EFFECT OF DEUTERIUM OXIDE (D_2O) ON VASCULAR SMOOTH MUSCLE

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BY

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ABSTRACT

The influence of deuterium oxide (D_2O) on aortic vascular smooth muscle was investigated. The effect on receptor- and voltage-operated calcium channels was studied with the use of phenylephrine and potassium chloride respectively. D_2O depressed the phenylephrine induced contraction response in a dose dependent manner with 50% inhibition of maximum contraction observed with 60% D_2O. The effect of 60% D_2O on phenylephrine induced contraction was reversible and not dependent on an intact endothelium. Sixty percent D_2O also reduced potassium chloride induced contractions by 50% indicating an effect on voltage operated calcium channels. Studies with Bay K 8644, an L-type calcium channel activator, and calcium free buffer studies, confirm an effect on utilization of extracellular calcium sources. Inhibition of sucrose induced contraction and acetylcholine and isoprenaline induced relaxation with 60% D_2O suggest an effect on intracellular calcium mobilization or on the contractile elements themselves. D_2O also affected both the pD_2' for nifedipine and the pA_2 for prazosin. Collectively, these data indicate that D_2O acts at multiple sites.

In vivo studies indicate that chronic oral administration of 25% D_2O for 12 days reduces the blood pressure of spontaneously hypertensive (SHR) and normotensive (WKY) rats.

However, the lowering of blood pressure did not result in a corresponding change in vascular smooth muscle contractility induced with phenylephrine and potassium chloride. These results support previous evidence that a reduction in blood pressure after hypertension has developed does not reduce vascular reactivity changes. A further acute study revealed that desensitization results from chronic exposure to D_2O such that 60% D_2O produces a significant depression of contraction only in aortic rings obtained from SHR and WKY rats which had not been chronically treated with twenty-five percent D_2O.

KEY WORDS

Aorta

Calcium channels

Contraction

Deuterium oxide

Hypertension

In vitro

In vivo

Relaxation

Reversibility

Vascular smooth muscle

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ABBREVIATIONS

ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
$CaCl_2$	Calcium chloride
cGMP	Cyclic guanosine monophosphate
D_2O	Deuterium oxide
EDRF	Endothelium derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
G-protein	Guanosine triphosphate binding protein
Hg	Mercury
IP_3	Inositol triphosphate
K^+	Potassium ion
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
M	Molar concentration
mm	Millimeters
mM	Millimolar concentration
$MgSO_4$	Magnesium sulphate
Na^+	Sodium ion
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
S.E.M.	Standard error of the mean
SHR	Spontaneously hypertensive rats
WKY	Wistar-Kyoto rats

CHAPTER 1

INTRODUCTION

1.1 Background information on deuterium oxide

Deuterium, the heavy stable isotope of hydrogen, was discovered by Urey et al. in 1932 and deuterium oxide (D_2O) was first made available by Urey in 1933. Since the addition of the extra proton to hydrogen practically doubles the mass, there are great differences between the original element and the isotope. The changes include an increase in the melting and boiling point by 3.82°C and 1.42°C respectively, a 25% increase in viscosity, lowering of vapor pressure and an increase in the specific gravity to 1.1074 (Barbour, 1937). There are also solubility changes such that carbon dioxide and oxygen are both about 10 percent less soluble in D_2O than in H_2O (Katz, 1960). Deuterium oxide is found as a natural component of ordinary water at a concentration of one part in 5000 or 0.02 percent (Barbour, 1937).

It is interesting to note that the high cost of D_2O was an obstacle for prolonged <u>in vivo</u> studies using D_2O during the thirty years following its discovery (Thomson, 1960) and even today the high cost is a limiting factor. Since there are large differences between hydrogen and deuterium, and hydrogen is a major constituent of mammalian tissues, early studies dealt with determining the effect of D_2O on mammals. Barbour

(1937) was one of the first researchers to conduct a study of this kind and one of his fundamental conclusions was that deuterium is a depressant of biological activity and that its effects can be reversible, or not, depending upon the concentration. From this study it was also determined that mice could not survive replacement of more than one third of body water by D_2O; metabolic rate is increased in the early stages of D_2O intoxication; and D_2O has a sympathomimetic action. A similar study was conducted by Thomson (1960) who found that rats died when about one third of their body water was replaced by D_2O. The effects of D_2O included impairment of kidney function, anaemia, disturbed carbohydrate metabolism, central nervous system disturbances, and wide effects on enzyme systems. It was concluded that the toxic action of D_2O was due to the additive effect of the many changes in rates of enzymatic reactions.

Chemical and biological studies conducted by Katz (1960) reinforced the fact that D_2O inhibits many enzymatic reactions. He determined that although the binding rate constants for H^+ and D^+ ions are similar, the rate constant for the unbinding of D^+ is much slower, demonstrating the increased stability of deuterium bonds and accounting for isotopic effects. Experimental rate constants supporting isotopic modifications due to H^+ and D^+ exchange have most recently been calculated by Michael et al. (1990).

Goodall (1958) suggested that the rate limiting process for vascular contraction may be proton transfer. He showed a three fold reduction in the development of isometric tension in frog sartorius muscle without a reduction in the amplitude of the action potential and concluded that D_2O acted on the contractile proteins with the involvement of possibly three protons. This finding sparked investigations into the effects of D_2O on the mechanical response of various muscle types.

Kaminer (1960) studied the effect of D_2O on the isolated atria of the frog and found that concentrations of heavy water between 99.8 and 25 percent decreased the force of contraction and the rate of spontaneous beating of the isolated atria. This data supports the result found by Barnes and Warren (1935) who were the first to show that 20% D_2O slows the frequency of pulsation of the excised heart of the frog. In order to elucidate the site of action of D_2O, Kaminer (1960) performed subsequent experiments on glycerol extracted psoas muscle fibres (where contractile proteins are isolated) and found that D_2O does not affect adenosine triphosphate (ATP) induced shortening of the muscle fibres. He then concluded that it is the excitation-contraction coupling which is affected by D_2O and not the contractile proteins.

Further studies on the inhibition of enzymes by Svensmark (1961) revealed that D_2O inhibited adenosine triphosphatase, an enzyme associated with muscular activity, by 50%. He then

investigated whether the inhibition was reflected in the mechanical response of skeletal muscle. Using frog semitendinous muscle he found that the amplitude of the action potential was unaffected by D_2O but there was a reduction of the frequency at which a complete tetanus could be maintained, suggesting an effect on the primary excitatory processes. There was also a reduction in twitch force and the rate of force development in D_2O which he suggested could be caused by a direct effect on the contractile elements. From this study it remained undecided whether the reduction in the force of contraction was due to a D_2O effect on the contractile elements or on excitation-contraction coupling. Also, due to inadequate controls, Svensmark argued that no definite conclusions could be drawn from the work of Goodall (1958) or Kaminer (1960).

The conflicting conclusions drawn from these studies led to increased interest in determining the site of action of D_2O. Kaminer and Kimura (1972) supported their previous conclusion by determining that the luminescent response of aequorin (a calcium sensitive luminescent protein) which accompanies contraction was abolished when H_2O was replaced by D_2O. However, it was again argued that suitable controls were lacking since the possible effects of D_2O on calcium sensitivity and kinetics of the aequorin reaction were unknown (Allen et al., 1984). Studies conducted by Allen et al. (1984) involved determining the influence of the substitution

of D_2O for H_2O on calcium transients and on contraction in both intact single skeletal muscle fibres injected with aequorin and in mechanically skinned fibres from frogs. These studies revealed that the calcium sensitivity of the myofibrils is reduced by D_2O substitution but that at saturating calcium concentrations the myofilaments generate more tension in D_2O than in H_2O. Results also indicated that calcium release from the sarcoplasmic reticulum was reduced in D_2O and it was concluded that this reduction in calcium release was a major factor in reducing twitch tension but reduced myofibrillar calcium sensitivity also played a role. Also, the rate of change of aequorin light emission brought about by D_2O substitution represented a 3 to 5 fold reduction in intracellular calcium concentration which was dependent on the concentration of D_2O and proportional to the fraction of H_2O replaced. This change in light emission, after solvent exchange, occurred too fast to be explained by exchange of intracellular water so it was postulated that the site whereby D_2O affected calcium release must be accessible to the extracellular space. It was concluded that there was time for D_2O to diffuse in or out of the T-tubules in time to produce the effects on calcium release and therefore it was proposed that the site of action of D_2O may be on excitation-contraction coupling in the T-tubules. Earlier studies (Sandow et al., 1976 and Yagi and Endo, 1976) also support D_2O effects on excitation-contraction coupling in skeletal muscle. Other evidence supporting an effect of D_2O on calcium

transients is found from the fact that D_2O reduces L-type calcium channel conductance in isolated myocytes (Prod'hom et al., 1987).

Despite wide interest in the study of the effects of D_2O on skeletal muscle, few experiments have been carried out using smooth muscle. The effect of D_2O on aortic vascular smooth muscle from spontaneously hypertensive rats (Vasdev et al., 1990a) and Dahl salt-sensitive hypertensive rats (Vasdev et al., 1990b) has been investigated. It was found that D_2O inhibited in vitro calcium uptake in aortae from these rats. Since D_2O has been demonstrated to reduce calcium uptake in vascular smooth muscle it might be expected that D_2O will also inhibit contraction of vascular smooth muscle. The purpose of the present study is to determine if this is so and to try to resolve the previously postulated mechanisms of action of D_2O on the contraction process.

The thoracic aorta was chosen to study the effect of deuterium oxide on vascular smooth muscle. The rat aorta was the vessel of choice since it is devoid of adrenergic nerves (Patil et al., 1972) and Fleisch (1974) reports that the rat aortic preparation is ideal when one wishes to investigate the effects of drugs on vascular smooth muscle devoid of nervous innervation. This condition is ideal since drugs not only act at receptors, producing an effect of their own, but may also act as nerves innervating the tissue and eliciting the release

of endogenous agonists resulting in additional tissue responses.

1.2 Mechanisms of vascular smooth muscle contraction

The most important step in the contraction or relaxation of vascular smooth muscle involves calcium mobilization (Somlyo and Somlyo, 1968). An increase in the intracellular calcium concentration at the level of the contractile proteins results in contraction of the muscle, whereas removal of calcium results in relaxation (Fleisch, 1974). Vascular smooth muscle cells may be activated either by agonists which activate receptor-operated calcium channels or by membrane depolarization which activates the opening of voltage-operated calcium channels.

There are three types of voltage dependent calcium channels. These are the T, L, and N channels which refer to transient (fast), long lasting (slow), and neuronal channels, respectively (Nowycky et al., 1985). However, only T- and L-type channels are found in vascular smooth msucle (Zelis and Moore, 1989).

In addition to receptor- and voltage-operated calcium channels there is also a leak channel which is the calcium entry pathway which allows a continuous entry of calcium into the cell that is not increased on stimulation by depolarization or agonists (Khalil et al., 1987).

Agonist activated increases in intracellular calcium concentration are mediated by a transducing guanosine triphosphate binding protein (G-Protein), which in turn activates enzymes, such as phospholipase C, to generate second messengers. Two of these second messengers are inositol triphosphate (IP_3) and Ca^{2+} which induce the sarcoplasmic reticulum to increase the release of calcium, thereby increasing the intracellular concentration of Ca^{2+} available for binding to and activation of calmodulin (Williamson, 1986). Calmodulin is a ubiquitous protein which is the major receptor of Ca^{2+} and appears to mediate most of the ion's activities as a second messenger (Wang and Waisman, 1979). The calcium-calmodulin complex then activates myosin light chain kinase. Myosin light chain kinase is the enzyme which catalyses the phosphorylation of myosin; a process important for the development of contraction in vascular smooth muscle (Adelstein et al., 1981). Activation of the kinase leads to rapid cycling of the cross-bridges between actin and myosin and this interaction of the contractile proteins results in contraction of vascular smooth muscle (Khalil et al., 1987).

Depolarization induced increases in intracellular calcium concentration occur by direct entry of calcium via the T- and L-type calcium channels (Zelis and Moore, 1989).

In this study phenylephrine, an alpha-adrenergic agonist, was chosen to study the D_2O effect on contraction induced via

receptor mediated increases in intracellular calcium. Although phenylephrine is not completely selective for receptor-operated calcium channels (Godfraind et al., 1986) it does act mainly on this type of channel to produce contraction of aortic vascular smooth muscle (Khalil et al., 1987). Potassium chloride acts mainly on the voltage-operated calcium channel (Meisheri et al., 1981) and was chosen to study the effect of D_2O on this channel type. The effects on this type of channel were also investigated using Bay K 8644 which enhances calcium influx via the voltage sensitive calcium channel under partial depolarizing conditions (Schramm et al., 1983). To further ensure no effect from receptor-operated calcium channels, they were blocked by the alpha-receptor antagonist phentolamine. Similarly, calcium free buffer studies were performed in an attempt to clarify the effects of D_2O on contraction induced by the influx of extracellular calcium.

Finally, the effect on sucrose induced contraction was studied to determine the effect of D_2O on intracellular calcium mobilization since it occurs independently of the level of membrane potential and in the absence of external calcium sources (Andersson et al., 1974).

1.3 Mechanisms of vascular smooth muscle relaxation

The two main proposed mechanisms of vascular smooth muscle relaxation involve an increase in either cyclic

adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) levels. In this study a representative was chosen from each group: isoprenaline, which is a beta-agonist and acts via an increase in cAMP levels; and acetylcholine, which results in relaxation via an increase in cGMP levels.

Various different mechansims are thought to result in vascular smooth muscle relaxation through an increase in cAMP; including beta-adrenergic stimulation, inhibition of phosphodiesterase, and regulation of myosin light chain kinase.

Although various mechanisms have been proposed for beta-adrenergic receptor mediated relaxation of vascular smooth muscle, all theories suggest the involvement of cAMP as the second messenger (Mueller and van Breemen, 1979). Relaxation of vascular smooth muscle in response to adrenergic stimulation is mediated by β_1, β_2, or both β_1 and β_2 adrenoceptors (Lucchesi, 1989). The beta-adrenergic receptor is coupled to adenylate cyclase by a stimulatory G-protein. Activation of adenylate cyclase converts ATP to cAMP and the cAMP in turn activates a protein kinase which catalyses a protein phosphorylation and leads to a reduction in intracellular free calcium (Mueller and van Breemen, 1979). To date, it has not been determined how the intracellular calcium concentration is reduced. The proposed mechanisms by

which cAMP may decrease the calcium ion concentration available to the contractile proteins include: increased Ca^{2+} sequestration into intracellular storage sites such as the sarcoplasmic reticulum (Mueller and van Breemen, 1979); inhibition of calcium influx (Meisheri and van Breemen, 1982); and increased calcium extrusion via increased activity of the Na^{+} - K^{+} pump (Scheid and Fay, 1984). At present it is thought that calcium sequestration by increased Ca^{2+} uptake into the sarcoplasmic reticulum probably plays the primary role in decreasing free intracellular calcium (Bülbring and Tomita, 1987).

There is also evidence to support a role for increases in cAMP via inhibition of phosphodiesterase. Kauffman et al. (1987) showed that a series of dihydropyridazones had vascular smooth muscle relaxant potencies which correlated directly with their ability to inhibit an isozyme of cyclic nucleotide phosphodiesterase located in the sarcoplasmic reticulum of cardiac muscle. They suggested that the vascular relaxation produced by these agents, which produced significant increases in cAMP, was related to their ability to inhibit a vascular enzyme similar or identical to this isozyme.

It is also suggested that increased cAMP levels may cause relaxation by the regulation of myosin light chain kinase. The activity of myosin light chain kinase is regulated by cAMP-dependent protein kinase which diphosphorylates the

enzyme and weakens its ability to bind to and be activated by calmodulin. Evidence supporting diphosphorylation of myosin light chain kinase has been found by Vallet et al. (1981) in vascular smooth muscle.

In recent years there is increasing evidence in support of a role for cGMP as a mediator of vascular smooth muscle relaxation. Cyclic GMP is thought to mediate vasorelaxation by endothelium derived relaxing factor (EDRF), atrial natriuretic factor, and the nitrovasodilators (Khalil et al., 1987). The increased cGMP levels are attained by activation of guanylate cyclase and the increased levels of cGMP lead to decreased intracellular calcium levels.

In 1980, Furchgott and Zawadzki discovered that the relaxation by acetylcholine of precontracted isolated vascular preparations is dependent on the presence of intact endothelial cells. It has been demonstrated that EDRF directly activates the soluble isozyme form of guanylate cyclase (Fosterman et al., 1986) and it is thought that EDRF is a reactive oxidized material, analogous to nitric oxide, that can give rise to a free radical which activates guanylate cyclase (Murad, 1986).

Unlike EDRF, atrial natriuretic factor selectively activates the particulate form of guanylate cyclase in vascular smooth muscle (Winquist et al., 1984) and has no

effect on the soluble form of the enzyme. The endothelium independent relaxant effect of atrial natriuretic factor is also thought to be mediated via increased tissue levels of cGMP (Winquist et al., 1984). However, Nakatsu and Diamond (1989) believe that cGMP turnover in the tissue may be more important than cGMP concentration.

Nitrovasodilators are also thought to mediate smooth muscle relaxation by activating the particulate form of guanylate cyclase and increasing intracellular concentrations of cGMP (Waldman and Murad, 1987). The exact mechanism of enzyme activation remains unknown but it has been suggested that these agents are all capable of generating nitric oxide and that this free radical is the activator of guanylate cyclase.

1.4 Competitive and noncompetitive inhibition

This study involved determining the effect of D_2O on the pA_2 value of the alpha-adrenoceptor antagonist prazosin which competitively blocks the response to the alpha-adrenoceptor agonist phenylephrine. Schild (1947) defined the pA_2 value as the negative logarithm of the molar concentration of a competitive antagonist which reduces the effect of a double concentration of agonist to that of a single one. The pA_2 value should also be an accurate indication of the affinity of an antagonist for its receptor (Muramatsu et al., 1990).

An agonist is a drug which when coupled with the receptor in an agonist-receptor complex will elicit a response. An antagonist on the other hand, may form an antagonist-receptor complex but it does not produce a cellular response. A competitive antagonist is one which will shift the dose response curve of an agonist to the right in a manner proportional to the antagonist concentration. However, the maximal response remains the same since the effect of the antagonist can be overcome by raising the agonist concentration. A pA_2 value may be determined by using a fixed concentration of antagonist along with graded concentrations of an agonist which acts at the same receptor.

Along with determining the D_2O effect on competitive inhibition the effect on noncompetitive inhibition was also studied. Noncompetitive inhibition is a type of antagonism which is not surmountable and therefore cannot be overcome by increasing the concentration of agonist or depolarizing agent. The noncompetitive antagonist used was nifedipine which is referred to as a calcium antagonist and blocks calcium entry via L-type calcium channels only (Zelis and Moore, 1989). Potassium chloride was used as the depolarizing agent.

Godfraind et al. (1986) define a calcium antagonist as a drug that alters the cellular function of calcium by inhibiting its entry or release or by interfering with its intracellular action. Although Church and Zsotér (1980)

suggest an intracellular action for nifedipine, Godfraind (1983) argues that the action of nifedipine is related to blockade of calcium entry.

To determine the effect of D_2O on the action of nifedipine, a pD_2' value was determined. The pD_2' is defined as the negative logarithm of the molar concentration of a noncompetitive antagonist which reduces the maximal effect of an agonist to 50% of its initial value (Ariens and van Rossum, 1957).

1.5 Hypertension

In order to gain knowledge in understanding the development of hypertension in man and to find methods for its control and treatment, studies have been carried out on various animal models, the most popular species of which is the rat. Hypertension is induced in animal models by a number of methods including deoxycorticosterone acetate administration and renal artery constriction with or without removal of the contralateral kidney (Field et al., 1972) but a number of genetic models of hypertension have also been developed. The most commonly used strains include the spontaneously hypertensive rat (SHR) which was developed by brother-sister inbreeding and the Dahl salt-sensitive rat which develops hypertension due to a sensitivity to increases in dietary sodium chloride (Triggle, 1989). One problem associated with using these genetic models of hypertension is

that there is no single adequate control for the models (Clineschmidt et al., 1970) and often the results depend on the type of normotensive control which is used for the study. Clineschmidt et al. (1970) suggested the use of more than one strain of rat as a control since they found that although aortae from SHR did not show a reduced contractile response to noradrenaline in comparison to National Institute of Health Wistar rats there was a depression of contraction in comparison to Carworth Farm Wistar rats.

A further problem associated with the use of genetically hypertensive rats arises from the fact that different strains show varying results. For example, Massingham and Shevde (1971) showed that aortae from the New Zealand strain of genetically hypertensive rat exhibited reduced reactivity to norepinephrine while Clineschmidt et al. (1970) showed that the sensitivity of SHR rats was the same as controls. Furthermore, the results obtained for any particular strain vary depending on whether the work is performed *in vivo* or *in vitro*. However, generally *in vitro* studies show a decrease in the sensitivity of tissues from SHR rats to contractile agents in comparison to normotensive controls (Triggle, 1989).

Although many problems exist with the use of these animal models information has been gained contributing to the knowledge of the disease process of hypertension in man and to the development of drugs for treatment.

It is widely accepted that the genetically hypertensive rat demonstrates vascular responses which are different from those of normotensive controls. Most research is geared toward investigating what determines these vascular reactivity changes and if they are a cause or a consequence of the hypertension. In this study an assessment was made as to the effect of D_2O on the blood pressure of spontaneously hypertensive rats and Wistar Kyoto normotensive rats (WKY), and on the contractile response to both phenylephrine and potassium chloride.

Previous studies by Vasdev et al. (1990a) using SHR rats show that D_2O reduces the blood pressure and high in vitro calcium uptake induced by phenylephrine via receptor-operated calcium channels and by potassium chloride via voltage-operated channels in aortae. Similar studies using Dahl salt-sensitive rats showed that D_2O normalizes both elevated blood pressure and in vitro aortic calcium uptake (Vasdev et al., 1990b). Since D_2O has been shown to normalize calcium uptake in SHR rats and reduce blood pressure it may be expected that the phenylephrine and potassium chloride induced contractions of SHR aortae from rats treated with D_2O will not differ from those of normotensive controls. In considering this hypothesis, however, it is worth noting that vascular reactivity changes using other hypotensive agents were not reversed once hypertension had developed (Triggle, 1989). Since this study involves the use of aortae from rats which

have already developed hypertension, this latter observation should be taken into consideration.

1.6 Aims of the study

1. To determine if D_2O has an effect on vascular smooth muscle contraction induced by phenylephrine which stimulates calcium entry via the receptor-operated calcium channel.

2. If there is a D_2O effect on phenylephrine induced vascular smooth muscle contraction, to determine if the effect is reversible.

3. Since vascular endothelium releases EDRF when stimulated by a range of pharmacological agents (Furchgott et al., 1983), to investigate whether the endothelium plays a role in the D_2O mediated effect on vascular smooth muscle contraction.

4. To determine if D_2O has an effect on vascular smooth muscle contraction induced by potassium chloride which stimulates calcium entry via voltage-operated calcium channels.

5. To investigate the effect of D_2O on vascular smooth muscle contraction induced by Bay K 8644 which enhances calcium influx via voltage-operated calcium channels under partial depolarizing conditions.

6. To determine the effect of D_2O on vascular smooth muscle contraction induced by extracellular calcium influx via the voltage-operated calcium channel after depletion of intracellular calcium.

7. To assess the effect of D_2O on sucrose induced vascular smooth muscle contraction which occurs independently of membrane potential and in the absence of extracellular calcium.

8. To determine whether D_2O will have a selective effect on vascular smooth muscle relaxation induced by either isoprenaline or acetylcholine which act by increasing intracellular concentrations of cAMP and cGMP, respectively.

9. To investigate whether D_2O will have a selective effect on either noncompetitive inhibition of potassium chloride induced contraction by nifedipine or competitive inhibition of phenylephrine induced contraction by prazosin.

10. To examine whether D_2O produces a hypotensive effect in SHR and WKY rats and, if so, to determine whether there will be corresponding vascular reactivity changes.

11. To attempt to clarify the previously postulated mechanisms of action of D_2O.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

For experimental procedures 2.4.1 to 2.4.11, male Sprague-Dawley rats weighing between 100-150 grams were purchased from Canadian Hybrid Farms, Nova Scotia, Canada. WKY and SHR rats (100-150 grams), for experimental procedures 2.4.12 to 2.4.14, were purchased from Charles River Inc., Montreal, Canada. At the time of sacrifice the rats weighed 300-400 grams.

The rats were maintained under standard light and temperature conditions (twelve hour light/dark period, 20-22 °C, 40-60% humidity). Housing was provided by rectangular shoe box cages, made of polycarbonate plastic, with a detachable metal rod lid (three rats per cage). The cages contained Beta-Chip wood chip bedding purchased from Charles River Inc. Diet consisted of free access to Purina Rat Chow #T-5012 (Ralston Purina Company) and tap water unless otherwise specified in the experimental procedures.

2.2 Drugs and reagents

The list of drugs and chemicals used throughout the experimental procedures is:

Acetylcholine chloride	Sigma Chemical Company, MO, USA
Bay K 8644	Research Biochemicals Inc., MA, USA
Calcium chloride	Sigma Chemical Company, MO, USA
Deuterium oxide	Sigma Chemical Company, MO, USA Atomic Energy of Canada Ltd., Ont., Can.
Dextrose	DIFCO Laboratories, MI, USA
Ethylenediaminetetraacetic acid	Sigma Chemical Company, MO, USA
Isoprenaline	Sigma Chemical Company, MO, USA
Magnesium sulphate	BDH Chemicals, Ont., Can.
Nifedipine	Sigma Chemical Company, MO, USA
Phentolamine	Sigma Chemical Company, MO, USA
Phenylephrine	Sigma Chemical Company, MO, USA
Potassium chloride	Fisher Scientific Co., Ont., Can.
Potassium dihydrogen phosphate	Fisher Scientific Co., Ont., Can.

Prazosin	Sigma Chemical Company, MO, USA
Sodium bicarbonate	Sigma Chemical Company, MO, USA
Sodium chloride	Fisher Scientific Co., Ont., Can.
Sucrose	Sigma Chemical Company, MO, USA

2.3 Preparation of aortic rings

2.3.1 Tissue isolation

Rats were killed by a blow to the head and exsanguinated. The chest cavity was then surgically opened and the thoracic aorta was removed and placed in Krebs solution of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 12.5, dextrose 11.1, and EDTA 0.01. These solutes were diluted in either deionized water or deuterium oxide, as specified in the experimental procedures, and aerated with 95% oxygen and 5% carbon dioxide. The aorta was then cleaned of all fat, blood and connective tissue and cut into sectional rings of approximately three mm in length.

2.3.2 Experimental apparatus

Aortic rings were stationed on metal hooks (made from insect pins) and attached to force transducers, by 5-0

silk thread, at one end while the other end was held stationary. Pairs of rings were lowered into 25 mL baths heated at 38°C, containing aerated Krebs solution. Tension measured by the force transducers was recorded on a Beckman R611 polygraph. A diagramatic representation of the experimental apparatus may be seen in Figure 2-1. One gram of tension was applied to the rings and they were equilibrated in the Krebs solution for a time specified in the experimental procedures.

2.4 Experimental procedures

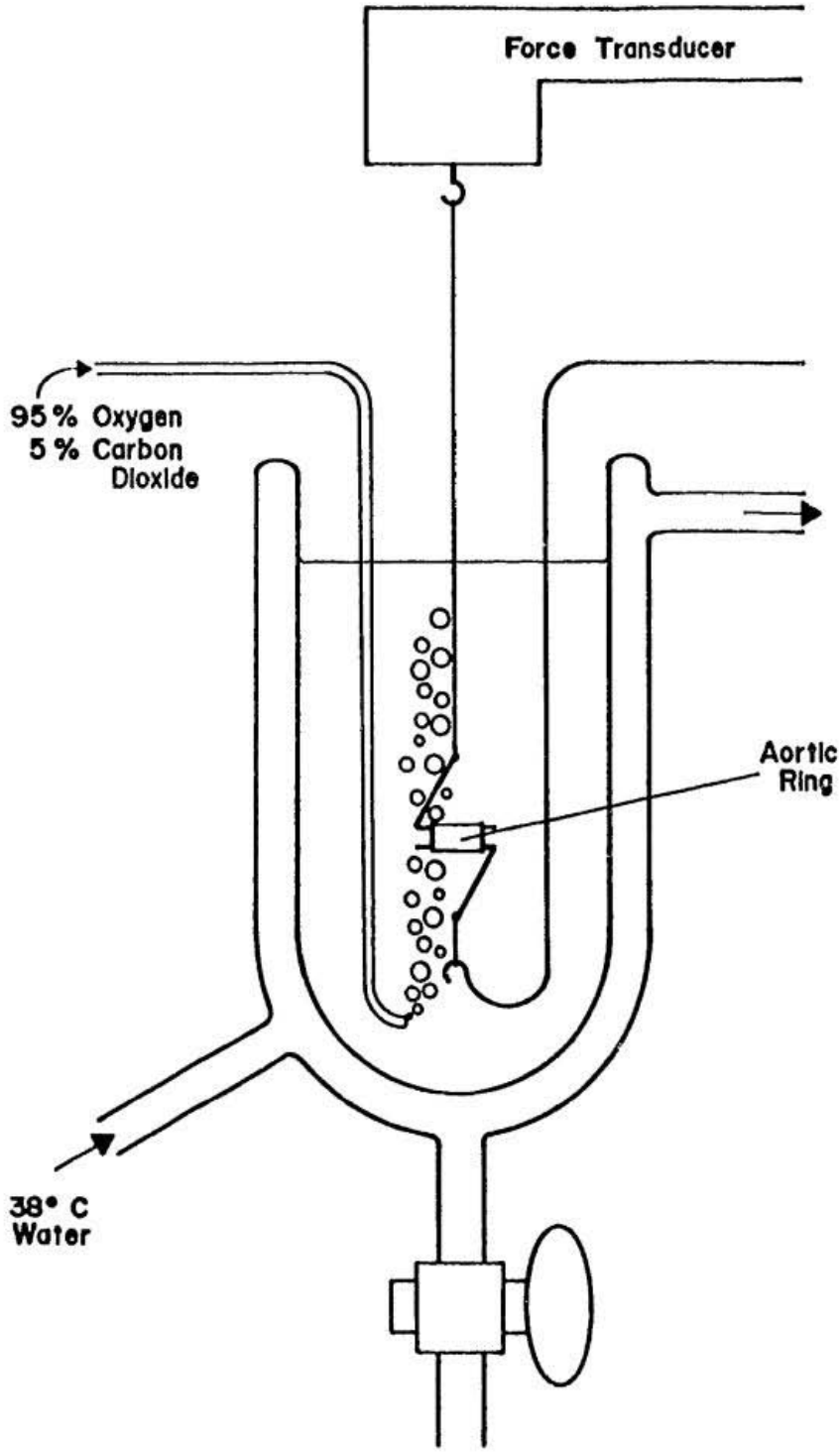
2.4.1 Phenylephrine dose response curves

Aortic rings were allowed to equilibrate for two hours in Krebs solution (made up in 0, 20, 40, 60, or 80% D_2O by volume), the solution being changed every thirty minutes. A dose response curve was then constructed by adding consecutive doses of phenylephrine ($1x10^{-12}M$ to $1x10^{-5}M$) to the bath containing the aortic rings once contraction to the previous concentration had reached a plateau. Following the contraction with phenylephrine, $1x10^{-5}M$ acetylcholine was added and a relaxation noted to confirm that the endothelium of the tissue was intact (also performed in protocols 2.4.2-2.4.4).

2.4.2 Reversibility

Rings were allowed to equilibrate in sixty percent D_2O Krebs for one hour; Krebs with zero percent D_2O was used

Figure 2-1: Experimental apparatus.


Force Transducer
95% Oxygen
5% Carbon
Dioxide
Aortic
Ring
38° C
Water

as a control. A phenylephrine dose response curve was then constructed using $1x10^{-12}M$ to $1x10^{-5}M$ phenylephrine. Rings which had been equilibrated in sixty percent D_2O Krebs were then re-equilibrated in regular Krebs for two hours. The second equilibration time was double that of the first because although the binding rate constants for H^+ and D^+ ions are similar, the rate constan for the unbinding of D^+ is slower by a factor of about 2.5 (Prod'hom et al., 1987). A second phenylephrine contraction dose response curve was then constructed.

2.4.3 Mechanical disruption of the endothelium

After the aorta had been removed, it was cut into two sections. One section had the endothelium mechanically disrupted by gently scraping the inside of the aorta with a tube of soft plastic. Three mm rings were then cut and a ring with an intact endothelium was equilibrated in the same bath as a ring with a disrupted endothelium. Tissues were set up in organ baths containing sixty percent D_2O Krebs or regular Krebs as the control. The tissues were allowed to equilibrate for two hours and then a phenylephrine dose response curve was performed. Acetylcholine induced relaxation was demonstrated to be absent to confirm that the endothelium scraping was effective.

2.4.4 Potassium chloride dose response curves

Rings were allowed to equilibrate in sixty percent

D_2O for two hours (regular Krebs for controls). Krebs solutions were then made up with varying concentrations of potassium chloride from 9.6 mM to 65.8 mM. The concentration of sodium chloride added was also simultaneously changed so as to maintain an isotonic solution. A dose response curve was then constructed by changing the bath solutions and substituting Krebs solution containing increasing concentrations of potassium chloride.

2.4.5 Calcium dose response curves

Aortic rings were permitted to equilibrate in either sixty percent D_2O Krebs or regular Krebs for two hours. A $1x10^{-6}M$ phenylephrine induced contraction was then measured to confirm the presence of the D_2O inhibitory effect. The rings were then washed in calcium free Krebs solution, containing 60 mM KCl, at fifteen minute intervals until no contraction response to KCl could be elicited (at least one hour); the calcium free Krebs was also diluted with either sixty percent D_2O or H_2O. A calcium dose response curve was then constructed by adding increasing concentrations of $CaCl_2$ from 0.5 mM to 5 mM.

2.4.6 Bay K 8644 dose response curves

Aortic rings were allowed to equilibrate in sixty percent D_2O Krebs or regular Krebs for two hours. The Krebs solution was then substituted for Krebs solution containing 10 mM KCl; $1x10^{-6}M$ phentolamine was added to block receptor

operated calcium channels. A Bay K 8644 dose response curve was then constructed using $1x10^{-9}$M to $1x10^{-5}$M Bay K 8644.

2.4.7 Sucrose induced contraction

Aortic rings were permitted to equilibrate in either sixty percent D_2O Krebs or regular Krebs for two hours. A sucrose contraction response was then performed using 250 mM sucrose.

2.4.8 Isoprenaline induced relaxation

Rings were permitted to equilibrate in either sixty percent D_2O Krebs or regular Krebs for one hour. A phenylephrine induced contraction response was then measured with $1x10^{-6}$M phenylephrine to confirm the presence of the D_2O inhibitory effect. After washing for one further hour the tissues equilibrated in regular Krebs were exposed to $1x10^{-6}$M phentolamine to bring the contraction levels to the same level as tissues incubated in sixty percent D_2O Krebs. A second $1x10^{-6}$M phenylephrine induced contraction was then performed and the precontracted tissues were relaxed with $1x10^{-4}$M isoprenaline.

2.4.9 Acetylcholine induced relaxation

Following the final phenylephrine control dose response curves in the absence of D_2O and in the presence of sixty percent D_2O in experimental procedure 2.4.11 an acetylcholine dose response curve was constructed using

$1x10^{-10}M$ to $1x10^{-4}M$ acetylcholine.

2.4.10 Determination of pD_2' for nifedipine

Tissues were equilibrated in either sixty percent D_2O Krebs or regular Krebs for two hours. A potassium chloride dose response curve was then constructed according to the method stated in experimental procedure 2.4.4. Subsequent response curves were constructed on the same tissues pre-incubated with three different concentrations of nifedipine: $5x10^{-9}M$, $1x10^{-8}M$, and $1x10^{-7}M$. Controls were performed simultaneously in which four dose response curves to potassium chloride were constructed without the addition of nifedipine. The pD_2' was calculated according to the following equation derived by Bickerton (1963):

$$pD_2' = pDx' + \log [Eam/ Eabm - 1]$$

where: pDx' = the negative logarithm of the molar concentration of nifedipine

Eam = the maximal contraction height in the absence of nifedipine

Eabm = the maximal contraction height in the presence of nifedipine

2.4.11 Determination of pA_2 for prazosin

Tissues were incubated in either sixty percent D_2O Krebs or regular Krebs for two hours. A phenylephrine dose

response curve was then constructed using $1x10^{-12}M$ to $1x10^{-5}M$ phenylephrine. Subsequent response curves were constructed on the same tissues pre-incubated with three different concentrations of prazosin: $1x10^{-9}M$, $1x10^{-8}M$, and $1x10^{-7}M$. Controls were performed simultaneously in which four dose response curves to phenylephrine were constructed without the addition of prazosin. The pA_2 was calculated according to the Schild Plot method as described by Schild (1947). The Schild Plot is a plot of log (X'/X)-1 versus -log (A) where:

X'/X= the agonist dose ratio

A= the antagonist concentration

For a competitive antagonist, the line has a slope of -1 and the intercept on the abscissa corresponds to pA_2.

2.4.12 Blood pressure measurements

Systolic blood pressure was measured by tail plethysmography according to the method described by Byrom (1969); signals were amplified using a model 5A amplifier (ITTC Life Science Instruments, California, USA). Four blood pressure measurements were recorded before the animals were sacrificed.

2.4.13 Chronic *in vivo* studies using twenty-five percent D_2O

WKY and SHR rats were treated as in section 2.1

until seven weeks of age and at that time the drinking water was replaced by either distilled water or twenty-five percent D_2O in distilled water for twelve days. The animals were then sacrificed and the thoracic aorta removed. Aortic rings were equilibrated in regular Krebs for two hours following which a dose response curve was constructed using either $1x10^{-12}M$ to $1x10^{-5}M$ phenylephrine or 9.6 mM to 65.8 mM potassium chloride by the methods stated in experimental procedures 2.4.1 and 2.4.4 (only regular Krebs solution was used).

2.4.14 Acute *in vitro* studies using sixty percent D_2O

WKY and SHR rats were treated as in section 2.4.13. Experimental procedures 2.4.1 and 2.4.4 were then performed (both regular Krebs solution and sixty percent D_2O Krebs solution were used).

2.5 Analysis of data

Results are expressed as mean $\pm$ standard error of the mean. Where no S.E.M. is indicated the level of error was too small to be detected with the means of recording and analysis employed. Tissue responses are expressed as percentage maximum contraction or relaxation, where the maximum contraction or relaxation (100%) corresponds to that of the control tissue in each group.

Maximum contractions between groups were compared using an unpaired student's t-test. Significance was accepted at a

level of $p < 0.05$.

Plots were constructed using SigmaPlot, Scientific Graph System. The pA_2 and EC_{50} values were calculated using Pharmacologic Calculation System, version 4.1 by Tallarida and Murray (Springer-Verlag New York Inc., 1987).

CHAPTER 3

RESULTS

3.1 Effect of D_2O on aortic smooth muscle contraction

3.1.1 Phenylephrine induced contraction

In normal Krebs solution contraction of aortic rings using $1x10^{-5}M$ phenylephrine resulted in a maximum control contraction of 1.08 ± 0.11 grams. Aortic rings incubated with increasing concentrations of D_2O had increasingly depressed phenylephrine induced maximal contractions (Figure 3-1). Twenty, forty, sixty, and eighty percent D_2O depressed the phenylephrine induced maximum contraction to 86.9% ± 12.6%, 74.3% ± 5.0%, 51.9% ± 6.5%, and 42.4% ± 3.7% of the control value respectively. The contractions were significantly depressed from forty percent to eighty percent D_2O.

Figure 3-2 shows the effect of varying concentrations of D_2O on the entire dose response curve constructed using $1x10^{-12}M$ to $1x10^{-5}M$ phenylephrine. Twenty, sixty, and eighty percent D_2O uniformly depress the dose response curve compared to the control curve. However, forty percent D_2O resulted in enhanced contraction from $1x10^{-11}M$ to $1x10^{-8}M$ phenylephrine. The dose response curve was depressed from $1x10^{-7}M$ to $1x10^{-5}M$ phenylephrine.

Figure 3-1: **Inhibition of $1x10^{-5}M$ phenylephrine induced aortic smooth muscle contraction with varying concentrations of D_2O.**

Columns represent mean percent maximum contraction and bars S.E.M. for eight tissues. An asterix represents a significant difference from the control contraction ($p < 0.01$).

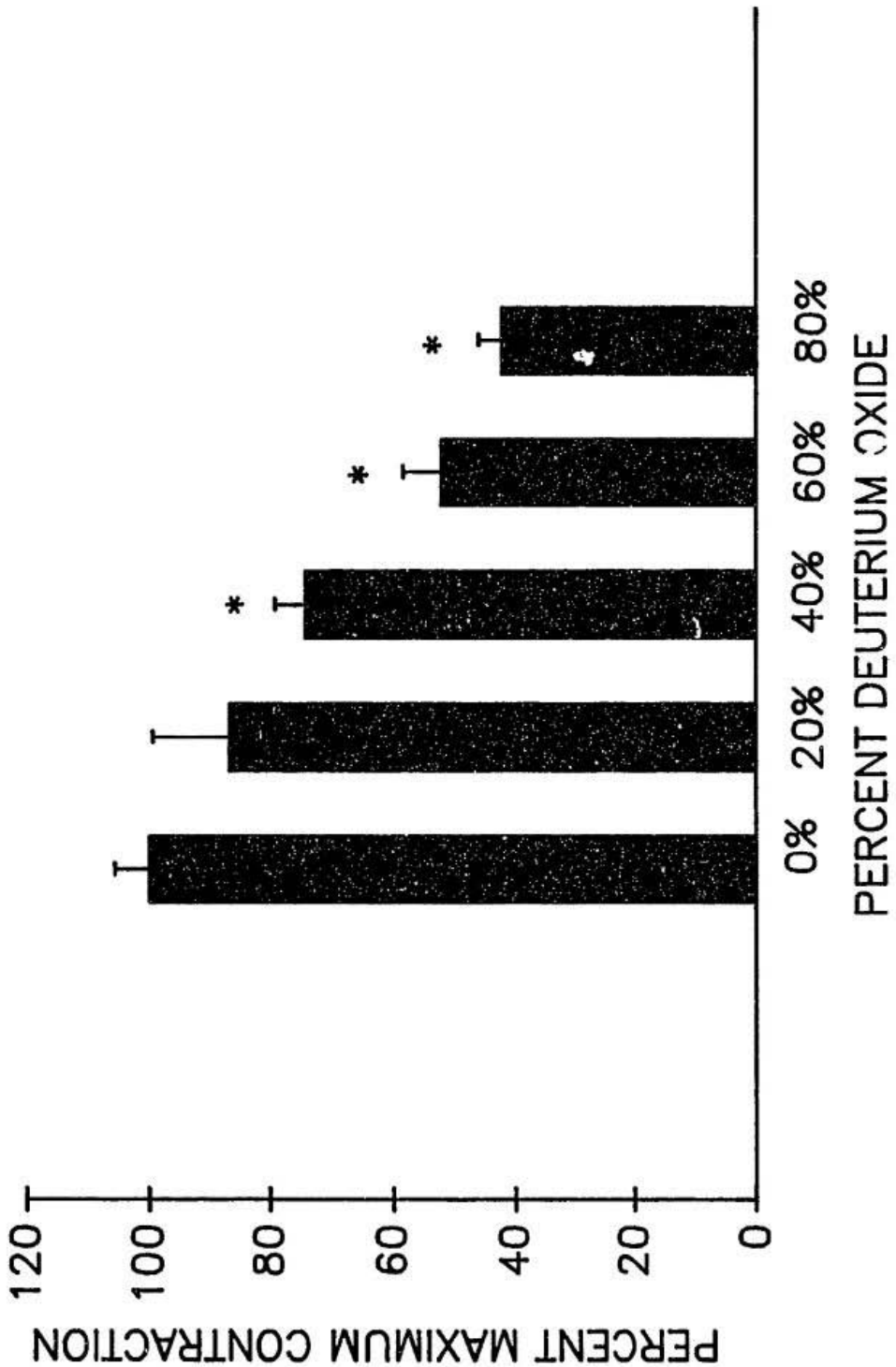
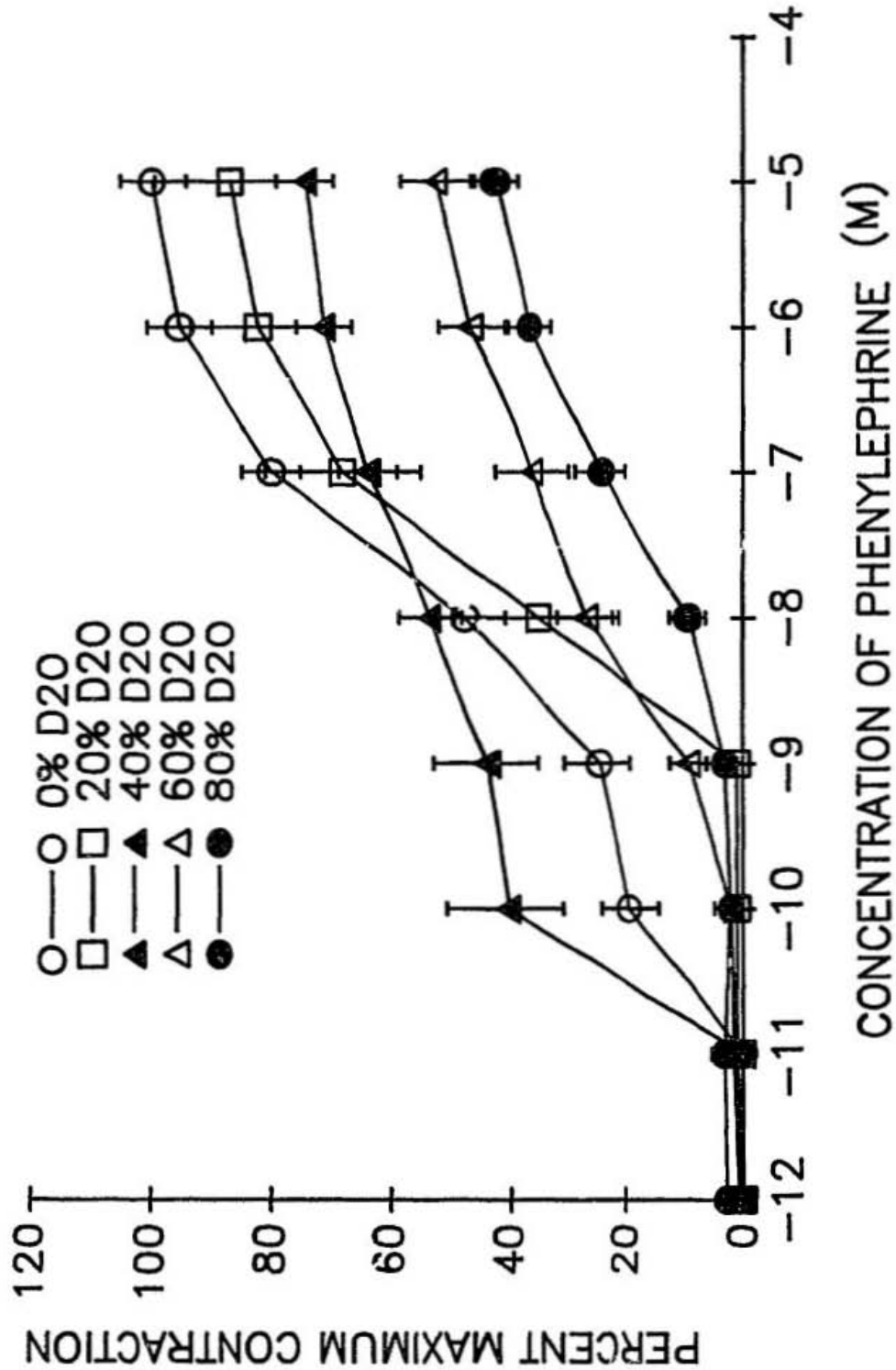

PERCENT MAXIMUM CONTRACTION
120
100
80
60
40
20
0
*
*
*
0%
20%
40%
60%
80%
PERCENT DEUTERIUM OXIDE

Figure 3-2: **The effect of varying concentrations of D_2O on phenylephrine induced aortic smooth muscle contraction.**

Dose response curves were constructed using $1x10^{-12}M$ to $1x10^{-5}M$ phenylephrine. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.


PERCENT MAXIMUM CONTRACTION
120
100
80
60
40
20
0
−12
−11
−10
−9
−8
−7
−6
−5
−4
CONCENTRATION OF PHENYLEPHRINE (M)
0% D2O
20% D2O
40% D2O
60% D2O
80% D2O

3.1.2 Reversibility

Reversal of the aortic smooth muscle inhibitory effect of sixty percent D_2O on phenylephrine induced contraction is shown in Figure 3-3. The first contraction dose response curves denoted by D_2Oa and H_2Oa are signficantly different ($p < 0.05$). H_2Oa corresponds to the first control curve constructed in the absence of D_2O. D_2Oa corresponds to the first dose response curve constructed in the presence of sixty percent D_2O. The D_2O has reduced the maximum contraction to 64.9% $\pm$ 9.6% of the control value. However, the second phenylephrine dose response curve two hours following removal of D_2O (D_2Ob; Figure 3-3) is not significantly different from the second control curve (H_2Ob; Figure 3-3).

3.1.3 Role of the endothelium

Mechanical disruption of the endothelium had no effect on the control phenylephrine dose response curves (Figure 3-4; H_2O intact/scraped) or on the depressed curves of tissues incubated in sixty percent D_2O Krebs solution (Figure 3-4; D_2O intact/scraped). A level of aortic contraction similar to that previously observed was demonstrated in all tissues incubated with either regular Krebs or sixty percent D_2O Krebs solution.

3.1.4 Potassium chloride induced contraction

Sixty percent D_2O depressed the dose response curve constructed using 9.6 mM to 65.8 mM potassium chloride (Figure

Figure 3-3: Reversal of the D_2O inhibitory effect on phenylephrine induced aortic smooth muscle contraction.

H_2Oa/b represent the first and second control curves respectively. D_2Oa represents the first curve of tissues exposed to sixty percent D_2O Krebs solution; D_2Ob represents the second curve after removal of D_2O. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.

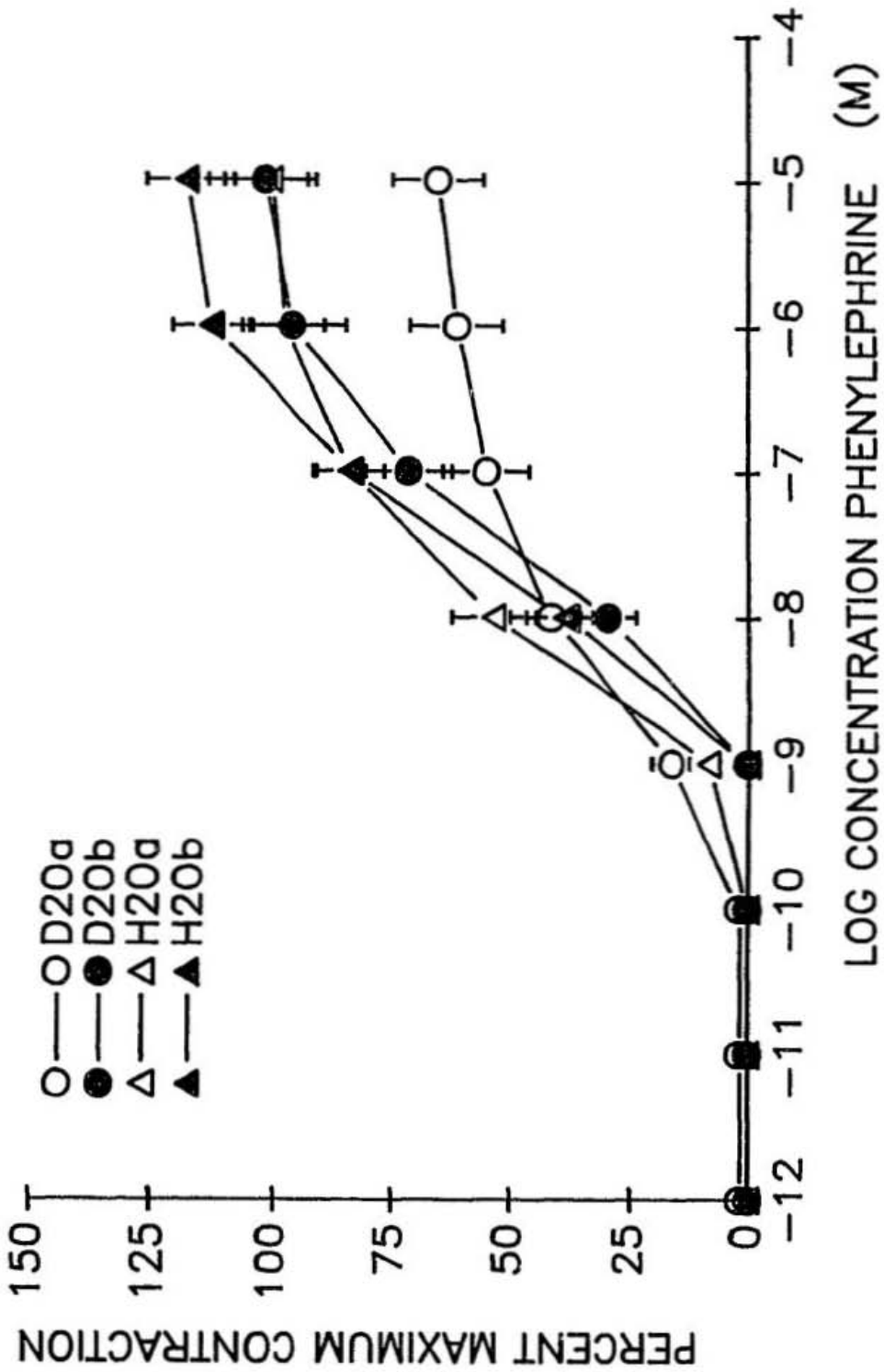
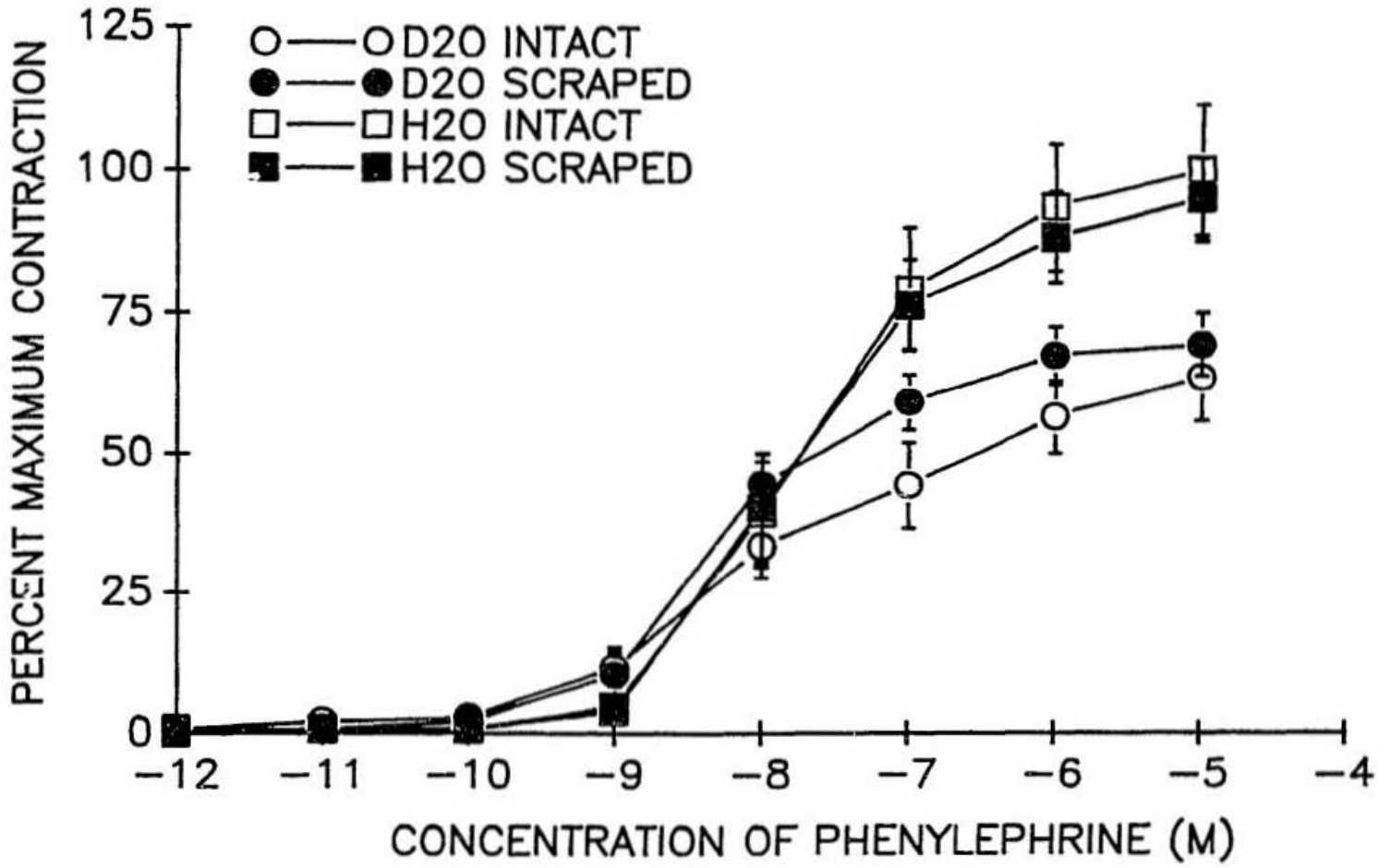

PERCENT MAXIMUM CONTRACTION
150
125
100
75
50
25
0
−12
−11
−10
−9
−8
−7
−6
−5
−4
LOG CONCENTRATION PHENYLEPHRINE (M)
D2Oa
D2Ob
H2Oa
H2Ob

Figure 3-4: **The role of the endothelium in the effect of sixty percent D_2O on phenylephrine induced aortic smooth muscle contraction.**

Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.


125
100
75
50
25
0
PERCENT MAXIMUM CONTRACTION
D2O INTACT
D2O SCRAPED
H2O INTACT
H2O SCRAPED
−12
−11
−10
−9
−8
−7
−6
−5
−4
CONCENTRATION OF PHENYLEPHRINE (M)

3-5). The control maximal contraction induced by 65.8 mM potassium chloride was 1.15 ± 0.10 grams and sixty percent D_2O depressed the potassium chloride induced maximum contraction to 51.9% ± 6.5% as compared to the control value. The difference in the potassium chloride dose response curve, constructed in D_2O Krebs, from the control curve, constructed in regular Krebs, was statistically significant ($p < 0.02$).

3.1.5 Calcium induced contraction

A calcium induced dose response curve was constructed after the contraction response to 60 mM KCl had been eliminated by washing aortic rings with calcium free Krebs solution. The level of the control maximum contraction at 5.0 mM calcium chloride was 0.93 ± 0.07 grams and sixty percent D_2O significantly ($p < 0.05$) reduced the maximum calcium contraction to 76.7% ± 5.9% as compared to control values (Figure 3-6).

3.1.6 Bay K 8644 induced contraction

The dose response curve constructed using $1x10^{-9}M$ to $1x10^{-5}M$ Bay K 8644 was significantly depressed by sixty percent D_2O ($p < 0.05$). The maximum control response in the absence of D_2O was 1.16 ± 0.11 grams. Sixty percent D_2O depressed this contraction response to 65.8% ± 11.2% as compared to the maximum control value (Figure 3-7).

Figure 3-5: **The inhibitory effect of sixty percent D_2O on potassium chloride induced aortic smooth muscle contraction.**

Dose response curves were constructed using 9.6 mM to 65.8 mM potassium chloride. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.

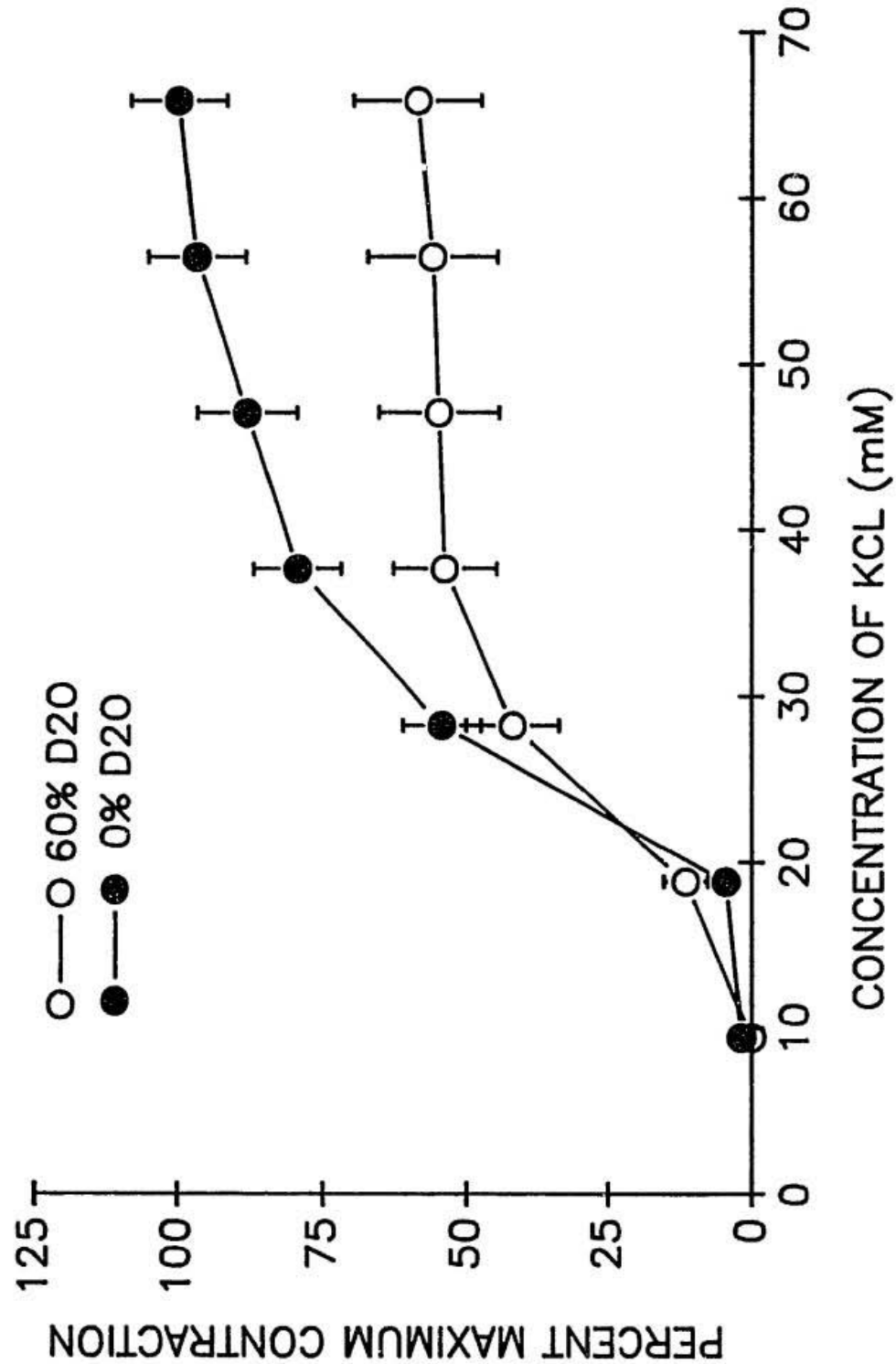

PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
0
10
20
30
40
50
60
70
CONCENTRATION OF KCL (mM)
O—O 60% D2O
●—● 0% D2O

Figure 3-6: **The inhibitory effect of sixty percent D_2O on the dose response curve of calcium in the presence of 60 mM potassium chloride.**

Dose response curves were constructed using 1.0 mM to 5.0 mM calcium chloride. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.

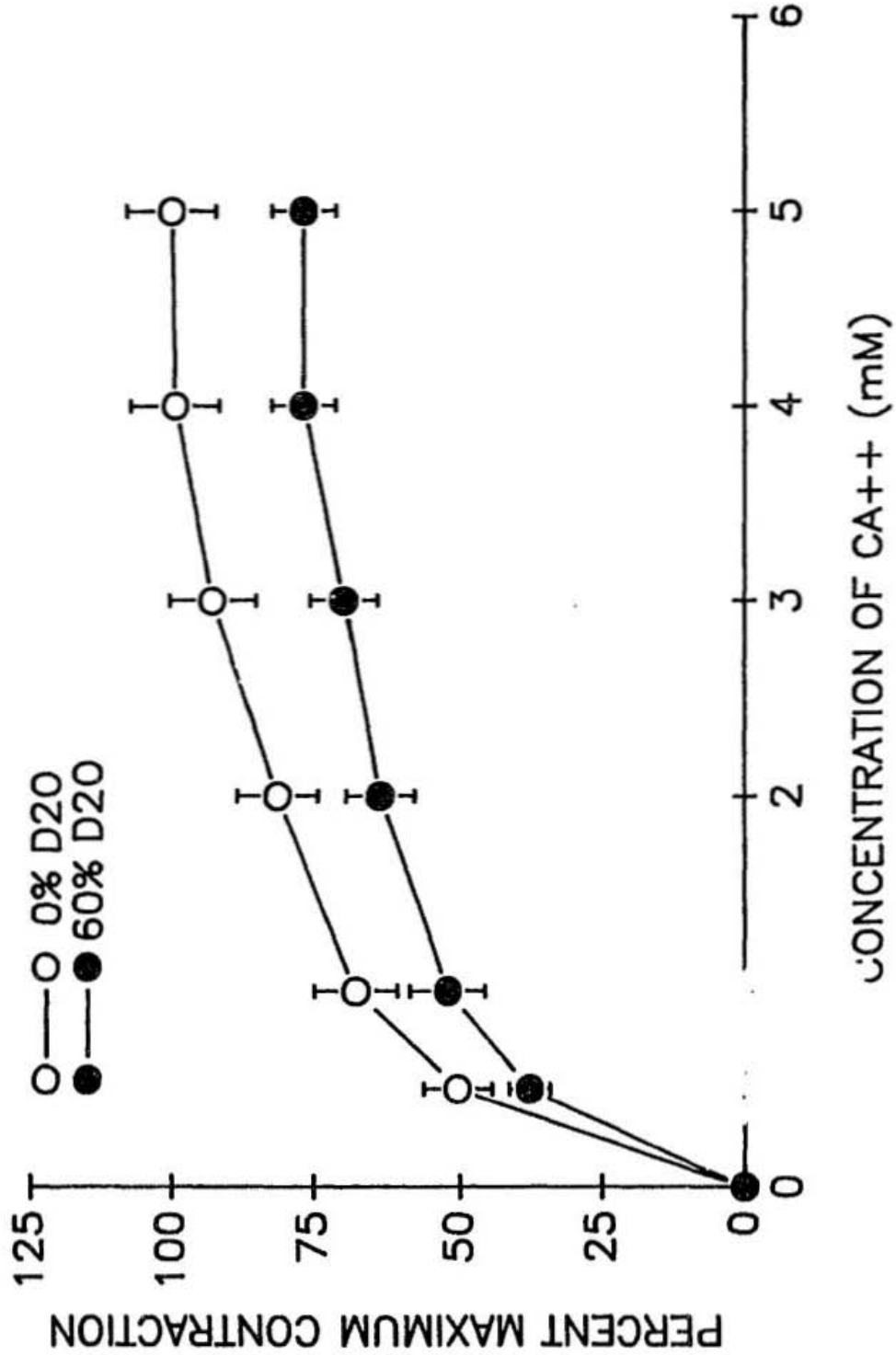
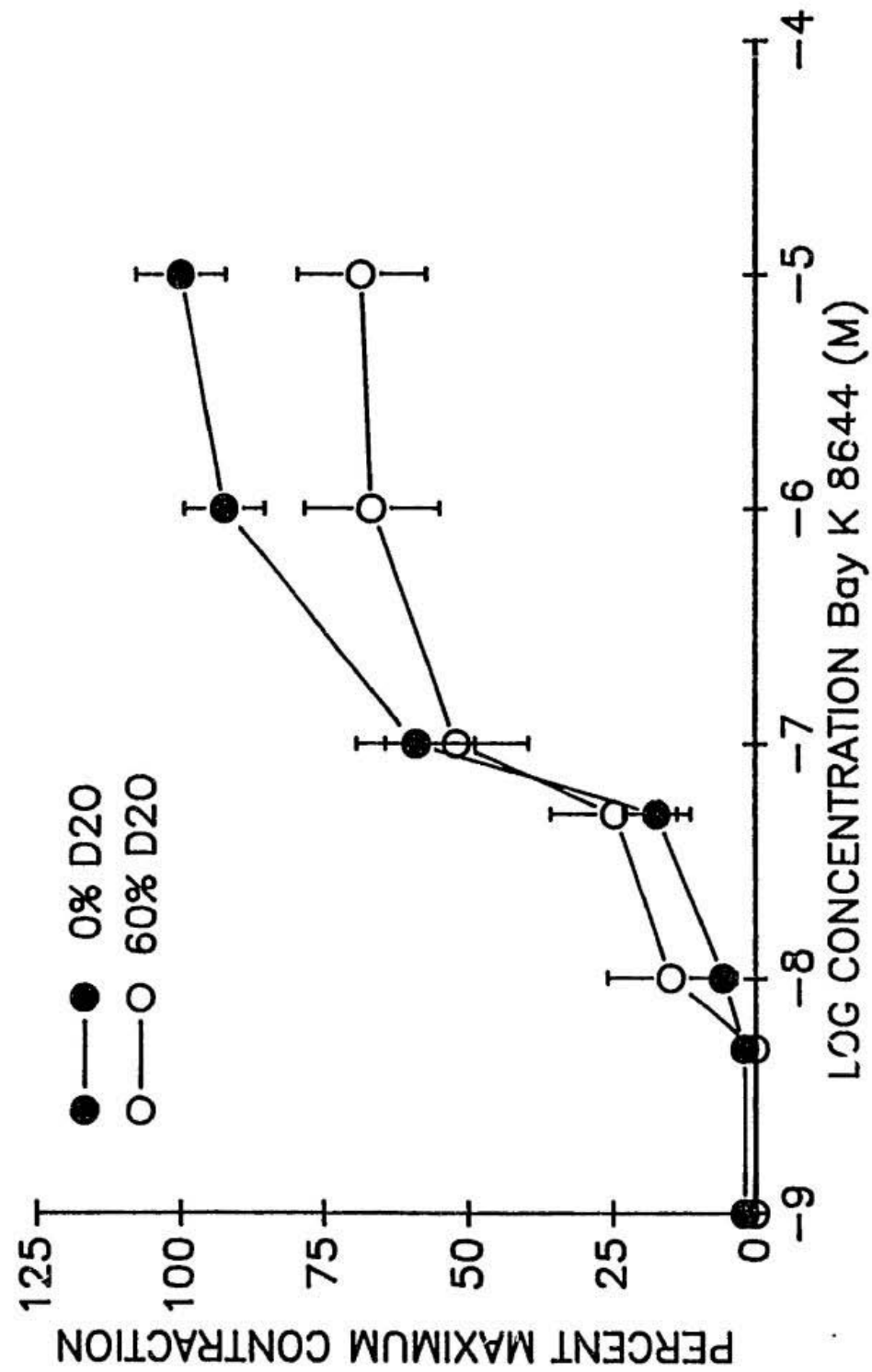

PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
0
2
3
4
5
6
CONCENTRATION OF CA++ (mM)
O 0% D20
● 60% D20

Figure 3-7: **The inhibitory effect of sixty percent D_2O on Bay K 8644 induced aortic smooth muscle contraction.**

Dose response curves were constructed using $1x10^{-9}M$ to $1x10^{-5}M$ Bay K 8644. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.


PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
0% D2O
60% D2O
−9
−8
−7
−6
−5
−4
LOG CONCENTRATION Bay K 8644 (M)

3.1.7 Sucrose induced contraction

Following incubation of aortic rings in regular Krebs a contraction response to 250 mM sucrose was performed. The amount of contraction of the control tissues to the sucrose was 0.23 ± 0.01 grams. Sixty percent D_2O significantly ($p < 0.01$) reduced the contraction to 55.6% ± 0.0% of the control value (Figure 3-8).

3.1.8 Synopsis

D_2O inhibited phenylephrine induced aortic smooth muscle contraction in a dose dependent manner with approximately 50% inhibition using 60% D_2O. This inhibitory effect was endothelium independent and reversible. Sixty percent D_2O also significantly inhibited potassium chloride, calcium, Bay K 8644, and sucrose induced aortic smooth muscle contraction.

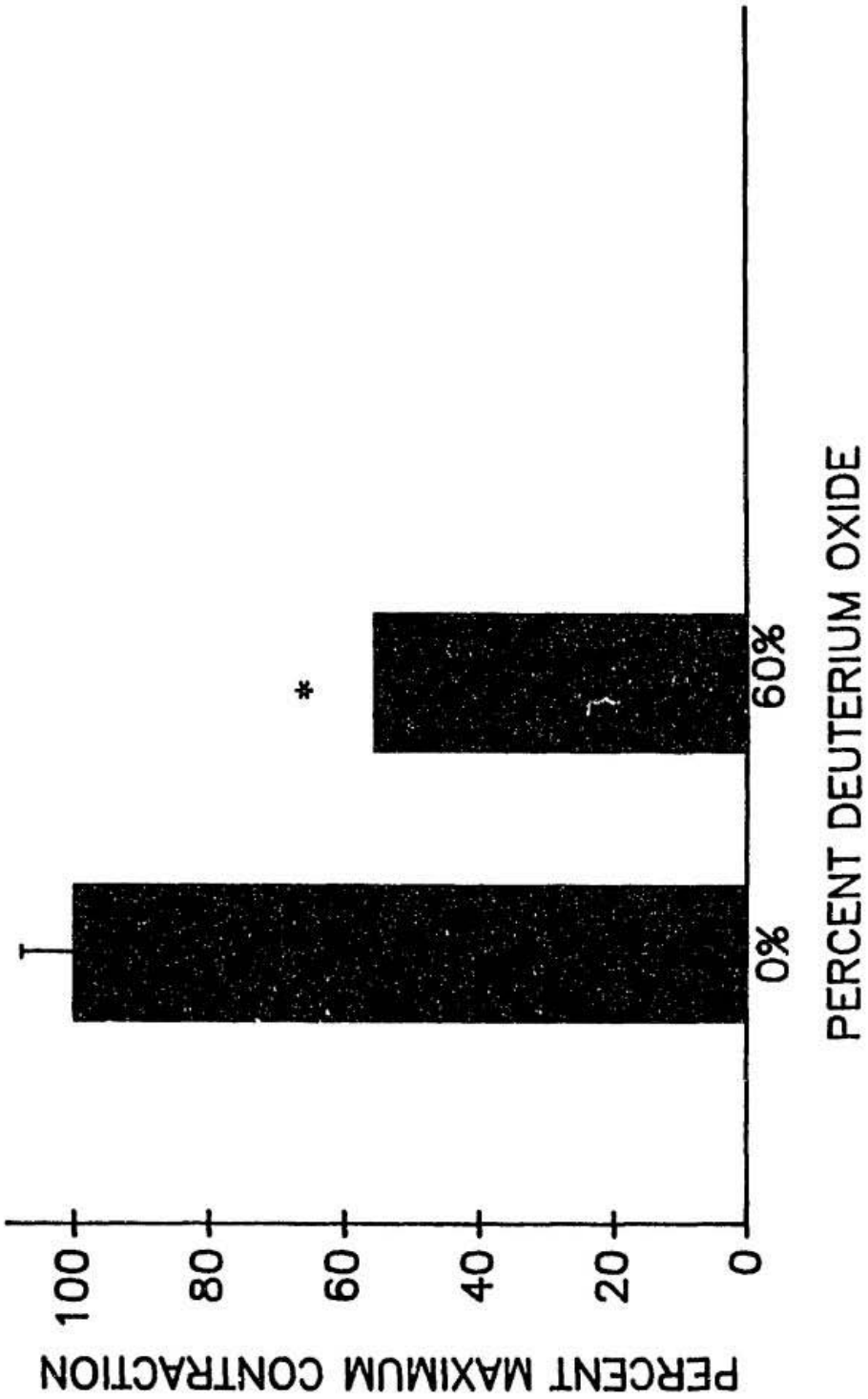
3.2 Effect of D_2O on aortic smooth muscle relaxation

3.2.1 Isoprenaline induced relaxation

Rings equilibrated in sixty percent D_2O Krebs and regular Krebs were precontracted with $1x10^{-6}M$ phenylephrine before relaxation with $1x10^{-4}M$ isoprenaline. The level of contraction of tissues incubated in regular Krebs was 0.69 ± 0.01 grams while that of tissues incubated in sixty percent D_2O Krebs was 0.46 ± 0.01 grams. Treatment of the rings equilibrated in regular Krebs with $1x10^{-6}M$ phentolamine

Figure 3-8: **The inhibitory effect of sixty percent D_2O on 250 mM sucrose induced aortic smooth muscle contraction.**

Columns represent mean percent maximum contraction and bars S.E.M. for four tissues. An asterix indicates a significant difference from control values ($p < 0.01$).


PERCENT MAXIMUM CONTRACTION
100
80
60
40
20
0
*
0%
60%
PERCENT DEUTERIUM OXIDE

resulted in a second contraction response to $1x10^{-6}M$ phenylephrine of 0.69 ± 0.01 grams. The second contraction response for tissues equilibrated in sixty percent D_2O Krebs was 0.66 ± 0.02 grams and this level of contraction was not significantly different from the second contraction response of tissues equilibrated in regular Krebs and phentolamine.

Sixty percent D_2O significantly ($p < 0.01$) reduced the relaxation response induced using $1x10^{-4}M$ isoprenaline to 50.3% ± 5.7% of the maximum control response (Figure 3-9).

3.2.2 Acetylcholine induced relaxation

Prior to relaxation with acetylcholine, aortic rings were precontracted with $1x10^{-5}M$ phenylephrine. For rings equilibrated in regular Krebs solution the level of contraction was 1.39 ± 0.08 grams; the level of contraction for rings equilibrated in sixty percent D_2O Krebs solution was 1.08 ± 0.07 grams. Sixty percent D_2O significantly ($p < 0.05$) reduced the maximum relaxation to 76.7% ± 7.7% as compared to the control value of tissues incubated in regular Krebs solution (Figure 3-10). Sixty percent D_2O also shifted the dose response curve of acetylcholine to the right. For tissues incubated in regular Krebs solution the EC_{50} was $9.62x10^{-8}M$ while for tissues incubated in sixty percent D_2O Krebs the EC_{50} was $5.64x10^{-7}M$.

Figure 3-9: **The inhibitory effect of sixty percent D_2O on $1x10^{-4}M$ isoprenaline induced aortic smooth muscle relaxation.**

Columns represent mean percent maximum relaxation and bars S.E.M. for eight tissues. An asterix indicates a significant difference from the control value ($p < 0.01$).

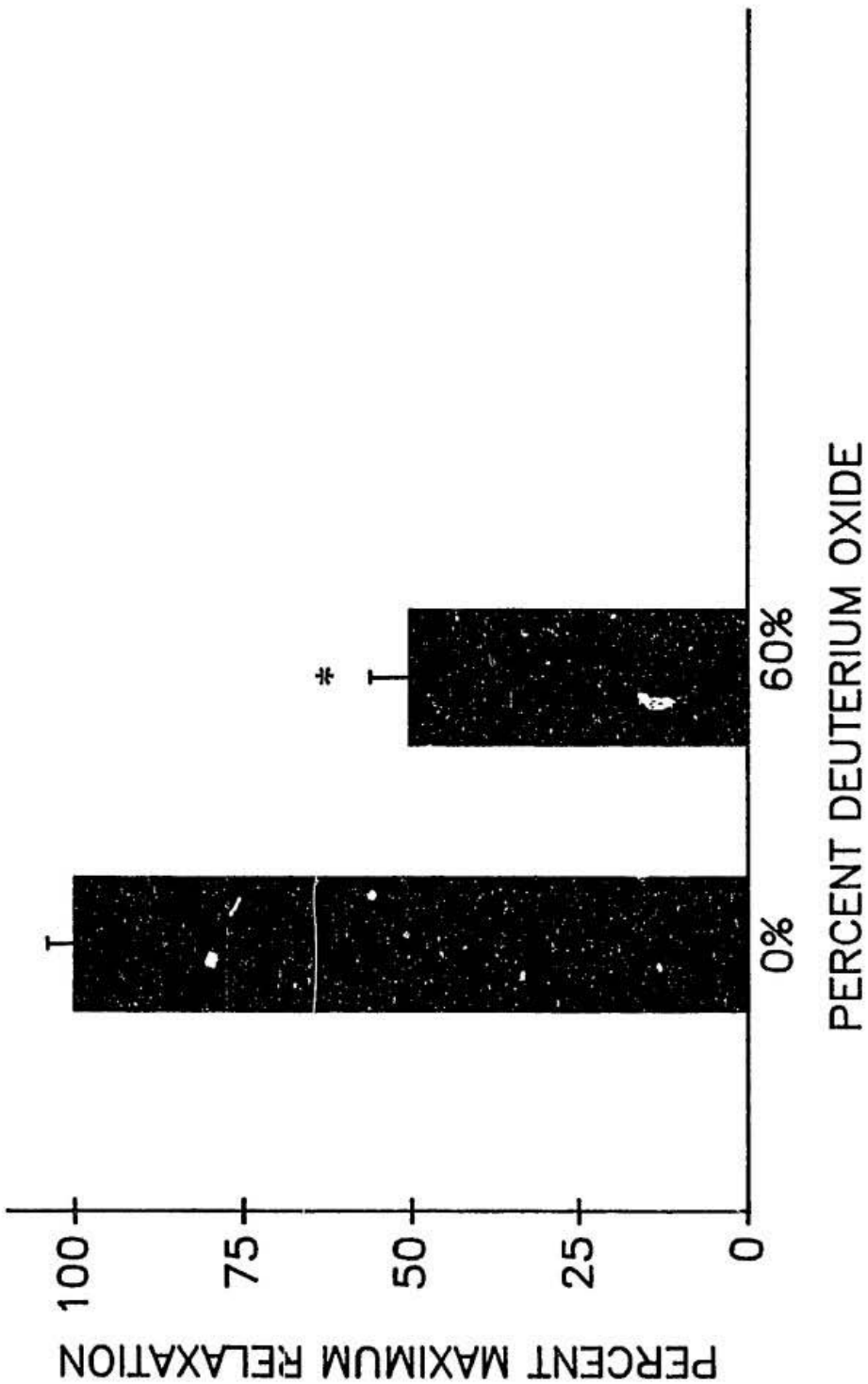
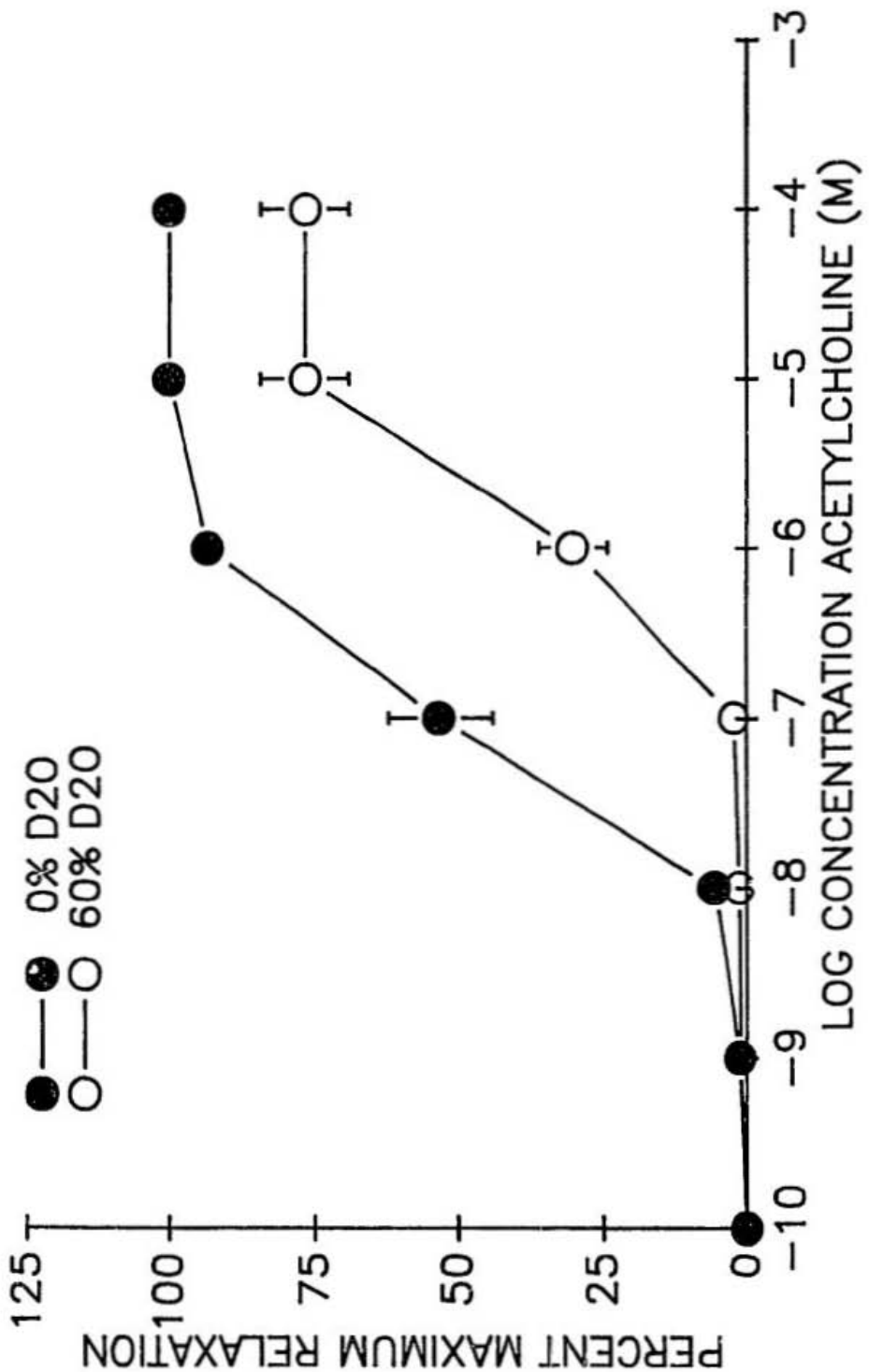

PERCENT MAXIMUM RELAXATION
100
75
50
25
0
*
0%
60%
PERCENT DEUTERIUM OXIDE

Figure 3-10: **The effect of sixty percent D_2O on the dose reponse curve constructed using $1x10^{-10}M$ to $1x10^{-4}M$ acetylcholine.**

Points represent mean percent maximum relaxation and bars S.E.M. for six tissues.


0% D2O
60% D2O
PERCENT MAXIMUM RELAXATION
125
100
75
50
25
0
−10
−9
−8
−7
−6
−5
−4
−3
LOG CONCENTRATION ACETYLCHOLINE (M)

3.2.3 Synopsis

Sixty percent D_2O reduced isoprenaline induced relaxation of aortic smooth muscle by approximately 50% and acetylcholine induced relaxation by 25%. D_2O also significantly changed the EC_{50} of the acetylcholine relaxation response.

3.3 Effect of D_2O on the affinity of an antagonist for its receptor

3.3.1 Noncompetitive inhibition

In determination of the pD_2' for nifedipine the four control curves in the absence of D_2O were not significantly different (Figure 3-11). Likewise, the four control curves in the presence of sixty percent D_2O were not significantly different (Figure 3-12). Nifedipine at concentrations of $5x10^{-9}M$, $1x10^{-8}M$, and $1x10^{-7}M$ depressed the maximum contraction response to 59.5% $\pm$ 7.1%, 38.9% $\pm$ 6.3%, and 8.3% $\pm$ 2.1% respectively of the control value in the absence of D_2O (Figure 3-13), and to 46.4% $\pm$ 5.2%, 21.4% $\pm$ 1.6%, and 3.6% $\pm$ 1.7% respectively in the presence of sixty percent D_2O (Figure 3-14).

The pD_2' for nifedipine in the absence of D_2O was 8.125 $\pm$ 0.001 whereas, the pD2' for nifedipine in the presence of sixty percent D_2O was 8.453 $\pm$ 0.002 (Table 3-1). The difference between these values was statistically significant

Figure 3-11: **Four consecutive dose response curves constructed using 9.6 mM to 65.8 mM potassium chloride in the absence of D_2O.**

Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.

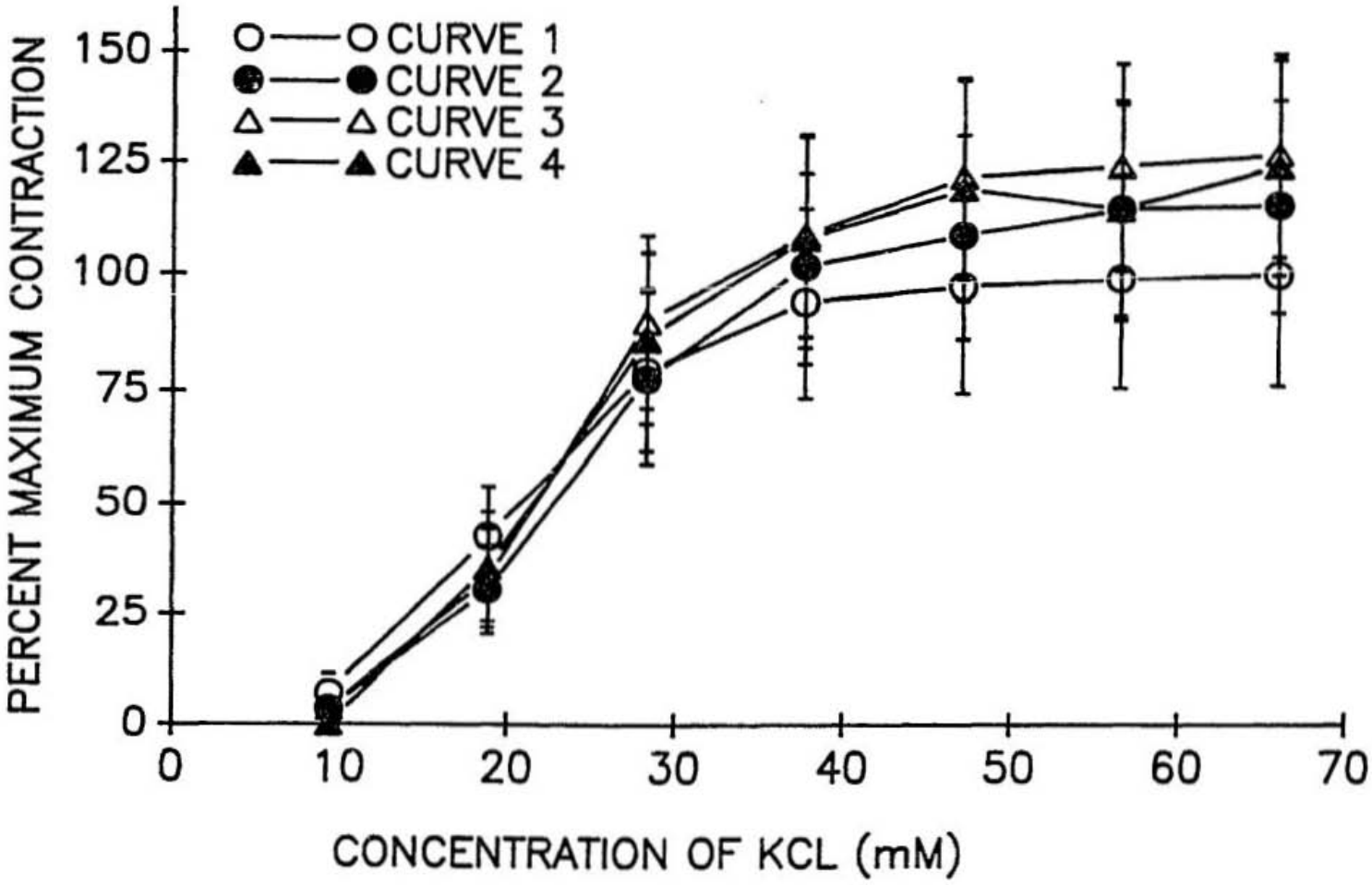

CURVE 1
CURVE 2
CURVE 3
CURVE 4
PERCENT MAXIMUM CONTRACTION
150
125
100
75
50
25
0
0
10
20
30
40
50
60
70
CONCENTRATION OF KCL (mM)

Figure 3-12: **Four consecutive dose response curves constructed using 9.6 mM to 65.8 mM potassium chloride in the presence of sixty percent D_2O.**

Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.

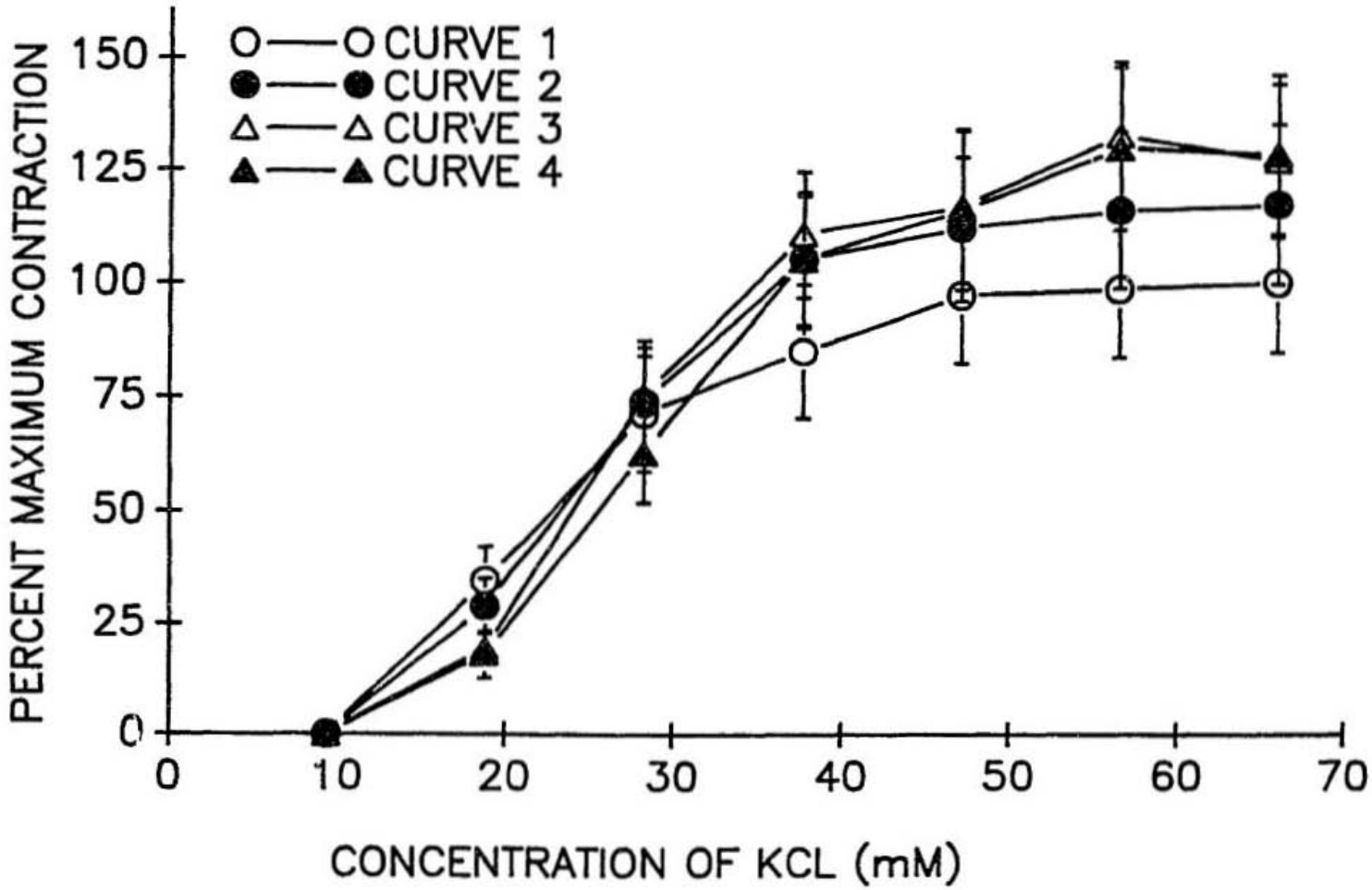

CURVE 1
CURVE 2
CURVE 3
CURVE 4
PERCENT MAXIMUM CONTRACTION
150
125
100
75
50
25
0
0
10
20
30
40
50
60
70
CONCENTRATION OF KCL (mM)

Figure 3-13: **Noncompetitive inhibition of potassium chloride induced aortic smooth muscle contraction in the presence of nifedipine and in the absence of D_2O.**

Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.

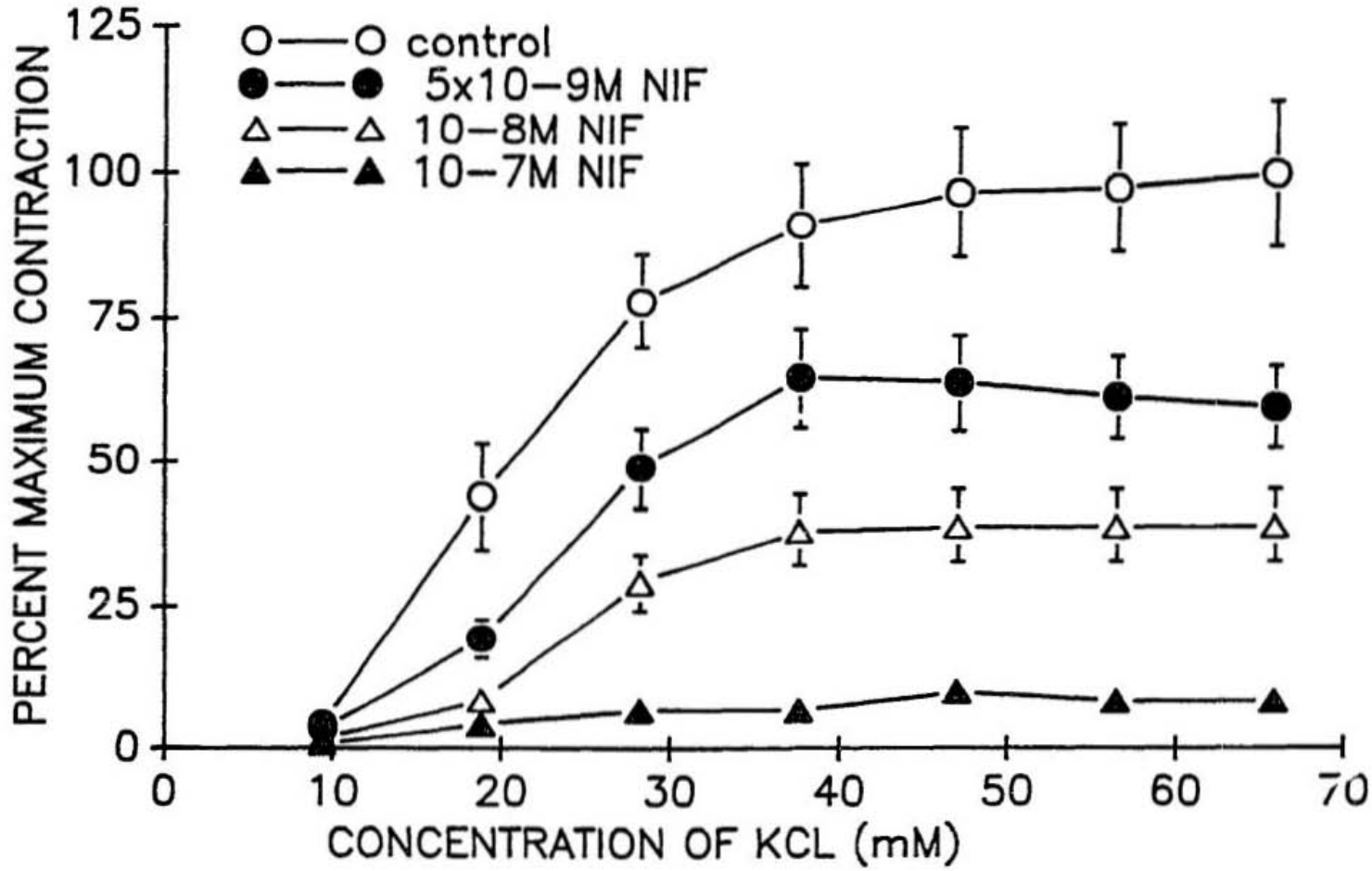

control
5x10−9M NIF
10−8M NIF
10−7M NIF
PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
0
10
20
30
40
50
60
70
CONCENTRATION OF KCL (mM)

Figure 3-14: **Noncompetitive inhibition of potassium chloride induced aortic smooth muscle contraction in the presence of nifedipine and sixty percent D_2O.**

Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.

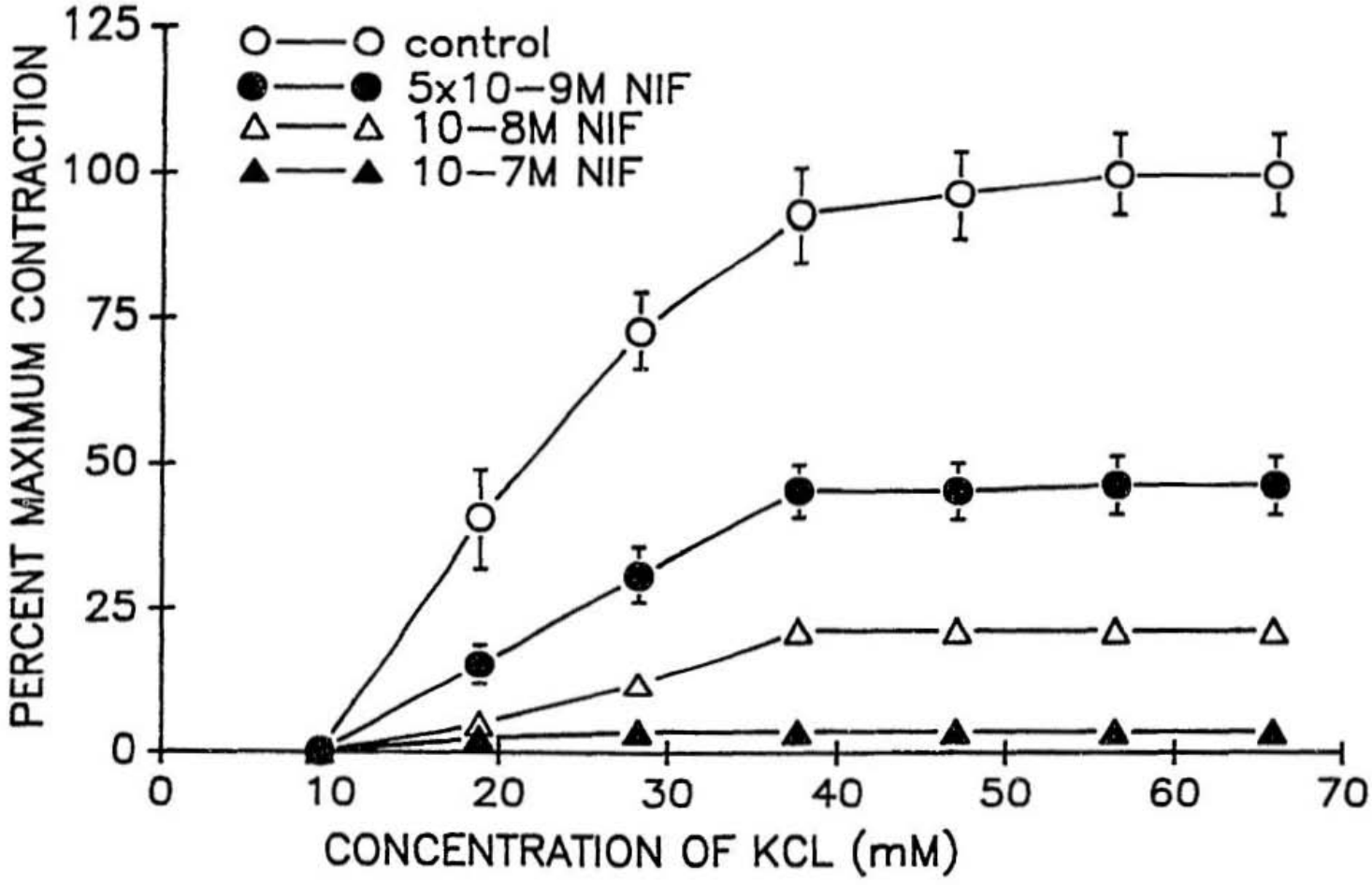

control
5x10−9M NIF
10−8M NIF
10−7M NIF
PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
0
10
20
30
40
50
60
70
CONCENTRATION OF KCL (mM)

Table 3-1: Effect of 60% D_2O on the pD_2' for nifedipine.

[nifedipine]	H_2O Krebs pD_2'	D_2O Krebs pD_2'
$5 \times 10^{-9}M$	8.134	8.363
$1 \times 10^{-8}M$	8.197	8.564
$1 \times 10^{-7}M$	8.045	8.432
mean $\pm$ S.E.M.	8.125 $\pm$ 0.001	*8.453 $\pm$ 0.002

*$p < 0.01$

($p < 0.01$).

3.3.2 Competitive inhibition

In the determination of the pA_2 for prazosin the four control curves in the absence of D_2O (Figure 3-15) were not significantly different and likewise, the four control curves constructed in the presence of sixty percent D_2O (Figure 3-16) were not significantly different. In the absence of D_2O and prazosin the EC_{50} was 1.08×10^{-7}M; 1×10^{-9}M, 1×10^{-8}M and 1×10^{-7}M prazosin changed the EC_{50} to 7.43×10^{-7}M, 1.86×10^{-5}M, and 1.01×10^{-4}M respectively (Figure 3-17). Consequently, the pA_2 value for prazosin in the absence of D_2O was 9.81 ± 0.38 and the slope of the Schild plot was -1.10 ± 0.21. In the presence of sixty percent D_2O and in the absence of prazosin the EC_{50} was 3.49×10^{-8}M; 1×10^{-9}M, 1×10^{-8}M, and 1×10^{-7}M prazosin changed the EC_{50} to 1.94×10^{-7}M, 1.23×10^{-5}M, and 3.16×10^{-4}M respectively (Figure 3-18). In determining the pA_2 for prazosin in the presence of sixty percent D_2O the slope of the Schild plot was -1.65 ± 0.14 and therefore no valid pA_2 value could be determined.

3.3.3 Synopsis

Sixty percent D_2O significantly changed both the pD_2' for nifedipine and the slope of the Schild plot for prazosin.

Figure 3-15: **Four consecutive dose reponse curves constructed using $1x10^{-12}M$ to $1x10^{-5}M$ phenylephrine in the absence of D_2O.**

Points represent mean percent maximum contraction and bars S.E.M. for six tissues.

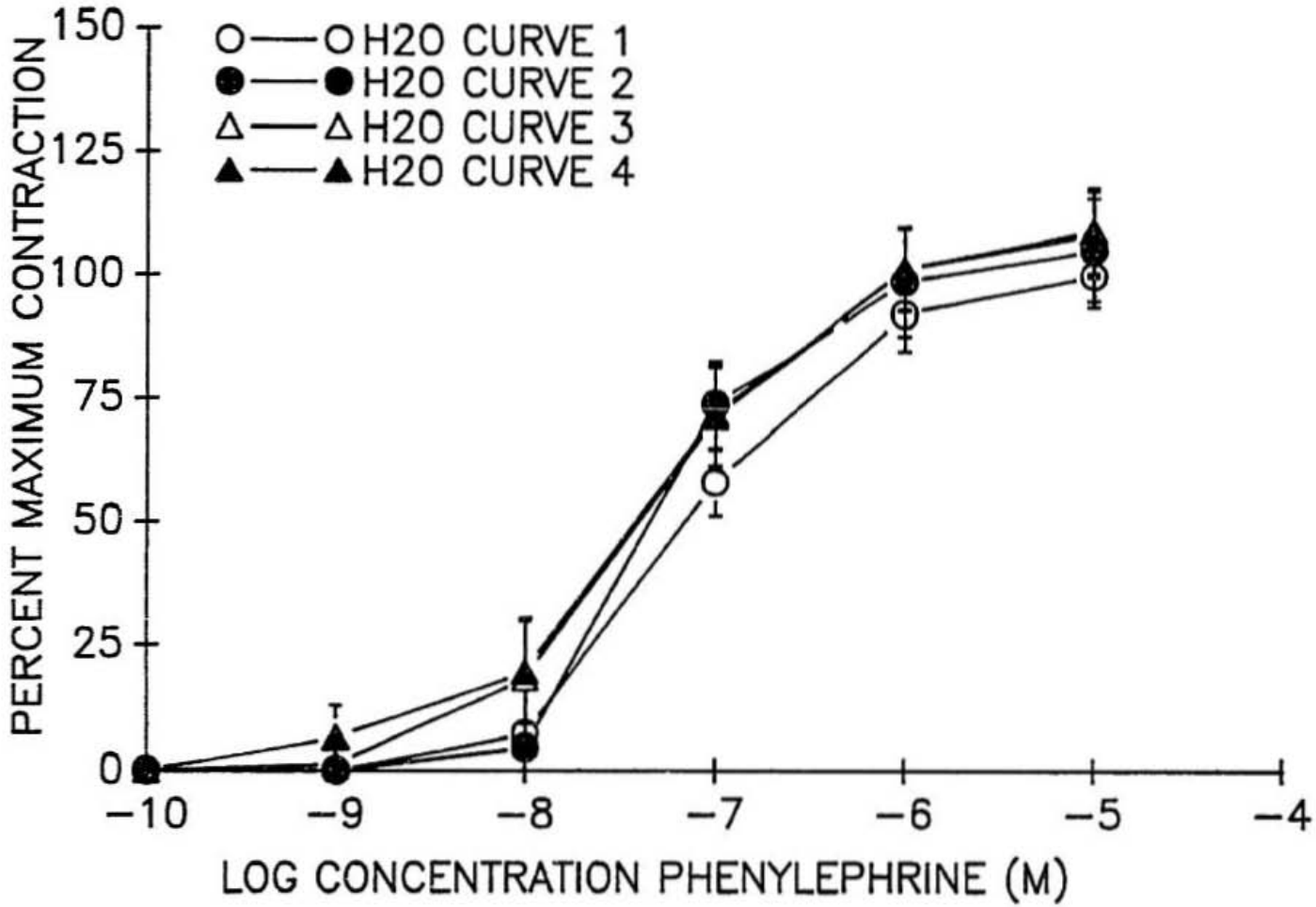

H2O CURVE 1
H2O CURVE 2
H2O CURVE 3
H2O CURVE 4
PERCENT MAXIMUM CONTRACTION
150
125
100
75
50
25
0
−10
−9
−8
−7
−6
−5
−4
LOG CONCENTRATION PHENYLEPHRINE (M)

Figure 3-16: **Four consecutive dose response curves constructed using $1x10^{-12}M$ to $1x10^{-5}M$ phenylephrine in the presence of sixty percent D_2O.**

Points represent mean percent maximum contraction and bars S.E.M. for six tissues.

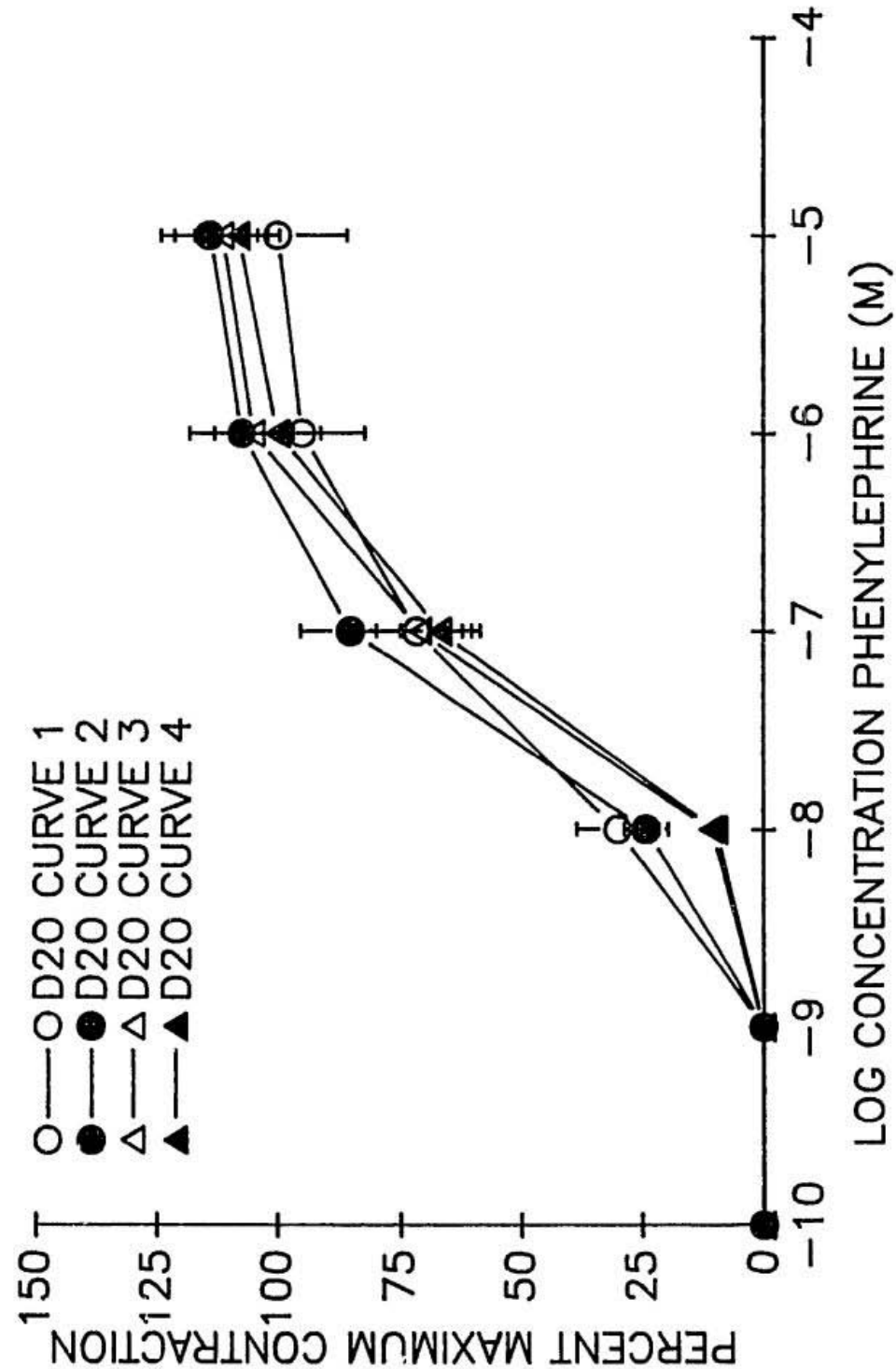

PERCENT MAXIMUM CONTRACTION
150
125
100
75
50
25
0
−10
−9
−8
−7
−6
−5
−4
LOG CONCENTRATION PHENYLEPHRINE (M)
O—O D2O CURVE 1
●—● D2O CURVE 2
Δ—Δ D2O CURVE 3
▲—▲ D2O CURVE 4

Figure 3-17: **Competitive inhibition of phenylephrine induced aortic smooth muscle contraction in the presence of prazosin and in the absence of D_2O.**

Points represent mean percent maximum contraction and bars S.E.M. for six tissues.

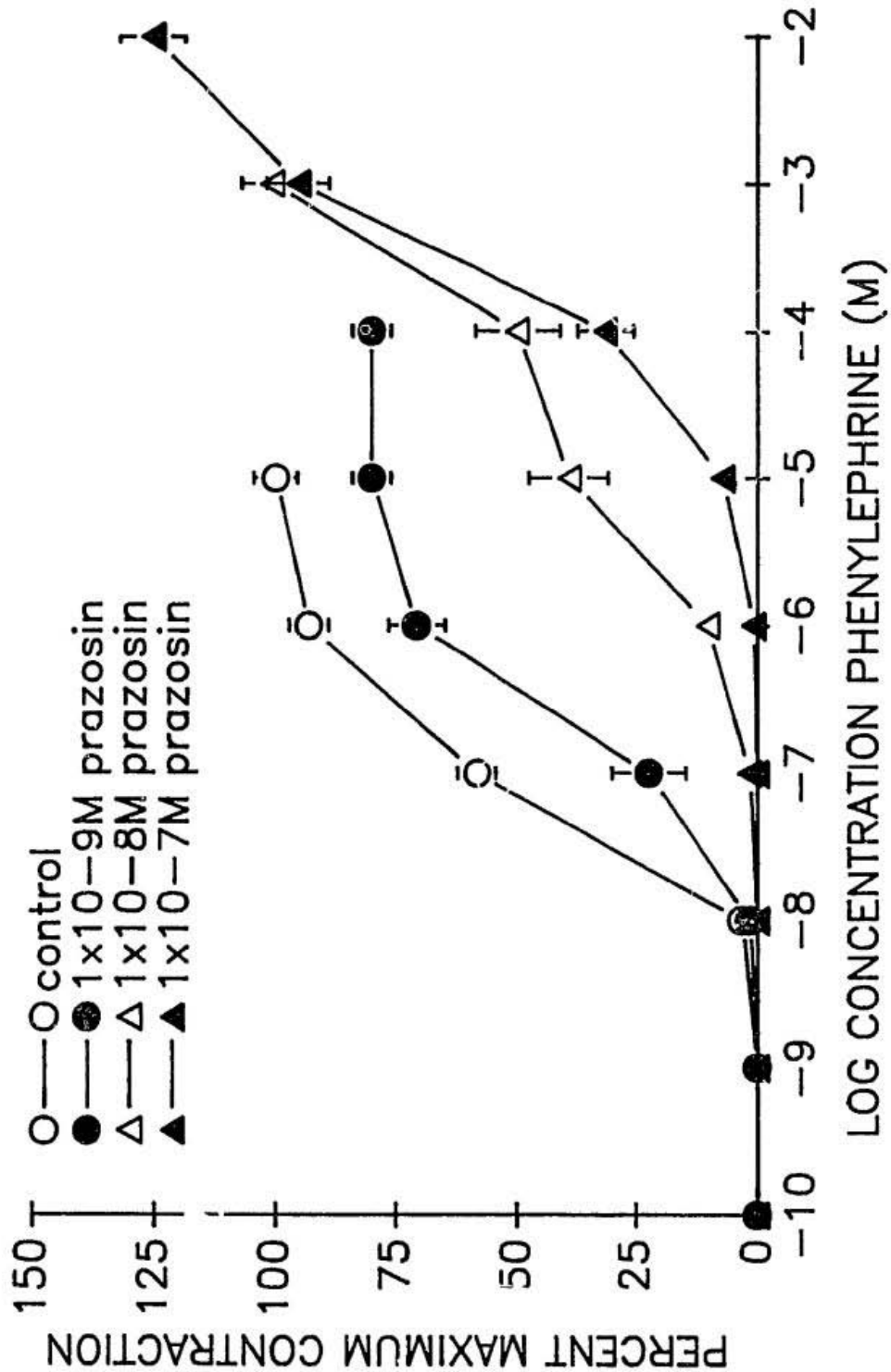
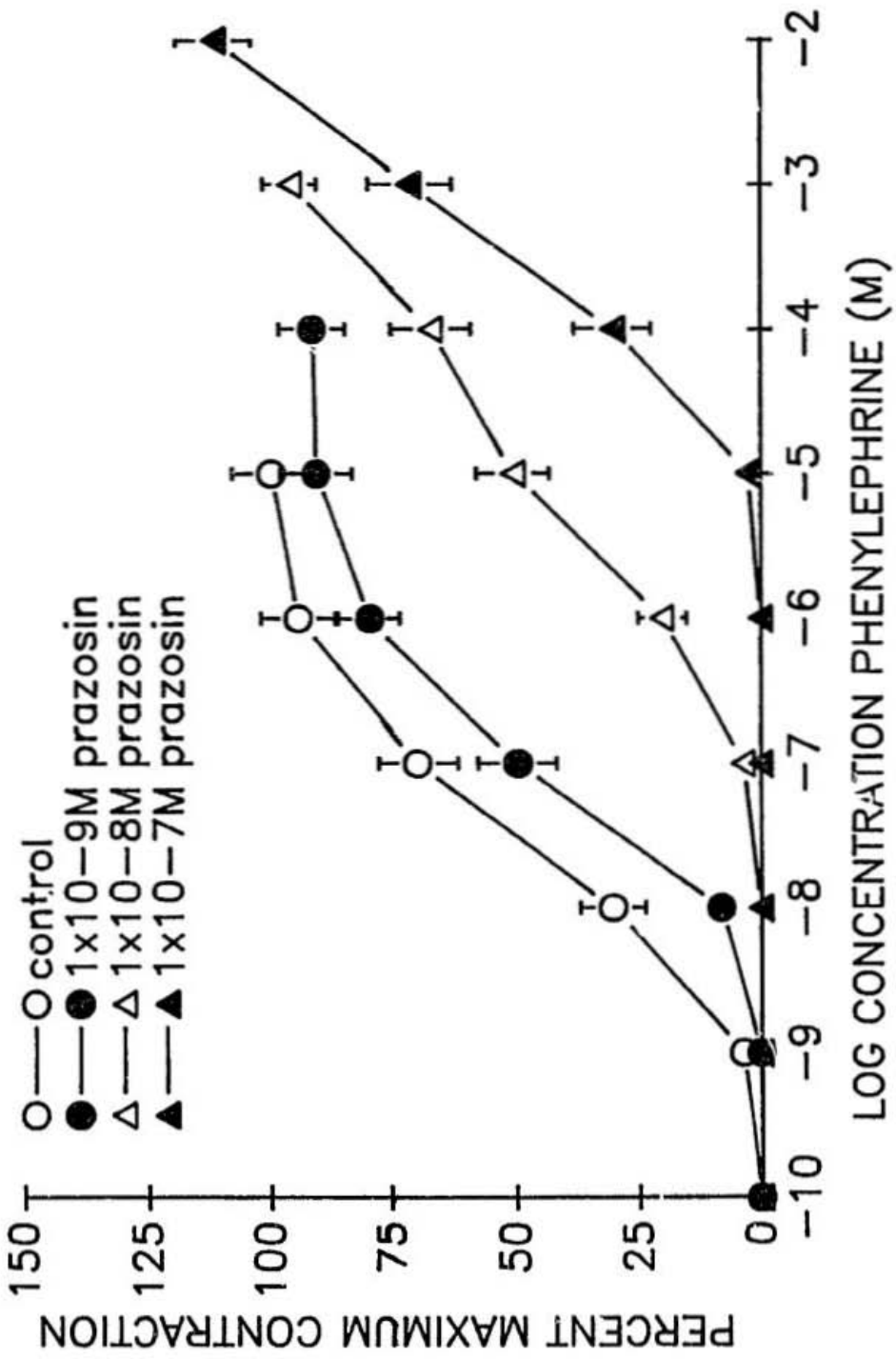

PERCENT MAXIMUM CONTRACTION
150
125
100
75
50
25
0
−10
−9
−8
−7
−6
−5
−4
−3
−2
LOG CONCENTRATION PHENYLEPHRINE (M)
O—O control
●—● 1x10−9M prazosin
Δ—Δ 1x10−8M prazosin
▲—▲ 1x10−7M prazosin

Figure 3-18: **Competitive inhibition of phenylephrine induced aortic smooth muscle contraction in the presence of prazosin and sixty percent D_2O.**

Points represent mean percent maximum contraction and bars S.E.M. for six tissues.


PERCENT MAXIMUM CONTRACTION
150
125
100
75
50
25
0
−10
−9
−8
−7
−6
−5
−4
−3
−2
LOG CONCENTRATION PHENYLEPHRINE (M)
O—O control
●—● 1x10−9M prazosin
Δ—Δ 1x10−8M prazosin
▲—▲ 1x10−7M prazosin

3.4 Effect of D_2O on aortic smooth muscle from WKY and SHR rats

3.4.1 Blood pressure levels of WKY and SHR rats

The blood pressure levels of WKY and SHR rats may be seen in Table 3-2. The blood pressures of WKY untreated (control) rats were significantly ($p < 0.01$) lower than those of age matched SHR control rats. Oral consumption of twenty-five percent D_2O for twelve days significantly ($p < 0.01$) reduced the systolic blood pressure of SHR and WKY rats in comparison to their respective controls. SHR rats consuming twenty-five percent D_2O had reduced blood pressures which were not significantly different from that of WKY controls, ie., 167 $\pm$ 5.3 and 161.2 $\pm$ 5.9 respectively.

3.4.2 Chronic <u>in vivo</u> effect of twenty-five percent D_2O

3.4.2.1 WKY/SHR: Potassium chloride induced contraction

The maximum level of KCl induced contractions for aortae of SHR control rats was significantly ($p < 0.02$) reduced to 64.4% $\pm$ 10.1% of the WKY control value of 0.65 $\pm$ 0.05 grams contraction (Figure 3-19). Chronic oral consumption of twenty-five percent D_2O, for twelve days by WKY rats had no significant effect on the <u>in vitro</u> potassium chloride induced contraction response curve when measured in normal Krebs solution. However, oral consumption of twenty-five percent D_2O by SHR rats produced a small but significant

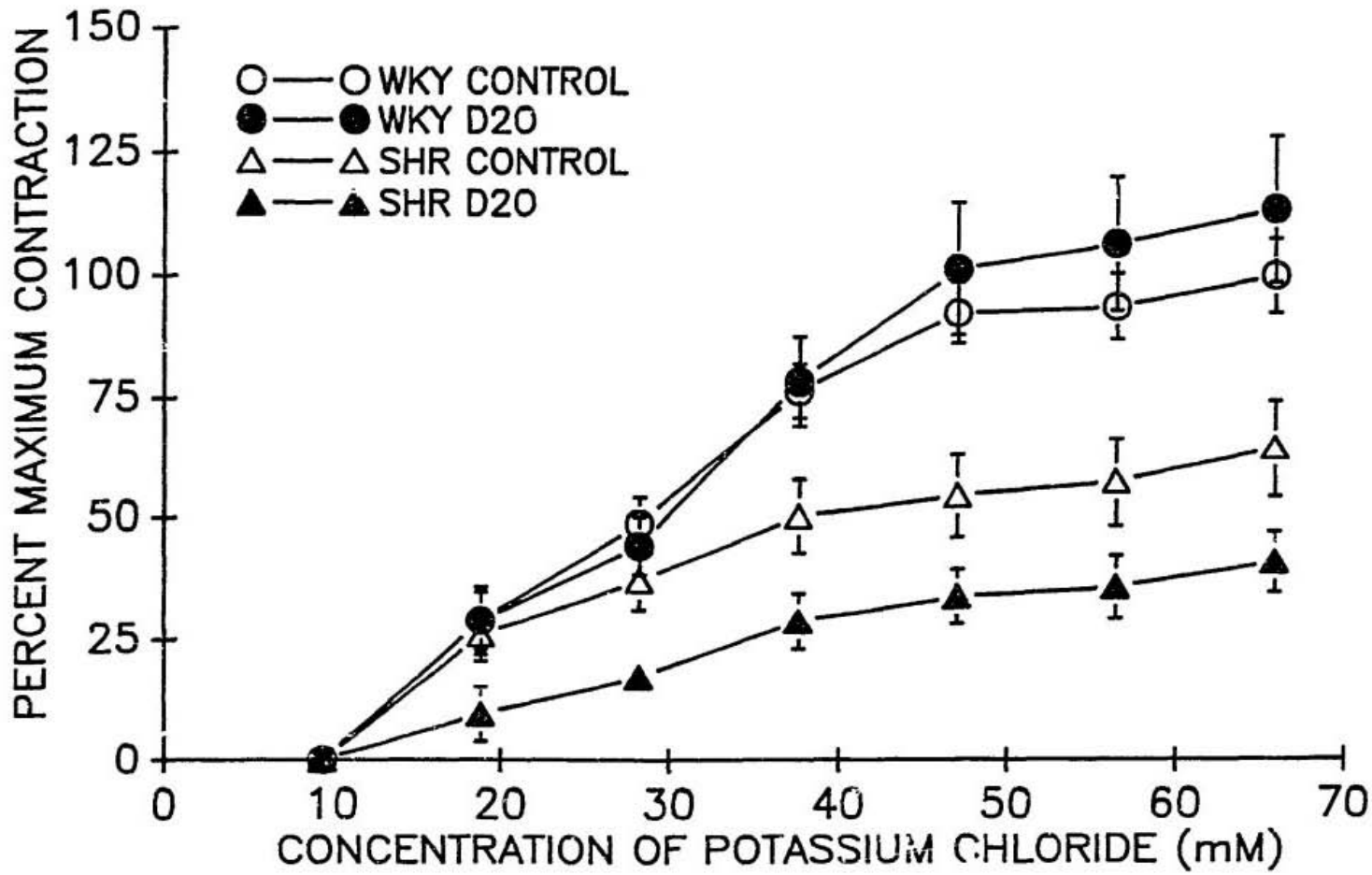
Table 3-2: Effect of 25% D_2O consumption for 12 days on the systolic blood pressures of spontaneously hypertensive (SHR) and normotensive (WKY) rats.

Type of rat	Type of water consumed	Systolic blood pressure (mmHg) mean ± S.E.M.*
SHR	H_2O	210.1 ± 4.3
SHR	25% D_2O	167.0 ± 5.3
WKY	H_2O	161.2 ± 5.9
WKY	25% D_2O	109.0 ± 5.6

*Each value represents an average of four measurements; each group consisted of six rats.

Figure 3-19: **Chronic *in vivo* effect of twenty-five percent D_2O on potassium chloride induced contraction of aortic smooth muscle from WKY and SHR rats.**

CONTROL corresponds to muscle from rats consuming distilled water and D_2O to rats consuming twenty-five percent D_2O. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.


WKY CONTROL
WKY D2O
SHR CONTROL
SHR D2O
PERCENT MAXIMUM CONTRACTION
150
125
100
75
50
25
0
0
10
20
30
40
50
60
70
CONCENTRATION OF POTASSIUM CHLORIDE (mM)

($p < 0.05$) depression of contraction, i.e., 41.0 $\pm$ 6.5% of the WKY control value.

3.4.2.2 WKY/SHR: Phenylephrine induced contraction

The maximum level of contraction induced using $1x10^{-5}$M phenylephrine was significantly ($p < 0.01$) different for aortic smooth muscle obtained from WKY and SHR rats (Figure 3-20). The level of contraction for SHR control rats was 63.4% $\pm$ 7.11% as compared to the WKY control value of 0.67 $\pm$ 0.06 grams.

Chronic oral administration of twenty-five percent D_2O had no significant effect on the phenylephrine induced contractions using aortic smooth muscle obtained from either WKY or SHR rats.

3.4.3 Acute <u>in vitro</u> effect of sixty percent D_2O

3.4.3.1 WKY: Potassium chloride induced contraction

The maximum level of contraction induced by 65.8 mM potassium chloride was not significantly different for aortic smooth muscle incubated in regular Krebs regardless of whether the WKY rats had previously orally consumed twenty-five percent D_2O (Figure 3-21; WKY CONT H_2O KREBS/WKY TREAT H_2O KREBS). In aortic smooth muscle obtained from WKY rats which had orally consumed twenty-five percent D_2O, sixty percent D_2O <u>in vitro</u> did not significantly alter the maximum contraction

Figure 3-20: **Chronic *in vivo* effect of twenty-five percent D_2O on phenylephrine induced contraction of aortic smooth muscle from WKY and SHR rats.**

CONTROL corresponds to muscle obtained from rats consuming distilled water and D_2O to rats consuming twenty-five percent D_2O. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.

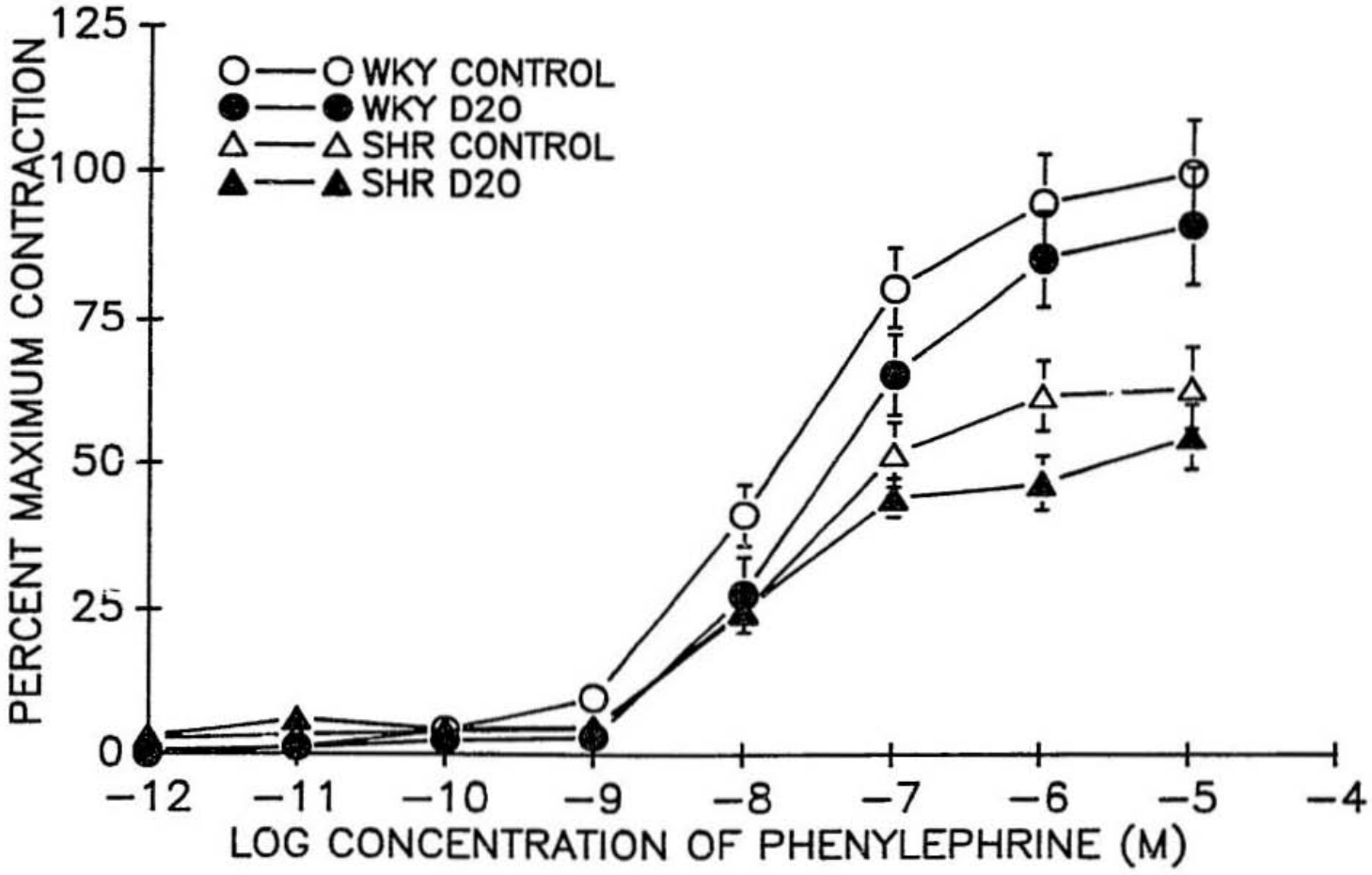
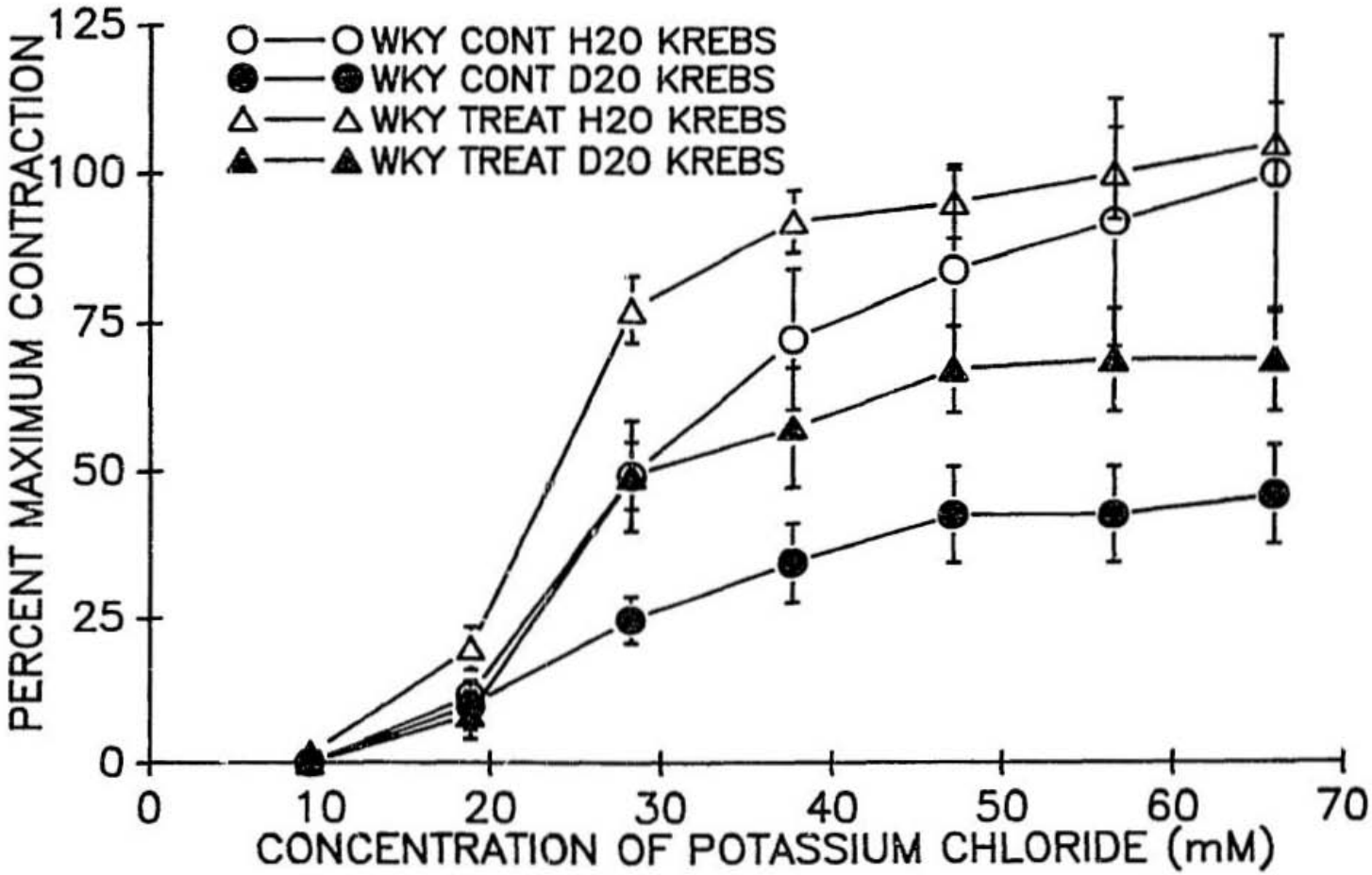

WKY CONTROL
WKY D2O
SHR CONTROL
SHR D2O
PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
−12
−11
−10
−9
−8
−7
−6
−5
−4
LOG CONCENTRATION OF PHENYLEPHRINE (M)

Figure 3-21: **Acute <u>in vitro</u> effect of sixty percent D_2O on potassium chloride induced contraction of aortic smooth muscle from WKY rats.**

CONT corresponds to rats consuming distilled water and TREAT to rats consuming twenty-five percent D_2O in distilled water. H_2O corresponds to aortic rings equilibrated in regular Krebs solution and D_2O to aortic rings equilibrated in sixty percent D_2O Krebs. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.


WKY CONT H2O KREBS
WKY CONT D2O KREBS
WKY TREAT H2O KREBS
WKY TREAT D2O KREBS
PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
0
10
20
30
40
50
60
70
CONCENTRATION OF POTASSIUM CHLORIDE (mM)

(Figure 3-21; WKY TREAT D_2O KREBS). In aortic smooth muscle obtained from WKY rats which had not been *in vivo* deuterated by twenty-five percent D_2O (Figure 3-21; WKY CONT D_2O KREBS), sixty percent D_2O Krebs significantly ($p < 0.05$) reduced the maximum contraction to 45.9% ± 8.5% as compared to the control value of 0.74 ± 0.01 grams.

3.4.3.2 WKY: Phenylephrine induced contraction

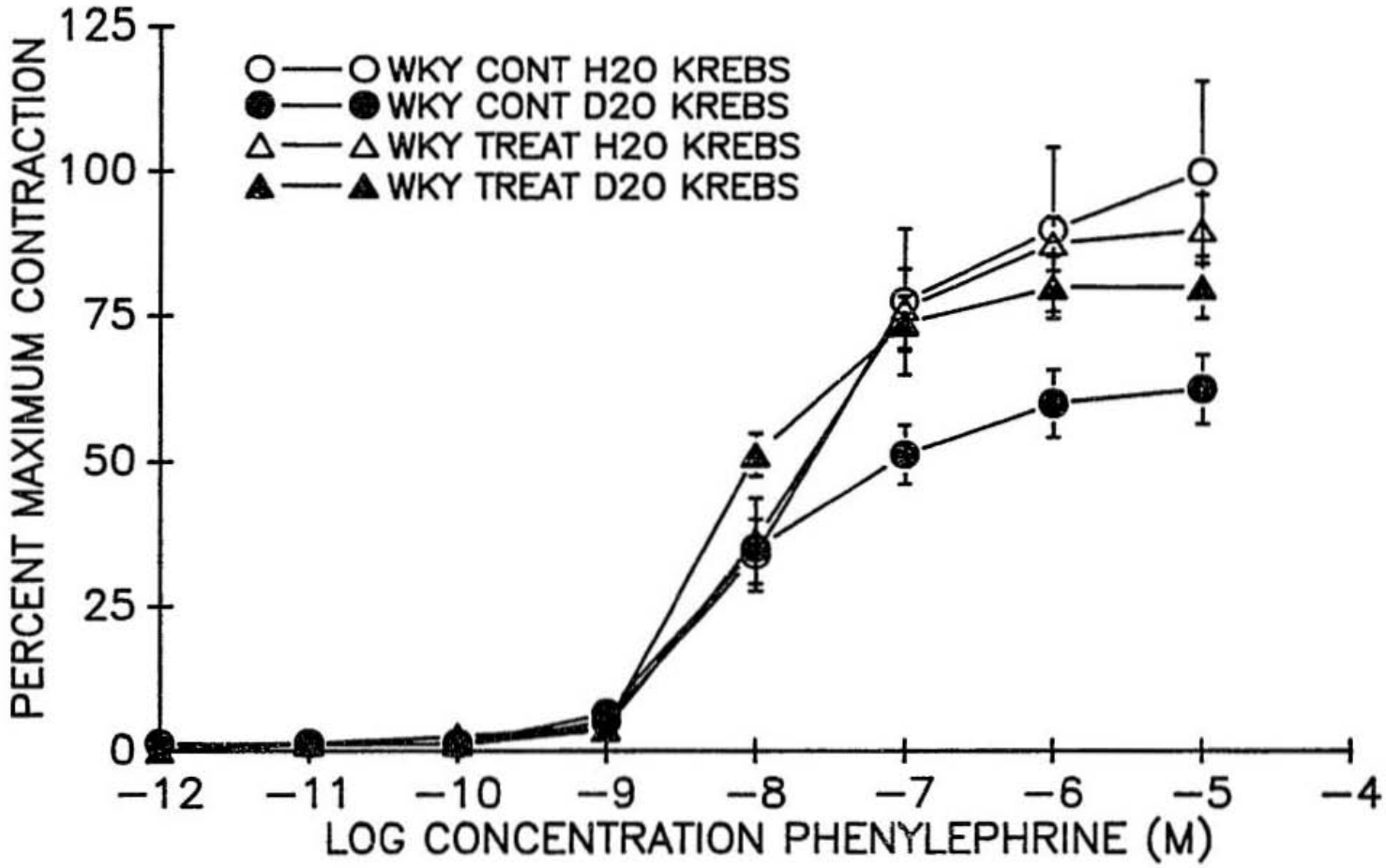
The maximum level of contraction induced by $1x10^{-5}M$ phenylephrine was not significantly different for aortic smooth muscle equilibrated in regular Krebs solution whether or not the WKY rats had been previously exposed to twenty-five percent D_2O (Figure 3-22; WKY CONT H_2O KREBS/WKY TREAT H_2O KREBS). In aortic smooth muscle obtained from rats exposed to twenty-five percent D_2O, sixty percent D_2O did not significantly reduce the maximum contraction (Figure 3-22; WKY TREAT D_2O KREBS). However, in aortic smooth muscle obtained from WKY rats which had not been previously exposed to twenty-five percent D_2O (Figure 3-22; WKY CONT D_2O KREBS), sixty percent D_2O significantly ($p < 0.05$) reduced the maximum contraction to 62.5% ± 6.0% as compared to the control value of 0.61 ± 0.07 grams.

3.4.3.3 SHR: Potassium chloride induced contraction

The maximum level of contraction induced using 65.8 mM potassium chloride (0.21 ± 0.02 grams) was not significantly different for aortic smooth muscle equilibrated

Figure 3-22: **Acute <u>in</u> <u>vitro</u> effect of sixty percent D_2O on phenylephrine induced contraction of aortic smooth muscle from WKY rats.**

CONT corresponds to rats consuming distilled water and TREAT to rats consuming twenty-five percent D_2O in distilled water. H_2O corresponds to aortic rings equilibrated in regular Krebs solution and D_2O to aortic rings equilibrated in sixty percent D_2O Krebs. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.


WKY CONT H2O KREBS
WKY CONT D2O KREBS
WKY TREAT H2O KREBS
WKY TREAT D2O KREBS
PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
−12
−11
−10
−9
−8
−7
−6
−5
−4
LOG CONCENTRATION PHENYLEPHRINE (M)

in regular Krebs solution whether or not the SHR rats had been previously exposed to twenty-five percent D_2O (Figure 3-23; SHR CONT H_2O KREBS/SHR TREAT H_2O KREBS). Sixty percent D_2O did not significantly change the maximum contraction of aortic smooth muscle obtained from either group of SHR rats.

3.4.3.4 SHR: Phenylephrine induced contraction

The maximum level of contraction induced using $1x10^{-5}M$ phenylephrine (0.28 $\pm$ 0.07 grams) was not significantly different for aortic smooth muscle equilibrated in regular Krebs solution whether or not the SHR rats had been previously exposed to twenty-five percent D_2O (Figure 3-24; SHR CONT H_2O KREBS/SHR TREAT H_2O KREBS). In aortic smooth muscle obtained from rats previously exposed to twenty-five percent D_2O, sixty percent D_2O did not reduce the maximum contraction (Figure 3-24; SHR TREAT D_2O KREBS). However, in aortic smooth muscle obtained from SHR rats which had not been previously exposed to twenty-five percent D_2O (Figure 3-24; SHR CONT D_2O KREBS), sixty percent D_2O significantly ($p < 0.05$) reduced the maximum contraction to 54.6% $\pm$ 7.4% as compared to the control value.

3.4.4 Synopsis

Oral consumption of 25% D_2O significantly reduced the systolic blood pressures of SHR and WKY rats, with a reduction of SHR blood pressure to a level not significantly different from that of WKY control rats. Oral consumption of 25% D_2O had no significant effect on phenylephrine induced

Figure 3-23: **Acute <u>in vitro</u> effect of sixty percent D_2O on potassium chloride induced contraction of aortic smooth muscle from SHR rats.**

CONT corresponds to rats consuming distilled water and TREAT to rats consuming twenty-five percent D_2O in distilled water. H_2O corresponds to aortic rings equilibrated in regular Krebs solution and D_2O to aortic rings equilibrated in sixty percent D_2O Krebs. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.

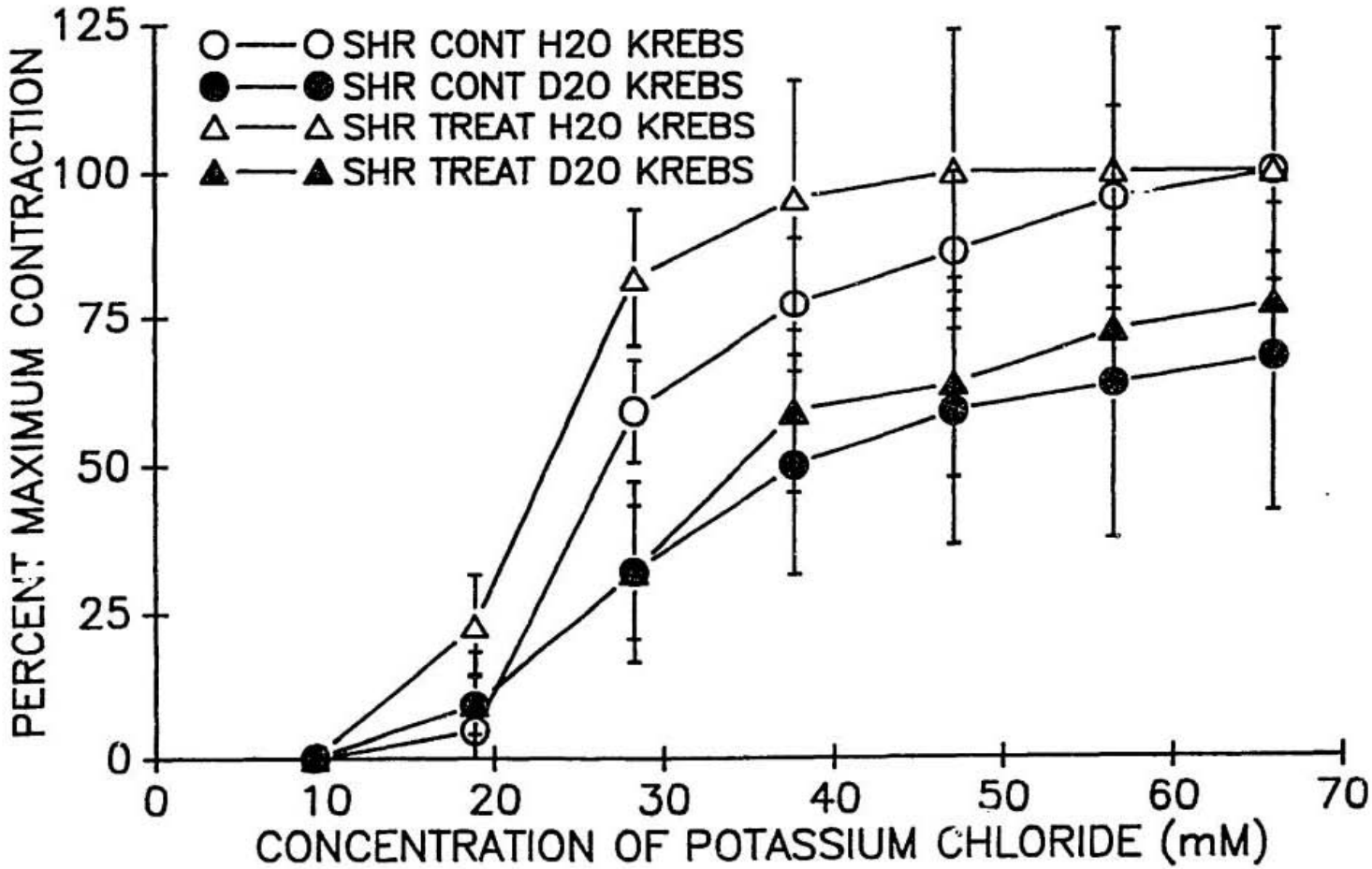
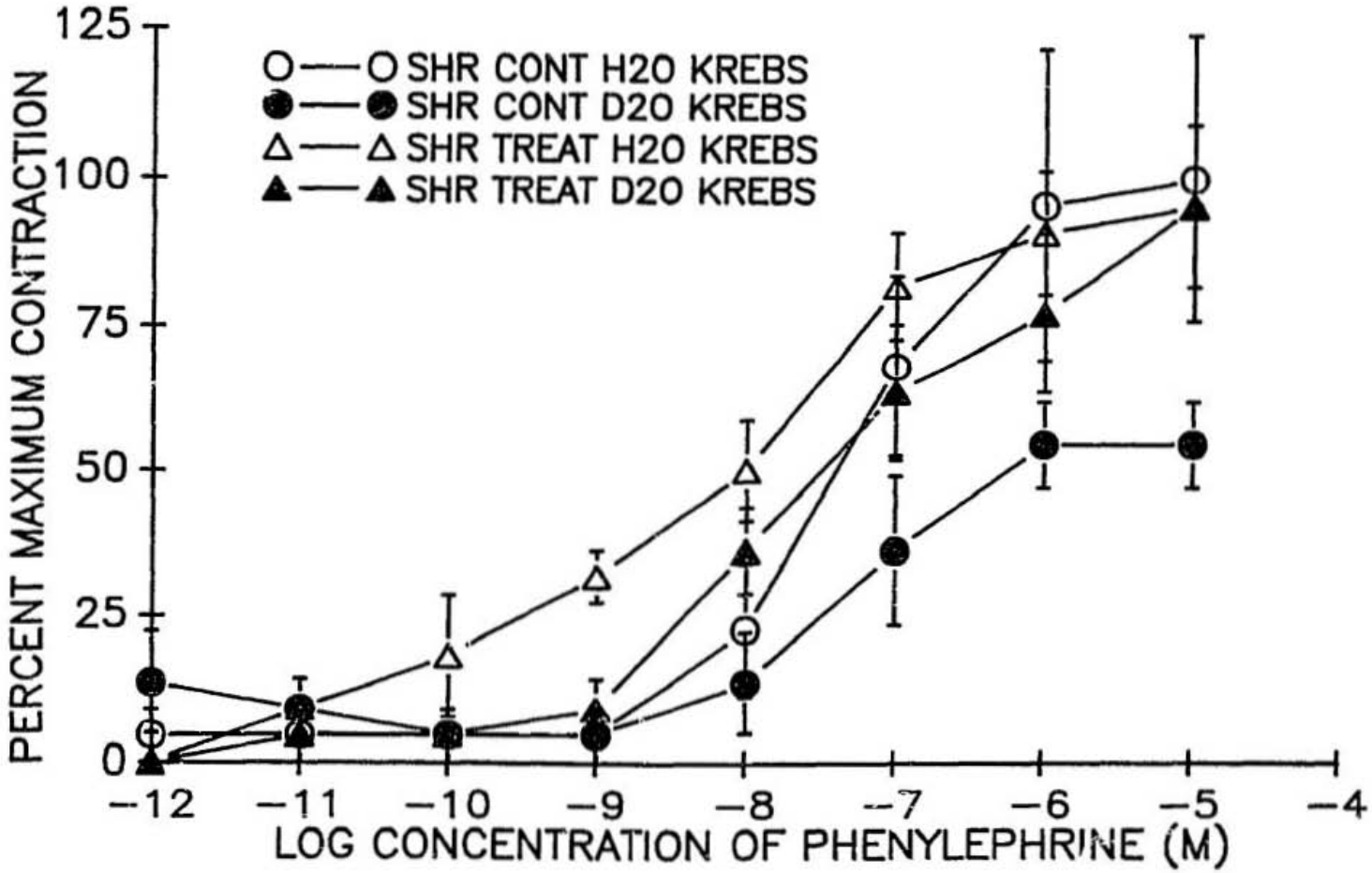

SHR CONT H2O KREBS
SHR CONT D2O KREBS
SHR TREAT H2O KREBS
SHR TREAT D2O KREBS
PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
0
10
20
30
40
50
60
70
CONCENTRATION OF POTASSIUM CHLORIDE (mM)

Figure 3-24: **Acute <u>in</u> <u>vitro</u> effect of sixty percent D_2O on phenylephrine induced contraction of aortic smooth muscle from SHR rats.**

CONT corresponds to rats consuming distilled water and TREAT to rats consuming twenty-five percent D_2O in distilled water. H_2O corresponds to aortic rings equilibrated in regular Krebs solution and D_2O to aortic rings equilibrated in sixty percent D_2O Krebs. Points represent mean percent maximum contraction and bars S.E.M. for eight tissues.


SHR CONT H2O KREBS
SHR CONT D2O KREBS
SHR TREAT H2O KREBS
SHR TREAT D2O KREBS
PERCENT MAXIMUM CONTRACTION
125
100
75
50
25
0
−12
−11
−10
−9
−8
−7
−6
−5
−4
LOG CONCENTRATION OF PHENYLEPHRINE (M)

contraction of aortae from SHR and WKY rats; there was a small depression of potassium chloride induced contraction in SHR rats only. Subsequent acute exposure of aortae from SHR and WKY rats to sixty percent D_2O demonstrated that significant inhibition of contraction occurred only in aortae obtained from rats which had not previously consumed 25% D_2O.

CHAPTER 4

DISCUSSION

4.1. Effect of D_2O on aortic smooth muscle contraction

Phenylephrine acts mainly on receptor operated calcium channels to produce contraction of aortic vascular smooth muscle (Khahil et al., 1987). Although phenylephrine is not completely selective for receptor operated calcium channels (Godfraind et al., 1986), it was used as a first approximation of the effects of deuterium oxide on agonist induced vascular smooth muscle contraction. Increasing concentrations of the D_2O depressed the maximal contraction in a dose-dependent manner (Figure 3-1) with an approximate IC_{50} at 60% D_2O. Similar effects have been shown where concentrations of D_2O between 25 and 99.8 per cent decreased the force of contraction and the rate of spontaneously beating, isolated atria of the frog (Kaiminer, 1960). The entire dose response curve using $1x10^{-12}M$ to $1x10^{-5}M$ phenylephrine (Figure 3-2) shows that varying concentrations of D_2O did not uniformly depress the lower end of the dose response curve; tissues exposed to forty per cent D_2O actually showed significantly increased contraction levels from $1x10^{-11}M$ to $1x10^{-8}M$ phenylephrine. However, although these tissues demonstrated increased sensitivity at these lower drug doses, the level of contraction at the maximum concentration of phenylephrine was

significantly depressed in comparison to control tissues. These data, showing inhibition of phenylephrine contraction, may suggest an effect of D_2O on calcium entry via the receptor-operated calcium channel.

The series of experiments demonstrating reversibility of the D_2O inhibitory effects on vascular smooth muscle contraction (Figure 3-3) indicate the absence of tissue injury. Similar studies using skeletal muscle also show reversibility of the D_2O effect on the force of contraction (Svensmark, 1961).

Previous studies have shown that the endothelium does have an effect on the enhanced second phenylephrine induced contraction in the rat isolated aortic strip when carrying out multiple dose response curves in one tissue (Demirel and Türker, 1989). These workers found no diminished effect of removal of the endothelium in their first phenylephrine contraction curve. Accordingly, by utilizing the first curve only, it was confirmed that the amount of contraction produced by phenylephrine in the 0% D_2O Krebs controls was the same whether or not the endothelium had been disrupted (Figure 3-4). Furthermore, aortic rings equilibrated in 60% D_2O Krebs displayed approximately the same percentage of decreased contraction, in relation to those of control tissues, whether or not the endothelium had been disrupted. Thus the endothelium does not appear to play a major role in the D_2O

mediated inhibitory effect of vascular contraction. Acetylcholine was used at the end of each experiment to ensure that the endothelium scraping was effective since it produces a relaxation effect in previously contracted intact rings but has no effect on endothelium-denuded aorta (Rapoport and Murad, 1983).

Potassium chloride dose response curves were constructed to show the effect of D_2O on voltage-operated calcium channels since KCl acts mainly on this type of calcium channel (Meisheri et al., 1981). Potassium chloride maximum contraction was reduced by 60% D_2O to a level similar to the inhibition of phenylephrine contraction (Figure 3-5). This suggests that D_2O also affects calcium uptake via voltage-operated calcium channels and supports the observations by Vasdev et al. (1990) of D_2O effects on ^{45}Ca uptake by rat aorta.

Studies conducted in calcium free Krebs help to differentiate effects on extracellular calcium influx from effects on intracellular calcium mobilization. In these experiments tissues were washed in calcium free Krebs until there was no contractile response to 60mM KCl. This was considered an indication that intracellular calcium stores had been exhausted since it has been shown that the calcium release induced by high doses of pharmacological contractile agents in the absence of extracellular calcium exhausts the

intracellular calcium stores (van Breemen, 1969). Subsequent calcium induced contraction was depressed in the presence of 60% D_2O (Figure 3-6) confirming an action of D_2O on extracellular calcium influx. However, it may have been more appropriate to ensure depletion of intracellular calcium sources by washing with calcium free Krebs solution containing phenylephrine since phenylephrine mediates vascular smooth muscle contraction by mobilizing intracellular calcium sources whereas potassium depolarization is not believed to mobilize intracellular calcium (Deth and van Breemen, 1974).

Sixty percent D_2O similarly reduced the maximum contraction of aortic rings with Bay K 8644 (Figure 3-7) which is dependent upon extracellular calcium and facilitates activation of the voltage dependent calcium channel (Su et al., 1984). Since this protocol was carried out in the presence of phentolamine, D_2O effects on receptor-operated calcium channels were excluded.

These results suggest that a major action of D_2O is on extracellular calcium influx through voltage-operated calcium channels.

These studies do not conclusively indicate whether D_2O is producing selective effects only on the voltage- and receptor-operated calcium channels. There appears to be an effect on both types of channel but D_2O could be acting at multiple

sites.

Evidence for actions of D_2O on intracellular calcium mobilization was obtained from studies of aortic contraction with sucrose. Andersson et al. (1974) have shown that a sucrose induced contraction of vascular smooth muscle occurs independently of membrane potential level and in the absence of extracellular calcium. D_2O depression of the sucrose contraction in this study (Figure 3-8) suggests further sites of action independent of extracellular calcium sources. However, conclusions from this study are limited since the precise mechanism of sucrose induced contraction is unknown and the absolute tension achieved was very low compared with other protocols in this study.

4.2. Effect of D_2O on aortic smooth muscle relaxation

Studies of the effect of D_2O on aortic smooth muscle relaxation were performed to further elucidate the mechanism of action of D_2O. For this purpose aortic smooth muscle relaxation with isoprenaline and acetylcholine was investigated. Since isoprenaline induced relaxation was measured on tissues precontracted with phenylephrine the contraction level of tissues equilibrated in 0% D_2O was reduced by use of the alpha-adrenoceptor antagonist phentolamine. The relaxation response with the β-adrenoceptor agonist, isoprenaline, in tissues equilibrated in 60% D_2O Krebs was significantly reduced compared to control tissues

(Figure 3-9). Sixty percent D_2O also depressed aortic smooth muscle relaxation with acetylcholine (Figure 3-10) which requires the presence of an intact endothelium and produces relaxation by the release of EDRF. Along with depressing the acetylcholine dose response curve, 60% D_2O has also shifted the curve to the right. This may indicate a combination of noncompetitive and competitive inhibition. These results indicate that the D_2O effect is not contraction specific but can also affect vascular smooth muscle relaxation, perhaps through an action on sarcoplasmic reticulum calcium mobilization or on the contractile proteins. The fact that D_2O affects relaxation using both isoprenaline and acetylcholine which act by distinct mechanisms (an increase in cAMP and cGMP, respectively) again suggests that D_2O acts at multiple sites.

4.3. Effect of D_2O on the affinity of an antagonist for its receptor

In studies to this point D_2O acted as an inhibitory agent in each protocol. As a further attempt to make a definite conclusion as to the site of action of D_2O, its effects on noncompetitive and competitive inhibition were studied using nifedipine and prazosin respectively. D_2O significantly changed the pD_2' for nifedipine (Table 3-1) which selectively inhibits calcium entry through voltage dependent calcium channels (Cauvin et al., 1983) producing a decreased vascular smooth muscle contraction. There is evidence that Bay K 8644

and nifedipine bind to the same binding site (Schramm et al., 1985) but whereas nifedipine blocks calcium entry, Bay K 8644 enhances it. Since the results showed that D_2O affected both Bay K 8644 contraction and the pD_2' for nifedipine this suggests a possible interaction at the shared binding site.

The pA_2 value for prazosin in the absence of D_2O was calculated as 9.81 $\pm$ 0.38 which was very similar to that determined by Muramatsu et al. (1990) for rat aorta. Sixty percent D_2O significantly changed the slope of the Schild plot and consequently no pA_2 value could be determined in the presence of D_2O. The change in the slope of the Schild plot may indicate an alteration in the kinetics of the interaction between prazosin and its binding site.

These data again suggest multiple sites of action for D_2O.

4.4. Chronic and acute effects of D_2O on aortic smooth muscle from WKY and SHR rats

The previous studies dealt only with acute in vitro effects of D_2O. Therefore the chronic in vivo effect of 25% D_2O on normotensive (WKY) and spontaneously hypertensive (SHR) rats was also studied.

Toal and Leenen (1985) have reported that significant differences in blood presure can be detected at four weeks of age and that SHR blood pressure progressively increases with

age. In this study, systolic blood pressure measurements confirmed that SHR control rats had significantly higher blood pressure than WKY control rats (Table 3-2). Seven weeks is clearly sufficient for the development of hypertension in the SHR rats and after that time D_2O treatment was started. Preliminary blood pressure measurements should have been made before D_2O treatment was started to ensure that WKY rats were normotensive and SHR rats were hypertensive. Since this procedure was not followed the D_2O treated rats were simply compared with control rats which did not receive D_2O. The blood pressure levels for the SHR and WKY rats in this experiment were measured by tail plethysmography but the differences in levels for SHR and WKY rats are similar to those measured by cannulae insertion into the aortic arch (Toal and Leenen, 1985). Oral consumption of 25% D_2O in the drinking water for 12 days reduced the blood pressure of SHR rats to a level not significantly different from that of WKY control rats. D_2O also reduced the blood pressure level of WKY rats. However, the blood pressure measurements of the WKY rats were high compared with those of other researchers (Vasdev et al., 1990a). The high blood pressure was probably due to the fact that the rats were not acclimatized before the blood pressure measurements were made.

The potassium chloride and phenylephrine induced contraction levels of control SHR rats were significantly lower than those of WKY rats (Figures 3-19 and 3-20). Although

in vivo studies usually indicate an increase in the contractility of vascular smooth muscle obtained from SHR rats, many in vitro studies have reported depressed contraction levels (Triggle, 1989). Spector et al. (1969) reported that aortic strips from spontaneously hypertensive rats were less responsive to contraction induced with norepinephrine, serotonin, and potassium chloride. Similarly, Field et al. (1972) reported decreased isometric tension produced in response to norepinephrine and potassium chloride in aortic rings of SHR rats compared to normotensive controls.

Oral consumption of 25% D_2O did result in a small depression in the in vitro potassium chloride dose response curve of aortae from SHR rats (Figure 3-19). However, after oral consumption of 25% D_2O there was no significant effect on subsequent in vitro potassium chloride induced aortic contractions from WKY rats (Figure 3-19) or phenylephrine induced contractions from WKY or SHR rats (Figure 3-20). Previous in vitro studies showed that 20% D_2O had no significant effect on phenylephrine induced aortic contraction (Figure 3-1), so it is not surprising that the in vivo effect of 25% D_2O was small. A higher concentration of D_2O was not used since Thomson (1960) found that rats died when about one third of their body water was replaced by D_2O.

Even though 25% D_2O reduced the blood pressure of SHR rats to the level of WKY control rats, the potassium chloride

and phenylephrine induced maximal contraction levels for the SHR rats were still significantly different from that of WKY control rats. It has been noted in several studies, that a reduction in blood pressure after hypertension has developed in SHR rats does not reverse vascular reactivity changes (Triggle, 1989). This may explain the apparent contradiction between blood pressure and aortic contraction following chronic D_2O treatment.

The acute studies revealed that maximum contraction with either phenylephrine or potassium chloride was not significantly different for aortic rings equilibrated in regular Krebs solution, regardless of whether the SHR or WKY rats had previously consumed 25% D_2O (Figures 3-21 to 3-24; H_2O Krebs). Even small chronic effects which were evident in the first part of the experiment (Figure 3-19; SHR D_2O) were no longer detectable after equilibration in regular Krebs solution for two hours. This may indicate that the D_2O effect is reversible as demonstrated in previous studies (Figure 3-3).

In all cases, aortic rings obtained from either SHR or WKY rats which had consumed 25% D_2O, did not show significantly decreased contraction levels when rings were equilibrated in sixty percent D_2O rather than normal Krebs (Figures 3-21 to 3-24). When potassium chloride was used as the contractile agent there was a trend towards a decrease in

contraction levels but it was not statistically significant (Figures 3-21 and 3-23). There is a very large standard error in the potassium chloride dose response curves of SHR rats (Figure 3-23) which may partly be due to the fact that the level of contraction was very small since SHR rats show decreased contraction levels, coupled with the inhibitory effects of 25% and 60% D_2O. However, from figures 3-21, 3-22, and 3-24, it is clear that rings obtained from SHR and WKY rats which had not previously consumed 25% D_2O showed significantly decreased potassium chloride and phenylephrine induced contraction levels in 60% D_2O Krebs solution. The level of inhibition in SHR and WKY rats was similar to that previously found in Sprague-Dawley rats (Figures 3-2 and 3-5). These data from the acute studies suggest that homologous desensitization or tolerance to D_2O may have occured since 60% D_2O has a reduced effect in aortic rings from rats with prolonged exposure to 25% D_2O. This would explain why D_2O produces a significant depression of contraction levels only in aortic rings obtained from rats not previously exposed to D_2O.

4.5. Summary and conclusion

From these studies it cannot be determined at which specific stage of the contraction process D_2O is producing its effect. In studies performed by Svensmark (1961) on frog skeletal muscle, it remained undecided as to whether force

reduction was due to the effect of D_2O on the contractile elements or on the excitation-contraction coupling. Similarly, no definite conclusions can be drawn from the work of Goodall (1958) on frog sartorius muscle or Kaiminer (1960) on glycerol extracted rabbit's psoas, as to the exact site of D_2O action. The level of inhibition was approximately 25% in the Bay K 8644 and calcium contraction study compared to 50% inhibition with the sucrose contraction and isoprenaline relaxation protocols. This difference suggests a greater effect of D_2O on intracellular calcium mobilization or other intracellular events, rather than on the influx of extracellular calcium. However, the observations that D_2O does inhibit contraction with phenylephrine, potassium chloride, Bay K 8644, and calcium all suggest that a major influence of D_2O is on extracellular calcium influx. Further evidence suggesting a nonspecific action for D_2O was obtained from the fact that D_2O inhibited relaxation with isoprenaline and acetylcholine, and affects both the noncompetitive inhibition of potassium chloride contraction with nifedipine and the competitive inhibition of phenylephrine contraction with prazosin. Taken together, the data indicate multiple sites of action of D_2O on vascular smooth muscle.

Data from the acute studies suggest that homologous desensitization to D_2O may have occured since D_2O produces a significant depression of potassium chloride and phenylephrine induced contraction levels only in aortic rings obtained from

SHR and WKY rats which had not previously consumed D_2O. It is not possible to isolate the mechanism of desensitization, such as down regulation of receptors, since the site of action of D_2O is unknown and appears to be nonspecific. Although chronic oral consumption of 25% D_2O reduced the systolic blood pressure in SHR and WKY rats, subsequent *in vitro* aortic contraction was not always similarly reduced. It may be concluded that the hypotensive effect of chronic D_2O administration does not entirely appear to be a consequence of vascular reactivity changes.

Tolerance to the effects of D_2O suggest that there have indeed been changes in membrane function and/or regulation or mobilization of calcium sources. There may be membrane receptor changes associated with D^+ and H^+ exchange since proton exchange is important in most biological reactions and isotopic effects due to D^+ and H^+ exchange have been shown to affect L-type calcium channel conductance (Prod'hom et al., 1987). Future studies should be directed toward performing receptor binding studies to determine if tolerance does occur due to down regulation of receptors. The effect of chronic *in vivo* exposure to D_2O on the pA_2 and pD_2' values of competitive and noncompetitive antagonists, respectively, may give some insight into whether receptor modifications occur due to D^+ and H^+ exchange. Finally, an analysis of calcium homeostasis in D_2O tolerant rats may demonstrate whether D_2O affects calcium regulation.

In summary, these studies indicate that deuterium oxide reduces vascular smooth muscle contraction induced through agonist and depolarization activation of calcium channels. This depression of contraction is reversible and not dependent on an intact endothelium. The action of D_2O appears not to be solely dependent on intracellular calcium mobilization or extracellular calcium utilization as determined by calcium free buffer studies and sucrose and Bay K 8644 induced contraction. Since D_2O has an effect on relaxation of vascular smooth muscle, sucrose induced contraction, and the action of both competitive and noncompetitive antagonists, this suggests multiple sites of action and perhaps an action on the contractile elements themselves. Chronic studies show that oral consumption of 25% D_2O reduces systolic blood pressure in hypertensive and normotensive rats but the hypotensive effect does not appear to be a consequence of vascular reactivity changes. Finally, chronic *in vivo* exposure to D_2O desensitizes aortic rings to subsequent inhibition by acute *in vitro* D_2O exposure.

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