that was also time dependent.

Maintenance of contraction after washout was dependent on continued receptor occupancy by epinephrine. Table 6 demonstrates that phentolamine given

after washout reduced relaxation $T_{1/2}$ much more than naloxone did. Adrenergic receptors must have had continued exposure to significant epinephrine concentrations throughout relaxation. Phentolamine can only initiate relaxation by displacing epinephrine from the receptor. Therefore, the rate of relaxation was dependent on the rate at which the concentration of epinephrine at the receptor site declined. The rate of relaxation after washout has been used extensively as an indirect measure of catecholamine efflux from uptake stores (62-64, 108).

Naloxone may inhibit extraneuronal uptake of epinephrine as originally suggested by Sasaki (94). Extraneuronal catecholamine uptake is selective for epinephrine over norepinephrine (64). Additionally, blockade of extraneuronal catecholamine uptake should augment epinephrine induced vasoconstriction and reduce relaxation $T_{1/2}$, just as did naloxone.

The concentration of epinephrine at the receptor site (biophase) is a function of its rate of accumulation minus its rate of removal. The vessels were in a bath with a set concentration of epinephrine which diffused into the tissue at a constant rate at equilibrium. The mechanisms of catecholamine uptake and degradation as well were constant at equilibrium. The biophase concentration of epinephrine remained constant as a balance between these influences. The vasoconstrictive tension was directly proportional to the epinephrine concentration at the receptor. The vessel wall tension was therefore constant while biophase epinephrine concentration remained in equilibrium. Introduction of an epinephrine uptake inhibitor resulted in a decrease in the rate of removal of epinephrine from the biophase. This shifted the equilibrium and induced a rise in epinephrine biophase concentration resulting in an augmented vasoconstriction. Once the epinephrine was washed from the bath, the biophase concentration of epinephrine was a balance between epinephrine diffusing

out of extraneuronal stores into the biophase compartment and epinephrine diffusing away from the biophase and into the bath. Blockade of extraneuronal uptake during vessel incubation with epinephrine prevented accumulation of epinephrine in extraneuronal uptake stores and decreased the amount of epinephrine available to diffuse from extraneuronal uptake stores into the biophase after washout. This allowed the biophase concentration of epinephrine to fall more rapidly. Therefore, the fact that naloxone selectively potentiated epinephrine and reduced its relaxation $T_{1/2}$ provides strong evidence for involvement of extraneuronal catecholamine uptake processes.

Catecholamine Uptake and Metabolism

Catecholamines induce changes in vascular tone by interacting with adrenergic receptors on the vascular smooth muscle. Catecholamines arrive at the receptor sites via release from sympathetic neurons directly onto the smooth muscle (predominantly norepinephrine) or are released from the adrenal glands and arrive via the circulation (predominantly epinephrine). Once in the proximity of the adrenergic receptors (biophase), catecholamines are eliminated by a combination of active uptake into the tissue and enzymatic degradation. There are two types of catecholamine uptake termed uptake$_1$ (neuronal) and uptake$_2$ (extraneuronal). Extraneuronal (uptake$_2$) preferentially takes up circulating epinephrine and neuronal (uptake$_1$) preferentially takes up norepinephrine released from nerve terminals (38).

There are two major pathways of enzymatic degradation as well. Monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT) enzymes are both active in vascular smooth muscle and are relatively nonselective between epinephrine and norepinephrine degradation (8, 38). MAO is predominantly found associated with

mitochondrial membranes in the sympathetic nerve terminals. MAO is, therefore, associated with neuronal catecholamine uptake. COMT is a ubiquitous cytosolic enzyme (38) which inactivates circulating and exogenous catecholamines. Investigators found that the effects of COMT inhibition are the same in vessels with nerves intact or absent (108). Therefore, cytosolic COMT is thought to be predominantly associated with extraneuronal catecholamine uptake. As a result of their association with specific uptake mechanisms, COMT degrades a higher percentage of epinephrine and MAO degrades a higher percentage of norepinephrine.

A variety of studies were employed to determine if the naloxone effect was the result of a change in catecholamine uptake or degradation. The effect of naloxone was compared to that of an extraneuronal uptake inhibitor (corticosterone) and a neuronal uptake inhibitor (desipramine). The effect of naloxone was also compared to that of a COMT inhibitor (pyrogallol) and a MAO inhibitor (pargyline).

It is unclear to what degree COMT is associated with the extraneuronal catecholamine uptake process. If COMT is entirely contained within uptake storage sites and extraneuronal uptake is dependent on continued COMT degradation of epinephrine, then both COMT and extraneuronal uptake inhibition would induce selective augmentation of epinephrine precontractions. If COMT is not isolated by an uptake process that is selective for epinephrine, then COMT inhibition would not selectively enhance epinephrine precontractions over norepinephrine because purified COMT degrades epinephrine and norepinephrine at equal rates (8).

Figures 24, 25 and 26 illustrate that corticosterone augmented epinephrine precontractions in a manner which is indistiguishable from naloxone and that desipramine had no affect on an epinephrine precontraction. This adds support to the hypothesis that naloxone may act to inhibit extraneuronal catecholamine uptake.

The lack of response to desipramine indicates that neuronal epinephrine uptake was negligible. Blockade of neuronal uptake could not induce the changes in epinephrine contractions observed with naloxone.

Figures 27, 28 and 29 illustrate that pyrogallol also augmented the precontractions of epinephrine in a manner indistinguishable from naloxone and that pargyline had no effect on epinephrine precontraction. Pyrogallol also augmented norepinephrine induced precontractions, but to a lesser degree than epinephrine. Figure 29 demonstrates that pyrogallol selectively potentiated epinephrine precontractions over norepinephrine in a fashion identical to the naloxone effect. The similarity between the effects of pyrogallol and naloxone suggest that naloxone may augment epinephrine induced vasoconstrictions by blocking COMT mediated enzymatic degradation. Since COMT does not degrade norepinephrine and epinephrine at differential rates *in-vitro*, the preferential augmentation of epinephrine induced vasoconstrictions by pyrogallol suggests that COMT is linked to extraneuronal catecholamine uptake in this tissue. COMT may have degraded epinephrine preferentially because extraneuronal uptake preferentially transports epinephrine to the site of enzymatic degradation.

Both extraneuronal catecholamine uptake blockade (corticosterone) and inhibition of COMT (pyrogallol) augmented epinephrine vasoconstrictions in a manner identical to naloxone. Both corticosterone and pyrogallol augmented epinephrine induced contractions and had no vasoconstrictive action alone.

COMT inhibition cannot explain the reduction in relaxation $T_{1/2}$ induced by naloxone. Past investigators have found that COMT inhibitors prolong relaxation time (62-64, 108), whereas extraneuronal catecholamine uptake inhibition reduces relaxation rate. Any interference in catecholamine degradation should increase the

existing concentration of epinephrine in the uptake storage reservoir and would tend to increase relaxation $T_{1/2}$.

Naloxone is structurally closer to corticosterone than to pyrogallol. This suggests that it is more likely that naloxone mimicked the effect of corticosterone than of pyrogallol.

It is possible that naloxone may have selectively augmented epinephrine induced vasoconstrictions via a mechanism unrelated to catecholamine uptake or degradation mechanisms or both. The naloxone effect may have simulated the effects of corticosterone and pyrogallol by chance alone. Although possible, this is unlikely.

Indirect studies could be employed to differentiate between extraneuronal uptake blockade and COMT inhibition as the mechanism of action of naloxone. The relaxation $T_{1/2}$s for epinephrine in the presence of corticosterone and pyrogallol could be compared to that for naloxone. I predict that corticosterone and naloxone would have a reduced relaxation $T_{1/2}$ and pyrogallol alone would increase relaxation $T_{1/2}$. Naloxone could also be tested directly for action as an inhibitor of COMT activity. An assay for COMT activity must be developed and then changes in the degradation of epinephrine or in the production of metanephrine examined in the presence of naloxone and a known COMT inhibitor. Development of this assay is underway. Therefore, this question currently remains unanswered.

Endothelial Dependency

The vascular endothelium is widely accepted as an important mediator of vasoconstriction and vasodilation. Figure 30 demonstrates that vessels stripped of endothelium elicited the naloxone effect in a manner indistinguishable from vessels

with endothelium intact. This indicates that the endothelium was not the principle site of catecholamine uptake or degradation responsible for the naloxone effect.

Opiate Receptor Interactions

A wide variety of opiate receptor agonists and antagonists, with both alkaloid and peptide structures were tested to determine if opiate receptors were responsible for the naloxone effect and if so, which subtypes. Each opioid was added in increments to a submaximal epinephrine precontraction to generate dose-response relationships. Data collection followed the pattern illustrated by the example in Figure 31. All opioids which induced a substantial change in vessel tension were also tested for vasoconstrictive activity in the absence of an epinephrine precontraction. None of the opioids which augmented epinephrine precontraction induced vasoconstriction alone. Figures 32 - 38 illustrate the dose-response curves generated from the data. Figure 39 summarizes these results as the augmentation (as a percent of KCL maximum) induced at the highest dose of each opioid (10 μM).

Analysis of the ED_{50}s for these data was impossible because many of the opioid dose-responses did not reach a maximum response. The higher doses required to complete all these responses were nor pursued for a variety of reasons including problems with relevance, solubility, availability and cost. ED_{50}s cannot be calculated on dose-response curves without knowledge of the maximum. Maximum responses can be estimated using a double reciprocal plot if several points are known on the linear portion of the dose-response curve. The data from the opioids was insufficient to accurately estimate a maximum response. Efficacy of each opioid to augment an epinephrine vasoconstriction was judged visually by inspecting superimposed dose-response curves. Response at maximum dose (10 μM) was

compared statistically using the response induced by the peak dose of the opioid (Figure 39).

This experiment was initiated in the belief that a high dose of naloxone was required to elicit the naloxone effect because it was operating at an opiate receptor for which it had relatively low affinity. If so, a survey of the opiate receptor subtype ligands should reveal a series of related compounds which initiates a response similar to the naloxone effect, but at a much lower dose. No such class of opiate receptor ligands was identified. Figures 32 - 38 group opioid dose-response curves by specific commonalities. Each figure includes a dose-response curve for naloxone for comparison.

Figures 32 and 33 illustrate opioid dose-response curves grouped by agonist and antagonist activity respectively. There is no consistent pattern. U50,488 was the best agonist and MR1,452 was the best antagonist. Both are kappa-opiate receptor ligands. Dynorphin 1-8 and 1-9 are kappa ligands as well, yet initiated no response.

Figures 34, 35 and 36 illustrate opioid dose response curves grouped by mu-delta- and kappa-opiate receptor subtype affinity respectively. The mu-receptor antagonists had a significant effect, whereas the mu-receptor agonists did not. Neither the delta-opiate receptor agonists nor antagonist had a significant effect. Figure 36 clearly depicts the seemingly contradictory data that both U50,488 (kappa-agonist) and MR1,452 (kappa-antagonist) demonstrated the greatest augmentation of an epinephrine precontraction, while the other peptide kappa-ligands have no effect.

Figures 37 and 38 illustrate opioid dose-response curves grouped by alkaloid and peptide structure respectively. Almost all of the opioids with alkaloid structures

augmented epinephrine precontractions. None of the peptide opioids augmented epinephrine precontraction except for dynorphin 1-9. Dynorphin 1-9 had an effect only at the highest dose and barely reaches significance. The general structure of the opioids correlated the best with their effects on epinephrine induced precontraction. Opioid alkaloids augmented epinephrine induced vasoconstriction and opioid peptides did not.

These experiments did not find a particular opiate receptor subtype which mediates the naloxone effect. Instead, the results indicate that the effect of naloxone is related more to its alkaloid structure than its activity as an opiate antagonist.

The effect of naloxone was compared to that of its opiate receptor inactive stereoisomer, (+)naloxone. Figure 40 demonstrates the relationship between (+) and (-)naloxone dose-response curves. Each naloxone enantiomer was added incrementally to an epinephrine precontraction to construct these dose-response curves. The (+)naloxone curve was displaced leftward and the ED_{50} for (+)naloxone was significantly lower than for (-)naloxone. (-)Naloxone has a greater affinity for opiate receptors than (+)naloxone. This data supports the conclusion of the previous experiments that naloxone did not augment epinephrine induced vasoconstriction via an opiate receptor mediated mechanism.

Sigma Ligands

Sigma receptors were originally classified as an opiate receptor subclass, but it is now well recognized that this poorly understood class of receptors has little in common with opiate receptors. Sigma ligands include compounds such as: a) dextrorotary benzomorphans such as (+)pentazocine; b) analogs of U50,488; c) PCP analogs; d) analogs of di-o-tolylguanidine (DTG); e) analogs of (+)-1-propyl-

3-(3-hydroxyphenyl)piperidine [(+)ppp]; f) steroids; and g) butyrophenones like haloperidol (115).

The fact that (+)naloxone had significantly greater efficacy than (-)naloxone to augment epinephrine induced vasoconstriction indicates that the naloxone effect was stereoselective. Stereoselectivity implies a receptor mediated mechanism of action. It is possible that the naloxone effect was mediated by a unknown receptor type that has greater affinity for (+) than (-)naloxone. Sigma receptors are classically described as naloxone insensitive. The naloxone effect occurred at a dose relatively high for naloxone's affinity for opiate receptors. Sigma receptors have greater affinity to the (+)stereoisomers of various benzomorphan opiate receptor ligands (Walker 90). Perhaps the naloxone effect was mediated via sigma receptors. (+)naloxone may have augmented epinephrine induced vasoconstriction at a lower dose than (-)naloxone because of the known selectivity of sigma receptors for (+)enantiomers of benzomorphan opiates.

Sigma receptors cross react with kappa-opiate receptor ligands (3, 4, 61, 115). MR1,452 and U50,488 are potent kappa ligands and had equal or greater efficacy than naloxone to induce the naloxone effect. Analogs of U50,488 are among the most potent sigma ligands available. MR2,034 and U50,488 induced effects comparable to known sigma ligands in rat and canine cerebral arteries (4). The fact that MR1,452 and U50,488 induced the naloxone effect suggests that sigma receptors may have mediated the response.

Corticosterone and other steroids are potent sigma ligands as well. Corticosterone augmented epinephrine induced vasoconstriction in a manner identical to that of naloxone. This effect of corticosterone suggests possible involvement of sigma receptors.

A series of sigma-receptor ligands were tested alone and in the presence of a submaximal epinephrine induced vasoconstriction. These agents included drugs from several different classes of sigma ligands. Each agent was administered incrementally to construct a dose-response relationship (10^{-9} - 10^{-5} M). Figure 31 exemplifies the manner in which these experiments were done. Figures 41 and 42 illustrate the dose-response curves grouped by tendency to have augmented or inhibited epinephrine induced vasoconstrictions. Figure 43 summarizes the change induced in the epinephrine precontraction by the highest dose of the sigma ligand.

Figure 41 demonstrates that (+)PPP and (-)pentazocine both augmented epinephrine precontractions as well or better than naloxone. (-)Pentazocine produced this effect at a much lower concentration than naloxone or (+)PPP. These sigma ligands had no vasoconstrictive activity in the absence of epinephrine precontraction. Since these sigma ligands enhanced epinephrine induced vasoconstriction yet had no independent vasoconstrictor activity, it is possible that they acted by the same mechanism as naloxone.

Figure 42 demonstrates that haloperidol, (+)pentazocine and (-)PPP inhibited epinephrine induced vasoconstriction most. These inhibitory sigma ligands were tested for inhibition of phenylephrine induced vasoconstrictions. The effects of these agents on phenylephrine and epinephrine are compared in Figure 44. These agents nonselectively inhibited epinephrine and phenylephrine vasoconstrictions. The naloxone effect enhanced epinephrine precontractions whereas these agents reversed epinephrine precontractions. Naloxone and these inhibitory sigma ligands may have acted via the same receptor to cause opposite effects. The naloxone effect, however, was selective between epinephrine and phenylephrine, unlike the inhibitory sigma ligands. The nonselectivity of these agents, therefore suggests that they were not mediated by the same mechanism as was naloxone.

Both PPP and pentazocine induced responses in epinephrine induced vasoconstriction that were stereospecific. Stereospecificity implies receptor mediation. Although it appears that most of these sigma ligands induced receptor specific responses, there are apparent inconsistencies in the data. For example (+)PPP and (+)pentazocine are both potent sigma ligands, yet induce opposite effects. DTG and rimcazole have high affinity for sigma receptors and induce little effect at all.

Two subtypes of sigma receptors have recently been isolated. $Sigma_1$-receptor subtype is labeled as 'high affinity' and $sigma_2$-receptor subtype is labeled 'low affinity'. The $sigma_2$-receptor has reversed affinity for benzomorphan stereoisomers. The $sigma_2$-receptor has high affinity for (-)benzomorphan stereoisomers and low affinity for the (+)benzomorphan stereoisomers. If the augmentation of epinephrine induced vasoconstriction was mediated by $sigma_2$-receptors, it would better explain why (-)pentazocine is effective where (+)pentazocine is not. DTG and (+)PPP retain high affinity at $sigma_2$-receptors (104, 105, 115).

(-)Pentazocine has a high affinity for kappa-receptors whereas (+)pentazocine does not (105). The stereoselective augmentation of epinephrine induced vasoconstriction by (-)pentazocine may be a result of its activity at the kappa-opiate receptor. (-)Pentazocine, U50,488 and MR1,452 are all potent kappa- and sigma2-receptor ligands. It remains unclear as to which, if either, receptor subtype mediates the similar effects induced by these agents.

Steroids and Steroid Metabolites

Steroids are proven sigma-receptor ligands (104, 105, 115). Progesterone binds to sigma receptors with the highest affinity (1.0 μM) followed by deoxycorticosterone and corticosterone. Many other steroids have similar or lower

sigma receptor affinity. The naloxone effect is closely mimicked by both corticosterone and several sigma-receptor ligands. It is possible that naloxone is a poor ligand at a sigma-receptor site which is a physiologic target for endogenous steroids.

The affinity of steroids for sigma ligand suggests that these receptors could not be activated by steroids at physiologic plasma concentrations. Only near the end of pregnancy does the plasma progesterone concentration approach that which is necessary for sigma receptor activation. Steroid metabolites may accumulate in the plasma in sufficiently high concentrations to activate sigma-receptors. One of the steroids or steroid metabolites may initiate a response similar to that of naloxone at a substantially lower dose so as to be physiologically relevant. A series of steroids and steroid metabolites were tested for the ability to augment epinephrine induced vasoconstriction at a much lower dose than required by naloxone, thereby suggesting physiologic relevance.

Figure 31 exemplifies the experimental protocol employed for each of the steroids tested. Figures 45 - 47 demonstrate the resultant dose-response curves grouped to illustrate important relationships. Figure 45 illustrates the dose-response relationships of various biologically active steroids added to vessels precontracted with epinephrine. The steroids with predominantly glucocorticoid activity (corticosterone and hydrocortisone) and mineralocorticoid activity (11-deoxycorticosterone) augmented epinephrine vasoconstrictions in a manner similar to naloxone. Progesterone (a progestin) augmented the epinephrine precontraction, but not to the same extent.

Figure 46 illustrates the dose-response curves generated by the biologically inactive steroid metabolites. Tetrahydrocorticosterone (THCS), tetrahydrocortisol (THCL) and pregnanalone (PGNL) are the biologically inactive, A-ring saturated

metabolites of corticosterone, hydrocortisone and progesterone respectively. A-ring saturation greatly reduced the ability of these compounds to augment epinephrine induced vasoconstriction. Pregnanalone and progesterone were equally poor at producing this response. Both glucocorticoid and mineralocorticoid activity seem to have potentiated the response.

11beta-hydroxysteroid dehydrogenase (11B-HSD) catalyzes the conversion of corticosterone to the biologically inactive 11-dehydrocorticosterone. Inhibition of 11B-HSD enhances glucocorticoid augmentation of adrenergic vasoconstriction in dermal arteries (106). 11B-HSD is found in vascular smooth muscle and the heart and has significant implications for cardiovascular responses to glucocorticoids (114).

11B-HSD protects nonselective mineralocorticoid steroid receptors from glucocorticoids in-vivo. 11B-HSD is concentrated around nonselective mineralocorticoid receptors. These receptors respond equally well to both glucocorticoids and mineralocorticoids. 11B-HSD uses only glucocorticoids for substrate. Therefore glucocorticoids approaching the nonselective mineralocorticoid receptor are inactivated by the 11B-HSD which surrounds the receptor site. The 11B-HSD is the 'guardian' of certain mineralocorticoid receptors in this manner (23). Corticosterone may lose its ability to augment epinephrine induced vasoconstriction once converted to 11-dehydrocorticosterone by 11B-HSD. If 11-dehydrocorticosterone is significantly less effective than corticosterone, there may be an important role for 11B-HSD in the in-vivo enhancement of steroid mediated augmentation of adrenergic vasoconstriction.

Figure 47 illustrates the dose-response curves generated by structural manipulations around the 11-hydroxyl on corticosterone. Oxidation of the 11-

hydroxyl to a carboxyl group (11-dehydrocorticosterone) and removal of the hydroxyl group (11-deoxycorticosterone) did not substantially reduce or increase the effect of corticosterone to augment an epinephrine precontraction. Although deoxycorticosterone retains potent mineralocorticoid activity, dehydrocorticosterone is biologically inactive. The significant ability of dehydrocorticosterone to have augmented epinephrine induced vasoconstriction despite biologic inactivity implies biologic steroidal activity may not mediate the effect. Perhaps the effect of steroids to mimic the naloxone effect was due to their general alkaloid structure as suggested by the data from the series of experiments on opioids.

Figure 48 summarizes the effect of the highest dose of various steroids and steroid metabolites on an epinephrine induced vasoconstriction. Corticosterone, hydrocortisone, dehydrocorticosterone and deoxycorticosterone all induced effects indistinguishable from that of naloxone. Tetrahydrocorticosterone was statistically similar to naloxone at $p < 0.01$, but not $p < 0.05$. Corticosterone, hydrocortisone and progesterone were tested for vasoconstrictive activity alone and they had none.

The most important finding of this series of experiments on steroids is that no steroid or steroid metabolite was more effective than naloxone. The high doses required for naloxone and the steroids imply that if there is an endogenous mediator of the naloxone effect, it is not one of the compounds surveyed here.

Future Investigations

Naloxone seems to have augmented epinephrine induced vasoconstriction by either inhibiting extraneuronal catecholamine uptake or inhibiting the enzyme catechol-o-methyl transferase (COMT). Several different studies could determine which mechanism mediates the response.

1. An indirect method exists which employs the same apparatus as used in these studies. Naloxone selectively augments epinephrine contractions over norepinephrine and induces a shortening of relaxation time after catecholamine washout. Collection of data detailing the effects of corticosterone and pyrogallol on epinephrine and norepinephrine vasoconstrictions as well as on relaxation times should clearly differentiate between extraneuronal uptake blockade (corticosterone) and COMT blockade (pyrogallol).

2. Direct quantitation of catecholamine uptake into vessels via radioimmuno assay (RIA) or via high pressure liquid chromatography (HPLC) would also determine if extraneuronal uptake was involved in the naloxone effect. If epinephrine uptake, vessel content, and efflux are measured acurately enough, catecholamine degredation could be determined as the difference between epinephrine uptake and the combined efflux and vessel content. $Degraded_{(total)} = Uptake_{(total)} - [Efflux_{(total)} + Content_{(total)}]$.

3. Pyrogallol, corticosterone and naloxone could be compared for relative ability to inhibit COMT once a system is operational which demonstrates consistent COMT mediated conversion of epinephrine to metanephrine. The epinephrine and metanephrine can be separated and quantitated using HPLC.

Additional studies are required to determine the relationship between the naloxone effect and sigma-receptor ligands. Once the mechanism is confirmed by which naloxone augments epinephrine induced vasoconstriction (catecholamine uptake vs degradation), sigma receptor ligands can be tested for activity at that site. For example, if extraneuronal uptake is confirmed to mediate the naloxone response; (+)PPP; U50,488; MR1,452 and (-)pentazocine can be tested for their capacity to inhibit extraneuronal catecholamine uptake.

Finally, the search should continue for endogenous substances which activate the naloxone effect at physiologic concentrations. There may be a physiologic, receptor activated, system which serves to potentiate adrenergic stimulation to the cardiovascular system. Naloxone may only mimick the action of an endogenous ligand to this postulated receptor. Although the steroids employed in these studies did not induce responses at physiologic concentrations, another endogenous substance may.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

Naloxone was studied extensively in order to characterize its effect on adrenergic vasoconstriction in canine skeletal muscle arteries and pursue the mechanism which mediates this effect. A summary of the significant findings is as follows.

1) Naloxone selectively potentiates epinephrine induced submaximal vasoconstriction.

2) Naloxone nonselectively potentiates submaximal vasoconstriction induced by a variety of adrenergic and nonadrenergic vasoconstrictors, but to a lesser degree than epinephrine.

3) Naloxone potentiates epinephrine induced submaximal vasoconstriction through alpha-adrenergic receptor activation.

4) Naloxone does not potentiate epinephrine induced submaximal vasoconstriction via beta-adrenergic receptor blockade.

5) Naloxone reduces the time required for precontracted vessels to relax once epinephrine has been removed from the bath.

6) Vessel relaxation time is dependent on efflux of intact epinephrine from catecholamine uptake storage.

7) Extraneuronal catecholamine uptake inhibition with corticosterone and catechol-o-methyl transferase inhibition with pyrogallol induce effects very similar to naloxone. Naloxone may augment epinephrine induced vasoconstriction via either of these mechanisms.

8) The vascular endothelium does not mediate the naloxone effect.

9) Naloxone does not augment epinephrine induced vasoconstriction via mu-, delta- or kappa-opiate receptor subtype activation.

10) Naloxone may augment epinephrine induced vasoconstriction via activity at a sigma receptor.

11) Inhibitory sigma ligands do not reverse epinephrine induced vasoconstriction via a mechanism related to the naloxone effect.

12) Steroids with glucocorticoid or mineralocorticoid activity augment epinephrine induced vasoconstriction in a manner similar to naloxone.

Conclusions

1) Naloxone augments epinephrine induced vasoconstriction by either extraneuronal catecholamine uptake inhibition or catechol-o-methyl transferase inhibition. The data best supports the former mechanism.

2) Many other compounds induce similar effects at micromolar doses because they share structural characteristics common to agents which inhibit catecholamine uptake or degradation.

3) The mechanism by which naloxone has its effect may be a regulated physiologic process with an as yet undiscovered endogenous modulator.

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