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The University of Arizona PH.D. 1982

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CHANGES IN VASCULAR ALPHA-ADRENERGIC RECEPTOR MECHANISMS DURING MATURATION

by

William Banner, Jr.

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN PHARMACOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by William Banner, Jr.

entitled Changes in Vascular Alpha-Adrenergic Receptor Mechanisms During Maturation

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Sue Piper Dubbs	7/28/82 Date
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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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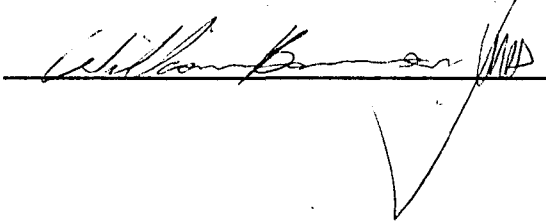
SIGNED: 

PREFACE

With the completion of this document I leave the nether world of the post-graduate-graduate student. In leaving it I have no regrets but some wonderment at why I chose this route to prepare myself for a career. Some would say pure ego, but I can assure them that while that may serve as a starting motivation, it would have been far more ego syntonic to finish with a Masters Degree and begin to pursue an academic career over a year ago. Perhaps the motivation to finish consisted of having to prove to some critics that a physician with clinical distractions can maintain a focus in research. That has been the most difficult thing to overcome and will continue for me to be a battle in the future.

This is not a perfect document. I think the flaws in research design I can see now are a reflection of the growth process and make this all that much more a special document to me. I offer my thanks to Dr. Sue P. Duckles who I know has asked herself whether she was walking the fine line between under and over supervision well enough - you were.

I thank Dr. Thomas Burks and Dr. Vincent Fulginiti for the emotional and economic support that has allowed me to continue as a graduate student. To the members of my committee, Dr. Kreulen, Dr. Yamamura, Dr. Perrier and Dr. Mayersohn, I express my gratitude for your patience and expertise throughout this process.

I would also like to offer a special note of thanks to Dr. John Gaines from the Section of Biostatistics. The learning experience from him has been tremendous. Throughout this document are references to analytic and graphic techniques that are referred to so casually that one might almost assume they are common knowledge. They are not! The patience of Dr. Gaines in instructing and encouraging me in their use has had a tremendous impact on my thinking and approach to science. I look forward to continuing this kind of learning experience. Also I would like to add a note of thanks to the people who have offered much more than technical and secretarial assistance: Jack Wiley, Gail Herod, Gary Lines, Elaine Rodriguez and Patrice Ferron.

Finally I thank Elizabeth G. Banner for suffering the slings and arrows of this dissertation without abandoning ship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

Abstract

The premature infant is subject to pathological alterations of the cardiovascular system leading to insufficient pulmonary blood flow and/or sudden surges of pressure to fragile cerebral blood vessels, conditions which are often associated with hypotension. Dobutamine appears to be a potentially useful agent to increase pulmonary blood flow and correct hypotension. In view of this potential, the alpha-adrenergic characteristics of dobutamine acts were studied in the isolated rabbit femoral and pulmonary arteries. Dobutamine on the post synaptic membrane of these tissues as a high affinity, low intrinsic activity alpha adrenergic agonist. No action of dobutamine to modulate stimulation evoked release of norepinephrine was found, although dobutamine did increase spontaneous release of norepinephrine. This effect was not blocked by antagonists of uptake 1 and uptake 2.

To further evaluate dobutamine for use in the neonate, contractile responses of the femoral and pulmonary arteries and aorta to dobutamine and norepinephrine were studied in dogs of various ages from newborn to 6 weeks. Maximal contractile responses to norepinephrine in the pulmonary artery and aorta increased with age. Dobutamine produced small or no responses in all newborn tissues studied and also showed increasing responsiveness with age.

To allow future study of the mechanism of these changes a method of radioligand binding was established for vascular smooth muscle using the ligands prazosin and rauwolscine in the dog and rabbit

pulmonary artery and aorta. This binding was found to demonstrate the properties of saturability, sterospecificity and rank order of potency.

Possible variables that could account for the observed changes in response during maturation were mathematically modeled to provide a theoretical basis for future studies combining measurement of contractile response and radioligand binding techniques.

Chapter 1

INTRODUCTION

The Problem

The transition occuring at the moment of birth is the single most dramatic physiologic event in the life of a human. In those few seconds, the pattern of circulation must change from one based on placental transfer of oxygen to one allowing pulmonary exchange of gases. These changes are primarily vascular in origin, resulting in vasodilation of the pulmonary vessels and constriction of the umbilical arteries and are uniquely characteristic of the infant born after 37 weeks gestation. At younger ages or in cases where the birth process is complicated by severe asphyxia, these vascular transitions may not occur or may be altered. This abnormal circulatory state has been termed persistent transitional (fetal) circulation.

A second condition associated with the premature birth process in infants involves the rupture of fragile blood vessels in a part of the brain called the germinal matrix layer. This process appears to be related to an inability to maintain vascular control in the neonate. Although rational therapy of these conditions would seem contingent upon the use of vasoactive drugs, there is no consensus on this therapy. A recently published poll (Yeager et al, 1980) revealed that while 73% of the academic neonatologists polled felt that dopamine was an appropriate adjunct to shock in the neonate, the remaining 27%

recommended a wide range of therapeutic interventions. This high level of uncertainty is primarily due to a lack of substantive information upon which to draw conclusions. It is the purpose of this dissertation to contribute to the body of knowledge on the actions of vasoactive drugs in the newborn animal, with the ultimate purpose of contributing to the ability of clinicians to care for disorders of circulation in the human neonate.

The Transitional Circulation

The normal gestational process lasts from 37 to 42 weeks. During this period the fetus is completely dependent on the placental circulation for oxygenation, nutrition, and waste exchange. Thus fetal blood distribution allows 33% of cardiac output for placental flow (Rudolph et al, 1971). Under these conditions blood returning from the placenta enters the portal venous circulation and bypasses the liver via the ductus venosus. Blood then enters the inferior vena cava. After entering the right ventricle, blood may shunt via the foramen ovale into the left atrium or enter the right ventricle. Right ventricular output, as studied in a primate model (Behrman et al, 1970), is largely shunted directly into the aorta by the ductus arteriosus with only 10.7% reaching the lung. At the instant of the first breath the resistance in the pulmonary vasculature drops dramatically and the ductus arteriosus closes. The mediators of this process have not been defined, although the compounds most commonly implicated are the prostaglandins. Evidence for a partial role of prostaglandins in "first breath" vasodilation has been described

(Leffler et al, 1978). The failure of indomethacin in this study to completely inhibit the "first breath" effect suggests that other mediators may also be involved.

Following the early dramatic decreases in pulmonary resistance in the neonate, a continuing process of decreasing medial wall thickness of the pulmonary arteries and arterioles is associated with progressive decreases in pulmonary artery pressure and pulmonary vascular resistance over the first two months of life. With the closure of the ductus arteriosus, the left ventricle becomes the primary source for systemic blood flow. Over the initial months of life a combination of increasing cardiac output and increasing vascular resistance result in a gradual increase in systemic arterial blood pressure. On the basis of these changes, the neonatal circulation is transformed from the fetal pattern to the pattern of blood flow that will be maintained with small alterations into adulthood.

Persistent Transitional Circulation

In contrast to the normal sequence of events, the preterm or asphyxiated infant may fail to completely make the transition to the extrauterine circulation. Rudolph (1980) has proposed three general classifications of persistent transitional circulation. These are 1) Acute pulmonary vasoconstriction with normal vascular development, 2) Increased medial thickness of pulmonary vascular smooth muscle. 3) Decreased cross sectional area of the pulmonary vascular bed.

Acute Pulmonary Vasoconstriction. In the syndrome of acute pulmonary vasconstriction there is an anatomically normal pulmonary vascular bed. Following an acute asphyxial episode there is a failure of the normal pulmonary vasodilation associated with the "first breath". This process may be difficult to reverse requiring more than correction of asphyxia. Systemic acidemia has been implicated as a contributing factor, but the cellular or chemical mediators of this process have not been identified. In the preterm infant, poor oxygen- ation associated with hyaline membrane disease may contribute to the decrease in pulmonary blood flow associated with this disease. In addition, unknown chemical mediators associated with prematurity may also lead to a failure of closure of the ductus arteriosus allowing a right to left shunt of blood further decreasing pulmonary blood flow.

Thus, in the acute pulmonary vasoconstriction syndrome, a suggested role for endogenous vasoactive compounds is based only on the circumstantial evidence of lack of an anatomic basis. Even with this concession, the potential effectiveness of pulmonary vasodilator therapy for the disorder provides a strong rationale for increasing our understanding of newborn pulmonary vascular development.

Increased Medial Thickness of Pulmonary Vascular Smooth Muscle. In the second of Rudolph's etiologic classifications are disorders associated with thickening of the media of the pulmonary resistance vessels. The two most frequent causes of this condition are chronic intrauterine hypoxia, and exposure to prostaglandin synthetase

inhibitors in utero. The mediators or mechanisms whereby this thickening occurs have not been defined. The result of this condition is a form of persistent transitional circulation that is poorly responsive to therapy and is associated with a poor outcome.

Decreased Cross-sectional Area of the Pulmonary Vascular Bed. In this disorder a failure of development of lung tissue due to growth failure results in an elevation of pulmonary vascular resistance due to a low total cross-sectional surface area of the vascular bed. Such congenital disorders of growth include Jeune's Thoracic Dystrophy and diaphragmatic hernia. In this situation the possibility of pharmacologic improvement is small, and the overall outcome limited.

It is apparent on the basis of this classification system that the term persistent transitional circulation is a generic term for many underlying disorders. It should also be apparent that the syndrome of acute pulmonary vasoconstriction presents an ideal situation in which a basic understanding of the pharmacology of pulmonary vascular smooth muscle in the newborn may offer useful therapeutic insights.

Intraventricular Hemorrhage

A pathologic condition unique to the premature newborn is the condition of subependymal germinal matrix hemorrhage. As a part of the development of the central nervous system the fetus forms a growth center for neuroglial elements in the subpendymal region below the third ventricle. This growth center is rich in rapidly dividing tissue and thus is rich in vascular supply. By the normal end of gestation

this growth center or germinal matrix has involuted and no longer possesses a rich vascular capillary bed. Thus the premature infant has a unique vascular structure that is absent at full term normal birth. On the basis of accumulated evidence (Lou et al 1979, Wigglesworth and Pape, 1978) it appears that this vascular bed is predisposed to hemorrhage into the ventricular system when subjected to asphyxial conditions followed by surges of cerebral blood flow. In a prospective study (Papile et al, 1978), evidence of hemorrhage in the subependymal area was present in 78% of all premature infants admitted to an intensive care nursery. This process of acute hemorrhage is associated with a high incidence of non obstructive hydrocephalus as well as focal and global neurologic deficits.

It was recognized that the full term newborn dog possesses an anatomic correlate to the subependymal germinal matrix layer until 2-3 weeks of age. Using this animal model (Goddard et al, 1980), it has been demonstrated that intraventricular hemorrage can be produced by asphyxia, hypertension and infusion of substances causing an increase in plasma volume and osmolality. This evidence has led Volpe (1979) to hypothesize that this richly vascularized area with a poorly developed supportive structure is injured by hypoxia and hypoperfusion. Following this injury any surge in blood flow to the area results in rupture of the capillary and precapillary vessels. By understanding the mechanisms by which the newborn controls regional distribution of blood flow a more rational approach to the use of vasoactive drugs in the neonate may help to control vascular instability and prevent surges

of cerebral blood flow which may cause hemorrhage in the subependymal area.

Current Therapeutic Approaches

The treatment of persistent transitional circulation has not been based on hemodynamic improvement. The lack of non invasive assessment of hemodynamic responses led to the random choice of agents with vasodilator properties as drugs of choice. The most common therapeutic end point used in clinical practice is an increase in arterial oxygenation. This highly variable end point has made critical clinical trials difficult and has given credibility to certain drugs without the benefit of comparative data.

Tolazoline is a frequently recommended drug for persistent transitional circulation despite the lack of comparative data. This drug has histaminergic, alpha adrenergic, and cholinergic properties (Ahlquist et al, 1946). In a review of the toxicity of tolazoline (Stevenson et al, 1979), it was found to have an 82% incidence of undesirable side effects, the most common being hypotension (67%). Only recently (Tripp et al, 1980) has it been appreciated that the vasodilator properties of tolazoline in fetal lambs may be largely due to stimulation of H1 and H2 receptors rather than its action as an alpha adrenergic antagonist. While some evidence suggests a selectivity of the pulmonary vasculature for this vasodilator effect, it is difficult to justify the use of this drug over more conventional vasodilators in view of its high rate of extravascular side effects.

Sporadic case reports on the successful use of drugs such as dopamine (Fiddler et al, 1980) and prostacyclin (Lock et al, 1979) suggest that these alternatives may be useful, but without systematic comparison, no conclusions can be drawn. Recognizing the high potential for hypotension associated with the use of tolazoline, in the poll previously cited (Yeager et al, 1980) respondents were asked to suggest therapy for this side effect. While 71% suggested dopamine the large variety of responses in the remaining 29% reflects the uncertainty with which these drugs are used. While continuing to fulfill the current criteria for efficacy (an increase in arterial oxygenation), the ideal choice of a drug for persistent transitional circulation would also use hemodynamic stability as a major factor.

Intraventricular Hemorrhage. Therapy with vasoactive substances should be directed at prevention of intraventricular hemorrhage rather than treatment. Elimination of practices such as the infusion of hypertonic bicarbonate may decrease the incidence of intraventricular hemorrhage, but without some systematic approach to maintaining vascular stability these catastrophic events will continue. Current practice in neonatology calls for the use of plasma volume expanders to increase cardiac output and consequently blood pressure in the hypotensive neonate. Only when this approach fails are agents that increase vascular resistance, such as dopamine, used to improve blood pressure. It may be that this initial approach to therapy increases systemic pressure without increasing cerebral vascular resistance, and thereby contributes to the development of intracerebral bleeding. In

the adult, large cerebral arteries appear to make a major contribution to total cerebral vascular resistance (Kontos et al, 1978). If this is true in the newborn, then by understanding drug effects in larger vessels, it may be possible to identify a drug that will increase systemic pressure and maintain cerebral blood flow constant.

Identification of Useful Therapeutic Agents. On the basis of the preceding discussion, certain characteristics can be identified that would be desirable in a vasoactive drug for the newborn: 1) the drug should maintain systemic vascular resistance, 2) the drug should reduce pulmonary vascular resistance, 3) the drug should act directly on vascular smooth muscle without the need to release endogenous vasoactive compounds.

On the basis of clinically available information it was determined that dobutamine may possess the desired attributes. With this background, it was felt to be clinically relevant to study the maturational changes in response to dobutamine as compared to a standard catecholamine such as norepinephrine. This would allow the evaluation of the potential use of dobutamine in neonatal persistent transitional circulation and as a vascular support in the prevention of intraventricular hemorrhage.

Dobutamine. Development of dobutamine came about as a result of a systematic search for dopamine analogs with useful vasoactive properties. Tuttle and Mills (1975) in evaluating inotropic derivatives of catecholamines, identified a hydroxyphenyl compound that

possessed inotropic activty with little chronotropic and vasopressor effect. On the basis of in vivo studies in the dog, these authors concluded that dobutamine possessed selective inotropic activity mediated via beta adrenergic receptors. They further suggested that dobutamine had no indirect effect to release norepinephrine from adrenergic nerves. This was on the basis of a failure of desmethylimipramine to alter the responses to dobutamine, although responses to dopamine were attenuated. Following this initial investigation a large series of clinical trials in humans (Leier et al, 1978, Stoner et al, 1977, Akhtar et al, 1975, Jewitt et al, 1974) demonstrated some consistent effects of dobutamine in adults. Dobutamine produced an increase in cardiac index, reduction in pulmonary capillary wedge pressure, and a decrease in both systemic and pulmonary vascular resistance but without the increase in heart rate and decrease in blood pressure associated with isoproterenol. These effects were attributed to beta adrenergic receptor effects although the striking differences between dobutamine and isoproterenol with respect to chronotropy and vasodepressor activity remain unexplained.

An additional clinical study by Kho et al, (1980) supported the earlier finding of Tuttle and Mills by demonstrating that serum norepinephrine concentrations were decreased during dobutamine treatment. They further demonstrated that dopamine treatment increased circulating levels of norepinephrine in patients with low cardiac

output. Another interesting observation of this study was an increase in plasma renin concentrations associated with dobutamine therapy.

Pediatric data on the use of dobutamine is limited. One study (Driscoll et al, 1979) compared the effects of isoproterenol, dopamine and dobutamine in the anesthetized newborn dog. This study demonstrated that both dobutamine and dopamine were capable of increasing systemic blood pressure and cardiac output, although dopamine was found to produce a greater increase in systemic vascular resistance. This study failed to address the effects of these drugs on pulmonary vascular resistance and also offers little information about the neonate with a compromised circulation. In a clinical trial in children (Bohn et al, 1980), dobutamine was administered to 11 children (age 2½ to 11 years) following cardiopulmonary bypass. Over this age range, dobutamine produced the expected increase in cardiac index and blood pressure, however these authors found an unexpected incidence of increased heart rate that precluded continuation of the study in three of the patients. The authors concluded that in children dobutamine does not have a selective positive inotropic effect and is less useful in this age group.

Dopamine. Dopamine is a naturally occurring catecholamine originally identified as a precursor of norepinephrine. Later studies (Goldberg et al, 1963, MacCannell et al, 1966) showed the potential of this drug for clinical use. At low doses (< 5 μg/kg/min) dopamine

produced a slight decrease in systemic vascular resistance and selective dilation of the renal, mesenteric, and coronary circulations (Goldberg, 1972). With increasing doses an increase in cardiac output and systemic vascular resistance results in an increase in blood pressure. At these doses the selective dilation seen at low doses is lost. The response of the pulmonary circulation to dopamine has also been studied in dog and man (Mentzer et al, 1976, Polumbo and Harrison et al, 1972). Both studies demonstrated a potent vasoconstrictor action of dopamine in the pulmonary vasculature. In the dog this response is blocked by phentolamine and is therefore ascribed to an alpha adrenergic receptor mediated effect. The effects of dopamine on the cerebral circulation are less well defined. Von Essen (1974) reported a decrease in cerebral blood flow at low doses of dopamine and an increase at high doses.

The maturational changes in response to dopamine have been studied in animals and humans. In the fetal lamb, Harris and Van Petten (1978) were able to demonstrate a shallow pressor dose response relationship to dopamine when compared to the adult. In a more comprehensive study in the neonatal lamb, Drummond et al (1981) showed that dopamine did not increase systemic vascular resistance or blood pressure at doses less than 20 μg/kg/min. At greater doses, there was a dose related increase in systemic vascular resistance and blood pressure. The striking finding in this study was a continuous increase in pulmonary vascular resistance from very low to very high doses of dopamine. Since no change in pulmonary blood flow was found, main

pulmonary artery pressure was increasing. Thus these authors suggested that pulmonary vascular resistance was preferentially increased by dopamine.

Driscoll et al, (1978) in a pediatric clinical trial in shock documented the usefulness of dopamine in increasing blood pressure and urine output, over a wide range of patient ages. A more detailed analysis of the cardiovascular effects of dopamine was undertaken by Lang (1980). In a group of 5 infants less than 2 years of age with repaired congenital heart disease, dopamine was shown to increase heart rate, arterial blood pressure, and cardiac index. The authors noted one infant with a dramatic increase in pulmonary vascular resistance from dopamine but overall could not find a significant difference in pulmonary resistance from this small group. Stephenson (1979) similarly studied 28 children over 3 months of age and found an increase in pulmonary vascular resistance that was not significant.

Thus these two clinically used drugs have never been systematically studied for use in the neonate for the conditions of persistent transitional circulation or prevention of intraventricular hemorrhage. On the basis of available evidence, dopamine appeared unsatisfactory in terms of the suggested criteria. Dopamine did not appear to decrease pulmonary vascular resistance and had well established indirect effects to release endogenous norepinephrine. Additionally in some critical vascular beds such as the cerebral circulation, dopamine failed to maintain systemic vascular resistance. Dobutamine, however, appeared in many systems to exhibit all of the desired properties for use in

persistent transitional circulation including production of pulmonary vasodilation with little effect on systemic blood pressure.

Development of Vascular Smooth Muscle

A study of changes in vascular smooth muscle reactivity during maturation must be designed with changes in the mechanical properties of the blood vessels in mind. Frist and Stenerman (1975) have reviewed the process of arterial development. The basic structure of the arterial wall is established during embryologic differentiation early in the first trimester. During the remainder of gestation there is only a change in the relative proportions of the various components. These changes are especially prominent in the third trimester and early neonatal period. Olivetti et al (1980) studied the rat aorta during the first few days of life. It was observed that the rat aorta thickened by increasing both the number and size of smooth muscle cells. Toda et al (1980) further observed that the smooth muscle cells changed from a rounded shape to a more irregular shape over the same age period. A more biochemical approach was undertaken by Stein et al, (1969), who demonstrated a changing phospholipid/DNA ratio in aortic vascular smooth muscle cells in both the rat and the rabbit. They felt that this increase in membrane content corresponded well with the observation of Toda that these cells change shape with age. In another study at the biochemical level, Seidel and Murphy (1979) studied the actomyosin content of rat aorta and demonstrated a dramatic increase during the first 5 weeks of life.

This increase in contractile elements corresponds well with the increasing pressure load these vessels are subjected to in the postnatal period. In support of this notion is a study by Nadasy et al, (1981), who studied the mechanical properties of double and single umbilical arteries. These authors took advantage of a rare congenital difference in the number of umbilical arteries in humans. In studying these tissues they drew the conclusion that the single umbilical artery by virtue of increased flow and pressure had an increased wall thickness. Although interesting, it is difficult to completely accept this conclusion in such a rare congenital abnormality, but the concept that increasing lumenal blood flow may affect the mechanical properties of arterial tissue is an important one.

Structural Changes in the Pulmonary Circulation

The normal course of maturational changes in the pulmonary circulation differs from that of the peripheral vasculature. As in the periphery, the basic structural elements of pulmonary blood vessels are present early in gestation (Hislop and Reid, 1972). However, the medial smooth muscle layer of pulmonary blood vessels begins to increase in thickness during the third trimester of pregnancy until the medial muscle mass per gram of lung at birth exceeds that of the adult. At the moment of birth, the ductus arteriosus closes and there is an immediate drop in pulmonary vascular resistance. This correlates well with the finding of Hislop and Reid (1973) of a decrease in medial thickness of small pulmonary arterioles. While all studies have supported the general concept of a decrease in pulmonary vascular

resistance, the mechanism of this is still in question. Sobin et al (1977) believed that the pulmonary arteriole shows a progressive decrease in smooth muscle thickness over a period of weeks. The larger pulmonary arteries may show these same changes. Another group (Levin et al, 1976) concluded that decreasing pulmonary vascular resistance in the first few weeks of life was related to an increase in the total number of vessels rather than to changes in smooth muscle thickness. In the pig (Haworth and Hislop, 1981), the decrease in pulmonary vascular resistance was characterized as consisting of three phases:

1) Vasodilation of small arteries and recruitment of small arteries within the acinar region of the lung.
2) Reduction in the amount of vascular smooth muscle in the pulmonary circulation, after the first 24 hour period.
3) After 2 postnatal weeks, a gradual re-modeling of the pulmonary arteries to allow for a low pressure system.

Despite some disagreement, it appears that the decreases in pulmonary vascular resistance following birth are associated with a decrease in the smooth muscle mass of these arteries. Thus, at a time when the systemic vascular resistance is increasing with appropriate increases in smooth muscle structure, the pulmonary circulation is showing a decrease in smooth muscle mass.

Clinical data have been gathered to support the concept that flow or pressure regulates the changes associated with maturation in vascular smooth muscle. Treatment of premature labor with

prostaglandin synthetase inhibitors has been found to cause premature closure of the ductus arteriosus. This leads to an increase in pulmonary blood flow that has been reported to cause an increase in the medial width of the pulmonary vascular smooth muscle (Levine et al, 1978).

Changes in Vascular Smooth Muscle Innervation

The innervation of the vasculature has also been shown to undergo changes during maturation. An early study by Silva and Ikeda (1971) identified structures suggesting adrenergic and cholinergic nerves in the fetal lamb aorta (using electron micrographs and acetylcholinesterase activity). In support of these observations, Gauthier et al, (1975) determined the norepinephrine content of heart, spleen, intestine, salivary glands, adrenal glands and ganglion of the maturing dog. The animals showed a progressive increase in norepinephrine content and an increase in the uptake of tritiated norepinephrine in the first 56 days of life in all tissues but the stellate ganglion. Ganglionic norepinephrine content was found to decrease over this age period. Bevan (1971) studied the ability of the rabbit aorta to incorporate tritiated norepinephrine from an incubation media. This uptake was separated into uptake that could not be blocked by phenoxybenzamine and that which could. It was found that there was a relatively constant phenoxybenzamine sensitive and phenoxybenzamine insensitive uptake of norepinephrine until day 5 of post-natal age. At that time the phenoxybenzamine sensitive component of uptake began to dramatically increase. Although it could be demonstrated in the guinea

pig aorta that a similar uptake mechanism existed, this same age related change in adrenergic nerve function could not be found.

Further work by Su et al, (1977B) studied the development of neuroeffector function in the carotid artery of the fetal lamb. These authors looked at the response of vascular smooth muscle to norepinephrine in the carotid artery as well as response to transmural nerve stimulation and uptake of tritiated norepinephrine. It was concluded that the postsynaptic response to norepinephrine preceded the development of neuronal uptake of norepinephrine. Further the response to transmural nerve stimulation when expressed as a percentage of the contractile response to norepinephrine was found to increase with age. This suggested that transmural nerve stimulation is the last process to develop in the chain of the neuroeffector response. In a similar manner, Stage and Ljung (1978) studied isolated portal veins from newborn and adult rats, rabbits, cats, and guinea pigs. They found a wide variation in the response to nerve stimulation between these animals, but the general pattern was of an increase in response to transmural nerve stimulation with age. These studies were only able to compare the neonate to the adult and thus lacked some of the ability of the previous study by Su et al, (1977B) to discern which component of the neuroeffector mechanism was developing. The functional role of endogenous norepinephrine in systemic blood vessels has not been fully investigated in vivo (vide infra), but in vitro studies (Ljung and Stage, 1975) have demonstrated sympathetic reflex activity in the first few days of life. A functional role for the sympathetic nerves in vascular development during maturation has been suggested by Bevan

(1975). This author found that tritiated thymidine incorporation into the smooth muscle cells of the rabbit ear artery decreases after sympathectomy. This suggests a regulatory role for sympathetic nerves in the proliferation of vascular smooth muscle.

Development of the nervous supply to the pulmonary blood vessels has been described for both the neonate and the adult. In the adult, vagal fibers containing acetylcholine innervate the pulmonary blood vessels at the adventitio-medial border. Adrenergic fibers arise from the thoracic ganglia and have been shown to innervate the pulmonary blood vessels down to 0.03 mm in diameter (Downing et al, 1980). However, functional studies could only identify adrenergic responses to transmural nerve stimulation in vessels larger than 0.6 mm (Su et al, 1978). Studies of the neonate are fewer. Silva and Ikeda (1971) identified cholinergic and adrenergic nerves in the pulmonary trunk of the fetal lamb in a similar manner as described above for their study in the aorta. The subjective impression of these authors was that this innervation was denser than that of the fetal aorta.

Age Related Changes in Vascular Smooth Muscle Reactivity

The literature on age related changes in the sensitivity of blood vessels to drugs is less clear cut. This subject has been extensively reviewed by Fleisch (1979). In reviewing this literature it is important to keep in mind current concepts of drug-receptor interaction. In this model, drug receptors are large protein complexes on the cell surface in the lipid matrix of the membrane. The

interaction of a drug with this protein complex triggers events that are only now beginning to be clearly understood. For the beta adrenergic receptor, for example, it appears that the adenylate cyclase system is coupled to this cell surface protein by a complex interaction involving membrane phospholipids (Hirata et al, 1979). The stimulation of the beta receptor therefore increases adenylate cyclase activity and triggers a cascade of events producing the intracellular response. Changes associated with maturation may depend upon several different sites of action of drugs. It has additionally been demonstrated that more than one receptor subtype may exist in a single tissue, and potentially even in a single cell. Thus, when dealing with supposed effects of drugs on vascular smooth muscle, the relative role of various receptors which respond to the same drug and the possible effects of age related changes in the coupling mechanism and/or the response mechanism must be taken into account. Probably the best receptor system studied in this regard has been the beta adrenergic receptor. This system shows a rise in function shortly after birth, followed by a relatively long plateau period of static activity, and then a gradual decline with senescence (Fleisch 1979).

Related to alpha adrenergic responses and the effect of age, there is less clarity in the literature. The most important tenet that has been violated has been the concept of multiple receptors in a given tissue. Often no effort has been made to eliminate the effects of beta adrenergic receptor activation and thus changes seen may not be due to alpha receptor activity. The other major objection to the majority of

studies thus far undertaken has been the failure to compare changes in alpha adrenergic receptor reactivity to changes in some non receptor mediated event. Thus, non specific alterations in smooth muscle structure and function may not have been taken into account. Despite these objections, in general, studies of alpha adrenergic receptor reactivity have found increases in response to drugs such as norepinephrine and phenylephrine with age.

The isolated rat aorta (Cohen and Berkowitz 1974, Tuttle 1966, Cohen and Berkowitz 1976) has an increased contractile response to norepinephrine with increasing age. Two studies of contractile response have purported to find a change in alpha receptor affinity with age (Su et al, 1977A, Petkov et al, 1975). Both of these studies used concentration response curves to define the ED50 (dose producing a 50% response). ED50 values showed no change with age in response to alpha adrenergic drugs in these studies. The authors therefore concluded that no changes in alpha adrenergic receptor affinity were occurring on the basis of these ED50 values. These authors have failed to take into account work by Ariens et al (1957) demonstrating that ED50 values do not represent values for half maximal receptor binding. Thus, no conclusions on alpha receptor affinity can be drawn from simple observation of unchanged ED50 values.

In the rabbit aorta, alpha receptor responses increased with age while serotonin produced decreasing responses with age (Hayashi and Toda, 1978). Park and Sheridan (1979) did compare responses to

phenylephrine as a percentage of maximal response to potassium chloride in the rabbit aorta. The authors concluded that no changes in response to phenylephrine occurred with age when the data was expressed in this manner. Another observation in this study was that the antagonist effect of phentolamine appeared to be greater in the younger rabbits. No explanation for the phenomenon could be found.

Studies on the newborn dog are few. Cox et al, (1976) studied the carotid, renal, mesenteric, and iliac arteries from dogs at various stages of development. The authors found an increasing maximal response to norepinephrine with age in all vessels studied. These authors used the ratio of the maximal response to potassium chloride over the maximal response to norepinephrine. This ratio was shown to decrease with age. The authors concluded that changes in excitation contraction coupling were responsible for the age-related changes, although no data to support this contention were offered. In the dog aorta (Gray, 1977) the ED50 did not change for potassium chloride but decreased for norepinephrine from the neonate to the adult. Taken over all, these studies offer little in terms of mechanistic understanding of the changes in alpha adrenergic receptor function with aging.

Despite the importance of the pulmonary artery to the ability of the newborn to survive, little is known about the functional development of drug receptors in the pulmonary artery. Su et al, (1977) studied large pulmonary arteries from the fetal lamb. The

responses to norepinephrine in the pulmonary artery were found to be small and showed no changes with age.

Studies in Vivo

The response of the anesthetized newborn animal to exogenous catecholamines has been more extensively studied than in vitro mechanisms. This in vivo type of data is somewhat helpful in determining clincal responses to adrenergic drugs but does little to contribute to understanding of mechanisms of action. Without extensively reviewing this method, some general comments can be made. Privitera et al, (1969) studied the effects of biogenic amines on heart rate and arterial blood pressure in vagotomized neonatal and adult dogs under general anesthesia. There was little change in alpha adrenergic receptor response with age. Potency ratios for norepinephrine, dopamine, and dopa were similar in the two age groups. Using tyramine it was demonstrated that the newborn dog was able to release endogenous norepinephrine to produce pressor responses in a similar manner to the adult.

In a similar study in the sheep, Barrett et al (1972) found that methoxamine was able to produce an increase in arterial blood pressure and decrease umbilical blood flow. Of interest in their study was the fact that blood flow to the kidney markedly decreased. Addressing more directly the question of systemic versus pulmonary hemodynamic response, Nuwayhid et al, (1975) demonstrated that

norepinephrine could produce an increase in both systemic and pulmonary vascular resistance in the fetal lamb. In support of this concept. Pagani et al (1979) found that the fetal lamb in vivo had increasing responsiveness of aortic smooth muscle to methoxamine. These authors also combined work on elastic stiffness of vascular smooth muscle and concluded that the elasticity of the neonate was less than that of the adult. The effects of catecholamine administration in the pig have also been described. Buckley et al (1979) found a general increase in chronotropic responses to norepinephrine and isoproterenol with age in the anesthetized pig.

The development of sympathetic nervous system responses in the neonate has also been extensively studied in vivo. Two of the earlier studies in this area were Boatman et al (1965, 1967) who demonstrated that blood pressure responses in the hind limb of neonatal dog to sympathetic stimulation appeared to initially have a cholinergic component which was then followed developmentally by a functional adrenergic vasoconstrictor response. The ability of these animals to alter blood pressure and cardiac output to sympathetic stimulation of the heart depended primarily upon increasing heart rate rather than inotropic responses. This suggested either some difference in contractility or a difference in the sympathetic activity of the newborn heart. More recent work by Assali et al (1977) has demonstrated that the newborn lamb has a functional sympathetic nervous system that can control, under normal conditions, heart rate and distribution of cardiac output. This appeared to have both cholinergic

and adrenergic components. Similarily, work by Gootman (1978) also established that the central nervous system has a regulatory effect on the sympathetic nervous system since both direct and reflex stimulation of the sympathetic nervous system are effective in the newborn pig. Thus while in vivo studies give little in the way of mechanistic insight into maturational processes, they do provide some useful clinical information. It can be concluded that the newborn in several species has a functional sympathetic nervous system. Extrapolation of this data to the human is difficult, but it has been demonstrated that reflex sympathetic activity exists in the newborn human (Young 1966).

On the basis of these data there is precedent for the hypothesis that maturational changes in the neuroeffector mechanism may be taking place. The relative contribution of neuronal elements, vascular smooth muscle reactivity, and receptor responsivity to these changes is not clear on the basis of previous studies.

A General Model of the Neuroeffector System

The following discussion is to provide a general background on the organization of the neuroeffector system. This should provide a rationale for the development of methods to study these mechanisms. To adequately characterize the effect of maturation on the vascular neuroeffector mechanism it is necessary to eliminate all of the components of the system except the one under study. The components of the neuro effector system are: 1) the neuron, 2) the smooth muscle

cell, and 3) the extranuronal tissue. All of these elements combine to produce sympathetic responses.

The neuronal component is a system of varicosities along the dendritic process of a sympathetic nerve. Each varicosity has vesicles containing norepinephrine. The nerve is capable of releasing norepinephrine into the synaptic cleft by 3 mechanisms:

1) release of norepinephrine by vesiculo-membrane fusion and exocytosis;
2) release of norepinephrine across the presynaptic membrane induced by drugs with low lipid solubility and blocked by cocaine and desmethylimipramine;
3) release of norepinephrine across the presynaptic membrane by highly lipid soluble drugs not blocked by cocaine and desmethylimipramine.

The release of norepinephrine from the vesicular storage site has been shown to be regulated by adrenergic receptors on the external surface of the presynaptic membrane. These receptors have been defined as alpha 2 on the basis of preference for drugs such as yohimbine and clonidine as compared to drugs such as prazosin. Other regulatory receptors for beta adrenergic drugs and angiotensin II have also been defined (Starke et al, 1981).

The second component of the neuroeffector system is the post synaptic or smooth muscle cell membrane. Receptors for a variety of drugs have been identified on this membrane such as those responsive to

adrenergic drugs, histamine and acetylcholine. The alpha adrenergic receptors that have been identified have been classified as alpha 1 on the basis of a preference for prazosin as opposed to yohimbine and clonidine. The presence of post synaptic receptors of the alpha 2 type has been suggested but the evidence for this appears tenuous at this time (Kobinger and Pichler, 1980, Ruffolo et al, 1982). The external smooth muscle cell receptor appears linked to 2 intracellular effects. The first is an increase in phosphatidylinositol turnover which in turn opens calcium channels in the membrane allowing the increase in intracellular calcium (Jones and Mitchell, 1978).

The third component of the neuroeffector system is the extraneuronal uptake system (Iversen et al, 1966). This is a relatively small non neuronal site of catecholamine storage and release which can be blocked with steroids such as desoxycorticosterone. These structural components of the neuroeffector system will be considered in evaluation of the maturation process. Various methods of study have been developed to isolate the systems of the neuroeffector mechanism for specific characterization of each.

Methods of Study

In view of confusion about the relative importance of various aspects of the neuroeffector mechanism and the growth process, it is important to distinguish between events occuring at the presynaptic area of the sympathetic nervous system and those that may occur on the postsynaptic membrane or even at the level of the contractile mechanism

itself. In the discussion that follows, methods used to define the different areas of the neuroeffector mechanism are reviewed and their applicability to study of the neonate discussed. For consistency throughout this text the following terms will be used for data obtained using the method listed:

1) K_B = Antagonist dissociation constant (Schild method)
2) K_A = Agonist dissociation constant (Furchgott method)
3) K_D = dissociation constant from direct radioligand binding
4) apparent K_D = dissociation constant from displacement of radioligand binding.

Smooth Muscle Contraction

The fundamental method for measuring smooth muscle contraction in vitro involves placing the tissue in a physiologic enviroment connected to a recording device. Three fundamental modes for study of smooth muscle contraction have been defined:

1. In the isotonic mode changes in smooth muscle length are measured while load is kept constant.
2. In the isometric mode developed force is measured while maintaining a fixed length.
3. In the auxotonic mode contractile force is measured while allowing length to change.

In the opinion of Paton (1975), isometric recording provides the advantages of allowing the choice of resting tension and of maintaining the length tension relationship in a constant position. The

disadvantage of this type of recording is that in repeated studies the resting tension response relationship may be altered, making multiple concentration-response curves less accurate as compared to isotonic recording.

Eliminating the effects of other interfering receptor systems may be more difficult. The major cross reactivity in the adrenergic system is between beta and alpha receptors. In studies of alpha receptors this potential cross reaction can be eliminated by the use of propranolol, a specific beta receptor blocker. Thus, by using pharmacologic techniques, the components of interest in a smooth muscle system can be isolated for study. Four basic types of stimulation are useful.

1. Potassium
2. Transmural nerve stimulation
3. Stretch
4. Drug

Stimulation due to potassium appears to be related to influx of calcium into cells (Weiss, 1975). This contraction is associated with membrane depolarization although the exact mechanism has yet to be delineated. The effects of potassium on vascular smooth muscle do appear to be primarily dependent on extracellular calcium concentration. Although generally felt to be independent of receptor function, the effects of potassium under some conditions can be decreased by high concentrations of phenoxybenzamine, suggesting that

although potassium releases norepinephrine from the noradrenergic neuron. This type of evidence may call into question the non receptor mediated mechanism of potassium induced contraction.

Transmural nerve stimulation is another method of studying the neuroeffector mechanism in vitro. Although originally designed for study of intestinal smooth muscle, this procedure has been extended to studies of vascular smooth muscle (Duckles and Silverman, 1980). In this method, square wave pulses of short duration are passed through the electrolyte solution surrounding the tissue. This avoids effects of current assymetrically spreading through tissues associated with point contact of electrodes with tissue. Stimulation of this type is felt to produce evoked release of neurotransmitter from presynaptic vesicles. The evidence for this neuronal release has been summarized by Paton (1975): 1) the threshold for producing a response to transmural nerve stimulation has a shorter pulse duration than that for smooth muscle contraction; 2) specific postsynaptic antagonists will block the response; 3) tetrodotoxin, an inhibitor of nerve depolarization, blocks the response to transmural nerve stimulation; and 4) denervated specimens show no response to transmural stimulation. Thus, this method provides a means of studying the release of endogenous neurotransmitters and the responses produced in vascular smooth muscle.

Drug induced stimulation can be used to produce concentration response curves with a characteristic hyperbolic shape. When using

measurement of smooth muscle contraction to study receptor mechanisms it is important to block other components of the neuroeffector system, so that only activity of a single receptor produces the response seen. In studies of the adrenergic system, the principle components to eliminate are uptake systems and other post synaptic receptors. Effects of indirectly acting adrenergic agonists such as tyramine to release norepinephrine must be blocked as this could cause an erroneously large response. In addition, if a drug is accumulated by neuronal or extraneuronal uptake mechanisms the concentration in the biophase will be reduced causing reduced agonist activity. These problems can be prevented by careful selection of the drugs studied and by use of a neuronal uptake blocker such as desoxycorticosterone.

Analysis of concentration response curves can be performed in several ways. The classic approach to a graphical display is to plot the logarithm of concentration versus the response. This generally produces a sigmoidal curve. Data plotted in this manner can be visually examined to determine two values: 1) ED50 (concentration producing a half maximal response) and 2) the maximal response. With these descriptors alone the curve can not be reproduced because no information on the relative slope of the curve is readily available. A more reproducible approach is to use a non linear regression program to systematically obtain values for equation (1) to describe the concentration response curve (Parker and Waud, 1971).

$$\text{Response} = \frac{\text{Max Response}}{1 + (\text{ED50}/\text{Concentration})^{N}} \qquad (1)$$

In this way a term of relative slope is introduced as "N". The term "N" has no apparent physiologic meaning but is merely a convenient term to allow graphical reproduction of the curve.

Since scientific uses of the concentration response curve will require replication of experiments, data analysis should allow for the determination of estimates of variability. Two methods have been commonly used. In the first method multiple responses at a single concentration are averaged to give a mean response at each concentration. From the curve obtained, an estimate of ED50 and maximal response is obtained either by visualization or by non linear regression. In the second method, individual concentration-response curves are constructed for each experiment and the ED50 and maximal response is determined for each experiment either by direct visualization or non linear regression. The multiple determinations of ED50 and maximal response can then be averaged.

The difference in these approaches has been discussed by Waud (1975). In the first method information about the steepness (slope) of the curves is biased. Averages of response tend to flatten the concentration response curve. For example, for two curves with the same maximal response and slope but differing ED50, the averaging method will produce a single curve with a different slope and no information on the variance of the ED50 to reflect the difference between the curves. On the other hand, if the curves are considered independently, the estimates of maximum and slope would be the same for both curves and ED50 would be

different. The average of these would produce a curve having the same slope and maximum with an intermediate value of ED50. The variance of the ED50 can be calculated from simple parametric statistics. Thus, while it would appear that the approach to analysis of concentration response curves may give similar data, in order to get a true estimate of the slope and variability of the data the method of analysis of each curve for a maximum, ED50, and slope will provide the most meaningful data. By using non linear regression, this analysis is further enhanced by removing experimenter bias or expectation from the analysis.

The methods of measuring smooth muscle isometric contractile force in response to drugs at varying concentration and analyzing the data using equation 1, provides only a minimum of information about relative maximal response, ED50 and slope. Since the latter two values have no real physiologic basis, the amount of data from these types of studies is limited. Historically ED50 was equated with KD (dissociation constant for drug receptor binding). As more studies were undertaken, it became apparent that a maximal response could be obtained with much less than maximal receptor binding. This was described as the "spare receptor" theory (Ariens et al, 1957). In a similar manner, the drug receptor interaction may produce a half maximal response at a drug concentration which would be expected to produce less than half maximal binding. Because of these limitations, techniques using competitive and non competitive drugs to measure agonist and antagonist binding constants have been developed.

The Schild Method. The Schild method allows calculation of an antagonist dissociation constant from repetitive concentration-response curves at varying antagonist concentrations. This method is based upon the assumption that drug receptor and antagonist receptor binding operate as in equation (2) where KA is the agonist dissociation constant, KB is the antagonist dissociation constant and A and B are drug and antagonist concentrations respectively (Ariens et al, 1957).

$$\text{Fractional agonist binding} = \frac{1}{1 + (1-B/KB)\ (KA/A)} \qquad (2)$$

If it is assumed that for the same fractional agonist binding, the same response will be obtained, then 3 or greater curves of concentration versus response in the presence and absence of increasing concentration of antagonist can be mathematically related. By taking concentrations producing equal responses (for example a half maximal response), and assuming these represent equal fractional agonist binding, equation (3) can be derived where A and A' are the concentration before and after the antagonist.

$$\frac{1}{1 + KA/A} = \frac{1}{1 + (1 + B/K_B)\ (K_A/A')} \qquad (3)$$

Algebraic rearrangement produces equation (4).

$$B/K_B = \frac{A'}{A} - 1 \qquad (4)$$

The ratio A'/A has been commonly termed the dose ratio. Substituting this expression and making a linear transformation gives equation (5).

$$\log B = \log K_B + \log (\text{dose ratio} - 1) \qquad (5)$$

Thus a plot of the logarithm of B versus the logarithm of the (dose ratio - 1) gives a line with a slope of 1 and an intercept of the K_B (Schild 1957). The negative logarithm of the intercept is commonly renamed pA_2 for the concentration producing a 2 fold shift in effect. Using this approach, relative values of the antagonist binding may be obtained for the tissue under study. This determination should be independent of the agonist used. Thus differences in pA_2 to a single antagonist as measured against 2 agonists, suggests multiple receptor types.

Furchgott Method. The second application of concentration response data was described by Furchgott (1966). This method was derived to fill the need to estimate agonist association constants from isometric contractile responses. Central to this method are proposed models of receptor binding and the relationship of this binding to response. The model used for this derivation assumes that fractional response is some undefined function of stimulus (S) as defined in equation (6) where A is drug concentration, K_A is the agonist association constant, and E is the efficacy.

$$\text{Fractional Response} = f(S) = f\left(\frac{A}{E \cdot K_A + A}\right) \qquad (6)$$

If the tissue is exposed to an irreversible inhibitor of the receptor such as dibenamine, only a fraction (q) of receptors remains and equation (7) is derived.

$$\text{Fractional Response} = f(S') = \frac{A'}{f(E \cdot q \cdot K_A + A')} \qquad (7)$$

By equating concentrations producing equal fractional response and rearranging terms, equation (8) is derived.

$$1/A = 1/qA' + (1 - q) / q K_A \qquad (8)$$

Thus, Furchgott has derived a linear equation relating the concentration producing equal responses before and after a non competitve blocker to an agonist dissociation constant. Practically, a plot of the reciprocals of these concentrations yields a line with a slope of 1/q and an intercept of $\frac{(1-q)}{q \cdot K_A}$.

Application of the Methods to Study the Alpha Adrenergic Response. Using the methods described for isometric contraction, many authors have reported studies on the alpha adrenergic receptor. Furchgott (1972) has reviewed the literature on relative potencies and antagonist dissociation constants. In all cases the agonist order of potency was epinephrine > norepinephrine > phenylephrine > isoproterenol. The observed pA_2 values for phentolamine were close to 8 for all of the agonists studied. Hence it appeared that the postsynaptic alpha adrenergic responses to agonists such as norepinephrine were mediated by a single homogenous receptor population.

More recently this notion has been questioned. Ruffolo (1981) constructed a Schild plot of yohimbine as an antagonist to phenylephrine in rat tissue. Different pA_2 values were found in different tissues (aorta, spleen, portal vein, bladder and vas deferens). These authors concluded that the postsynaptic receptor in rat aorta was alpha 2 adrenergic in nature. This concept has been supported by others (Kobinger and Pichler, 1980, Ruffolo et al, 1982), but the significance of this finding is unclear.

Application of Methods to the Study of Dobutamine. Early studies of dobutamine in vivo (vida supra) suggested that the actions of dobutamine were mediated by beta adrenergic receptors. The alpha adrenergic nature of dobutamine was not recognized until in vitro studies on vascular smooth muscle were undertaken. Morishita (1980) has studied isolated femoral and pulmonary arteries from the dog. In the femoral artery, dobutamine produced a contractile response with an ED50 value intermediate to norepinephrine and dopamine but with a lower maximal response. This maximum increased slightly if propranolol was added to the preparation but still retained a lower maximum than norepinephrine. In the pulmonary artery no response was found to dobutamine but norepinephrine and dopamine produced typical sigmoidal curves. When concentration response curves to norepinephrine were done in the presence of dobutamine, the curves shifted to the right and it appeared that the maximal response was depressed. In this model the response to potassium chloride was not altered by dobutamine.

Kenakin (1981) has also studied smooth muscle responses to dobutamine. In the rat anococcygeus muscle this author found K_A values of phentolamine that were similar for dobutamine and norepinephrine suggesting that they were acting through similar receptors. They further determined relative efficacy by using the Furchgott method and comparing the response to norepinephrine and dobutamine at similar receptor occupancies. They reported a 40 fold higher efficacy for norepinephrine as compared to dobutamine. In the rabbit aorta, this author found a linear Schild plot for phentolamine against dobutamine with a value similar to that of the rat anococcygeus. One observation that the author reported was that the 95% confidence limits using the Furchgott method to determine K_D showed a 4 fold increase in variance for dobutamine as compared to norepinephrine. A recent and important observation of the properties of dobutamine (Ruffolo et al, 1981) was that the (+) and (-) isomers of dobutamine possess differing activity. The isomers possessed equal affinities for the alpha receptor but only the (-) isomer has partial agonist activity. In concentration-response curves of the (-) isomer and the racemate, these 2 attained the same maximal response and the ED50 only varied slightly.

Thus it appears from these few studies, that dobutamine possesses alpha agonist activity principally as the (-) isomer. It does not appear that dobutamine is able to generate responses to the same maximum as norepinephrine, although receptor occupancy appears similar to norepinephrine. Classically, agonists of this type have been called partial

agonists (Furchgott 1972). This describes a drug with high affinity for a receptor but a low ability to generate response.

Methods of Study of Presynaptic Neuroeffector Mechanisms

Studies of presynaptic neuroeffector mechanisms have evolved in two separate areas:

1) receptor mediated modulation of catecholamine release
2) spontaneous release of stored norepinephrine.

While different mechanisms are implicit in the data from these studies, the general method of study is the same. On the basis of earlier work Su and Bevan (1970) have described a superfusion technique applicable to vascular smooth muscle. In this preparation, a spiral strip of tissue is cut and suspended between two platinum electrodes. A slow continual flow of physiologic salt solution bathes the tissue and is then collected. Two methods of determining catecholamine release are possible in this system. A specific radioenzymatic assay of norepinephrine is possible although the concentrations of norepinphrine obtained from studies of small tissue segments may be below the limits of this assay (Duckles and Rapoport 1979). Under conditions of low catecholamine outflow, the problem of sensitivity can be circumvented by pre incubation of the tissue with tritiated norepinephrine (Su and Bevan 1980). Under experimental conditions release of total or fractionated tritium can be used as a marker of release of norepinephrine and metabolites. Using these techniques to determine norepinephrine release, presynaptic mechanisms can be studied.

Receptor Mediated Modulation of Catecholamine Release. On the basis of many studies there appears to be a regulatory receptor on the presynaptic membrane. Using the apparatus described above and transmural nerve stimulation, the release of norepinephrine per stimulation pulse can be determined. If this release is modified by the addition of drugs to the superfusate, a receptor mediated process may be suspected. Confirmation of the receptor nature of the effect can be found in stereospecificity and rank order of potency experiments. The independence of these processes from known neuronal transport processes is further evidence for a cell surface mediated process. In a recent review by Starke (1981) 63 different regulatory receptor types on various neuronal elements were compiled. The alpha receptor located on adrenergic nerves has been found to inhibit the release of norepinephrine (Gothert 1977, Gothert et al, 1979, Dixon et al, 1979). Despite this evidence, the actual physiologic role of these presynaptic receptors has been questioned (Kalsner et al, 1980). The current overall model for presynaptic regulation has been reviewed by Westfall (1977). In this model, norepinephrine released from neuronal vesicles by adrenergic stimulation enters the synaptic cleft and acts at the postsynaptic membrane. As the concentration of neurotransmitter increases, it begins to act also on presynaptic receptors which decrease further release of norepinephrine. It also appears that substances from other neurons and/or the post synaptic membrane may act in a similar manner to inhibit or augment release. Based on rank order of potency (Starke et al, 1975) the presynaptic alpha receptor appears to show a different preference for drugs than the post synaptic receptor. Drugs such as clonidine and yohimbine show a

preference for presynaptic receptors whereas prazosin appears to prefer the postsynaptic alpha adrenergic receptor. On this basis the term alpha 2 has been applied to the presynaptic alpha adrenergic receptor. The ability of dobutamine to modulate norepinephrine release by presynaptic alpha 2 adrenergic receptors has not been studied.

Spontaneous Release of Stored Norepinephrine. Besides diffusion neuronal and extra neuronal accumulation constitute the primary means of removal of norepinephrine from the synaptic cleft. There appears to be 3 distinct processes involved in this uptake.

1) carrier mediated neuronal uptake
2) non carrier mediated neuronal uptake
3) extra neuronal uptake

Drugs may act using these three processes to produce alterations in the spontaneous efflux of norepinephrine from the nerve terminal.

Carrier mediated uptake of norepinephrine was first studied by Axelrod et al (1959) using tritiated norepinephrine. The dramatic loss of this uptake process by sympathectomy suggested a role of neuronal elements (Iversen et al, 1966A). This same author (1966B) demonstrated that alpha tyramine was transported into the neuron. Callingham (1967) further demonstrated that this uptake and subsequent release of norepinephrine was blocked by drugs such as desmethylimipramine. On the basis of this evidence there appears to be a specific membrane transport for norepinephrine that can be inhibited by desmethylimipramine and

cocaine. Tyramine competes with norepinephrine for transport and produces a net efflux of norepinephrine. Whether this efflux is from vesicular or extra vesicular sites of norepinephrine storage in the neuron is unclear, as has been reviewed by Trendelenburg (1979).

The available structural data on dobutamine suggests that sterically it would not be taken into the neuron by this uptake process (Tuttle and Mills 1975). These authors supported this assumption by finding in dogs in vivo that desmethylimipramine did not alter the dose-response curve to dobutamine, although responses to dopamine were altered. The ability of dobutamine to alter spontaneous release of catecholamines from neuronal stores has not been systematically studied.

The ability of some drugs such as amphetamine to alter spontaneous release of norepinephrine from neurons does not appear to be dependent on the same carrier system described for tyramine (Thoenen et al, 1968). The ability of compounds with high lipid solubility (e.g. amphetamine, ephedrine, phenylpropanolamine) to release norepinephrine is speculated to be on the basis of passive diffusion into the neuron, although Trendelburg (1979) has pointed out the difficulties in identifying a saturable transport process for compounds such as these that distribute widely in tissue. Thus, there exists a poorly understood mechanism by which some drugs increase spontaneous release of norepinephrine in a manner that is not blocked by desmethylimipramine or cocaine. The ability of dobutamine to increase release of norepinephrine in a manner similar to amphetamine has not been explored.

In addition to the transport system blocked by desmethylimipramine in the neuron (uptake 1) a system of catecholamine uptake has been identified in the non neuronal elements of many tissues. Cardiac muscle, smooth muscle, brain vascular endothelium, collagen, and elastin have all been reported to accumulate norepinephrine (Gillespie 1973). This accumulation or uptake 2 is blocked by hydrocortisone and related compounds (Graef and Trendelenberg 1974, Kalsner 1975, Draskoczy and Trendelenberg 1970). The physiologic role for this uptake system has not been defined although the intracellular presence of catechol-O-methyl transerase suggests that this may be a pathway of elimination for norepinephrine. The effect of dobutamine on this uptake process has not been described.

Methods of Study: Radioligand Binding

The technique of radioligand binding is a method of direct characterization of binding of drugs to receptor components of membranes (Snyder 1978). The general approach in measuring binding of receptors while eliminating binding to non specific tissue elements involves homogenization and purification of a tissue sample to provide a relatively pure cell membrane preparation. This membrane preparation is incubated with a drug that has been made with a high specific activity and is specific for the receptor under study. The drug under these conditions will bind to receptor and tissue elements of membrane. The bound radioactivity is then separated from the drug remaining free by techniques such as filtration or centrifugation. A second similar sample is prepared and incubated with the ligand and a very high concentration

of a non radioactive drug specific for the receptor under study. This second drug should displace nearly all ligand from receptors but not compete for the ubiquitous non-saturable tissue binding sites. The difference in binding in the presence and absence of the non radioactive drug is considered to be specific receptor binding.

If the case of a single receptor type is considered, the binding process at equilibrium can be mathematically described as equation (1) (Williams and Lefkowitz 1978), where B_{max} is maximal binding and K_D is the drug dissociation constant.

$$\text{Bound} = \frac{B_{max}}{1 + K_{D/Free}} \qquad (1)$$

Equation (1) resembles the standard Michaelis-Menten equation. This equation can be readily expanded for different receptor types and/or cooperativity between receptors. Using this model (equation 1), data for the relationship of free to bound drug can be analyzed to provide characterization of binding affinity and the number of binding sites.

To further characterize receptor binding, studies of ligand displacement may be used. In these studies, ligand binding is determined in the presence of increasing concentrations of competing drug (Williams and Lefkowitz 1978). The concentration of displacer producing a 50% decrease in specific binding is termed the IC_{50}. By determining the IC_{50} for a drug displacing a ligand with known K_D and a free concentration

(L), equation (2) can be applied to determine the inhibitory dissociation constant (K_I) for a drug.

$$K_I = \frac{I_{50}}{1 + (L)/K_D} \qquad (2)$$

Using this method, a rank order of receptor affinity for a series of drugs can be established. This technique may enable the investigators to distinguish receptor types on the basis of differing relative orders of potency.

A third form of study of receptor binding is to determine the reaction kinetics of ligand association and dissociation. In this technique the rate of the processes described in equation (3) can be determined, where L is ligand and R is a receptor.

$$L + R \underset{K_{-1}}{\overset{K_1}{\rightleftarrows}} LR \qquad (3)$$

The time course of approaching equilibrium can be analyzed in this model using an approximation based on the differential equation derived from (3). This approximation is valid when free ligand (L) does not change with time. The resulting approximation (equation 4) allows the determination of an observed constant (K_0) which is the sum of K_1 (L) and K_{-1} in equation 3.

$$\text{Bound} = (\text{Bound at equilibrium})\ (1 - e^{-K_0 t}) \qquad (4)$$

If the binding process is allowed to reach equilibrium and the K_1 process terminated by the addition of a large amount of a competitor, then the semi-logarithmic decay of the K_{-1} process can be determined.

These basic techniques have been used to characterize receptors of varied types in a large number of tissues. Specific work in vascular smooth muscle has been limited by high non specific binding and lack of ligands with a high specific activity to allow work with small tissue amounts. Tsai and Lefkowitz (1978) reported saturable binding to alpha adrenergic receptors in canine aortic tissue. This ^3H dihydroergocryptine binding was found to be reversible and stereospecific with a rank order of potency suggesting that alpha adrenergic receptors were being assayed. Similarily Colucci et al (1980) have characterized ^3H dihydroergocryptine binding in rat mesenteric artery. This binding was also specific, saturable and was felt to be alpha 2 adrenergic on the basis of rank order of potency. These same authors (Colucci et al, 1981), applied this method to demonstrate regulation of alpha adrenergic receptors by measuring ^3H WB4101 binding in rat mesenteric arteries. In rats sympathectomized with 6 hydroxydopamine or reserpine, a decrease in K_D was observed with no change in B_{max}. In rats treated with epinephrine for 4 days, a decrease in B_{max} was observed with no alteration in K_D. Some recent evidence (Hoffman and Lefkowitz 1980) would question the specificity of these results by finding that WB4101 labeled both alpha 1 and alpha 2 receptors. In view of this problem, it has been suggested that the ligand ^3H prazosin may have a higher specificity for alpha 1

adrenergic receptors (Karliner et al, 1979). Using this ligand, Bobik (1981) has demonstrated stereospecific saturable binding in dog arteries. This author used a series of ultracentrifugation steps to purify vascular membranes. Another important finding was that binding with ^{3}H-yohimbine was also demonstrated. In comparing the binding of ^{3}H-prazosin and ^{3}H-yohimbine, the differences in rank order of potency of alpha adrenergic competitors suggested that this binding was to alpha 1 and alpha 2 adrenergic receptors respectively. Evidence of specificity (Tanaka and Starke 1979) and the recent availability of ^{3}H-rauwolscine may make this an even more useful ligand for studying alpha 2 receptor binding.

The technique of radioligand binding has not been directly applied to the problem of vascular development. Studies of development in lung and myocardium have been reported. Whittsett et al (1982) demonstrated ^{3}H-prazosin binding with an increase in B_{max} with increasing gestational age in the rat lung. Similarly Noguchi (1982) found ^{3}H-prazosin binding to vary only in increasing B_{max} with age in rat heart. In both studies K_D showed no change with age. Thus a practical method for radioligand binding in vascular smooth muscle has been described, but has not been applied to the problem of vascular development.

Summary

The management of persistent fetal circulation and intraventricular hemorrhage represent major areas of confusion for clinicians. A knowledge of the nature of the developmental process of vascular smooth muscle would be useful in understanding the responses of

the cardiovascular system to drugs such as norepinephrine and dobutamine during development. In order to understand the interaction of the components of the neuroeffector system in the response to dobutamine, it is necessary to first study the alpha adrenergic properties of this drug, using in vitro techniques. Having defined those properties, a study of vascular smooth muscle during development in the dog would utilize the standard techniques of isometric contraction to evaluate the changes occurring with aging.

To augment these studies the development of a useful technique of radioligand binding in vascular smooth muscle may allow future studies of development at the receptor level to be performed. An important adjunct to this work will be to hypothesize a mathematical model for the process of development. This will lay the foundation for future work in the areas of receptor development.

On the basis of these 4 general areas, the following questions will serve as a format to provide continuity for the remainder of this dissertation paper:

1) What are the alpha adrenergic characteristics of dobutamine?
2) How do alpha adrenergic receptor mediated responses change during maturation?
3) Can a method for radioligand binding in vascular smooth muscle be developed?
4) Can the maturation of alpha-adrenergic mediated responses be mathematically modeled?

CHAPTER 2

METHODS

The methods section is organized under the four main questions listed at the end of the introduction.

What Are the Alpha Adrenergic Properties of Dobutamine?

All studies in this section were performed on mature New Zealand white rabbits (2-3 kgs) or mongrel dogs. Rabbits were sacrificed by decapitation and immediately dissected for the vascular tissue under study. Dogs were sacrificed with pentobarbital overdose. Anatomically the femoral artery refers to the segment of this vessel in the femoral triangle. The pulmonary artery was considered to be the extrapulmonary segment of the right and left pulmonary arteries not including the main pulmonary artery. The aorta includes both thoracic and abdominal components. Tissue segments were cut from the most distal end. This series of studies as well as some of the subsequent work in this dissertation uses the general method of measurement of smooth muscle contraction.

Measurement of Vascular Smooth Muscle Contraction

To measure developed force in blood vessels with an internal diameter greater than 0.3 mm, ring segments from 2-5 mm in length are cut. A stainless steel wire is passed through the vessel lumen and

connected to a micrometer. A second piece of wire is also placed through the lumen of the vessel and mounted on a force transducer. The micrometer can be used to adjust resting length and, therefore, force on the vessel. The entire assembly is placed in a 37^{o} C oxygenated Krebs' solution. The composition of this solution in mM is as follows: Na^{+} 147.6, K^{+} 6.4, Ca^{++} 1.6, Mg^{++} 1.2, Cl^{-} 130, HCO_3^{-} 26, SO_4^{2-} 1.2, H_2PO_4 1.2, glucose 11, disodium ethylenediamine tetracetate 0.027. Preliminary studies were undertaken with each vessel of a particular size or age to establish the optimum resting force and standardize the resting state of the vascular smooth muscle. This is accomplished by administering a standard dose of norepinephrine in sequence at various resting lengths. The minimum resting force which allows the greatest response is considered to be optimal and was used for subsequent experiments on similar tissues.

Following the initial stabilization of the vessel, concentration-response curves were obtained by adding cumulative concentrations of the drug to be studied. This prevents randomization of sequence but allows a relatively greater amount of information to be derived from a single tissue. In all studies of postjunctional effects desmethylimipramine (10^{-6}M) was present in the bath in order to prevent presynaptic uptake of catecholamines and release of norepinephrine via a tyramine-like mechanism. In all studies of postjunctional alpha adrenergic receptors for drugs having mixed alpha and beta receptor activity, propranolol (10^{-6}M), was also added to the bath in order to eliminate the response of beta adrenergic receptors.

At the end of each cumulative dose response curve the maximum contractile response of the vessel was determined by adding a high concentration (120mM) of potassium chloride.

Concentration response curves were fitted using the general equation 1.

$$\text{Response} = \frac{\text{Max Response}}{1 + \left(\frac{\text{Concentration}}{ED_{50}}\right)N} \qquad (1)$$

In equation 1, ED_{50} is the concentration producing 50% of a maximal response. The SPSS nonlinear regression program using this equation takes each concentration-response curve and gives estimates of the equation variables.

Antagonist Dissociation Constants. The reversible competitive antagonists, such as phentolamine, can be used for characterization of agonist responses by determination of the antagonist dissociation constants for a receptor population (K_B). This method was first described by Schild (1957) and will be referred to as the Schild method. This method calls for use of the general method of vascular smooth muscle contraction measurement. The general format includes performing concentration response curves in the presence and absence of increasing concentrations of a specific antagonist. The shift in ED_{50} value that occurs in the antagonist treated concentration response curve is considered to be due to the competitive antagonism for a

specific receptor. By dividing the agonist ED_{50} in the presence of antagonist by the same ED_{50} value in the absence of antagonist, a dose ratio can be obtained. Schild has used a plot of the logarithm of the (dose ratio -1) plotted against the logarithm of antagonist concentration to produce a linear plot intercepting the X axis at the logarithm of K_B value. In more straightforward mathematical terminology the K_B, or antagonist dissociation constant can be calculated by equation 2.

$$K_B = \frac{\text{antagonist concentration}}{\text{dose ratio } -1} \qquad (2)$$

Two approaches to the use of antagonists to differentiate the effects of dobutamine and norepinephrine were used. Single point determinations of K_B using equation 2 were performed for a series of drugs, and additionally Schild plots were constructed for a more limited series of drugs. Since antagonists operating on a single population of receptors should have K_B values that are independent of the agonists used, any differences between norepinephrine and dobutamine may be attributable to some other effect.

Agonist Dissociation Constants. The dissociation constant of a receptor population for a given agonist (K_A) was obtained by using a technique described by Furchgott (1972). In this technique, an irreversible blocking agent such as phenoxybenzamine, acting on alpha-adrenergic receptors, is used. A paired series of vessels are mounted

in the manner described for measuring concentration response curves in vascular smooth muscle. One vessel is exposed to phenoxybenzamine for a measured period of time in order to reduce the total number of alpha receptors present. Following this period, phenoxybenzamine is washed out of the bath, and the tissue and mate are allowed to re-equilibrate for 1 hour under the same conditions. Following this, a concentration response curve to the agonist is done in both the control and the treated vessel. The phenoxybenzamine treated vessel should have a lower maximal response because of the fewer number of alpha receptors present. By plotting the reciprocal of the concentrations that will produce equal responses in both the treated and untreated vessel, a plot with the relationship shown in equation 3 can be produced.

$$\frac{1}{A} = \frac{1 - q}{q K_D} + \frac{1}{q A'} \qquad (3)$$

In this equation q is the fraction of receptors remaining active after phenoxybenzamine, K_A is the agonist dissociation constant and A and A' are the concentrations of agonist producing equal responses before and after phenoxybenzamine. Algebraic rearrangement of equation 3 produces equation 4 allowing a direct solution of K_D values from the slope and intercept of the double reciprocal plot.

$$K_D = \frac{\text{the slope} - 1}{\text{intercept on the } 1/A \text{ axis}} \qquad (4)$$

Thus by using the method of Furchgott and inactivating a fraction of the total receptors present, an estimate of the dissociation constant for the agonist can be obtained.

Presynaptic Characterization of the Effect of Dobutamine

To characterize the presynaptic effects of dobutamine the general method of tissue superfusion and transmural nerve stimulation was used (Su and Bevan 1970). In this method the rabbit ear artery is cut into a spiral strip and suspended by threads between two parallel platinum electrodes. The upper end of the strip is attached to a force transducer. After a period of equilibration, the vessel strips can be stretched to their optimal resting tension determined by methods described previously. The vessel strips are then superfused by constant flow (1 ml/min) of Krebs' solution at 37^{o}C. Following a further equilibration period, drugs may be added to the superfusion reservoir and thus expose the tissue in a constant fashion. This general method has been applied to the study of both spontaneous release of norepinephrine and stimulation evoked release of norepinephrine as these are altered by dobutamine.

Effects of Dobutamine on Spontaneous Norepinephrine Release. To assess the effects of dobutamine on spontaneous release of presynaptic norepinephrine not in response to nerve stimulation, the general superfusion technique described was applied. Prior to mounting the tissue, a period of incubation in a Krebs' solution containing

10^{-7}M l- 7-^{3}H (N) -norepinephrine (New England Nuclear, specific activity 21.4 Ci/mmol) was allowed for 1 hour. Following an equilibration period the tissues were superfused at a constant rate of 1 ml/min. The superfusate was collected at 1 min intervals and analyzed for total tritium by liquid scintillation techniques. The standard sequence in the study was to allow a 60 minute washout period followed by a 5 minute collection period with no drug exposure. Dobutamine 10^{-5}M or 10^{-6}M was then added directly to the superfusate reservoir and samples collected for 10 minutes. After an additional 10 minute exposure to the drug, the superfusate was collected for another 5 minute period. Dobutamine was then washed out for 20 minutes, the superfusate collected for a baseline period of 5 minutes and during a single 60 second transmural electrical stimulation of the tissue at 8 Hz with a S4 grass stimulator (vide infra). This study was performed at 2 concentrations of dobutamine and in the presence and absence of cocaine (10^{-6}M) and desoxycorticosterone (10^{-6}M). These data were analyzed by comparing tritium release/minute above the average of the control period as a percentage of the total tissue content of tritium.

Effect of Dobutamine on Stimulation-Evoked Release of Norepinephrine. Using the superfusion technique described above, the ability of dobutamine to modulate stimulation-evoked norepinephrine release was evaluated. In this methodology, the use of transmural nerve stimulation provided a sufficiently large concentration of norepinephrine overflow to allow direct determination of endogenous norepinephrine. The general format of the study was to superfuse the

tissue with a Krebs' solution containing 10^{-5}M cocaine and $4 \cdot 10^{-5}$M desoxycorticosterone. After tissue stretching and equilibration periods, transmural nerve stimulation was performed. This stimulation was performed with a Grass S4 stimulator with a constant voltage coupler (Duckles and Silverman 1980). For these studies a supramaximal voltage was applied (6 volts across electrodes) for a pulse duration of 0.3 msec. A fixed frequency of 8 Hz for 60 seconds was used. Following a control stimulation period the test drug, dobutamine, was added to the superfusate, equilibrated for 30 minutes and a second stimulation administered. The Krebs solution was then changed to remove the dobutamine and after equilibration a third stimulation period was performed. The superfusate in these experiments was collected in 3 ml aliquots directly into polypropylene test tubes containing acetic acid. The superfusate was then stored at -20^{o}C. At the same time, standards were mixed into 3 ml aliquots of Krebs' solution and similarly stored. An assay of the superfusate was performed using the method described by Duckles and Rapoport (1979). The tubes were thawed and mixed with 0.3 ml of 2M Tris buffer containing 5% EDTA and 0.1 mM dithiothreitol with 28 mg of acid washed alumina in each tube. After continued washing of the alumina an 0.2 ml sample of each tube was transferred to a second tube and assayed for norepinephrine using a phenylethanolamine-N-methyl transferase (PNMT) radioenzymatic assay. This assay is accomplished by allowing the specific enzyme PNMT to methylate norepinephrine to epinephrine adding a tritiated methyl group from tritiated S-adenosylmethionine. Following this, the mixture can be extracted with alumina and counted

using standard liquid scintillation techniques. The background counts per minute for each of the assays is equivalent to 50 pgm of norepinephrine. The results will be finally expressed as picomoles/gm/pulse during control, treatment and subsequent control periods.

In Vitro Denervation Using 6 Hydroxydopamine

To further investigate whether release of norepinephrine from adrenergic nerves may contribute to the contractile response to dobutamine, the method of in vitro denervation using 6-hydroxydopamine was applied to the rabbit femoral and pulmonary arteries (Aprigliano and Hermsmeyr 1976). Preliminary studies were undertaken to determine the stability of 6-hydroxydopamine in the buffer solution used. It was confirmed that an unbuffered Krebs solution was necessary with the addition of glutathione to further prevent oxidation. These assessments were dependent upon spectrophotometric changes associated with 6 hydroxydopamine oxidation. Having prevented the oxidation of 6-hydroxydopamine during the incubation process, tissues from the rabbit femoral and pulmonary arteries were cut in half and either incubated in plain unbuffered Krebs solution or unbuffered Krebs solution containing glutathione (10^{-6}M) and 6 hydroxydopamine (10^{-6}M) for 30 minutes. Additional control studies were done to determine the effects of glutathione alone. Following a 30 minute incubation in this solution, the tissues were rinsed and transferred to the standard buffered Krebs solution at 37^{o}C and maintained in a well oxygenated state. Following equilibration and stretching to normal resting

tension, the tissues were exposed to transmural nerve stimulation to determine the effects of the denervation procedure (15 volt supramaximal stimulation, 0.3 msec duration, 8Hz for 1 min). In all tissue studied there was no response to nerve stimulation in the 6-hydroxydopamine treated group and a positive response in the control group. Following the return to baseline a standard concentration response curve was done in these tissues in the presence of propranolol (10^{-6}M). The data were analyzed using the standard method of non linear regression to determine maximal contractile responses and ED_{50} values.

Summary

The two general techniques of smooth muscle contraction and tissue superfusion were used to evaluate the alpha adrenergic characteristics of dobutamine. All the data accumulated was compared parametrically using the SPSS version 9 package for paired or unpaired t-tests or one-way analysis of variance models. In the case of the studies of release of tritiated norepinephrine by dobutamine in the presence and absence of uptake blockers, a multivariate analysis of variance was required to separate the multiple effects.

What are the Age Related Differences in Response to Norepinephrine and Dobutamine in the Maturing Dog?

In this series the general characteristics of contractile responses to alpha adrenergic drugs in the dog maturing from birth to 6

weeks of age were determined. The animals selected were from mongrel litters delivered vaginally in the animal resources section of the University of Arizona. All deliveries were spontaneous and there was no evidence of premature delivery. Although special precautions were taken to isolate the mother and offspring, a high rate of infant mortality was observed from infectious etiologies. In view of this, it was difficult to obtain paired litter mates for study and therefore, unless otherwise specified, the observations were made in a random sequence to avoid the effects of intra litter variability. In all experiments, the animals were sacrificed using barbiturate overdose and tissue specimens were promptly removed and oxygenated. The tissues were mounted and tested as previously described for studies of vascular smooth muscle contraction. In all cases at the end of each study, a single dose of potassium chloride (120mM) was introduced in order to obtain a maximal response. The results were excluded if no or a minimal response was obtained to potassium chloride. An additional safeguard to eliminate the possible technical difficulties in dealing with extremely small tissue segments was the use of a non adrenergic drug, serotonin, which produced a relatively large contractile response. This provided assurance of tissue viability. Initial studies were performed characterizing the femoral artery and pulmonary artery. All studies were performed in the presence of desmethylimipramine and propranolol with the exception of studies of the effect of denervation on these tissues. Confirmation of the alpha adrenergic nature of the responses observed was obtained by the use of specific alpha blockers, prazosin and phentolamine. To determine the relative

importance of presynaptic effects, a series of denervation experiments as described previously were also undertaken using 6 hydroxydopamine in vitro in the 4-6 week age dog and the 0-2 week age dog. When dobutamine failed to produce a response, the affinity of the drug for the receptor under study was evaluated by exposing to dobutamine and then performing a concentration response curve to norepinephrine.

Can a Method for Radioligand Binding in Vascular Smooth Muscle be Developed?

Animals were sacrificed and tissues were rapidly removed by dissection for all binding studies. Tissue was frozen at $0^{o}C$ for periods less than 1 week after it was demonstrated that no deterioration occurred in this time period. Multiple specimens were obtained and simultaneously thawed and homogenized in 20 ml of ice cold 50mM Tris HCl buffer with 1 mM $MgCl_2$ (pH 7.5 at $23^{o}C$). The tissue was homogenized using a polytron homogenizer at speed 7 for 20 seconds. After filtration through 2 layers of gauze the resulting homogenate was centrifuged at 3,000 x g for 5 minutes to remove large particulate matter. The supernatant from this low speed spin was then placed in ultracentrifuge tubes and re-centrifuged at 120,000 x g for 1 hour. The pellet resulting from this procedure was resuspended in 2 ml of ice cold Tris HCl buffer and again homogenized. The resulting homogenate was layered over a 2M Sucrose-50mM Tris buffer solution and placed in an ultracentrifuge tube. The resulting gradient was centrifuged at 120,000 x g for 2 hours. The material collecting at the interface was resuspended in 50mM HCl Tris buffer with .25M Sucrose and centrifuged

at 120,000 x g for 1 hour. The resulting pellet was resuspended in Tris HCl Mg Cl_2 buffer and centrifuged at 120,000 x g for 30 minutes. This final pellet was resuspended in Tris HCl $MgCl_2$ buffer to produce a concentration of 1 to 2 mg of protein/ml as measured by a Lowry assay. The resulting tissue suspension was homogenized one final time and used for the following experimental procedures.

Equilibrium Binding

To determine the equilibrium binding constant a 0.2 ml aliquot of membrane preparation was incubated with increasing concentrations of tritiated radioligand either in the presence or absence of 0.25 x 10^{-3}M norepinephrine. The two ligands studied were ^3H prazosin and ^3H rauwolscine. Furoyl-5-^3H -prazosin was obtained from New England Nuclear at a specific activity of 17.1 Ci/mmol (4/18/80). Methyl-^3H - Rauwolscine was also obtained from New England Nuclear at a specific activity of 84.4 Ci/mmol (6/4/81). The final total incubation volume for this series of studies was 0.4 ml. After incubation at 23^{o}C for 30 minutes 3 mls of ice cold Tris HCl $MgCl_2$ buffer was added followed immediately by filtration through Whatman GF/C filters in a vacuum filtering apparatus designed to filter 5 ml of liquid in 2 seconds. The filters were then rinsed with 3 x 3 ml aliquots of the same buffer. Filters were then placed in scintillation vials along with a scintillation cocktail made of omniflour, Triton X100, and Toluene. The filters were extracted for 6 hours and then counted in an unrefrigerated scintillation counter.

Specific binding data was defined as the difference between binding in the presence and absence of norepinephrine. The data was standardized as femtomoles of ligand bound/mg protein as determined by Lowry procedure. Data analysis was performed using nonlinear regression on the raw data counts using equations 1 and 2.

$$\text{Nonspecific Binding} = M \cdot L \qquad (1)$$

$$\text{Total Binding} = (M \cdot L) + B_{max}/(1 + \frac{K_D}{L}) \qquad (2)$$

Bmax was the maximal number of binding sites, K_D was the dissociation constant and L was the ligand concentration.

Determination of Association and Dissociation Kinetics

For this series of experiments a 0.2 ml aliquot of membrane preparation was also used. This membrane preparation was incubated with 1.2 nM prazosin or 2.5 nM ^3H rauwolscine in the presence or absence of norepinephrine. The reaction was terminated at .5, 1, 2, 4, 8, 15 and 30 min following the start of incubation. The terminated reaction was filtered and filters counted as previously described. In one series of experiments the reaction was incubated for 30 minutes in the absence of norepinephrine and then .25 x 10^{-3}M norepinephrine was added to the reaction vessels. These tubes were then continued in their incubation process and terminated at 30.5, 31, 32, 34, 38, 45 and 60 minutes following the initial incubation period. The data were analyzed using nonlinear regression and the equations detailed in

the introduction (Williams and Lefkowitz 1978). On the basis of these experiments an association rate constant and dissociation rate constant for the ligands studied can be determined.

Antagonist Dissociation Constants

To determine inhibition constants for a series of antagonists, a 0.2 ml aliquot of membrane preparation was incubated with either 1.2 nM ^{3}H prazosin or 2nM ^{3}H rauwolscine. To the reaction vessel the antagonist to be studied was added in increasing concentrations from $10^{-9}M$ to $10^{-3}M$. The reaction was incubated to equilibrium for 30 minutes and then filtered in the manner described above. The I_{50} was the value of antagonist concentration producing 50% inhibition of ligand binding. On the basis of these values, equation (3) can be used to determine a K_I value for a known ligand concentration (L).

$$K_I = \frac{I_{50}}{1 + L/K_D} \qquad (3)$$

Drugs Used

The following drugs were used in this series of experiments: cocaine HCL, Merck Co; desoxycorticosterone, Aldrich Chemical Co,; dobutamine HCL, Eli Lilly Co.; norepinephrine bitartrate, yohimbine HCl, dopamine HCl, propranolol HCl, glutathione, 6-hydroxydopamine HCl, and serotonin, Sigma Chemical Co.; phenoxybenzamine HCl, Smith Kline Co.; desmethylimipramine HCl, USV Pharmaceutical; prazosin HCl, Pfizer Inc.; clonidine HCl, Boehringer Ingelheim; phentolamine, Ciba

Pharmaceutical Co.Stock solutions of catecholamines were made in 0.001 N HCl, all other drugs were dissolved in distilled water.

Can the Maturation Process of Alpha Adrenergic Receptors be Mathematically Modeled?

This question was answered theoretically but graphed for comparative purposes using two computer systems. Initial work was done on the Cyber 6400 (Control Data Corporation) with graphics routines run in Fortran IV language and executed using the Cal Comp plotter. This was a relatively cumbersome system with a 24 hour delay in reproduction. For the final graphics used in association with mathematical modeling, the MLAB system on the DEC-20 computer (Digital Equipment Corporation) was used. This was writtten by Gary Knott and Douglas Reece at the National Institutes of Health. This program uses the Marquardt-Levenberg algorithm for nonlinear regression. This part of the system was used extensively for data analysis in the radioligand binding section. By using the ability of MLAB to manipulate functions and matrices and then display the results graphically, the mathematical modeling section of the dissertation was produced.

CHAPTER 3

RESULTS

What are the Alpha Adrenergic Properties of Dobutamine?

Smooth Muscle Contraction

For consistency it was felt that a single method of contraction response data presentation should be selected. As an initial data reduction method, all concentration response data were expressed as the developed force produced by drug, calculated as a percentage of the maximal response to 120 mM potassium chloride. Figures 1 and 2 each illustrate examples of three possible modes of data presentation: (1) the single nonlinear regression line was determined for all the concentration response data taken together; 2) values of ED50, maximum and slope were obtained from individual nonlinear regression on each set of data and averaged; 3) average percent developed force was determined for each drug concentration. The observation of Waud (1975) that data analyzed as in method 3 tends to misrepresent data by flattening the shape of the sigmoidal concentration response curve, (see introduction) seems also to be valid for using nonlinear regression on grouped data. This effect is prominent in figure 1. Figure 2 suggests that for some data sets the methods produce similar results. In view of the possible bias introduced by methods 1 and 3, method 2 seems capable of avoiding these problems and providing reliable error estimates of the parameters under study.

Using this approach multiple concentration response curves to norepinephrine and dobutamine in the rabbit femoral and pulmonary arteries were obtained in the presence of propranolol and desmethylimipramine. Figures 3 and 4 show the percent developed force to dobutamine and norepinephrine. Dobutamine in both tissues produced a lower maximal response ($p < .001$, unpaired t-test) when compared to norepinephrine. There was no difference in ED50 values between drugs. In the dog femoral and pulmonary artery, the maximal response to dobutamine was also less than the maximal response to norepinephrine (Fig. 5 and 6). This difference was statistically significant (unpaired t-test, $p < .025$) in the pulmonary artery. The difference in maximal response to dobutamine and norepinephrine could not be statistically analyzed in the dog femoral artery because of the failure of parameter estimates to converge in the nonlinear regression. The competitive affinity of dobutamine for the alpha adrenergic receptor was suggested by the ability of dobutamine to decrease the response to norepinephrine in concentrations which produce no contractile response (Figure 7).

Antagonist Dissociation Constants

The value of the antagonist dissociation constant for a particular drug, such as phentolamine, should be independent of the agonist used in the determination. If dobutamine and norepinephrine are producing contractile responses via different receptors, values of antagonist dissociation constants would vary with the agonist used. To evaluate this possibility, a series of these studies were performed.

By comparing concentration response curves in a control tissue to a concentration response curve in the presence of an antagonist a series of antagonist dissociation constants were calculated (Table 1).

A more comprehensive evaluation of antagonist effects on contractile responses to norepinephrine and dobutamine was performed using the method of Schild (1957). The standard approach of repetitive concentration response curves in a single tissue with increasing antagonist concentration was found to be unsatisfactory. As shown in figure 8, with repetitive exposure to norepinephrine and dobutamine in a single tissue, a shift to the right for norepinephrine and a decrease in maximal response to dobutamine occurred. To overcome this difficulty, concentration response curves for antagonists were obtained in both a paired and unpaired fashion. Figure 9 shows an example of a plot of the logarithm of (dose ratio -1) versus the logarithm of antagonist concentration obtained from average ED50 values in unpaired data. In the rabbit femoral artery the value of pA_2 (-logarithm of antagonist dissociation constant) for phentolamine using dobutamine obtained by the unpaired method was 8.9. In a similar manner the pA_2 values of phentolamine and prazosin in the rabbit pulmonary artery were 7.5 and 9.5 respectively (Figure 10). The pA_2 of prazosin with norepinephrine as agonist in this same tissue was 9.5 (Figure 11).

By simultaneously studying 4 paired tissue segments with differing concentrations of antagonists a single Schild plot can be obtained (see example figure 12). This produced a mean pA_2 for

dobutamine of 7.75 $\pm$ .38 in the rabbit femoral artery (N = 3). A summary of pA_2 values for prazosin and phentolamine is shown in Table 2.

Agonist Dissociation Constants

As a part of studying the properties of dobutamine, a comparison of the dissociation constants for norepinephrine and dobutamine using the general method of Furchgott (1972) was applied. A representative data set is shown in figure 13. Paired tissue segments were incubated in plain buffer or phenoxybenzamine for varied times. Concentration response curves for each tissue were then obtained. Analysis of this data was performed in three ways: 1) nonlinear regression, 2) a limited or constrained nonlinear regression and 3) the classical approach. The classical approach using a double reciprocal plot is demonstrated in figure 14. An alternative approach is to use nonlinear regression to analyze the same data. In method 1, the equation, response = $(1-(1-FB)^{R})^{N}$, was used where FB is the fraction bound, and R and N are exponential variables (Boeynaems and Dumont 1980). The fraction bound is a function of drug concentration, receptor number and q, the fraction of receptors remaining after phenoxybenzamine. In method 2, the variables N and R were fixed to 1 in the nonlinear model.

Tables 3 and 4 show a detailed breakdown of the analytic approaches used by tissue and drug. Out of 34 experiments, 32% could not be graphically analyzed by the classical method. The unconstrained

method (1) failed to converge on a solution in 26%. Method 2 arrived at a solution in all the data sets studied.

Figure 13 of a raw data set additionally shows the best fit lines from the simultaneous nonlinear regression of method 1. A compilation of the double reciprocal plots from the classical method used in this series of experiments is shown in figure 15.

A method combining nonlinear regression of the individual data sets before and after phenoxybenzamine and the double reciprocal plot is shown in figure 16. By fitting the standard sigmoidal function to the data, accurate values of agonist concentration for response can be obtained and used in a double reciprocal plot. A series of points obtained in this manner is shown in figure 17 with the superimposed line of best fit. Correcting the data to conform to this line and reimposing it on the concentration response curve previously derived gives figure 18. The similarly poor convergence of this model after many attempts led to exclusion of this in the comparison.

Figures 19 and 20 show a comparison of the estimates of K_A and q obtained by the three methods for dobutamine and norepinephrine. The standard error of K_A for the limited regression approach was the lowest for the three methods. Thus, although dobutamine and norepinephrine appear to have similar agonist dissociation constants, these methods show great variability and would not discern small differences.

Presynaptic Effects

The presynaptic effects of dobutamine were sudied in three ways. The first method was the spontaneous release of ^{3}H norepinephrine by dobutamine. The second method studies the regulation by dobutamine of stimulation-evoked norepinephrine release. The third method utilized in vitro denervation with 6 hydroxydopamine followed by determination of concentration response curves to dobutamine.

Spontaneous Release of ^{3}H Norepinephrine. Experiments to study release of tritium as norepinephrine and metabolites were undertaken in the general format shown in figure 21. Counts/minute of tritium per fraction are measured during a control period, a period of drug exposure and then following a washout. A final transmural nerve stimulation period is used as an index of norepinephrine release for comparison. Figure 22 shows comparative raw data for tritium release as average blank, total above blank with dobutamine, the return to control, and nerve stimulation. The effects of dobutamine at 2 concentrations and in the presence and absence of cocaine and desoxycorticosterone are shown. Each value is the mean of 4 separate studies. Expressing the data as tritium release in excess of blank per minute as a percentage of total tritium content produces the results in figure 23. Multivariate analysis revealed that dobutamine at $10^{-5}M$ and $10^{-6}M$ both produced significant increases in tritium release ($p < .05$) and there was a significant effect of increased dobutamine concentration ($p < .05$). Cocaine ($10^{-6}M$) and desoxycorticosterone ($10^{-6}M$) had no effect on dobutamine induced release of tritium. These

results suggest that dobutamine can increase the spontaneous release of norepinephrine. The failure of cocaine and desoxycorticosterone to alter this increase suggests that uptakes 1 and 2 are not involved.

Modulation by Dobutamine of Stimulation-Evoked Norepinephrine Release. The ability of dobutamine to alter the amount of norepinephrine released during nerve stimulation at 8 Hz was studied. Figure 24 shows mean data for dobutamine 10^{-6}M and $5 \cdot 10^{-6}$M (N = 3). Following a control stimulation, dobutamine was added to the tissue perfusate, followed by a second period of transmural nerve stimulation. A final period of return to control completes the study. Using one-way analysis of variance dobutamine did not significantly alter stimulation-evoked norepinephrine release.

In vitro denervation. Studies of presynaptic effects were undertaken using 6 hydroxydopamine to denervate tissue in vitro. Paired tissue segments were exposed to buffer or 6 hydroxydopamine for 30 minutes followed by measurements of contractile responses to norepinephrine and dobutamine in the usual manner. Denervation was confirmed by a loss of response to transmural nerve stimulation in the treated group. Figure 25 shows the effect of 6- hydroxydopamine on norepinephrine concentration response curves in the rabbit femoral artery. The mean logarithm of ED50 for the treated group (N = 6) was $-6.67 \pm .24$ compared to a control group value of $-6.35 \pm .18$. This difference was not statistically significant (paired t-test). A similar series of curves for dobutamine in the rabbit femoral artery is

shown in figure 26. The logarithm of ED50 for the treated group (N = 9) was -6.33 $\pm$ .15 and the control group -6.32 $\pm$ .19 (N.S. paired t-test). Contractile responses to dobutamine in the denervated rabbit pulmonary artery (N = 4) are shown in figure 27. The mean logarithm of ED50 in the treated group was -6.97 $\pm$ .07 compared to -6.87 $\pm$ .12 in the control pairs.

How do Alpha Adrenergic Receptor Mediated Responses Change During Maturation?

The ability of drugs to produce contractile responses mediated via alpha adrenoceptors was studied in the dog during maturation. The first 6 weeks of life in the dog are characterized by rapid growth and development. Figure 28 shows the rapid weight gain associated with this period. In a similar manner smooth muscle structures may be growing over this period independent of changes in receptor stuctures. Figures 29 and 30 show the relationship of developed force in response to potassium chloride as a function of weight and age respectively in the mongrel dog femoral artery. These same relationships within a single litter are shown in figure 31 for the aorta. To correct for this variance, all data for drug induced responses are expressed as a percentage of the maximum contractile response to potassium chloride.

Femoral Artery

The responses to alpha adrenergic drugs in the dog femoral artery from birth to 14 days of age are shown in figure 32. All studies were performed in the presence of desmethlyimipramine and

propranolol. The specificity of this response as being alpha adrenergic in nature was suggested by the ability of phentolamine to block norepinephrine induced responses as shown in figure 33. Since the response to dobutamine was minimal, the ability of dobutamine to bind to the alpha adrenergic receptor was supported by the antagonist effect of dobutamine on norepinephrine responses (Figure 34). Denervation with 6-hydroxydopamine did not alter responses to norepinephrine in the 0-2 week age dog (Figure 35). The mean values of logarithm of ED50 were not statistically different (paired t-test), although the shift in response suggests some small role for presynaptic uptake of norepinephrine.

Responses to this same group of alpha adrenergic agonists in the 2-4 week age dog are shown in figure 36. Phentolamine and dobutamine also blocked responses to norepinephrine in the femoral artery from this group of dogs (Figure 37 and 38). Additionally the specific blockade of the response to dobutamine by prazosin is shown in figure 39.

The 4-6 week age dog femoral artery was also studied using the alpha adrenergic drugs norepinephrine, clonidine and dobutamine as shown in figure 40. Data showing serotonin as a contractile agonist is included. The effects of phentolamine and prazosin as blockers of norepinephrine are shown in figures 41 and 42. Antagonism of dobutamine by phentolamime is shown in figure 43. Denervation of paired tissue samples by 6 hydroxydopamine produced the curves in

figure 44. The differences in ED50 were not significant (paired t-test). Summary data for the effect of drugs by age are shown in figures 45, 46 and 47. These aging trends were analyzed using one-way analysis of variance and showed no significant changes in maximal response with age.

In summary, the dog femoral artery in the first 6 weeks of life shows no significant changes in maximal responses to alpha adrenergic drugs. Although not significant, dobutamine appeared to show an increase in maximal contractile response. Surprisingly, norepinephrine produced large responses even in the youngest animals studied.

Pulmonary Artery

In contrast to the femoral artery, the pulmonary artery of the newborn showed striking changes in norepinephrine response. These responses in segments of the dog pulmonary artery in the first 14 days of life are shown in figure 48. All studies were performed in the presence of desmethylimipramine and propranolol. Because of the minimal responses obtained, a positive control of contraction to serotonin was used and is shown for comparison.

Similarily the 2-4 week age dog had minimal responses to alpha adrenergic drugs but did respond well to serotonin (Figure 49).

The 4-6 week age group began to show increasing response to alpha adrenergic drugs as shown in figure 50. Age related trends in

response to norepinephrine are shown in figure 51. Using one-way analysis of variance the progression with age was highly significant ($p < .001$). The same trends for dobutamine (Figure 52) were suggestive ($p < .06$) but not significant as were the trends for clonidine (Figure 53). Serotonin produced large responses early in life as shown in figure 54.

Aorta

The contractile responses of the aorta to adrenergic drugs also showed dramatic increases with age. The response to alpha adrenergic drugs in the aorta from a sequence of litter mates is shown in figures 55, 56 and 57. As previously, the response to serotonin was used in the very small animals to demonstrate the responsiveness of the tissue.

Summary Data

Maximal responses to receptor mediated processes when expressed as a percentage response to potassium chloride appear to provide a useful method of profiling the growth process. In figures 58 and 59 these changes are summarized. In the dog femoral artery the changes did not prove statistically significant. This same data for the pulmonary artery showed the statistically significant trend of increasing responsiveness to norepinephrine. The responses in the aorta also showed statistically significant increases with age ($p < .01$). This series of studies shows that changes in contractile responses of dog vascular smooth muscle to alpha adrenergic drugs do take place with maturation, but that these changes may vary from one tissue to another.

While these observations are useful and relevant they do not imply any underlying mechanisms relating to these changes. Initial plans to further characterize maturational changes using the methods of Schild and Furchgott were not deemed feasible in view of the small responses observed.

Can a Method of Radioligand Binding Be Developed for Vascular Smooth Muscle?

To study radioligand binding in vascular smooth muscle alpha adrenergic receptors a ligand for alpha 1 receptors, prazosin was studied initially.

Prazosin

A useful ligand assay should show characteristics of saturability, stereospecificity, and appropriate rank order of potency. Attempts were made to characterize these properties in rabbit aortic membranes prepared as described in the method section. One initial problem encountered was that of a dramatic loss of ^{3}H prazosin from the predicted values of standard curves. This was especially striking at lower concentrations of prazosin suggesting that adherance to glass and/or plastic was responsible for this process. By using coated glassware, this problem was overcome and the result of subsequent standard curves is shown in figure 60.

Binding Characteristics in the Rabbit Aorta. The general characteristics of binding of prazosin to rabbit aortic membranes is

shown in figure 61. This figure shows total and nonspecific binding as a function of free ligand concentration. The superimposed curves are from the simultaneously fit data using nonlinear regression. Figure 62 shows the specific binding transformed into femtomoles of ^{3}H prazosin bound per mg of protein. Figure 63 shows the same data set transformed in the traditional Scatchard plot. The results of estimates of K_D and B_{max} for these two methods were similar.

A useful assay will be linear with respect to the protein concentration in the preparation. By studying specific binding at various protein concentrations the linearity of this process can be determined. Figure 64 shows this plot for 3 ligand concentrations with the linear regression of the data.

Another property of the binding process relates to the kinetics of association and dissociation. These rate constants for k_1 and k_{-1} can be determined for the binding of prazosin to rabbit aortic membranes as shown in figure 65. The progression of the total and non specific binding process and the alteration of this process by the sudden introduction of a large amount of a specific competitor can be mathematically transformed into the specific rate constants and a final estimate of K_D. These determinations for 2 separate studies are shown in table 5. Rank order of displacement and sterospecificity of ^{3}H prazosin binding were also demonstrated. As shown in figure 66, the ability of yohimbine to displace ^{3}H prazosin was less than that of prazosin. Similarly figure 67 shows the stereospecific displacement of

^3H prazosin binding. Figures 68 and 69 show displacement curves for dobutamine, phentolamine and norepinephrine. Inhibition constants (K_I) or apparent K_D calculated from these studies are shown in Table 6.

Having demonstrated the practicality and accuracy of this binding technique, the binding of ^3H prazosin was studied in the rabbit and dog pulmonary artery and aorta. These data are shown in figures 70 and 71 and summarized in Table 7.

Rauwolscine

The alpha 2 adrenergic ligand, ^3H rauwolscine was also used to characterize binding in vascular smooth muscle. Initial concerns over binding of ligand to plastic and glass was overcome by using coated glassware. The recovery of ligand as standard curves is shown in figure 72.

Characteristics. The general characteristics of ^3H rauwolscine binding in the rabbit aorta are shown in figure 73 as total and nonspecific binding for duplicate samples with the superimposed lines of best fit. Figure 74 shows the specific binding for this data with the regression curve. Figure 75 shows the data from figure 74 transformed into the traditional Scatchard plot. Both the methods produced similar results.

In a manner similar to ^3H prazosin binding, the binding of ^3H rauwolscine was characterized by the rank order of potency for

displacement. Figure 76 shows the displacement of ^{3}H rauwolscine by yohimbine. Prazosin could not be shown to displace ^{3}H rauwolscine. Figure 77 shows the displacement of ^{3}H rauwolscine by norepinephrine and dobutamine. The data for displacement and summary data for this ligand are included in Tables 6 and 7.

Summary

These studies have demonstrated the applicability of radioligand binding methods to the study of alpha adrenergic receptors of vascular smooth muscle. The binding of ^{3}H prazosin appeared to be a simple monomolecular process with preferential binding to alpha 1 receptors. In contrast ^{3}H rauwolscine showed specificity for alpha 2 adrenergic receptors.

Table 1: Values of pA2 - Single Points

	Agonist	
	Dobutamine	Norepinephrine
RABBIT		
Pulmonary Artery		
WB4101	7.5	7.9
Prazosin	8.6	9.6
Phentolamine	5.9	5.6
Femoral Artery		
Prazosin	8.0	7.3
Phentolamine	6.3	6.0

Table 2: pA2 Values from Schild plots

		Unpaired	Paired
RABBIT PULMONARY ARTERY			
Antagonist:	Prazosin		
Agonist:	Dobutamine	9.4	8.6
	Norepinephrine	9.5	9.6
Antagonist:	Phentolamine		
Agonist:	Dobutamine	7.6	7.3
	Norepinephrine	-	8.3
RABBIT FEMORAL ARTERY			
Antagonist:	Phentolamine		
Agonist:	Dobutamine	8.9	7.75 $\pm$ .38 (N = 3)

TABLE 3: METHODS OF CALCULATION OF AGONIST DISSOCIATION CONSTANTS - LOGARITHM OF K_A

	MEAN	STD DEV	NUMBER SUCCESSFULLY ANALYZED
FEMORAL ARTERY			
Norepinephrine (N = 5)			
Regression	-8.76	1.07	2
Limited Regression	-6.93	0.39	5
Double Reciprocal Plot	-5.78	0.80	4
Dobutamine (N = 6)			
Regression	-6.93	1.03	6
Limited Regression	-7.47	0.21	6
Double Reciprocal Plot	-6.93	0.26	2
PULMONARY ARTERY			
Norepinephrine (N = 5)			
Regression	-7.92	0.87	5
Limited Regression	-7.35	0.34	6
Double Reciprocal Plot	-6.46	0.10	2
Dobutamine (N = 8)			
Regression	-7.58	0.46	8
Limited Regression	-7.21	0.33	8
Double Reciprocal Plot	-7.04	0.33	5
AORTA			
Norepinephrine (N = 8)			
Regression	-6.87	1.31	7
Limited Regression	-6.31	0.66	8
Double Reciprocal Plot	-5.41	0.11	2

TABLE 4: METHODS OF CALCULATION OF AGONIST DISSOCIATION CONSTANTS - FRACTION OF RECEPTORS REMAINING - q

	MEAN	STD DEV	NUMBER SUCCESSFULLY ANALYZED
FEMORAL ARTERY			
Norepinephrine (N = 5)			
Regression	0.90	0.12	2
Limited Regression	0.48	0.25	5
Double Reciprocal Plot	0.09	0.08	4
Dobutamine (N = 6)			
Regression	0.19	0.29	6
Limited Regression	0.30	0.15	6
Double Reciprocal Plot	0.25	0.19	2
PULMONARY ARTERY			
Norepinephrine (N = 6)			
Regression	0.82	0.16	5
Limited Regression	0.68	0.10	6
Double Reciprocol Plot	0.28	0.16	2
Dobutamine (N = 8)			
Regression	0.56	0.24	8
Limited Regression	0.37	0.24	8
Double Reciprocol Plot	0.33	0.15	5
AORTA			
Norepinephrine (N = 8)			
Regression	0.77	0.40	7
Limited Regression	0.72	0.15	8
Double Reciporcal Plot	0.30	0.31	2

Table 6: Values for Competitive Inhibition of Binding (Apparent K_D)

	Ligand	
	3H Prazosin (nM)	3H Rauwolscine (nM)
Prazosin	8.25	NA*
Yohimbine	700	4300
l Norepinephrine	600	6800
d Norepinephrine	5900	NA*
Dobutamine	100	4300
Phentolamine	14.9	

* No Apparent Displacement

Table 5: Rates of Formation (k_1) and Breakdown (k_{-1}) for ^{3}H Prazosin Binding

		Study 1	Study 2	Mean
K_0	(observed)	.537	.572	
K_1	($min^{-1}nM^{-1}$)	.375	.447	.406
K_{-1}	(min^{-1})	.068	.025	.046
K_D	(nM)	.181	.057	.119

Table 7: Summary of Binding Data

	Ligand			
	3H Prazosin		3H Rauwolscine	
	K_D (nM)	B_{max}*	K_D (nM)	B_{max}*
Rabbit Aorta	.11 ± .02	27.4 ± 4.9 (7)	15.2 ± 5.4	64.6 ± 13.2 (2)
Rabbit Pulmonary Artery	.02 ± .01	21.2 ± 4.3 (4)	15.93	55.7 (1)
Dog Aorta	.08 ± 0.2	73.2 ± 22.5 (2)		
Dog Pulmonary	.08 ± .08	64.1 ± 34.2 (2)		

Values are Mean ± S.E.M. (N)

*Femtomoles/Mg Protein

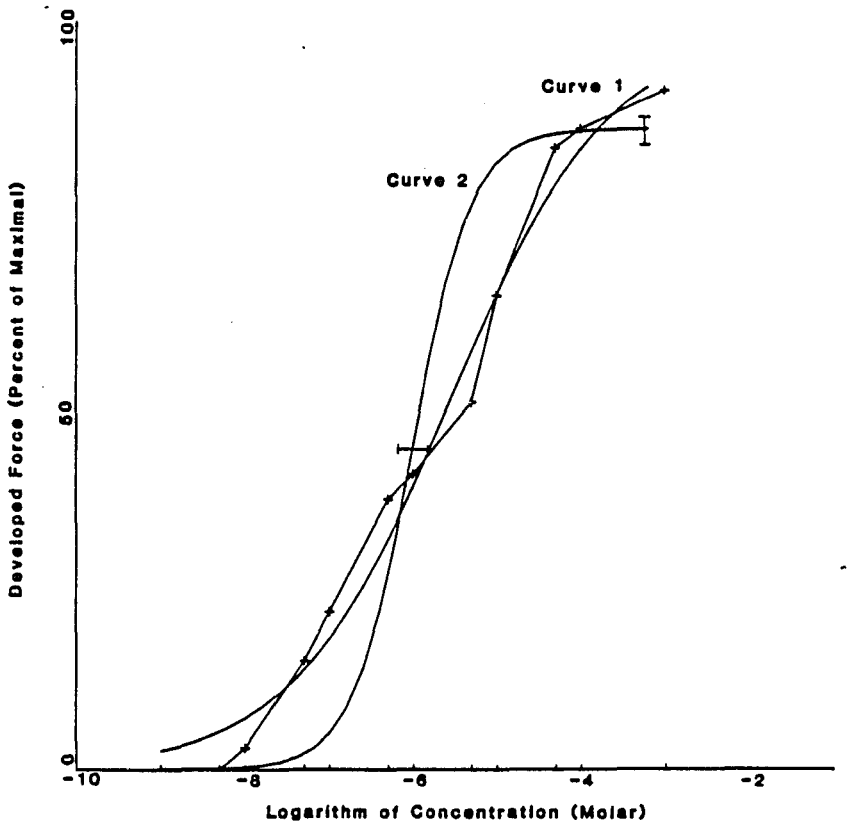


Fig. 1. Three Methods of Analysis of Concentration Response Curves in the Rabbit Femoral Artery.

Concentration response data to norepinephrine in the rabbit femoral artery were analyzed by three different methods (see text). The (+) sign represents average responses at each concentration. Curve 2 represents the 15 individual non linear regression analyses of concentration response data with average parameters (+ S.E.M.). Curve 1 is the result of a single nonlinear regression of all the data.

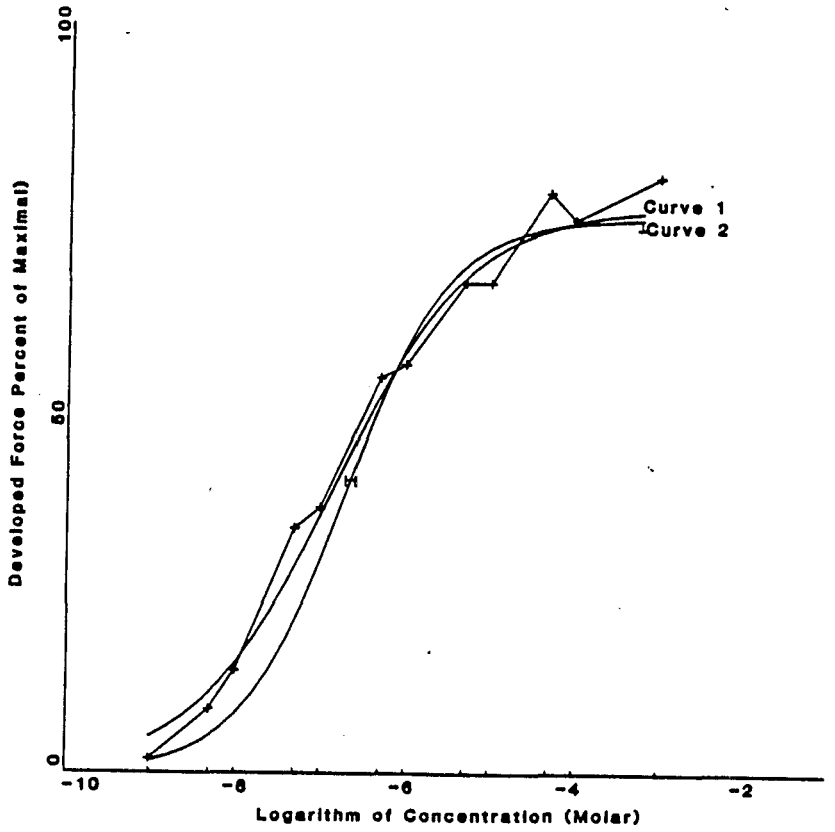


Fig. 2. Three Methods of Analysis of Concentration Response Curves in the Rabbit Pulmonary Artery.

Concentration response data to norepinephrine in the rabbit pulmonary artery were analyzed by three different methods (see text). The (+) sign represents average responses at each concentration. Curve 1 represents the non linear regression fit of all the accumulated data and curve 2 represents 34 individual non linear regression analyses of concentration response data with average parameters ($\pm$ S.E.M.).

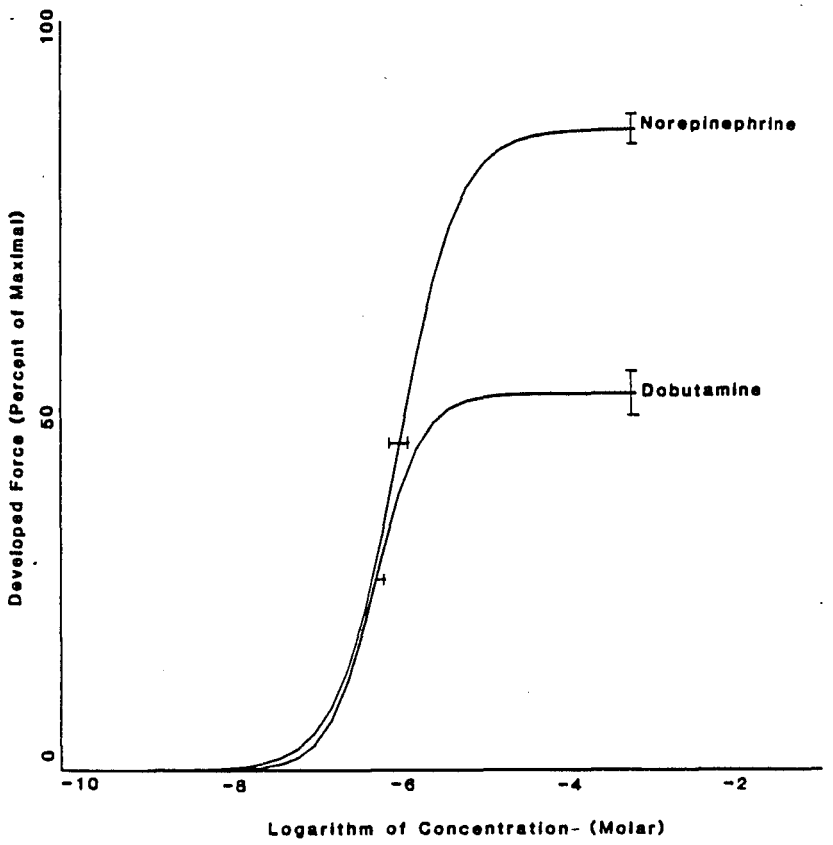


Fig. 3. Concentration Response Curves for Norepinephrine and Dobutamine in the Rabbit Pulmonary Artery.

Developed force as a percent of the maximal response to potassium chloride is shown as the single curve representing the average of fitted parameters of concentration response data in the rabbit pulmonary artery. Responses to norepinephrine (N = 34) and dobutamine (N = 34) are shown with ED50 and maximal response (± S.E.M.).

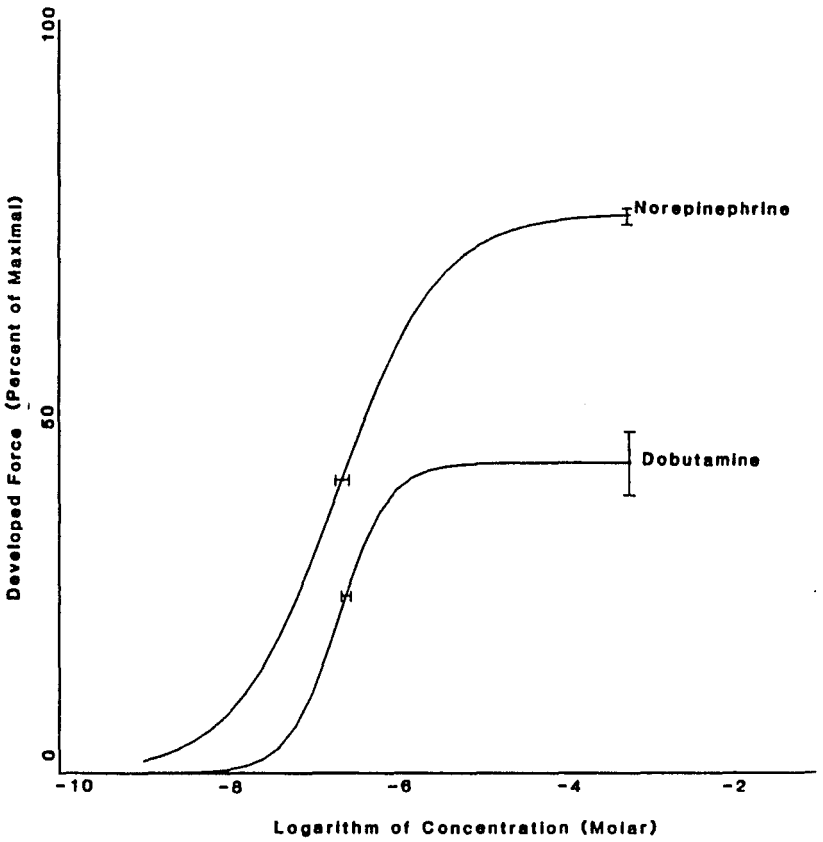


Fig. 4. Concentration Response Curves for Norepinephrine and Dobutamine in the Rabbit Femoral Artery.

Developed force as percent of maximal response to potassium chloride is shown as a single curve representing the average of fitted parameters in the rabbit pulmonary artery. Norepinephrine (N = 15) and dobutamine (N = 20) are shown with ED_{50} and maximal response ($\pm$ S.E.M.).

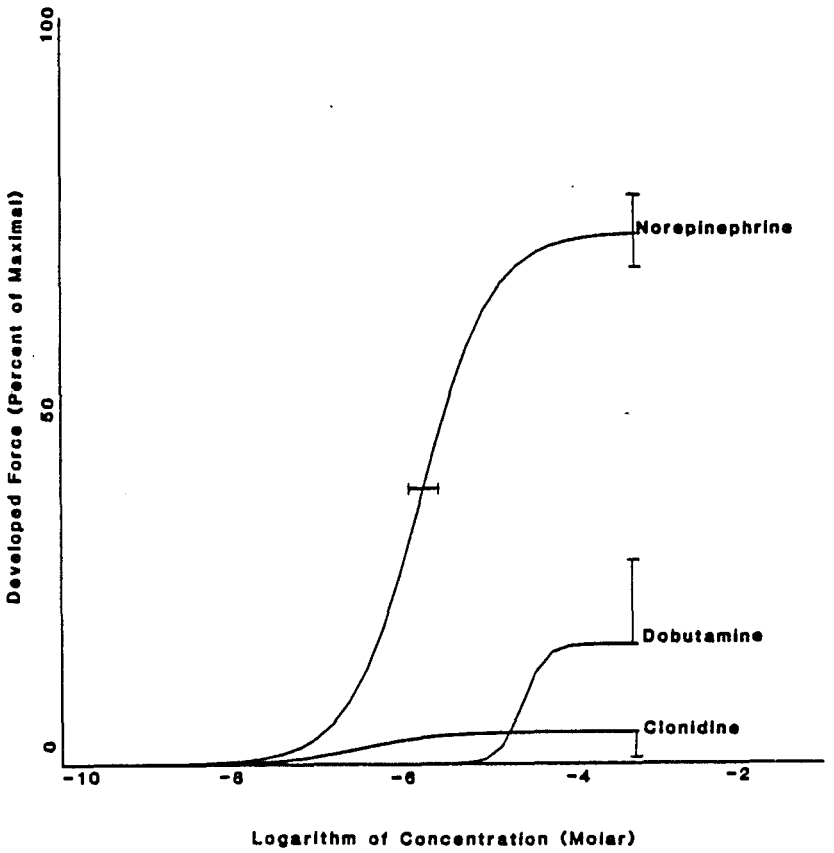


Fig. 5. Concentration Response Curves for Norepinephrine, Dobutamine, and Clonidine in the Adult Dog Pulmonary Artery.

Responses produced by norepinephrine (N = 6), dobutamine (N = 3), and clonidine (N = 2) based on average parameter estimates of ED50 and maximal response are shown (± S.E.M.).

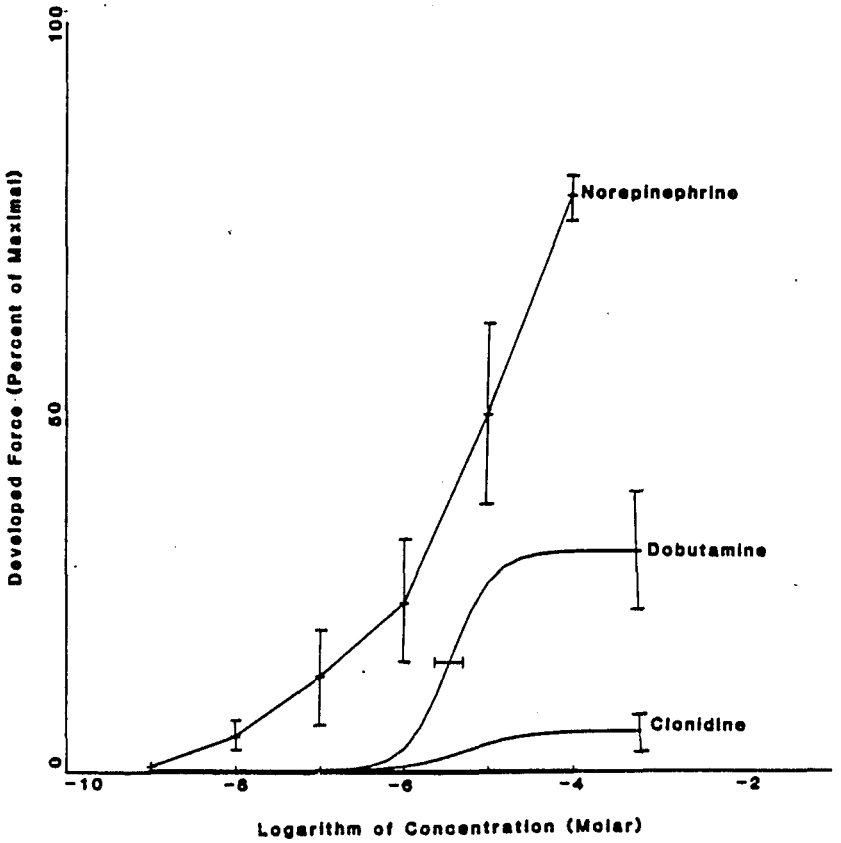


Fig. 6. Concentration Response Curves for Norepinephrine, Dobutamine and Clonidine in the Adult Dog Femoral Artery.

Concentration response curves for norepinephrine (N = 4), dobutamine (N = 5), and clonidine (N = 3) are shown. The concentration response data for norepinephrine are shown as average responses at each concentration because of a failure to converge on nonlinear estimates of ED50 and maximal response. The other curves are shown as parameter averages (± S.E.M.).

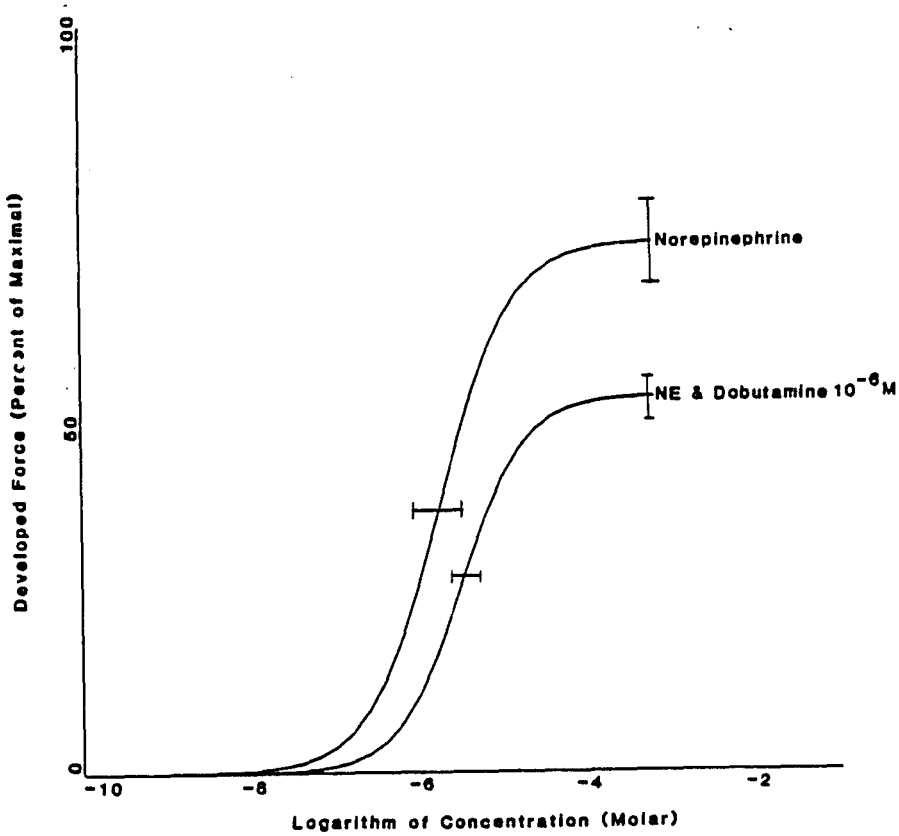


Fig. 7. Concentration Response Curves for Dobutamine Acting as an Antagonist to Norepinephrine.

Dobutamine at a concentration not producing a response in the adult dog pulmonary artery was added to the tissue bath. A concentration response curve to norepinephrine was then done. The results (n = 2) are shown in comparison to the standard curve for norepinephrine.

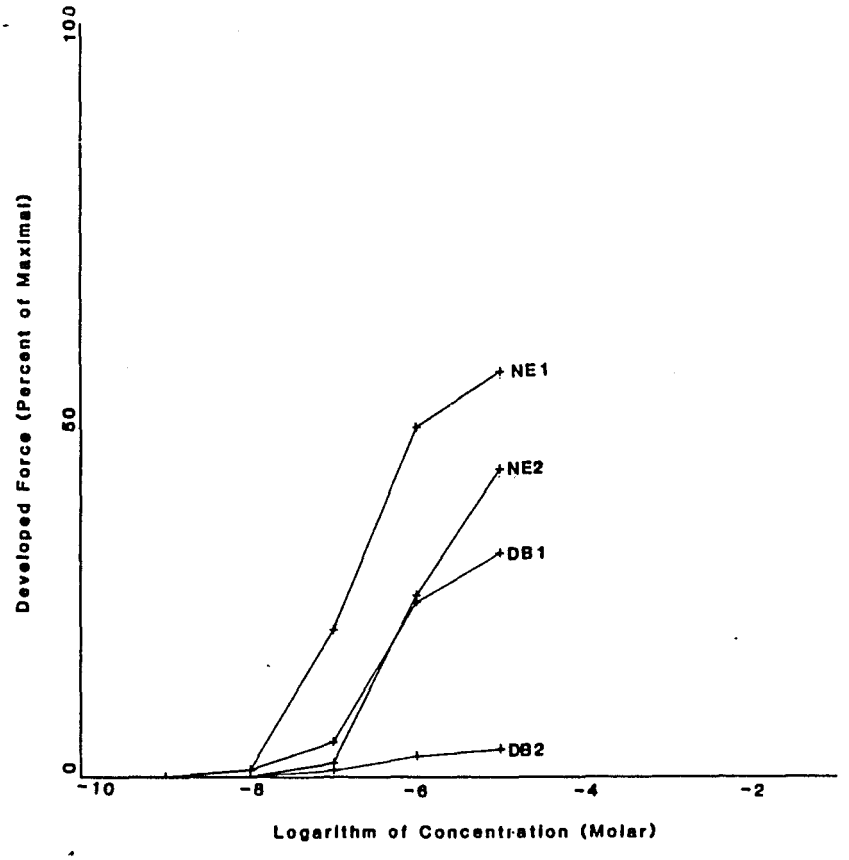


Fig.8. Concentration Response Data for Repetitive Single Tissue Experiments.

The four data sets illustrated demonstrate the effect of repetitive concentration response curves in a single tissue. Curves NE1 and NE2 are to norepinephrine and curves DB1 and DB2 to dobutamine. The repetitive curves were alternated between drugs with a 30 minute washout period between each.

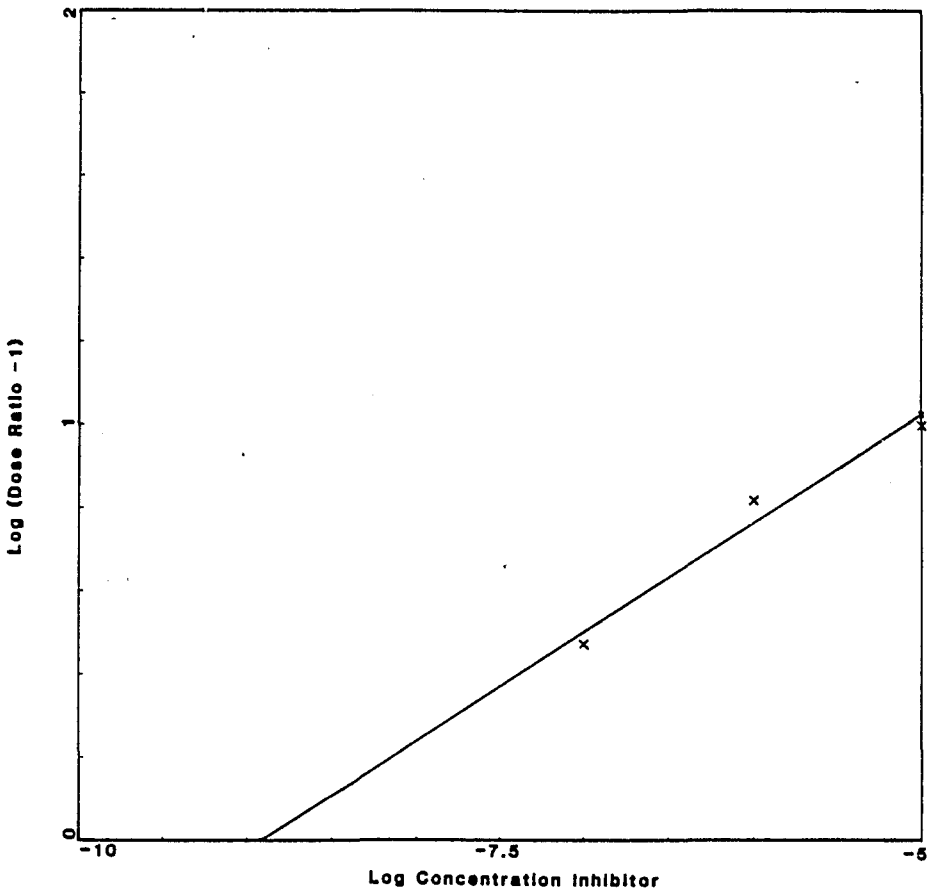


Fig. 9. A Schild Plot for Phentolamine Using Dobutamine as an Agonist in the Rabbit Femoral Artery.

This Schild plot represents average ED50 values obtained for dobutamine in the presence of increasing concentrations of phentolamine in the rabbit femoral artery. The average ED50 values were used with the average ED50 values for dobutamine in the femoral artery to obtain a dose ratio.

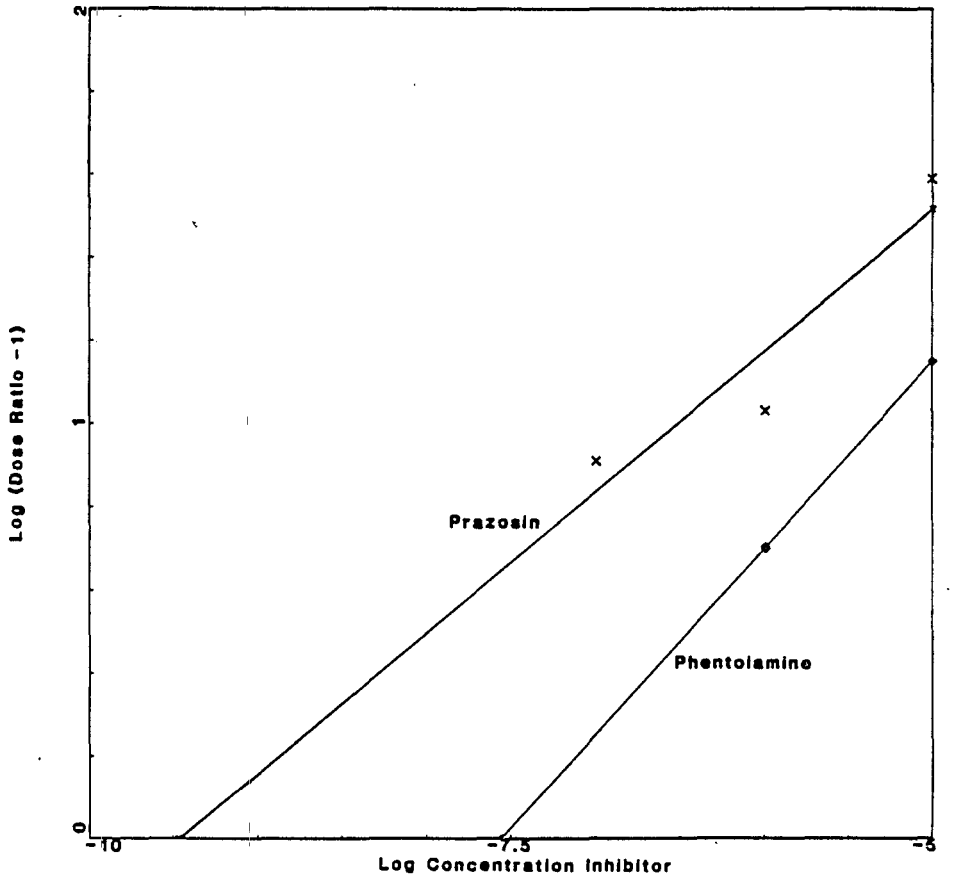


Fig. 10. A Schild Plot for Phentolamine and Prazosin Using Dobutamine as an Agonist in the Rabbit Pulmonary Artery.

This set of Schild plots was obtained by using average values of ED50 for concentration response curves obtained in the presence of increasing concentrations of antagonists. The resulting average values were contrasted with an average ED50 value obtained for dobutamine in the rabbit pulmonary artery to obtain a dose ratio.

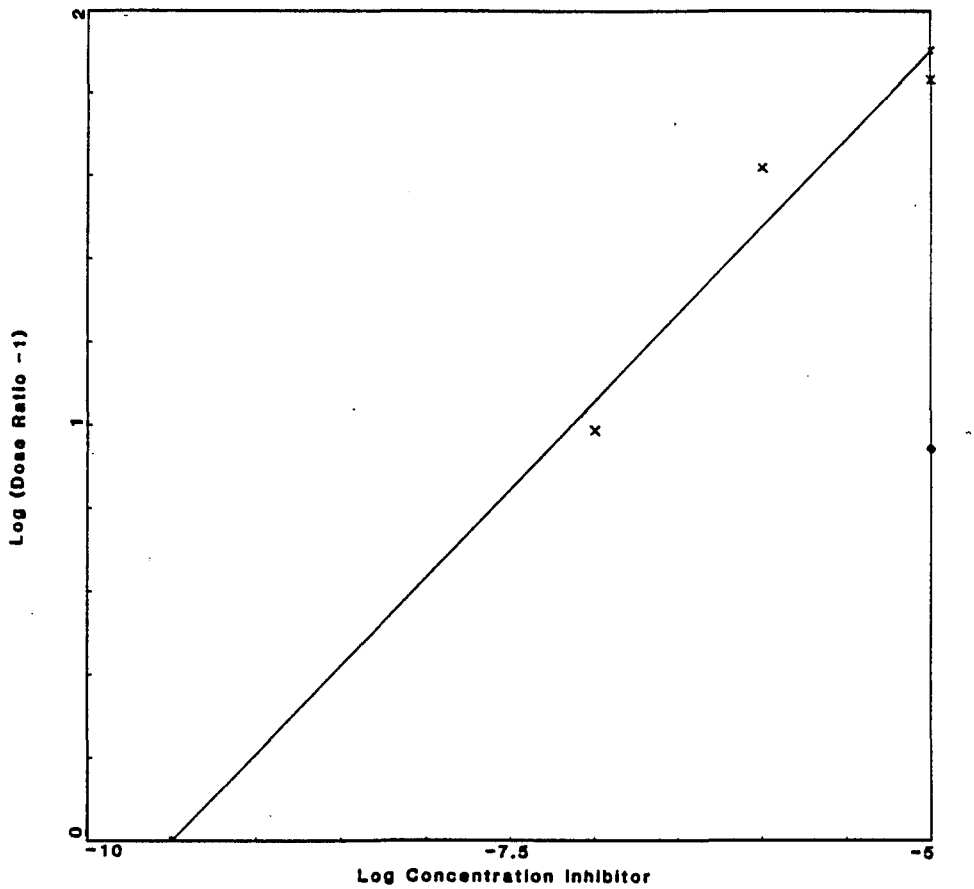


Fig. 11. A Schild Plot for Prazosin Using Norepinephrine as an Agonist in the Rabbit Pulmonary Artery.

This Schild plot represents a plot using average ED50 values obtained for concentration response curves to norepinephrine in the presence of increasing concentrations of prazosin. These average values were compared to an average ED50 value obtained for norepinephrine in the rabbit pulmonary artery to obtain the dose ratio.

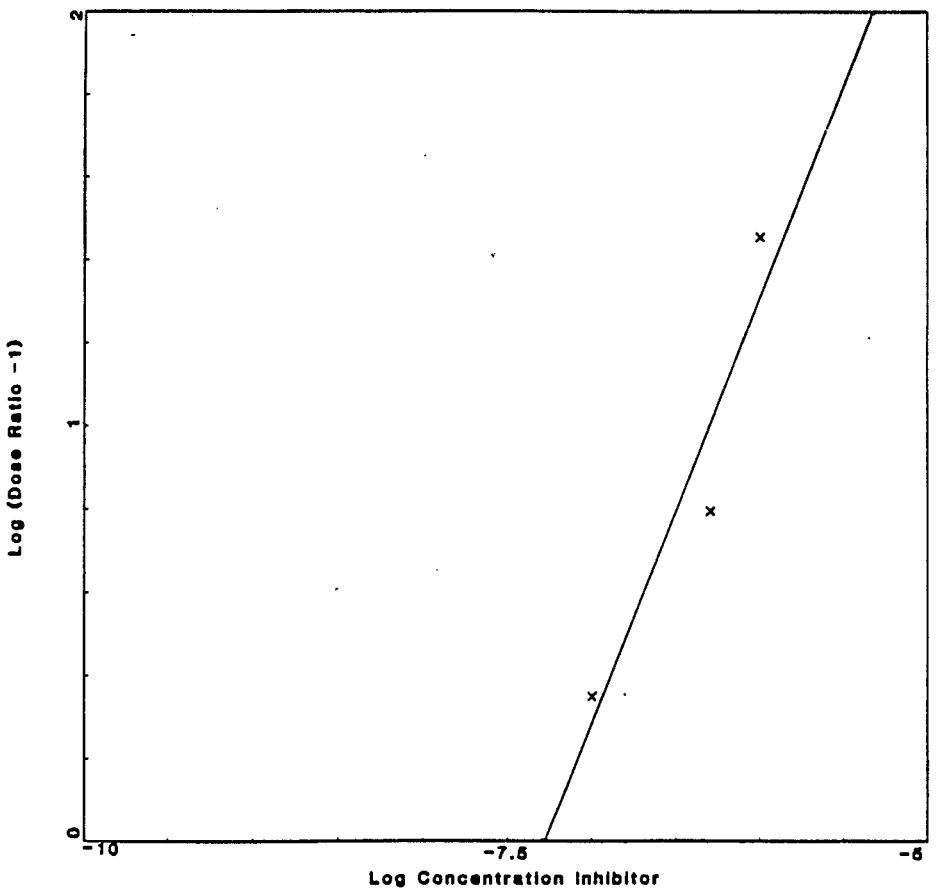


Fig. 12. A Schild Plot Using Four Paired Tissues with Increasing Antagonist Concentration.

This Schild plot was obtained by simultaneously doing concentration response curves to paired tissue sets in the presence of increasing concentrations of antagonist. In this example phentolamine is used as an antagonist to dobutamine.

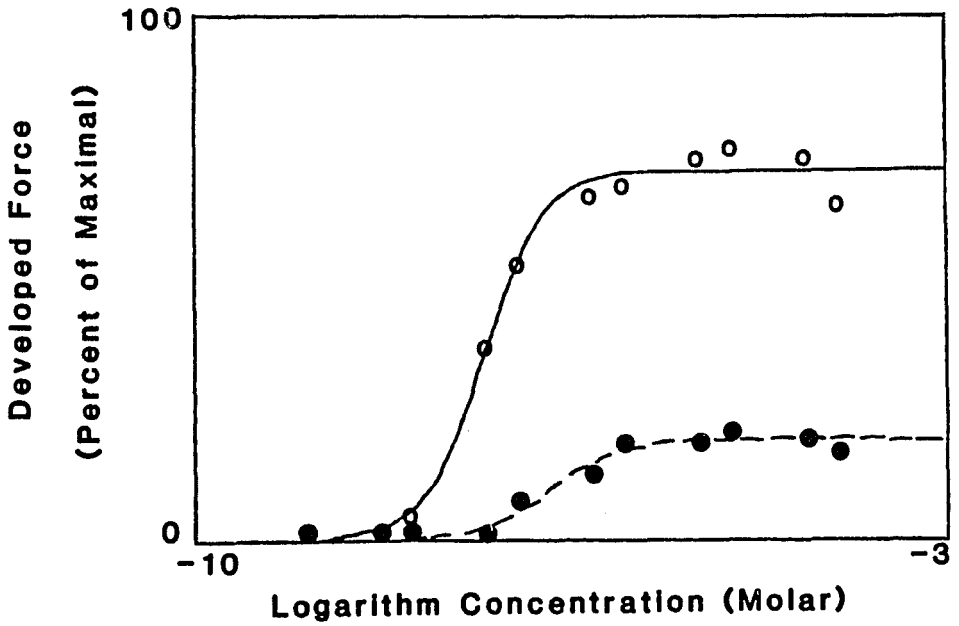


Fig. 13. An Example of a Data Set Used to Determine an Agonist Dissociation Constant.

The two concentration response curves shown are to dobutamine before and after the non competitive alkylating agent, phenoxybenzamine. A double reciprocal plot of concentrations producing equal responses in this method can be used to determine an agonist dissociation constant.

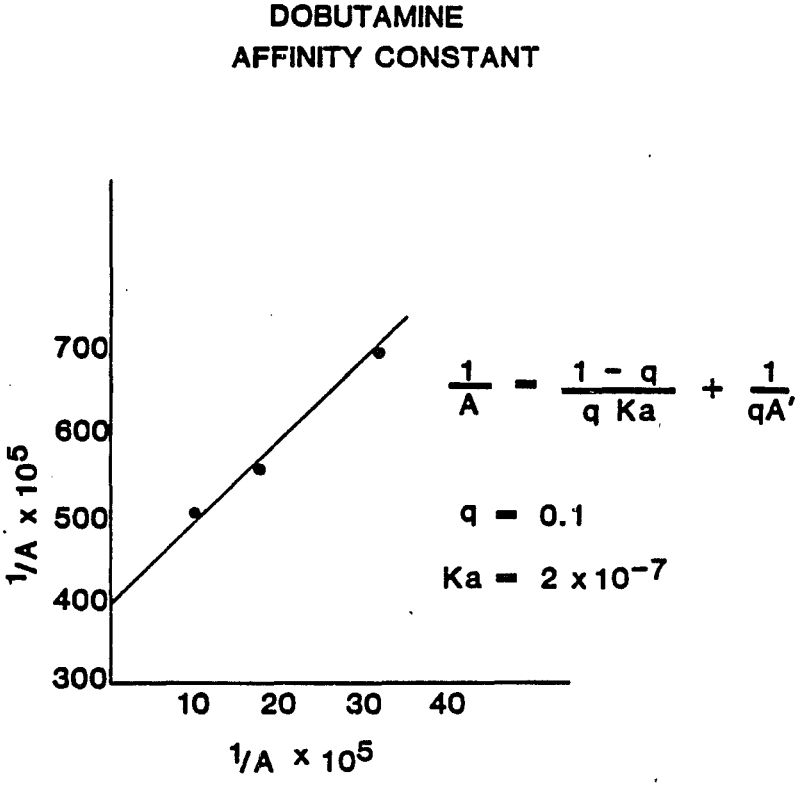


Fig. 14. An Example of the Method of Furchgott to Determine Agonist Dissociation Constants.

The use of a double reciprocal plot of agonist concentrations producing equal responses before and after an irreversible blocking agent is demonstrated. Analysis of the slope and intercept of the best fit lines through these points can be used to calculate an agonist dissociation constant

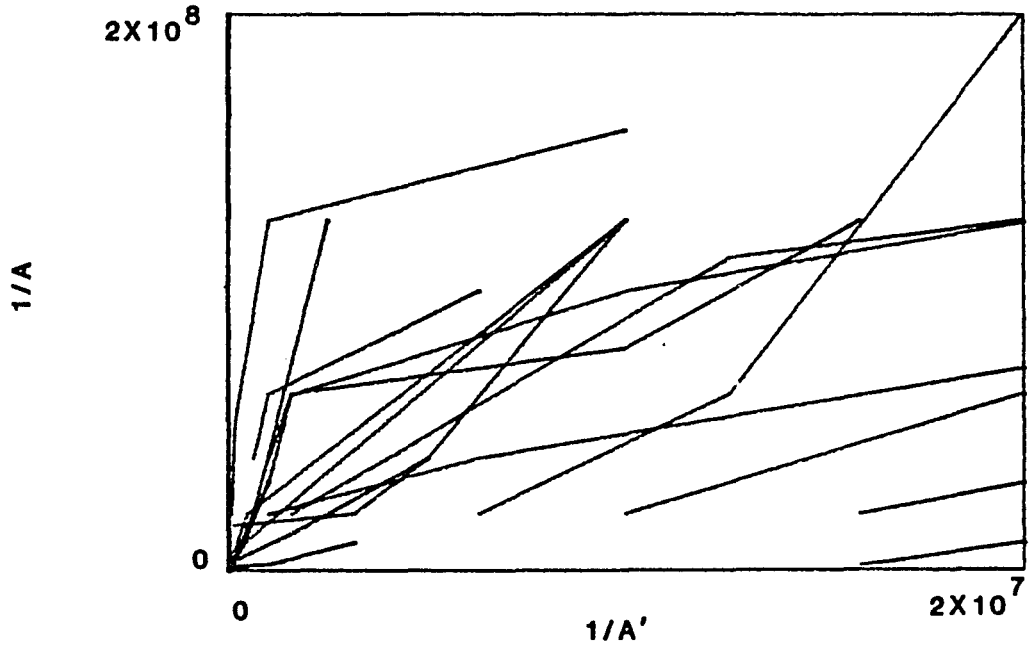


Fig. 15. Double Reciprocal Plots for Determination of Agonist Dissociation Constants Using the Method of Furchgott.

A large series of double reciprocal plots in the method of Furchgott are shown to demonstrate the wide variability in range and linearity of the plots.

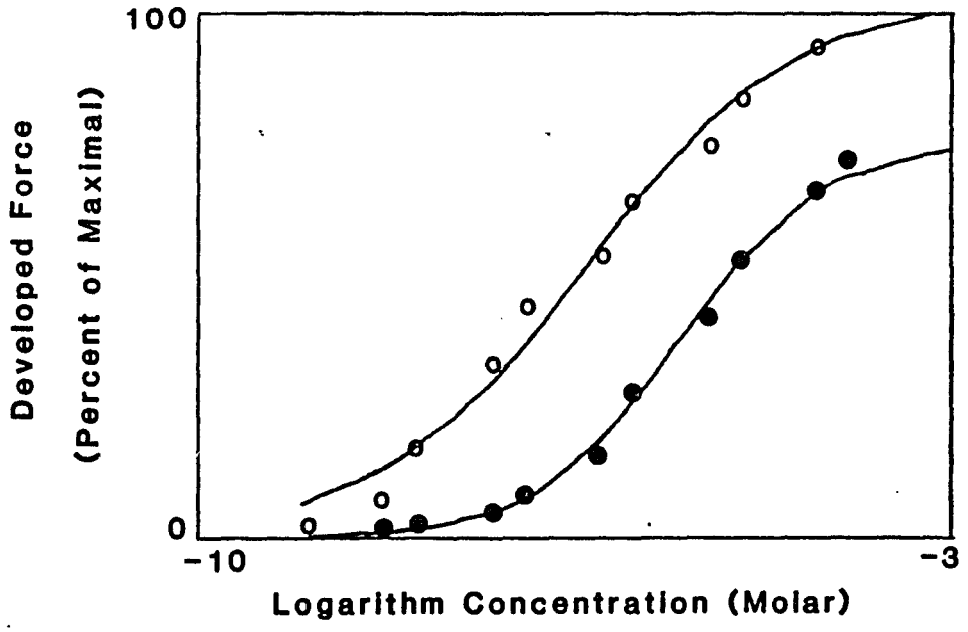


Fig. 16. A Nonlinear Regression Approach to the Method of Furchgott.

Concentration response data for norepinephrine were independently fitted using nonlinear regression. On the basis of the fitted equations, agonist concentrations producing equal responses were plotted in a double reciprocal manner (see fig. 17).

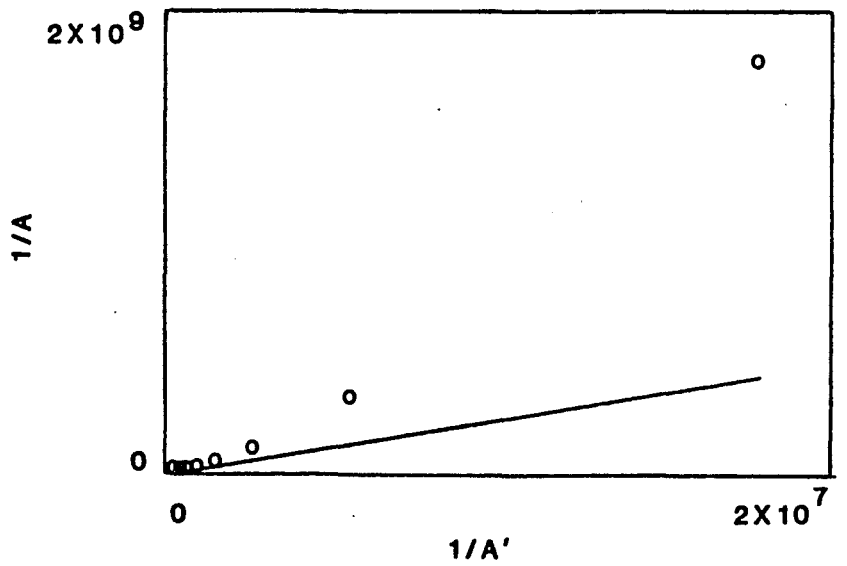


Fig. 17. Double Reciprocal Plot of Concentrations Producing Equal Response Using Nonlinear Regression.

On the basis of figure 16, double reciprocals of the concentration producing equal responses from nonlinear regression were plotted. Using linear regression, the line of best fit is shown for the points determined.

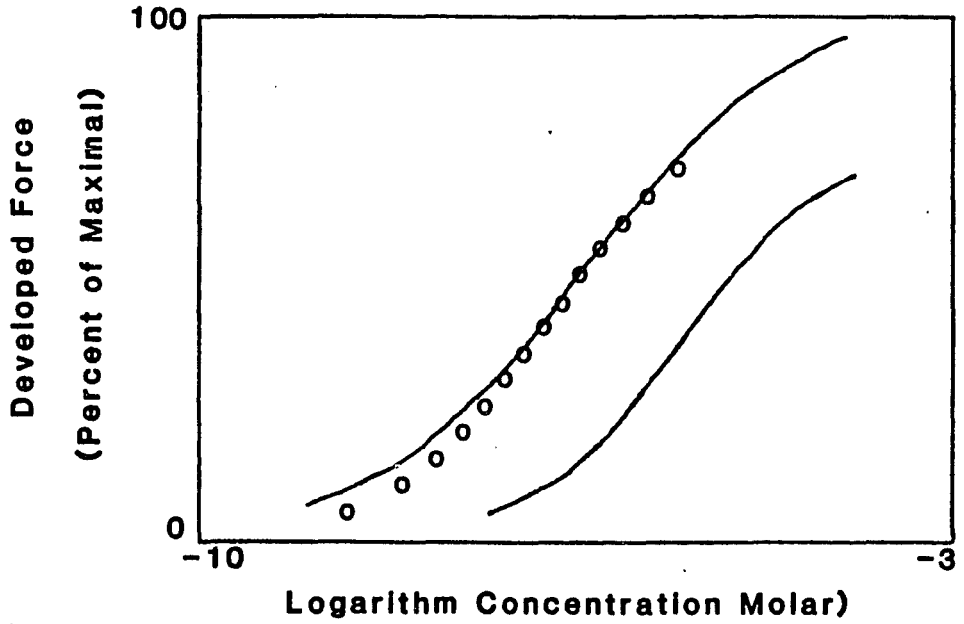


Fig. 18. A Linearly Constrained Data Set Using the Method of Furchgott.

By assuming the linear relationship shown in figure 17, a series of corrected concentration response data points (O) is shown plotted against the original nonlinear regression curve for the data set.

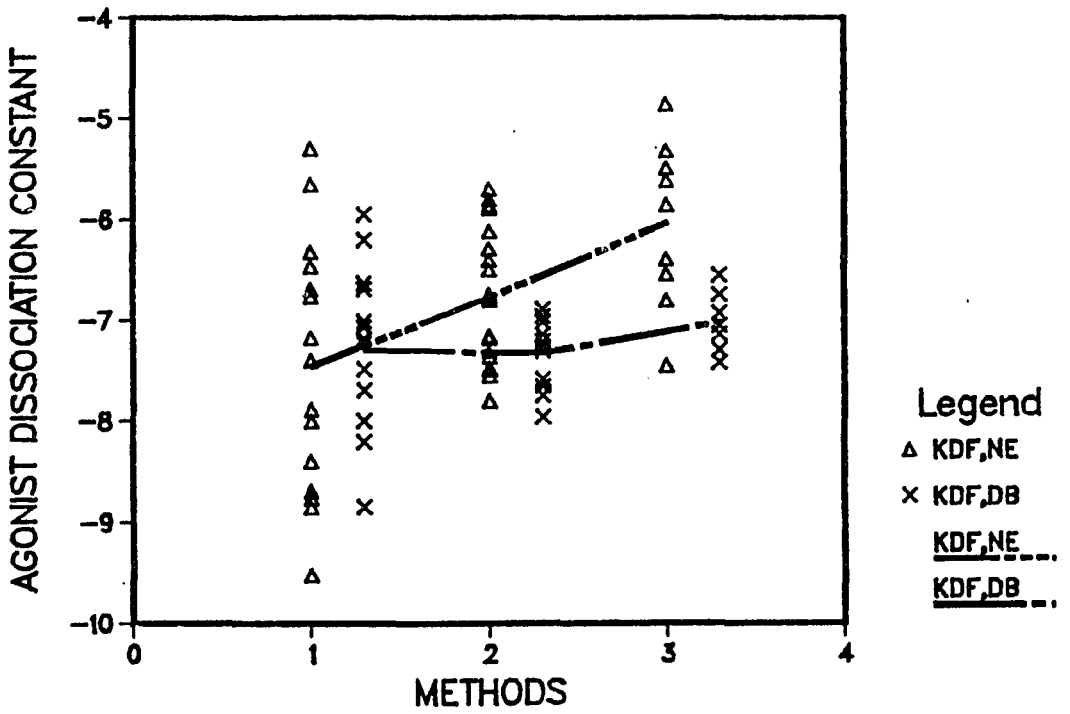


Fig. 19. A Distribution Plot of Values of Agonist Dissociation Constants Obtained by the Method of Furchgott in Comparison to Methods Utilizing Nonlinear Regression.

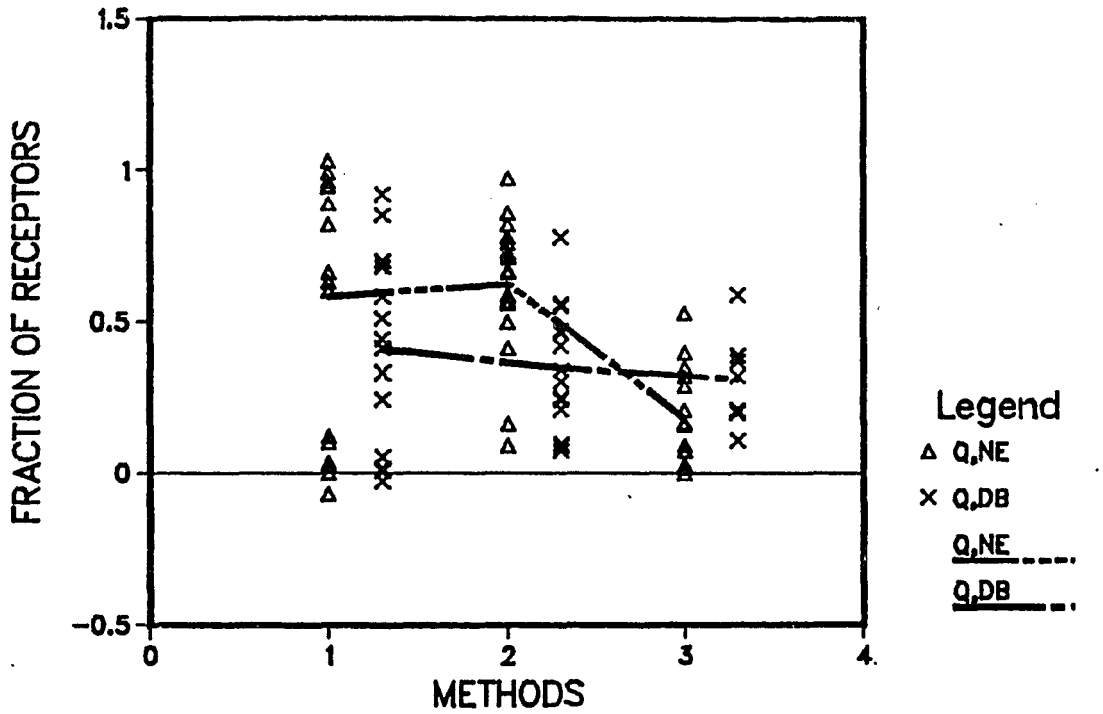


Fig. 20. The Distribution Plot of Values for Fractional Receptor Removal by Phenoxybenzamine Comparing the Methods of Furchgott and Nonlinear Regression.

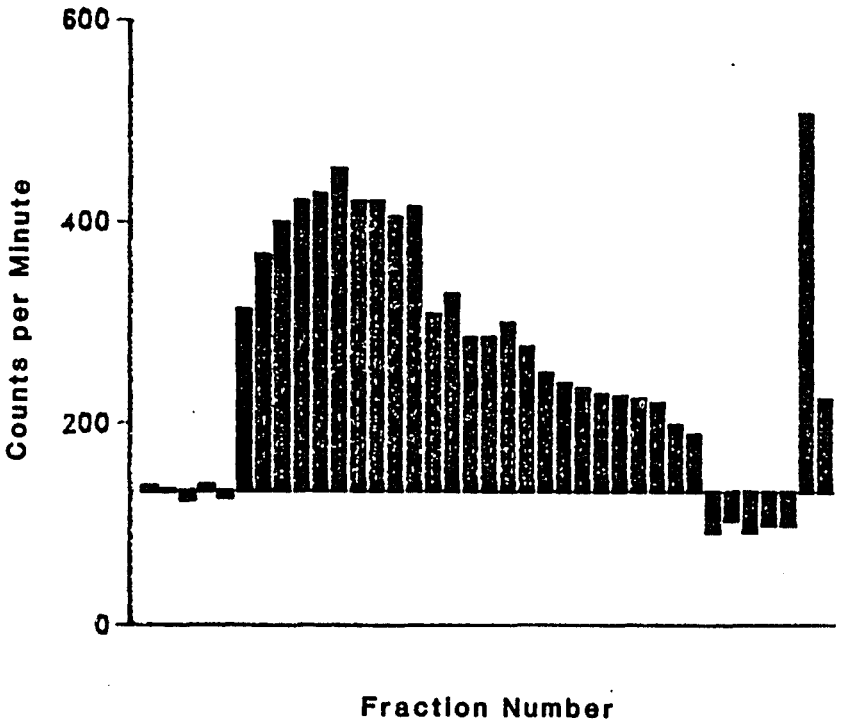


Fig. 21. General Method for the Study of Drug Induced Release of Tritiated Norepinephrine and Metabolites.

This plot shows the general method for study of release of tritiated norepinephrine by dobutamine. Following a control period represented as an average by the baseline for the plot, release of total tritium can be shown to increase by the addition of dobutamine. A washout period is allowed and a return to control followed by response to transmural nerve stimulation.

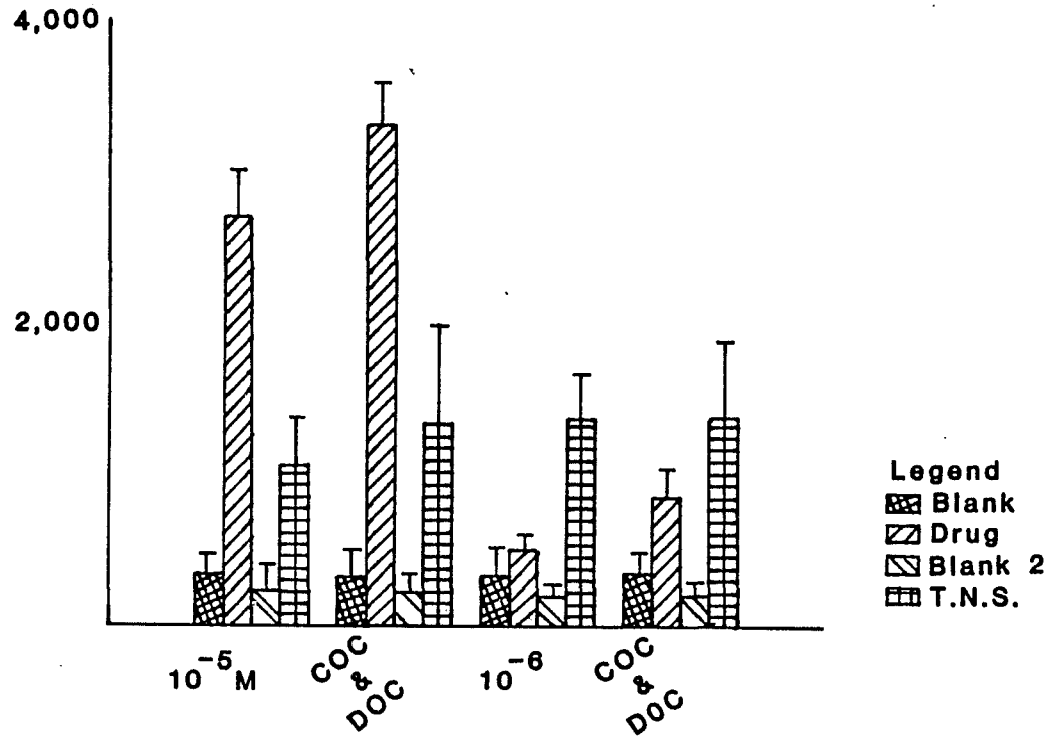


Fig. 22. Raw Data Showing the Effects of Doses of Dobutamine and Uptake Blockers on Release of Tritiated Norepinephrine and Metabolites.

The raw data for total release of tritiated norepinephrine and metabolites by dobutamine over a 10 minute collection period, is shown in comparison to release of tritium during control periods and transmural nerve stimulation (N = 4). The four separate plots represent the relationship of dose of dobutamine and the effect of the blockers cocaine and desoxycorticosterone on drug induced release.

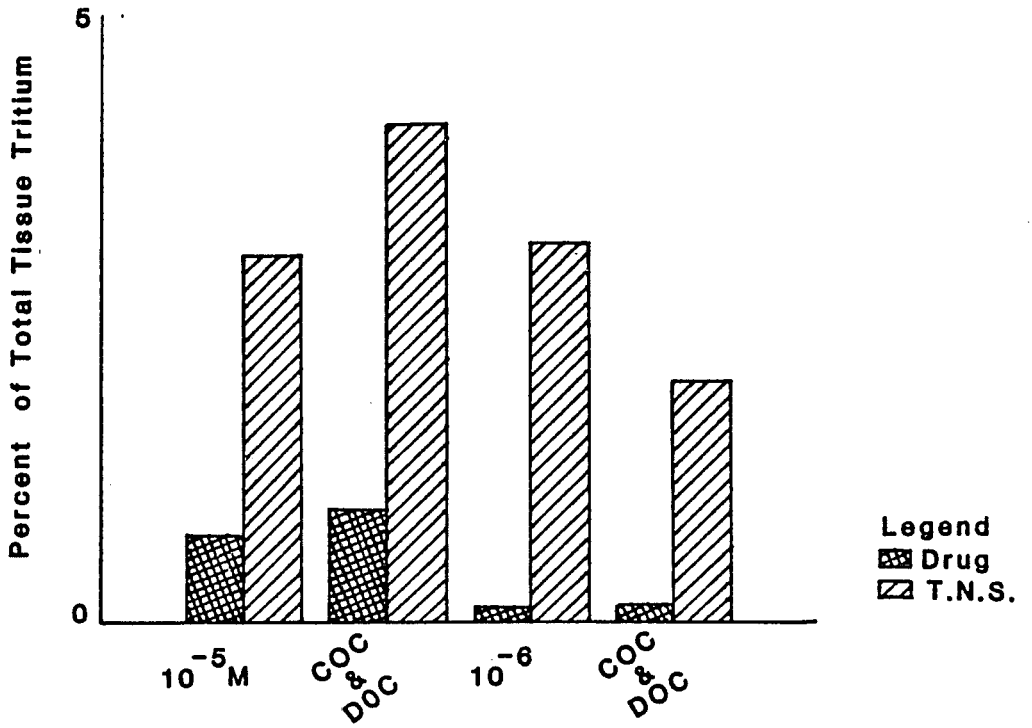


Fig. 23. Dobutamine Induced Release Per Minute as a Fraction of Total Tissue Content of Tritiated Norepinephrine and Metabolites.

This graph shows data from figure 22 re-expressed as fractional release of tritium compared to total tissue tritium content.

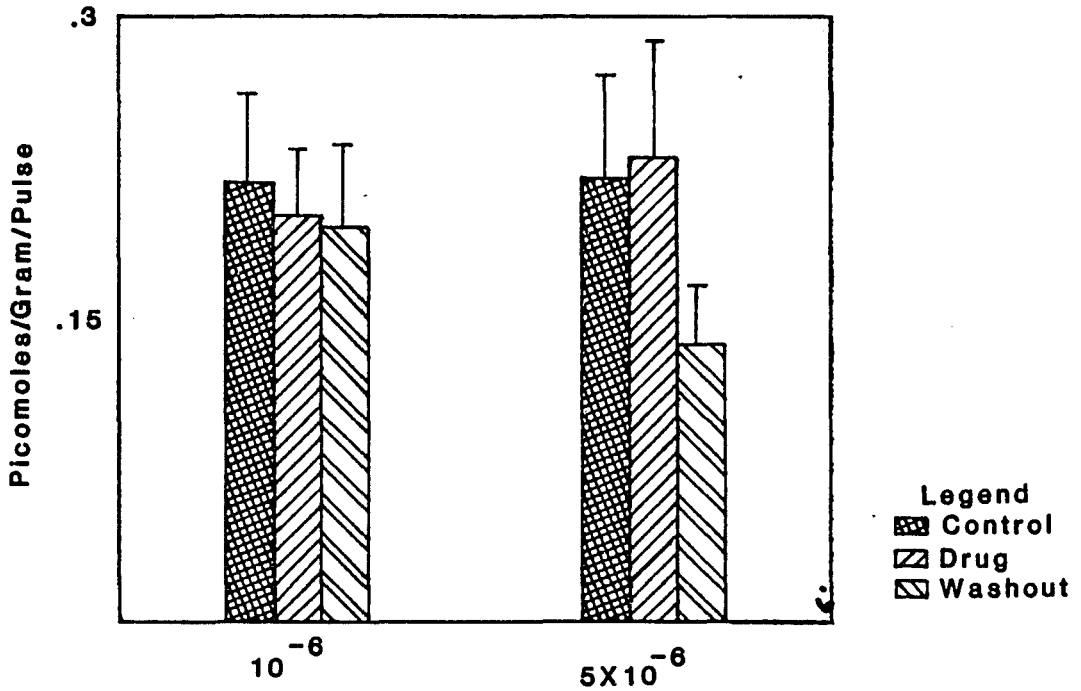


Fig. 24. Effect of Dobutamine on Stimulation-Evoked ^{3}H Norepinephrine Release.

The release of tritiated norepinephrine by transmural nerve stimulation during a control period is compared to stimulation evoked release of norepinephrine in the presence of dobutamine at two concentrations.

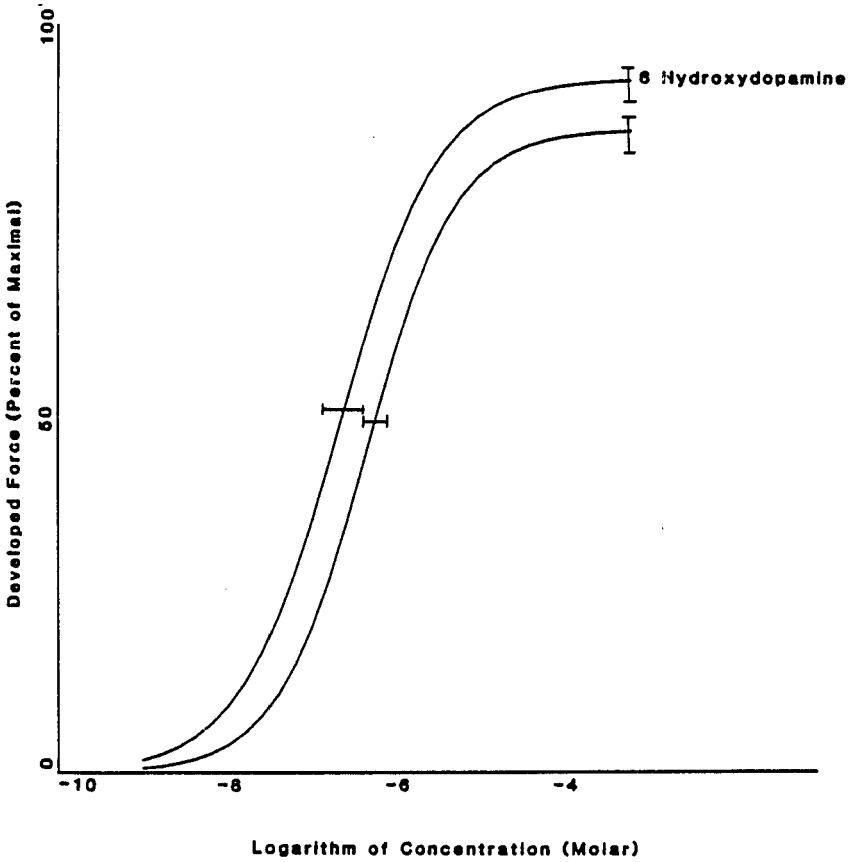


Fig. 25. The Effect of 6- Hydroxydopamine on Contractile Responses to Norepinephrine in the Rabbit Femoral Artery.

Paired concentration response curves (N = 6) before and after exposure to 6- hydroxydopamine in the rabbit femoral artery are shown based on average parameter estimates (± S.E.M.).

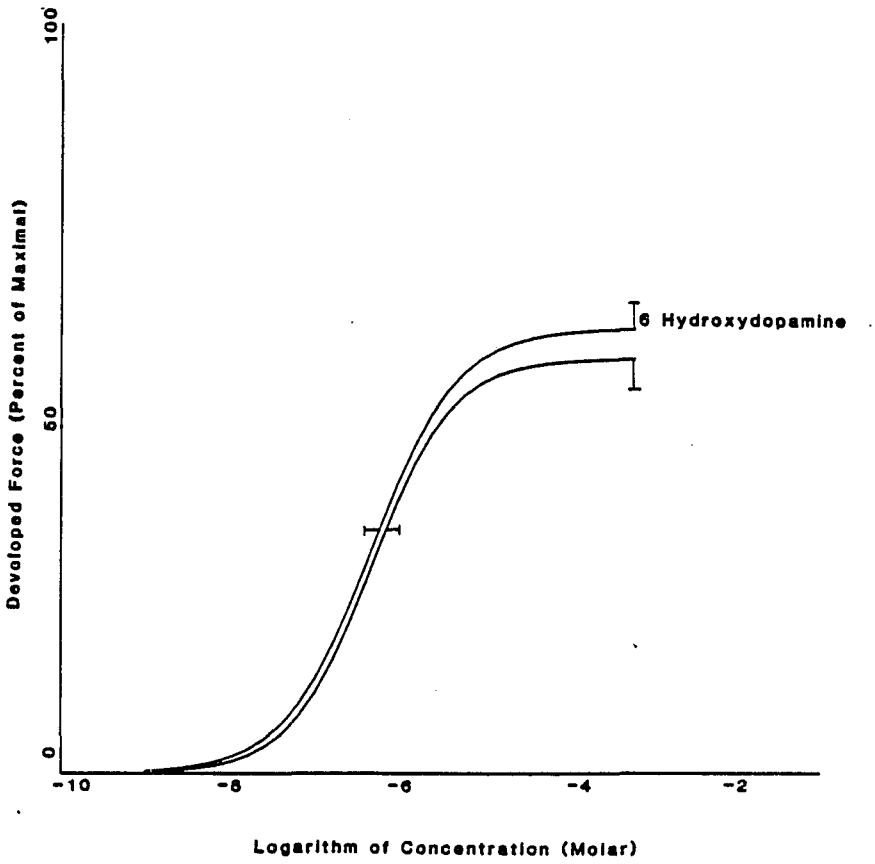


Fig. 26. The Effect of 6- Hydroxydopamine on Contractile Responses to Dobutamine in the Rabbit Femoral Artery.

Paired concentration response curves (N = 9) before and after exposure to 6- hydroxydopamine in the rabbit femoral artery are shown based on average parameter estimates (± S.E.M.).

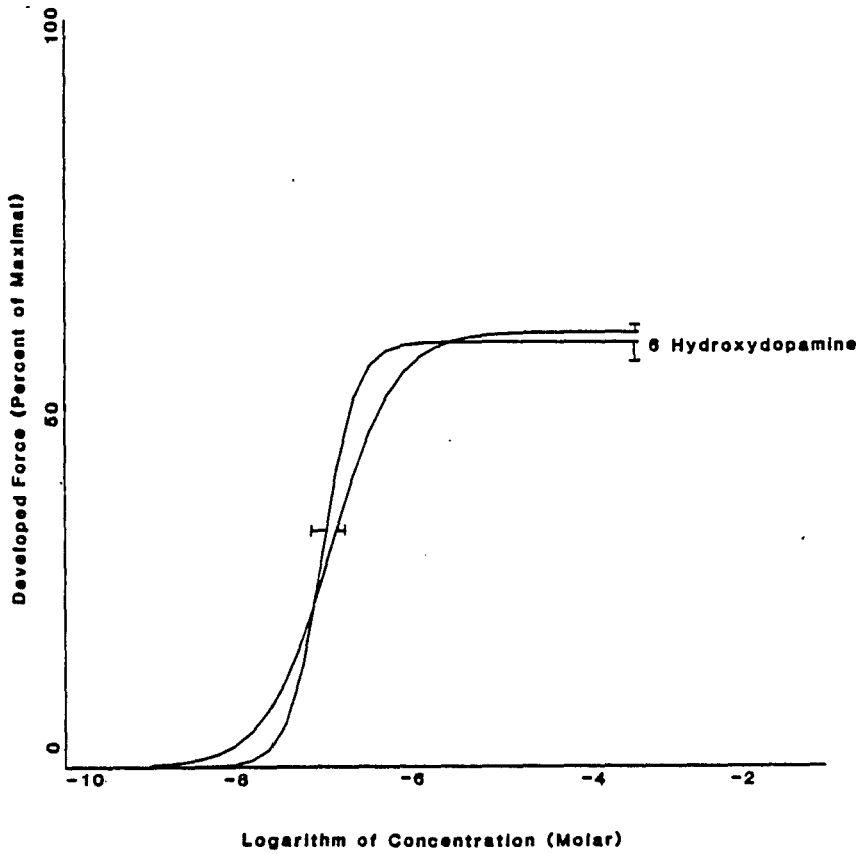


Fig. 27. The Effect of 6- Hydroxydopamine on Contractile Responses to Dobutamine in the Rabbit Pulmonary Artery.

Paired concentration response curves (N = 4) before and after exposure to 6- hydroxydopamine in the rabbit femoral artery are shown based on average parameter estimates (+ S.E.M.).

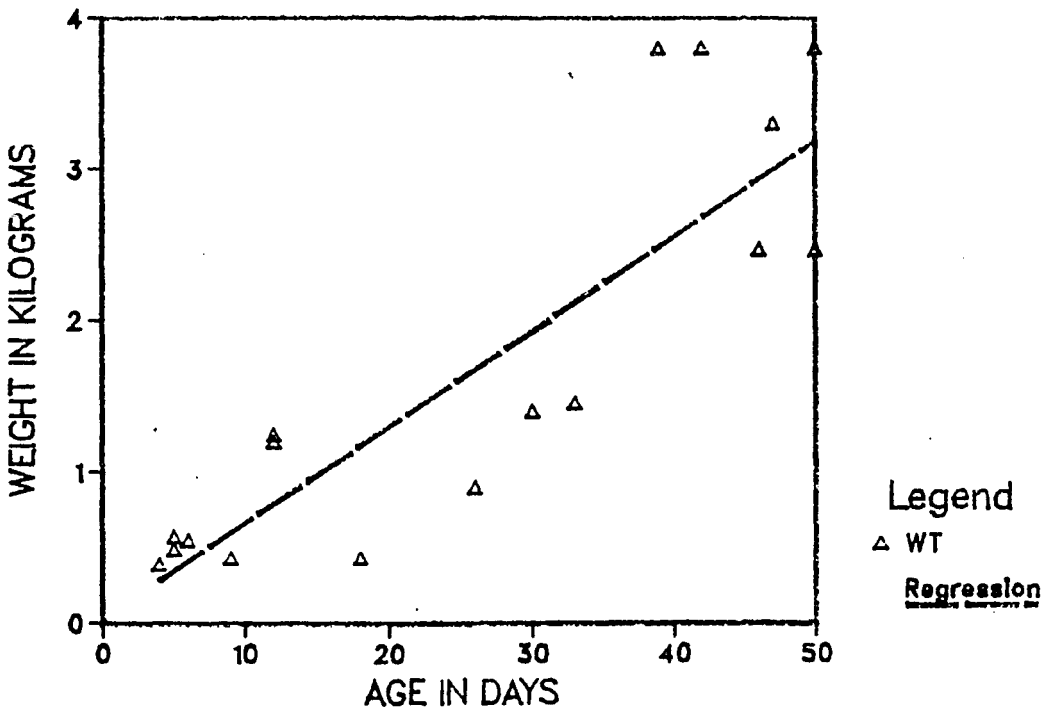


Fig. 28. Body Weight of the Newborn Dog as a Function of Age.

Body weight in kilograms is plotted versus age in days for a population of newborn dogs. The linear regression line is also shown.

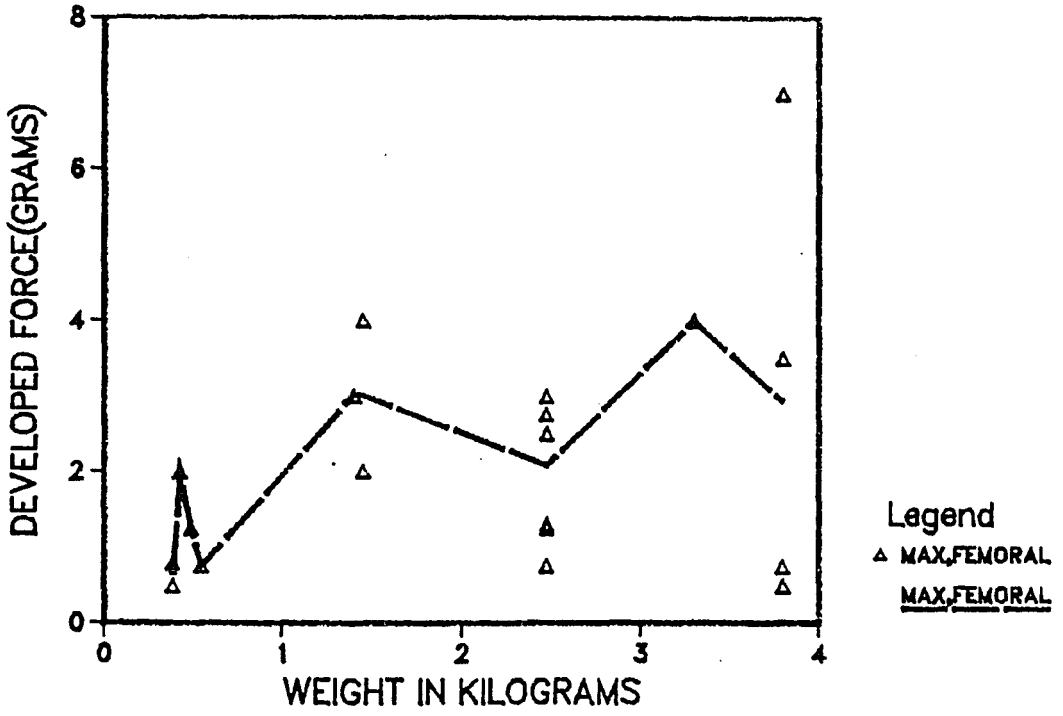


Fig. 29. Maximum Contractile Response of the Dog Femoral Artery as a Function of Body Weight.

Developed force in grams to potassium chloride in the dog femoral artery is plotted as a function of body weight. The data is shown as individual values with an average value.

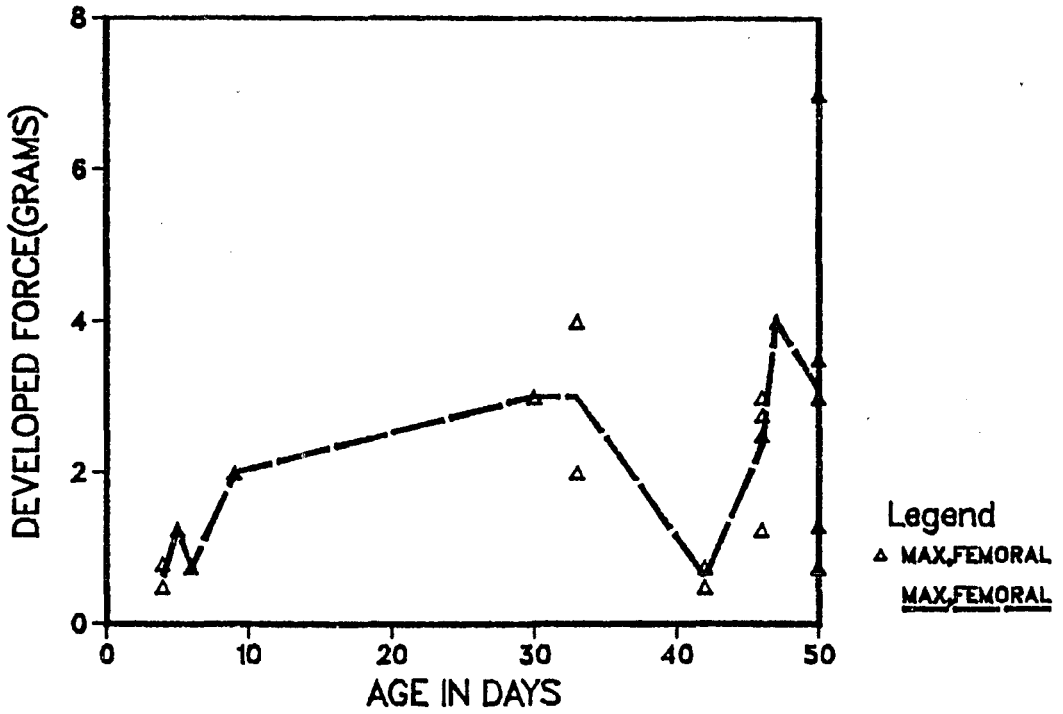


Fig. 30. Maximum Contractile Response of the Dog Femoral Artery as a Function of Age.

Developed force in grams to potassium chloride is plotted as a function of age in the dog femoral artery. Absolute values are plotted with the mean response for each group.

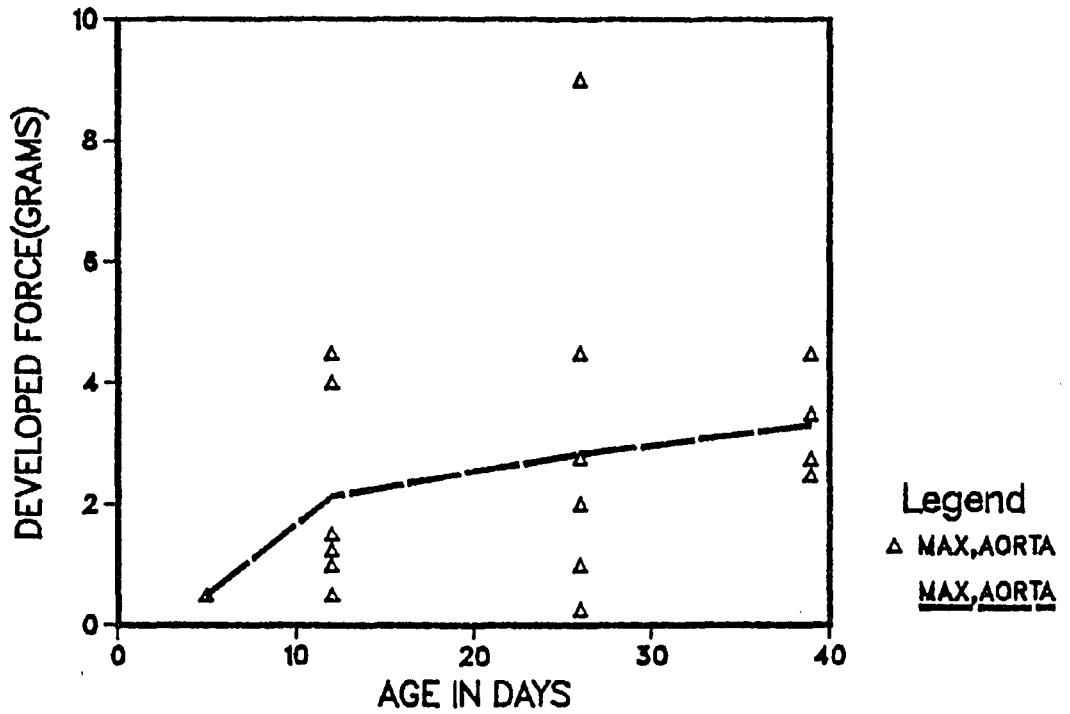


Fig. 31. Maximum Contractile Response in the Dog Aorta as a function of Age.

The maximum contractile force in grams in response to potassium chloride is plotted versus the age in days. The data is shown as individual values and a mean maximal response of litter mates sacrificed sequentially.

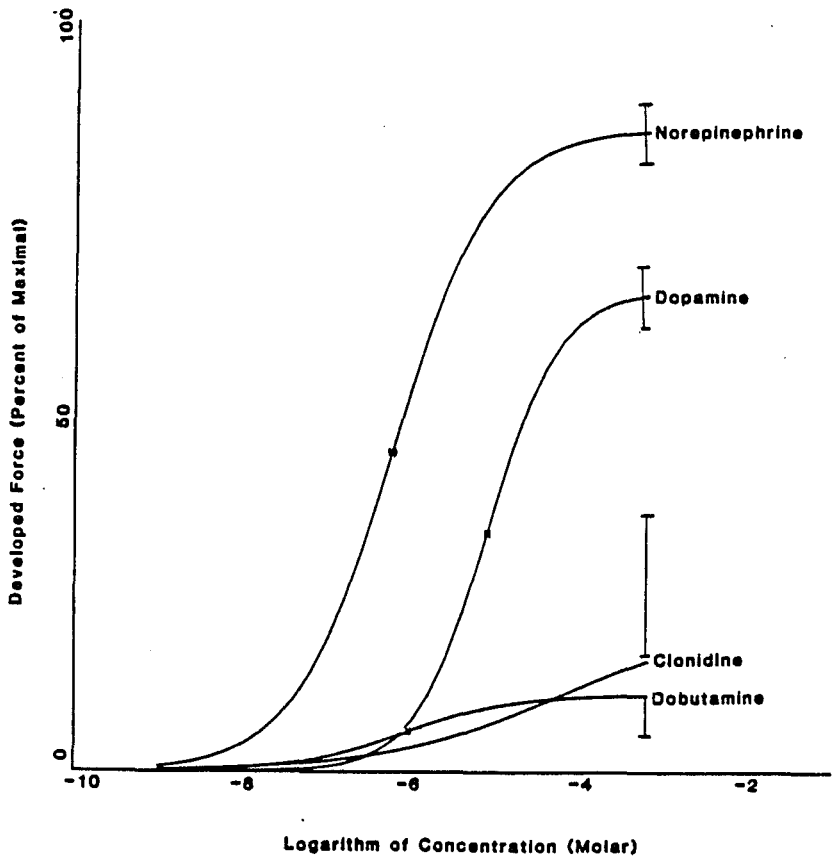


Fig. 32. Comparative Responses of the Dog Femoral Artery at 0-2 Week Age.

Developed force as a percent of maximal response to potassium chloride in the femoral artery is shown for the 0-2 week age dog in response to norepinephrine (N = 11), dopamine (N = 2), clonidine (N = 3), and dobutamine (N = 9).

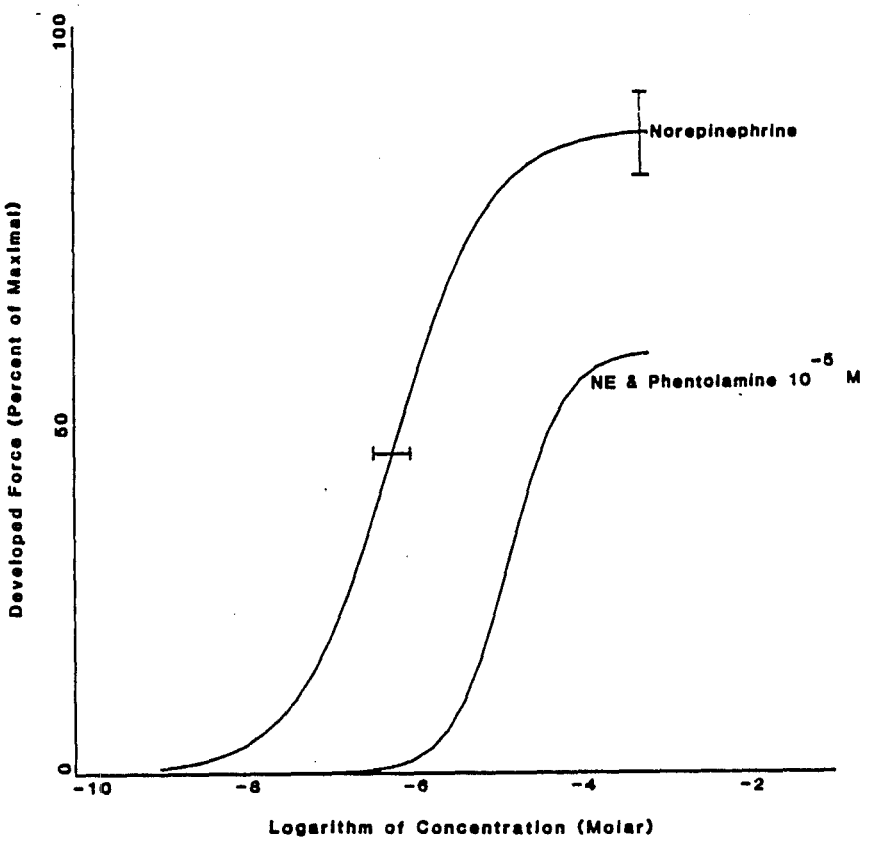


Fig. 33. The Effect of Phentolamine on the Response to Norepinephrine in the 0-2 Week Age Dog.

Developed force as percent of maximal response to potassium chloride is shown in the presence and absence of phentolamine 10^{-5}M in the femoral artery of the 0-2 week age dog.

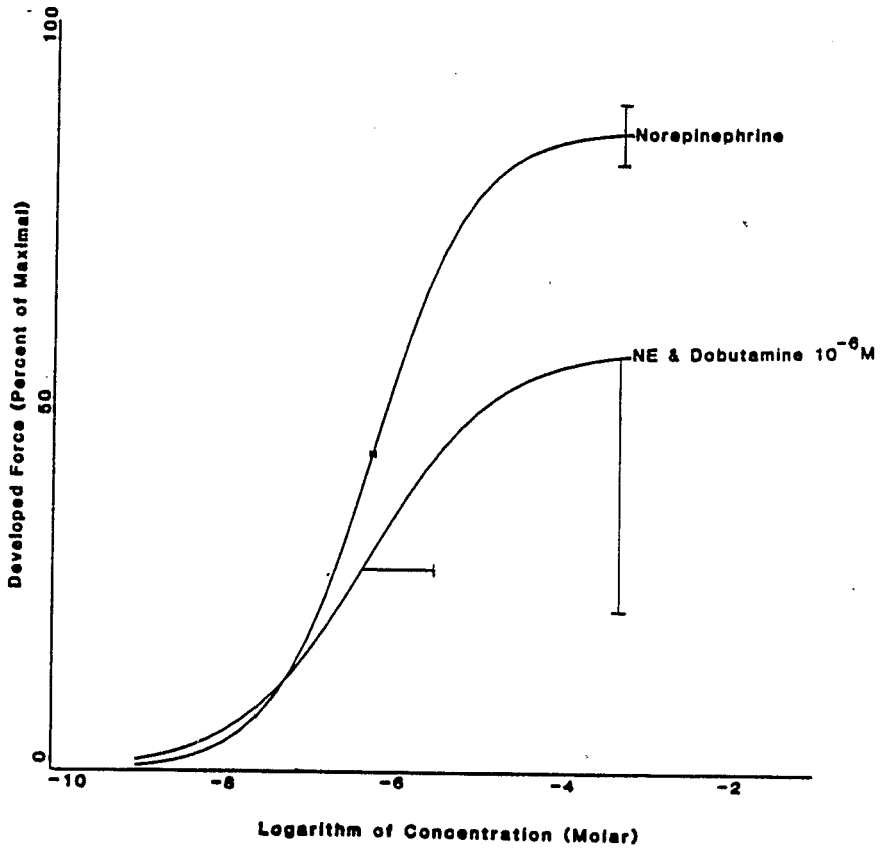


Fig. 34. The Effect of Dobutamine on the Responses to Norepinephrine in the 0-2 Week Age Dog Femoral Artery.

Developed force as a percent of the maximal contraction to potassium chloride is shown for increasing concentrations of norepinephrine in the presence and absence of dobutamine (N = 2).

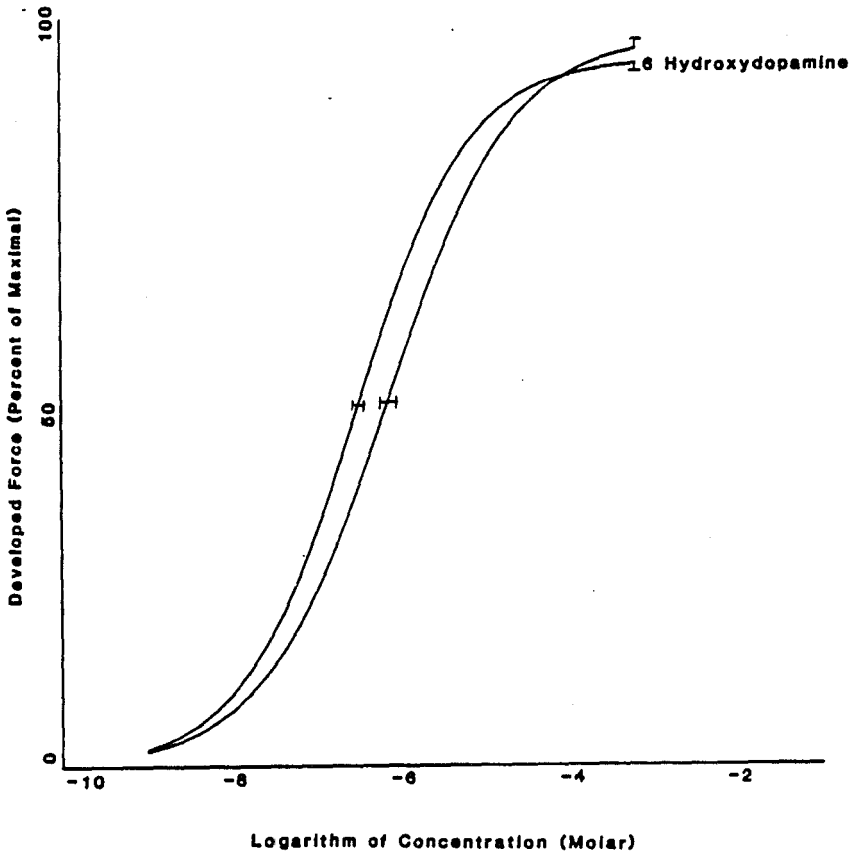


Fig. 35. The Effect of 6- Hydroxydopamine on Responses to Norepinephrine in the 0-2 Week Age Dog.

Developed force as a fraction of the maximal response to potassium chloride is shown in paired tissues in the presence (N = 2) and absence of incubation with 6- hydroxydopamine.

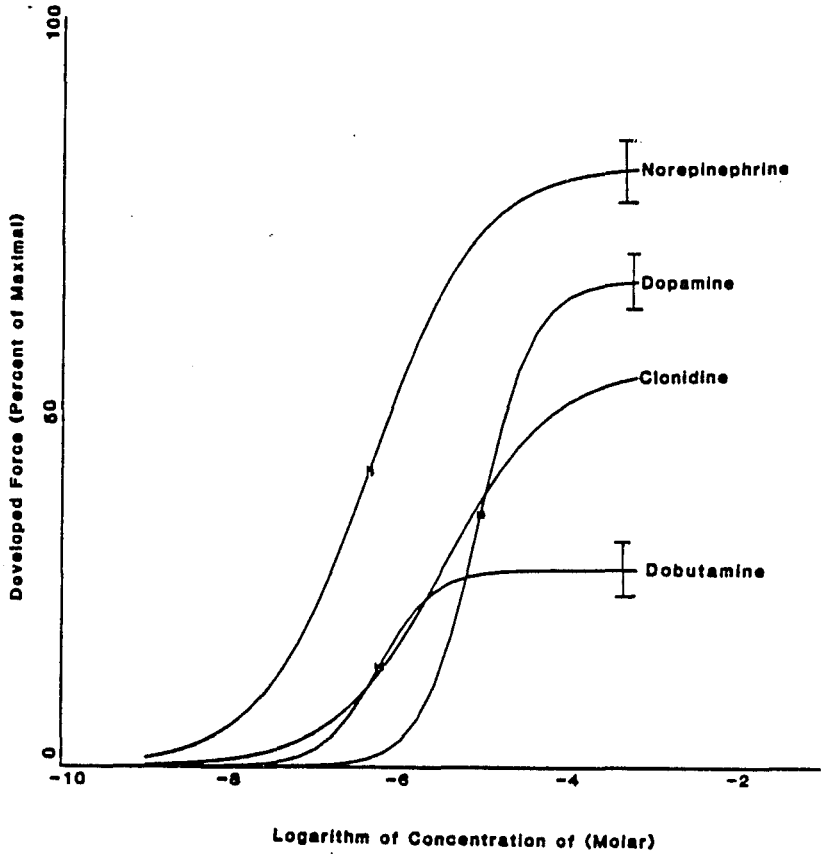


Fig. 36. Contractile Responses of the Dog Femoral Artery at 2-4 Weeks of Age.

Developed force as a fraction of a maximal contractile force to potassium chloride is shown for the drugs norepinephrine (N = 4), dopamine (N = 2), clonidine (N = 1), and dobutamine (N = 10) in dog femoral artery from 2-4 weeks of age.

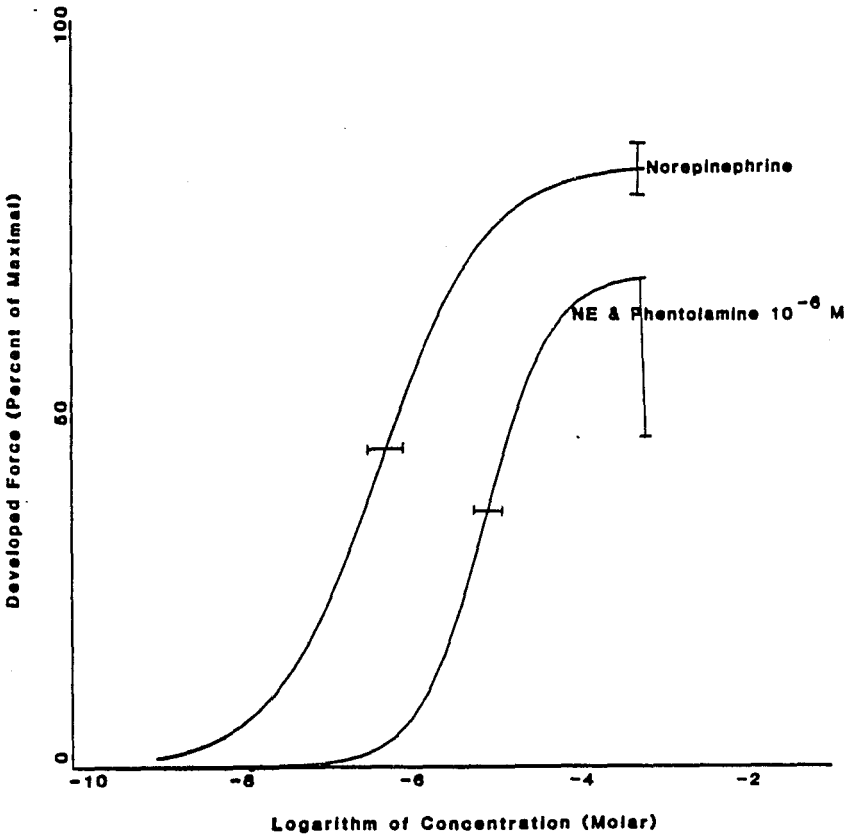


Fig. 37. The Effects of Phentolamine on Contractile Responses to Norepinephrine in the 2-4 Week Dog Femoral Artery.

Developed force as a fraction of the maximal contractile response to potassium chloride is shown for norepinephrine in the presence of phentolamine (N = 2) in comparison to control.

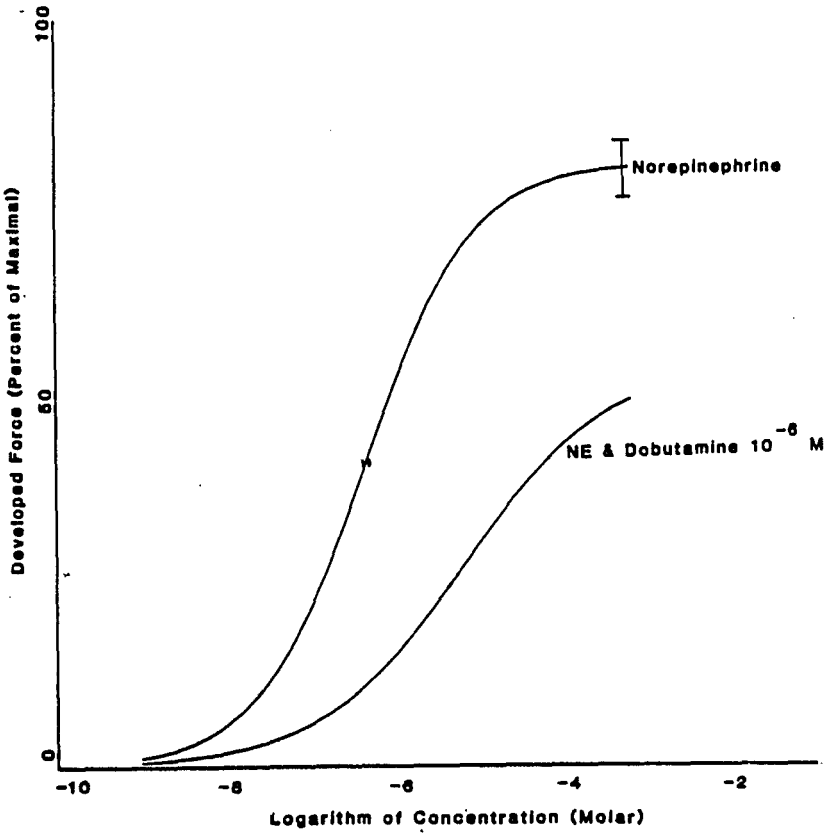


Fig. 38. The Effect of Dobutamine on the Contractile Response to Norepinephrine in the Dog Femoral Artery 2-4 Weeks of Age.

Developed force as a percentage of maximal contractile force to potassium chloride is shown for norepinephrine in the presence and absence of dobutamine in the dog femoral artery at 2-4 weeks of age.

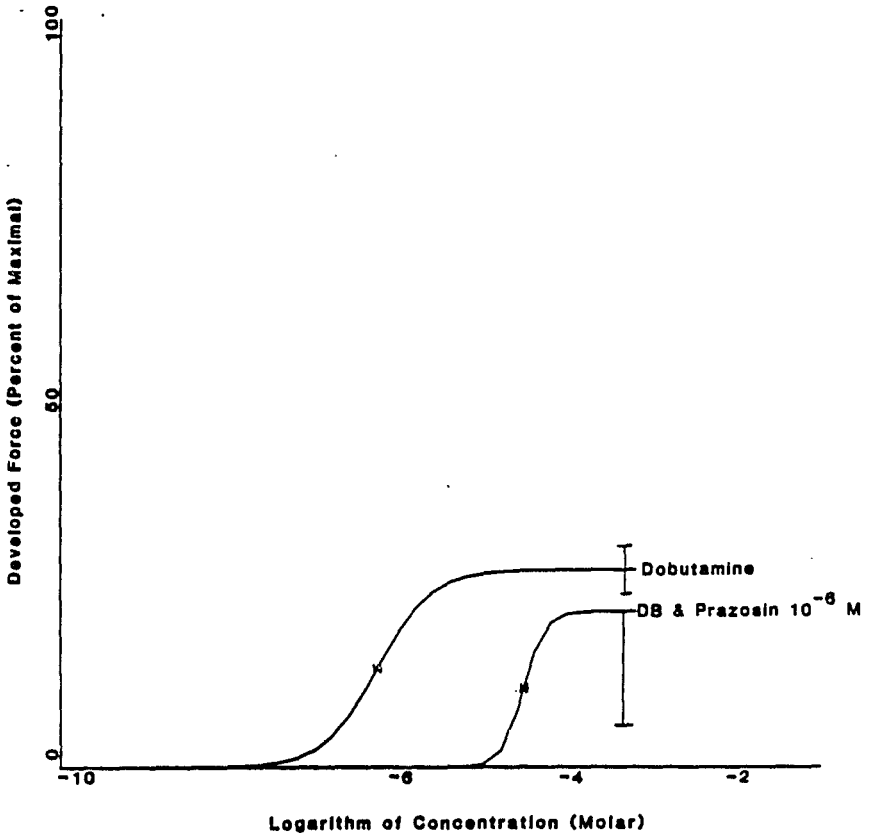


Fig. 39. The Effect of Prazosin on Contractile Responses to Dobutamine in the Dog Femoral Artery 2-4 Weeks of Age.

Developed force as a percentage of the maximal contractile force to potassium chloride is shown for dobutamine in the presence and absence of prazosin (N = 2) in the dog femoral artery from 2-4 weeks of age.

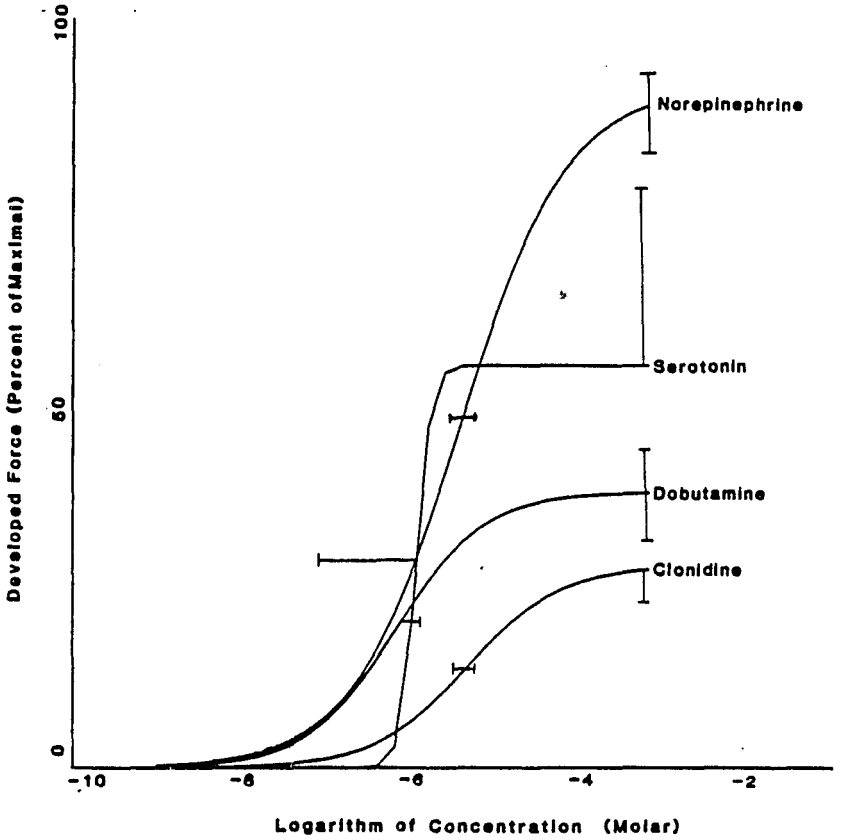


Fig. 40. Contractile Responses of the Dog Femoral Artery at 4-6 Weeks of Age.

Developed force as a percentage of the maximal contractile force to potassium chloride is shown for the drugs norepinephrine (N = 7), serotonin (N = 2), dobutamine (N = 2) and clonidine (N = 3) in the dog femoral artery from 4-6 weeks of age.

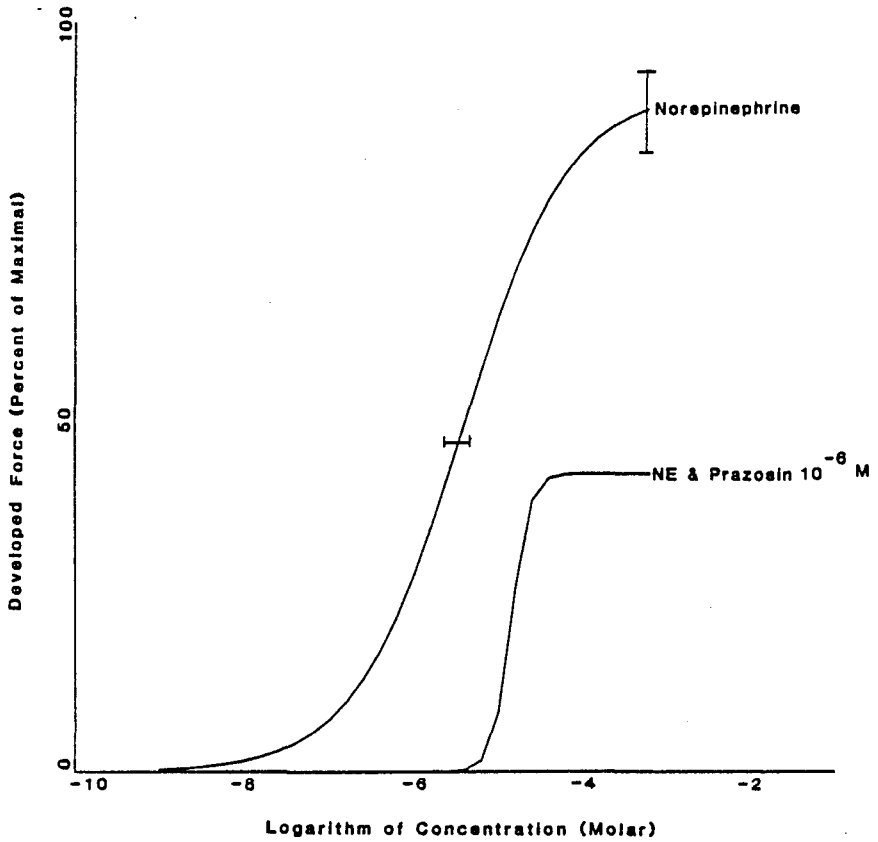


Fig. 41. The Effect of Prazosin on Contractile Responses to Norepinephrine in the Femoral Artery of the 4-6 Week Age Dog.

Developed force as a fraction of maximal contractile force to potassium chloride is shown for norepinephrine in the presence and absence of prazosin in the dog femoral artery from 4-6 weeks of age.

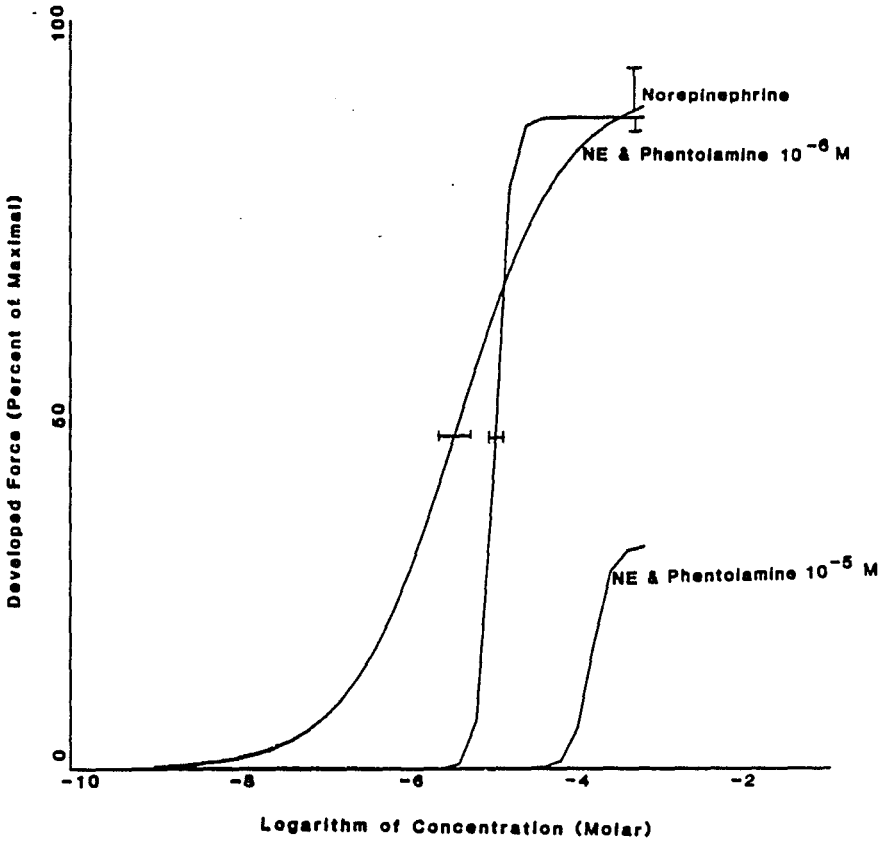


Fig. 42. The Effect of Phentolamine on Contractile Responses to Norepinephrine in the Femoral Artery of the 4-6 Week Age Dog.

Developed force as a fraction of maximal contractile force to potassium chloride is shown for norepinephrine in the presence and absence of increasing concentrations of phentolamine in the dog femoral artery at 4-6 weeks of age.

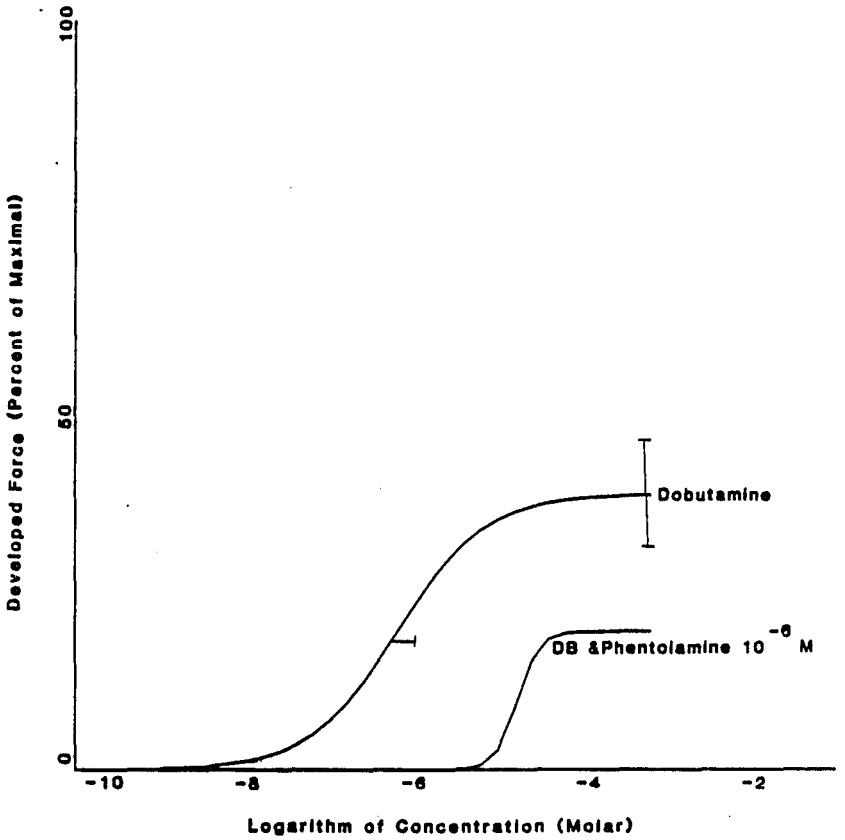


Fig. 43. The Effect of Phentolamine on Contractile Responses to Dobutamine in the Femoral Artery of the 4-6 Week Age Dog.

Developed force as a fraction of maximal contractile response to potassium chloride is shown for dobutamine in the presence and absence of phentolamine in the dog femoral artery at 4-6 weeks of age.

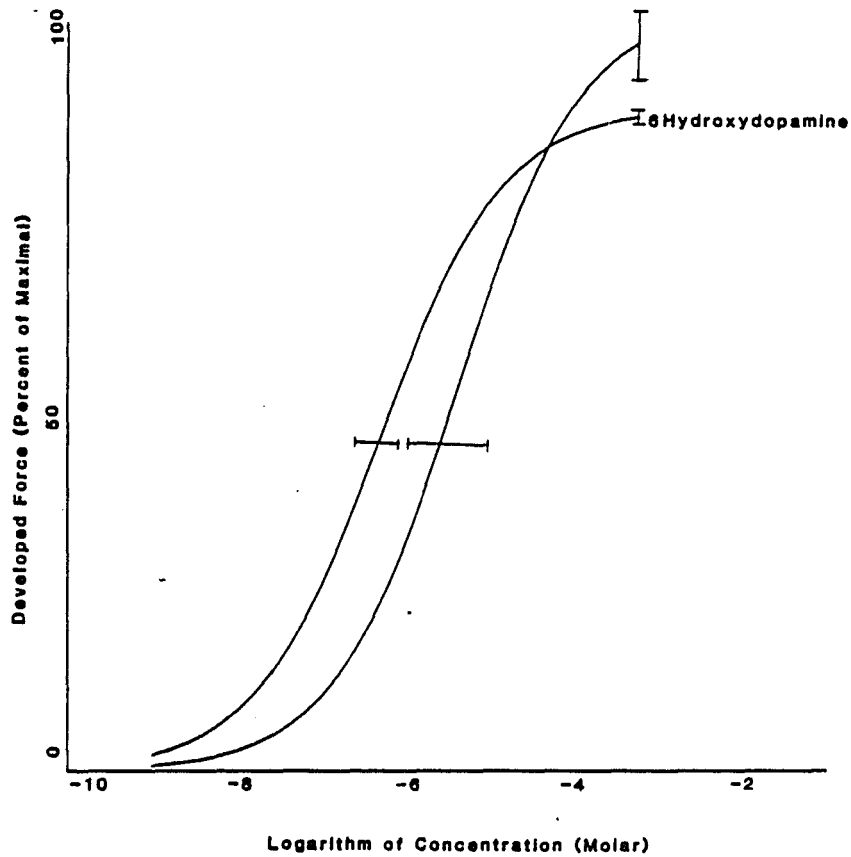


Fig. 44. The Effects of 6- Hydroxydopamine on the Contractile Responses to Norepinephrine in the 4-6 Week Age Dog Femoral Artery.

Developed force as a percentage of the maximal contractile response to potassium chloride is shown for paired vessels (N = 2) following exposure to 6- hydroxydopamine in the femoral artery of the 4-6 week age dog.

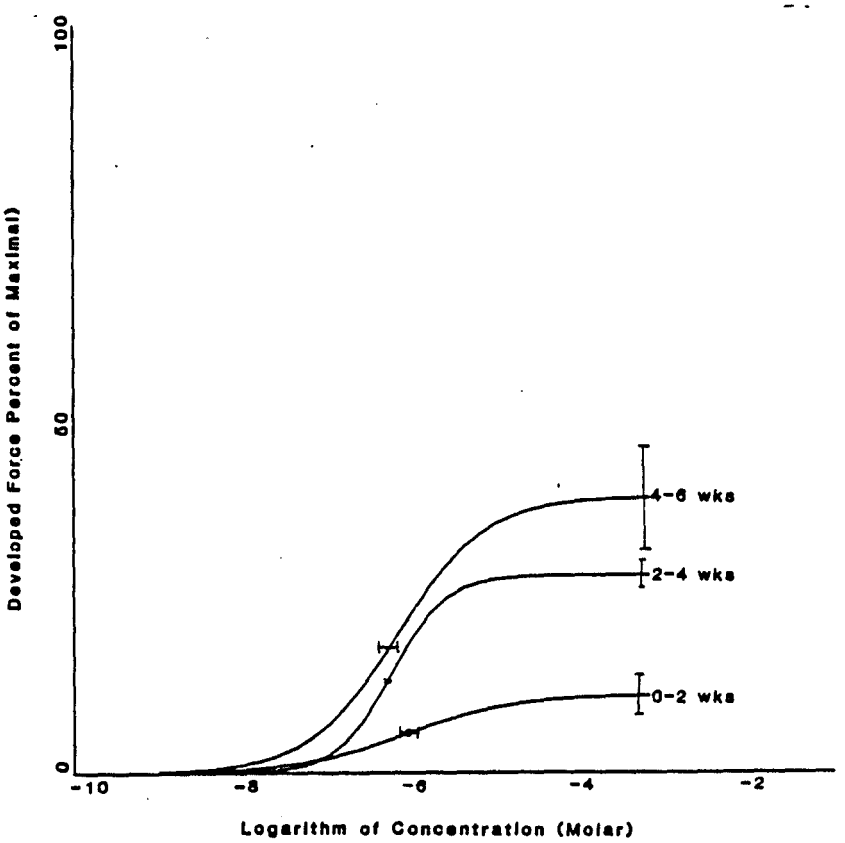


Fig. 45. Contractile Responses of the Dog Femoral Artery to Dobutamine at Various Ages.

Developed force as a percent of maximal contractile response to potassium chloride is shown for dobutamine in the dog femoral artery at increasing ages.

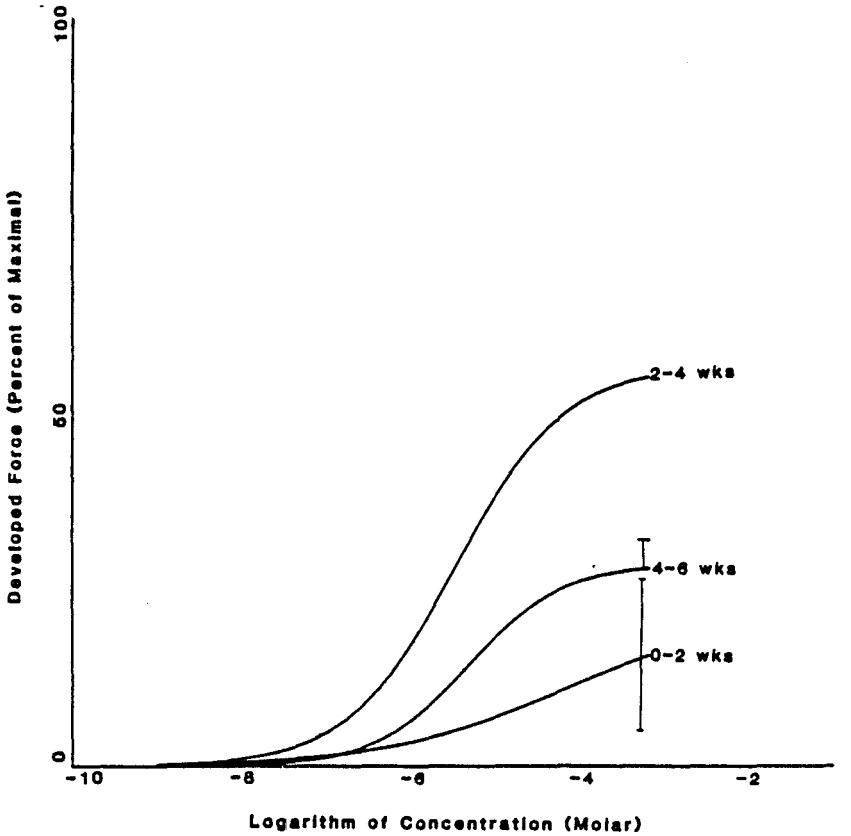


Fig. 46. Contractile Responses of the Dog Femoral Artery to Clonidine at Various Ages.

Developed force as a percent of maximal contraction to potassium chloride is shown for clonidine at 3 different ages in the dog femoral artery.

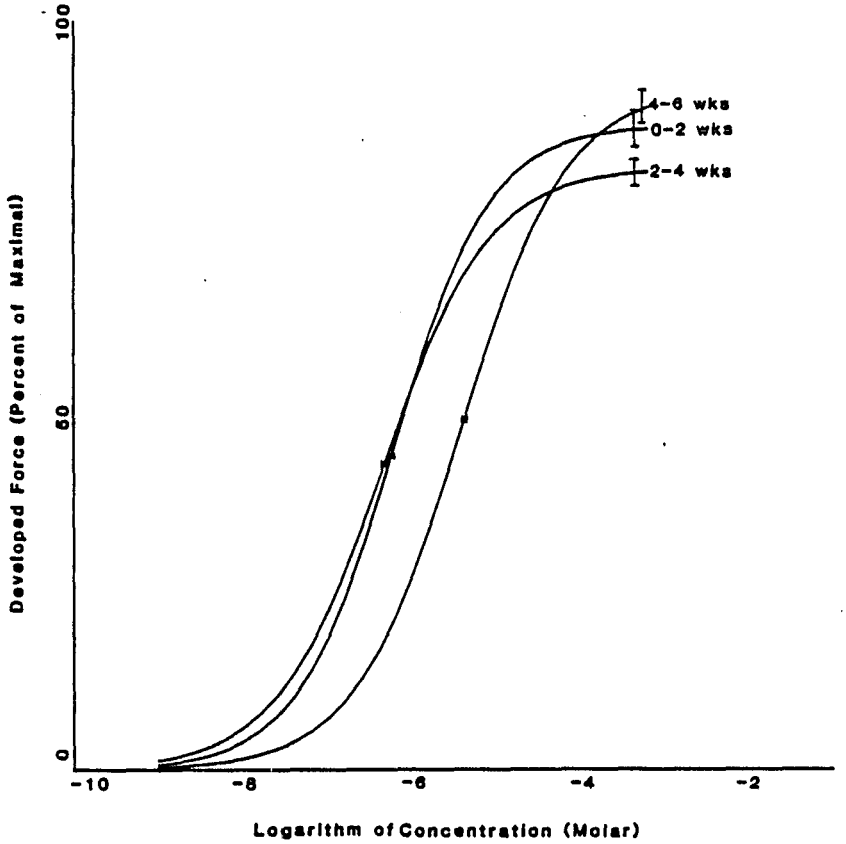


Fig. 47. Contractile Responses of the Dog Femoral Artery to Norepinephrine at Various Ages.

Developed force as a percent of maximal contractile response to potassium chloride is shown for norepinephrine at 3 different ages in the dog femoral artery.

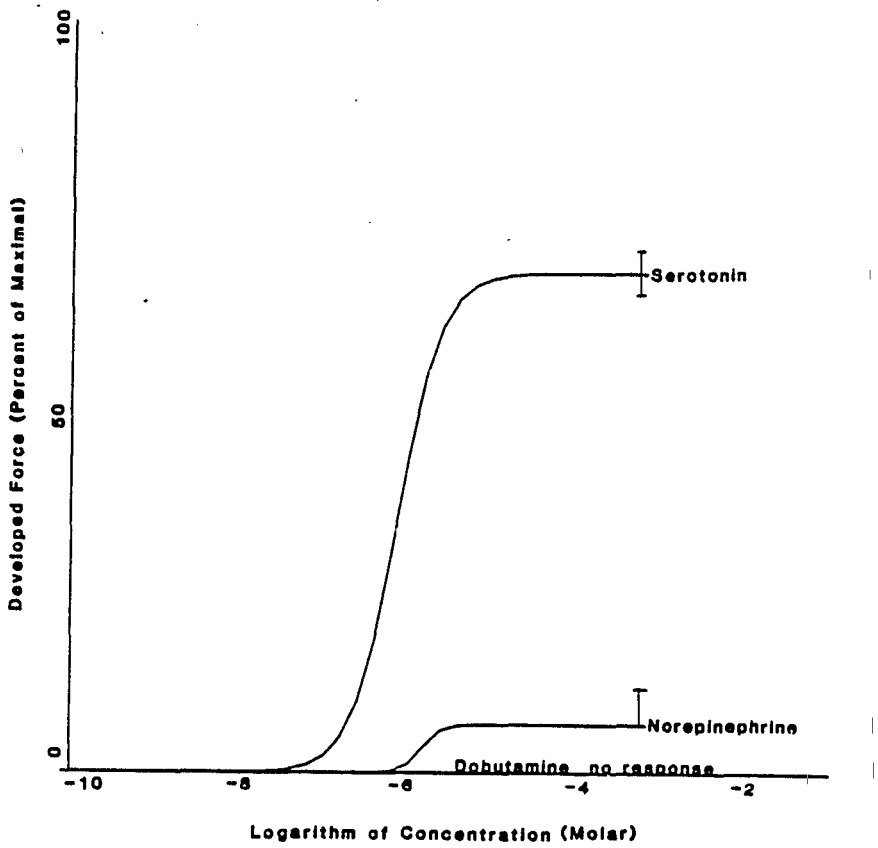


Fig. 48. Contractile Responses for the Pulmonary Artery of the 0-2 Week Age Dog.

Developed force as a percent of maximal contractile response to potassium chloride is shown for serotonin (N = 8) and norepinephrine (N = 8) in pulmonary artery of the 0-2 week age dog. Dobutamine (N = 9) produced no response in any of the tissues studied at this age.

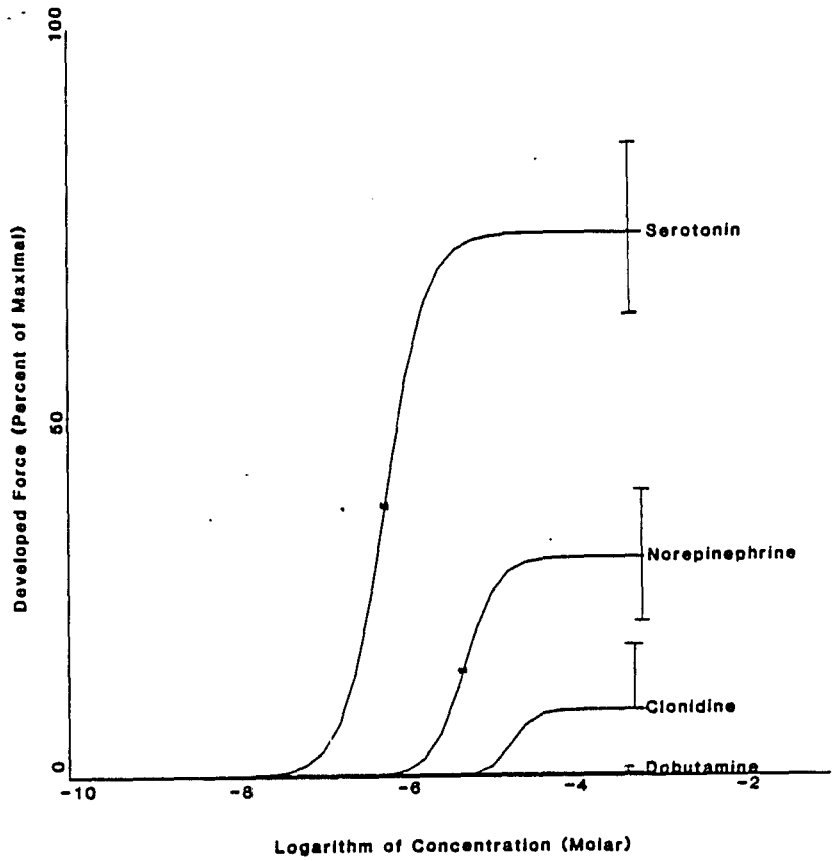


Fig. 49. Contractile Responses for the Pulmonary Artery of 2-4 Week Age Dog.

Developed force as a percent of a maximal contractile response to potassium chloride in the dog pulmonary artery from 2-4 weeks of age is shown for serotonin (N = 4), norepinephrine (N = 13), clonidine (N = 2) and dobutamine (N = 7).

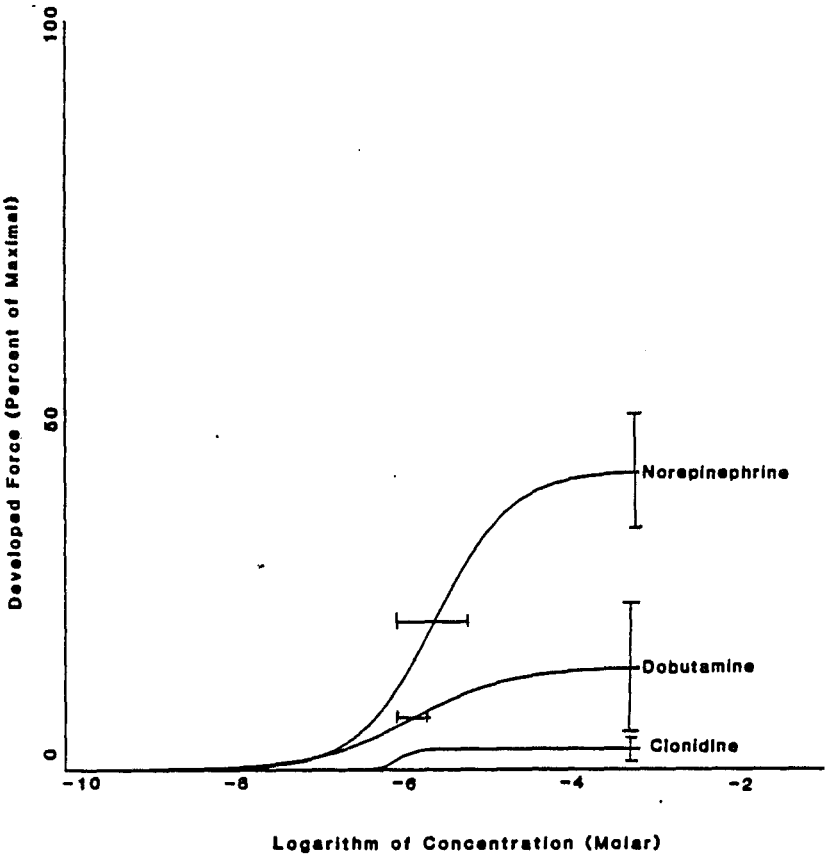


Fig. 50. Contractile Responses for the Pulmonary Artery of the 4-6 Week Age Dog.

Developed force as a percent of a maximal contractile response to potassium chloride in the dog pulmonary artery from 4-6 week of age is shown for norepinephrine (N = 5), dobutamine (N = 2), and clonidine (N = 3).

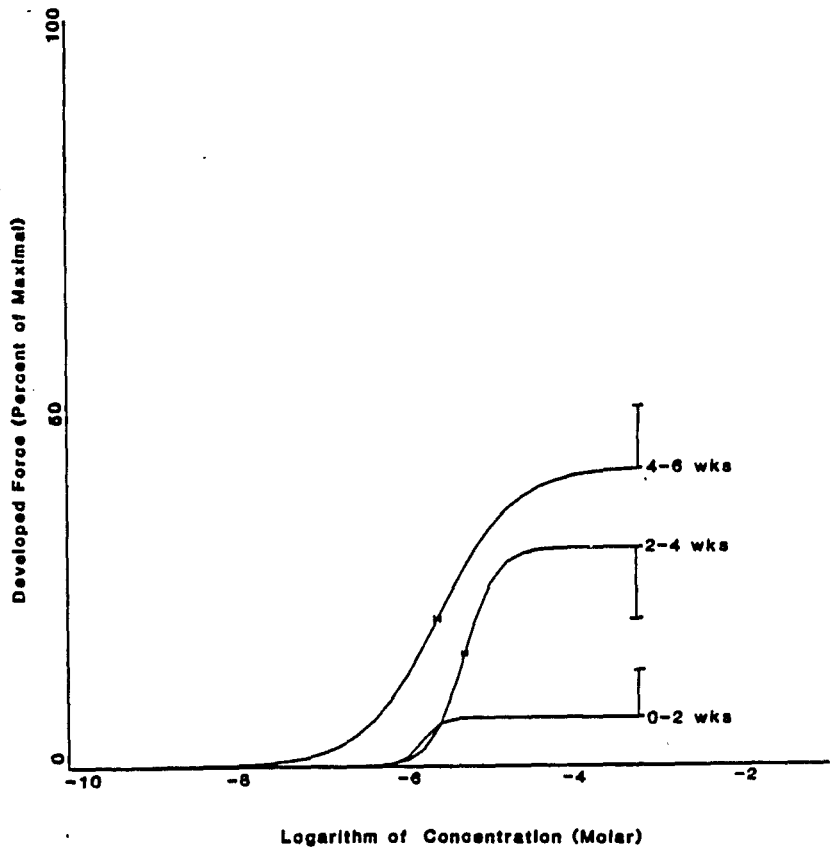


Fig. 51. Contractile Responses to Norepinephrine in the Pulmonary Artery of the Dog at Various Ages.

Developed force to norepinephrine as a percent of maximum contractile force to potassium chloride as a function of age in the dog pulmonary artery is shown.

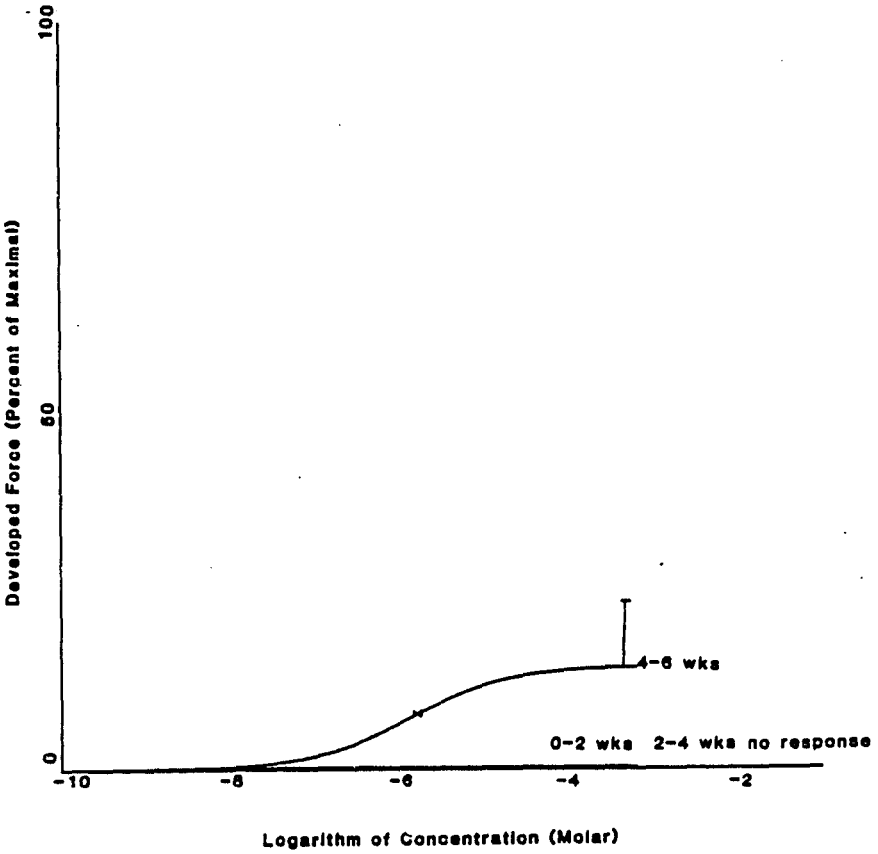


Fig. 52. Contractile Responses to Dobutamine in the Pulmonary Artery of the Dog at Various Ages.

Developed force to dobutamine as a percent of maximal contractile force to potassium chloride in the dog pulmonary artery is shown as a function of age.

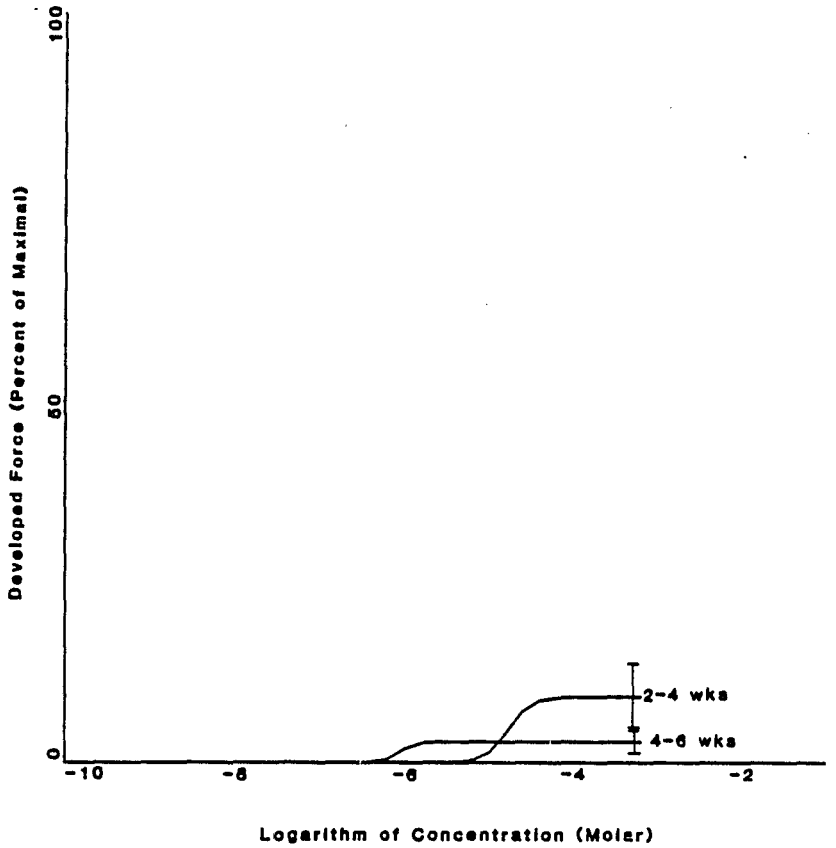


Fig. 53. Contractile Responses to Clonidine in the Dog Pulmonary Artery at Various Ages.

Developed force as a percent of the maximal contractile force to potassium chloride is shown for clonidine in the dog pulmonary artery as a function of age.

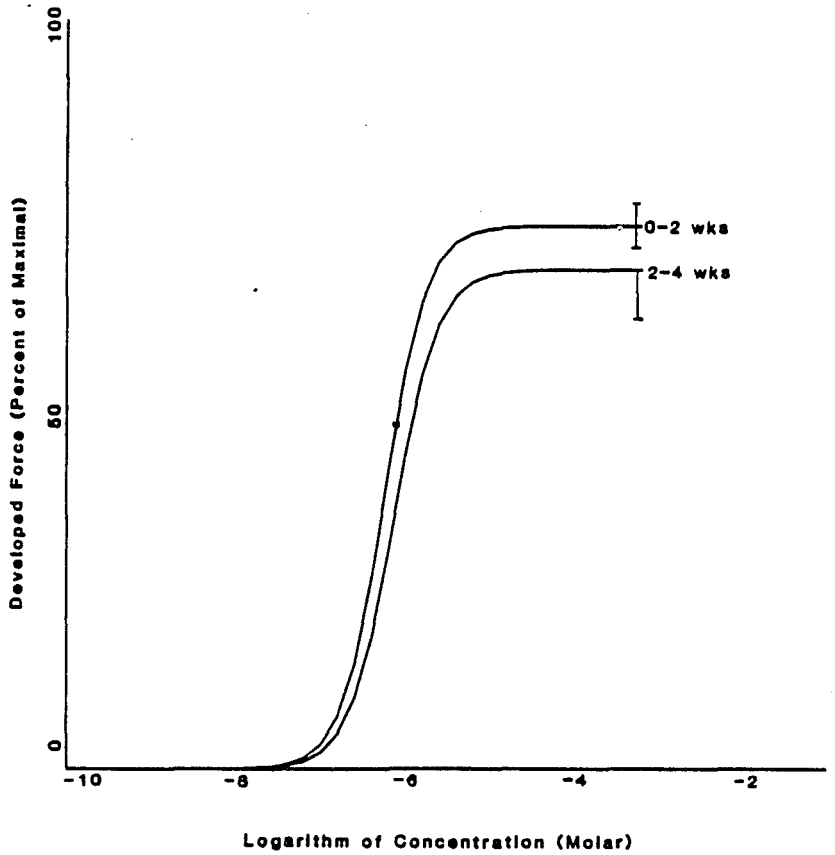


Fig. 54. Contractile Responses to Serotonin in the Dog Pulmonary Artery at Various Ages.

Developed force as a percent of the maximal contractile force to potassium chloride is shown for serotonin in the dog pulmonary artery as a function of age.

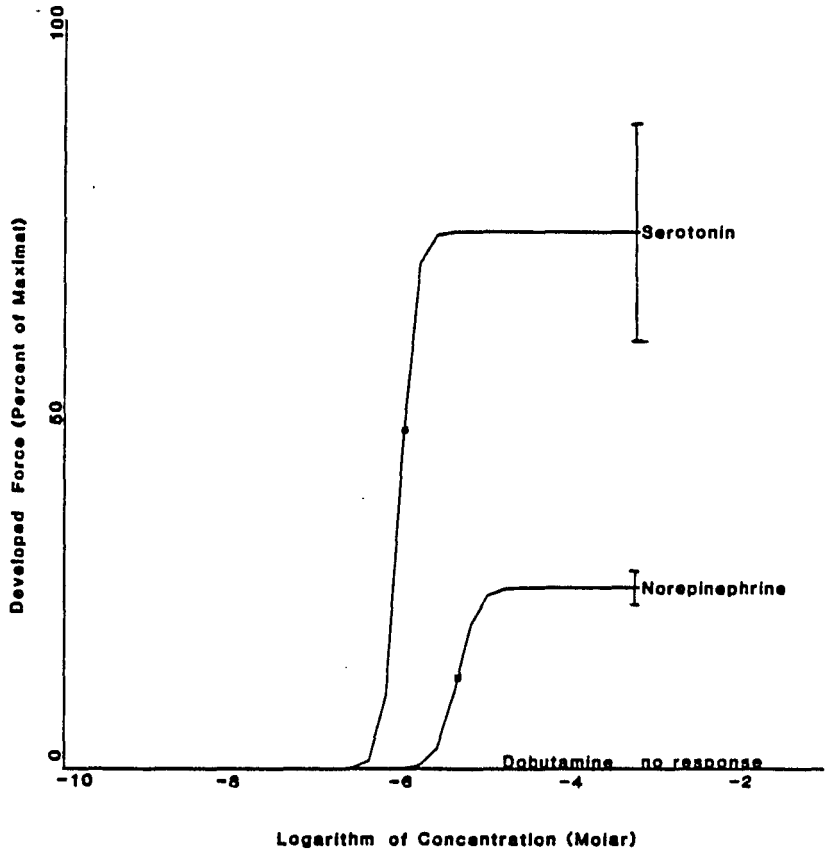


Fig. 55. Contractile Responses to Dobutamine in the Dog Aorta at 0-2 Weeks of Age.

Developed force as a fraction of maximal contractile response to potassium chloride is shown for serotonin (N = 2) and norepinephrine (N = 2) in the 0-2 week age dog aorta. Dobutamine produced no response in any of the tissues tested (N = 2). These data are from litter mates.

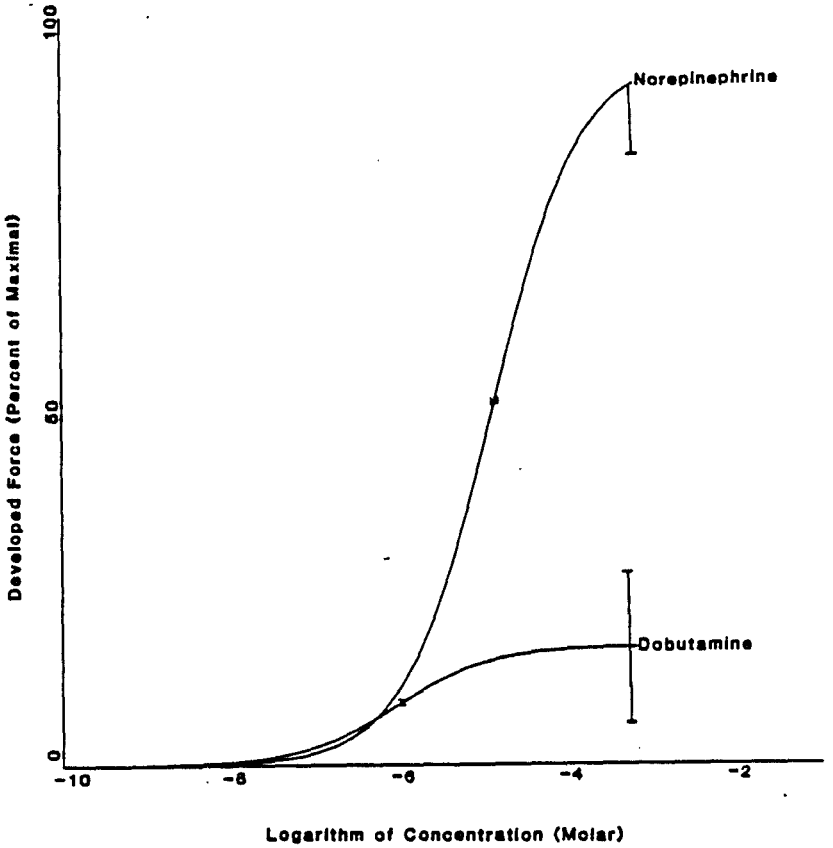


Fig. 56. Contractile Responses in the 2-4 Week Age Dog Aorta.

Developed force as percent of maximal contractile response to potassium chloride is shown for norepinephrine (N = 2) and dobutamine (N = 2) in the 2-4 week age dog aorta. These data are for litter mates.

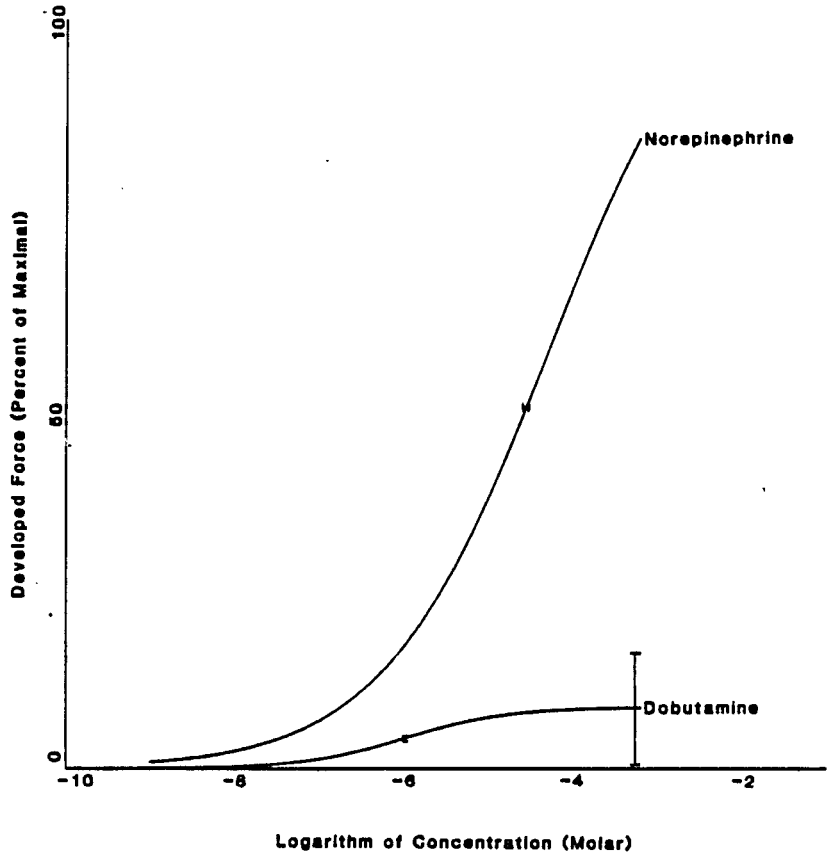


Fig. 57. Contractile Responses in the 4-6 Week Age Dog Aorta.

Developed force to norepinephrine (N = 2) and dobutamine (N = 2) as a percent of maximal contractile response to potassium chloride in the dog aorta from 4-6 weeks of age is shown. These data are for litter mates.

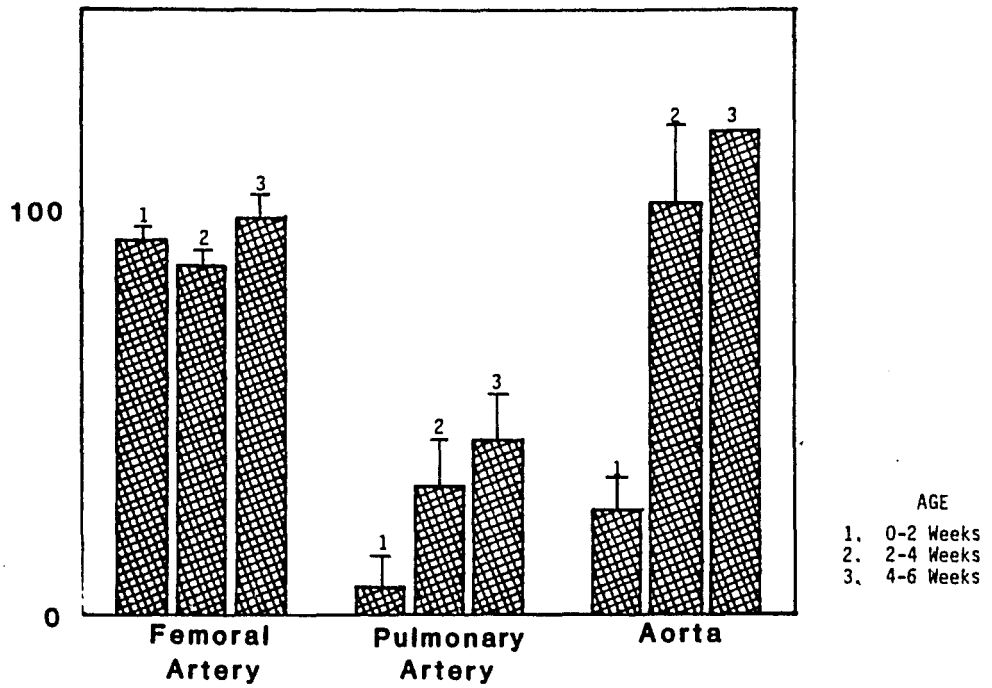


Fig. 58. Summary of Maximal Responses to Norepinephrine as a Function of Age in the Dog Femoral and Pulmonary Arteries and Aorta.

Maximal Responses to norepinephrine from the previous data are combined for the three tissues studied.

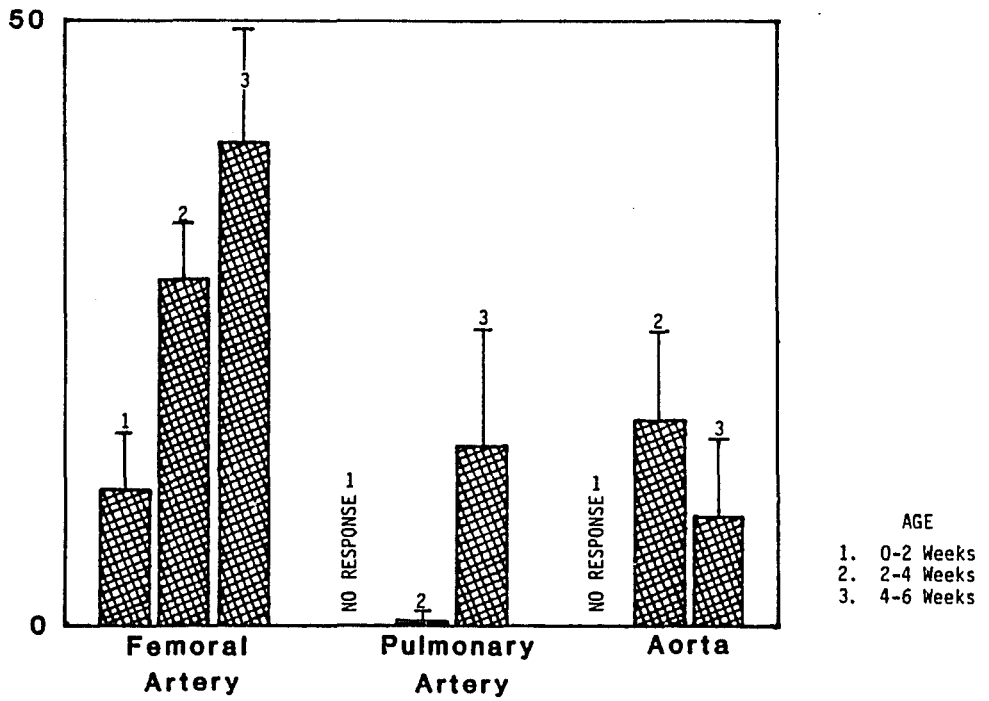


Fig. 59. Summary of Maximal Responses to Dobutamine in the Dog Femoral and Pulmonary Arteries and Aorta.

Maximal responses to dobutamine from the previous data are combined for the three tissues studied.

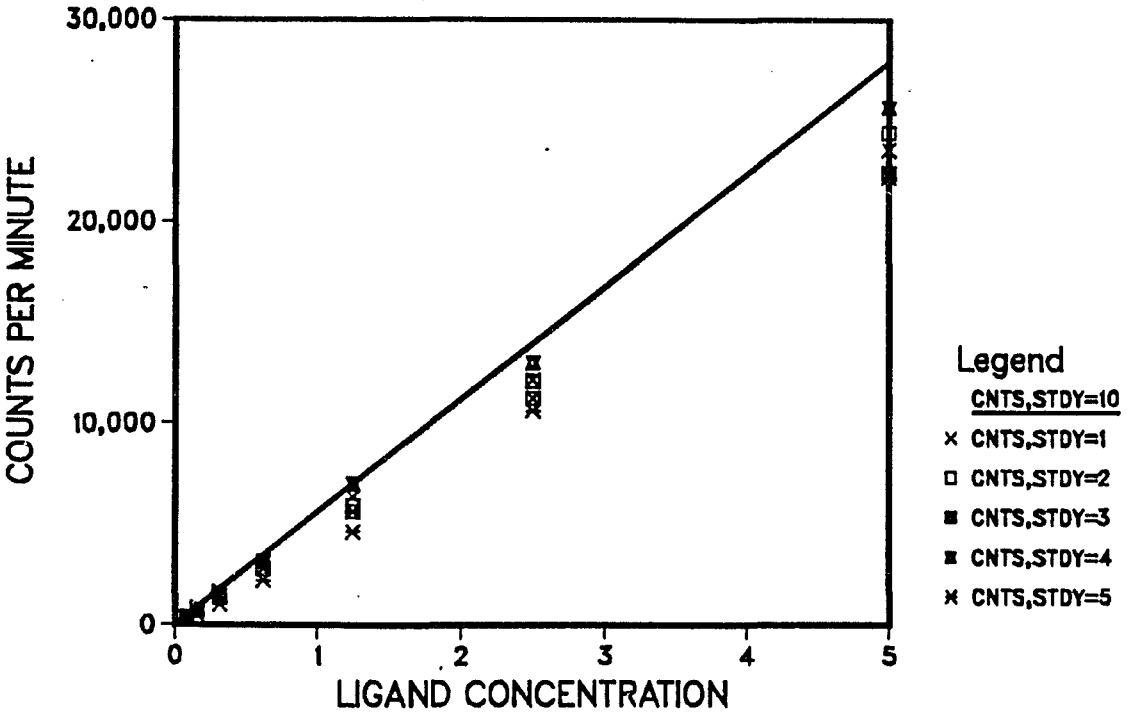


Fig. 60. Recovery of 3H Prazosin in Standard Curves.

The ability to produce standard ligand concentrations in the nanomolar range for the ligand prazosin is shown. The straight line gives the exact predicted count per minute based on manufacturers specific activity and concentration data.

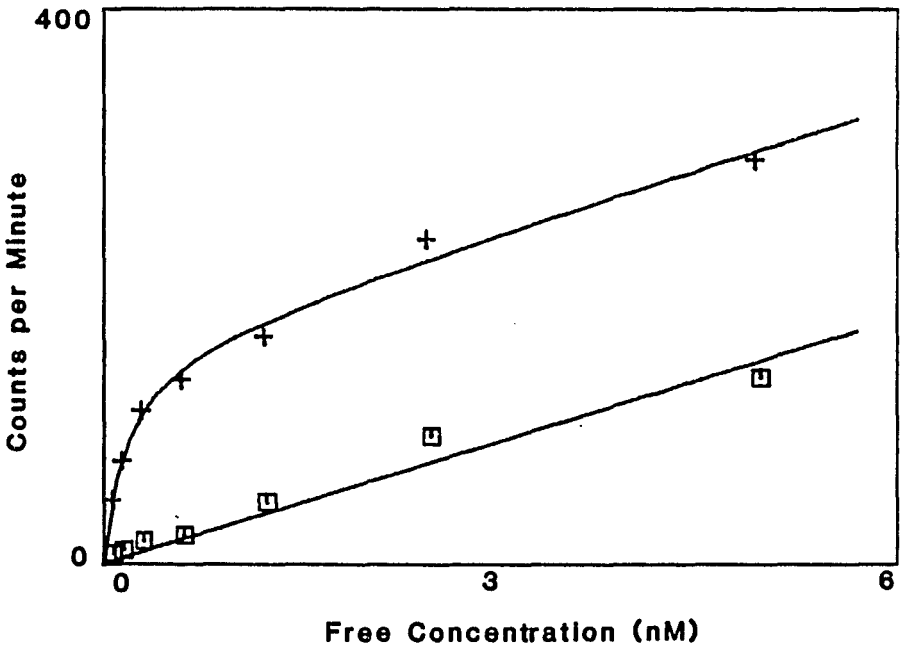


Fig. 61. Total and Non Specific Binding Activity for Prazosin in Rabbit Aorta.

A raw data set for the rabbit aorta is shown as counts per minute of bound prazosin in the presence and absence of norepinephrine. These curves were fitted simultaneously to produce the lines of best fit as shown.

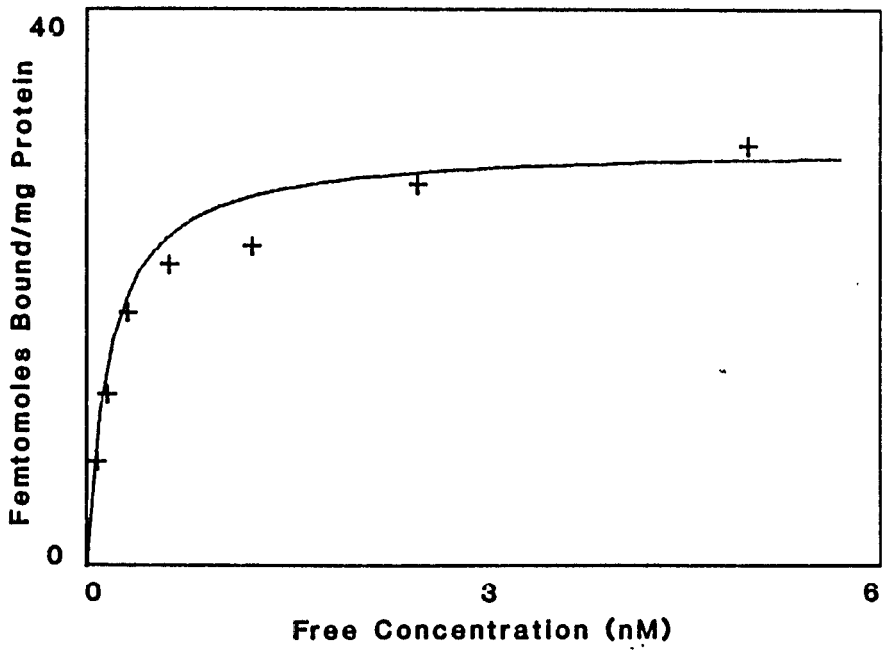


Fig. 62. Specific Binding of Prazosin to Rabbit Aorta.

The data from figure 61 is converted to femtomoles of prazosin bound/mg membrane protein versus free ligand concentration producing a simple saturable curve.

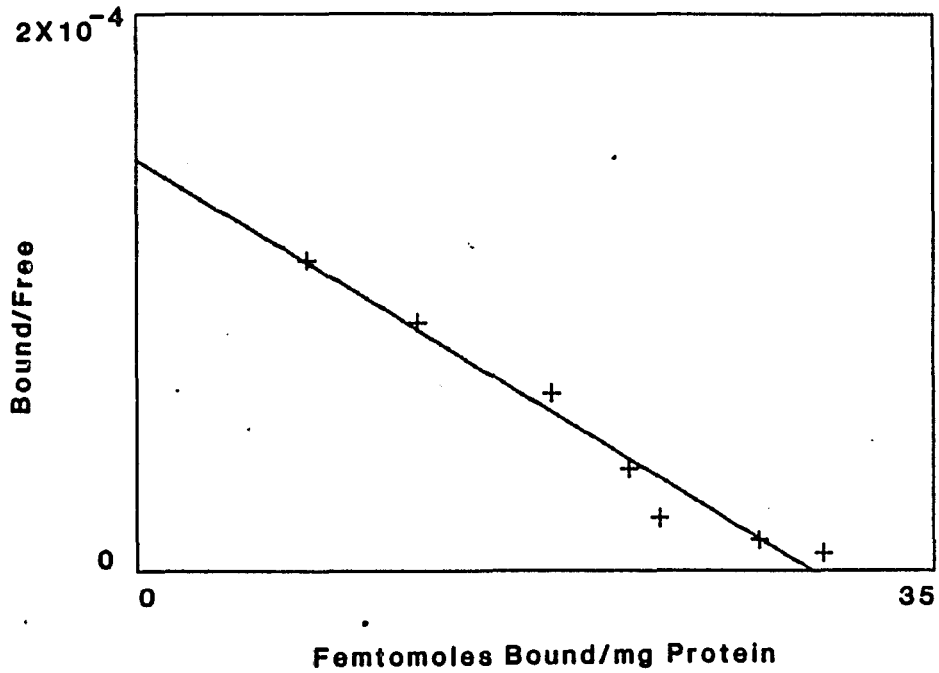


Fig. 63. Scatchard Analysis of Prazosin Binding to Rabbit Aorta.

A Scatchard plot for data from figure 62 is shown.

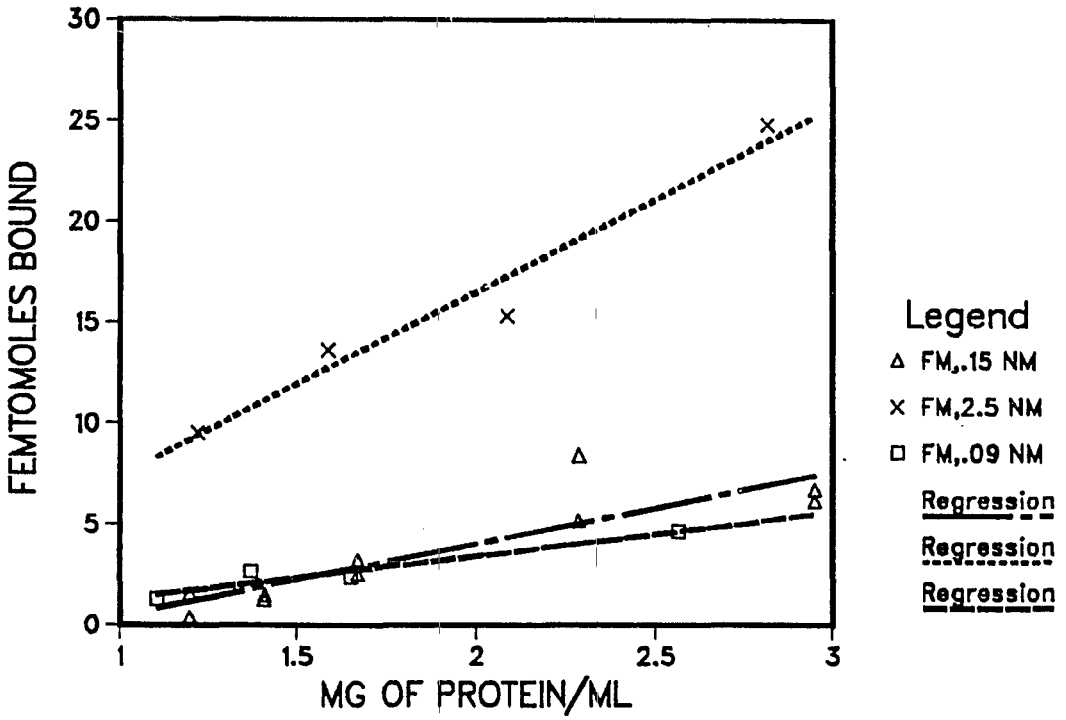


Fig. 64. Specific Binding as a Function of Protein Concentration.

The data points and regression curves for 3 ligand concentrations are shown expressed as specific binding of prazosin versus protein concentration.

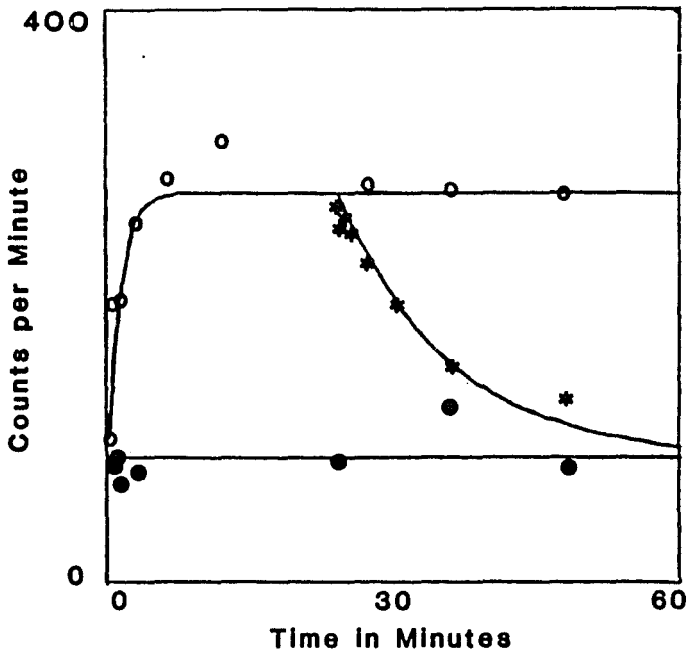


Fig. 65. Studies on the Rate of Formation of the Prazosin Receptor Complex.

Binding of prazosin as counts per minute is shown as a function of time for non specific binding ( ) total binding (o) and the displacement of bound prazosin by norepinephrine (*). Rates for complex formation and breakdown were calculated from these rate plots.

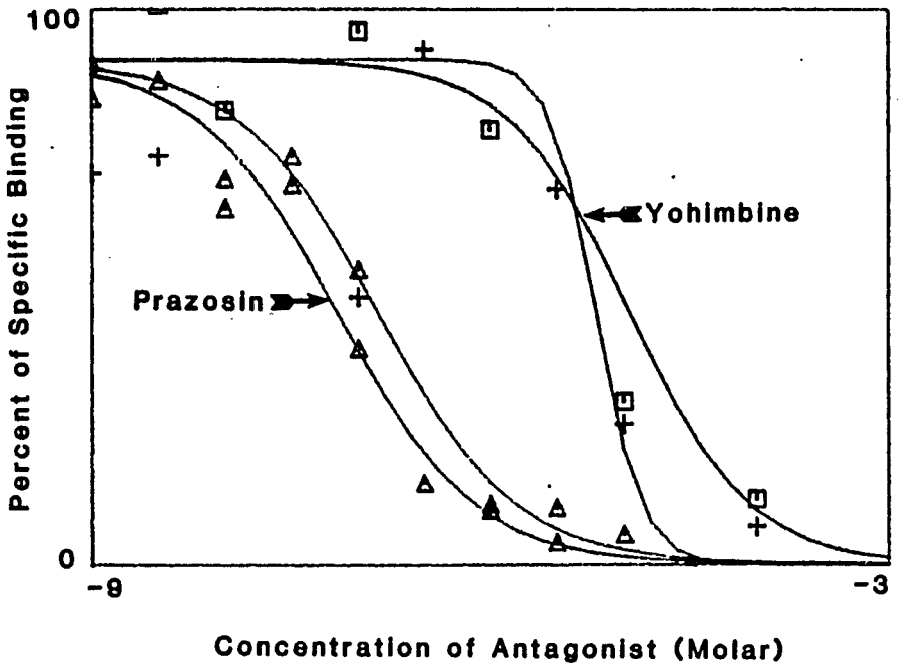


Fig. 66. Rank Order of Potency for Displacement of 3H Prazosin from Rabbit Aortic Membranes.

The displacement of prazosin from rabbit aortic membranes is shown as a percent of specific binding versus concentration of antagonist. Prazosin displaced 3H prazosin more effectively than yohimbine.

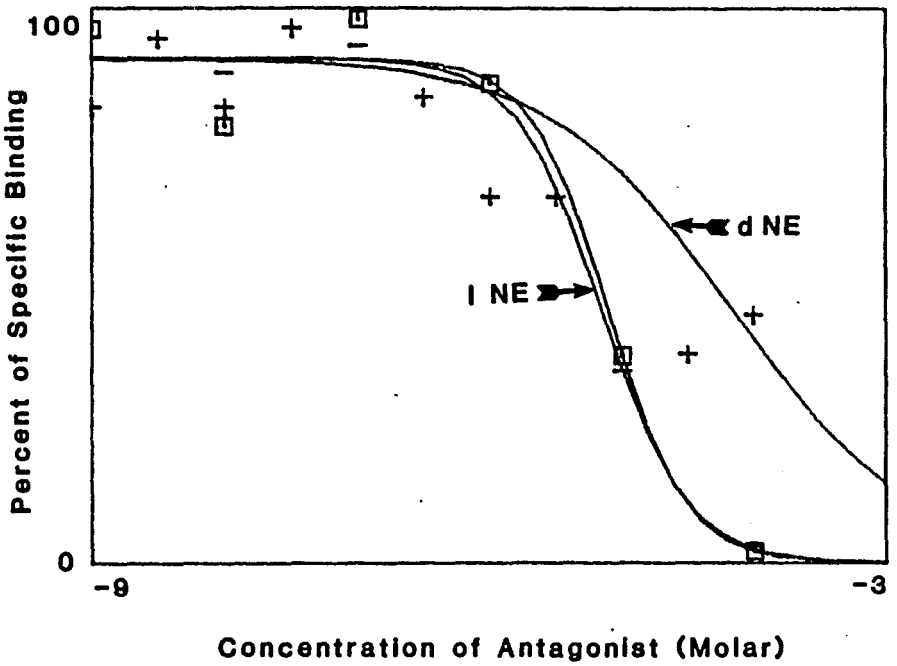


Fig. 67. Displacement of 3H Prazosin from Rabbit Aorta by l and d Norepinephrine.

Displacement of specific binding of prazosin to rabbit aortic membranes by l and d norepinephrine is shown.

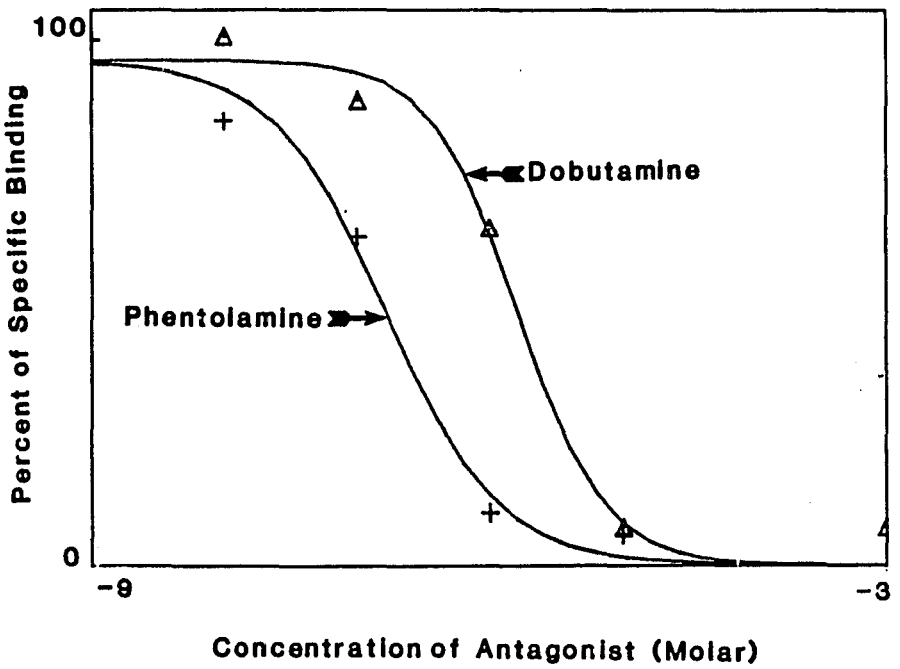


Fig. 68. The Displacement of 3H Prazosin in Rabbit Aorta by Phentolamine and Dobutamine.

Specific binding of 3H prazosin is shown in the response of increasing concentrations of dobutamine and phentolamine.

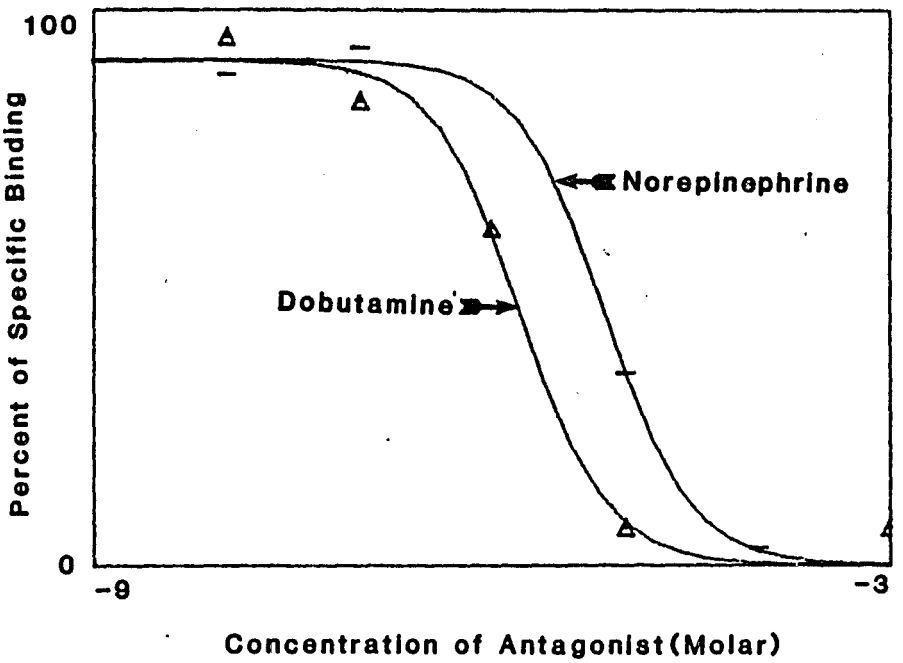


Fig. 69. Displacement of 3H Prazosin Binding by Dobutamine and Norepinephrine.

Specific binding of prazosin to rabbit aortic membranes is shown in the presence of increasing concentrations of dobutamine and norepinephrine.

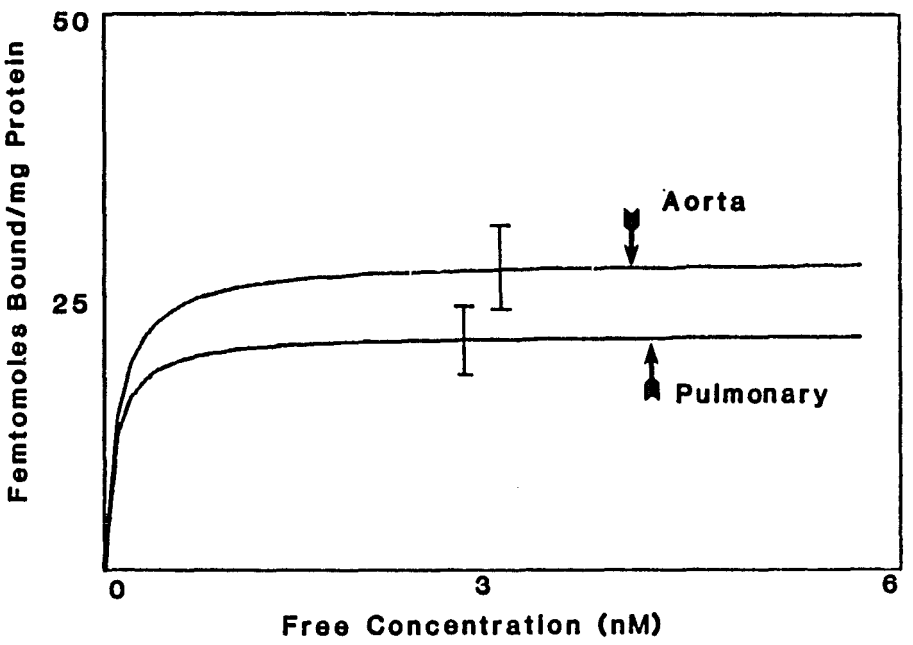


Fig. 70. Summary Data for Binding of 3H Prazosin to Rabbit Vascular Membranes.

Specific binding of 3H prazosin to the rabbit pulmonary artery (N = 4) and the rabbit aorta (N = 7) is shown as a function of ligand concentration. B_{max} is shown ($\pm$ S.E.M.)

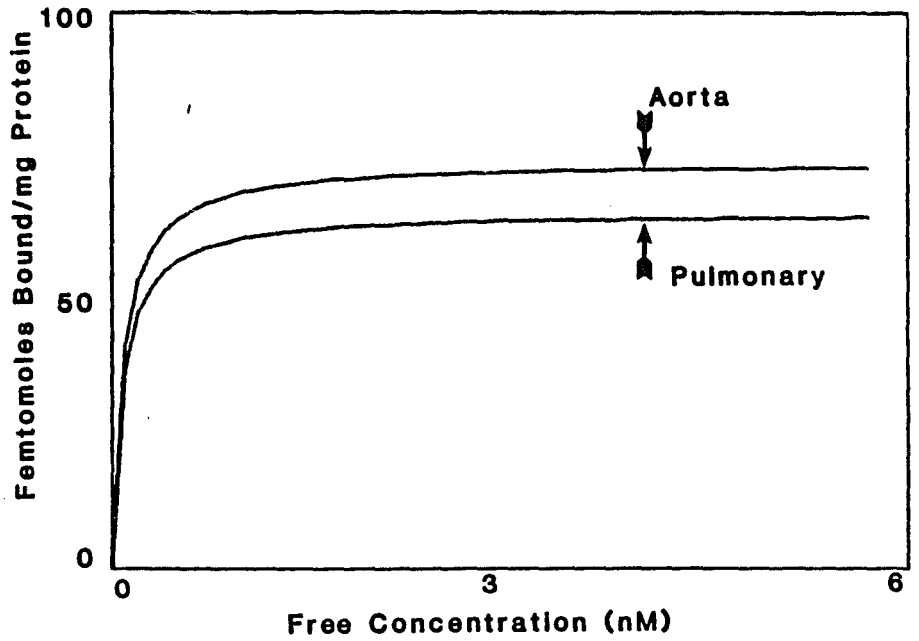


Fig. 71. Summary Data for Binding of 3H Prazosin in Dog Vascular Membranes.

Specific binding of 3H prazosin in the dog pulmonary artery (N = 2) and aorta (N = 2) is shown as a function of free ligand concentration.

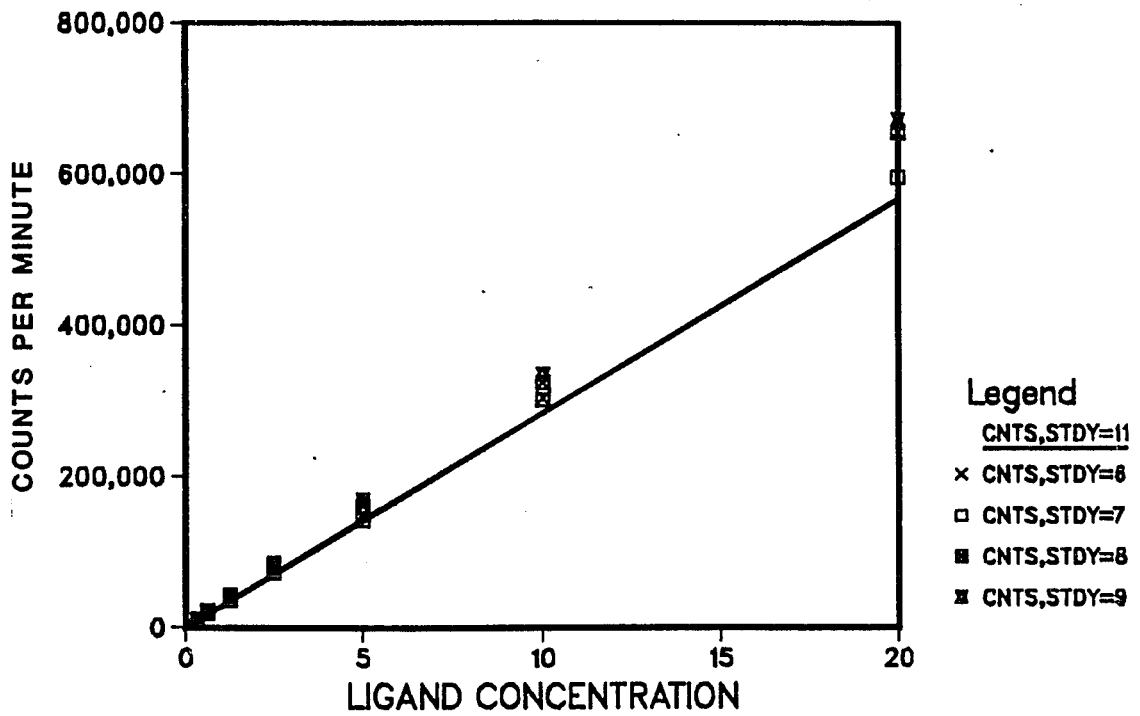


Fig. 72. Standard Curves for 3H Rauwolscine.

Recovery and reproducibility of 3H rauwolscine in standard curves is shown as counts per minute as a function of ligand concentration. The staight line is the predicted standard curve based on specific activity and concentration.

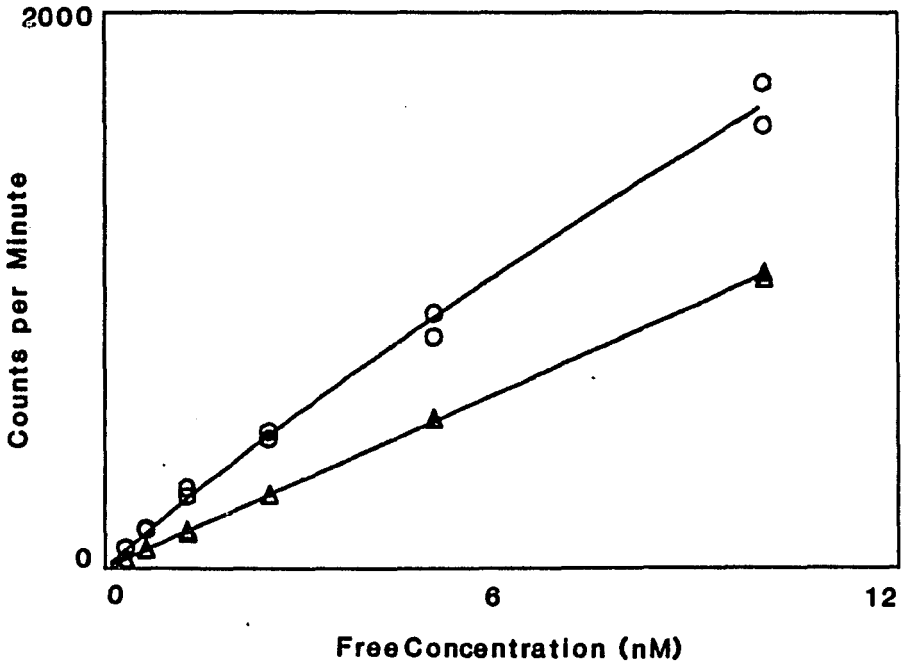


Fig. 73. Total and Non Specific Binding of 3H Rauwolscine to Rabbit Aorta.

The total and non specific binding of 3H rauwolscine is shown as a function of free ligand concentration for duplicate samples from the rabbit aorta. The lines represent nonlinear regression analysis of this data.

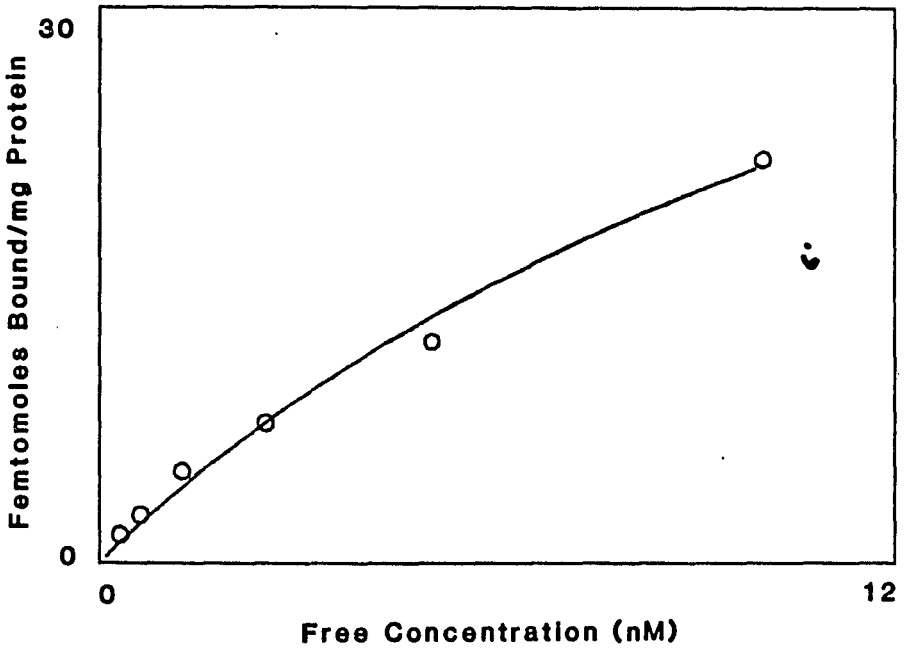


Fig. 74. Specific Binding of 3H Rauwolscine to Rabbit Aorta.

Specific binding in femtomoles/mg protein is shown for 3H rauwolscine as a function of free ligand concentration in the rabbit aorta. The line shows the non linear regression of this data.

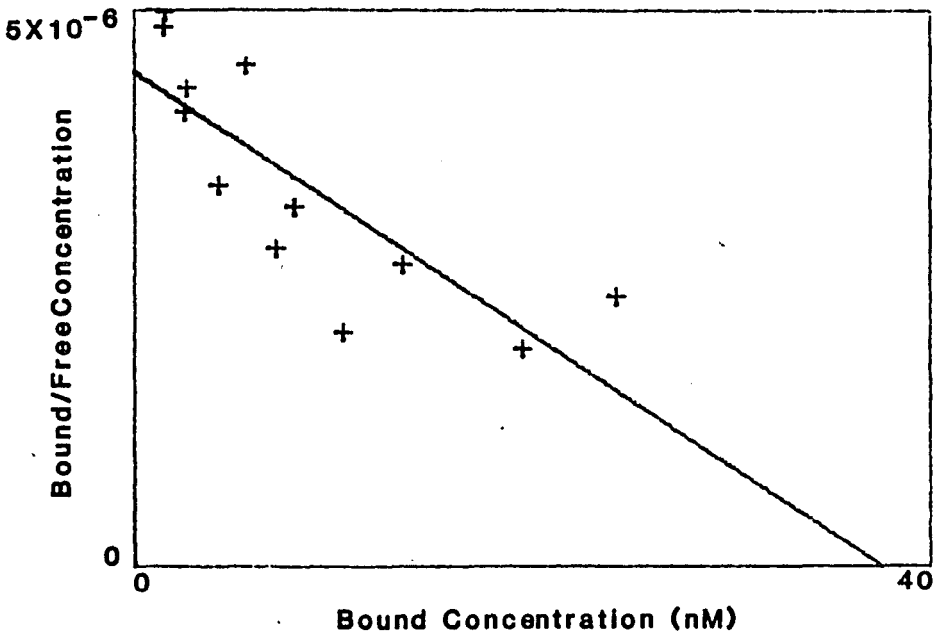


Fig. 75. Scatchard Analysis of 3H Rauwolscine Binding in Rabbit Aorta.

A Scatchard plot of the data from figure 74 is shown.

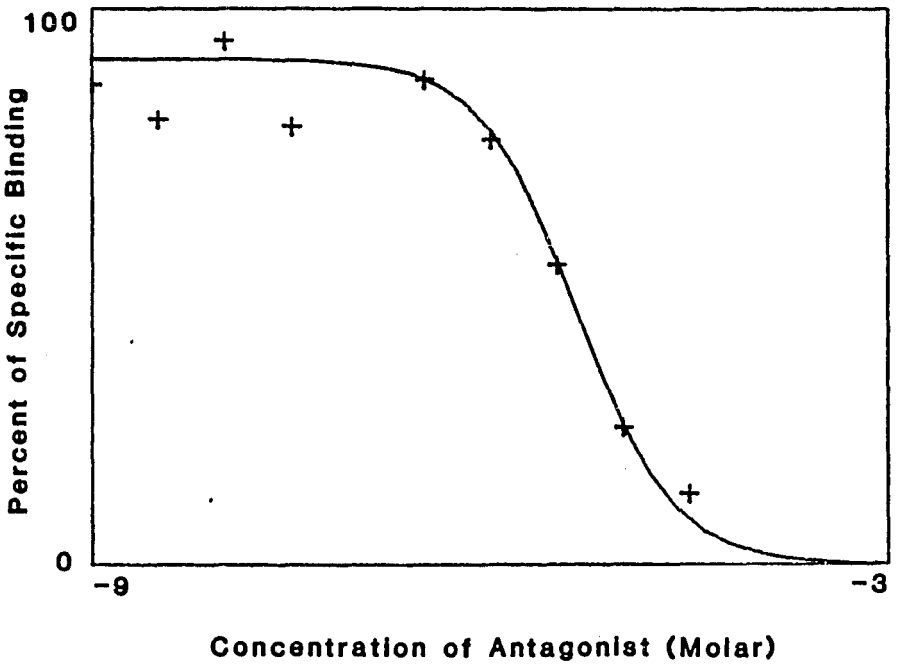


Fig. 76. Displacement of 3H Rauwolscine Binding by Yohimbine.

Displacement by yohimbine of specific binding of 3H rauwolscine is shown. Prazosin used in a similar manner produced no discernable displacement (N = 3).

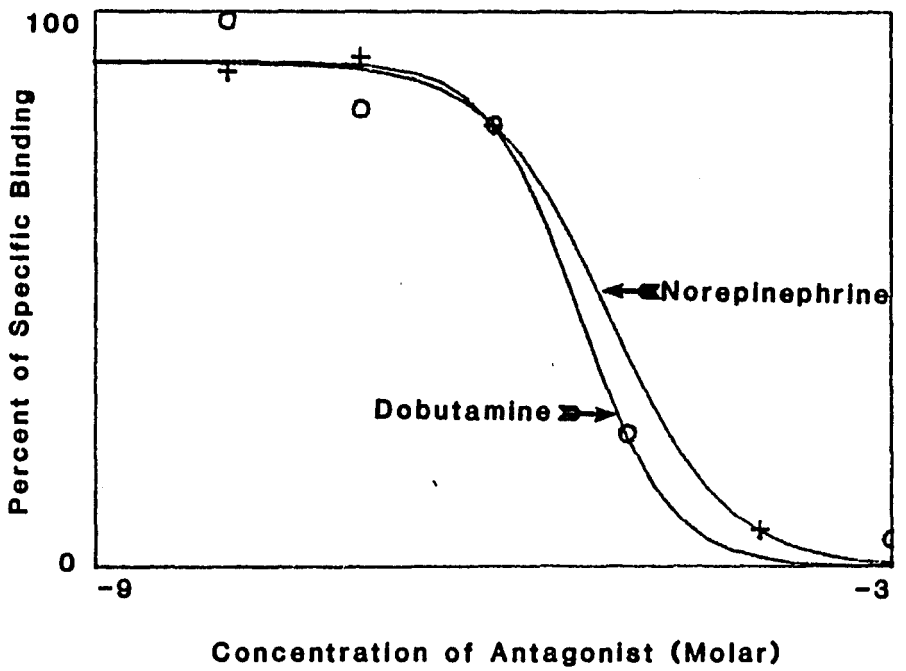


Fig. 77. Displacement of 3H Rauwolscine by Dobutamine and Norepinephrine.

Displacement by dobutamine and norepinephrine of specific binding of 3H rauwolscine is shown as a function of concentration.

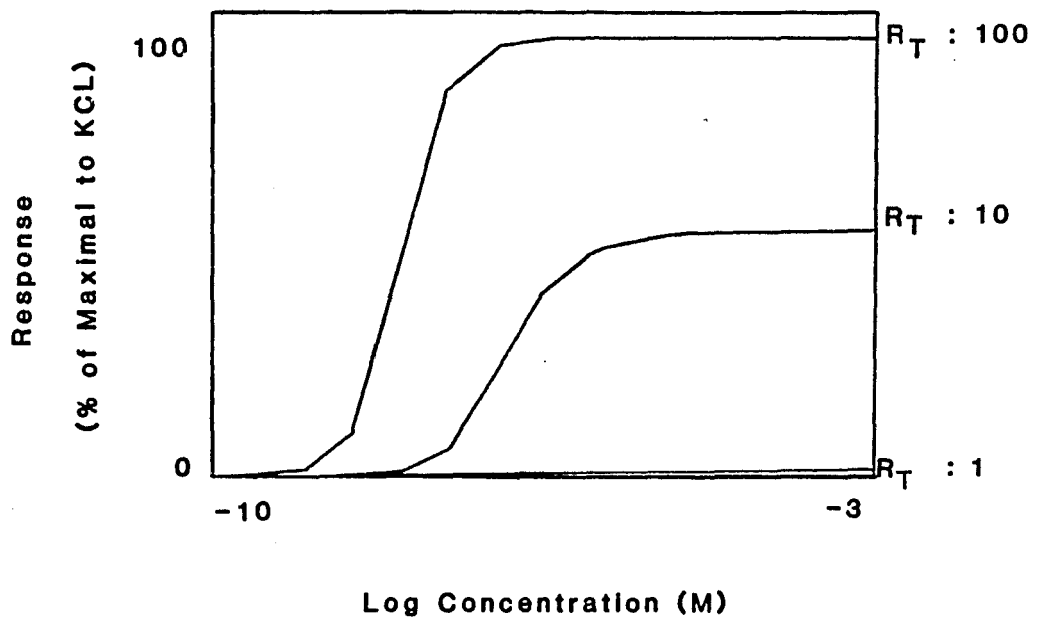


Fig. 78. A Model of the Concentration-Response Relationship for an Agonist with High Intrinsic Activity.

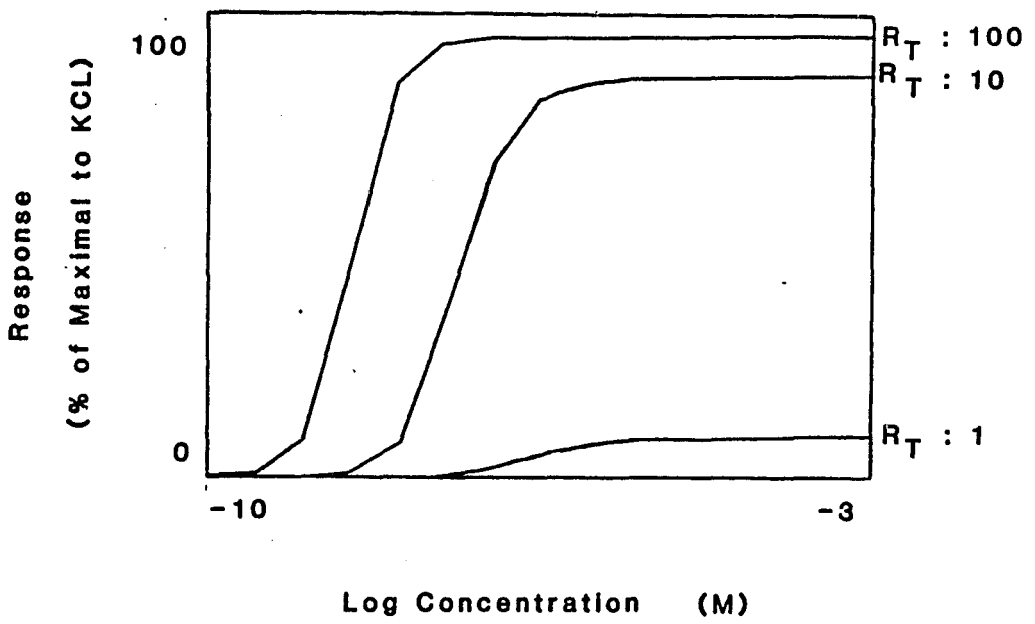


Fig. 79. A Model of the Concentration-Response Relationship for an Agonist with Low Intrinsic Activity.

A plot of response as a percentage of maximum versus the logarithm of concentration. This plot uses equation (21) with values of K_{RAE} = 80, N = 1, and $K_D = 1 \cdot 10^{-7}$ M. The value of RT is varied as shown.

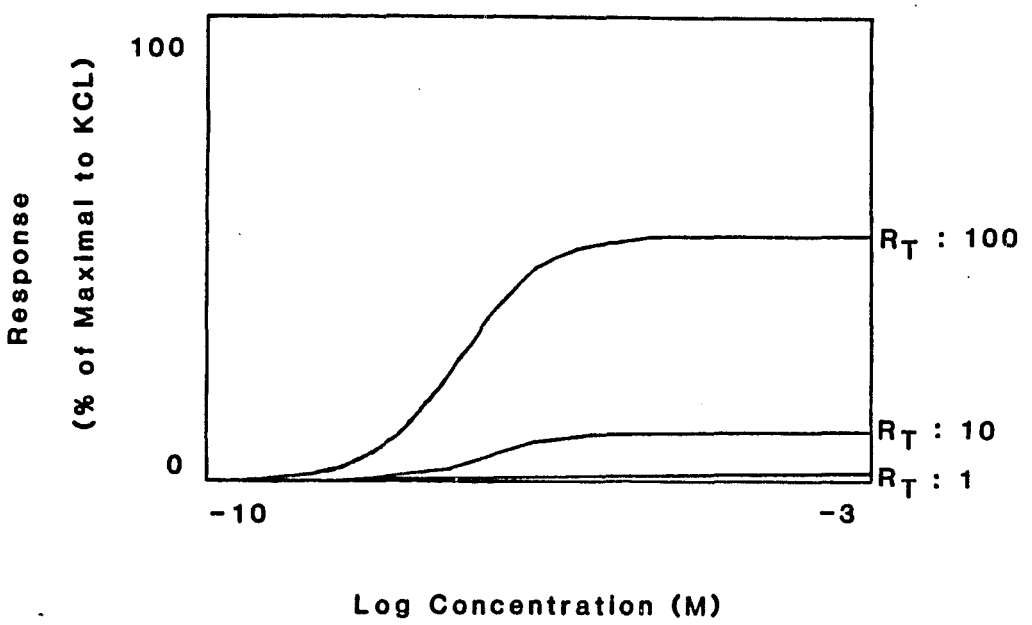


Fig. 80. A Concentration-Response Model with Positive Cooperativity.

A plot of response as a percentage of maximum versus the logarithm of agonist concentration. This plot uses equation (21) with values of K_{RAE} = 10, N = 2, and $K_D = 1 \cdot 10^{-7}$ M. The value of RT is varied as shown.

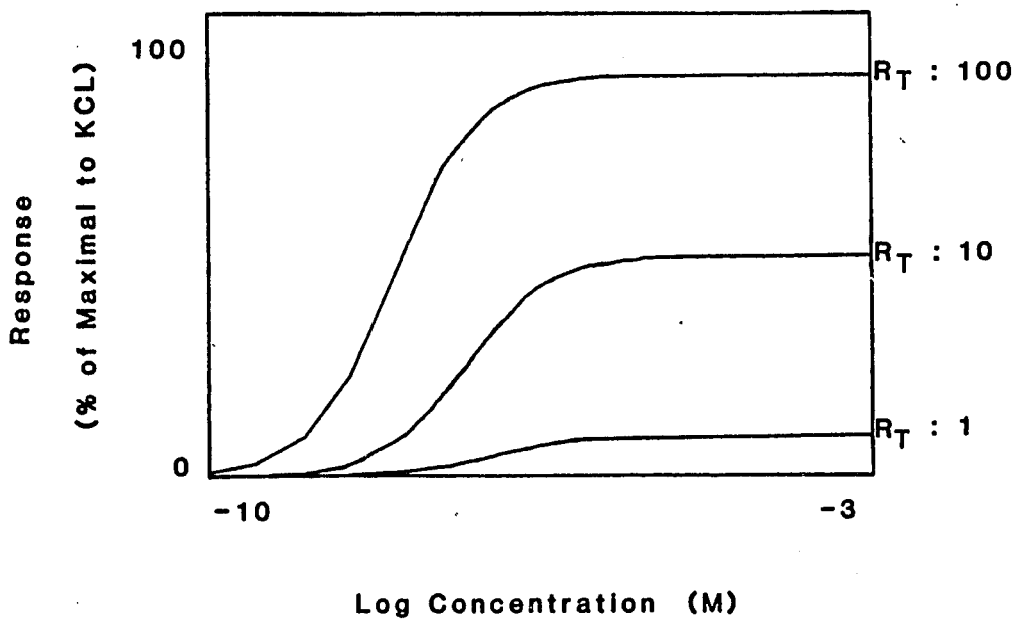


Fig. 81. A Concentration-Response Model with Negative Cooperativity.

A plot of response as a percentage of maximum versus the logarithm of agonist concentration. This plot used equation (21) with values of K_{RAE} = 10, N = 0.5, and $K_D = 1 \cdot 10^{-7}$ M. The value of RT is varied as shown.

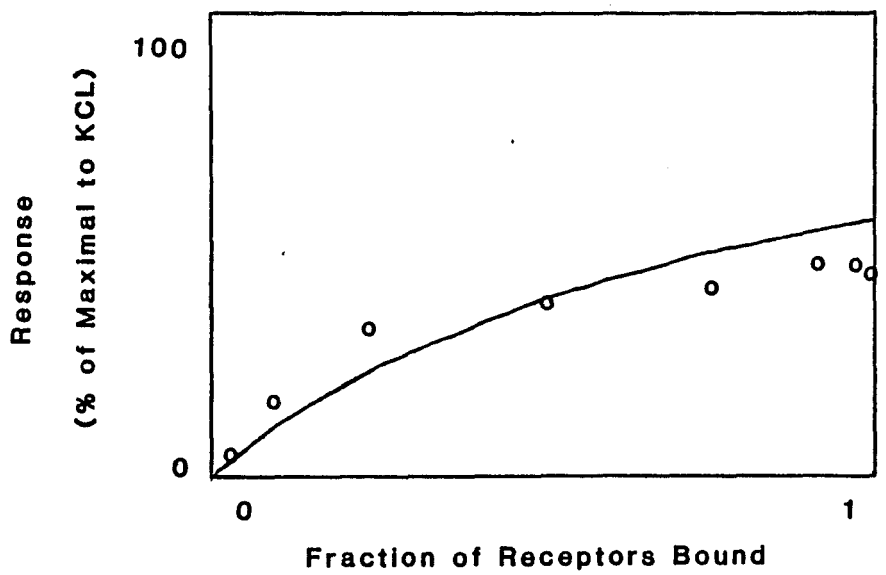


Fig. 82. The Relationship of Receptor Binding to Response for a Drug with Low Intrinsic Activity.

The relationship between fractional receptor binding and fractional response is plotted. The theoretical data plotted is for an agonist with a low intrinsic activity as in Figure 79. The superimposed data points represent actual response data obtained for dobutamine with the K_D value obtained by the method of Furchgott.

CHAPTER 4

DISCUSSION

Each question posed at the end of the introduction will first be discussed individually, followed by a summary to relate all the findings.

What are the Alpha Adrenergic Properties of Dobutamine?

In view of the important role of the concentration response curve in this dissertation, the choice of methods of analysis of these curves needs strong consideration. The approach of averaging responses for each concentration is obviously unacceptable because it fails to provide any method for comparison of curves. Maximal responses are based solely on the mean and standard deviation of the single response point rather than the entire data set. An ED_{50} value can be estimated but without determination of variability.

Analyzing an entire set of data points by nonlinear regression produces a single curve with estimates of variability of the parameters, but as shown in figure (1) the slope of this curve may be biased as described by Waud (1975) for averaged data. In the example of figure 1 the concentration response curves were also individually fitted, and the parameters averaged yielding a single curve. As can be seen the slope of the curves differed depending upon the method used

for analysis. Figure 2 shows that this difference in analytic approach may have only a minimal influence on some data sets. Thus it would seem that the biased effect of averaged data observed by Waud can also occur if nonlinear regression is used on grouped data. With this in mind all concentration response curves in the dissertation were fitted independently and estimates from each curve treated as though normally distributed.

Contractile Responses

Dobutamine in the presence of propranolol caused vascular smooth muscle to contract. In comparison to norepinephrine, dobutamine produced a consistently lower maximum in the femoral artery of both rabbits and dogs. Similar results were found in the rabbit pulmonary artery, while the differences in maximum response were even more striking in the dog pulmonary artery. Two possible lines of explanations for this finding are: 1) norepinephrine and dobutamine are acting via different receptor types or 2) dobutamine has the characteristics of a partial agonist on the alpha adrenergic receptor.

Current techniques of in vitro study do not allow direct confirmation of a drug as a partial agonist. Support for a drug as a partial agonist is based upon excluding multiple receptors, indirect drug actions or non specific effects of the drug on vascular smooth muscle. When these have been excluded, a drug producing a lower maximal response is thought to be a partial agonist.

Support for the idea that dobutamine acts via alpha adrenergic receptors is shown in figure 7. Responses to norepinephrine are antagonized by dobutamine in the presence of propranolol, suggesting that dobutamine can occupy alpha adrenergic receptors although in some tissues it may not have sufficient efficacy to produce a resonse on its own. The pattern of antagonism in this figure is not the classical pattern of competitive inhibition with a shift to the right of the concentration response curve and no change in maximal response. This divergence from classical antagonism has been well described although not understood (Goldstein et al, 1974). This author has reviewed the effect of a high affinity antagonist on a weak agonist and the failure of this competitive inhibition to fit classical antagonism. There exists at this time no mathematical model that would explain this phenomenon for a competitive inhibition.

As further evidence for the action of dobutamine and norepinephrine on the same receptor, determination of pA_2 values was undertaken. Determination of antagonist dissociation constants should be independent of the agonist used if the agonists are acting on the same receptor population (Schild 1957). While this is an accepted principle the question arises as to what magnitude of difference is large enough to support the concept of multiple receptors. Ruffolo et al (1982) have used 95% confidence intervals to define differences in pA_2 values. This requires large numbers of studies. In general differences of less than 1 log unit were not significant. While this is not rigorous evidence it can provide some criteria for expected variations. Table 1 shows a series of values obtained from a single

set of paired concentration response curves in the presence of a large amount of antagonist. These appeared to show no difference between dobutamine and norepinephrine. No differences of the magnitude seen by Ruffolo were observed in the data in table 1. This data set while showing interval consistency produced values for pA_2 that were lower than the values reported by Kenakin (1981). This may have been a reflection of non specific effects of the high concentrations of antagonists used in our studies.

In view of this discrepancy the more definitive Schild plot was used to determine pA_2 values (figures 9, 10, 11, 12). These studies produced values consistent with those reported elsewhere and also showed the same consistency between norepinephrine and dobutamine (Table 2).

Further proof that dobutamine acts at a single postsynaptic receptor site would be to demonstrate similar agonist dissociation constants in several tissues using the method of Furchgott. An assessment of the reproducibility of this method was undertaken for inter and intra tissue variability. As can be seen in table 3 and figure 19 the classical method of Furchgott appears to produce widely variable estimates of K_A for both norepinephrine and dobutamine although no striking differences between the drugs are apparent. Attempts at a nonlinear model to simultaneously fit the data sets similarly produced widely variable results unless the model was constrained to exponential values of 1 as can be seen in figure 19.

This simplification while greatly reducing the variability of the estimate of K_A values for both norepinephrine and dobutamine, depends on a model similar to that of Clark (1933) and thus does not allow for the concept of spare receptors described by Ariens (1957). The use of nonlinear regression to fit the independent data sets (figures 16, 17, 18) and transform the data using the method of Furchgott (1972) met with equally irreproducible results.

Despite the wide acceptance of the Furchgott method, critical reviews of the variability of the approach have not been found. As can be seen in figure 15, the general applicability of this method to a large number of data sets may be limited as it does not always produce a true linear double reciprocal relationship. A system of simultaneous nonlinear fitting using a series of equations similar to those suggested by the unconstrained nonlinear regression model seems the most reasonable approach however, without independent confirmation of the underlying relationship of receptor binding to response, it is difficult to justify. The method of Furchgott suggests that dobutamine has an agonist dissociation constant of the same order of magnitude as norepinephrine; however, the results show wide variability and require independent confirmation.

Effects of Dobutamine on Norepinephrine Release

Data on the direct release of norepinephrine by dobutamine was surprising. Based on the observations in vivo of Tuttle and Mills (1975) in the dog and Kho (1980) in the human dobutamine was not felt

to be capable of releasing endogenous norepinephrine. These authors found that the effects of dobutamine were not altered by pretreatment with desmethylimipramine, and thus concluded that dobutamine does not release norepinephrine. Thus a tyramine-like norepinephrine release was ruled out, but the possibility that dobutamine could act like amphetamine was not considered. In the present series of experiments dobutamine was shown to increase spontaneous tritium release from tissues prelabeled with ^{3}H norepinephrine (figures 22 and 23). This effect was not blocked by cocaine and desoxycorticosterone. These findings suggest that dobutamine may be an indirectly acting sympathomimetic like amphetamine. The clinical significance of this increase of norepinephrine efflux is unknown; however, a decrease in circulating norepinephrine has been shown in response to dobutamine (Kho 1980) suggesting that reflex mechanisms may play an important role in the in vivo effects of this drug.

Denervation using 6-hydroxydopamine appears to demonstrate some differences between norepinephrine and dobutamine. While the mean logarithm of ED_{50} values for dobutamine in treated and untreated tissue differed only by 0.01; the same values for norepinephrine differed by an average of 0.32. Thus while presynaptic reuptake appears to be an important influence on the concentration response curve to

norepinephrine it does not appear to alter the available concentration of dobutamine in the synapse. Further, the effect of dobutamine to release transmitter from the nerve as demonstrated in tissues prelabeled with ^{3}H norepinephrine is not apparent in this study. One explanation of this discrepancy relates to the mechanism of action of tyramine. This compound acts only on uptake and release of norepinephrine but has little postsynaptic effect. Therefore it loses efficacy in denervated states. In contrast, dobutamine has been shown to have direct effects on the post synaptic membrane, and based on the Furchgott method, appears to have a K_A value in the same range as norepinephrine. Thus the presence of dobutamine in sufficient quantities to provoke norepinephrine release in the untreated tissue, would allow dobutamine to act as a competitive antagonist to the norepinephrine released. Thus, dobutamine-provoked release of norepinephrine can only be assessed by the superfusion technique and not by a concentration response curve. An alternative explanation is that the superfusion technique was deonstrating overflow of tritiated metabolites rather than norepinephrine. This explanation is supported by the observtion that, during measurement of stimulation-evoked endogenous norepinephrine radioenzymatic assay did not increase. This suggests that spontaneous release of unchanged norepinephrine did not increase. Reserpine by acting at the vesicular level produces findings similar to this. Some caution, however, must be taken in interpreting the results of the radioenzymatic assay because of the sensitivity of this assay at the very low concentrations being studied.

A further possible mechanism for modulation of norepinephrine release by dobutamine involves presynaptic receptors. The possibility that dobutamine may affect this system was studied in vitro by measuring endogenous norepinephrine release in response to transmural nerve stimulation. At a stimulation frequency of 8 Hz dobutamine did not alter endogenous norepinephrine release. This suggests that dobutamine acts as neither an agonist or antagonist at presynaptic receptors. Even with this evidence, the possibility exists that the modulating effect may only be apparent at lower frequencies (Duckles, in press, Medgett et al, 1978).

Summary

In summary, characterization of the vasoconstrictor properties of dobutamine showed that this drug is a partial agonist in alpha adrenergic receptors of vascular smooth muscle. Schild plots suggested that if multiple post synaptic receptors were being acted upon, norepinephrine and dobutamine act in the same manner, or more likely that a single post synaptic receptor population was responding. The use of the Furchgott method demonstrated wide variability in predicted values of K_D for both norepinephrine and dobutamine, although the average values for both drugs were similar. The Furchgott method may be amenable to techniques of nonlinear regression, however, these methods (and even the classical Furchgott method) require independent verification of the relationship between receptor number and response in order to be more useful. Dobutamine was found to be capable of releasing norepinephrine in a dose dependent manner that was not

blocked by cocaine and desoxycorticosterone. In contrast to this action, no effect of dobutamine on stimulation evoked release of norepinephrine could be found, and denervation with 6-hydroxydopamine did not alter the contractile response to dobutamine.

Despite the ability of dobutamine to release norepinephrine in vitro, this drug remained a reasonable candidate for use in the neonate. The ability of dobutamine to produce a greater maximal response in the dog femoral artery than in the dog pulmonary artery suggested that dobutamine may be able to preferentially exert beneficial effects in the newborn pulmonary vascular bed and that the in vitro model might be able to predict these effects. Thus, the actions of dobutamine in vascular smooth muscle from the neonate were studied.

How Do Alpha Adrenergic Receptor Mediated Responses Change During Maturation?

Studies of the maturational process are often confounded by multiple variable. As can be seen in figure 28, the first weeks of life are a time of rapid growth for the dog . This growth is accompanied by structural and functional development. While the rapidity of this process in the dog may make it a species more subject to confounding variables, it was chosen for this study on the basis of many factors. The dog at birth has structures resembling the premature human such as the subependymal germinal layer and therefore might tend to reflect other changes associated with the development of the

premature human. The animals are relatively large at birth in contrast to cats and rabbits but do not have the high cost disadvantages of primates or other large animals. In addition, current studies have demonstrated the ability of the newborn dog (Goddard et al, 1980) to model vascular disturbances similar to the premature human. Should future work with vasoactive drugs be undertaken this model may be useful. The disadvantage to us was the presence of a heterogenous mongrel dog population as a variable. As figures 29 and 30 show, the response of tissue from these animals was widely variable. To minimize these differences and study only receptor mediated changes, the concept of comparison to a maximal response to potassium chloride was used. While the response to potassium chloride may in part be due to release of norepinephrine, this response appears to be more related to an effect directly on the post synaptic membrane. In retrospect, our findings support the notion that norepinephrine release is not primarily responsible for the response to potassium chloride since tissues that did not respond to norepinephrine did contract to potassium chloride. With the support of these observations, the data were all expressed as a percentage of the maximal contraction to potassium chloride, and, in tissues unresponsive to adrenergic drugs, serotonin was used to demonstrate that the tissue was capable of producing receptor mediated contractions.

The results of this series of experiments were striking in the three tissues studied. In response to norepinephrine, the femoral artery showed little change with age whereas the pulmonary artery and

aorta showed increases from threshold level to near maximal responses during the first 6 weeks of life. For dobutamine similar trends of increasing maximal response were noted. In all cases dobutamine produced a lower maximal response than norepinephrine. Because of this relationship, in tissues where a response occurred to norepinephrine and none to dobutamine, dobutamine could be shown to act as an antagonist to norepinephrine. In the 0-2 week age group contractile responses to dobutamine in the pulmonary artery and aorta were minimal. Even in the more active femoral artery only a small response was obtained. The alpha adrenergic specificity of the responses to norepinephrine in these tissues was confirmed by the use of either phentolamine or prazosin as an antagonist. It is of interest that a large number of these competitive inhibition studies showed a decrease in maximal response as well as an increase in ED_{50} value. This finding is not explainable in terms of current receptor response coupling theories and emphasizes the need for an increased understanding of this process.

In view of the minimal responses to dobutamine obtained in the tissues of the very young, several postulates can be suggested. One model would be to ascribe the difference in dobutamine and norepinephrine at various ages to multiple receptor populations. To distinguish these receptors, antagonist dissociation constants as measured by the Schild method would vary by agonist used and perhaps would change with age suggesting a relative preponderance of one receptor type at a given age. In view of the near threshold responses to dobutamine seen in the

very young and the general lack of reproducibility of the Schild method in very labile systems such as the rabbit pulmonary artery, conventional approaches to answering this question would not be applicable.

A second possible explanation would be that the affinity of the receptor changes as a function of age. To examine this possiblity the method of Furchgott could be used to determine K_A as a function of age. In the most mature tissue studied (4-6 wks of age) this method was attempted 6 times and produced paired concentration response curves that could not be analyzed by the classical double reciprocal plot because of either too much or too a small reduction in response due to phenoxybenzamine. Considering these failures and the wide variability of the Furchgott method as demonstrated in figure 19, this approach did not seem realistic in terms of practicality and/or reliability.

The third and fourth alternative explanations for alterations observed with aging would be a change in density of receptors responding to the drug and/or a change in the intracellular mediation (coupling) of the response. Some precedent for increased receptor density as a possible mechanism have been set in works by Noguchi (1982) and Whittsett et al (1982) who found in rat heart and lung respectively, an increase in B_{max} for radioligand binding to alpha adrenergic ligands during maturation. None of the current techniques depending upon in vitro evaluation of contractility will allow an assessment of these phenomena of receptor density or coupling.

With these limitations it was recognized that alternative methods of study would be needed in order to further evaluate the possibilities outlined above. The most reasonable alternative method appeared to be radioligand binding.

Can a Method for Radioligand Binding in Vascular Smooth Muscle Be Developed?

To achieve an in depth understanding of the relationship of growth to changing vascular smooth muscles responses, a method for evaluating total receptor number and binding affinity independent of response was critical. Reported methods in small vessels (Colucci et al 1980, 1981) did not seem applicable to the problem of maturation in view of the large number of animals required to provide adequate tissue amounts. Tsai and Lefkowitz (1978) reported radioligand binding using a membrane preparation of aortic tissue requiring only a single ultracentrifugation step but still using a relatively large amount of tissue. Neither of these methods had been applied to the pulmonary artery which has relatively less available tissue. The method of Bobik (1982) for membrane preparation appeared to offer a useful approach to the purification of homogenized vascular smooth muscle to achieve a greater yield. Using this concept of differential ultracentrifugation a preparation of membrane protein was derived in our laboratory.

Having obtained a membrane preparation the choice of ligands was of importance. The studies of Colucci et al (1980) and Tsai and Lefkowitz (1978) used ^{3}H-dihydroergocryptine. Colucci et al (1981)

began to use ^{3}H WB4101 because of a higher alpha 1 adrenergic receptor preference. Hoffman and Lefkopwitz (1980) however, have questioned this selectivity. Prazosin (Karliner et al 1979) in the rat heart has been shown to exert a high specific preference for binding sites characterized as alpha 1 adrenergic and therefore seemed a reasonable ligand for use. To characterize alpha 2 adrenergic receptors, rauwolscine has been reported (Tanaka and Starke 1979) to exhibit high preference for receptors and was therefore selected. The first major difficulty was the dramatic loss of radioactivity in the aliquot of ligand used as a standard. This was particularly striking at lower ligand concentrations. By using glassware treated with dimethylsilane, this problem was overcome as shown in figures 60 and 72.

Using ^{3}H prazosin the binding properties of rabbit aortic membranes were studied with respect to saturability, stereospecity and rank order of potency. The data analysis in all cases was performed using non linear regression. This approach has been compared to more traditional data transformation methods by DeLean et al (1981) and has been shown to have greater statistical validity. In all the data shown for binding studies the computer drawn best fit curve is also shown.

^{3}H-prazosin and ^{3}H-rauwolscine both appear to demonstrate simple non cooperative binding with distinctly different binding preferences (Table 6). The values obtained for the K_D of prazosin by these techniques is in agreement with the data of Karliner et al (1979) for the myocardium and the data of Bobik (1982) for dog aortic

membranes. Similar data was obtained for the rabbit and dog pulmonary arteries (Table 7). Rauwolscine binding in the rabbit aorta and pulmonary arteries were also obtained, although in two cases the binding of this ligand did not demonstrate saturability as assessed by nonlinear regression over the range of ligand studied (.15 - 10nM.

Since one of the orginal purposes of this dissertation was the evaluation of dobutamine, the apparent $K_D$for this drug against prazosin and rauwolscine was determined. These findings supported the earlier findings of K_A values for dobutamine. Dobutamine appeared to have a greater affinity for alpha adrenergic receptors than norepinephrine. Taking the average values of K_A in the rabbit pulmonary artery and comparing these to the apparent K_D values, dobutamine had the same affinity measured by both methods. Norepinephrine values also showed strong agreement suggesting that the K_A value obtained using the Furchgott method was not biased by alpha 2 receptors. It appeared from apparent K_D values to rauwolscine that dobutamine had a high affinity for alpha 2 adrenergic receptors that was not apparent from studies on stimulation-evoked norepinephrine release.

Can the Maturation of Alpha-Adrenergic Mediated Responses Be Mathematically Modeled?

The Classical Model

It has long been recognized that responses of smooth muscle to drugs can be described as a saturable function. (Parker and Waud 1971).

$$\frac{\text{Response}}{\text{Max Response}} = \frac{1}{1 + \frac{ED_{50}}{A}^{N}} \qquad (1)$$

Equation (1), where A is agonist concentration ED_{50} is the concentration producing 50% response and N is a constant describing slope, defines the relationship of drug concentration to response. When response is plotted versus the logarithm of drug concentration the general shape of a sigmoidal curve is assumed. With the introduction of the receptor as a mediator of drug action, attempts were made to mathematically describe the relationship between receptor-drug binding and response. The first of these models was suggested by Clark (1933). This is also known as the occupancy theory. Assumptions central to this model are: 1) there is a reversible reaction of receptor and drug leading to a response, 2) only a single receptor population is responsible for drug binding, 3) response is linearly proportional to drug binding, 4) response determinations are all made at equilibruim, 5) the drug exists only in the free state or bound to a receptor complex, and 6) only a negligible fraction of total drug is bound at any time to the receptor. Using the symbol "R" for receptor number and "A" for drug concentration, the underlying relationship of this model is shown in equation (2).

$$R + A = RA = \text{Response} \qquad (2)$$

Using the relationships of the law of mass action, equation (3) can be derived.

$$\frac{R \cdot A}{RA} = K_D \qquad (3)$$

On this basis, Clark derived a general equation (4) where R_T is the total number of receptors in the system.

$$RA = \frac{R_T \cdot A}{K_D + A} \qquad (4)$$

In this model since effect or response is a linear function of RA, equation (5) logically follows, where K_e is a proportionality constant of effect

$$\text{Response} = K_e \cdot RA \qquad (5)$$

Further, the maximal response is equal to $K_e \cdot R_T$. Thus, the fractional response compared to the maximal response is simply $\frac{RA}{R_T}$.

When comparing this relationship to the previously described relationship for the general sigmoidal curve, it should be apparent that Clark's model would apply when N is assumed to be 1 and the ED_{50} equals K_D.

While this model does describe a sigmoidal dose-response curve, it fails to describe adequately some of the recognized properties of smooth muscle. After two groups (Ariens et al, 1957, Stephenson 1956) observed that the maximal response to drugs in a single system could vary, the concept of intrinsic activity was introduced as shown in equation (6) where α is the intrinsic activity.

$$\text{Response} = \alpha \cdot RA \qquad (6)$$

This equation still implied a proportional relationship of response to RA. The recognition that many drugs will produce large responses in vascular smooth muscle while binding only a small fraction of the total

receptors, led Ariens et al (1957) to recognize that binding was not a linear function of effect. These authors used the equation (7) to explain the observed discrepancy between binding and response data.

$$\text{Response} = f\left(\alpha \frac{RA}{R_T}\right) \quad (7)$$

While this function took into account observed phenomena, it was not a useful mathematical model to attempt to predict response in terms of a definable relationship to binding.

A Model of Nonlinear Coupling

To extend the more classical models described, Boynaems and Dumont (1977) have developed a model to account for the spare receptors described by Ariens. This concept of receptor excess was modeled on the assumption that receptors are linked to effector units such that binding of one receptor unit (in a cluster of N receptors) will fully activate the effector unit. With this assumption these authors derived equation (8).

$$\frac{\text{Effect}}{\text{Effect Max}} = 1 - \left(1 - \frac{A}{K_D + A}\right)^N \quad (8)$$

This model is useful in describing the spare receptor theory, but it fails to incorporate the concept of intrinsic activity. In equation (8) as fractional binding approaches 1, effect/effect max approaches 1. As a development of this model Boynaem and Dumont used plots of fractional receptor binding versus fractional response to demonstrate the characteristics of their derived function. These plots provide a useful graphical method of demonstrating the relationship of binding to effect in proposed models.

Models Of Drug-Receptor Binding

To find models useful in describing binding-response relationships, it is first necessary to describe the relationship of agonist concentration to drug receptor binding. The simplest functional form of receptor binding to drug is expressed by the mathematical relationship in equation (9).

$$\frac{RA}{R_T} = \frac{1}{1 + K_D / A} \qquad (9)$$

This model assumes a single receptor population with a simple non-interactive binding pattern. For a system involving 2 receptor sites with differing dissociation constants, the relationship in equation (10) has been described (Boeynaems and Dumont 1980).

$$RA = \frac{R_{T1}}{1 + \frac{k_1}{A}} + \frac{R_{T2}}{1 + \frac{K_2}{A}} \qquad (10)$$

The general equation for binding with N different binding sites can be derived in the following form where N is the number of binding sites (11).

$$RA = \sum_{\iota=1}^{N} R_T i \; / \; 1 + \frac{K_{Di}}{A} \qquad (11)$$

Another conceptual relationship in the study of drug-receptor binding is the concept of cooperativity (Hill, 1910). The simplest model in this particular relationship involves increasing affinity of receptor for drug in the presence of other receptor drug complexes. This is referred to as positive cooperativity and is mathematically described by equation (12).

$$\frac{RA}{RT} = \frac{A^N}{A^N + K_D} \qquad (12)$$

Development of a Model for the Binding-Response Relationship

While more complex models of binding have been proposed (Monod, 1966, Koshland, 1966), data described for vascular binding (Bobik, 1981, Colucci, 1981) have demonstrated that the relationships in equation (9) or (10) may satisfactorily describe drug receptor binding. On the basis of this fundamental understanding of the observed relationship of binding to response, it may be possible to define criteria and derive a more specific functional relationship for a given system of response in terms of binding. The criteria for an effective model of the receptor-response relationship in vascular smooth muscle should include the following: 1) maximal response should be a dependent function of total receptor number, 2) the concept of intrinsic activity should be incorporated, and 3) there should be a specific relationship with definable parameters between the standard sigmoidal curve of response and the sigmoidal relationship of binding. To derive a useful model of the binding-response relationship the simplest model of the binding process will be used as in equation (9), although substitution of more complex binding models may be easily made. Algebraic rearrangement of equation (9) produces equation (13).

$$RA = \frac{R_T}{1 + \frac{K_D}{A}} \qquad (13)$$

In the context of the lipid bilayer theory of membrane structure it has been postulated that receptors on the external cell surface may interact with an effector structure (E) in a reversible manner that is dependent upon drug-effector interaction in both the absence and presence of drug as shown in equation (14).

$$\begin{array}{ccc} R + E & \rightleftarrows & RE \\ + A \uparrow\downarrow & & \uparrow\downarrow + A \\ RA + E & \rightleftarrows & RAE \end{array} \qquad (14)$$

If the assumption that drug is required for receptor-effector interaction is made, as suggested by Boeynaems and Dumont (1977), the equation (14) simplifies to equation (15).

$$RA + E = RAE \qquad (15)$$

Effect is then a function of RAE as in equation (16).

$$\text{Effect} = K_{eff} \cdot RAE \qquad (16)$$

Developing further, it may be postulated that this process is saturable and can be described by equation (17) where E_T is the total number of effector units and K_{RAE} is the concentration of RA at half maximal binding of effector units.

$$RAE = \frac{E_T \cdot RA}{RA + K_{RAE}} \qquad (17)$$

Effect may than be shown to be related to binding combining equations (16) and (17) to give equation (18).

$$\text{Effect} = \text{Keff} \cdot E_T \cdot \frac{RA}{RA + K_{RAE}} \qquad (18)$$

In a system where effect can be described as a fraction of non receptor mediated effect then Keff $\cdot$ E_T = Max effect (for example potassium

chloride induced contraction). When defined in this way, the following relationship becomes apparent.

$$\frac{\text{Effect}}{\text{Max Effect}} = \frac{RA}{RA + K_{RAE}} \qquad (19)$$

Thus when contraction is expressed as a fraction of non receptor mediated maximal contraction, effect is a function of A, R_T, K_D, and K_{RAE}. In a given system a relative value of R_T can be defined as a function of maximal binding of a radioligand. K_D is also definable by this method, independent of concentration-response data. It should also be apparent that the term k_{RAE} would serve to relate activities of varying drugs as a form of "intrinsic activity".

On the basis of the model of Hill (1910), cooperativity of binding of RA to the effector unit would yield a more general form of equation (19) in which N is the positive exponent of cooperativity as shown in equation (20).

$$\frac{\text{Effect}}{\text{Max Effect}} = \frac{RA^N}{RA^N + K_{RAE}} \qquad (20)$$

Combining equation (13) and (20) and rearranging produces equation (21) relating effect to drug concentration.

$$\frac{\text{Effect}}{\text{Max Effect}} = \frac{1}{1 + \dfrac{K_{RAE}}{\dfrac{R_T^{\ N}}{1 + \dfrac{K_D}{A}}}} \qquad (21)$$

In the case where N = 1 (no effector cooperativity) equation (20) simplifies to (22).

$$\% \text{ Effect} = \frac{1}{1 + \frac{K_{RAE}\left(1 + \frac{K_D}{A}\right)}{R_T}} \qquad (22)$$

Expression (21) and (22) provide a functional relationship between drug concentration and % effect with variable K_{RAE}, K_D, R_T and with the possibility of cooperativity through the term N. Therefore, this model will fulfill the criteria previously defined for a useful model. Making use of equation (21) and the graphics of MLAB, substitution of estimated values of K_D, R_T and K_{RAE} will allow a demonstration of the mathematical properties of this model.

Application of the Model

An estimated value of K_D (1×10^{-7}M) as obtained using the method of Furchgott for norepinephrine is used for this analysis. As shown in fig. (78), by varying values for R_T in equation (21) where K_{RAE} is relatively low (or intrinsic activity is high), for example norepinephrine as the agonist, the relationship of maximal response to total receptor number is apparent. Holding all other variables constant and changing K_{RAE} to reflect a low intrinsic activity (for example, dobutamine), the concentration-response curves as shown in fig. 79 are obtained. The model appears on this basis to describe the features of a partial agonist.

Without invoking cooperativity, this model system would require large changes in total receptor number to account for changes over a wide response range. As seen in fig. 80 and 81 these same two agonists may also be modeled with positive cooperativity (N=2) or negative cooperativity (N=.50). These changes in cooperativity are demonstrated to alter the relationship of changes in R_T to response and may produce large changes in the response as a percent of maximum.

A commonly plotted relationship in proposed models is fractional receptor binding versus response. This graphic relationship applied to our model for a compound with low intrinsic activity such as dobutamine can be seen in fig. 82. The superimposed data points represent experimentally obtained response data points for dobutamine plotted against fractional receptor binding for dobutamine. This assumes simple saturable binding with a K_D of 1×10^{-7}M. Although not absolutely correlated, the relationship between the modeled line and the data points demonstrates the utility of the derived formulae.

Thus the derived model fulfills defined criteria for a useful model of the concentration-response relationship. With this model the process of alpha adrenergic maturation can be studied in relationship to the equations described. Since the final relationship of equation (22) is on the basis of data expressed as a fraction of maximal response to a non-receptor mediated response (for example contraction to potassium chloride), changes in smooth muscle mass or contractile

elements will be eliminated and only receptor mediated changes will be apparent. By considering growth as a function of R_T, K_D, K_{RAE} and N, the maturation process may be viewed as dependent upon changes in receptor number, changes in binding affinity, alterations in effector coupling and/or changes in cooperativity. Keeping these factors in mind, a rational approach to research design can attempt to define each variable possibly involved in the growth process.

Conclusion

This dissertation has attempted to answer 4 specific questions involving adrenergic amines and the process of maturation. The alpha adrenergic characteristics of dobutamine appear to be that of a high affinity, low intrinsic activity vasoconstrictor acting directly on the post synaptic alpha adrenoceptor. In the dog dobutamine and norepinephrine were both demonstrated to produce increasing responses with age in some vascular tissues. The femoral artery of the dog had a well developed contractile response at all ages. The pulmonary artery was found to have surprisingly little contractile response in the neonate even to norepinephrine. This observation, if valid in the human, provides an important insight into the possible roles of non adrenergic vasoconstrictors in the pathogenesis of persistent fetal circulation. This suggests even more strongly that dobutamine may be an important drug to act as a specific peripheral vasopressor without worsening the constriction of the pulmonary vascular bed.

Ligand binding further demonstrated that dobutamine binds to alpha 1 adrenoceptors. This supports the concept of dobutamine as a drug with high affinity and low activity. Using the estimate of K_I from displacement studies as an estimate of K_D and calculating receptor occupancy producing equal responses in the rabbit femoral artery, norepinephrine was found to be 17 times as efficacious as dobutamine in eliciting contractile responses. Comparing the dog and rabbit pulmonary artery another interesting insight can be made. On the basis of radioligand binding these tissues have similar receptor affinities to prazosin, but the dog pulmonary artery has a greater receptor density. In contractile response studies, however, the dog pulmonary artery had a greater ED50 value and depressed maximal response to both norepinephrine and dobutamine. In the model proposed, this difference would be accounted for on the basis of a difference in coupling mechanisms for the two tissues.

By combining the techniques of radioligand binding and contractile response future work on closely paired tissues from a controlled animal population should allow even further insight into the role of changes in coupling and receptor density during maturation.

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