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ROLE OF ENDOTHELIUM, LIPOXYGENASE PRODUCTS AND MEMBRANE DEPOLARIZING FACTORS IN HYPOXIC CONTRACTION OF THE CANINE ISOLATED BASILAR ARTERY

by

DOUGLAS ALLEN ELLIOTT

A thesis submitted to the Faculty of Graduate Studies

University of Manitoba

in partial fulfillment of the requirements

for the degree of Master of Science

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DEDICATIONS

For Sonia and our children

who stood by me through the good times and the rough times,

and for doing without me so much

To my Mother who never gave up hope

I love you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

1. Rings of canine basilar artery, suspended *in vitro*, contract reversibly in response to hypoxia (95% N_2/5% CO_2). These contractions are calcium-dependent because they are blocked by reduction of external Ca with EGTA ($2.5x10^{-3}$ M) or by treatment of the preparation with the calcium entry blocker, verapamil ($5x10^{-6}$ M). This is in contrast to cerebral vessels *in vivo* which **dilate** in response to hypoxia. My first objective in these studies was to better characterize this hypoxic contraction by examining the effects of various agents and manipulations. There is some controversy regarding the role of endothelial cells in this response. Therefore, my second objective was to determine the role of endothelium in hypoxic contraction.

2. Mechanical or chemical removal of the endothelium, did not abolish hypoxic contractions. Indeed hypoxic contraction of the canine isolated basilar artery is only partially, if at all, dependent on the endothelium. Effectiveness of the removal of endothelium was confirmed by the reversal of vasodilator responses to vasopressin and acetylcholine, and by scanning electron microscopy.

3. Experiments were undertaken to determine the role of altered ionic concentrations on hypoxic contraction. Withdrawal of sodium from the buffer bathing the tissue prevented hypoxic contractions completely, and this was reversed by readdition of sodium. Whether absence of Na^+ *per se* or a secondary reduction in membrane depolarization resulting from this is the mechanism, is not certain.

4. Studies were conducted to determine the effect of lipoxygenase inhibition on hypoxic contraction. Contractions were blocked by treatment of the vessel with inhibitors of lipoxygenase. Nor-

dihydroguaiaretic acid (NDGA, $5x10^{-6}$ M) treatment reduced hypoxic contraction. This suggests that hypoxic contraction is some how mediated by products of the lipoxygenase pathway. The augmentation of KCl ($2.5x10^{-2}$ M)-induced contraction by hypoxia, however, was not significantly affected by lipoxygenase inhibition. This demonstrates that contraction due to partial depolarization of the muscle membrane is additionally increased by hypoxia.

5. In an effort to relate *in vitro* hypoxic contraction to the *in vivo* response, the effect of a neurohumoral vasodilator on this contraction was studied. Of clinical importance is the observation that hypoxic contraction is prevented and even reversed by treatment with adenosine, an agent known to be released *in vivo* during hypoxia. Adenosine ($5x10^{-6}$ M) applied at the peak of hypoxic contraction caused a complete relaxation of the vessel. Pretreatment with adenosine also inhibited hypoxic contraction in a dose-dependent manner.

6. Contractions due to hypoxia are augmented by pretreatment of the muscle with the dihydropyridine calcium channel agonist, BAY K 8644 ($5x10^{-11}$ M), indicating that voltage-gated calcium channels play some role in this contraction. Hypoxic contractions are not inhibited by phentolamine, propranolol, 6-OH dopamine, methysergide or indomethacin (10^{-5} M). However they are abolished by treatment with hydroquinone ($5x10^{-5}$ M), an agent reported to abolish endothelium-mediated relaxant responses in vascular preparations.

7. The resting tone of the vessel is primarily passive and, therefore, not dependent on calcium flux. Upon hypoxia, breakdown of membrane phospholipids occurs. It could be reasoned that the potassium efflux and subsequent sodium influx seen during hypoxia causes depolarization leading to some influx of calcium through voltage-gated channels, and this might trigger activation of phospholipase A_2 which may lead to generation of arachidonic acid. This stimulates 5-

lipoxygenase, which produces some unknown mediator (e.g. leukotriene(s))which further increases calcium influx. Perhaps this mediator also partially depolarizes the membrane and this facilitates the large depolarization produced at the muscle membrane by hypoxia. The combined effect produces a contraction which is not wholly dependent on endothelial factors, but is dependent on sodium influx and is blocked by hydroquinone, lipoxygenase inhibition, and by adenosine. It is possible that the release of adenosine *in vivo* during anoxia counteracts the effect of this lipoxygenase-produced mediator and masks the constriction with a more powerful dilatation. This would become important if adenosine production was compromised or blocked during an hypoxic episode.

INTRODUCTION

OBJECTIVES

Knowledge of the effects of hypoxia on the cerebral vasculature is important for understanding the pathophysiology of altered cerebral blood flow (CBF) after hypoxia/ischemia.

Tissue is considered to be in a state of hypoxia when its oxygen supply is reduced below normal, although, perfusion of the tissue may be normal. Ischemia is the condition of decreased oxygen supply due to decreased perfusion (Braunwald & Sobel, 1980).

The objectives of this thesis are:

1. To assess the pharmacological agents that influence the responses of basilar artery segments to hypoxia. This is important because the vascular responses to hypoxia are different in the *in vitro* compared to the *in vivo* condition.

2. To determine the effect of hypoxia on the responses of basilar artery segments to vasoactive agents.

3. To determine the role of endothelium in hypoxic contraction of this vessel and of the role of K channels in the endothelially-mediated response.

Factors which affect Cerebral blood flow (CBF)

Factors that affect the diameter of cerebral arteries and arterioles intimately affect blood flow to the brain. Cerebral vascular tone and hence CBF is influenced by the partial pressure of carbon dioxide (P_aCO_2), the partial pressure of oxygen (P_aO_2), and metabolic end products

in the environment of the cerebral vessels. Intrinsic myogenic responses to pressure and flow, and extrinsic neurogenic influence through innervation may also alter cerebrovascular tone.

Reduction in P_aCO_2, **hypocapnia**, leads to vasoconstriction and decreased CBF (Raper *et al.*, 1971; Busija *et al.*, 1981), whereas elevation in P_aCO_2, **hypercapnia**, results in vasodilatation and increased CBF (Raper *et al.*, 1971; Busija & Heistad, 1981). These responses occur as a result of changes in extracellular fluid (ECF) hydrogen ions [H^+], caused by CO_2 diffusing across the blood-brain barrier. Acidosis has been shown to dilate, while alkalosis has been shown to constrict cat pial arteries (Kuschinsky *et al.*, 1972; Schneider *et al.*, 1977).

An hypoxic episode caused by a fall in P_aO_2, leads to cerebral vasodilatation and a concomitant increase in CBF in newborns and adults alike (Jones *et al.*, 1978, 1981; Busija & Heistad, 1981). Such a response occurs within 30-60 s of onset of hypoxia (Nilsson *et al.*, 1975; Busija & Heistad, 1981). This process helps to maintain oxygen delivery to cerebral tissue.

Adenosine

Adenosine levels in cerebral tissues increase during hypoxia (Nordstrom *et al.*, 1977; Winn *et al.*, 1979, 1981; Phillis *et al.*, 1987). Adenosine also has potent vasodilatory properties on rat pial arterioles (Morii *et al.*, 1986; Winn *et al.*, 1981), cat pial arteries (Wahl & Kuschinsky, 1976; Haller & Kuschinsky, 1987), and human arterioles (as judged by mean arterial pressure and PET scan measurements; Sollevi *et al.*, 1987). All of this evidence suggests a role for adenosine in the hyperemia associated with cerebral hypoxia and ischemia. In support of this hypothesis are reports that the increase in CBF during hypoxia, is attenuated by the adenosine antagonist, theophylline (Emerson & Raymond, 1981; Morii *et al.*, 1983). Topical application of adenosine deaminase, the enzyme that metabolizes adenosine, greatly attenuates cerebral hypoxic vasodilatation (Wei & Kontos, 1981).

Before assessing the direct effect of hypoxia on cerebral vascular smooth muscle, the general physiological regulation of smooth muscle function will be reviewed.

SMOOTH MUSCLE

Ultrastructure and types of smooth muscle

Smooth muscle is composed of three different muscle filament types: thick (myosin, 14 nm), thin (actin, 6-8 nm), and intermediate (desmin or vimentin, 1 nm). The actin/myosin ratio is approximately 15:1, which is much higher than in striated muscle (Somlyo *et al.*,1984). The two major types of mammalian smooth muscle are: multiunit and single unit. The visceral smooth muscle is characterized by fibres which have close appositions with each other due to gap junctions. Thus any stimulus is easily conducted to the nearby fibres. This type of smooth muscle is found in most of the hollow organs of the body, e.g., the gut, the uterus, the ureters. Multiunit smooth muscle is composed of discrete muscle fibres which are usually separately innervated. This type of smooth muscle is found in the blood vessels and the iris of the eye (Guyton, 1966). The remainder of this discussion will focus mainly on the activation of vascular tissue although many of the concepts are similar for all smooth muscle.

Vascular smooth muscle contraction

Activation of vascular smooth muscle is regulated by events occurring at the muscle plasma membrane. For instance, depolarization of the muscle cell increases extracellular calcium influx which results in tension development (Bolton 1979; Horn 1977; Van Breemen *et al.*, 1979), agonist stimulation causes either a change in membrane potential or increased ion conductances via voltage-gated or receptor-operated channels (Casteels *et al.*, 1977a; Harder & Sperelakis, 1978, 1979). Changes in E_m prior to agonist stimulation markedly affects the sensitivity of the muscle (Casteels *et al.*, 1977b; Haeusler 1978; Hermsmeyer *et al.*, 1981).

Tension development in arterial smooth muscle is closely related to changes in E_m, such that small membrane voltage changes can induce large tension changes (Harder & Waters, 1984). Some agonists cause calcium influx through receptor operated channels which are independent of depolarization.

Excitation-Contraction Coupling and energy metabolism in vascular smooth muscle.

In contrast to visceral smooth muscle, spike discharges rarely, if ever, occur in arterial smooth muscle, therefore, spike discharges play little role in arterial tone (Fleckenstein, 1983). Vascular smooth muscle contraction is initiated by an increase in intracellular calcium leading to the formation of a calcium-calmodulin complex and subsequent activation of myosin light chain kinase (Kamm *et al.*, 1987). This enzyme phosphorylates the light chain of myosin such that it can interact with actin to hydrolyze ATP and cycle the crossbridges to cause shortening or force (Johns *et al.*, 1987).

The three primary mechanisms postulated for the entry of activator calcium are, the calcium leak, the voltage-gated calcium channel, and the receptor-operated calcium channel (Johns *et al.*, 1987). The first mechanism is partially blocked by inorganic calcium antagonists such as Mn^{2+} and La^{3+} and can be responsible for contraction in smooth muscle if the calcium sequestering system is compromised (Loutzenhiser *et al.*, 1985). The uptake of calcium can be regulated by the potential across the membrane and this uptake is blocked by extracellular calcium withdrawal or calcium chelation with EGTA. These voltage-gated calcium channels are of two types: T (transient) and L (long-lasting) and are very sensitive to organic calcium antagonists like nisoldipine (Worley *et al.*, 1986). BAY K 8644 is a derivative of the 1,4-dihydropyridine calcium channel antagonists such as nitrendipine, which actually enhances calcium entry and contracts smooth muscle (Schramm *et al.*, 1983). Reports indicate that this

enhancement of calcium current occurs as a result of the promotion of mode 2, the open mode, of the voltage-gated calcium channel by the drug (Hess *et al.*, 1984). In rabbit isolated aortic rings this drug, BAY K 8644 (10^{-9}- 10^{-7} M), caused dose-dependent contractions when the preparation was partly depolarized with subthreshold KCl ($1.5x10^{2}$ M) (Franckowiak *et al.*, 1985). The receptor-operated channels are stimulated by various chemical agonists released from nerve terminals e.g., norepinephrine, or present in blood e.g., 5-hydroxytryptamine, prostaglandins. This allows inward flux of calcium or causes release of a second messenger which can increase permeability of the membrane to calcium or cause release of calcium from internal stores. Two main sources of intracellular calcium release are the plasma membrane (PM) and the sarcoplasmic reticulum (SR) with the SR being the major source (Bond *et al.*, 1984; Kowarski *et al.*, 1985) and the inner surface of the PM contributing a minor component once activation has begun (Saida & Van Breemen, 1987). It has been suggested that the Na^{+} - Ca^{2+} exchange mechanism, which plays a large role in calcium handling in cardiac tissue, does not play an important role in tension regulation in smooth muscle (Casteels *et al.*, 1985).

The maintenance of tone in smooth muscle requires a tight coupling between metabolism and contractility because the store of ATP and phosphocreatine is small relative to the rate of utilization during contraction (Paul *et al.*, 1984). Without metabolic synthesis of phosphagen, the energy pool would be exhausted in the first few moments of the contraction and the force for tone could not be sustained (Paul *et al.*, 1984). Oxidative phosphorylation is the main generator of energy, in the form of ATP, in vascular smooth muscle (Paul, 1980).

Some vessels display spontaneous electrical activity, e.g., mesenteric artery and portal vein, while others are electrically quiescent, e.g., pulmonary arteries (Speden, 1970). The canine

basilar artery is electrically quiescent with a resting membrane potential of 49 mV with a very minor contribution from an electrogenic Na^+ ,K^+ -pump (Fujiwara, *et al.*, 1982).

The low density of alpha-adrenoceptors in cerebral vessels (Bell *et al.*, 1985) probably accounts for the lack of vasoconstrictor responses to norepinephrine (Fujiwara *et al.*, 1982). Cerebral vessels exhibit relaxant responses to catecholamines precontracted vessels (Edvinsson & Owman, 1974). Treatment of basilar arteries with 5-HT ($1x10^{-8}$ M) or ATP ($1x10^{-5}$ M) depolarized the membrane, decreased the membrane resistance and produced dose-dependent contractions (Fujiwara, *et al.*, 1982).

HYPOXIA AND SMOOTH MUSCLE

Effects of hypoxia on non-cerebral vascular smooth muscle.

The reported effects of hypoxia on systemic vessels are varied and include attenuation of agonist-induced contractions, transient relaxations followed by contractions, potentiation of agonist-induced contractions, and direct activation of isolated vessels. Hypoxia and substrate (glucose) depletion caused attenuation of contractions in rabbit aortic strips to epinephrine (Furchgott, 1955). From this it was theorized that even under anaerobic conditions, a potent stimulus can still contract a vessel if enough glucose is present. A similar finding was reported for potassium-induced contractions in the bovine carotid artery (Laszt, 1960). Hypoxia was shown to significantly attenuate contractile responses to epinephrine, KCl, angiotensin II, histamine, 5-hydroxytryptamine, acetylcholine, and $BaCl_2$, in rabbit aortic strips (Shibata & Briggs, 1967; Needleman & Blehm, 1970; Altura & Altura, 1976). Isolated rabbit aortic strips, precontracted with phenylephrine (10^{-6}M) (Namm & Zucker, 1973) or epinephrine (10^{-6} g·l^{-1}) (Detar & Bohr, 1968), were reversibly relaxed by exposure to hypoxia. This relaxation became significant only when the PO_2 was dropped below 70 mm Hg.

Isolated hypoxic rabbit aortic rings exposed to transient normoxia, exhibited an initial relaxation followed by contraction (Griesemer & Coret, 1960). In isolated hypoxic helical strips of rabbit aorta, exposure to epinephrine ($3x10^{-6}$ M) caused contraction which was subsequently relaxed on exposure to increasing PO_2 (100 mm Hg) (Detar & Bohr, 1972). This relaxation was reversed upon re-exposure to hypoxia and was referred to as hypoxia-induced contraction and was either unaffected, or slightly potentiated, by uncoupling electron transport and oxidative phosphorylation with 2,4-dinitrophenol (10^{-3} M). Isolated anoxic (0% O_2) canine coronary artery rings precontracted with K^+ ($2x10^{-2}$ M), exhibited further increases in tension compared to K^+ -induced contractions in the presence of 20% O_2 (Van Nueten *et al.*, 1980). These same vessel segments exhibited transient but significant (1.35 $\pm$ 0.67 g) increases in tension when made anoxic. In canine coronary rings precontracted with 5-HT ($4x10^{-7}$ M) and norepinephrine ($9.5x10^{-7}$ M), hypoxia (95% N_2/5% CO_2) caused significant augmentation of these responses (Van Nueten & Vanhoutte, 1980). In unstimulated strips of canine coronary artery, severe hypoxia (PO_2= 2mm Hg) induced a transient relaxation followed by a sustained contraction, and this was accompanied by marked increases in potassium and norepinephrine efflux (Borda *et al.*, 1980).

The response of veins to hypoxia has also been studied. Isolated strips of canine mesenteric and saphenous veins, precontracted with norepinephrine ($1.5x10^{-8}$ M- $6x10^{-7}$ M) or KCl ($3x10^{-2}$ M- $5x10^{-2}$ M), were depressed by hypoxia (PO_2 mm Hg), as were mesenteric strips precontracted with acetylcholine (ACh; $5x10^{-7}$- $5x10^{-6}$ M). However, contractions to ACh in saphenous vein strips were potentiated by hypoxia (Vanhoutte, 1976). The apparent contradictions here may be explained by the nature of the vessels i.e., arteries versus veins.

The response of pulmonary vessels to hypoxia is also vasoconstriction. von Euler and Liljestrand (1946) first suggested that this was an inherent property of the pulmonary vasculature which resulted in decreased arterial pulmonary blood flow in the intact cat upon hypoxia. They concluded that the decreased blood flow was the result of a direct action of anoxia on the blood vessel wall. Hypoxia did not always induce contractions in isolated pulmonary vessels. Isolated helical strips of 4^{th} and 5^{th} order pulmonary arteries (1- 3 mm diameter), suspended in physiological salt solution (PSS), exhibited no increase in contractility when exposed to hypoxia (5.8% - 0% O_2), and the contractile responses to electrical stimulation, 5-HT, norepinephrine, angiotensin II, acetylcholine, and KCl, were all depressed during hypoxia (Lloyd, 1967). In a follow up study by Lloyd (1968), pulmonary arteries associated with a thin layer of parenchyma, did contract when exposed to hypoxia. This suggested that hypoxic pulmonary constriction was secondary to some unknown hypoxic disturbance of the parenchyma. Strips of rabbit pulmonary artery and aorta immersed in inert fluorochemicals or humidified gas, to minimize washout of water-soluble materials from the tissue, contracted upon hypoxia in a reversible fashion (Lloyd, 1970). In another study, isolated rabbit pulmonary artery and aortic strips equilibrated at 100 mm Hg for 2 hrs, exhibited anoxic (PO_2= 0 mm Hg, measured with an oxygen macroelectrode) relaxation of epinephrine (10^{-7} M)-induced contractions. In strips equilibrated at a PO_2 of 20 mm Hg, anoxia (PO_2= 0 mm Hg) augmented contraction to epinephrine only in the pulmonary strips (Detar & Gellai, 1971). Studies by Harder (1985a, 1985b) demonstrated that isolated rings of small ($3x10^{-4}$ m) pulmonary arteries from cat developed active tension when exposed to hypoxia (PO_2= 30-50 mm Hg), and that these contractions resulted from direct smooth muscle membrane depolarization. These findings tend to rule out any role for the parenchyma in the hypoxic pressor response. In another study isolated rabbit lobar pulmonary artery ring segments (1.5- 2.5 mm diameter)

contracted upon hypoxia (PO_2= 11 mm Hg) and these contractions were not blocked by procaine (10^{-3} M), phentolamine (10^{-6} M), isoproterenol (10^{-8} M), diphenhydramine, PGE_1, & atropine (10^{-6} M), or nitroglycerin (10^{-5} M) (Ohe *et al.*, 1986). Isolated rat lung showed hypoxic pressor responses when perfused with blood or plasma, but decreased or no pressor reactivity when perfused with PSS (Berkov, 1974; McMurtry, 1984). Since dexamethasone, a synthetic glucocorticoid, significantly potentiated hypoxic vasoconstriction in PSS-perfused isolated rat lungs, a role for glucocorticoids in the response to hypoxia in this model was proposed (Herget & M^{c}Murtry, 1987). A reversible hypoxia-induced fetoplacental vasoconstriction has been demonstrated in sheep (Stock *et al.*, 1980) and in isolated human placental cotyledons (Howard *et al.*, 1984; 1987), indicating a role for hypoxic vasoconstriction in local fetoplacental blood flow regulation. Strips of saphenous vein incubated for 75 min in glucose-free medium contracted upon exposure to hypoxia (PO_2mm Hg) in a calcium-independent manner (Vanhoutte, 1976).

Reversible development of tension by hypoxia and substrate depletion has also been demonstrated, in canine trachealis (Kroeger & Stephens, 1971; Bose, 1976) and guinea pig taenia coli (Bose & Bose, 1975; Knull & Bose, 1975). This increase in tension in the guinea pig taenia coli (Bose & Bose, 1975; Knull & Bose, 1975) and in the canine trachea (Bose, 1976) is insensitive to calcium chelation and has been postulated to be due to the development of rigor. Similarly, in glucose-depleted isolated rabbit pulmonary artery rings, hypoxia (PO_2= 30-50 mm Hg) produced a contraction which was not blocked by calcium channel blockade with nifedipine (10^{-6} M) or calcium chelation with EGTA (10^{-3} M) (Ohe *et al.*, 1986)

In summary several unstimulated isolated non-cerebral vessel segments from dog, rabbit, cat, and rat, exhibit increases in tension when made hypoxic. In prestimulated vessels the

evidence indicates that hypoxia causes augmentation in canine vessels, but attenuation in rabbit vessels. Other smooth muscles such as taenia coli and trachea also respond to hypoxia with a small increase in basal tension, but diminished responses to stimulating agonists.

Effects of hypoxia on cerebral vascular smooth muscle

Little work has been done on the effects of hypoxia on isolated cerebral arteries. All of the previous studies except one indicate that hypoxia induces or augments cerebral vascular contraction. In isolated canine basilar artery hypoxia augmented contractions to 5-HT ($6.3x10^{-10}$ g·ml^{-1}). This effect was significantly attenuated by pretreatment with the calcium antagonist flunarizine ($4x10^{-8}$ g·ml^{-1}) (Van Nueten *et al.*, 1982). In isolated rings of canine basilar artery, 10 min of hypoxia (PO_2= 1 mm Hg) induced an increase in tension. This hypoxic contraction was not abolished by phentolamine or propranolol (10^{-5} M), ketanserin (10^{-6} M), apyrase (0.8 U·ml^{-1}), indomethacin or meclofenamate (10^{-5} M) (Katusic & Vanhoutte, 1986). Contractions to PGF_{2a} (10^{-6} M), 5-HT (10^{-7} M), UTP (10^{-5} M), and KCl ($3x10^{-3}$ M) were potentiated by hypoxia, on the other hand, relaxation to vasopressin (10^{-7} M) was completely abolished by hypoxia while relaxation to thrombin (1 U·ml^{-1}) was converted to a contraction by hypoxia (Katusic & Vanhoutte, 1986). Mallick *et al.*, (1987) demonstrated that in basilar rings pretreated with the K^+ -conductance inhibitor, 4-aminopyridine (10^{-3}- $6x10^{-3}$ M), hypoxia (PO_2 mm Hg) induced an increase in tension which was reversible and calcium dependent. These hypoxic contractions were not affected by pretreatment with methysergide, atropine, or indomethacin (10^{-5} M), phentolamine (10^{-6} M), or ouabain ($3x10^{-6}$ M). In another study, isolated canine basilar artery rings exhibited hypoxic augmentation of contractile responses to KCl ($2.5x10^{-2}$ M), PGF_{2a} (10^{-5} M), and haemoglobin (10^{-6} M). This augmentation was not antagonized by guanethidine, methysergide, prazosin, or diphenhydramine (10^{-5} M) (Nakagomi *et al.*, 1987). The fact that hypoxia can augment contractile responses to

haemoglobin and possibly other spasmogenic agents may be clinically important with regard to the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage. If hypoxia and the products released from lysed red blood cells e.g., haemoglobin, K^+ , etc., constrict these vessels in an additive fashion, vasospasm may ensue.

Simeone & Vinall (1980) demonstrated that isolated bovine middle cerebral arteries incubated in an anoxic medium (0% O_2) for 30 min, produced diminished contractile responses to 5-HT (10^{-6} M) and rabbit and human whole blood when compared with control. The normal tonic and long-lasting contractions became phasic and quickly relaxed to baseline. The dose response curve to 5-HT was significantly shifted to the right in oxygen-deprived medium. These findings may represent the events that occur after a more prolonged period of hypoxia.

To summarize, cerebral vessels exposed to acute hypoxia exhibit augmentation of agonist-induced contractions and, although, these contractions are calcium-dependent they are not inhibited by many of the known receptor blockers.

FACTORS AFFECTING SMOOTH MUSCLE

Effects of red blood cell hemolysate

Cerebral arteries are contracted by exogenously applied blood (Echlin, 1965; Simeone *et al.*, 1968; Echlin, 1971). It was thought that this contraction was caused by serotonin released from platelets (Simeone & Vinall, 1975). One of the first reports to focus on the role of the erythrocyte in this contraction was the work of Osaka in 1977. He demonstrated that the exposed basilar artery of the cat *in situ* was significantly constricted by the topical application of various fresh blood fractions including hemolysate from lysed red blood cells. Using canine isolated basilar arteries, Ozaki & Mullan, (1979) demonstrated contraction induced by one of the fractions of lysed red blood cells run on a Sephadex G-25 column. The contractile activity

of this fraction was stable for up to 7 days and it possessed a molecular weight over 5000 daltons. In canine isolated basilar artery, contraction induced by haemoglobin (10^{-8}- 10^{-4} M; molecular weight about 67000 daltons) was first demonstrated by Tanishima, 1980. In cerebral vessels, haemoglobin (red blood cell hemolysate) causes contractions which are independent of the endothelium (Fujiwara *et al.*, 1984; Conner & Feniuk, 1987) and may be mediated by prostaglandins (Lang & Maron, 1988).

Role of the vascular endothelium

For many years the vascular endothelial layer was thought of merely as a barrier between blood and the underlying smooth muscle cells. The endothelial cell is now regarded as an important metabolic and endocrine organ and not just a physical barrier to blood-borne humoral compounds. The endothelial cells, which line the intimal layer of blood vessels, are responsible for several important functions. These include plasma lipid transport regulation, hemostatic control, and modulation of vascular smooth muscle reactivity through the release of various chemical mediators. Many vasoactive substances such as 5-HT, adenosine, and bradykinin are inactivated by the endothelium, while others, e.g., angiotensin II, are generated (Vane *et al.,* 1987). These cells can also synthesize and, upon precise stimulation, secrete various vasoactive substances. Substances released from the endothelium include a group of endothelium-derived relaxing factors (EDRF), which are released when the endothelial cells are stimulated by acetylcholine (ACh), ATP, and other agents (Furchgott, 1984).

For years the observation that ACh was a relaxant *in vivo* but a constrictor *in vitro* had puzzled researchers (Furchgott & Zawadzki, 1980). In helical strips of rabbit aorta dilatation to inorganic nitrites, nitroprusside, organic nitrates, and other known vasodilators was demonstrated. However, only contractions were elicited with ACh or carbachol. With aortic

ring preparations it was discovered that ACh could induce both relaxation and contraction. Lack of relaxing response in the strips ocurred after unintentional destruction of the intimal surface (Furchgott *et al.*, 1979). In other words the relaxing response to ACh was dependent on the presence of endothelial cells which, upon muscarinic activation, release a relaxing substance (perhaps nitric oxide) that acts on the smooth muscle cell (Furchgott & Zawadzki, 1979).

Factors that affect endothelium-dependent relaxation

Many agents have been shown to induce endothelium-dependent relaxations in arteries from a variety of species. Relaxations to adenosine triphosphate **(ATP)** and adenosine diphosphate **(ADP)** are endothelium-dependent in canine femoral artery (DeMey & Vanhoutte, 1980) and rabbit aorta (Furchgott & Zawadzki, 1980), as are those to **bradykinin** in a variety of canine arteries (Cherry *et al.*, 1982; Chand & Altura, 1981), **vasopressin** in canine basilar artery (Katusic *et al.*, 1984), **substance P** in rabbit, canine, and cat arteries (Zawadzki *et al.*, 1981), **arachidonic acid** in canine arteries (DeMey *et al.*, 1982), the calcium ionophore **A23187** in various vessels (Furchgott *et al.*, 1981), uridine triphosphate **(UTP)** and uridine diphosphate **(UDP)** in human pial vessels (Hardebo *et al.*, 1987), as well as a few others (For an extensive review see Furchgott, 1983).

Rubbing of the intima can abolish EDRF-mediated relaxations. **Anoxia/hypoxia** can inhibit ACh-induced relaxations in canine femoral artery (DeMey & Vanhoutte, 1980). Relaxations elicited by ACh (Furchgott & Zawadzki, 1980) and A23187 (Zawadzki, *et al.*, 1980) in rabbit aorta, as well as ACh and bradykinin relaxations in canine arteries (Furchgott, 1983) are also inhibited by hypoxia.

Several enzyme inhibitors also inhibit EDRF-mediated relaxations. The compound **5, 8, 11, 14-eicosatetraynoic acid (ETYA)** which is an inhibitor of 5-lipoxygenase and a free radical scavenger, inhibits ACh-induced relaxations in rabbit aorta (Furchgott & Zawadzki, 1980; 1981), canine arteries (Cherry *et al.*, 1982; DeMey *et al.*, 1982), and rat aorta (Van de Voorde & Leusen, 1983). ETYA also inhibits relaxations elicited by A23187, ATP, and substance P in rabbit aorta (Furchgott & Zawadzki, 1980; Zawadzki *et al.*, 1980), by histamine and A23187 in rat aorta (Rapoport & Murad, 1983), by bradykinin in canine arteries (Cherry *et al.*, 1982). The phospholipase A_2 inhibitor, **quinacrine**, inhibits relaxations produced by ACh in rabbit aorta (Singer & Peach, 1983), canine arteries (DeMey *et al.*, 1982), and rat aorta (Rapoport & Murad, 1983), relaxations produced by substance P in rabbit aorta (Zawadzki *et al.*, 1981), and by bradykinin in canine arteries (Cherry *et al.*, 1982).

The free radical scavenger **hydroquinone** inhibits relaxations elicited by ACh and A23187 in rabbit aorta (Furchgott, 1981) in a reversible manner after a short exposure (5- 10 min.). This effect became irreversible after longer exposure and was thought to indicate damage to the endothelium (Furchgott *et al.*, 1981). The relaxation-inhibiting property of hydroquinone was demonstrated *in situ* in the rabbit with no evidence of endothelium damage (Bing *et al.*, 1987). The mechanism of action of hydroquinone is thought to be through the formation of superoxide radicals to inactivate EDRF (Moncada *et al.*, 1986).

The breakdown products of erythrocytes have many actions on the vasculature. Haemoglobin (Hb) inhibits relaxation of bovine retractor penis muscle (Bowman & Gillespie, 1981). In rabbit basilar artery, ACh and ATP (10^{-7}- 10^{-4} M) relaxations of 5-HT (10^{-6} M)-induced contractions were completely abolished by Hb (10^{-7}- 10^{-5} M) (Fujiwara *et al.*,

1986). In canine middle cerebral artery, angiotensin II (10^{-7} M)-induced relaxations were inhibited by hemolysate (Hb; $1.6x10^{-5}$ M) (Toda, 1988).

Role of endothelium in the response of non-cerebral vascular smooth muscle to hypoxia

The role of endothelium in the response of vascular smooth muscle to hypoxia has been studied in many tissue preparations and has proven controversial. There is even contradictory evidence from the same laboratory about the relationship between endothelium and hypoxia.

In canine femoral artery (DeMey & Vanhoutte, 1981; 1982; 1983), pulmonary artery, saphenous artery, and splenic artery (DeMey & Vanhoutte, 1982), contractions produced by $BaCl_2$ (10^{-3} M), norepinephrine ($2x10^{-8}$- $2x10^{-6}$ M), and KCl ($2.5x10^{-2}$ M), were augmented by hypoxia (PO_2 mmHg), to a greater degree if the endothelium was present. Rings and strips of canine coronary arteries without endothelium exhibited anoxic (95% N_2/5% CO_2) augmentation of contractions induced by 5-HT ($5x10^{-7}$ M), KCl ($2x10^{-2}$ M), and PGF_{2a} ($2x10^{-6}$ M), only when in contact with a strip containing a functional endothelium layered on top (Rubanyi & Vanhoutte, 1985). The layering experiments were presumed to indicate the release of a diffusible vasoconstrictor substance from the endothelial cells in response to hypoxia/anoxia. Contractions of porcine coronary rings induced by media obtained from cultured bovine aortic endothelial cells again indicated the existence of a diffusible coronary vasoconstrictor substance, although, hypoxia was not required for release of this polypeptide (Hickey *et al.*, 1985). In unstimulated canine coronary arteries, hypoxia caused Ca^{2+} -dependent contractions even in the absence of endothelium, but, such contractions were abolished by lipoxygenase inhibition (Rimele & Vanhoutte, 1986).

Rings of rat tail artery and side branches of canine femoral artery exhibited endothelium-dependent dilatation when exposed to intraluminal hypoxia (PO_2= 40 mm Hg) (Busse *et al.*, 1983).

Role of endothelium in the response of cerebral vessels to hypoxia

To date two studies have reported on the role of endothelium in the hypoxic response of cerebral vessels, but with somewhat conflicting views. Rings of canine basilar artery with functional endothelium exposed to hypoxia (95% N_2) responded with a contraction (Katusic & Vanhoutte, 1986). Presence of endothelium was indicated by relaxation in the presence of vasopressin (10^{-7} M). In this vessel, anoxia caused contraction in the presence of indomethacin, even in the absence of the endothelium. The authors proposed that anoxia causes contraction of canine basilar artery through endothelium-dependent means, and through direct activation of previously stimulated smooth muscle cells. In these preparations, anoxia caused augmentation of contractions to PGF_{2a} (10^{-6} M), KCl ($3x10^{-2}$ M), 5-HT (10^{-7} M), and UTP (10^{-5} M). On the other hand, in rings without endothelium this augmentation was decreased to less than half compared to those with endothelium. In contrast, another study examining the effects of hypoxia on canine basilar artery, demonstrated that removal of the endothelium did not alter the hypoxic augmentation of contractions to KCl ($2.5x10^{-2}$ M), PGF_{2a} ($3x10^{-7}$ M), and haemoglobin (10^{-6} M) (Nakagomi *et al.*, 1987). One explanation may be the different resting tensions used; 1 g in the study by Nakagomi, and 3 g in the study by Katusic & Vanhoutte. Another possiblity is that the latter investigators used CaEDTA ($2.6x10^{-5}$ M) in their buffer. The exact role of the endothelium in the response of cerebral vessels to hypoxia/anoxia is, therefore, still controversial.

Calcium antagonists and the response of vascular smooth muscle to hypoxia

The tension of vascular smooth muscle changes in response to altered levels of cytoplasmic free calcium. As explained above, there are two main sources of this intracellular calcium release as well as three primary sources of calcium entry. The role of calcium during hypoxia has been studied by many investigators, but the source of this activator calcium is still uncertain. In isolated canine coronary arterial rings, anoxia (PO_2= 0mm Hg) potentiated contractions produced by 5-HT ($4x10^{-7}$ M) and this augmentation was significantly attenuated by the calcium antagonists verapamil (10^{-6} M), diltiazem (10^{-5} M), nifedipine (10^{-7} M) (Barrett *et al.*, 1986) and lidoflazine ($5x10^{-7}$ M) (Van Nueten & Vanhoutte, 1980). Isolated strips of canine saphenous vein precontracted with ACh ($5x10^{-7}$- $5x10^{-6}$ M) were further contracted by anoxia (PO_2= 1 mm Hg). This potentiation was attenuated by calcium channel blockade with iproveratril ($2x10^{-5}$- 10^{-4} M) (Vanhoutte, 1976). Hypoxic contraction in isolated rings of pulmonary artery was blocked by verapamil (10^{-6} M) and potentiated or attenuated when calcium levels were raised or lowered respectively, thus indicating that it was dependent on extracellular calcium influx (Harder *et al.*, 1985). The hypoxia-induced contraction in isolated canine basilar artery rings was abolished by calcium channel blockade with diltiazem (10^{-5} M) (Katusic & Vanhoutte, 1986), D600 (10^{-5} M) (Mallick *et al.*, 1987), and calcium chelation with EGTA $2.5x10^{-2}$ M (Mallick *et al.*, 1987) or 10^{-3} M (Nakagomi *et al.*, 1987). The calcium antagonist nicardipine (10^{-8}- 10^{-7} M), which has been shown to block entry of calcium through the voltage-dependent calcium channels in canine basilar artery (Yamamoto *et al.*, 1983), effectively abolished the hypoxic augmentation of contractions to KCl ($2.5x10^{-2}$ M), and PGF_{2a} ($3x10^{-7}$ M), and greatly attenuated the hypoxic augmentation to haemoglobin (10^{-6} M).

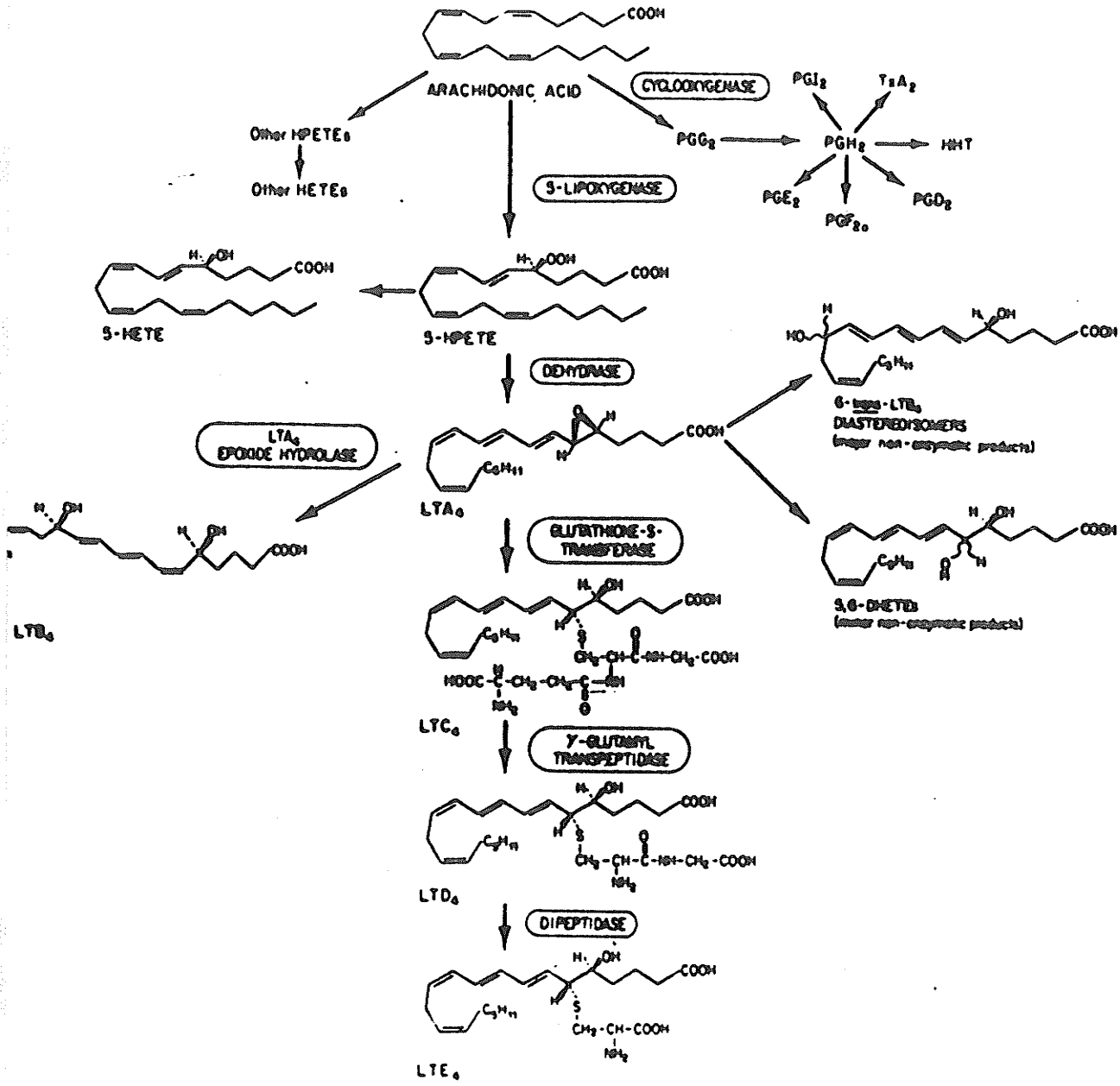
These findings indicate that the effects of hypoxia on isolated vascular smooth muscle are dependent on extracellular calcium influx. What is still unclear is the importance of calcium

entry through voltage-gated channels versus that through receptor-operated channels. Another question is the role of endothelial factors that may be released during hypoxia.

Leukotrienes and vascular smooth muscle

Leukotrienes (LT) A_4- E_4 (Fig. 1) are biologically active products of arachidonic acid (AA). With hypoxia phospholipases and lipases cleave AA from membrane phospholipids. AA then serves as a substrate for the cyclooxygenases to produce a myriad of biologically active products such as prostaglandins, thromboxanes and prostacyclin. AA can also serve as a substrate for 5-lipoxygenase, which produces various leukotrienes and their metabolites (Lewis & Austen, 1984). Inhibitors of the lipoxygenases include diethylcarbamazine (Orange & Austen, 1968), ETYA, and a variety of antioxidants including nordihydroguiaretic acid (NDGA) (Higgs & Flower, 1981; Louis-Flamberg *et al.*, 1988). LTC_4 in a dose of $4x10^{-9}$- $6x10^{-9}$ g produced a 40-50% reduction in blood flow to guinea pig skin (Williams & Piper, 1980). Leukotrienes are also potent constrictors of cranial arteries. LTD_4 (10^{-9}- $1.5x10^{-8}$ mols·kg^{-1}) constricted rat internal and external carotid *in vivo* (Tagari *et al.*, 1983). LTD_4 ($2x10^{-9}$- $2x10^{-7}$ M) also contracted human isolated basilar and vertebral arteries (Tagari *et al.*, 1983). Leukotrienes B_4, C_4, and D_4 ($3x10^{-8}$- $4x10^{-8}$ M) applied topically to the surface of exposed mouse brain, caused dose-related constriction of the pial arterioles (Rosenblum, 1985). Leukotrienes can also contract other smooth muscles such as human isolated airway and pulmonary smooth muscle (Haana *et al.*, 1981), and guinea pig isolated pulmonary arteries (Hand *et al.*, 1981).

During cerebral ischemia induced in gerbils by bilateral carotid artery occlusion, both saturated and unsaturated free fatty acids (FFA) increased 10 fold over the preischemic levels (Yoshida *et al.*, 1980). Another study in the gerbil showed that 5- 15 min of ischemia produced significant increases in the levels of LTC_4 and LTD_4 (Moskowitz *et al.*, 1984). Rehncrona *et*



From: Lewis, R.A., & Austen, K.F.

The biologically active leukotrienes.

(1984) J. Clin. Invest. 73:889-897

al., (1982) and Yoshida *et al.*, (1983) have also reported increased FFA upon complete or incomplete ischemia with the largest increases in arachidonic acid (AA).

Decreased CBF after 10 or 20 min of forebrain ischemia in gerbils was associated with significant increases in LTB_4 levels (Dempsey *et al.*, 1986a). This increase as well as the resulting cerebral edema was blunted by pretreatment with lipoxygenase inhibitor, nor-dihydroguiaretic acid (1.2 $mg \cdot kg^{-1}$) (Dempsey *et al.*, 1986b). One of the first studies to investigate the role of leukotrienes in hypoxic pulmonary vasoconstriction (HPV), *in vivo*, demonstrated that leukotriene antagonists FPL-57231 and FPL-55712 (Sheard *et al.*, 1982) both prevented and reversed HPV in anaesthetized sheep (Ahmed & Oliver, 1983). Blockers of leukotriene synthesis or action inhibited the hypoxic presser response in rat isolated lung preparations whether given before or during hypoxia (fraction of inspired air, FIO_2= 3%) (Morganroth *et al.*, 1984). In rat isolated lungs (Morganroth *et al.*, 1984) and anesthetized dogs (Lonigro *et al.*, 1988), hypoxia (PO_2= 25- 30 mm Hg) caused an increase of LTC_4. Rings of canine coronary arteries, with or without endothelium, contracted during hypoxia (PO_2mm Hg).These contractions were abolished by lipoxygenase inhibition (pretreatment with phenidone or BAY G6575, 10^{-6} M) but not by cyclooxygenase inhibition (pretreatment with indomethacin, 10^{-6} M) (Rimele & Vanhoutte, 1983,1984). In rings of canine coronary arteries, anoxia (PO_2mm Hg) caused augmentation (44.5 $\pm$ 7.5%) of contractions to PGF_{2a} ($2x10^{-6}$ M) which were not affected by inhibition of cyclooxygenase (indomethacin, 10^{-6} M) or lipoxygenase (phenidone, $5x10^{-5}$ M, or NDGA 10^{-5} M) (Rubanyi & Vanhoutte, 1985). Therefore, lipoxygenase inhibition altered only the effects of hypoxia on unstimulated vessels. These data indicate that hypoxia-mediated contractions or augmentations of vascular smooth muscle tone may be partially mediated by products of the lipoxygenase pathway.

The effect of changing extracellular ion concentrations on smooth muscle.

Responses of smooth muscle to various stimuli are altered by changes in extracellular ion concentrations. In frog sartorius muscle, baseline tension is increased when the muscle is bathed in low ionic strength medium (Gordon *et al.*, 1973). Contracture in guinea pig isolated taenia coli strips by equilibration in high-K^+ ($1.43x10^{-1}$ M) normal sodium Krebs was not relaxed, but, further contracted after wash with Na^+ -free (sucrose-substituted), normal K^+ ($5.9x10^{-3}$ M) Krebs medium until the external Na^+ concentration was raised (Katase & Tomita, 1972). X-ray diffraction data from Weber & Murray, (1973) indicate that Mg-ATP causes dissociation of actin and myosin in low ionic strength medium. In guinea pig isolated taenia coli strips, equilibration in Na^+ -free medium (sucrose-substituted Krebs) caused a rapid contraction which came back to baseline in 15- 20 min (Ma & Bose, 1977). Upon addition of KCl ($7x10^{-2}$ M) a sustained contraction was produced which was not relaxed by washing with Na^+ -free medium containing normal-K^+ , but, was relaxed when external sodium was reintroduced.

In canine isolated basilar artery strips, substitution of one-half of the NaCl with choline chloride caused a sustained contraction which was potentiated by the cardiac glycoside ouabain ($2x10^{-7}$ M) and abolished by exposure to Ca^{2+} -free medium (Toda, 1978). This same preparation contracted when exposed to low-K^+ and these contractions were abolished by pretreatment with ouabain ($2x10^{-7}$ M), and by exposure to Ca^{2+} -free medium. Reduction of Cl^- caused a moderate transient contraction which was attenuated by exposure to verapamil ($2x10^{-7}$- 10^{-6} M). Vascular smooth muscle is very sensitive to changes in extracellular ion concentrations, therefore, the function of these muscles may be closely studied using Na^+ -free solutions.

Ion fluxes during hypoxia

Studies on changing ion permeability during hypoxia in cardiac and smooth muscle have indicated that intracellular calcium and sodium concentrations increase while there is a net efflux of potassium. In isolated canine coronary arteries, hypoxia (PO_2mm Hg) induced transient relaxation followed by sustained contraction in the unstimulated vessel. Measurements with ^{42}K indicate that the contraction was accompanied by a significant increase in K^+ efflux (Borda *et al.*, 1980). Similarly in cardiac muscle from rabbit (Conrad *et al.*, 1979; Crake *et al.*, 1987), dog (Lowry *et al.*, 1942), and pig (Friedrich *et al.*, 1981), there was a loss of potassium upon hypoxia which was due to increased efflux rather than decreased influx (Kleber, 1984). Electrophysiological studies in cat papillary muscle (Vleugels *et al.*, 1978), and in rat myocardium (Conrad *et al.*, 1983), indicated that hypoxia caused a decrease in the action potential duration as well as an increase in steady state outward current. Hypoxia (PO_2mm Hg), acidosis (pH= 6.77), and elevated K^+ levels ($1.15x10^{-2}$ M) caused a decrease in membrane potential and an increase in intracellular sodium activity in guinea pig papillary muscles (Wilde & Kleber, 1986). Studies in pulmonary arteries of cats showed that reducing PO_2 from 400 to 30 mm Hg produced Ca^{2+} -dependent action potentials and contractions and resulted in membrane depolarization without increased K^+ efflux (Harder *et al.*, 1985a, 1985b). The authors speculated that perhaps the membrane depolarization was due to Na^+ or Ca^{2+} influx.

PROPOSAL

With the aforementioned properties of vascular smooth muscle applied to the canine basilar artery, I intended to study in detail the mechanism by which hypoxia causes canine isolated basilar artery to contract *in vitro*, and test the hypotheses that this response:

1) is not wholly mediated by the endothelium. This was addressed in experiments involving removal of the endothelial cells by several methods and through verification using pharmacological means and scanning electron microscopy.

2) is dependent on extracellular calcium influx through voltage-gated calcium channels. This was studied using the calcium channel agonist BAY K 8644 and Ca channel blockade.

3) can occur as a result of membrane depolarization. This was assessed with the use of sodium-free buffer and through the use of agents which partially depolarized the membrane.

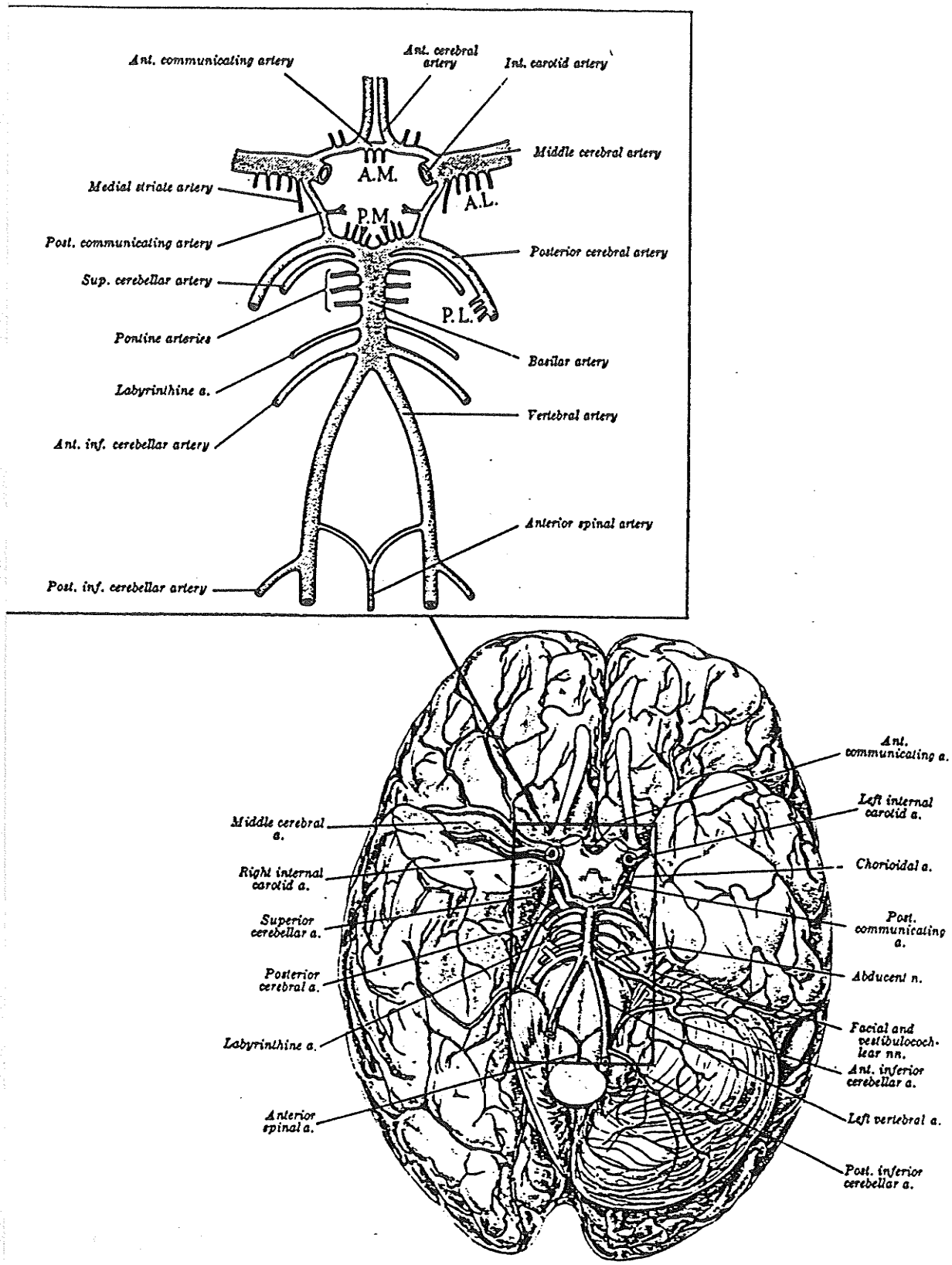
4) can be mediated through activation of the lipoxygenase pathway. Inhibitors of 5-lipoxygenase were used to elucidate the role of leukotrienes in hypoxic contraction.

MATERIALS & METHODS

Animal and tissue

In the rat, the arterial vasculature and the microvasculature are equally important in regulating cerebral blood flow (Harper *et al.*, 1984). In the cat, the larger cerebral vessels extending from the circle of Willis are largely responsible for adjustments in cerebral blood flow over large pressure changes (Kontos *et al.*, 1978). The basilar artery of the dog is a primary vessel in the adjustment of CBF to changes in oxygen tension. This artery is easily obtained and is fairly amenable to experimental manipulation. The basilar artery is formed by the junction of the two vertebral arteries and extends anteriorly along the ventral surface of the pons to divide into the posterior cerebral arteries which supply the occipital and temporal lobes, and the transverse branches which supply the pons and adjacent regions of the brain (Fig. 2).

Mongrel dogs of either sex weighing between 5-14 kg were used as the source of cerebral arteries. Once anaesthetized with sodium pentobarbital (30 mg/kg I.V.), a portion of the skull was removed by an oscillating saw and the brainstem to midbrain was excised, *en masse*, and placed in a dissection dish containing physiological salt solution (PSS) aerated with 95% O_2/5% CO_2. The basilar artery was dissected clear of the brain stem, under magnification, freed of adhering meninges, and all branching arteries were severed close to the main artery. Two succesive ring segments (5- 10 mm long) were cut approximately 5 mm upstream from the branch of the two vertebral arteries and this was the tissue used in the experiments.



From: Gray's Anatomy (1964).

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Experimental Setup

The rings were suspended in 20 ml aerated baths of PSS maintained at 37^0C with the aid of two 'L'-shaped stainless steel wires (30 AWG) passed through the vascular lumen (Refer to Fig. 3). One wire was connected to a stationary post and the other to a micromanipulator and force transducer (Grass FT03) such that isometric tension development could be measured. A resting tension of 2 g was established and the vessel was allowed to equilibrate for 1 hr. This resting tension has been demonstrated to be optimum in the canine basilar artery for contractions produced by 5-HT, PGF_{2a}, and KCl (Allen *et al.*, 1974). The viability of the preparation was assessed by measurement of a contraction in response to a maximal depolarizing dose of KCl (10^{-1} M). Tension changes were recorded on a Grass Model 7 Polygraph.

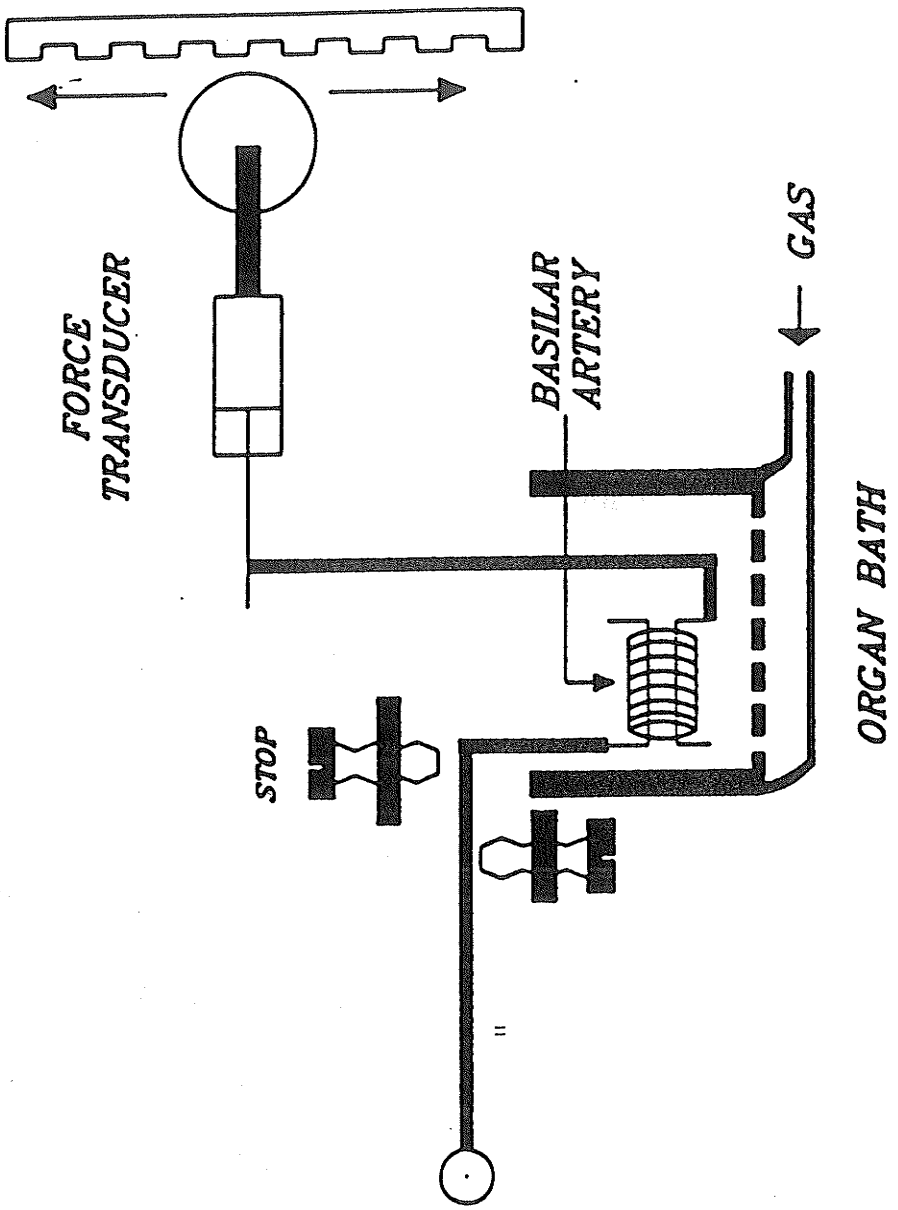
Protocols

1. Response of canine isolated basilar artery to hypoxia.

Hypoxia was initiated by changing the gas mixture bubbling through the bath to 95% N_2/5% CO_2 for a period of 10 min. Once a positive response (as outlined in results section) was observed, the effects of various agents including hydroquinone ($4x10^{-5}$ M), 4-aminopyridine ($3x10^{-3}$ M), nordihydroguiaretic acid ($5x10^{-6}$ M) and adenosine ($5x10^{-6}$ M) and endothelium removal on hypoxic contraction were determined.

2. Effect of calcium channel blockade.

The vessel was exposed to either a calcium entry blocker (verapamil, 10^{-6} M) or calcium chelator EGTA, ($2.5x10^{-3}$ M) and the response to hypoxia was compared to control.


FORCE TRANSDUCER
BASILAR ARTERY
GAS
ORGAN BATH
STOP

3. Removal of Endothelium

To test for the presence of a functional endothelium, contraction of the vessel ring was first produced by the application of 5-hydroxytryptamine (5-HT, 10^{-7} M). Subsequent addition of acetylcholine (ACh, $5x10^{-5}$ M) and/or arginine^{8}vasopressin (AVP, 10^{-8} M) to the bath caused relaxation of vascular tone. This is indicative of the presence of endothelium and the release of endothelium-dependent relaxing factor(s) (Furchgott & Zawadzki, 1979). Once baseline responses were determined the endothelium of the vessels was removed and the responses to the various drugs and hypoxia were observed and compared to their own controls. The response of the vessels to high extracellular K^{+} (10^{-1} M) was recorded before and after removal of endothelium.

The endothelium was removed by either intimal rubbing with a 23 gauge stainless steel needle or intraluminal exposure to 0.1% Triton X 100 for 1 min. Triton treatment has been documented to effectively remove the endothelial layer from small cerebral vessels (Conner & Feniuk, 1987). In some experiments the artery was gently everted before mounting. This facilitated the subsequent removal of the endothelium by gentle rubbing of the outer surface of the vessel. In some cases though, eversion of the vessel alone abolished the relaxing response to ACh and AVP, indicating damage to the endothelium. Therefore for these experiments there were no internal controls even though they contracted in response to hypoxia.

4. Scanning electron microscopy

In each of 3 experiments 2 vascular preparations were first mounted in the bath and tested for relaxant response to acetylcholine and arginine^{8}vasopressin, as indicated above. One of the vessels was fixed for histological examination at this point. The lumen and outer surface of the remaining vessel was treated with 0.1% Triton X 100 for 1 min. After washout of the

deterdent, abolition of relaxant response to the two agents was always observed. At this point the vessel was removed and fixed for electron microscopy. The vessel was transversely cut in 1 mm lengths and fixed for 24 hrs at 4^0C in a solution of 2.5% glutaraldehyde in $1.2x10^{-1}$ M phosphate buffer with $2x10^{-5}$ M $CaCl_2$ added. This was followed by 3 rinses in a solution containing 8% dextrose in phosphate buffer and osmication in 2% phosphate-buffered OsO_4. The preparations were then dehydrated in increasing concentrations of ethanol and critical point dried from CO_2. The specimens were mounted with conductive silver paint onto studs and examined with a JEOL JSM 35-C scanning electron microscope. The accelerating voltage was 20 KV and the magnification was 2000 X unless otherwise stated.

5. Effect of calcium channel agonist

Before and after addition of a subthreshold dose of the calcium channel agonist BAY K 8644 (10^{-11} M) to the bath the response to hypoxia was determined in 5 muscles.

6. Effect of sodium-free medium

The response of the 15 vessels to hypoxia was observed in normal Tris buffer and in sodium-free Tris buffer (reagents section). In these experiments normoxia involved bubbling the bath with 100% O_2 and hypoxia involved bubbling with 100% N_2.

7. Effect of potassium conductance blockers.

A series of experiments involving the potassium-conductance blocker 4-aminopyridine (4-AP) was carried out in 20 vessels. The response to hypoxia in the presence of 4-AP was observed and compared to control without. In a separate study the response of canine basilar artery to relaxations induced by ACh in the presence and absence of 4-AP was determined.

8. Effect of hydroquinone.

The response of the vessel to hypoxia and to 5-HT (10^{-7} M), KCl (10^{-1} M), and haemoglobin, ($2x10^{-7}$ M) was determined in 7 vessels in the presence and absence of hydroquinone.

8. Effect of adenosine.

The effect of exogenously added adenosine (10^{-7}- 10^{-5} M) on hypoxic contraction was determined in 6 vessels. The adenosine was added either prior to or during hypoxia and the results noted.

9. Statistical evaluation

Data are shown as means $\pm$ S.E.M. and were analyzed by one way analysis of variance, unless otherwise stated, and Duncan's multiple range test with p indicating statistical significance.

10. Reagents

The following chemicals were used: 5-hydroxytryptamine, 4-aminopyridine, tetraethylammonium chloride, acetylcholine chloride, arginine[8] vasopressin, nordihydroguiaretic acid (NDGA), 5,8,11,14-eicosatetraynoic acid (ETYA), (all from Sigma Chemical Co.), hydroquinone (J. T. Baker Chemical Co.), sodium nitroprusside (Hoffmann-La Roche Ltd.), barium chloride, Triton X 100 (both from Fisher Sci. Co.), BAY K 8644 (Bayer A.G.), canine red blood cell hemolysate.

Blood (10 ml) was obtained from dogs at the same time as the vessel, via a major artery and collected in heparin-containing tubes. It was then centrifuged @ 5000X g for 10 min and the buffy coat aspirated to leave the red blood cells (RBC), which were washed 2 times with

10 ml isotonic saline and centrifuged @ 15000X g for 20 min. This left about 5 ml of RBC which were diluted to 30 ml in distilled water and 10^{-6} l aliquots ($2x10^{-7}$ M, haemoglobin) were added to the bath.

SOLUTIONS

Physiological Media (concentration in 10^{-3} M)

	Krebs Hensel	Tris Sol'n	Na^+ -free Sol'n
NaCl	120.0	120.0	0.0
Sucrose	0.0	0.0	240.0
KH_2PO_4	1.4	1.4	1.4
KCl	4.7	4.7	4.7
$MgSO_4$	1.2	1.2	1.2
$CaCl_2$	2.5	2.5	2.5
Glucose	11.0	11.0	11.0
Tris-HCl	0.0	10.0	10.0
$NaHCO_3$	25.0	0.0	0.0

Electron Microscopy Fixative:

paraformaldehyde 5%		200 ml
glutaraldehyde 50%		25 ml
phosphate buffer 0.4 M	150 ml	

$CaCl_2$ 0.5%		2 ml
DD water to make		Total 500 ml
Filter through # 44 filter paper		

Phosphate buffer (0.4 M):

$NaH_2PO_4.H_2O$	5.3 g
K_2HPO_4	28.0 g
DD water to make	500 ml

Paraformaldehyde solution (5%):

paraformaldehyde	10 g
DD water	200 ml
1 N NaOH	4-6 drops

Dextrose rinse solution:

dextrose		80 g
$CaCl_2$ 0.5%		4 ml
phosphate buffer 0.4 M	300 ml	
DD water to make		Total 1000 ml

Double strength buffer:

dextrose		7 g
phosphate buffer 0.4 M	30 ml	
DD water to make		Total 50 ml

OsO_4 buffer:

Double strength buffer	15 ml	
$CaCl_2$ 0.5%		0.15 ml
4% OsO_4 in DD water		15 ml

RESULTS

Effect of endothelium removal on contractions and relaxations elicited by various agonists

Isolated canine basilar artery rings contracted (0.76 ± 0.1 g) when exposed to 5-HT (10^{-7} M) and subsequently relaxed when exposed to ACh ($5x10^{-5}$ M) or AVP (10^{-8} M) (Figure 4a & 5). This was indicative of a functional endothelial layer, capable of releasing endothelium-derived relaxing factors. When the vessel was made hypoxic (PO_2 mm Hg) a contraction was produced which was relaxed upon restoration of normoxia (PO_2 = 600 mm Hg) (Figure 4b.). Removal of the endothelium by rubbing or with the use of the detergent, Triton X 100, had no significant effect on contractions elicited by 5-HT or KCl (Table 1). However, endothelium-dependent relaxations produced by ACh and AVP, were either abolished or converted to small contractions (Figure 4c & 5). The relaxations were 31.3 ± 2.8% for ACh and 67.7 ± 9.2% for AVP in the presence of a functional endothelium and -1.03 ± 1.63% and -9.5 ± 4.6% respectively in the absence of endothelium (Table 2)

Contractions elicited by hypoxia were still present after endothelium removal but were partially reduced in size (Figure 6 & Table 1). The absence of endothelium was confirmed pharmacologically (Figure 4b) and with the use of Scanning Electron Microscopy (Figure 7).

Effect of sodium-free medium on hypoxic contraction

The response of the canine basilar artery to hypoxia in the presence and absence of sodium was determined in a series of 9 dogs. Hypoxia produced a contraction of 0.71 ± 0.10 g (n= 15) in normal Tris buffer, while the response in Na^+ -free (sucrose) buffer was 0 g (n= 15). After

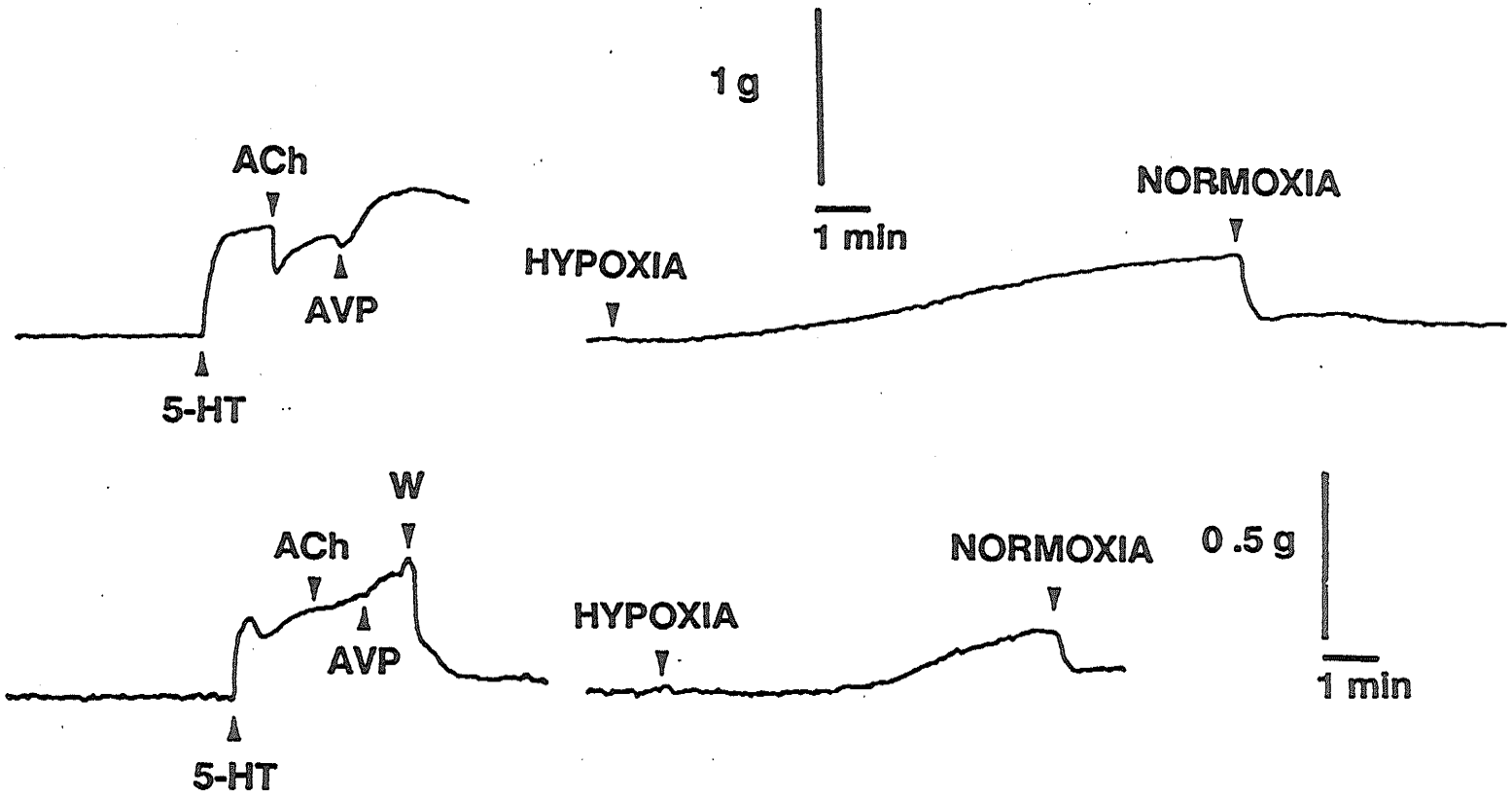

1 g
ACh
AVP
5-HT
1 min
HYPOXIA
NORMOXIA
W
ACh
AVP
5-HT
HYPOXIA
NORMOXIA
0.5 g
1 min

Figure 4.

Hypoxic increase in isometric tension tracing from a canine basilar artery ring in the presence or absence of endothelium.

Top Trace: Contraction due to 5-HT (10^{-7} M). Subsequent relaxation by ACh ($5x10^{-5}$ M) and AVP (10^{-8} M) indicates presence of functional endothelium. 10 min of hypoxia caused a contraction which was reversed by normoxia.

Bottom Trace: Same tissue as above after intraluminal exposure to 0.1% Triton X 100 for 1 min. ACh and AVP relaxations were converted to contractions, indicating absence of functional endothelium. Hypoxic contraction is present after endothelium removal.

Hypoxia-induced contraction appears to be indepedent of the endothelium.

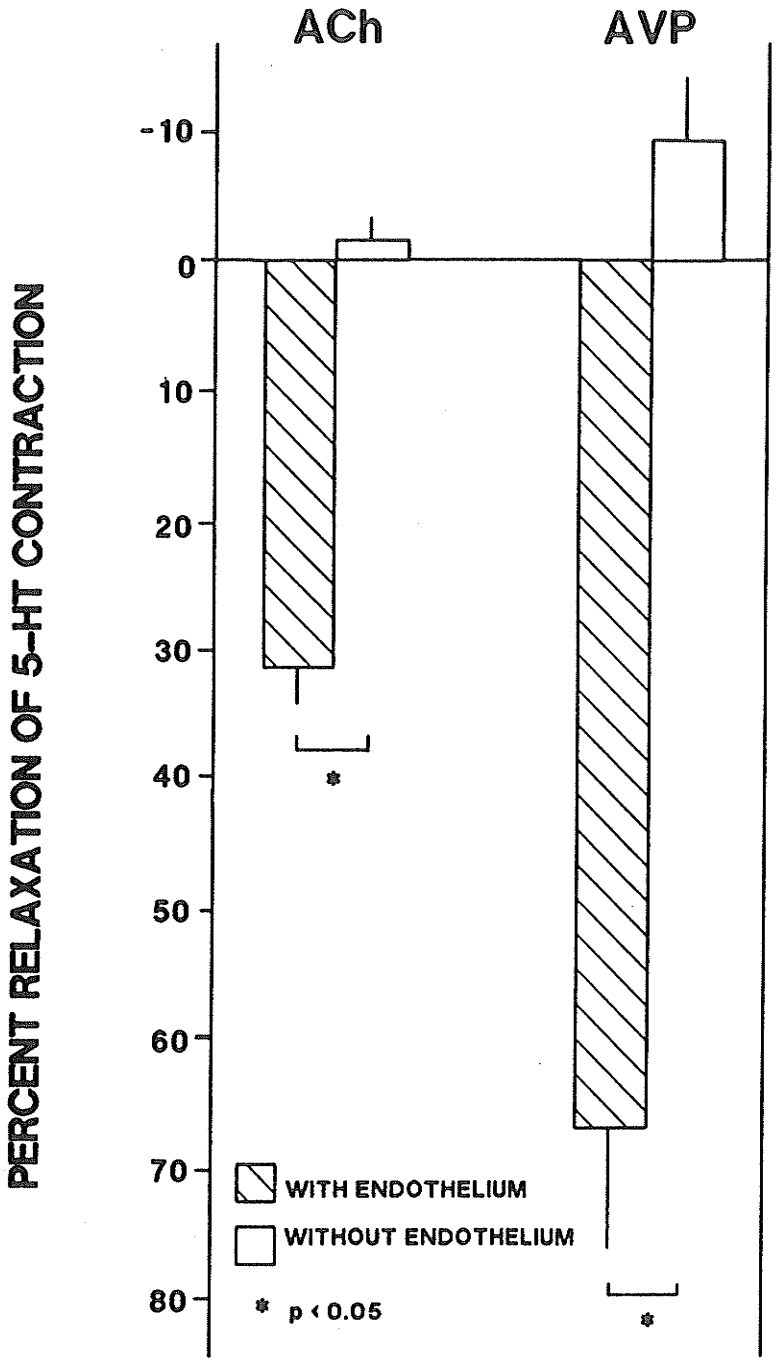

ACh
AVP
PERCENT RELAXATION OF 5-HT CONTRACTION
-10
0
10
20
30
40
50
60
70
80
WITH ENDOTHELIUM
WITHOUT ENDOTHELIUM
* p < 0.05
*
*

Figure 5.

Plot of percent relaxation for ACh and AVP in the presence and absence of endothelium.

The relaxations of 5-HT-induced (10^{-7} M) contractions were produced by ACh ($5x10^{-5}$ M) and AVP (10^{-8} M) in the presence and the absence of endothelium.

The bars represent means $\pm$ S.E.M. of 5-10 experiments. The asterisk represents statistical significance at $p \leq 0.05$. Details in Table 2.

Table 1. Effect of Presence or Absence of Endothelium on Tension Increase due to Hypoxia, KCL or 5-HT

	Developed Isometric Tension (g)								
	HYPOXIA			KCL (100 mM)			5-HT (0.1 uM)		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
+ Endothelium	0.73	0.11	8	0.95	0.36	3	0.76	0.10	6
- Endothelium	0.30 *	0.06	8	0.66	0.14	3	0.54	0.13	6

* $p < 0.05$

Table 2. Effect of Presence or Absence of Endothelium on Acetylcholine (ACh) or Arginine Vasopressin (AVP)-induced Relaxation

	Percent Change in Active Tension with					
	ACh (50 uM)			AVP (0.01 uM)		
	Mean	SEM	n	Mean	SEM	n
+ Endothelium	-31.34	2.81	10	-67.65	9.18	6
- Endothelium	1.03 *	1.63	10	9.46 *	4.63	5

* $p < 0.05$

washout of sucrose buffer and equilibration in normal Tris the response to hypoxia returned 0.62 ± 0.12 g, (n= 10). The responses to KCl (10^{-1} M) and 5-HT ($2.5x10^{-7}$ M) were also determined. The contraction produced by KCl was 1.35 ± 0.23 g (n= 7) in normal Tris, and 0.68 ± 0.14 g (n= 7) in sucrose buffer, and 0.98 ± 0.17 g (n= 3) after washout of sucrose buffer. 5-HT produced a contraction of 0.87 ± 0.17 g (n= 6) in normal Tris, and 0.11 ± 0.03 g (n= 8) in sucrose buffer, and 1.0 ± 0.14 g (n= 2) after washout of sucrose buffer. This is summarized in Figure 8.

Effect of 4-AP on hypoxic contraction

The response of the canine basilar artery to hypoxia in the presence and absence of 4-AP ($3x10^{-3}$ M) was determined in 14 dogs. Without 4-AP present hypoxia produced a contraction of 0.17 ± 0.03 g (n= 8), and with 4-AP present the contraction was 0.42 ± 0.05 g (n= 8).

Effect of BAY K 8644 on hypoxic contraction

The response to hypoxia before, during, and after exposure to a subthreshold dose of the calcium channel agonist BAY K 8644 ($5x10^{-11}$ M) was determined in 5 dogs. Treatment with BAY caused an augmentation of hypoxic contraction when compared to control (Figure 9). Before BAY treatment, hypoxia produced a contraction of 0.08 ± 0.02 g (n= 5). In the presence of BAY the contraction increased to 0.11 ± 0.03 g (n= 5). When compared to control and depicted as percent of control contraction the augmentation produced by BAY treatment was significant.

Inhibition of lipoxygenase and the effect on hypoxic contraction

There is much evidence to support the contention that the leukotrienes and other eicosanoids may play a role in cerebral ischemia and cerebral hypoxia (see Introduction). Our

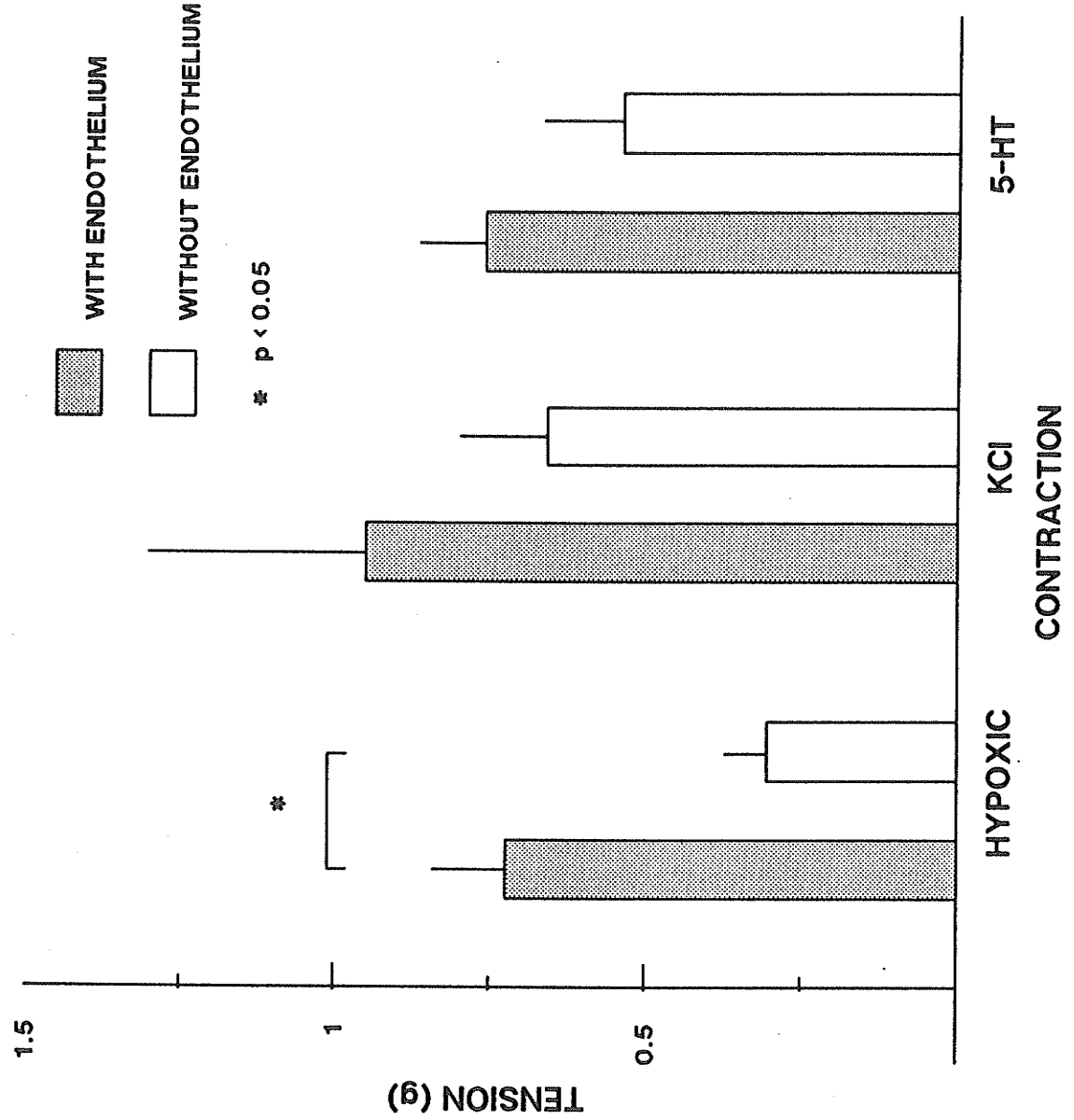
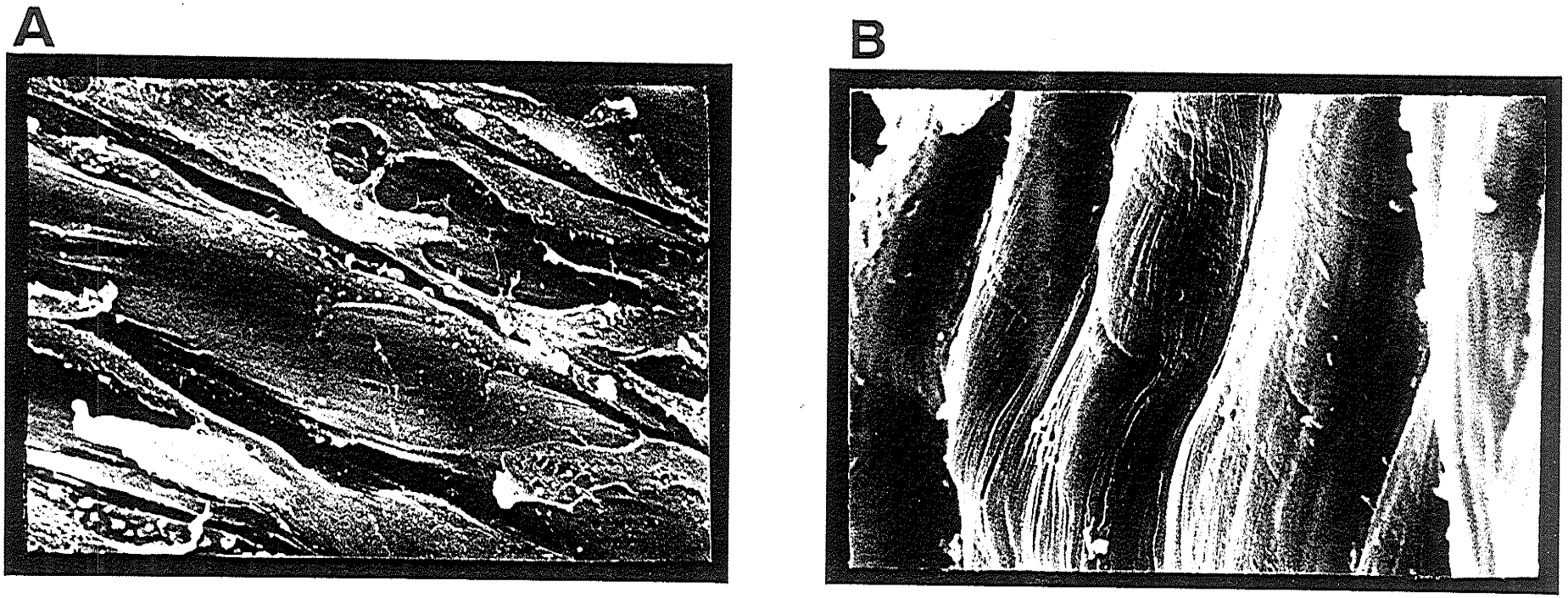

WITH ENDOTHELIUM
WITHOUT ENDOTHELIUM
* p < 0.05
TENSION (g)
1.5
1
0.5
*
HYPOXIC
KCl
5-HT
CONTRACTION

Figure 6.

A representative figure of Table 1. This plot of tension generated by hypoxia, KCl (10^{-1} M), and 5-HT (10^{-7} M) in the presence and absence of endothelium is a summary of experiments in 8 dogs. The asterisk indicates significance at $p \leq 0.05$.


A
B
10 μm

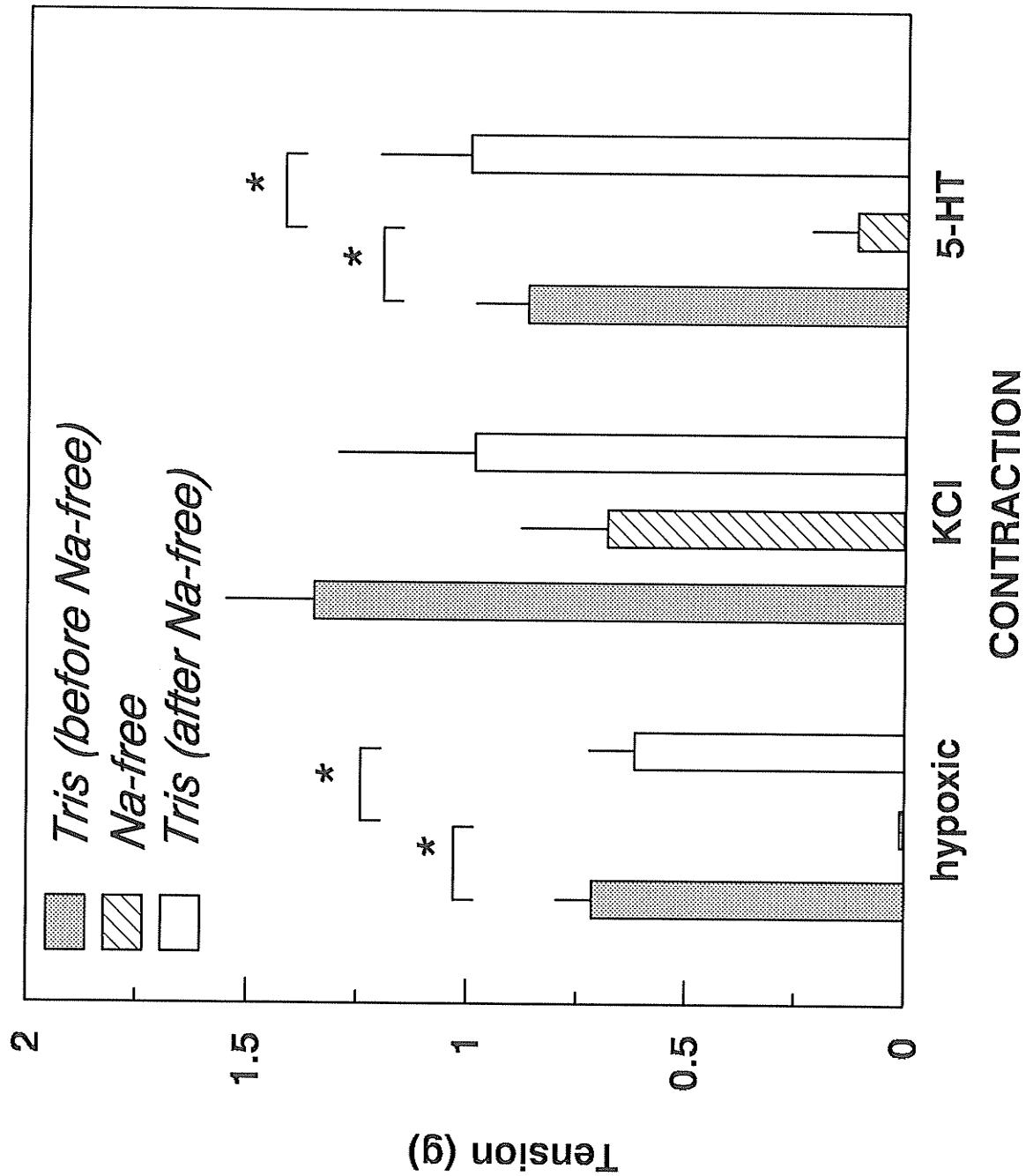

Tris (before Na-free)
Na-free
Tris (after Na-free)
Tension (g)
2
1.5
1
0.5
0
*
*
*
*
hypoxic
KCl
5-HT
CONTRACTION

Figure 8.

Plot of tension generated by hypoxia, KCl, and 5-HT in normal and sodium-free medium. The effects of the 3 agents were recorded in normal Tris buffer followed by that in Na^+-free Tris buffer. This was followed by washout of Na^+-free medium. The hypoxic contractions were 0.71 $\pm$ 0.1 g (n=15), 0 g (n=15), and 0.62 $\pm$ 0.12 g (n=15) for control, sodium-free and washout control mediums respectively. Contractions elicited by KCl (10^{-1} M), and 5-HT (10^{-7} M) were also compared in the 2 media to determine the specificity of the action. The KCl contractions were not significantly affected by sodium-free medium while those to 5-HT were. The bars represent mean values $\pm$ S.E.M. of 15-20 experiments with * indicating significance at $p \leq 0.05$.

HYPOXIC CONTRACTION (% CONTROL)
160
140
120
100
80
60
40
20
0
+ BAY K 8644 (0.05 nM)
CONTROL
+ BAY K 8644 (0.05 nM)
CONTROL
* p < 0.05
n = 5
.16
.14
.12
.1
.08
.06
.04
.02
0
HYPOXIC CONTRACTION (g)

Figure 9.

Plot of percent control hypoxic contraction with and without the presence of BAY K 8644. Pretreatment with BAY K 8644 ($5x10^{-11}$ M) caused augmentation of hypoxic contraction. The bars represent mean values $\pm$ S.E.M. for 5 dogs with * indicating significance at $p \leq 0.05$.

intent in this study was to try and elucidate the role of lipoxygenase products in hypoxia-induced contraction in the canine isolated basilar artery.

Hypoxia produced a contraction in the vessel of 0.32 $\pm$ 0.078 g (n= 4) and pretreatment of the vessel segment with the antioxidant and lipoxygenase inhibitor NDGA ($5x10^{-6}$ M) reduced this contraction to -0.011 $\pm$ 0.011 g (n= 4; Table 3). Application of NDGA at the peak of hypoxic contraction completely relaxed the vessel in 2 preparations. Pretreatment with the preferential lipoxygenase inhibitor ETYA ($2.5x10^{-6}$- $5x10^{-6}$ M) significantly blocked the response to hypoxia (Figure 10) and application post-hypoxia completely reversed the contraction. Contractions elicited by KCl (10^{-1}- $2.5x10^{-2}$ M) were not significantly affected by NDGA (Fig. 11) or ETYA. Hypoxia augments contractions elicited by low KCl ($2.5x10^{-2}$ M, Figure 11) and this potentiation is not significantly affected by concentrations of NDGA or ETYA that block the response to hypoxia alone. The combined KCl/hypoxic contraction was 1.057 $\pm$ 0.34 g (n= 6) and 0.625 $\pm$ 0.09 g (n= 6) with and without NDGA respectively. These results suggest that the response to hypoxia in the canine isolated basilar artery is a multi-faceted event involving not only direct membrane stimulation, but, also products of the lipoxygenase pathway.

Effect of hydroquinone on hypoxic contraction

Work by Furchgott and others has shown that endothelial function and endothelium-dependent dilatation of vascular smooth muscle is inhibited by hydroquinone (HQ). Our purpose in this study was to determine if hypoxia-induced contractions in the canine isolated basilar artery were dependent on the endothelial layer and if HQ treatment could prevent or alter this response.

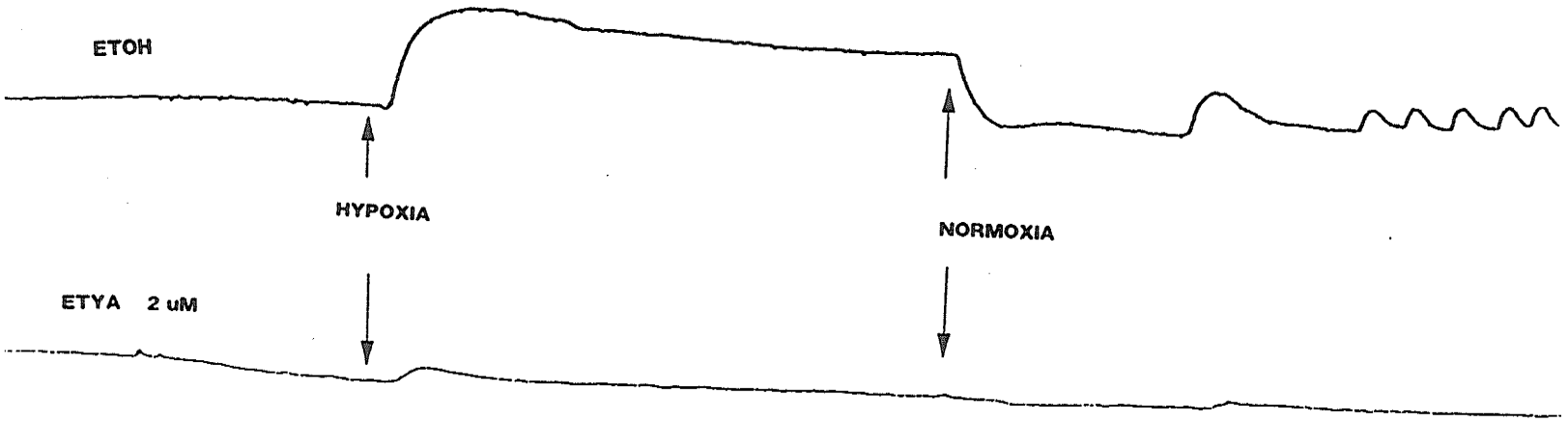

ETOH
HYPOXIA
NORMOXIA
ETYA 2 uM

Figure 10.

Typical basilar artery contractile response to hypoxia in the absence and presence of ETYA. Pretreatment with the specific lipoxygenase inhibitor ETYA ($2x10^{-6}$ M) significantly blocked the response to hypoxia.

Table 3. Hypoxic Contraction and the effect of NDGA (5×10^{-6} M)

	MEAN	SEM	n
HYPOXIC	0.32	0.07	4
HYPOXIC & NDGA	-0.01*	0.01	4

* $p = 0.019$

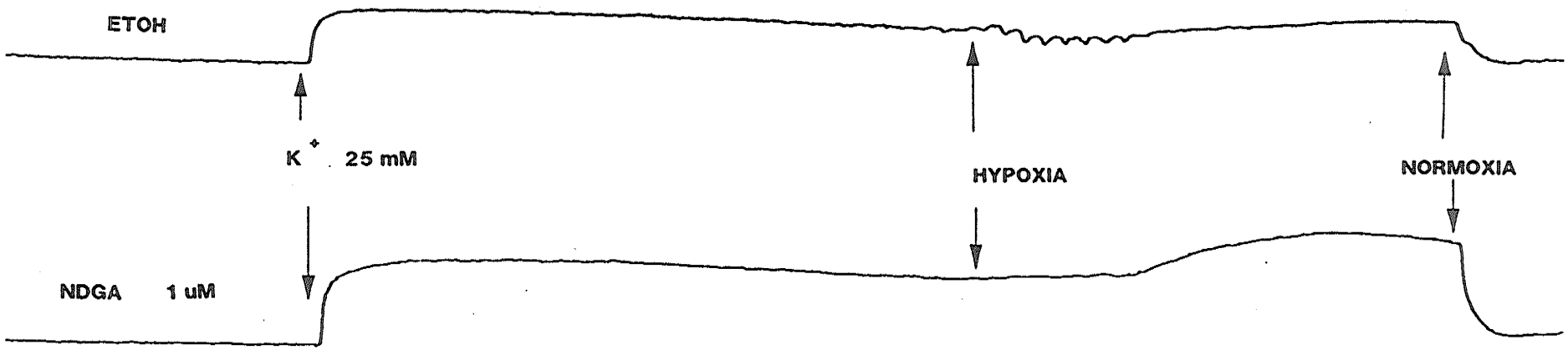

ETOH
K+ 25 mM
HYPOXIA
NORMOXIA
NDGA 1 uM

Figure 11.

Typical basilar artery response to hypoxia and KCl with and without NDGA. Hypoxia augments contractions elicited by low KCl (2.5×10^{-2} M) and this potentiation was not significantly affected by concentrations of NDGA (10^{-6} M) that block the response to hypoxia alone. The combined KCl/hypoxic contraction was 1.057 $\pm$ 0.34 g (n=6) and 0.625 $\pm$ 0.09 g (n=6) with and without NDGA respectively.

Pretreatment of basilar artery rings with hydroquinone ($4x10^{-5}$ M) significantly attenuated contraction due to hypoxia or red blood cell hemolysate without affecting those due to KCl or 5-HT (Figure 12). The mean contraction sizes were 0.69 $\pm$ 0.04 g, 1.23 $\pm$ 0.16 g, 0.51 $\pm$ 0.06 g, and 0.39 $\pm$ 0.05 g for hypoxia, KCl, 5-HT, and haemoglobin respectively before HQ (Table 4). In the presence of HQ the contractions were 0.01 $\pm$ 0.01 g, 1.3 $\pm$ 0.27 g, 1.12 $\pm$ 0.21 g, and 0.01 $\pm$ 0.01 g respectively. HQ potentiated the contractile effects of 5-HT, but this finding was not further investigated in this study. In vessel segments devoid of endothelium, i.e., no relaxation in response to ACh or AVP, the hypoxia-induced contraction was still effectively blocked by pretreatment with HQ. The fact that HQ attenuated endothelium-independent haemoglobin-induced contractions as effectively as those produced by hypoxia in both endothelium-intact and endothelium-devoid preparations, suggests that HQ may have a direct smooth muscle locus of action in addition to its more well known effect on the endothelium.

Hypoxic contraction and the effect of adenosine

The hypoxic/anoxic-induced release of adenosine and the concomitant vasorelaxant properties in the cerebral vasculature are well documented (see Introduction). In this series of experiments we attempted to determine the effect of exogenously applied adenosine on hypoxic-induced contraction in canine isolated basilar artery.

Canine basilar artery rings contracted by hypoxia were completely relaxed by exposure to adenosine ($5x10^{-6}$ M, Figure 13). This relaxation was striking and actually proceeded well past the initial resting tone of the vessel segment, indicative of the vasorelaxant properties of adenosine. Pretreatment with adenosine also attenuated hypoxic contraction in a dose-dependent manner. Adenosine ($5x10^{-6}$ M) decreased the hypoxia-induced contraction by 92% (n= 6, Figure 14). The experimental points were fitted by a 4th order polynomial regression.

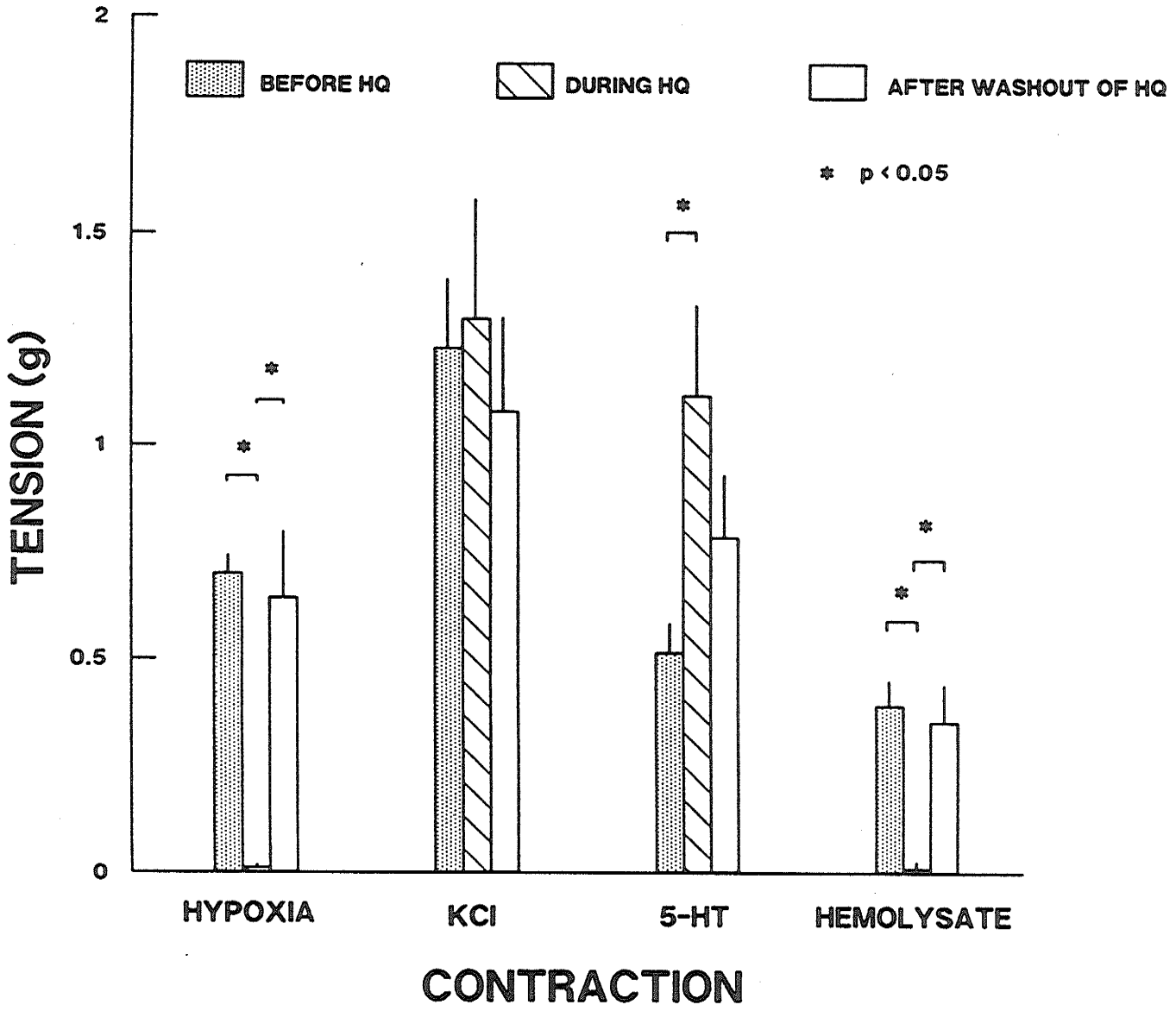

BEFORE HQ
DURING HQ
AFTER WASHOUT OF HQ
* p < 0.05
TENSION (g)
2
1.5
1
0.5
0
HYPOXIA
KCl
5-HT
HEMOLYSATE
CONTRACTION

Figure 12.

Plot of isometric contraction with hypoxia, KCl, 5-HT, and haemoglobin in the absence and presence of hydroquinone. The contraction elicited by hypoxia and haemoglobin ($2x10^{-6}$ M) were completely abolished by pretreatment with hydroquinone ($4x10^{-5}$ M) while those to KCl (10^{-1} M) were unaffected and 5-HT (10^{-7} M) were augmented. The bars represent means $\pm$ S.E.M. with * indicating significance at $p \leq 0.05$. This plot is summarized in Table 4.

Table 4. Effect of Hydroquinone (HQ; 50 uM) on Contraction due to Hypoxia, KCL, 5-HT or Hemoglobin

	Developed Isometric Tension (g)											
	HYPOXIA			KCL (100 mM)			5-HT (0.1 uM)			HEMOLYSATE (0.2 uM hemoglobin)		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
Before HQ	0.69	0.04	7	1.23	0.16	7	0.51	0.06	4	0.39	0.05	6
During HQ	0.01*	0.01	7	1.30	0.27	4	1.12*	0.21	3	0.01*	0.01	7
After Wash	0.64	0.15	5	1.08	0.21	6	0.78	0.14	4	0.35	0.08	4

* $p < 0.05$

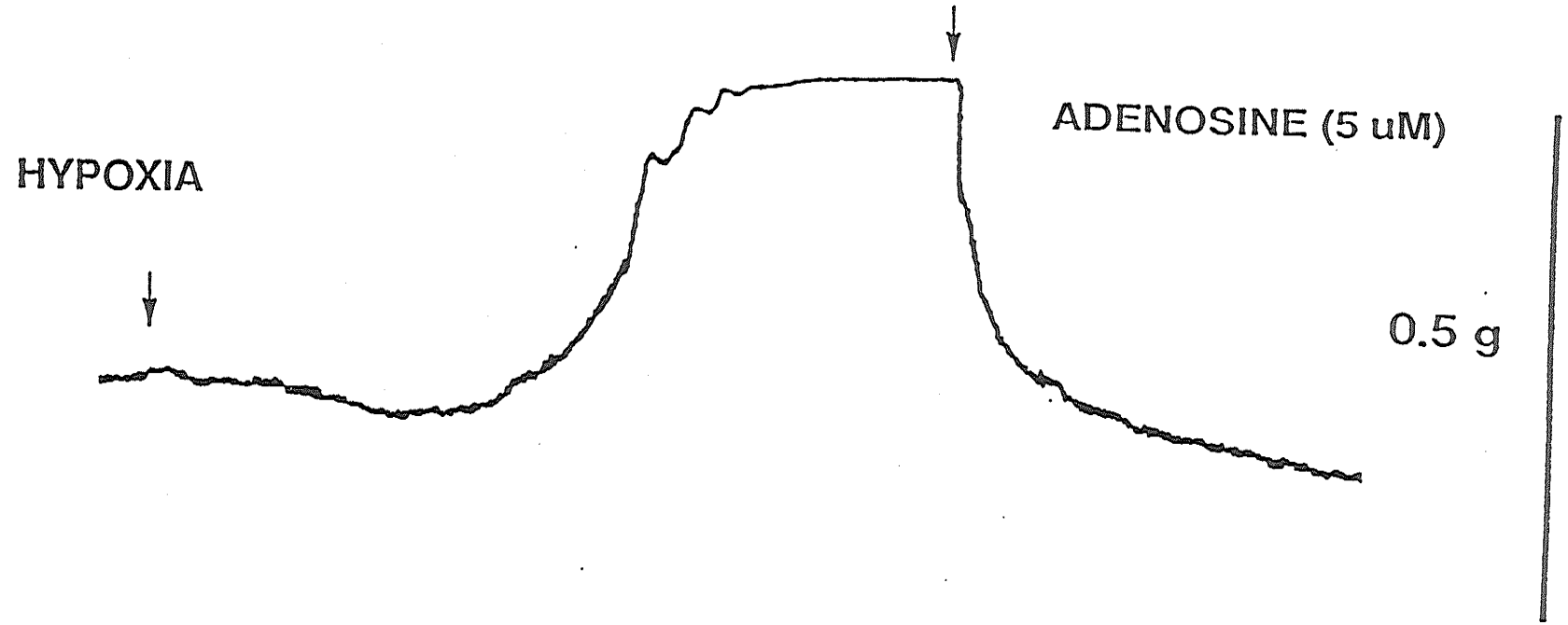

HYPOXIA
ADENOSINE (5 uM)
0.5 g

Figure 13.

Typical response of basilar artery to exposure to adenosine at the peak of hypoxic contraction. Hypoxic contraction is completely relaxed upon addition of adenosine ($5x10^{-6}$ M) to the bath. This is typical of 5 such experiments.

PERCENT OF CONTROL ACTIVE TENSION
160
140
120
100
80
60
40
20
0
0
0.1
1
10
ADENOSINE CONCENTRATION (uM)

Figure 14.

Plot of percent control tension in the basilar artery versus adenosine concentration. The inhibitory effect on hypoxic contraction is seen. Pretreatment with adenosine (1.25×10^{-7}- 10^{-5} M) produced dose-dependent effects on hypoxic contraction in 8 dogs. Low dose adenosine (1.25×10^{-7} M) caused a 27 $\pm$ 30% augmentation of hypoxic contraction while high dose (10^{-5} M) attenuated contraction by 93 $\pm$ 3%. Each point on the plot represents the mean $\pm$ S.E.M. indicated by error bars. The points were fitted by 4th order polynomial regression.

For the pre-hypoxic effect of adenosine, the area under the resulting contraction curve was measured.

Effect of 4-AP on ACh-induced relaxation

Figure 15 -1a depicts a typical example of a canine basilar artery ring contracted with 5-HT and relaxed with subsequent addition of ACh. Pretreatment of the rings with 4-AP ($3x10^{-3}$ M) caused a variable augmentation of 91.5 $\pm$ 16.3% (n= 7) of the contractile response to 5-HT (10^{-7}- $5x10^{-7}$ M) and abolished the relaxation due to ACh (Figure 15 1b). ACh in concentrations of $2.5x10^{-5}$, $5x10^{-5}$,and 10^{-4} M elicited relaxations of 25 $\pm$ 5.7% (n= 4), 35.6 $\pm$ 3.2% (n= 5), and 33.6 $\pm$ 4.0% (n= 5), respectively in the absence of 4-AP, while the same concentrations caused relaxations of 0% (n= 4), 0% (n= 5), and 1.12% $\pm$ 0.7% (n= 5), respectively with 4-AP present (Figure 16).

Effect of 4-AP on electrogenic sodium pump-mediated relaxation

Basilar artery rings exposed to K^+ -free medium exhibited a transient contraction with subsequent relaxation to baseline. Such a preparation contracted in response to 5-HT (2.5 x 10^{-7} M). Upon addition of K^+ to the vessel in K^+ -free medium, which makes the sodium pump electrogenic (Bose & Innes, 1973), there was a prompt relaxation (Figure 15-2a.). This was not affected by the presence of 4-aminopyridine (Figure 15-2b.). Potassium ($1.5x10^{-3}$ & $4.5x10^{-3}$ M) produced relaxations of 53.8 $\pm$ 4.5% (n= 6) and 98 $\pm$ 1.8% (n= 5) respectively without 4-AP present and 62 $\pm$ 4.8% (n= 6) and 98.4 $\pm$ 1.4% (n= 5) respectively with 4-AP present (Figure 16).

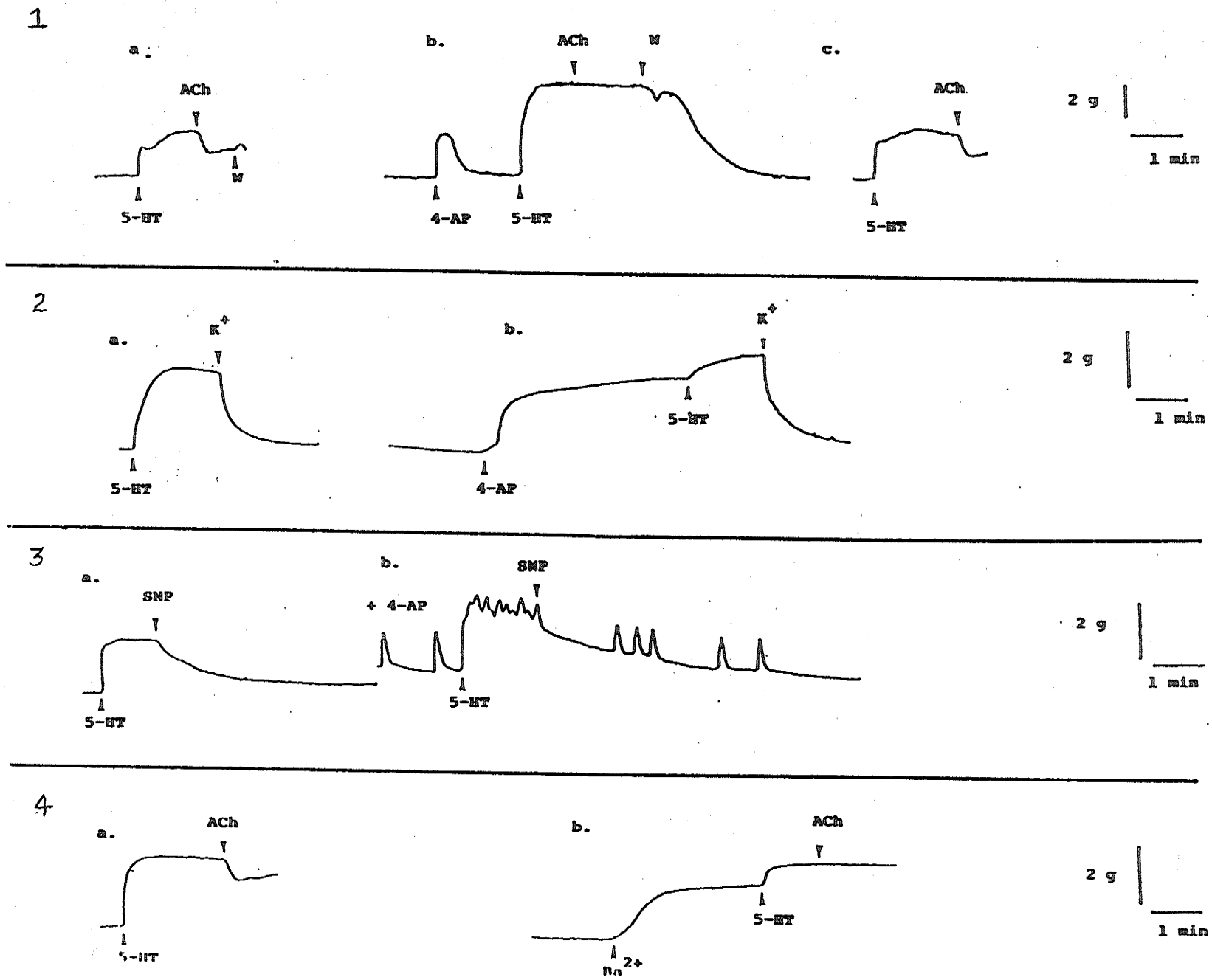

1
a.
ACh
W
5-HT
b.
ACh
W
4-AP
5-HT
c.
ACh
5-HT
2 g
1 min
2
a.
K+
5-HT
b.
K+
5-HT
4-AP
2 g
1 min
3
a.
SNP
5-HT
b.
+ 4-AP
SNP
5-HT
2 g
1 min
4
a.
ACh
5-HT
b.
ACh
5-HT
Mn2+
2 g
1 min

Figure 15.

Typical contractile responses of basilar artery to various relaxants in the absence and presence of 4-AP. Effect of 4-aminopyridine on ACh-, K^+-, and SNP-induced relaxation of 5-HT-induced contraction of canine basilar artery.

1a. Basilar artery ring precontracted with 5-HT (2.5×10^{-7} M) is relaxed by addition of ACh (5×10^{-5} M).

1b. Previous vessel after exposure to 4-AP (3×10^{-3} M) contracts to 5-HT but now relaxation to ACh is abolished.

1c. Same vessel now relaxes upon exposure to ACh after washout of 4-AP.

2a. Canine basilar artery ring, in K^+-free medium, precontracted by 5-HT (2.5×10^{-7} M) is relaxed by addition of K^+ (4.5×10^{-3} M) to the bath.

2b. Previous vessel pretreated with 4-AP (3×10^{-3} M) still displays relaxation produced by K^+.

3a. Canine basilar artery ring precontracted with 5-HT (2.5×10^{-7} M) is relaxed of SNP (10^{-7} M).

3b. Previous vessel pretreated with 4-AP ($3x10^{-3}$ M) still displays relaxation after addition of SNP. The rhythmicity observed in this vessel is due to 4-AP.

3c. Same vessel after washout of 4-AP displays unaltered SNP-induced relaxation.

4. Effect of Ba^{2+} pretreatment on ACh-induced relaxation of a vessel precontracted with 5-HT.

4a. Canine basilar artery ring precontracted with 5-HT ($2.5x10^{-7}$ M) is relaxed by the addition of ACh ($5x10^{-5}$ M).

4b. Previous vessel pretreated with Ba^{2+} (10^{-3} M) displays an increase in tension. Contraction produced by 5-HT is not relaxed by addition of ACh in the presence of Ba^{2+}.

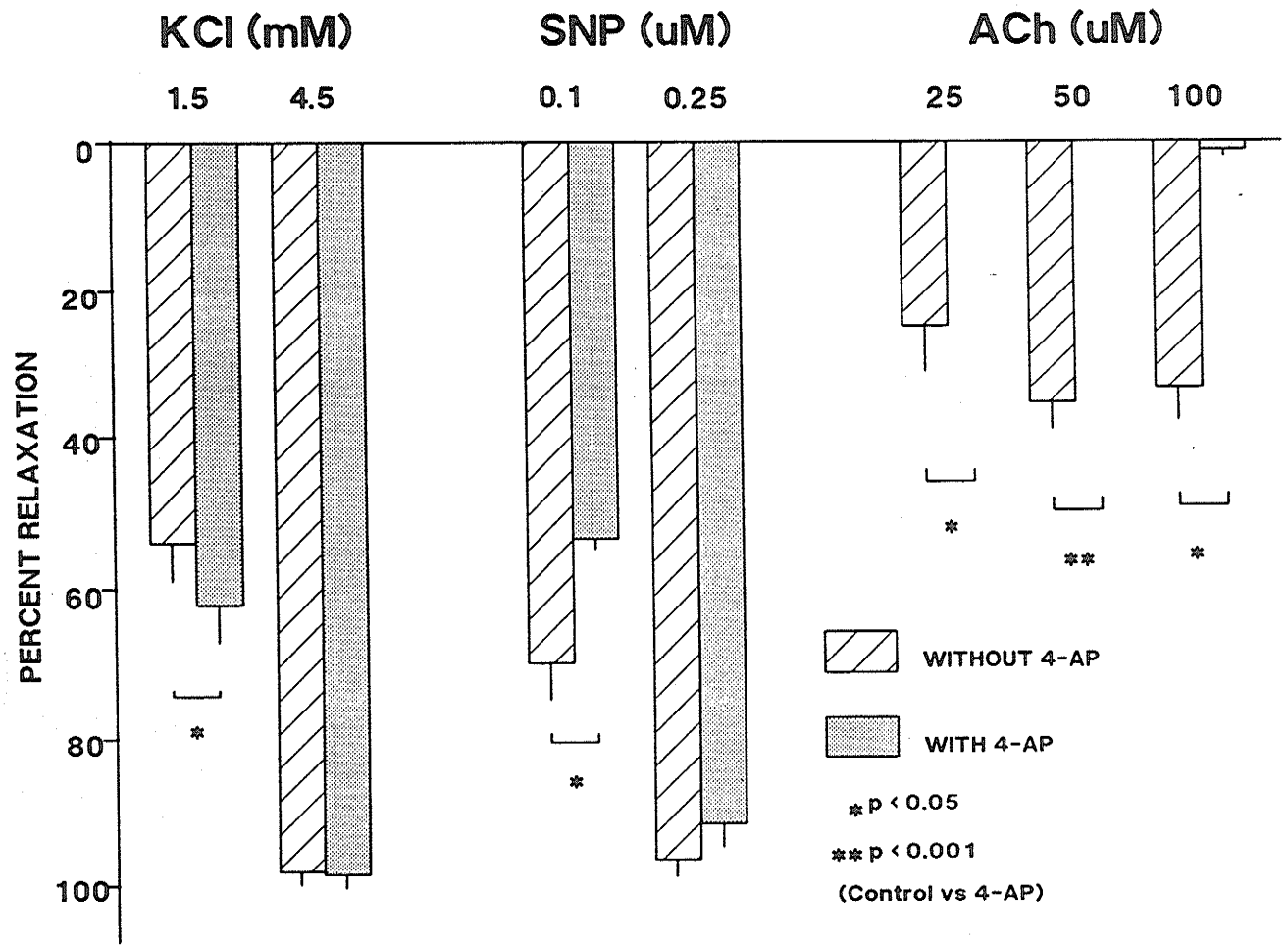

KCl (mM)
SNP (uM)
ACh (uM)
1.5
4.5
0.1
0.25
25
50
100
PERCENT RELAXATION
0
20
40
60
80
100
WITHOUT 4-AP
WITH 4-AP
* p < 0.05
** p < 0.001
(Control vs 4-AP)

Figure 16.

Plot of percent relaxation with KCl, SNP, and ACh versus dose with or without 4-AP. The effect of 4-AP was determined on relaxations elicited by various concentrations of KCl ($1.5x10^{-3}$ & $4.5x10^{-3}$ M), SNP (10^{-7} & $2.5x10^{-7}$ M) and ACh ($2.5x10^{-5}$, $5x10^{-5}$, 10^{-4} M).

The bars represent means $\pm$ S.E.M. with * indicating significance at $p \leq 0.05$, ** indicating $p \leq 0.01$, and *** indicating $p \leq 0.001$.

Effect of 4-AP on sodium nitroprusside-induced relaxation

Sodium nitroprusside (SNP) caused relaxation of 5-HT-contracted rings in a dose dependent manner (Figure 15-3a). This relaxation time was variable, from animal to animal and from treatment to treatment in the same preparation. Therefore time was allowed in each case for the vessel to relax fully (back to baseline) or to plateau to a new baseline. Concentrations of 10^{-7} M, and $2.5x10^{-7}$ M SNP produced relaxations of 69.8 $\pm$ 4.5% (n= 6) and 96.4% $\pm$ 2.2% (n= 7) respectively. After pretreatment with 4-AP ($3x10^{-3}$ M) the same concentrations produced relaxations of 53.5 $\pm$ 1.3% (n= 6) and 91.6 $\pm$ 3.1% (n= 7) respectively (Figure 16). The amount of relaxation obtained with the higher concentration of nitroprusside was not significantly affected by pretreatment with 4-aminopyridine.

Effect of Ba^{2+} on ACh-induced relaxation

Pretreatment of basilar artery rings with Ba^{2+} (10^{-3} M) produced a sustained contraction. Arterial rings precontracted with 5-HT relaxed upon exposure to ACh in the absence of Ba^{2+} (Figure 15-4a) but did not relax with Ba^{2+} present (Figure 15-4b). ACh concentrations of $5x10^{-5}$ and 10^{-4} M produced relaxations of 55.2 $\pm$ 5.0% (n= 5) and 35.6 $\pm$ 3.8% (n= 5) respectively in the absence of Ba^{2+}, and 0% (n= 5), and 0% (n= 5) respectively with Ba^{2+} present.

DISCUSSION

The *in vivo* response of the cerebral blood vessels to hypoxia is vasodilation (Raper *et al.*, 1971; Jones *et al.*, 1978, 1981; Busija and Heistad, 1981) which is thought to be due to local release of adenosine (Nordstrom *et al.*, 1977; Winn *et al.*, 1979, 1981; Phillis *et al.*, 1987). Other parts of the vascular tree react to hypoxic conditions with either contraction or dilation, depending on whether the preparation is *in vivo* or *in vitro*. Isolated cerebral vessels (Katusic & Vanhoutte, 1986; Mallick *et al.*, 1987; Nakagomi *et al.*, 1987) as well as some isolated cardiac vessels (Van Nueten *et al*, 1980; Detar & Bohr, 1972) and all pulmonary vessels (Lloyd, 1970; Harder, 1985a, 1985b) exhibit contractions when made hypoxic. These responses are calcium-dependent and reversible. The endothelium, which regulates many vasoactive responses of blood vessels (Furchgott, 1983), has been suggested as a requirement in the response to hypoxia of canine isolated basilar artery (Katusic & Vanhoutte, 1986) and canine coronary artery (Rubanyi & Vanhoutte, 1985). Other evidence suggests that it plays only a minor role at most in the response to hypoxia in canine isolated basilar artery (Nakagomi *et al.*, 1987) and canine coronary artery (Rimele & Vanhoutte, 1986).

In our experiments involving endothelium removal and the effect on hypoxic contraction, it is clearly evident that the hypoxic contraction still persisted after the 5-HT-contracted vessel segments failed to relax in the presence of ACh and/or AVP. This direct pharmacological evidence of a non-functional or absent endothelium was supported by electron micrographs which showed an unmistakeable difference between vessel segments with and without endothelium. Therefore, in the canine isolated basilar artery hypoxia produces a contraction which can be elicited even when the endothelium of the vessel is absent.

Our results indicate that the endothelium plays only a minor role in the response of canine isolated basilar artery to hypoxia, and that this response is regulated by at least 2 other factors. These factors include direct membrane depolarization and stimulation via mediators of the lipoxygenase pathway.

The dependency of the hypoxic contraction on extracellular calcium influx is well documented. However, exactly how this calcium enters the cell is unclear. Findings by Nakagomi *et al.*, (1987) depicting the block of hypoxic augmentation of KCl contractions by nicardipine indicate hypoxia facilitates the entry of calcium through voltage-gated calcium channels. In experiments with the calcium channel agonist BAY K 8644 we have demonstrated that contractions elicited by hypoxia in the canine isolated basilar artery are significantly potentiated by subthreshold doses of BAY K which have been shown to promote the open mode of the voltage-gated calcium channel (Hess *et al.*, 1984).

The suggestion that hypoxia causes membrane depolarization is supported by the findings of Harder (1985a,b) and by the findings of our experiments involving sodium-free buffers. We demonstrated that withdrawal of sodium from the buffer bathing the tissue, prevented hypoxic contractions completely, and that this was reversed by readdition of the physiological concentration of sodium. Therefore, hypoxia causes, and the resulting contraction is dependent on, sodium influx and resultant membrane depolarization in canine isolated basilar artery rings. Studies by us also demonstrate that hypoxic contractions are augmented by agents which partially depolarize the membrane, such as 4-aminopyridine, and submaximal doses of KCl.

In an effort to better understand the mechanism of hypoxic contraction we endevoured to discover the agents that could attenuate or block this response. A number of diverse receptor blockers had no effect on the contraction and some manipulations even potentiated this effect.

In our search we discovered 3 agents which were effective in blocking hypoxic contraction in the canine isolated basilar artery. The first of these was hydroquinone, which is reported to be a free-radical scavenger, and the other 2 were agents proposed to inhibit the lipoxygenase enzymes; nordihydroguiaretic acid (NDGA) and 5,8,11,14-eicosatetraynoic acid (ETYA).

Hydroquinone is generally used as a tool to block endothelium-mediated relaxations. Its mechanism is believed to be either a disruption of endothelial integrity (Furchgott, 1984), or production of free radicals leading to the breakdown of an endothelial factor (Moncada *et al.*, 1986). Superoxide dismutase or hydroquinone modify endothelium-mediated responses (Moncada *et al.*, 1986; Rubanyi & Vanhoutte, 1986; Furchgott, 1981). The effect of hydroquinone becomes irreversible upon longer exposure (Furchgott, 1981). In our experiments, hydroquinone was able to reversibly block hypoxic contraction in normal as well as endothelium-denuded preparations of the canine basilar artery. This finding is a novel one considering that this is the first indication that hydroquinone causes alterations in contractile properties of vascular smooth muscle. The ability to block the response of the artery to hypoxia without attenuating the response to membrane depolarization (high K^+) or to a vasoactive agent (5-HT) suggests a specific action against the effect(s) of hypoxia.

Work by Rimele & Vanhoutte (1983/4) demonstrated that canine isolated coronary artery rings exhibited hypoxic contractions which were not affected by cyclooxygenase inhibition, but, were blocked by inhibition of the lipoxygenase pathway. Katusic & Vanhoutte (1986) demonstrated that hypoxic contraction in canine isolated basilar artery rings was not affected by cyclooxygenase inhibition, but failed to look at the effect of lipoxygenase inhibition, stating only that inhibition of cyclooxygenase pathway by anoxia diverts liberated arachidonic acid (AA) into the lipoxygenase pathway producing a mediator which somehow potentiates calcium

entry. Hypoxic/anoxic conditions have been demonstrated to increase AA (Rehncrona *et al.*, 1982) as well as leukotrienes (Moskowitz *et al.*, 1984) and increase activity of phospholipase A_2 (Kawaguchi & Yasuda *et al.*, 1988). It is also well demonstrated that leukotrienes cause vasoconstriction (Tagari *et al.*, 1883; Rosenblum, 1985).

We decided to examine the effects of lipoxygenase inhibition with the agents NDGA and ETYA. Our results demonstrate that lipoxygenase inhibition blocks the response of this vessel to hypoxia. It can, therefore, be postulated that hypoxia causes phospholipid breakdown which in turn stimulates lipoxygenase activity and leads to production of a mediator which, may depolarize the membrane, or directly increase calcium influx leading to contraction. In experiments involving partial membrane depolarization and lipoxygenase inhibition, we demonstrated that vessel segments stimulated with submaximal concentrations of KCl, contracted in response to hypoxia whether or not lipoxygenase was inhibited. In other words, once the membrane is depolarized to a critical level, the contraction occurs even if the lipoxygenase-produced mediator is not being liberated, or its effects are being blocked.

The fact that both HQ and NDGA are proposed free-radical scavengers and that both seem to act similarily to block hypoxic contraction, suggests a possible role for HQ as a lipoxygenase inhibitor. This suggestion is supported by evidence from a recent publication which showed that an hydroquinone derivative of a substituted naphthalene, U-66,858 (IC_{50} $1.2x10^{-7}$ $g{\cdot}ml^{-1}$), selectively inhibited 5-lipoxygenase *in vitro* and inhibited pulmonary bronchoconstriction *in vivo* (Johnson & Stout, 1988).

Role of K Channels in Endothelium-mediated Relaxation

We have examined the effect of 4-aminopyridine on endothelium-dependent (ACh-induced) and endothelium-independent (sodium nitroprusside-induced and electrogenic

sodium pump-mediated) relaxations of canine basilar artery. The results of our study clearly show that 4-aminopyridine blocks ACh-induced relaxation without altering the relaxant responses to sodium nitroprusside or to the electrogenic sodium pump. The fact that Ba^{2+} also blocks ACh-induced relaxation supports the notion that blockade of voltage-sensitive K^+ channels is the mechanism of action of 4-aminopyridine.

Recently, Gebremedhin *et al.* (1987) suggested that quinine, tetramethylammonium ion (TMA) and tetraethylammonium ion (TEA) can inhibit the endothelium-dependent relaxations of rabbit aorta by ACh and the calcium ionophore A23187, through blockade of voltage-insensitive Ca^{2+} -activated K^+ channels. This report also indicated that these agents had no effect on endothelium-independent relaxations elicited by sodium nitrite. If an increase in intracellular Ca^{2+} is required for release of EDRF (Zawadzki *et al.*, 1980), then perhaps blockade of these channels might lead to an inhibition of EDRF-mediated relaxation. In contrast, endothelium-dependent relaxation in porcine aorta elicited by bradykinin, ATP, and the calcium ionophore A23187, all induce Ca^{2+} -activated K^+ efflux, whereas that elicited by ACh does not (Gordon & Martin, 1983). 4-aminopyridine is a potent blocker of voltage-sensitive K^+ channels (Meves & Pichon, 1977) and in concentrations of 10^{-4}- $3x10^{-4}$ M, inhibits the voltage-insensitive Ca^{2+} -activated K^+ channels (Bartschat & Blaustein, 1985). However, higher concentrations of 10^{-3}- $2x10^{-3}$ M (Bartschat & Blaustein, 1985), or $5x10^{-3}$ M or greater (Hermann & Gorman, 1981) actually stimulate Ca^{2+} -activated K^+ efflux. Since the concentration of 4-aminopyridine used in our experiments ($3x10^{-3}$ M) is in the range believed to stimulate voltage-insensitive Ca^{2+} -activated K^+ channels and if in the canine basilar artery, as in porcine aorta, ACh-induced relaxations are not linked with Ca^{2+} -activated K^+ efflux, then the attenuation of this relaxation by 4-aminopyridine is different from that caused in rabbit aorta by quinine, TEA, & TMA.

In canine trachealis muscle 4-aminopyridine mimics the agonist-like actions of ACh and the mechanical and electrical responses to 4-aminopyridine are blocked by atropine (Kannan *et al.*, 1983). This suggests that some effects of 4-aminopyridine may be mediated either through a direct muscarinic receptor activation or enhanced ACh release from cholinergic nerves via Ca^{2+} influx (Lundh & Thesleff, 1977). If 4-aminopyridine acts as an agonist at the muscarinic receptor, as in canine trachealis, then it is unlikely that it will also block the action of ACh at its receptor. The antagonism of ACh-induced relaxation may therefore occur at the level of EDRF release or on the action of EDRF on the smooth muscle cell. The former site seems probable because 4-aminopyridine, in the concentrations used, has no effect on nitroprusside and K^+ induced relaxations which do not act through EDRF or the endothelium. An alternate, but less likely, possibility may be that the mechanism of action of EDRF on the vascular smooth muscle is different from those of nitroprusside or the electrogenic sodium pump.

Ba^{2+} has been shown to block voltage-sensitive K^+ channels (Hermann & Gorman, 1979). Since Ba^{2+} also inhibits the ACh-induced relaxations, the most likely explanation for 4-aminopyridine and Ba^{2+} inhibition of ACh-induced relaxation is by blockade of these same voltage-sensitive K^+ channels. Electrophysiological studies of this phenomenon may help to answer this question.

Studies on EDRF-mediated relaxations involving the use of 4-aminopyridine must be reassessed. Relaxations to acetylcholine have been used in many preparations to determine presence of a functional endothelium. Since 4-aminopyridine, or similar agents, are used to increase myogenic tone, the absence of the endothelium may be mistakenly assumed in the presence of these agents.

Proposed Model

From the data collected by us and others, we propose the following model to indicate the chain of events which leads to contraction when the canine isolated basilar artery is made hypoxic.

The resting tone of the vessel is largely passive and, therefore, not totally dependent on calcium flux. During hypoxia breakdown of membrane phospholipids occur. It could be reasoned that the potassium efflux (Borda *et al.*, 1980; Conrad *et al.*, 1979) and subsequent sodium influx seen during hypoxia, leads to a slight influx of calcium through voltage-gated channels, and this trigger calcium activates phospholipase A_2 and leads to generation of fatty acid hydroperoxides such as AA. This causes stimulation of 5-lipoxygenase, and production of some unknown mediator which further increases calcium influx. Perhaps this mediator also partially depolarizes the membrane and this trigger depolarization facilitates the larger depolarization produced at the muscle membrane by hypoxia alone and the combined forces produce a contraction which is not dependent on endothelial factors, but, is dependent on sodium influx and blocked by hydroquinone, lipoxygenase inhibition, and by adenosine. It is possible that the release of adenosine *in vivo* during hypoxia counteracts the effect of this lipoxygenase-produced mediator and masks the constriction with a more powerful dilatation. This would become important when adenosine production is compromised or blocked during an hypoxic episode.

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