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An Intramural, Tension-modulating Reflex in the Rat Caudal Artery

Greg G. Geary

ABSTRACT

AN INTRAMURAL, TENSION-MODULATING REFLEX IN THE RAT CAUDAL ARTERY

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Electrical field stimulation (EFS) of preconstricted arteries causes relaxation. This relaxation is of either neuronal, vascular smooth muscle, and/or endothelial origins. We have shown that EFS-induced relaxation of intact, phenylephrine (PE) preconstricted rat tail artery (RTA) rings was dependent upon the extracellular concentration of Ca^{++}. Inhibiting either nitric oxide (NO) synthesis with N-nitro-L-arginine methyl ester (L-NAME), or the synthesis of cyclic guanosine monophosphate (cGMP) with methylene blue (MB) reduced the EFS-induced relaxation. In addition, inhibition of $K_{Ca}{}^{++}$-dependent hyperpolarization with tetraethylammonium (TEA), or K_{ATP} hyperpolarization with $BaCl_2$ or glibenclamide also reduced EFS-induced relaxation. L-arginine reversed the effect of L-NAME. When either MB and KCl, L-NAME and KCl, or L-NAME and $BaCl_2$ were used, the EFS-induced relaxation was completely inhibited. The EFS-induced relaxation was not inhibited by tetrodotoxin, a voltage-operated Na^+ channel antagonist. EFS-induced relaxation was partially inhibited by endothelium

denuding. The remaining EFS-induced relaxation in the denuded RTA was inhibited by $BaCl_2$ and KCl. EFS-induced relaxation in the intact, KCl preconstricted RTA was inhibited by L-NAME and MB. Effluent from freshly isolated, bovine aortic endothelial cells (BAEC) exposed to EFS relaxed denuded RTA and could be blocked by L-NAME, MB, KCl, and TEA. L-arginine reversed the effect of L-NAME as in the ring experiments. EFS-induced relaxation of intact, pressurized, and PE preconstricted RTA was frequency and voltage-dependent, and inhibited by either L-NAME, $BaCl_2$, or the voltage-operated Ca^{++} channel antagonist diltiazem. As in all the other studies, L-arginine reversed the effect of L-NAME. Membrane potential recordings of EFS-induced relaxation showed a mean membrane hyperpolarization of -20 mV simultaneously with relaxation. A positive correlation was shown to exist between the initial level of tone and the EFS-induced relaxation. It can be concluded that the vascular smooth muscle contains an endogenous hyperpolarization mechanism that regulates initial changes in arterial tone. Higher arterial tension causes the release of NO and another hyperpolarization factor from the endothelium which function to further regulate arterial tone.

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Each person whose signature appears below certified that this dissertation in his opinion is adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy.

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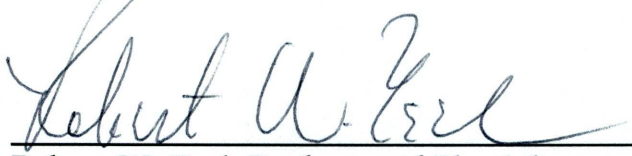

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INTRODUCTION

General Characteristics of Vascular Smooth Muscle

Precapillary vessels within the cardiovascular system are required to distribute the blood in such a way that each capillary is provided with blood in the correct amount and at the correct pressure. This role is accomplished through the ability of the cardiovascular system to adjust the resistance of each vessel. The control of vessel resistance is determined by changes in lumen diameter. Lumen diameter, in turn, is a function of the amount and arrangement of the vascular wall materials and, in particular, of the level of tone in the vascular smooth muscle. Control of lumen diameter is a function of the sympathetic innervation and the vasoactive substances released from the endothelium (Rhodin, 1980; Bevan and Laher, 1991).

The vessels that contribute most to the precapillary resistance are known as resistance arteries. Formerly, it had been assumed that the resistance arteries consisted of the arterioles alone, defined anatomically as being those precapillary vessels with no more than one complete layer of smooth muscle, that is arterioles with diameters less than 30-50 μm. It is now clear that about one-half of the precapillary resistance lies proximal to the arterioles. The vessels concerned are known as small arteries and may be further defined as those vessels proximal to the arterioles with diameters ranging between 100 μm-500 μm (Johnson and Hanson, 1962).

The general structure of arteries and arterioles is similar. Each has a layer of endothelial cells lining the internal surface of the vessel (tunica intima). Beneath the endothelium lies a layer of fine connective tissue (lamina propria) and a thicker internal elastic lamina. These layers are prominent in arteries but less prominent in arterioles. Circumferentially arranged smooth muscle cells make up the media of the vessel (tunica media). In large arteries, the media contains up to 40 layers of smooth muscle cells. Fine distributing arterioles have only a single layer of smooth muscle cells. Arterioles give way to capillaries that are devoid of smooth muscle cells. An outer or adventitial layer (tunica adventitia) that contains collagen fibers wraps around the medial layer of the artery. Varicose sympathetic nerve axons, which innervate arteries, are usually confined to this layer (Rhodin, 1980).

The following sections are a detailed discussion of the anatomical and physiological characteristics of each of these layers.

Tunica Adventitia

The adventitia of the small arteries contains connective tissue, (elastin and collagen), fibroblasts, mast cells, macrophages, and occasional Schwann cells with associated nerve axons. The available evidence suggests that the nerves terminals are all contained within the adventitial layer and do not penetrate the media. The adrenergic nerves within the adventitial

layer consists of axons with varicosities (2 μm long and 1 μm across) interspersed at 3-10 μm intervals. The axons are unmyelinated and surrounded by Schwann cells. In the rat tail artery, sympathetic innervation is extremely dense, containing 40,000 varicosities per mm^2. The minimum distance between the nerve and muscle junction is rarely less than 100 nm. However, reports of varicosity-to-smooth muscle distances of approximately 20 nm support the concept of specialized neuromuscular junctions similar to the neuromuscular junctions of skeletal muscle (Rhodin, 1980; Bao *et al.*, 1993).

Adrenergic Regulation of Vascular Smooth Muscle

The involvement of different mechanisms during sympathetic nerve stimulation is best explained by the results of Cheung (1984). A small brief stimulus, which excited only a portion of the rat tail artery sympathetic nerve, caused a smooth muscle membrane depolarization with two components. A short time after the stimulus (20 ms) a rapid membrane depolarization of 10 mV, lasting for 1 second, was recorded. This depolarization is termed an excitatory junctional potential (EJP). The EJP was followed by a slow depolarization [neurogenic alpha-depolarization (NAD)] of 2 mV that lasted for seconds. Tension recordings indicated that the muscle started to constrict prior to the NAD. The constriction, like the NAD, lasted for many seconds (greater than 30 seconds), although the membrane depolarization far

outlasted constriction. Alpha-adrenoceptor antagonists abolished the slow depolarization and constriction without influencing the EJP. Purinoceptor (P_{2x}) and voltage-operated Ca^{++} channel (VOCC) antagonists inhibited the initial EJP and increased the latency between the onset of stimulus and the beginning of the EJP (Bao *et al.*, 1993).

When more axons were recruited by a slight increase in stimulus strength, the EJP amplitude also increased and triggered a smooth muscle action potential. The NAD was also larger but of insufficient amplitude to initiate an action potential. The tension recorded now had two components: a short-latency, brief component that followed the muscle action potential and a larger component associated with the NAD. Again, only the slow depolarization and slow constriction were inhibited by alpha-antagonists while the EJP and short-latency constriction were inhibited by purinoceptor and VOCC antagonists (Bao *et al.*, 1993).

With a further increase in stimulus strength, the EJP still triggered an action potential and associated constriction but now the NAD was of sufficient magnitude to trigger an action potential. The action potential superimposed on the NAD produced an additional transient tension increase. The EJP action potential and constriction were still sensitive to purinoceptor and VOCC antagonism. However, now the alpha-sensitive

depolarization and constriction were sensitive to both receptor and VOCC antagonists (Bao *et al.*, 1993).

From the work of Cheung (1984) and Bao *et al.* (1993), it can be concluded that sympathetic nerve activation resulted in the following activity: 1) sympathetic nerves release ATP which activates the P_{2x}-purinoceptor on the post-junctional membrane; 2) the resulting EJP does not cause constriction. If sufficient numbers of ATP receptors are activated, the resulting depolarization opens VOCC, which generate an action potential and initiate constriction (electromechanical coupling); 3) the sympathetic nerves also release norepinephrine (NE), which activates alpha-adrenoceptors; 4) the alpha-mediated constriction always precedes the alpha-mediated depolarization. Changes in membrane potential are not necessarily involved in this mode of tension development (pharmacomechanical coupling; described below). Presumably Ca^{++} has been released from intracellular or membrane-bound stores; 5) activation of sufficient alpha-adrenoceptors can produced a threshold depolarization for VOCC. Tension is then produced by Ca^{++} arriving from two separate sources, internal release and extracellular influx.

Tunica Media

The tunica media, or smooth muscle layer, of small arteries is bound on the luminal side by a well-defined internal elastic lamina, although the

external elastic lamina is fragmented or absent. Smooth muscle cells have lengths of 40-60 μm and a diameter of 4 μm near the nucleus. The number of smooth muscle cell layers within the media of small arteries decrease with decreasing vessel diameter. In the rat tail artery, there are 12 to 15 cell layers or a total thickness of approximately 150 μm of smooth muscle. Within the media, the smooth muscle cells are circumferentially arranged, with the pitch angle approximately 80° from the path of the vessel. Each cell has a nucleus, several mitochondria, and sarcoplasmic reticulum (SR). Their membranes are not smooth but contain many caveoli or invaginations. The smooth muscle cells are electrically coupled through membranous contacts (gap junctions) allowing the smooth muscle cells to behave as an electrical syncytium (Rhodin *et al.*, 1980).

Resting Membrane Potential of Vascular Smooth Muscle

The resting membrane potential of the rat tail artery (RTA), when determined in vitro, has been found to range between -62 mV to -66 mV (Jobbling and McLachlan, 1992). The concentration difference and the resting fluxes of ions across the cell wall of arterial smooth muscle have been estimated. As with all cells, the arterial muscle cells maintain large ionic gradients across their membranes. The intracellular Na^+ $[Na^+]_i$ concentration is (10-20 mM) much lower than that of the extracellular fluid $[Na^+]_o$ (150 mM). The $[K^+]_i$ is much higher (130-160 mM) than the $[K^+]_o$ (3-5 mM). The $[Cl^-]_i$

(40-70 mM), although less than that of the $[Cl^-]_o$ (140 mM) is higher than that of many excitable cells. The free $[Ca^{++}]_i$ is maintained at very low concentration (0.1 μM) compared with the $[Ca^{++}]_o$ (2 mM). The equilibrium potentials of each of these ions are E_{Na} = +50 mV; E_K = -90 mV; E_{Cl} = -20 mV; and E_{Ca} = >+150 mV (Casteels, 1981).

Each ion will make a larger or smaller contribution to the resting membrane potential depending on the relative conductance of each ion and their electrochemical gradient. There is general agreement that the resting arterial membrane has a higher permeability to K^+ than to Cl^-, Na^+, or Ca^{++}. Taken together, this observation indicates that the resting membrane potential is primarily regulated by the transmembrane K^+ gradient and the resting conductance of K^+ (Casseels, 1981).

Most arterioles are directly innervated and can respond directly to released neurotransmitter. The sympathetic innervation at the arterial level is confined to the adventitiomedial border. If the membrane potentials of all cells are to be changed during sympathetic neuromuscular transmission, then the junctional current generated in the innervated cells must in some way be passed to the non-innervated cells deeper in the media. The passive spread of current in smooth muscle preparations results from individual smooth muscle cells being electrically coupled to neighboring cells to form a syncytium (Mekata, 1986). The techniques most frequently used to describe

the electrical characteristics of vascular smooth muscle are those of Tomita (1968). A length of smooth muscle is placed between two polarizing plates, and a voltage is applied across them. A proportion of the resulting current flows through the extracellular fluid, and the remainder flows across the smooth muscle membrane causing a membrane potential change in the smooth muscle. This potential change is largest near the polarizing plates and becomes progressively smaller away from the plates. However, membrane potential changes can be recorded from sites much greater than the length of individual smooth muscle cells, implying that current flows through low-resistance pathways between cells.

Electrical coupling between vascular smooth muscle cells has been demonstrated in many different preparations (Abe and Tomita, 1968; Bolton, 1974; Hirst and Neild, 1980; Fujiwara *et al.*, 1982). Hirst and Neild (1980) placed two independent intracellular electrodes in smooth muscle cells separated along the length of an artery by several hundred microns. Because individual cells have circumferential organization, the two electrodes could not have been in the same cell. When a small current was passed through one electrode, it caused a membrane potential change at the second electrode. Again, some of the injected current must have flowed to the recording point via low-resistance pathways between cells.

Finally, Abe and Tomita (1968) showed that a strip of smooth muscle subjected to a polarizing extracellular field behaved as a simple infinite linear cable. When a membrane potential change was induced at one of the polarizing plates, the steady-state amplitudes of the potentials decreased exponentially with distance from the polarizing plates. The distance at which the potential decreased to 37% of the value at the polarizing electrodes was defined as the electrical length constant. The length constant is a measure of the ease with which current or charge will flow along the cytoplasm compared with the ease with which it will flow across the membrane. With a multicellular preparation, the length constant largely reflects the resistance to current flow across the membranes of individual cells compared with the resistance to flow from cell to cell through gap junctions. A typical input resistance across the cell membrane is 10,000 MΩ while the resistance to current flow from cell to cell is 1 MΩ. This supports the view that smooth muscle cell membranes have a very low absolute permeability to ions in comparison to coupling between cells.

In summary, the resting membrane potential of smooth muscle cells is determined by the electrical and chemical gradients of ions across the cell membranes. Individual arterial smooth muscle cells are electrically coupled to cells around them to form a syncytium. When a current is injected into an individual cell as a result of transmitter action, that current will flow through

gap junctions into adjacent cells. Therefore, at rest all arterial smooth muscle cells have high specific membrane resistances and the density of open channels in their membrane must be low. The high membrane resistance means that current will preferentially flow between individual cells via gap junctions rather than across the cell membrane

Pharmacomechanical Coupling

In 1964, Su *et al.* found that NE appeared to produce contraction without any change in the membrane potential. Furthermore, NE caused both a phasic followed by a tonic increase in intracellular Ca^{++} $[Ca^{++}]_i$ and tension. Tonic elevation of Ca^{++} and tension was inhibited by VOCC antagonists (nifedipine, diltiazem, verapamil) while the phasic components are attenuated by antagonists which alter the refilling and storage of Ca^{++} in the sarcoplasmic reticulum (SR; caffeine and thapsigargin).

Upon NE release from the nerve terminal, NE binds to the adrenoceptor on the post-junctional smooth muscle membrane causing activation of a membrane GTP-binding protein (G-protein). G-protein activation results in the subsequent activation of membrane-bound phospholipase C (PLC). PLC catalyzes the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2). This results in the formation of inositol 1,4,5-triphosphate (IP_3), which moves into the cytosol, and a lipid product, 1,2-diacylgylcerol (DAG), which remains in the membrane. DAG activates a

specific Ca^{++}-phospholipid-dependent protein kinase termed protein kinase C (PKC) (Van Breemen, 1989).

IP_3, a water soluble lipid metabolite, diffuses through the cytosol and binds to receptors on the SR causing release of intracellular Ca^{++}. Cytosolic Ca^{++} rises from a basal level of 120-270 nM to 500-700 nM following IP_3 binding. Four Ca^{++} ions bind to calmodulin resulting in a Ca^{++}-calmodulin (Ca_4^{++}-CM) complex. The Ca_4^{++}-CM complex induces a conformational change in CM with exposure of binding sites for myosin light chain kinase (MLCK). Ca_4^{++}-CM binds to MLCK resulting in a ternary complex, Ca_4^{++}-CM-MLCK, which represents the active form of the kinase. The activated MLCK catalyzes the phosphorylation of serine 19 in each of the two 20-kd myosin light chains (MLC). MLC phosphorylation triggers cross-bridge cycling and the development of force at the expense of adenosine triphosphate (ATP) hydrolysis. Relaxation generally occurs upon removal of Ca^{++} from the cytosol, which results in dissociation of the Ca_4^{++}-CM-MLCK complex and regeneration of the inactive MLCK enzyme. Myosin that was phosphorylated during the contractile phase of the cycle is dephosphorylated by one or more MLC phosphatases. (Somlyo and Somlyo, 1968; Hokin, 1985; Rasmussen, 1987; Andrea and Walsh, 1992; Labell *et al.*, 1992).

In studies where intracellular concentrations of Ca^{++} and MLC phosphorylation were measured, addition of an appropriate agonist, resulted

in an immediate, transient increase of both intracellular Ca^{++} and myosin phosphorylation. The Ca^{++} levels fell to just above basal levels within 2-5 minutes. Phosphorylation fell more slowly, requiring 15-30 minutes to reach levels indistinguishable from baseline, although contraction was sustained. This sustained contraction in the face of transient increases of intracellular Ca^{++}, and in the content of MLC-phosphorylation, led to the latch bridge hypothesis for tension maintenance (Kamm and Stull, 1985).

In this model, initiation of contraction resulted from the formation of cycling actin-myosin crossbridges. A shift in the property of these crossbridges occurred when the MLC was dephosphorylated. A new type of long-lasting actin-myosin bridge formed, called the latch bridge. The latch bridge formation was more sensitive to Ca^{++} than the initial activation of MLCK and less energy dependent. When Ca^{++} concentrations returned to resting values, the latch bridges completely dissociated, returning tension to a basal state (Kamm and Stull, 1985).

Several mechanisms of PKC regulation of vascular smooth muscle have been suggested. Phorbol esters (tumor promoters which substitute for DAG in PKC activation) were shown to induce contractions by elevation of $[Ca^{++}]_i$ with consequent phosphorylation of MLC at serine 19. In this context, potential sites of PKC-catalyzed phosphorylation would include the L-type voltage-dependent sarcolemmal Ca^{++} channel, sarcolemmal receptor-operated

Ca^{++} channel, the SR IP_3 receptor/Ca^{++} release channel, the sarcolemmal and SR Ca^{++}-ATPase, and the sarcolemmal Na^{+}/Ca^{++} exchanger. The explanation for a phorbol ester-induced increase in $[Ca^{++}]_i$ would require phosphorylation of one of the Ca^{++} entry mechanisms and/or inactivation of the Ca^{++} removal mechanism (Rasmussen *et al.*, 1987).

PKC has been shown to affect the sustained component of smooth muscle contraction. This has led investigators to suspect alternative intermediate (thin-filament) proteins regulated by PKC phosphorylation (caldesmin, desmin, and synemin). Alternatively PKC may directly phosphorylate either MLCK or MLC at the level of serine 19. However, phosphorylation at either one of these sites would be more conducive to relaxation than contraction (Rasmussen *et al.*, 1985).

In summary, NE binding to a receptor on the smooth muscle membrane and causes the synthesis of IP_3 and DAG from the membrane phospholipid proteins. IP_3 diffuses through the cytosol to bind to a receptor on the SR, releasing intracellular Ca^{++}. DAG stays within the membrane causing the activation PKC. The released Ca^{++} binds to CM and activates MLCK which phosphorylates MLC. Smooth muscle contraction results from the phosphorylation of MLC and cross-bridge cycling. Ca^{++} elevation and MLC phosphorylation are transient processes which return to values just above basal levels although muscle tension is still maximal (latch). PKC

influences contraction by undetermined mechanisms although the sensitivity of the myofilaments to Ca^{++}, Ca^{++} release or influx, and Ca^{++} sequestration are possibilities. Relaxation is associated with the return of Ca^{++} levels returning to baseline values and the dissociation of myosin from actin.

Tunica Intima

The tunica intima, or endothelial layer, is squamous in structure, and forms a continuous cover separating the vessel lumen from the smooth muscle layer. The endothelial cells are approximately 0.2-2 μm thick, 10-20 μm wide, and 30-50 μm long with tapering ends and orientated with the long axis parallel to the direction of flow. The luminal surface of the vascular endothelial cells contains a negatively charged plasma membrane. The plasma membrane consists of gylcosaminoglycans, oligosaccharides, glycoproteins, glycolipids and sialoconjugates. Proteins bound to this surface include $alpha_2$-macroglobulins, lipoprotein-lipases, fibronectin, albumin, antithrombin III, heparin cofactor II, protein-c and protein-s. In contrast, the abluminal surface is primarily a support structure. The abluminal surface provides anchoring fibers which link endothelial cells with the smooth muscle basal lamina. Small processes penetrate the internal elastic lamina to form myoendothelial junctions with smooth muscle cells. Gap junctions between the endothelium and smooth muscle are an important observation in view of the endothelium's ability to affect vascular contractility. The

surface coat covering the endothelial cells consists of branching fibrils associated with the cell surface, which extend 200 nm into the lumen of the vessel towards the basement lamellae, and partially filling the intercellular clefts (Rhodin, 1980; Wehrmacher, 1988).

The vascular endothelium is considered to be the largest and most productive paracrine organ in the body (Dinnerman et al., 1990). Endothelial cells synthesize and release; connective-tissue components, vasodilators endothelium-derived relaxing factor (EDRF)/nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (PGI_2); Busse *et al.*, 1988; Shepherd *et al.*, 1991; Rubanyi *et al.*, 1991), procoagulants, anticoagulants, fibrinolytic substances and prostanoids which contribute to normal vasomotion and thromboresistance.

Endothelium-Derived Relaxing Factor (EDRF)

In 1980, Furchgott and Zawadzki reported that relaxation of smooth muscle by acetylcholine (ACh) was dependent on the presence of endothelial cells in the preparation. Removal of the endothelial layer inhibited the ability of ACh to induce relaxation and in most arteries, caused contraction, especially at higher concentrations. Furchgott (1983) termed this vasoactive substance endothelium-derived relaxing factor (EDRF). The chemical and pharmacological properties of EDRF are now known to be indistinguishable from nitric oxide (NO; Ignarro *et al.*, 1987). Ignarro and co-workers used

chemiluminescence to detect NO and EDRF reaction with ozone. Their results showed; 1) both vasodilatory factors are unstable and possess short-half lives (less than 10 seconds); 2) both activate soluble guanylate cyclase by a heme-dependent mechanism; 3) hemoproteins inhibit the dilatory effects on smooth muscle; 4) both relax venous as well as arterial smooth muscle; 5) both relaxing factors have been shown in bioassay systems to possess the same rate of loss of activity with increased transit time; 6) both inhibit platelet adhesion and aggregation; and 7) both NO and EDRF were potentiated by superoxide dismutase (Furchgott, 1989; Ignarro *et al.*, 1987; Moncada *et al.*, 1991).

Nitric Oxide Synthesis

Vascular endothelial cells generate NO from the terminal guanidine nitrogen atom of L-arginine. NO formation also requires a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme system, Ca^{++}, calmodulin, tetrahydrobiopterin, nitric oxide synthetase and superoxide dismutase catalyzes (Ignarro, 1991).

Mechanism of Action and Half-Life of Nitric Oxide

Nitric oxide is synthesized in the vascular endothelium and elicits its principle biological action on underlying smooth muscle. Due to the small molecular size and high lipophilicity of NO, it diffuse out of the endothelium, penetrate the associated vascular smooth muscle and convert

soluble magnesium guanosine 5-triphosphate (GMP) to cyclic GMP (cGMP) through activation of soluble guanylate cyclase (Ignarro, 1991). NO's inherent ability to bind hemoglobin, myoglobin and other reduced hemoproteins and its inactivation by superoxide anions contributes to an extremely short half-life and prevents any widespread vascular effects (Mugge *et al.*, 1991).

NO passively enters the cell and initiates signal transduction by binding to the heme (reduced Fe^{++}) group of soluble guanylate cyclase and disassociates it from the enzyme, creating a nitrosyl-heme-enzyme ternary complex. The binding of NO to the heme group on soluble guanylate cyclase causes a 200 fold increase in the conversion rate of magnesium guanosine 5'-triphosphate substrate to cGMP. Signal transduction is terminated when the heme (reduced Fe^{++}) axial ligand is replaced on guanylate cyclase and NO decomposes into nitrite and nitrate (Ignarro, 1991). cGMP activates a cyclic G-protein kinase which leads to vasodilation by: 1) reducing phosphatidlyinositol turnover (reduced IP_3, diacylglycerol and protein kinase C activation); 2) inhibiting stored Ca^{++} release (Lang and Lewis, 1991; Ahlner *et al.*, 1990; Ahlner *et al.*, 1991); 3) reducing the rate of myosin light-chain kinase phosphorylation (Drazsnin *et al.*, 1983); or 4) regulating the influx of extracellular Ca^{++} through voltage-operated channels (Auch-Schwelk and Vanhoutte, 1991).

Conclusions based on the recent NO research indicates that the

substrate required for NO synthesis is L-arginine which is located in the cytosolic fraction. NO Inhibitors include the analogs N-nitro-L-arginine methyl ester (L-NAME), N-methyl-L-arginine (L-NMA), N-nitro-L arginine (L-NNA) These analogs appear to compete with L-arginine for the enzyme required for the synthesis of NO. The precise mechanism of NO synthesis and release is unknown, however NO synthesis requires the elevation of intracellular concentrations of Ca^{++}, either through regulation of extracellular calcium influx or intracellular release. Intracellular Ca^{++} binds to calmodulin which initiates the synthesis of NO. The membrane potential may play a role in the transmembrane influx of Ca^{2+}. NO is a paracrine hormone which affects the smooth muscle though simple diffusion. The half-life of NO is estimated between 1 to 10 seconds. A variety of endogenous agents increase the synthesis and release of NO, which then causes an increase in vascular smooth muscle cGMP and vasorelaxation. One stimulatory agent, ACh, appears to increase NO synthesis and release, as well as other endothelium-derived relaxing agents (EDHF and PGI_2).

Endothelial-Derived Hyperpolarizing Factor

An endothelium-dependent, nonprostanoid hyperpolarizing factor identified by Chen (1988) and Chen and Suzuki (1989) acts through K^+ ion channels to mediate hyperpolarization and relaxation in vascular smooth muscle. Feletou and Vanhoutte (1988) demonstrated that ACh induced

transient hyperpolarization and relaxation of vascular smooth muscle, but was not inhibited by indomethacin (a cyclooxygenase inhibitor of prostacyclin synthesis). The hyperpolarization was completely reversed by either ouabain (an inhibitor of sodium-potassium pump) and incubation in potassium-free solution; however, the ACh-mediated relaxation was unaffected. Therefore, a transiently acting, endothelially-mediated, hyperpolarizing factor was suggested to regulate the vascular smooth muscle sodium-potassium pump and hyperpolarization, while another endothelial factor was responsible for a more sustained vascular relaxation effect. The sustained component which modifies vascular resistance has been identified as NO by use of hemoglobin and methylene blue, selective inhibitors of NO synthesis and cGMP synthesis (Ignarro *et al.*, 1991). However, the agent and specific action of the hyperpolarizing factors seem more complex.

Chen *et al.* (1988) and Chen and Suzuki (1990) characterized the electrophysiological and pharmacological differences between EDHF and NO as follows; 1) mechanical responses to NO are sustained whereas those to EDHF are mostly transient; 2) inhibitors of NO (L-NMMA, methylene blue and hemoglobin) do not block the action of ACh-induced hyperpolarization; 3) NO relaxes vascular smooth muscle without an alteration in membrane potential; 4) ACh-induced relaxation is diminished following repeated exposure while the effects of ACh on hyperpolarization are not; and

5) stimulation by ACh generates two separate hyperpolarization effects (transient followed by sustained) but only in intact vessels. The transient hyperpolarization is not as sensitive to extracellular calcium concentration as the sustained component of hyperpolarization. Vessels without endothelium do not demonstrate a sustained hyperpolarization, therefore establishing the necessity for endothelial tissue in the synthesis of EDHF. Finally, transient hyperpolarization requires 3-7 minutes to completely affect the tissue.

EDHF's chemical identity is as yet undetermined. The physiological signal which stimulates the secretion of EDHF involves agonist binding to the membrane receptor. Receptor/agonist interaction causes phosphatidylinositol turnover, IP_3 synthesis, increased Ca^{++} efflux from the $[Ca^{++}]_i$ stores (sarcoplasmic reticulum) and Ca^{++} influx from the extracellular medium. EDHF may exert its primary effect on the endothelium. EDHF increases endothelial K^+ efflux current through either a Ca^{++}- or ATP-activated channel. This current is then conducted through myoendothelial gap junctions to the associated smooth muscle to cause smooth muscle hyperpolarization. Alternatively, EDHF may act as a humoral substance, secreted by the endothelium to cause activation of a smooth muscle Ca^{++}- or ATP-sensitive K^+ channels. K^+ channel activation would hyperpolarize the vascular smooth muscle, reduce Ca^{++} influx through

voltage operated channels, and contribute to vascular relaxation. An elevated extracellular concentration of K^+ would attenuate the ACh-mediated hyperpolarization without affecting NO-mediated relaxation. This stresses the importance of K^+ efflux (Nagao and Vanhoutte, 1991).

Endothelial Regulation of Arterial Tone

Frank and Bevan (1983) were the first to report that norepinephrine preconstricted ring segments from pulmonary arteries relaxed in response to electrical field stimulation (EFS). The precise mechanism was not determined at that time, although removal of the endothelium completely inhibited the relaxation. Recent studies have now identified the L-arginine/NO/cGMP pathway as the primary regulatory mechanism mediating endothelium-dependent EFS relaxation (Buga and Ignarro, 1992; Van Riper and Bevan, 1992).

In 1988, Kotecha and Neild used EFS to relax 5-HT and norepinephrine preconstricted rat tail artery (RTA) ring segments. EFS caused hyperpolarization and relaxation by an endothelium-independent mechanism. The correlation between degree of dilation and hyperpolarization was statistically significant. The relaxation was sensitive to KCl and tetraethylammonium (TEA), suggesting a Ca^{++}-regulated K^+ channel. Antagonists to histamine (cimetidine); to free radicals (ascorbate); and NO (oxyhemoglobin) had no effect. It is not known why they were unable to

identify an endothelium-dependent component.

The physiological role of EFS vasodilation is unknown. However, Vargus *et al.* (1990), using perfused rat carotid arteries, found that the degree of vascular tone regulated the release of endothelial NO. Under constant perfusion rates, phenylephrine (PE) caused increased pressure that was enhanced with inhibition of NO synthesis (L-NNA). If the animal was pithed, the L-NNA response was significantly attenuated during PE infusion. When PE infusion was elevated to levels that raised the arterial pressure to that of intact animals, the L-NNA response returned to values seen in the non-pithed animals. Raising PE infusion to reach hypertensive pressures raised the L-NNA inhibitory response. They concluded that the changes in arterial tone directly stimulated the release of endothelial NO. They further speculated that the modulation of NO may serve as a reflex mechanism to sustain blood flow in the presence of intense sympathetic neural transmission. The signaling transduction mechanism was not investigated in this study, although sensitivity to NO and shear stress was suggested.

Miller *et al.* (1984), Hynes *et al.* (1988), Topouzis *et al.* (1991), Auch-Schwelk *et al.* (1991), Thompson *et al.* (1993), and Thorin *et al.* (1994) used either EFS stimulation to release neuronal NE, or applied exogenous NE to constrict perfused RTA. Their data showed that endothelium denuding or inhibition of the L-arginine/NO/cGMP pathway increased the degree of

EFS- and NE-induced constriction. EFS constriction was sensitive to tetrodotoxin (TTX) which supported the neuronal origin of NE as the cause of constriction. Although direct evidence was not presented, the diffusion of NE through 150 μm of smooth muscle to directly stimulate endothelial release of NO was ruled out (Hynes *et al.*, 1988). Alpha-receptor antagonism with prazosin (alpha-1) and yohimbine (alpha-2) suggested that binding efficacy was inversely related to NO release (Hynes *et al.*, 1988). Miller *et al.* (1984) and Topouzis *et al.* (1991) used BHT-920 and clondine (alpha-2 agonists) and phenylephrine (alpha-1 agonist) to show that alpha-2 agonists increased the release of NO greater than alpha-1 agonist. Auch-Schwelk *et al.* (1991) also related NE binding efficacy to NO release and suggested that Ca^{++} influx from the extracellular fluid was the signal that caused the release of NO. This research showed that low efficacy agonist binding of NE increased cytosolic Ca^{++} by influx through VOCC more than by release from intracellular stores via the phosphotidylinositol (IP_3) system. It was suggested that NO would then modulate Ca^{++} entrance through VOCC and thereby vascular tone.

EFS-mediated vasoconstriction causes an increase in shear stress which may be responsible for the release of nitric oxide (Tesfamariam *et al.*, 1987, 1988; Reid *et al.*, 1991; Vo *et al.*, 1991; Vo *et al.*, 1992; and Thorin *et al.*, 1994). Perfusion methods were similar to those described by Hynes *et al.* (1988). EFS caused a reduction in luminal diameter as smooth muscle tone increased.

When NO metabolic inhibitors (L-NAME and oxyhemoglobin) were perfused through the lumen, the degree of muscle tone caused by EFS increased. The neuronal release of NE following EFS was not influenced by an NO-dependent system. The basal smooth muscle tone was not enhanced by these substances, suggesting that basal release of NO was not responsible for the modulation of EFS constriction. These authors concluded that a shear stress mechanism is primarily responsible for the release of NO during sympathetic neuronal transmission.

In summary, EFS causes smooth muscle constriction through the neuronal release of NE. The degree of constriction is modulated by NO released from the endothelium. The signaling mechanisms for the release of NO are undefined. Some possible mechanisms that have been discussed are: 1) NE diffusion through the smooth muscle layers. NE binds to alpha-2 receptors on the endothelium which causes the release of NO; 2) NE starts a signal transduction mechanism by binding to the alpha-adrenoceptor on the adventitiomedial layer. Receptor bound NE generates an electrochemical signal carried by Ca^{++}. This signal is propagated through the smooth muscle to the endothelium causing the release of NO; 3) NE causes an increase in arterial tone and a subsequent reduction in lumen diameter. The reduction in lumen diameter increases the shear of the blood on the endothelial surface causing the release of NO. Although the signal transduction mechanism

responsible for regulation of arterial tone by NO has eluded investigators, an integration network between the nerve, smooth muscle and endothelium is clearly at work to control arterial tone.

The aim of our study was to investigate the local regulatory mechanisms which modulate smooth muscle tone. Specifically, we wanted to determine if EFS causes the release of NO from the preconstricted rat tail artery. This has led to the following hypotheses:

Statement of Hypotheses:

1) EFS-induced relaxation of a preconstricted rat tail artery ring is endothelium dependent.

2) EFS-induced relaxation of a preconstricted rat tail artery ring is sensitive to NO antagonism.

3) EFS-induced relaxation of a preconstricted rat tail artery ring exhibits a hyperpolarization component.

4) EFS of isolated endothelial cells causes relaxation in denuded rat tail artery rings.

5) EFS of isolated endothelial cells is sensitive to antagonists of NO.

6) EFS of isolated endothelial cells is sensitive to antagonists of hyperpolarization.

7) EFS-induced relaxation of a preconstricted and pressurized (closed) rat tail artery segment is sensitive to antagonists of NO.

8) EFS-induced relaxation of a preconstricted and pressurized (closed) rat tail artery segment exhibits a smooth muscle-specific hyperpolarizing component.

9) EFS causes smooth muscle membrane hyperpolarization prior to smooth muscle relaxation.

10) Membrane potential recordings of EFS-induced relaxation shows a hyperpolarization-independent relaxation sensitive to NO antagonists.

MATERIALS and METHODS

Preparation of Rat Tail Artery Rings

Sprague Dawley rats weighing between 250-350 grams were decapitated, and a segment of tail artery was removed and placed in aerated (95% O_2: 5% CO_2) Krebs' solution. The composition of the Krebs' solution was (in mM): NaCl, 118; KCl, 5.4; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 12.5; $MgSO_4$, 1.12; and glucose, 10.0. Three cm sections of proximal tail arteries were removed, cut into 3 mm segments (rings) and mounted on platinum wires. They were immersed in 5 ml tissue baths filled with Krebs solution and maintained at 37°C. After the initial one hour equilibration, the tissues were stretched to a basal tension of 1.0 gram (g), which was determined to be the optimal length for force development (Price *et al.*, 1981). An additional one hour equilibration was allowed before the experiments began. Isometric contractions were recorded using Grass PT-3 force transducers. The resulting electrical signals were digitized on a MacLab analog-digital converter and were recorded by a Macintosh computer. Endothelium denuding was performed by threading a # 6.0 silk thread through the lumen and gently rolling the artery on a paper towel moistened in Krebs solution. Denuding was considered successful if the artery did not relax in the presence 1 μM ACh following preconstriction by 1 μM PE.

Preparation of Pressurized Arteries

Rat tail arteries were prepared as previously described, except that the 3 cm proximal section was removed and cannulated, intact, between two 20

gauge stainless steel needles within a horizontal 25 ml bath aerated (95% O_2 and 5% CO_2) and maintained at 37° C. The artery was triple tied on either end and attached to a micrometer. The artery was then stretched to adjust the arterial length to the *in-vivo* length. The lumen was flushed for three minutes with fresh Krebs solution to remove residual blood cells before elevation of intraluminal pressure. The down-stream cannula was connected in series with a pressure transducer and two-way stopcock, while the upstream cannula was connected to a 20 ml syringe and two-way stop-cock. Albumin, 3.2 g/L, was added to the luminal Krebs solution to increase the oncotic pressure and reduce filtration during elevation of intraluminal pressure.After closing the downstream stop-cock, intraluminal pressure was increased by forcing Krebs solution into the lumen with the upstream 20 ml syringe. Once pressure had reached 100 mm Hg the upstream stop-cock was shut and the system was now essentially closed. An incubation period of one hour preceded all experiments.

Electrical Field Stimulation Studies

Electrical field stimulation (EFS) was conducted through two platinum electrodes placed approximately 5 mm apart on each side of the tissue. Arterial tension was first raised to approximately 70% of maximum by PE (30 μM) with TTX (1 μM) present in the bath. EFS was applied with a current amplifier and stimulator. EFS parameters were 10-50 v, pulses of 0.1-1.0 ms

duration, frequencies were between 0.125-128 Hz, current was between 100-500 mA, and delivered as 5 second trains. Pulse durations and train lengths were limited to a maximum of 1.0 ms and 5 seconds, respectively, to avoid the possibility of generating free radicals and direct stimulation of smooth muscle voltage operated channels. The EFS parameters chosen caused constriction only in the absence of TTX (1 μM), identifying neuronal release of neurotransmitter (Hynes *et al.*, 1988; Bao *et al.*, 1993).

The optimal amplitude and duration parameters for EFS-induced relaxation of intact and denuded RTA were 0.1 ms at 30 v. Following PE-induced preconstriction (30 μM, ED_{70}), a frequency-response curve of EFS-induced relaxation (0.25-32 Hz, 30 v, 0.1 ms, 5 seconds) was determined with TTX (1μM) in the bath. Rings were washed 7 times and allowed to equilibrate for 30 min after EFS to permit complete recovery from the previous EFS.

Preparation of Bovine Aorta Endothelial Cell

Bovine aorta endothelial cells (BAEC) were harvested as described by Bing *et al.* (1991). Briefly, 6-12 aortas were acquired from a local slaughter house, placed in ice cold Krebs solution and transported within 15 minutes to the laboratory. Intercostal branches were tied and excess connective tissue was removed from the vessels. The lumen of each vessel was then filled with collagenase solution (1 mg/ml) dissolved in Krebs solution. Vessels

were incubated at 37°C for one hour. After incubation, the aortas were cut lengthwise and one end was suspended on a rack to expose the flat intimal surface. The aortas were then washed with Krebs solution, gently rubbed with a rubber policeman and washed again with fresh Krebs solution. The washes were collected in a 500 ml flask and then transferred into 15 ml centrifuge tubes for 5 minutes of centrifugation at 1000 RPM. The supernatant fluid was discarded and the pellet was resuspended in fresh Krebs solution.

Microcarrier beads were prepared in the following manner: 20 mg of cytodex-3 beads were placed into 10 ml of calcium-magnesium free Krebs solution to produce quelling. The solution was incubated for one hour at 37°C, with occasional stirring. After incubation, the calcium-magnesium free Krebs solution was decanted and replaced with normal Krebs solution. Endothelial cells were incubated (37°C) with the beads for one hour to promote adherence. The BAEC pellet was placed on filter paper 105 μm mesh size (Spectrum Medic Industries Inc. Los Angeles CA). The BAEC pellet was then placed in a 20 ml bath constantly perfused with Krebs solution (37°C). Indomethacin (10 μM) was added to prevent prostaglandin synthesis. PE (30 μM) was also added to the BAEC bath in order to replace the PE initially used to preconstrict the denuded RTA. BAEC were first stimulated with EFS and the effluent was run over a denuded, preconstricted RTA in order to

generate a frequency-tension response. The BAEC response to EFS on denuded RTA was then determined following 30 minutes of incubated with L-NAME (100 μM). Following the response to L-NAME, a 30 minute L-Arginine (1 mM) BAEC incubation period was conducted. Subsequent BAEC stimulation with EFS followed the L-arginine incubation and the response was determined in the denuded, preconstricted RTA. Effluent flowed from the BAEC bath through a segment of polyethylene tubing to the bath containing the denuded RTA strip. The total transit time was less than one second.

Chemicals and Preparation of Solutions

Type 1 collagenase, phenylephrine hydrochloride, indomethacin, acetylcholine chloride, methylene blue, L-arginine, N-nitro-L-arginine methyl ester (L-NAME), tetrodotoxin (TTX), atropine sulphate, tetraethylammonium chloride (TEA), ascorbic acid, diltiazem, and cytodex-3 beads were purchased from Sigma Chemical Company (St. Louis, MO). Double-distilled water was used to make all the solutions. Indomethacin, which inhibits the synthesis of prostaglandins, was dissolved in an equimolar concentration of Na_2CO_3. Glibenclamide, a specific antagonist of the K_{ATP} channel, was dissolved in DMSO and then diluted to a final concentration of less than 0.1% DMSO. TEA (1 mM) is a specific antagonist of the Ca^{++}-sensitive K^+ channels (Nelson, 1993). L-NAME inhibits the synthesis of

NO by acting as a false substrate of L-arginine. Methylene blue inhibits NO synthesis of cGMP from soluble guanylate cyclase in the vascular smooth muscle. Ca++-free medium was created by replacing $CaCl_2$ with 2 mM EDTA. Solutions with elevated potassium concentrations were made by replacing NaCl with equimolar amounts of KCl. Incubation with indomethacin, atropine, methylene blue, L-NAME, L-arginine, and tetrodotoxin was for 30 minutes.

In the pressurized experiments, intraluminal infusion and incubation with L-NAME and L-arginine were conducted to modify the synthesis of nitric oxide. All other antagonists ($BaCl_2$, TEA, KCL, and diltiazem) were applied to the 25 ml bath 30 minutes before the start of the experiment. After each procedure, repeated washing and 30 minutes of incubation in fresh Krebs solution preceded the next experiment.

Statistical Analysis

All data in the text and figures are given as means ± SEM in grams. Means were compared by Student's paired and unpaired t-test or by a one way analysis of variance (ANOVA).

RESULTS

RTA Ring and BAEC Studies

A log dose-response curve for phenylephrine (PE) and a frequency-response curve for electrical field stimulation (EFS), 0.1 ms pulse duration and 30 v with and without tetrodotoxin (TTX; 1 μM) are shown in Figure 1A. Figure 1B shows that EFS-induced constriction, unlike constriction with PE (Figure 1A), was inhibited by TTX.

Figure 2 shows the 0.1 ms, 30 v, and 5 second frequency-tension curves in 4 sets of different experiments: 1) in the PE preconstricted intact RTA ; 2) in the PE preconstricted denuded RTA; 3) KCl preconstriction in the intact RTA; and 4) in the denuded, PE preconstricted RTA following exposure to effluent from electrical field stimulated freshly isolated bovine aortic endothelial cells (BAEC). The maximal change in tension (relaxation) to EFS in the intact, PE preconstricted segment was -0.9 ± 0.08 grams (g) and -0.95 ± 0.07 g for 4 Hz and 16 Hz, respectively. Since the difference between 4 Hz and 16 Hz did not appear to be statistically significant, therefore 4 Hz was taken to be the maximal stimulus.

Identical EFS parameters were used to stimulate the intact and denuded RTA (Figure 2). The maximal change in tension (relaxation) was achieved between 16 Hz (-0.11 ± 0.02 g) and 32 Hz (-0.12 ± 0.09 g; $p>.05$). Comparison of EFS-induced (4 Hz, 0.1 ms, 30 v) relaxation of endothelium-intact versus endothelium-denuded RTA shows a significant ($p<.005$) decrease in EFS-induced relaxation after endothelium

Figure 1. (A) The dose-tension curve in grams (g) for phenylephrine (PE) in the rat tail artery with and without the voltage-operated Na^+ channel antagonist tetrodotoxin (TTX). (B) The frequency-tension curve (g) for electrical field stimulation (EFS, 1.0 ms, 30 v) with and without TTX. TTX inhibited only the EFS-induced contraction.

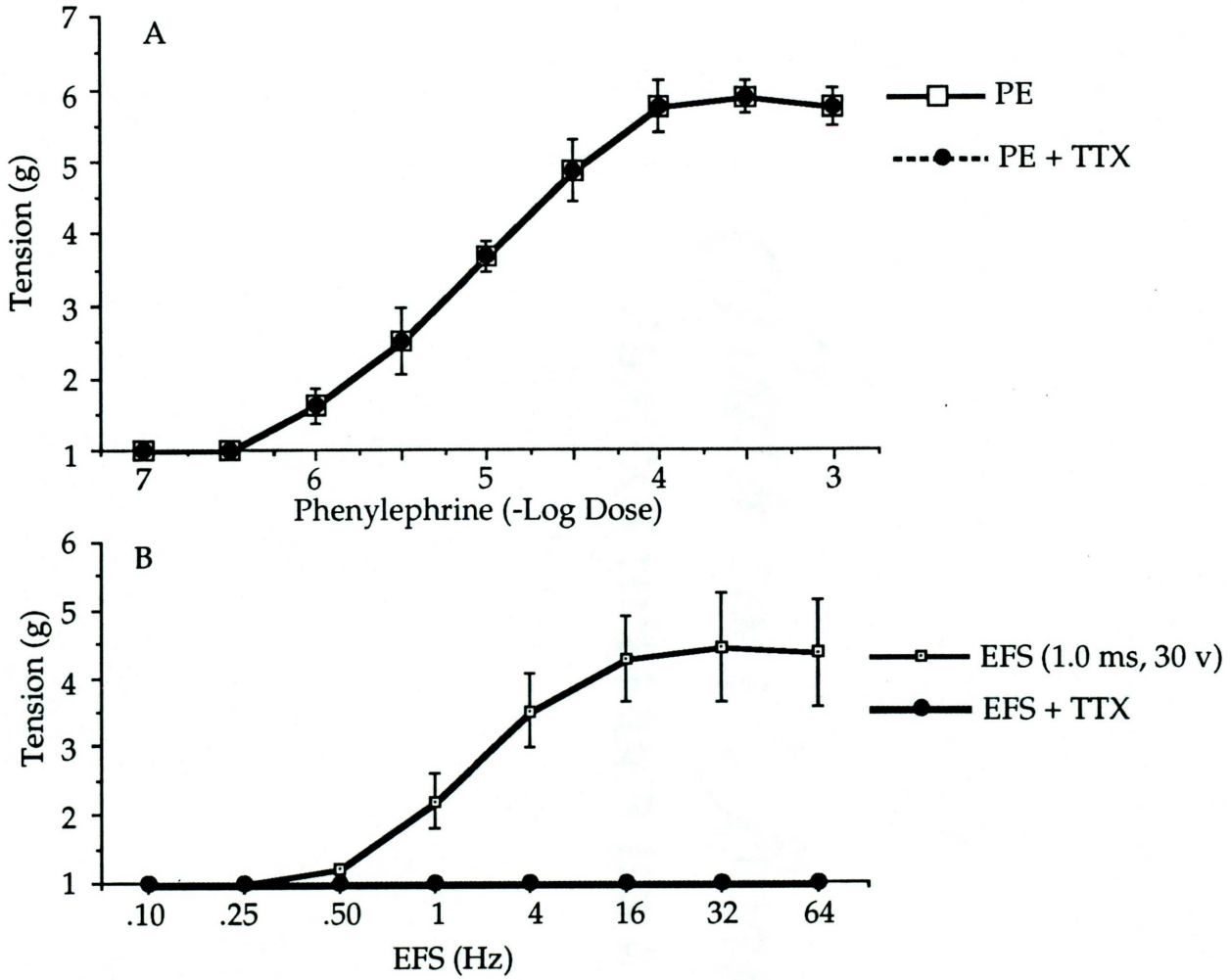
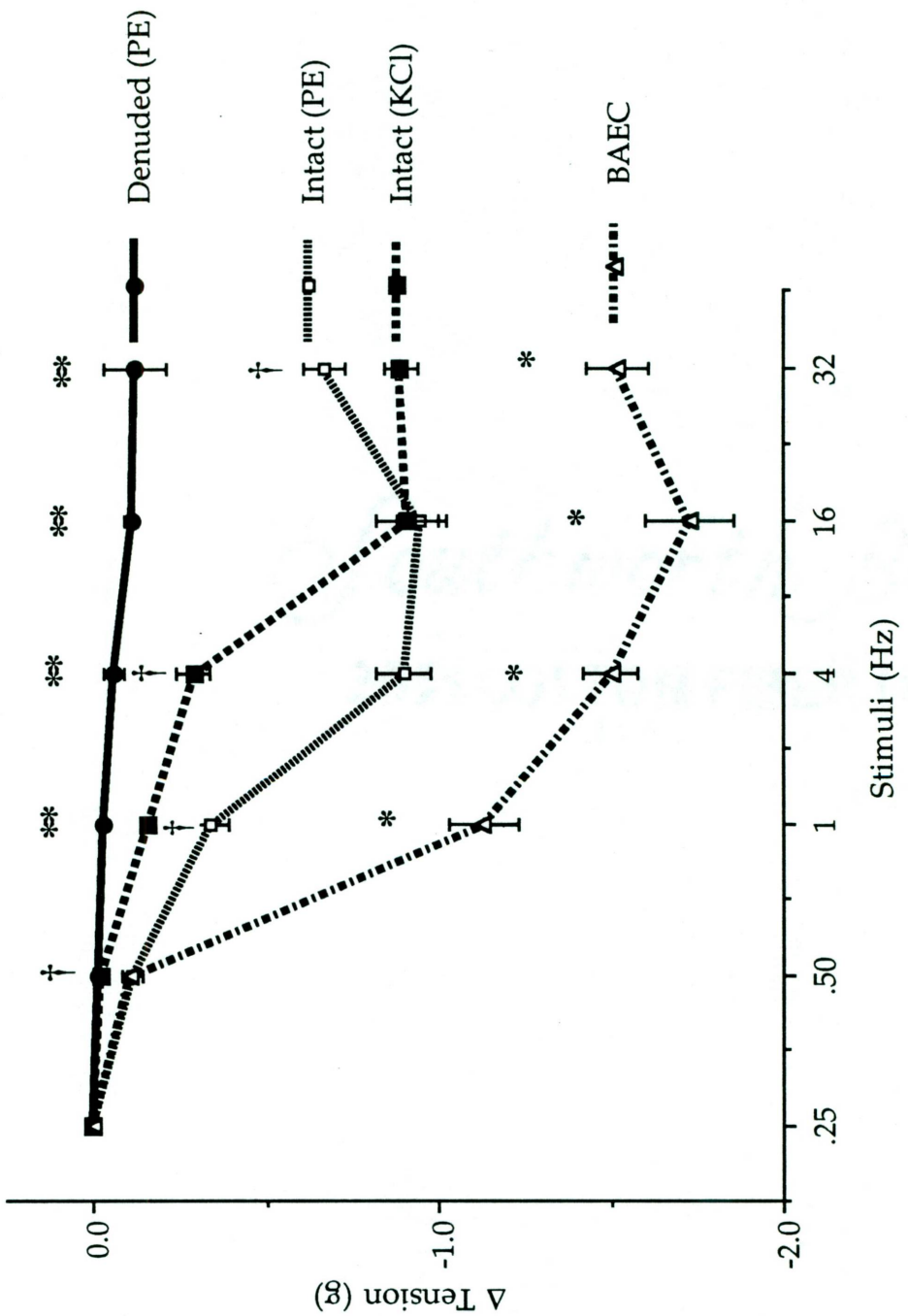

A
Tension (g)
7
6
5
4
3
2
1
7
6
5
4
3
Phenylephrine (-Log Dose)
PE
PE + TTX
B
Tension (g)
6
5
4
3
2
1
.10
.25
.50
1
4
16
32
64
EFS (Hz)
EFS (1.0 ms, 30 v)
EFS + TTX

Figure 2. The frequency-tension curves for electrical field stimulation-induced (EFS; 0.1 ms, 30 v) change in tension in grams (g) in the intact and denuded rat tail arteries (RTA). RTA were preconstricted with either 30 μM phenylephrine (PE) or (120 mM) KCl prior to EFS. The change in tension of the denuded RTA after superfusion with effluent from isolated, freshly harvested, bovine aorta endothelial cells (BAEC) following EFS is shown. Statisical differences from the intact, PE values are indicated as follows: †$p<.05$; *$p<.01$; **$p<.005$.


Denuded (PE)
Intact (PE)
Intact (KCl)
BAEC
Δ Tension (g)
0.0
-1.0
-2.0
.25
.50
1
4
16
32
Stimuli (Hz)

removal. All EFS-induced (0.5-32 Hz, 0.1 ms, 30v) relaxation values in the denuded vessels were significantly less than the EFS-induced (0.5-32 Hz, 0.1 ms, 30v) relaxations in the intact vessels, ($p<.05$). Notice the residual relaxation which is present although the endothelium has been removed.

In Figure 2, KCl preconstriction was done to eliminate any EFS-dependent hyperpolarization while unmasking the pure NO component. Following KCl preconstriction, the EFS-induced relaxations were significantly reduced from the PE preconstricted values at all EFS frequencies except for 16 Hz (PE control -0.95 ± 0.70 g; 16 Hz KCl -0.91 ± 0.090 g). A dual component at lower frequencies (0.50-4 Hz) was noted with KCl preconstriction which was absent at 16 Hz. This suggests that a K^+ efflux mechanism (hyperpolarization) is present at lower frequencies.

The effect that BAEC effluent had on denuded RTA after EFS (0.1 ms, 30 v) is also shown in Figure 2. The effluent from electrical field stimulated BAEC caused significant relaxation in the denuded RTA. These responses were shown to be frequency-dependent; (16 Hz; -1.730 ± 0.130 g, and 32 Hz; -1.520 ± 0.09 g; $p<.05$ comparing BAEC responses to 4 Hz intact response).

Figure 3 shows the effect of EFS-induced relaxation on the intact, PE preconstricted RTA in the presence of NO and hyperpolarization antagonists. L-NAME (100 μM) significantly reduced the 4 Hz EFS-induced relaxation

Figure 3. The control (4 Hz, 0.1 ms, 30 v) response to electrical field stimulation-induced (EFS) change in tension in grams (g) in the intact, phenylephrine (PE) preconstricted rat tail arteries. The control 4 Hz-induced change in tension is shown in comparison to the 4 Hz-induced change in tension in the presence of the NO antagonist L-NAME (NM); the cGMP antagonist methylene blue (MB); the K_{ATP} hyperpolarization antagonist $BaCl_2$ (Barium); the nonspecific hyperpolarization antagonist KCl; the K_{Ca}^{++} hyperpolaraization antagonist tetraethylammonium (TEA); and in Ca^{++}-free Krebs solution. All values (*$p<.05$; **$p<.01$) are significant as compared to the control response.

Δ Tension (g)
1
0
-1
-2
Control
NM
ARG
KCl
TEA
Barium
MB
Ca++Free
-.90
*
-.27
*
-1.27
*
-.26
*
-.53
*
-.21
*
-.25
**
+.18

from control values of -0.9 ± 0.08 g to -0.27 ± 0.04 g. L-arginine (1 mM) reversed L-NAME's inhibition to relaxation; -1.27 ± 0.15 g. Increased extracellular KCl (120 mM) decreased EFS-induced relaxation to -0.26 ± 0.03 g as did TEA (1 mM), -0.53 ± 0.01 g. $BaCl_2$ (100 μM) attenuated EFS-induced relaxation to -0.21 ± 0.05 g. Methylene blue (MB, 10 μM) inhibited EFS-induced relaxation to -0.25 ± 0.20 g. In a Ca^{++}-free medium EFS-induced relaxation was reversed from a control value of -0.9 ± 0.08 g to a constriction state of +0.18 ± 0.19 g.

Figure 4 shows the effect of EFS-induced (16 Hz, 0.1 ms, 30v) relaxation on denuded RTA in the presence of NO and hyperpolarization antagonists. The EFS response was not affected by L-NAME (control -0.11 ± 0.02 g; L-NAME -0.12 ± 0.04 g) or 1 mM TEA (control -0.11 ± 0.02 g; TEA -0.12 ± 0.05 g). The specific K_{ATP} antagonists, $BaCl_2$ (100 μM) and glibenclamide (1 μM), along with increased extracellular KCl (120 mM), reversed the EFS-induced relaxation response to constriction (control -0.11 ± 0.02 g, $BaCl_2$ +0.14 ± 0.05 g, glibenclamide +0.06 ± 0.10 g, and KCl +0.22 ± 0.06 g).

In Figure 5, EFS-induced (16 Hz, 0.1 ms, 30 v) relaxation of intact RTA following KCl preconstriction was reversed to constriction with the nitric oxide antagonists L-NAME (+0.40 ± 0.21 g compared to control -0.91 ± 0.09 g) and methylene blue (+0.80 ± 0.11 g compared to control -0.91 ± 0.09 g). L-arginine reversed L-NAME's constriction response to

Figure 4. The control (16 Hz, 0.1 ms, 30 v) response to electrical field stimulation-induced (EFS) change in tension in grams (g) in the denuded, phenylephrine preconstricted rat tail arteries. The control change in tension is shown in comparison to the 16 Hz-induced change in tension in the presence of the NO antagonist L-NAME (NM); the K_{ATP} hyperpolarization antagonists $BaCl_2$ (Barium) and glibenclamide (GLB); the nonspecific hyperpolarization antagonist KCl; and with the K_{Ca}^{++} hyperpolarization antagonist tetraethylammonium (TEA). All values (*$p<.05$) are significant as compared to control response.

Δ Tension (g)
0.50
0.25
0.00
-0.25
-0.50
Control
NM
Barium
GLB
TEA
KCl
-.11
-.12
+.14
*
+.06
*
-.12
+.22
*

Figure 5. The control (16 Hz, 0.1 ms, 30 v) response to electrical field stimulation-induced (EFS) change in tension in grams (g) in the intact, KCl preconstricted rat tail arteries. The control change in tension is shown in comparison to the 16 Hz-induced change in tension in the presence of either the NO antagonist L-NAME (NM); the cGMP antagonist methylene blue (MB); or in combination with both L-NAME and the NO substrate L-arginine (ARG). All values (**$p<.01$) are significant as compared to the control response.

Δ Tension (g)
1.0
0.0
-1.0
-2.0
Control
MB
NM
NM/ARG
-.91
+.80
**
+.40
**
-.97

relaxation (L-NAME +0.40 ± 0.09; L-arginine -0.97 ± 0.12 g; $p<.01$ compared to 16 Hz in the absence of NO antagonists).

Figure 6 shows the intact, 4 Hz EFS-induced relaxation in the presence of either voltage-operated Ca^{++} channel (VOCC) antagonist, nitric oxide antagonists, or hyperpolarization antagonists. The 4 Hz response is also shown in the presence of diltiazem. The 4 Hz EFS-induced relaxation was blocked and constriction occurred in all treatment groups. The VOCC blocker diltiazem (100 μM) increased tension to +0.14 ± 0.03 g; L-NAME (100 μM), with either elevated KCl or $BaCl_2$, increased arterial tone (+0.20 ± 0.05 g and +0.08 ± 0.15 g, respectively). Methylene blue (MB; 10 μM), with elevated KCl (120 mM), increased tone to +0.22 ± 0.05 g. Ascorbic acid (A-ACID; 100 μM) had no effect on EFS-induced relaxation (control -0.90 ± 0.08 g, ascorbic acid -1.19 ± 0.14 g). All values are significant at the $p<.05$ level as compared to the intact 4 Hz response.

The response that effluent from electrical field stimulated BAEC had denuded RTA was also subjected to NO and hyperpolarization antagonists (Figure 7). Figure 7 shows the 16 Hz EFS control response was (-1.73 ± 0.13 g) reversed by methylene blue (MB; 10 μM) and L-NAME (100 μM), +0.91± 0.18 g and +0.60 ± 0.24 g, respectively. Glibenclamide (1 μM; GLB-R) and $BaCl_2$ (100 μM), both K_{ATP} inhibitors (in the denuded RTA bath) and KCl (120 mM) preconstriction caused a significant reduction in BAEC effluent-induced

Figure 6. The control (4 Hz, 0.1 ms, 30 v) response to electrical field stimulation-induced (EFS) change in tension in grams (g) in the intact, phenylephrine preconstricted rat tail arteries. The control change in tension is shown in comparison to the 4 Hz change in tension in the presence of the voltage operated Ca^{++} channel antagonist diltiazem; the NO antagonist L-NAME (NM); the cGMP antagonist methylene blue (MB); the K_{ATP} hyperpolarization antagonist $BaCl_2$; the nonspecific hyperpolarization antagonist KCl; and the free radical scavenger ascorbic acid (A-ACID). All values ($p<.05$) are significant as compared to the 4 Hz response.

Δ Tension (g)
1
0
-1
-2
Control
Diltiazem
MB/KCl
NM/KCl
NM/Barium
A-Acid
-.90
+.14
*
+.22
*
+.20
*
+.08
*
-1.19

Figure 7. The change in tension of the endothelium-denuded rat tail arteries (RTA) after exposure to effluent from 16 Hz (0.1 ms, 30 v) electrical field stimulated bovine aorta endothelial cells (BAEC). The control (16 Hz) change in tension in grams (g) was compared to 16 Hz-induced change in tension in the presence of the NO antagonist L-NAME (NM) placed in the BAEC bath; the cGMP antagonist methylene blue (MB) placed in the RTA bath; the K_{ATP} hyperpolarization antagonist glibenclamide (GLB) placed in either the denuded RTA bath (R) or in the BAEC bath (E); and the K_{Ca}^{++} antagonist tetraethylammonium (TEA) placed in the RTA bath. The L-NAME response was compared to the L-NAME with the NO substrate L-arginine (ARG+NM); placed in the BAEC bath. All values (*$p<.05$; **$p<.01$) are significant as compared to the control response.

Δ Tension (g)
2.0
1.0
0.0
-1.0
-2.0
-3.0
Control
KCl
TEA
MB
GLB (R)
GLB (E)
NM
Bariur ARG+NM
-1.73
* -1.10
-1.56
** +.91
* -1.37
-1.50
** +.60
* -1.27
-1.54

relaxation (16 Hz control -1.730 ± 0.130 g; GLB-R -1.37 ± 0.07 g; $BaCl_2$ -1.27 ± 0.06 g; and KCl -1.10 ± 0.07 g; respectively). TEA (1 mM) had no significant effect when placed in the denuded RTA bath. L-arginine (1 mM) reversed the constriction caused by L-NAME (L-NAME +0.60 ± 0.24 g L-arginine -1.54 ± 0.13 g. Glibenclamide (1 μM; GLB-E) in the endothelial cell bath had no effect.

Pressurized Experiments With EFS (1.0 ms, 30 v)

Figure 8 shows a log dose-pressure curve for PE (Figure 8A) and a frequency-pressure curve for EFS (1.0 ms, 30 v; Figure 8B) in a pressurized RTA. The pressurized arterial response to PE alone is compared with PE plus L-NAME. The pressurized arterial response to EFS alone and following exposure to TTX and L-NAME (100 μM) are also shown. These figures demonstrate that EFS-induced constriction is completely inhibited by TTX. Furthermore, the level of tone is increased following L-NAME treatment.

Figure 9 shows the EFS-induced (1.0 ms, 30 v) change in pressure (relaxation) following PE preconstriction (30 μM) in a pressurized RTA. The minimum EFS-induced relaxation response was -6.32 ± 0.24 mm Hg at 0.50 Hz while the 64 Hz maximal response was -90.99 ± 12.29 mm Hg (open squares). Figure 9 also shows that following PE preconstriction, EFS caused a biphasic response at higher frequencies (closed triangles). As stimulus frequency increased, the EFS-induced response changed from a monophasic decrease in

Figure 8. (A) The dose-pressure curve (mm Hg) for phenylephrine (PE) in the intact, pressurized rat tail arteries with and without the NO antagonists L-NAME (NM). (B) The frequency-pressure curve (mm Hg) for electrical field stimulation (EFS, 1.0 ms, 30 v) in the intact, pressurized rat tail arteries with and without the NO antagonist L-NAME (NM) and the voltage operated Na+ channel antagonists tetrodotoxin (TTX).

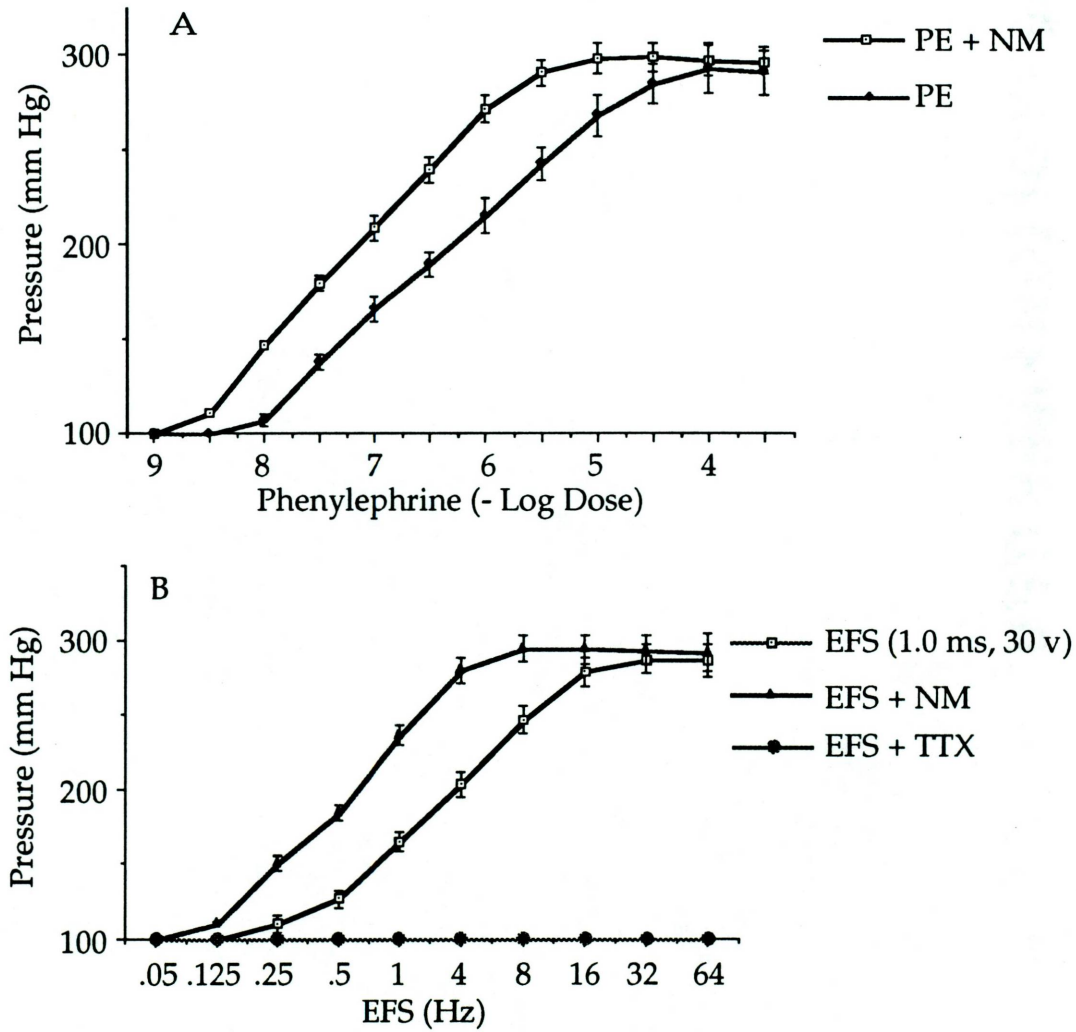
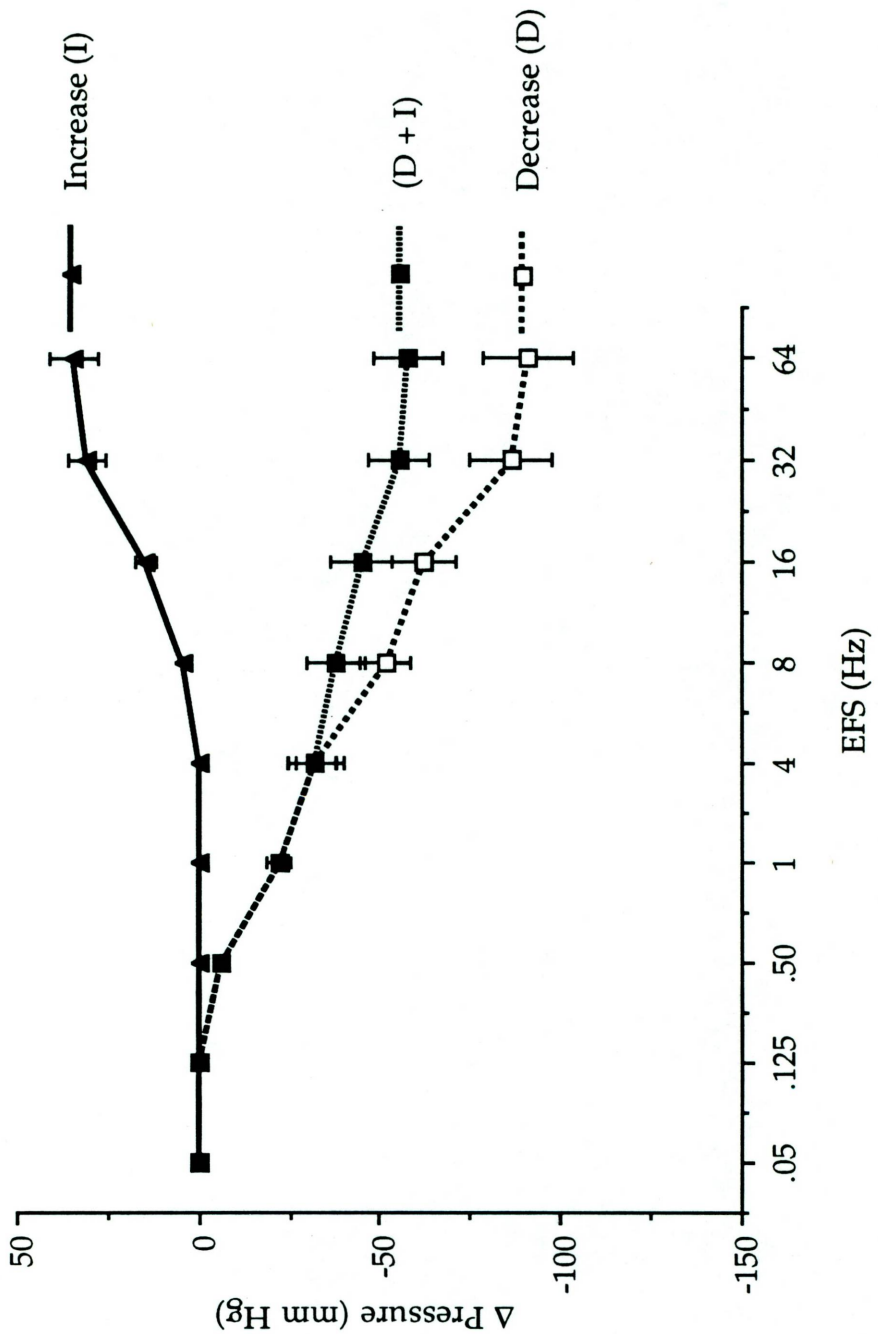

A
300
200
100
Pressure (mm Hg)
9
8
7
6
5
4
Phenylephrine (- Log Dose)
PE + NM
PE
B
300
200
100
Pressure (mm Hg)
.05 .125 .25 .5 1 4 8 16 32 64
EFS (Hz)
EFS (1.0 ms, 30 v)
EFS + NM
EFS + TTX

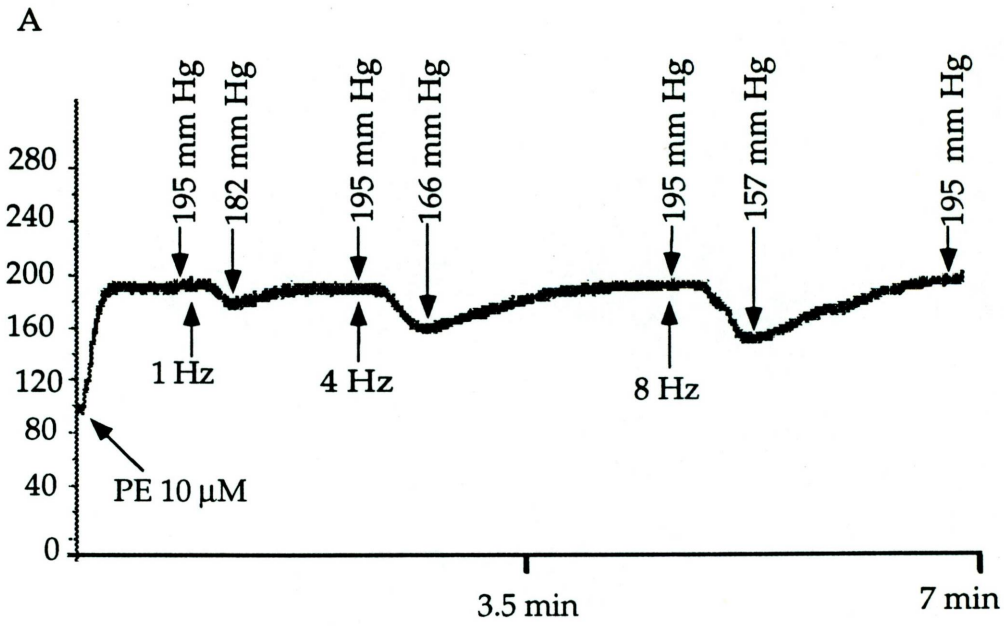
Figure 9. The frequency-pressure curves (mm Hg) for electrical field stimulation-induced (EFS; 1.0 ms, 30 v) change in pressure in the intact, pressurized, and phenylephrine precontricted rat tail arteries. At 8 Hz, the monophasic decrease in pressure (D; open squares) is preceded by an increase in pressure (I; closed trangles). The difference between these changes in pressure are shown in the decrease pressure plus increase pressure curve (D+I; closed squares).

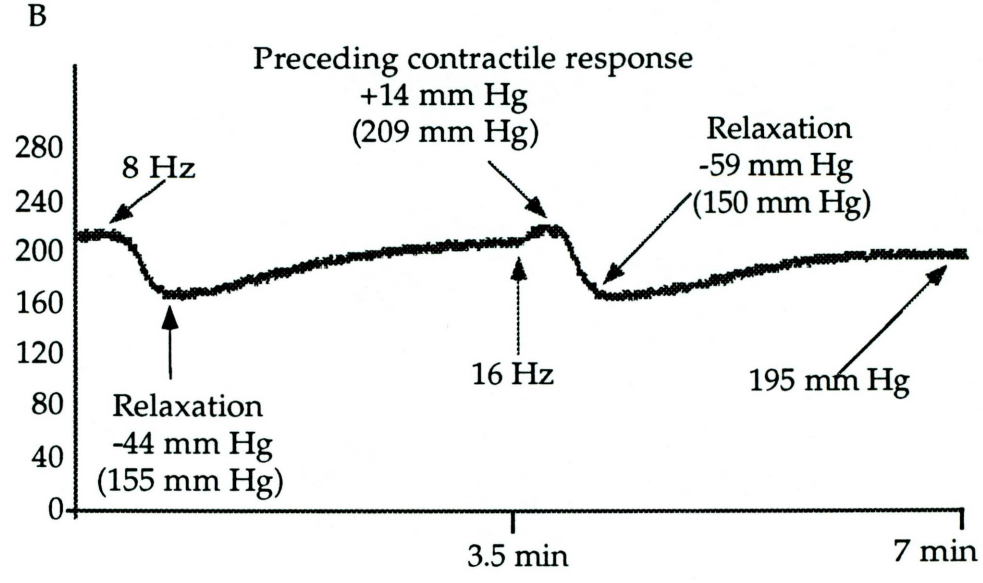

Increase (I)
(D + I)
Decrease (D)
Δ Pressure (mm Hg)
50
0
-50
-100
-150
.05
.125
.50
1
4
8
16
32
64
EFS (Hz)

pressure to a biphasic increase in pressure then decrease in pressure. The monophasic relaxation response was evident between 0.50 Hz and 4.0 Hz and ranged from -6.32 ± 0.24 mm Hg to -32.25 ± 5.58 mm Hg of relaxation. The biphasic response first appeared between 4 Hz and 8 Hz and continued through 64 Hz. The biphasic response was characterized by an increase in pressure (I), ranging from +4.13 ± 0.41 mm Hg to +34.48 ± 6.75 mm Hg, preceding the decrease in pressure (D), ranging between -51.70 ± 6.95 mm Hg to -90.99 ± 12.29 mm Hg. The mean difference in pressure between these two responses is represented by curve D+I curve (closed squares). The mean pressure values ranged from -6.32 ± 0.24 mm Hg to -57.50 ± 9.50 mm Hg. Frequencies were limited to 64 Hz to avoid damage to the smooth muscle.

Figure 10 shows the EFS-induced decrease and increase pressure change (relaxation and constriction, respectively) in a typical experiment with the intact, pressurized and preconstricted rat tail artery. In Figure 10A, the rat tail artery was pressurized to 100 mm Hg and then stimulated with 10 μM phenylephrine (PE). The EFS-induced (1.0 ms, 30 v) relaxation following PE preconstriction are in response to 5 seconds of EFS at 1 Hz, 4 Hz, and 8 Hz, respectively. At 1 Hz, the vessel responded with a relaxation of -13 mm Hg. The relaxation required approximately 3 seconds. An additional 3-4 seconds are required for the pressure to return to basal stimulated levels. The 4 hz-induced relaxation response was similar in onset (5-6 seconds) to the

Figure 10. (A) The decrease in pressure (mm Hg) of an intact, pressurized, and phenylephrine preconstricted rat tail artery following electrical field stimulation (EFS, 1.0 ms, 30 v). (B) The decrease in pressure following EFS (8 Hz, 1.0 ms, 30 v) and the increase and decrease in pressure following EFS (16 Hz, 1.0 ms, 30v). The 16 Hz-induced change in tension illustrates the biphasic characteristic of higher frequency EFS in the pressurized RTA.


A
195 mm Hg
182 mm Hg
195 mm Hg
166 mm Hg
195 mm Hg
157 mm Hg
195 mm Hg
280
240
200
160
120
80
40
0
1 Hz
4 Hz
8 Hz
PE 10 µM
3.5 min
7 min

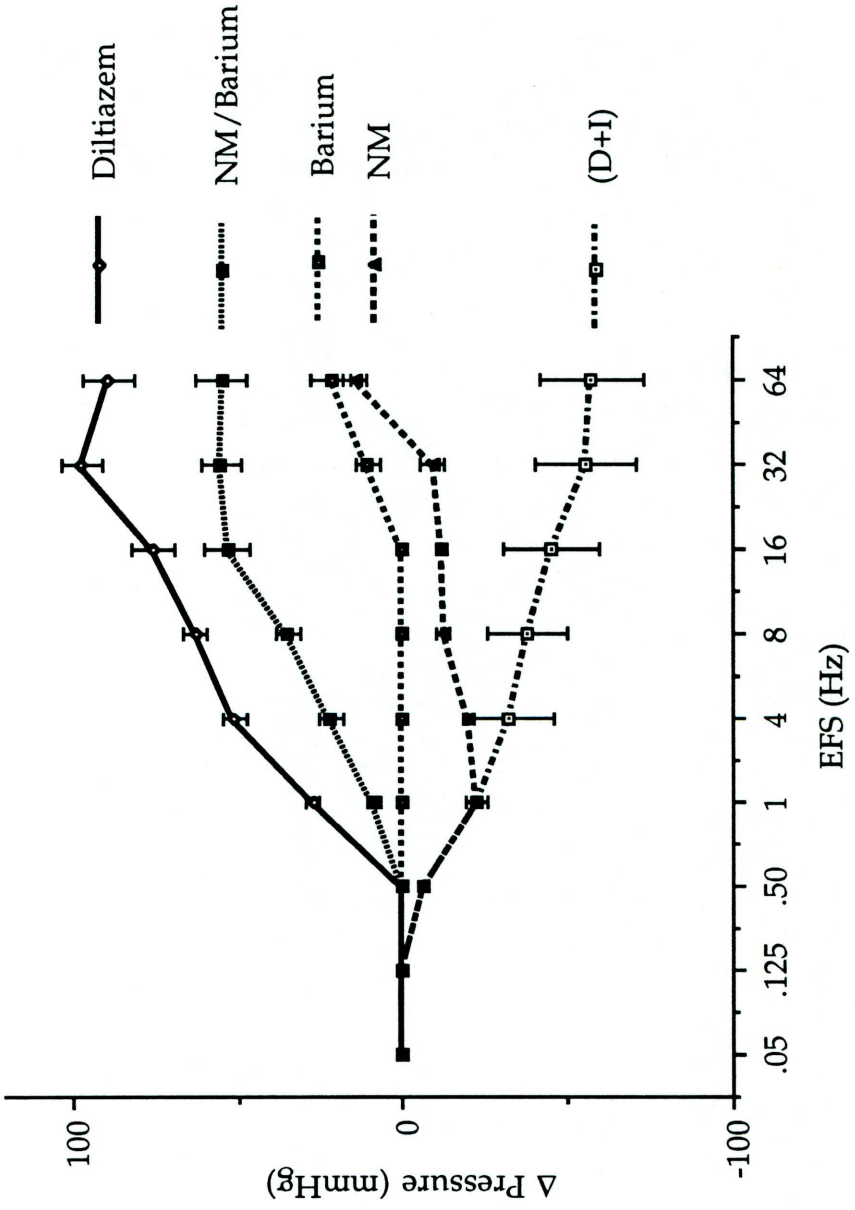

B
Preceding contractile response
+14 mm Hg
(209 mm Hg)
Relaxation
-59 mm Hg
(150 mm Hg)
8 Hz
280
240
200
160
120
80
40
0
16 Hz
195 mm Hg
Relaxation
-44 mm Hg
(155 mm Hg)
3.5 min
7 min

1 Hz-induced response although time to return to prestimulated levels was longer, between 12-14 seconds. The EFS-induced relaxation at 4 Hz was also greater than the 1 Hz response, -29 mm Hg at 4 Hz versus -13 mm Hg at 1 Hz. The last illustration in Figure 10A shows the 8 Hz EFS-induced relaxation. The time to maximal relaxation was approximately 4 seconds with the maximal response of -38 mm Hg. The time required for return to pre-stimulated levels was 15 seconds.

In 10B, another 8 Hz response is shown with similar times to peak relaxation (6 seconds), maximal relaxation (-44 mm Hg) and time to return to pre-EFS values (25 seconds). Again, this response is characteristic of a monophasic EFS-induced relaxation. Figure 10B also shows the 16 Hz EFS-induced (1.0 ms, 30 v) relaxation. Unlike the preceding relaxation, the 16 Hz-induced relaxation was preceded by an increase in pressure (constriction), to approximately +14 mm Hg, for the duration of the EFS period (5 seconds). Following cessation of EFS, the vessel relaxed a total of -59 mm Hg from the peak of the EFS constriction. The time to maximal relaxation was 5 seconds and the time to return to the pre-EFS levels was approximately 25 seconds. This is a typical experiment with EFS, although variations in the absolute magnitudes of pressure change did occur.

The EFS-induced relaxation was analyzed in the presence of nitric oxide, hyperpolarization and VOCC antagonists (Figure 11). The effects of

Figure 11. The frequency-pressure (D+I) curve (mm Hg) for electrical field stimulation-induced (EFS; 1.0 ms, 30 v) in the intact, pressurized, and phenylephrine preconstricted rat tail arteries. The control (D+I) curve is shown with the (D+I) curves in the presence of either the NO antagonist L-NAME (NM); the K_{ATP} hyperpolarization antagonist $BaCl_2$ (Barium); with a combination of L-NAME and $BACL_2$ (NM/Barium); and with the voltage operated Ca^{++} channel antagonist diltiazem. All points between 4-64 Hz are significantly ($p<.05$) different from the (D+I) curve. At 64 Hz, the L-NAME and BaCl2 response were not statistically significantly from each other.


Diltiazem
NM/Barium
Barium
NM
(D+I)
100
0
-100
Δ Pressure (mmHg)
.05
.125
.50
1
4
8
16
32
64
EFS (Hz)

pharmacological agents on RTA pressure were compared to the mean relaxation curve (D+I). Mean relaxations were between -6.32 ± 0.24 mm Hg (0.05 Hz) and -57.50 ± 9.50 mm Hg (64 Hz). Luminal infusion with 100 μM L-NAME had no effect at lower frequencies (between 0.05 Hz-1.0 Hz). As stimulus frequency increased, the EFS-induced relaxation was decreased from the control 4 Hz-induced relaxation of -32.25 ± 7.58 mm Hg to -19.90 ± 1.30 mm Hg. The maximal 64 Hz-induced relaxation was reduced from -57.50 ± 9.50 to +14.39 ± 3.87 mm Hg. The 8 Hz to 64 Hz-induced responses in the presence of L-NAME were significantly different from the intact mean (D+I) response ($p<.05$). Addition of 100 μM $BaCl_2$ prior to PE preconstriction completely inhibited the EFS-induced relaxation between 0.50 Hz to 16 Hz; in other words, the vessel maintained its pre-EFS level of pressure throughout the EFS period. However, the 32 Hz and 64 Hz-induced relaxation changed to constriction following $BaCl_2$ exposure (32 Hz, -55.40 ± 8.59 mm Hg to +10.59 ± 3.70 mm Hg; 64 Hz, -57.50 ± 9.50 mm Hg to +34.10 ± 3.84 mm Hg). All responses in the presence of $BaCl_2$ were significantly different from intact mean (D+I) responses ($p<.05$). The EFS-induced relaxation was reversed to higher levels of pressure in the presence of diltiazem (100 μM), and following combined treatment with L-NAME and $BaCl_2$, (Figure 11). All responses in the presence of diltiazem,

and L-NAME plus $BaCl_2$ are significantly different from the intact mean (D+I) response ($p<.05$).

Figure 12 shows the maximal (64 Hz) EFS-induced (1.0 ms, 30 v) relaxation in a pressurized, intact and preconstricted RTA. Figure 12 also shows the sensitivity of this response to antagonists of NO, hyperpolarization and VOCC. The 64 Hz-induced relaxation was -90.99 ± 3.29 mm Hg. Diltiazem (10 μM), KCl (60 mM), and $BACL_2$ (100 μM), applied independently, reversed the relaxation from -90.99 ± 3.29 mm Hg to +89.02 ± 7.51 mm Hg; +21.69 ± 3.71 mm Hg and +34.10 ± 3.84 mm Hg, respectively. The NO antagonist L-NAME (100 μM) increased the pressure in the RTA to +14.39 ± 3.87 mm Hg. The 64 Hz-induced response with L-NAME returned to control levels following exposure to 1 mM L-arginine (-74.06 ± 4.018 mm Hg), a value insignificant from the control value. In the presence of L-NAME (100 μM) and $BaCl_2$ (100 μM) arterial pressure increased to +54.88 ± 7.51 mm Hg. All responses in the presence of diltiazem, KCl, $BaCl_2$, and L-NAME were significantly different from the 64 Hz control response.

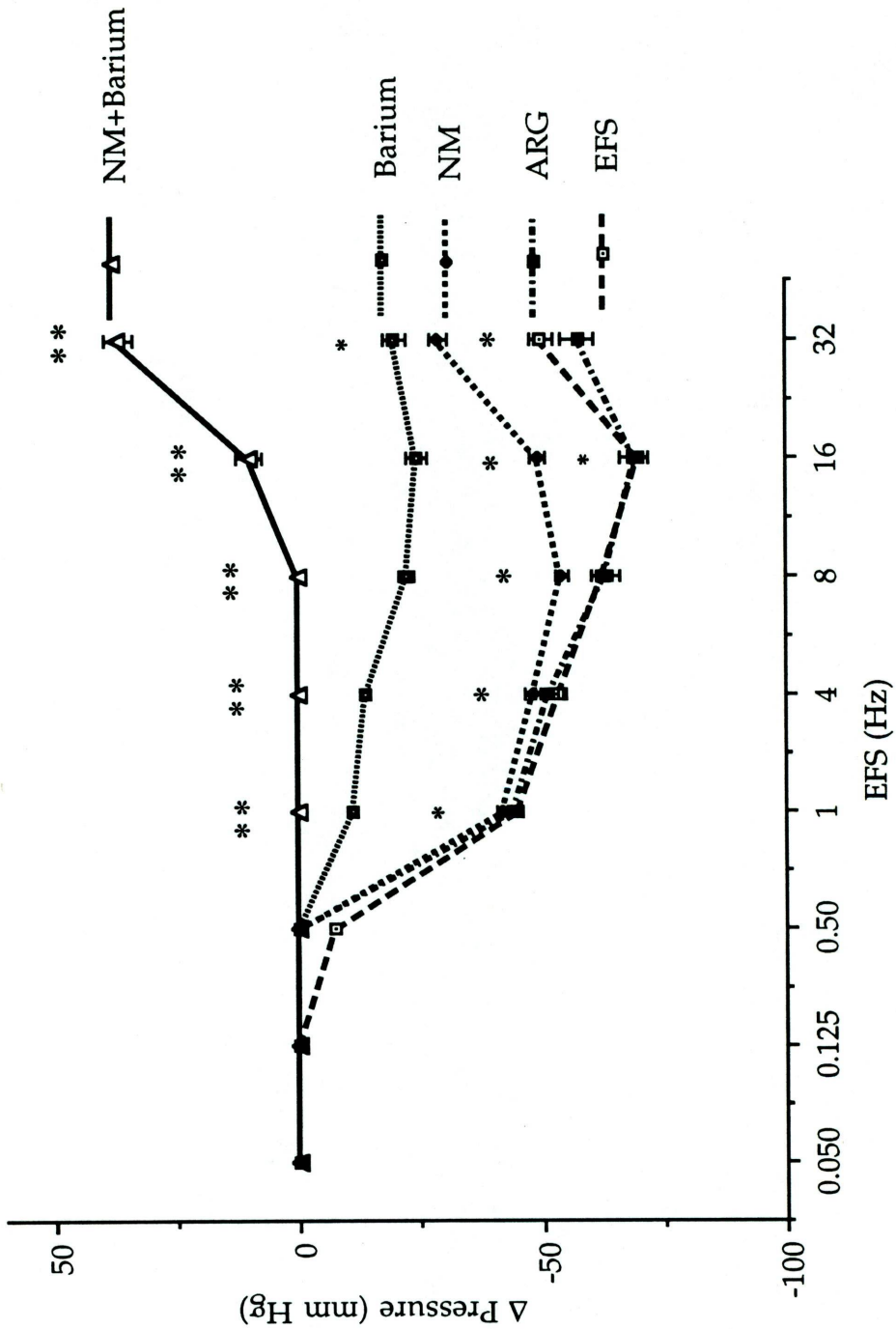
Pressurized Experiments With EFS (0.1 ms, 50 v)

Figure 13 shows the EFS-induced (0.1 ms, 50 v) relaxation following PE (30 μM) preconstriction of an intact, pressurized RTA. The minimum EFS-induced relaxation was -7.71 ± 1.09 mm Hg at 0.50 while the 16 Hz-induced relaxation was maximal (-69.97 ± 2.17 mm Hg). The 1 Hz, 4 Hz

Figure 12. The control (64 Hz, 1.0 ms, 30 v) electrical field stimulation-induced change in pressure (mm Hg) in the intact, pressurized, and phenylephrine preconstricted rat tail arteries. The control change in pressure is shown in comparison to the 64 Hz-induced responses in the presence of either the NO antagonist L-NAME (NM); the K_{ATP} antagonist $BaCl_2$ (Barium); the nonspecific hyperpolarization antagonist KCl; after L-NAME (NM) replacement with the NO substrate L-arginine (ARG); and in combination with L-NAME and $BaCl_2$ (NM/Barium). All values are significant ($p<.05$) as compared to the control response.

Δ Pressure (mm Hg)
300
200
100
0
-100
-200
-300
Control
Diltiazem
KCl
Barium
NM
ARG
NM/Barium
-90.99
+89.02
+21.69
+34.10
+14.39
-74.10
+54.88
*
*
*
*
*
*

Figure 13. The frequency-pressure curve (mm Hg) for electrical field stimulation-induced (EFS; 0.1 ms, 50 v) change in pressure in the intact, pressurized, and phenylephrine preconstricted rat tail arteries. The frequency-pressure control curve for electrical field stimulation-induced change in pressure is shown along with the EFS-induced curves in the presence of either the NO antagonist L-NAME (NM); the K_{ATP} hyperpolarization antagonist $BaCl_2$ (Barium); after L-NAME replacement with the NO substrate L-arginine (ARG); and with L-NAME and $BaCl_2$ (NM/Barium) in combination. All values are significant (*$p<.05$; **$p<.01$) as compared to EFS curve.


NM+Barium
Barium
NM
ARG
EFS
Δ Pressure (mm Hg)
50
0
-50
-100
0.050
0.125
0.50
1
4
8
16
32
EFS (Hz)

and 8 Hz responses were: -44.68 ± 1.33 mm Hg; -53.47 ± 1.65 mm Hg; and -62.29 ± 1.96 mm Hg, respectively. The 32 Hz-induced relaxation (-49.93 ± 2.29 mm Hg) was significantly less than the 16 Hz-induced relaxation ($p<.05$). Unlike the EFS relaxation with 1.0 ms and 30 v, the 0.1 ms and 50 v parameters did not cause a biphasic response (Increase and Decrease pressure changes) at the higher stimulation frequencies (8 Hz to 32 Hz). Figure 13 also shows the frequency-pressure curves (mm Hg) for antagonists of nitric oxide and hyperpolarization. Luminal infusion with 100 μM L-NAME had no significant effect at lower frequencies (between 0.05-8 Hz). However, the EFS-induced relaxation following 16 Hz and 32 Hz were significantly decreased following exposure to L-NAME (control 16 Hz, -69.97 ± 2.17 mm Hg, 16 Hz with L-NAME -25.17 ± 2.09 mm Hg; control 32 Hz, -49.93 ± 2.29 mm Hg, and 32 Hz with L-NAME -28.68 ± 1.89 mm Hg; $p<.05$). Replacing the luminal L-NAME with L-arginine (1 mM) caused the 16 Hz and 32 Hz EFS-induced relaxation to return to control values (-63.050 ± 2.90 mm Hg; -57.39 ± 3.26 mm Hg, respectively). Addition of $BaCl_2$ (100 μM) prior to PE preconstriction, significantly inhibited the EFS-induced relaxation between 0.50 and 32 Hz (Figure 13; $p<.05$). When L-NAME and $BaCl_2$ were used in combination, the EFS-induced relaxations at lower frequencies (0.50 Hz to 8 Hz) were completely inhibited, while the response at higher frequencies (16 Hz to 32 Hz) was greater pressure ($p<.01$).

Figure 14 shows the maximum response to EFS (0.1 ms, 50 v) in an intact, pressurized, and preconstricted RTA occurred at 16 Hz. Figure 14 also shows the 16 Hz response with nitric oxide, hyperpolarization and VOCC antagonists. The EFS-induced relaxation at 16 Hz was -69.97 ± 2.17 mm Hg. Luminal infusion of L-NAME reduced the EFS-induced pressure change to -49.05 ± 1.71 mm Hg ($p<.05$). Replacing luminal L-NAME with L-arginine (1 mM) caused the EFS-dependent relaxation to return to control values (-63.05 ± 2.90 mm Hg). In a separate set of experiments, addition of either 100 μM $BaCl_2$ or 60 mM KCl reduced the 16 Hz-induced relaxation to -25.17 ± 2.09 mm Hg and -17.80 ± 2.09 mm Hg of relaxation, respectively ($p<.05$). Diltiazem (a VOCC antagonist) reversed the 16 Hz-induced relaxation to +33.80 ± 3.77 mm Hg of constriction ($p<.01$). When L-NAME and $BaCl_2$ were added in combination, the 16 Hz-induced relaxation reversed into a contractile response (+21.41 ± 2.6 mm Hg) ($p<.01$).

Simulated Passive Membrane Experiment With EFS

Figure 15 shows the effects of EFS on membrane potential with two different membrane time constants. The long pulse duration parameter caused the simulated passive membrane to summate between 8 Hz and 16 Hz. The threshold membrane potential changed from 240.70 mV to 477.48 mV (150 ms time constant) and from 186.00 mV to 371.10 mV (500 ms time constant) between the 8 Hz and 16 Hz EFS parameters. The

Figure 14. The control (16 Hz, 0.1 ms, 30 v) electrical field stimulation-induced (EFS) change in pressure (mm Hg) in the intact, pressurized, and phenylephrine preconstricted rat tail arteries. The control change in pressure is shown with the 16 Hz-induced responses in the presence of either the NO antagonist L-NAME (NM); the K_{ATP} hyperpolarization antagonist $BaCl_2$ (Barium); the nonspecific hyperpolarization antagonist KCl; after L-NAME replacement with the NO substrate L-arginine (ARG); with the voltage operated Ca^{++} channel antagonist diltiazem; and with L-NAME and $BaCl_2$ (NM/Barium) in combination. All values are significant (*$p<.05$; **$p<.01$) as compared to the control response.

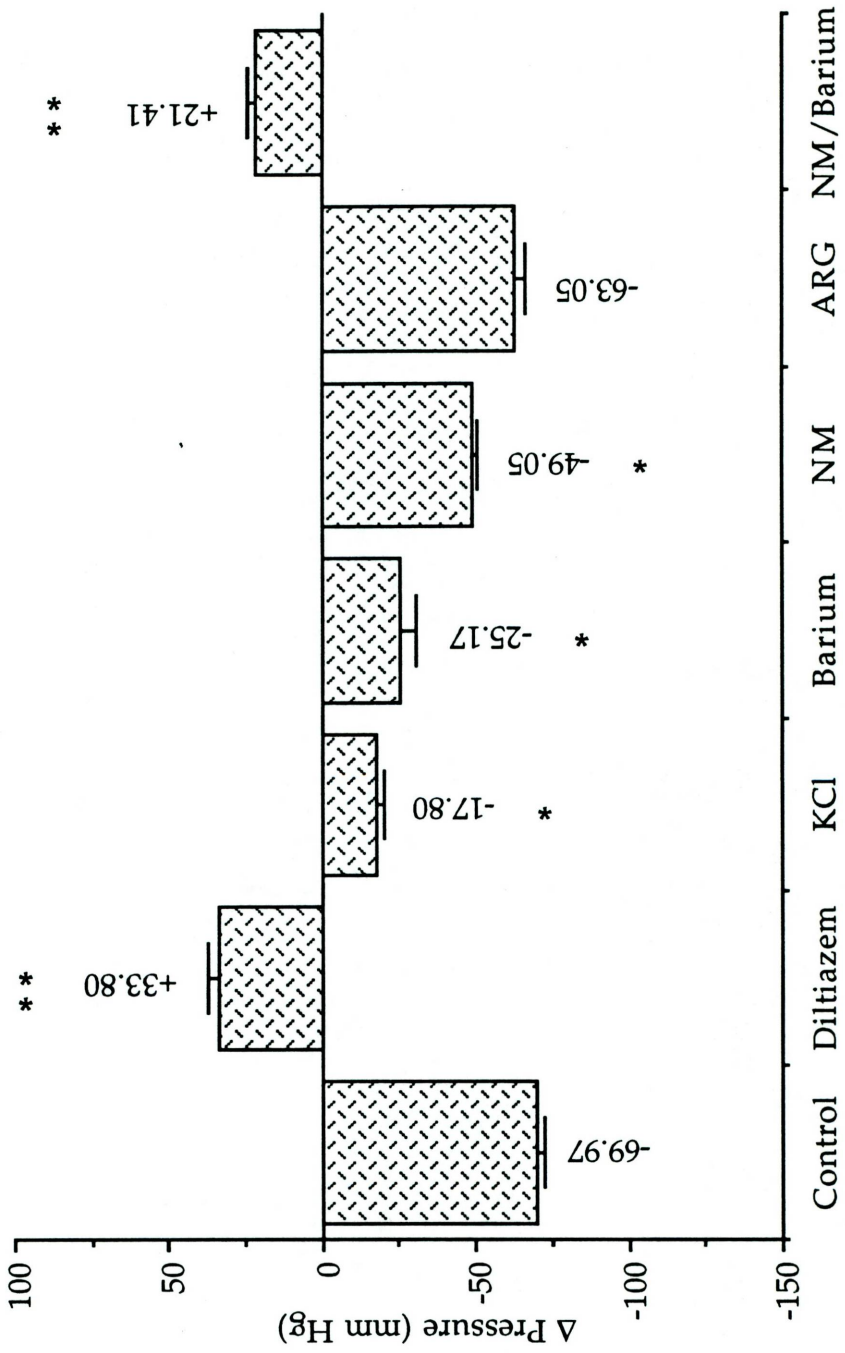
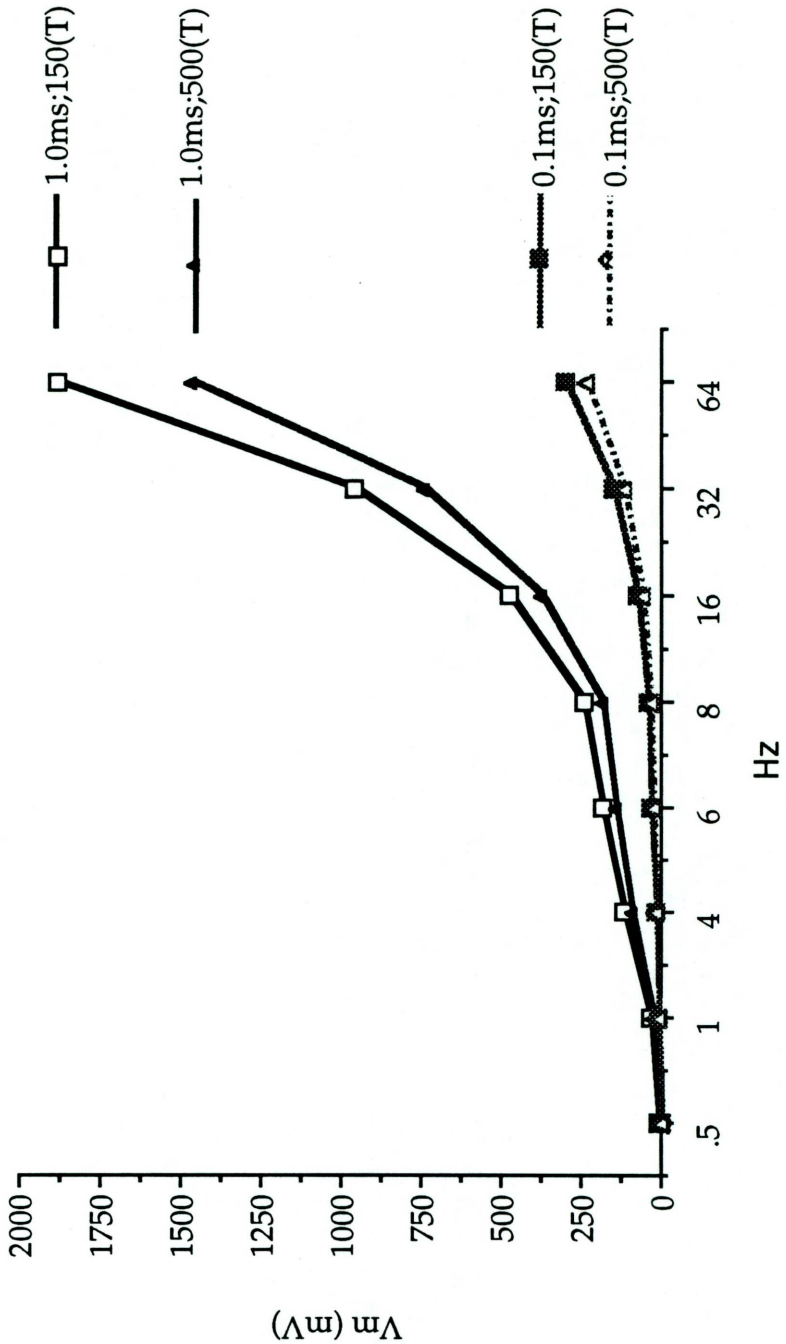

Δ Pressure (mm Hg)
100
50
0
-50
-100
-150
Control
Diltiazem
KCl
Barium
NM
ARG
NM/Barium
-69.97
+33.80
**
-17.80
*
-25.17
*
-49.05
*
-63.05
+21.41
**

Figure 15. The influence of pulse duration and membrane time (T) constant on temporal summation and the membrane potential. Two membrane time constants (150 ms and 500 ms) were used with either the long (1.0 ms) or short (0.1 ms) pulse duration during changes in EFS frequencies.


1.0ms;150(T)
1.0ms;500(T)
0.1ms;150(T)
0.1ms;500(T)
Vm (mV)
2000
1750
1500
1250
1000
750
500
250
0
.5
1
4
6
8
16
32
64
Hz

short pulse duration (0.1 ms) parameter caused the artificial membrane to summate between 16 Hz and 32 Hz. The threshold membrane potential changed between 16 Hz and 32 Hz from 75.87 mV to 153.90 mV (150 ms time constant) and from 59.70 mV to 120.23 mV (500 ms time constant).

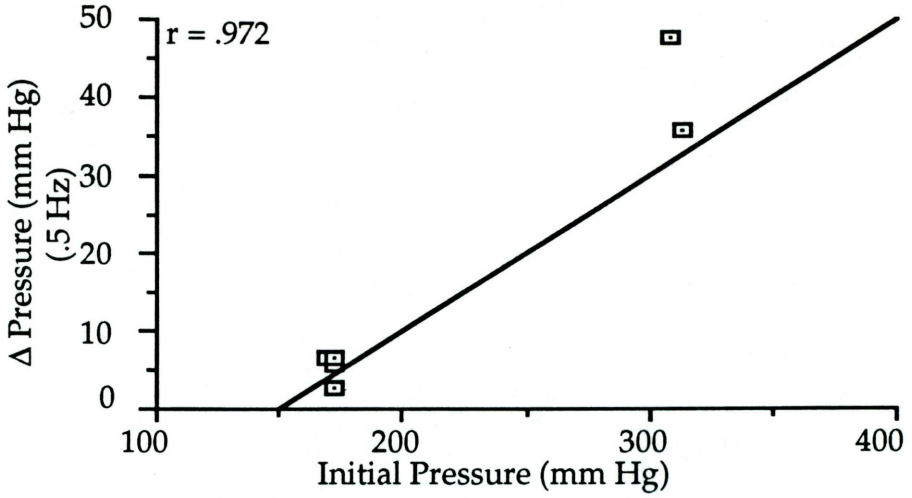
Pressure/Relaxation Relationship

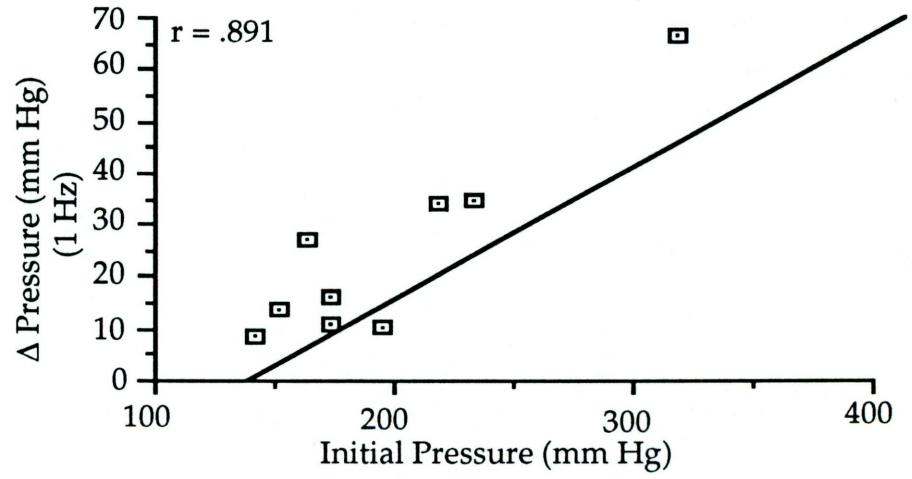
A significant correlation was seen between the level of preconstriction (pressure in mm Hg) and degree of relaxation following EFS stimulation. Figure 16 and Figure 17 shows 6 scatter diagrams relating initial tension to EFS-induced (0.50-32 Hz, 1.0 ms, 30 v) relaxation. The coefficient of linear correlation (r) are: 0.50 Hz, .972; 1 Hz, .891; 4 Hz, .644; 8 Hz, .693; 16 Hz, .664; and 32 Hz, .712, (all scatters have between 6-21 points from 7 arteries).

Membrane Potential Recordings

The relationship between the smooth muscle membrane hyperpolarization and pressure changes in the intact, pressurized, and PE preconstricted (30 μM) RTA are presented in Figure 18. In 18A, the vessel had been preconstricted with 30 μM PE causing an increase in pressure from 100 mm Hg (resting level) to 186 mm Hg. The pre-EFS membrane potential (E_M) decreased from a resting potential of -63 mV to -26 mV following the PE preconstriction. A six pulse EFS (0.5 Hz, 1.0 ms 30 v) train caused the membrane to hyperpolarize from the -26 mV preconstricted level to -57 mV.

Figure 16. Scatter diagrams showing the relationship between electrical field stimulation-induced (1.0 ms, 30 v) change in pressure (mm Hg) and initial pressure.


r = .972
Δ Pressure (mm Hg)
(.5 Hz)
50
40
30
20
10
0
100
200
300
400
Initial Pressure (mm Hg)


r = .891
Δ Pressure (mm Hg)
(1 Hz)
70
60
50
40
30
20
10
0
100
200
300
400
Initial Pressure (mm Hg)

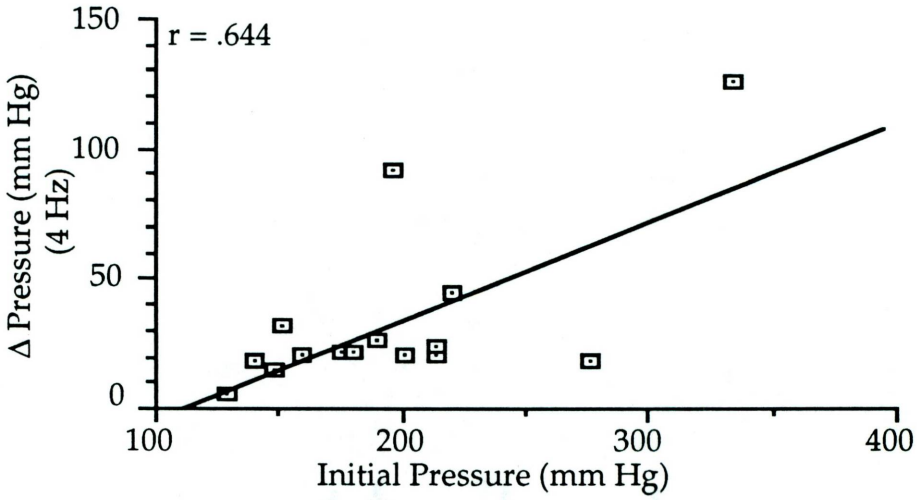
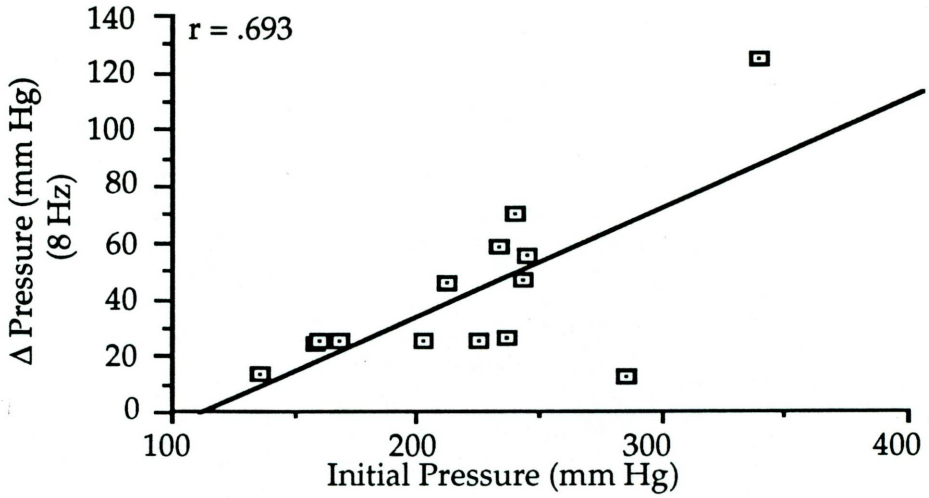
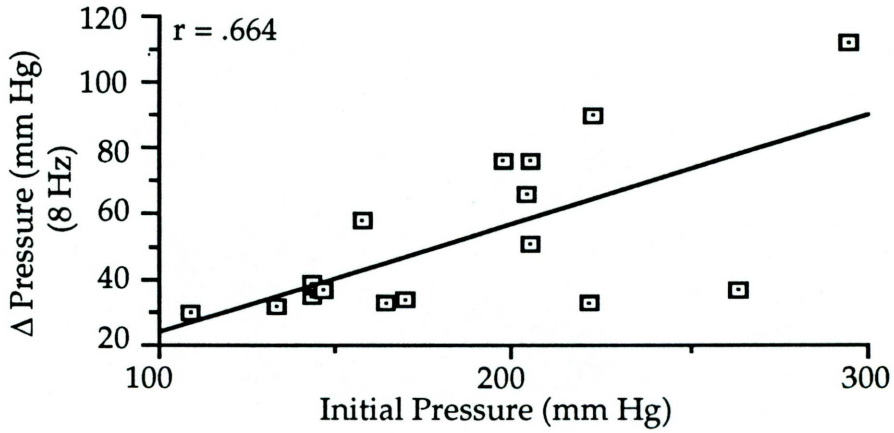

r = .644
Δ Pressure (mm Hg)
(4 Hz)
150
100
50
0
100
200
300
400
Initial Pressure (mm Hg)

Figure 17. Scatter diagrams showing the relationship between electrical field stimulation-induced (1.0 ms, 30 v) change in pressure (mm Hg) and initial pressure.


r = .693
Δ Pressure (mm Hg)
(8 Hz)
140
120
100
80
60
40
20
0
100
200
300
400
Initial Pressure (mm Hg)


r = .664
Δ Pressure (mm Hg)
(8 Hz)
120
100
80
60
40
20
100
200
300
Initial Pressure (mm Hg)

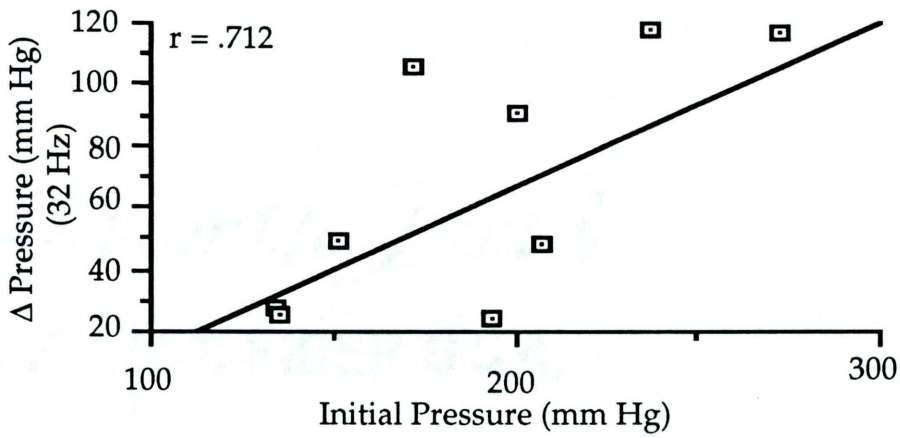
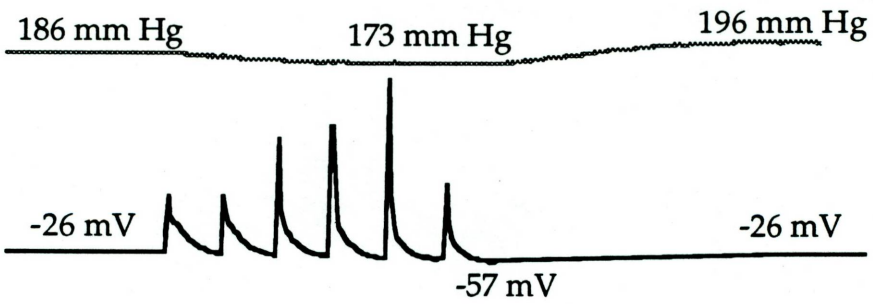
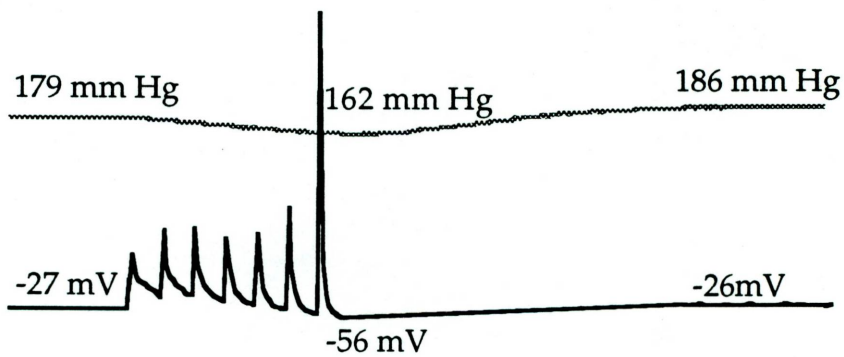

r = .712
Δ Pressure (mm Hg)
(32 Hz)
120
100
80
60
40
20
100
200
300
Initial Pressure (mm Hg)

Figure 18. Three typical experiments illustrating the relationship between smooth muscle membrane hyperpolarization and relaxation in the intact, pressurized, and phenylephrine preconstricted, rat tail arteries. (EFS = A. 0.5 Hz, 1.0 ms, 30 v; B. 1.0 Hz, 1.0 ms, 30 v; C. 0.5 ms, 1.0 Hz, 50 v). The top trace units are in mm Hg. The bottom trace units are in millivolts (mV). Spikes correspond to the number of stimulus pulses at each EFS parameter.

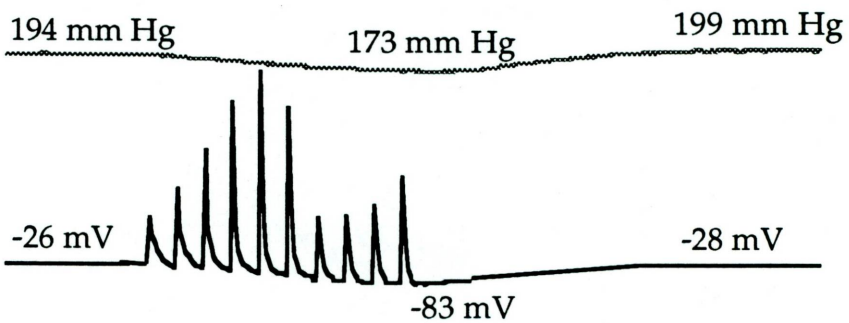
A


186 mm Hg
173 mm Hg
196 mm Hg
-26 mV
-26 mV
-57 mV

B


179 mm Hg
162 mm Hg
186 mm Hg
-27 mV
-26mV
-56 mV

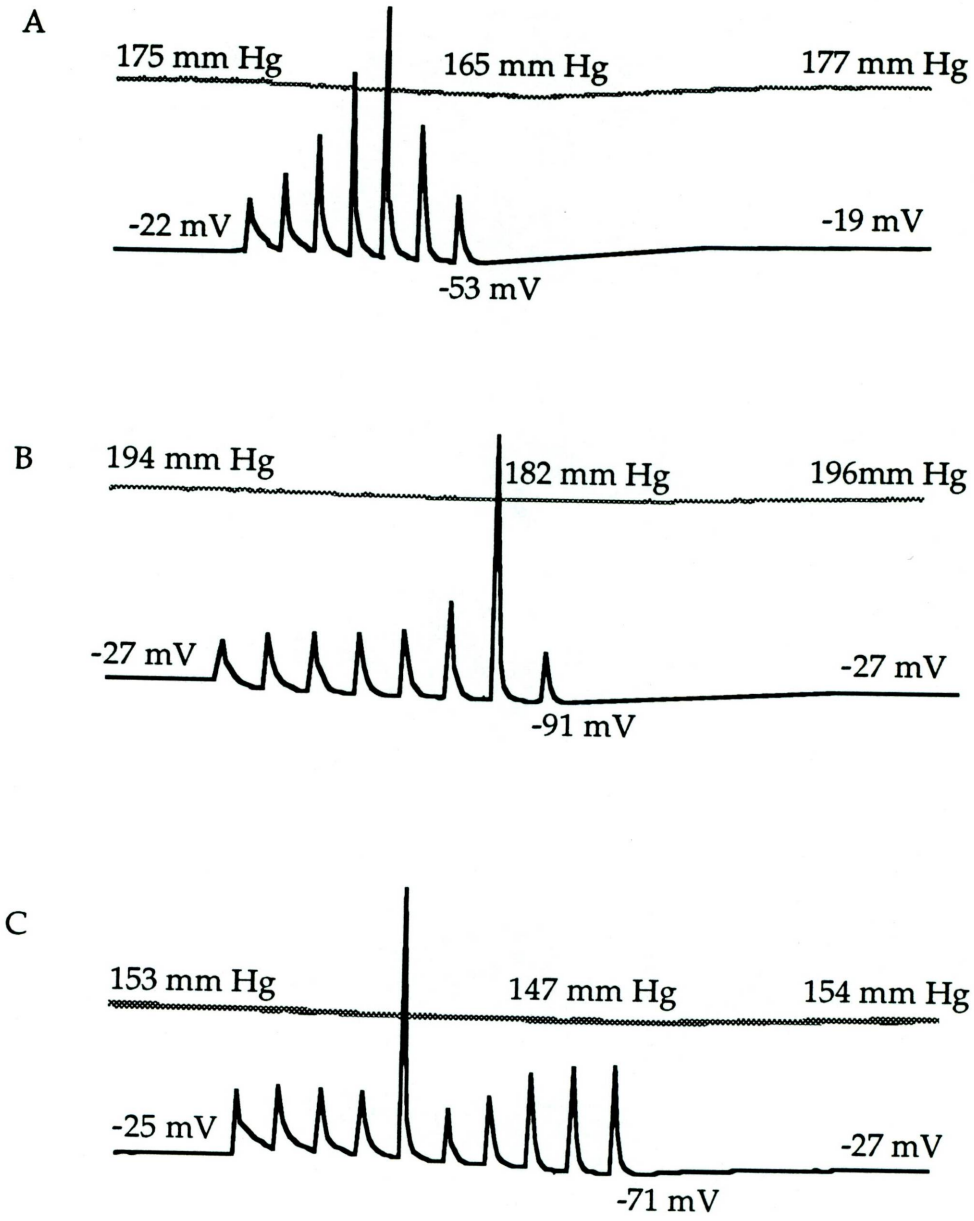
C


194 mm Hg
173 mm Hg
199 mm Hg
-26 mV
-28 mV
-83 mV

The first pulse caused the membrane to hyperpolarize to -31 mV, pulses two through five caused further hyperpolarization. The corresponding change in intraluminal pressure was from 186 mm Hg to 173 mm Hg, a -13 mm Hg relaxation. Following EFS, vessel pressure increased to 196 mm Hg, a value greater than the original value, in approximately four seconds. Membrane potential returned to the control value (-26 mV) eight seconds after cessation of stimulation. In 18B, the PE preconstriction increased intraluminal pressure to 179 mm Hg and caused E_M to depolarize to -27 mV. A seven pulse train of EFS (1 Hz, 1.0 ms, 30 v) resulted in a 29 mV hyperpolarization (-56 mV) and a subsequent -17 mm Hg pressure change (162 mm Hg). The E_M returned within six seconds to -26 mV while intraluminal pressure required eight seconds to return to 186 mm Hg, slightly more pressurized than control. In 18C, PE preconstriction caused intraluminal pressure to increase 94 mm Hg to 194 mm Hg. E_M depolarized to -26 mV from a resting value of -66 mV. Ten pulse of EFS (0.5 ms, 50 v, 1 Hz) caused the E_M to hyperpolarize 47 mV to -83 mV. The corresponding relaxation was -21 mm Hg, or to a pressure of 173 mm Hg. E_M returned to the control value (-28 mV) in eight seconds. Intraluminal pressure required ten seconds to reach an intraluminal pressure of 199 mm Hg, slightly more pressurized than control.

Figure 19 again shows the relationship between membrane hyperpolarization and pressure following EFS. In 19A, the preconstricted

Figure 19. Three typical experiments illustrating the relationship between smooth muscle membrane hyperpolarization and relaxation in the intact, pressurized, and phenylephrine preconstricted, rat tail arteries. (EFS = A. 1.0 Hz, 1.0 ms, 30 v; B. 1.0 Hz, 0.5 ms, 30 v; C. 1.0 Hz, 1.0 ms, 30 v). The top trace units are in mm Hg. The bottom trace units are in millivolts (mV). Spikes correspond to the number of stimulus pulses at each EFS parameter.


A
175 mm Hg
165 mm Hg
177 mm Hg
-22 mV
-19 mV
-53 mV
B
194 mm Hg
182 mm Hg
196mm Hg
-27 mV
-27 mV
-91 mV
C
153 mm Hg
147 mm Hg
154 mm Hg
-25 mV
-27 mV
-71 mV

pressure is 175 mm Hg with a E_M of -22 mV. A seven pulse EFS (1.0 ms, 30 v, 1 Hz) caused the intraluminal pressure to decrease -10 mm Hg to 165 mm Hg. E_M increased during EFS from the pre-EFS of -22 mV to -53 mV, a 31 mV hyperpolarization. Cessation of stimulation returns the E_M to -19 mV (slightly more depolarized than control) in 11 seconds. Intraluminal pressure returned to 177 mm Hg, a pressure slightly greater than control, in 14 seconds. In 19B, the preconstricted E_M is -27 mV with a corresponding intraluminal pressure of 194 mm Hg. An eight pulse EFS (0.5 ms, 30 v, 1 Hz) caused the E_M to hyperpolarize to -91 mV, a 64 mV increase. This trace shows E_M reached a maximum of -91 mV. After the EFS period, E_M returned to -27 mV in 10 seconds. Intraluminal pressure decreased -12 mm Hg during EFS to 182 mm Hg. Upon cessation of EFS, intraluminal pressure rose to 196 mm Hg within twelve seconds. In 19C, the preconstricted E_M was -25 mV with a corresponding intraluminal pressure of 153 mm Hg. EFS (1.0 ms, 30v, 1 Hz) caused the E_M to hyperpolarize to -71 mV As was evident in B above, the membrane reached a maximal hyperpolarization. Intraluminal pressure decreased -16 mm Hg to 137 mm Hg. Upon cessation of EFS, E_M required nine seconds to reach a E_M of -45 mv. After four additional seconds, the E_M reached -27 mV. Intraluminal pressure rose from 137 mm Hg to 154 mm Hg within 14 seconds.

DISCUSSION

Frank and Bevan (1983) first described the vasorelaxing effects of electrical field stimulation (EFS) on a precontracted artery. They did not define the mechanism, although removal of the endothelium eliminated the response. Furthermore, opposing the endothelial surface of an intact vessel against the luminal surface of a denuded artery restored EFS-induced relaxation despite the presence of tetrodotoxin (TTX), atropine, propranolol, indomethacin, or verapamil. On the other hand, Kotecha and Neild (1988) showed an EFS-induced relaxation that was endothelium-independent and inhibited by TEA and KCl. Short trains of EFS in the presence of ascorbic acid did not alter this relaxation, thereby excluding the possibility of free radicals as the cause of relaxation. An EFS-induced relaxation equally dependent upon hyperpolarization and NO was shown by Buga and Ignarro (1992). They concluded that endothelial hyperpolarization increased the Ca^{++} influx into the endothelium. This Ca^{++} signal caused the synthesis and release of endothelial NO and subsequent relaxation of the vascular smooth muscle.

The physiological role of EFS-induced relaxation is unknown. However, Hynes *et al.* (1988), Bucher *et al.* (1992), and Vo *et al.* (1992) showed that the level of EFS-induced constriction was increased by denuding the vessel of endothelium or inhibiting the synthesis or effect of NO. Bucher *et al.* (1992) reported that treatment with L-arginine reversed the effects of N-nitro-L-arginine methyl ester (L-NAME), that is induced relaxation.

Bucher *et al.* (1992) concluded that nitric oxide derived from endothelial cells modulated the vasoconstrictor response to nerve stimulation. However, the signal transduction mechanism between the smooth muscle and endothelium was not determined.

Thus evidence exists which clearly defines two regulatory mechanisms controlling vascular smooth muscle tone: (1) hyperpolarization; and, (2) NO secreted from the endothelium.

Hyperpolarization and The Control of Arterial Tone

I showed that EFS caused a frequency-dependent relaxation of preconstricted vessels. At low frequencies of stimulation, relaxation was inhibited by the hyperpolarization antagonists KCl, TEA, $BaCl_2$, and glibenclamide. These hyperpolarization antagonists are known to inhibit K_{ATP} and K_{Ca}^{++} channels (Nelson, 1993). When membrane potential and arterial pressure were measured simultaneously, the EFS-induced relaxation was always preceded by membrane hyperpolarization. I found this hyperpolarization and relaxation to be endothelium-independent and similar to that identified by Kotecha and Neild (1988), and further supported by Furspan (1990). Furspan (1990) identified a large conductance (194 pS) K_{ATP} channel in the RTA that was sensitive to voltage, $BaCl_2$ and ATP. In addition, Kalsner (1992) described a similar endothelium-independent, smooth muscle specific relaxation mechanism. In Kalsner's study arterial

tension was decreased by EFS through direct stimulation of the K_{ATP} sensitive channel and an increase in membrane potential. The increase in membrane potential caused voltage-operated Ca^{++} channels (VOCC) to close on the smooth muscle membrane and reduce Ca^{++} influx. The Na^{+}/Ca^{++} exchanger was then able to work with greater efficiency to extrude intracellular Ca^{++} and thereby cause relaxation. Kalsner (1992) showed that EFS-induced relaxation was inhibited and contraction occurred in a Ca^{++}-reduced environment. These data are identical to those shown in our research. Kalsner (1992) speculated that a reduced Ca^{++} concentration at the inner face of the Na^{+}/Ca^{++} exchanger would make it less able to compete with intracellular Na^{+}. They also suggested that destabilization and depolarization of vascular cell membranes in reduced Ca^{++} lowers the activation of the Na^{+}/Ca^{++} exchanger, whereas high Ca^{++} stabilizes the vascular cell membranes and increases transporter activity.

The physiological role of the low frequency hyperpolarization and relaxation in the RTA is to rapidly modulate minor increases in arterial tone. Any elevation of cytosolic Ca^{++}, change in purine concentration, or depolarization of the smooth muscle membrane would be the stimulus for K^{+} efflux. The K^{+} efflux and subsequent membrane hyperpolarization would close VOCC on the smooth muscle membrane and reduce the entrance of extracellular Ca^{++}. The reduced influx of Ca^{++} allows the extrusion

mechanisms (Ca^{++}-ATPase, Na^{+}/Ca^{++} exchanger) to reduce cytosolic Ca^{++} more efficiently. A reduction in cytosolic Ca^{++} would cause Ca^{++} to dissociate from calmodulin, inactivate MLCK, dephosphorylate serine 19 on myosin light chain, and cause the muscle to relax.

Nitric Oxide and The Control of Arterial Tone

The results of my study show that as frequency of stimulation increases, EFS-induced relaxation is largely due to the release of NO and less to hyperpolarization. These results suggest that EFS can cause endothelial release of NO which modulates the degree of arterial tension. NO synthesis requires an increase in endothelial cytosolic Ca^{++}. The EFS-dependent mechanism that causes the cytosolic Ca++ to increase and leads to the release of NO is still undetermined. A possible mechanism involves EFS-dependent opening of VOCC located on the endothelium. The existence of endothelial VOCC is controversial in light of the overwhelming evidence against them (Takeda *et al.*, 1987; Adams *et al.*, 1989; Luckhoff *et al.*, 1990; Campbell *et al.*, 1991; Dolar *et al.*, 1992). However, an underlying characteristic of all of the above cited studies is their use of cultured endothelial cells, which change morphologically and functionally during subculturing (Busse *et al.*, 1988; Bossu *et al.*, 1989; 1992). Bossu *et al.* (1989; 1992) identified L- and T-type VOCC in freshly isolated endothelial cells. The peak conductance of each was 20.5 pS and 2.8 pS, respectively. These Ca^{++}

conductance values are similar to conductance values for receptor-operated Ca^{++} channels (26 pS and 20 pS) that are known to stimulate NO synthesis (Nilius 1991; Adams *et al.*, 1989).

My data show that freshly isolated BAEC release NO in response to EFS. Effluent from stimulated BAEC caused a frequency-dependent relaxation in preconstricted, endothelium-denuded RTA. This relaxation was inhibited by L-NAME and methylene blue. To our knowledge, no one has shown that EFS caused the release of NO from freshly isolated BAEC. Furthermore, these data provide indirect evidence that EFS causes the endothelium to depolarize and VOCC to open. The entrance of Ca^{++} through endothelial VOCC causes cytosolic Ca^{++} to increase. The subsequent elevation of endothelial cytosolic Ca^{++} is the stimulus that causes the synthesis of NO.

A possible answer to the function of endothelial VOCC may reside with the studies that use EFS to contract resting, perfused arteries. EFS-induced contractions have been shown to cause various degrees of smooth muscle depolarization (Neild and Kotecha, 1988). Furthermore, EFS-induced contraction has also been shown to cause the immediate release of NO from the endothelium (Bucher *et al.*, 1992). This EFS-induced release of NO serves to modulate the degree of arterial tone caused by sympathetic nerve release of norepinephrine. Therefore, sympathetic activation and subsequent vascular smooth muscle depolarization also causes the

endothelium to depolarize. Endothelial depolarization opens VOCC on the endothelial cell membrane and allows extracellular Ca^{++} to enter the cell. The increase in cytosolic Ca^{++} stimulates the synthesis of NO. Although NO release during EFS-induced contractions is well defined, the transduction mechanism, from nerve through vascular smooth muscle to endothelium, is not known. There are three possible mechanisms by which electrical field stimulation may cause simultaneous arterial constriction and nitric oxide release: 1) shear stress; 2) endothelial alpha-2 receptor activation by neuronal NE release; and, 3) an electrochemical signal, generated within the smooth muscle and propagated to the endothelium which stimulates the synthesis of NO. Since our pressurized system was static, the shear stress hypothesis was not a factor.

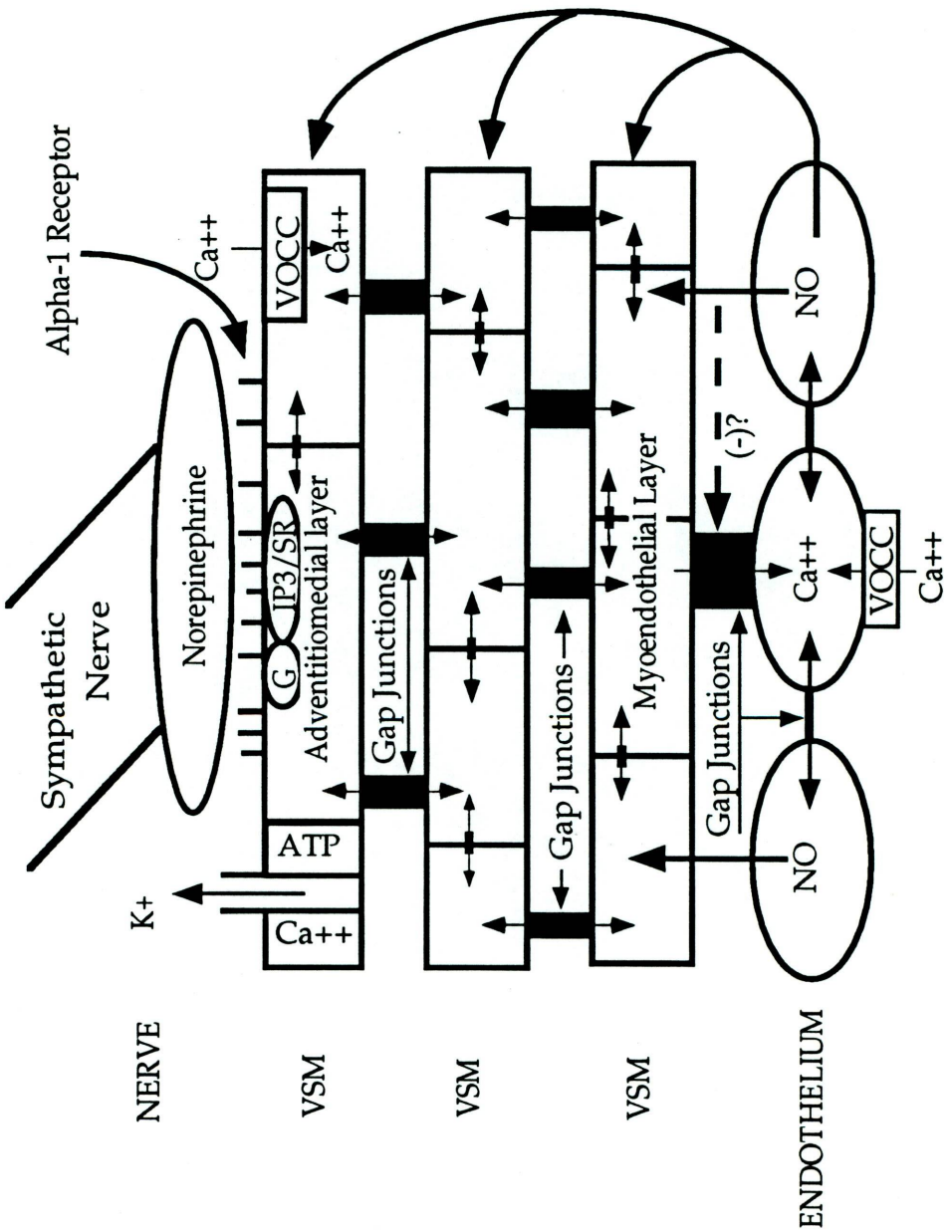
The second hypothesis postulates that norepinephrine diffuses from the nerve varicosities through the 150 μm (12-15 cell layers) of RTA to the intima. Once at the intima, NE binds to the alpha-2 receptor on the endothelium which causes the release of NO. However, NE reaches the intima only after a significant delay, and after the EFS-induced contraction has waned (Bao, 1993). Hynes *et al.* (1988), Vargus *et al.* (1990), Auch Schwelk *et al.* (1991), and Bucher *et al.* (1992) all showed that nitric oxide is released immediately upon EFS-induced contraction. Therefore, the released nitric oxide serves as an instantaneous modulator of arterial tone,

and the norepinephrine diffusion hypothesis is inadequate to explain nitric oxide's rapid ability to regulate arterial tone.

My hypothesis, and the third possibility, is that EFS-induced relaxation is caused by a rapidly propagated electrochemical signal that transverses the smooth muscle layers to the endothelium. This electrochemical signal, the result of extracellular Ca^{++} influx into the smooth muscle cell, would result in two consequences: 1) to further depolarize and possibly constrict the vascular smooth muscle; and, 2) to signal the underlying endothelium to release NO. My results show that EFS-induced relaxation in RTA ring and pressurized segments is mediated by endothelial NO. In order for EFS to cause relaxation in a preconstricted vessel as hypothesized, EFS would have had to depolarize the smooth muscle sufficiently to propagate an electrochemical signal to the endothelium resulting in opening endothelial VOCC. The endothelium senses the level of smooth muscle depolarization by an electrochemical signal propagated through low impedance junctions situated between smooth muscle layers and between smooth muscle and endothelial layers (Figure 20).

Indirect evidence for an electrochemical signalling mechanism was shown in the pressurized experiments. In these experiments, low to moderate frequencies of EFS (4 Hz, 30 v) and long pulse duration (1.0 ms) caused NO release from preconstricted RTA. At these frequencies, the arterial pressure decreased immediately upon cessation of EFS. It then rose to control

Figure 20. Our model proposes that the sympathetic nerve releases norepinephrine which binds to alpha-1 receptors on the post-junctional membrane. Ligand/receptor binding releases intracellular (SR) Ca^{++} via G-protein activation and IP_3 synthesis. Elevated intracellular Ca^{++} depolarizes the vascular smooth muscle (VSM) membrane allowing additional extracellular Ca^{++} to enter the muscle through voltage-operated Ca^{++} channels (VOCC). Entrance of extracellular Ca^{++} induces an action potential (AP) that is propagated through gap junctions in the VSM to the underlying endothelium. NO synthesis is stimulated by either a direct effect of the AP-induced endothelium depolarization or by the AP-induced endothelium depolarization and subsequent Ca^{++} entrance through VOCC in the endothelium. NO diffuses from the endothelium to the VSM modulating the level of tension. Depolarization, ATP levels and/or elevated intracellular Ca^{++} causes VSM K^+ efflux and membrane hyperpolarization. Hyperpolarization will increase the membrane potential and close VSM VOCC. Reduced Ca^{++} influx through VOCC will modulate the level of tension.


Sympathetic Nerve
Norepinephrine
Alpha-1 Receptor
Ca++
VOCC
Ca++
G
IP3/SR
Adventitiomedial layer
ATP
K+
Ca++
Gap Junctions
Gap Junctions
Myoendothelial Layer
Gap Junctions
(-)?
NO
NO
Ca++
VOCC
Ca++
NERVE
VSM
VSM
VSM
ENDOTHELIUM

10-20 seconds after stimulation. Although NO is released at these low frequencies, the causative mechanism is still unexplained. When the frequency of EFS was increased (8 Hz) without changing the pulse duration, another change in arterial pressure occurred. At the onset of EFS and throughout the 5 seconds of stimulation, the arterial pressure increased. Immediately upon cessation of EFS, arterial pressure decreased more than occurred during low frequency EFS. After 20-30 seconds, arterial tension again increased to control. These results suggest that at low to moderate frequencies of EFS, extracellular Ca^{++} enters the preconstricted smooth muscle. At this point, the initial tone created by preconstriction is great enough to override any further increases in pressure generated by EFS. However, the increased levels of intracellular Ca^{++} caused the smooth muscle membrane to further depolarize, which generated a propagated electrochemical signal (Neild and Kotecha, 1987). This electrochemical signal is the primary mechanism for communication between the smooth muscle and the endothelium. At the level of the myoendothelial junction, this electrochemical signal is transmitted to the endothelium which leads to an increase in the endothelial Ca^{++}. The Ca^{++} signal either stimulates nitric oxide synthesis directly or causes endothelial depolarization. Endothelial depolarization opens VOCC on the endothelial membrane which allows Ca^{++} to enter the endothelium. Under these conditions, the entrance of

extracellular Ca^{++} is the stimulus for NO synthesis. With higher frequencies of EFS, smooth muscle is further depolarized enhancing Ca^{++} influx. The smooth muscle tone will increase above the level caused by PE preconstriction because now the additional Ca^{++} can cause further activation of myosin light chain kinase and phosphorylation of myosin light chain. A contractile response therefore precedes the relaxation response. Since the magnitude of Ca^{++} influx is greater, the magnitude of the electrochemical signal is also greater. This larger electrochemical signal causes greater endothelial depolarization, greater Ca^{++} influx, greater cytosolic concentrations of Ca^{++}, greater NO synthesis, and greater smooth muscle relaxation.

When pulse duration is decreased from 1.0 ms to 0.1 ms an interesting phenomenon occurs. As frequency of EFS (16 Hz to 32 Hz) is increased, the NO-dependent relaxation increases, but not to the magnitude observed during the experiments with long pulse duration. In addition, the preceding contractile response seen in the long pulse duration experiments is absent throughout the short pulse duration experiments. These results can be explained by the results obtained from the simulated passive membrane. We used previously established smooth muscle membrane time constants (Hirst and Edwards, 1989) to determine the frequency required to reach the threshold for discharging the membrane capacitance. With the long pulse

duration (1.0 ms) and a membrane time constant of either 150 ms or 500 ms, temporal summation first appeared between 1 Hz and 4 Hz. However, the steepest increase in the membrane potential difference occurred between 4 Hz and 8 Hz. With the same membrane time constant values, 150 ms and 500 ms, our short pulse duration (0.1 ms) showed the same magnitude of temporal summation between 16 Hz and 32 Hz. These results show that low frequencies of EFS can depolarize the smooth muscle membrane sufficiently enough to propagate through the muscle layer to the endothelium to cause the first detectable signs of NO release. As the frequency of EFS increases, greater membrane depolarization occurs resulting in greater Ca++ influx. The additional Ca^{++} influx causes the muscle to contract prior to the EFS-induced relaxation. The shorter pulse duration causes a lesser magnitude of depolarization per pulse; however, the threshold frequency for temporal summation in the short pulse duration falls within the 8 Hz to 16 Hz range where we see the first sign of a NO-dependent relaxation occurring. The results from our passive and pressurized experiments establish that NO release is absolutely dependent upon the discharging of the smooth muscle capacitance. The subsequent entrance of extracellular Ca^{++} into the smooth muscle causes additional smooth muscle depolarization which serves as the regulatory signal for endothelial release of NO. These results also explain the absence of a preceding contraction in response to short

pulse and higher frequencies of EFS. The short pulse duration did not depolarize the membrane to the same magnitude as that of the long pulse duration. If we had increased the frequency of EFS beyond 32 Hz, the stimulus may have summated to reach the threshold necessary for further contraction seen in the long pulse duration experiments. Therefore, short pulse EFS-induced temporal summation caused a electrochemical signal to reach the endothelium by way of low impedance junction in the smooth muscle (Figure 20) but insufficient to raise arterial pressure above the PE-induced precontraction.

The Local Control Reflex

The data from EFS-induced relaxation in ring and pressurized vessels suggest that hyperpolarization and NO work synergistically to fine tune the intraluminal pressure. Inhibition of either one impairs or overwhelms the other from maintaining optimal wall tension, resulting in general failure of the system to modulate tone. Support for this position has been offered by Buga and Ignarro (1992). They identified an endothelium-dependent relaxation induced by EFS that was sensitive to Ca^{++}, calmodulin, L-arginine analogs (L-NAME), methylene blue (MB), TEA, and KCl. Pretreatment with either MB or L-NAME inhibited the EFS-induced relaxation response between 80-90%. In separate experiments, application of TEA (K_{Ca}^{++}-specific antagonist) or KCl (30-60 mM) to the bath reduced the EFS-induced relaxation

to 70% of control. They did not determine the precise mechanism by which electrical current is coupled or gated to the endothelial/NO pathway; however, Buga and Ignarro (1992) did speculate that EFS may induce endothelial hyperpolarization through the K_{Ca}^{++} channel. Endothelial hyperpolarization increases the inward driving force for extracellular Ca^{++} to enter the endothelium. The elevated intracellular Ca^{++} causes the synthesis and release of NO which modulates arterial tension. This hypothesis is further supported by Luckhoff and Busse (1990) who have shown that endothelial hyperpolarization increases the inward Ca^{++} driving force into the endothelium. Elevated intracellular Ca^{++} stimulates NO synthesis and release.

Although our data agree with those of Buga and Iganrro (1992) we propose an alternate hypothesis (Figure 20) which states that Ca^{++} entrance into the smooth muscle generates an electrochemical signal that is propagated to the underlying endothelium. Equally important in our hypothesis is that the propagated electrochemical signal is transmitted from smooth muscle to endothelium by way of low impedance junctions. This electrochemical signal causes either the direct synthesis of endothelial NO or endothelium depolarization. Endothelial depolarization causes Ca^{++} to enter the endothelium through VOCC and stimulate the synthesis of NO. The NO would act back upon the smooth muscle to inhibit Ca^{++} influx through

VOCC. This would in turn inhibit the electrochemical signal and attenuate NO release, a classical negative feedback system (Auch Schwelk *et al.*, 1991; Von Der Weid *et al.*, 1994). Alternatively, NO might act on the low impedance junctions connecting the medial and endothelial layer. NO would cause the signal transduction pathway to close and decrease the endothelial stimulus (Saéz *et al.*, 1987). The K^+ efflux (K_{Ca}^{++}-sensitive channel) and membrane hyperpolarization stimulated by Ca^{++} influx would provide another mechanism for regulation of intraluminal pressure. Together, these systems would provide substantial regulation available to the smooth muscle to modulate sympathetic nerve transmission and arterial tone.

Vargus *et al.* (1990) were the first to associate the level of arterial tension with nitric oxide release. They showed that the level of basal or agonist stimulated tone is directly related to the release of NO. Furthermore, alteration in the sympathetic innervation impairs sympathetically-induced vasomotor tone and the release of endothelial NO.

Vo *et al.* (1992) and Thorin *et al.* (1994) also found a relationship between arterial tension and endothelial release of nitric oxide in the rat tail artery. Both studies showed that in a perfused rat tail artery, EFS-induced constriction was elevated following L-NAME, methylene blue, and hemoglobin treatment. Furthermore, Vo *et al.* (1992) determined that

the rise in intraluminal pressure caused by PE, 5-HT, endothelin or KCl was also increased following inhibition of NO; however, application of L-NAME during basal flow conditions had no affect. Vo *et al.* (1992) concluded that a significant relationship existed between the level of arterial tone and the release of NO. NE diffusion was not responsible for nitric oxide release because NO release was induced by agonists that do not stimulate the adrenergic receptor. Because L-NAME had no effect during the resting condition, basal release of nitric oxide did not influence intraluminal pressure. Finally, the smooth muscle did not become more sensitive to NO, because the NO donor sodium nitroprusside (SNP) was less effective at high intraluminal pressure. They speculated that the changes in arterial tension caused an increase in wall shear stress. An increase in shear stress would lead to the direct stimulation and release of nitric oxide from the endothelium and relaxation of the smooth muscle. However, we have shown that shear is not necessary.

Low Impedance Pathways

The mechanism of signal transduction from adventitial to the endothelium layer requires that a propagated signal transverse the smooth muscle layers. Overwhelming evidence in the literature supports the idea that signalling between smooth muscle cells occurs through low impedance junctions. These low impedance or gap junctions cause the smooth muscle

to behave as a functional syncytium (Mekata *et al.*, 1986; Hirst and Edwards, 1989; Auch-Schwelk *et al.*, 1991; Christ *et al.*, 1992; Von Der Weid *et al.*, 1994).

Direct evidence for communication between smooth muscle and endothelial cells is not as abundant. Myoendothelial junctions or gap junctions, similar to those found between smooth muscle cells, have been identified between smooth muscle and endothelial cells (Larson *et al.*, 1987; 1985; Davies *et al.*, 1988; Wehrmacher, 1988; Asada and Lee, 1991). Indirect evidence presented by Von Der Weid *et al.* (1993) has shown that smooth muscle membrane oscillations in pig coronary artery are transmitted to the endothelium. These transmitted smooth muscle oscillations were shown to be precisely synchronized with the oscillations in the endothelium. Although dye transfer techniques from the endothelium to the smooth muscle did not locate the low impedance pathway, only a rapid communicating transduction mechanism could be responsible for the precise membrane synchronization. Von Der Weid *et al.* (1993) concluded that an electrochemical signal, of smooth muscle origin, is propagated to the endothelium by way of myoendothelial junctions. This smooth muscle electrochemical signal is a one-way signal, from the smooth muscle to the endothelium. Once the signal has stimulated the endothelium, NO is released and enters the smooth muscle by simple diffusion.

In support of the hypothesis by Von Der Weid *et al.* (1993), work done by Hynes *et al.* (1988), Auch Schwelk *et al.* (1991), and Bucher *et al.* (1992) have shown indirect evidence for a smooth muscle to endothelium communication mechanism. EFS was used to constrict perfused, intact vessels. They showed that the EFS-induced increase in intraluminal pressure was enhanced following inhibition of nitric oxide. Although the relationship between arterial tension and nitric oxide release was not discussed by Hynes *et al.* (1988), they showed that the intraluminal pressure in denuded RTA increased as EFS frequency was increased. Indirectly, these data suggest that the effect of the nitric oxide regulatory mechanism increases as intraluminal pressure increases. However, unlike our pressurized system, shear stress in a perfused system may have been the mechanism coupling arterial tension with endothelial release of nitric oxide. Despite flow, the responses of our pressurized system are identical to those responses in the perfused system. Since shear stress is still only speculation, we conclude that perhaps both systems are at work to regulate arterial tension during sympathetic nervous system transmission.

Physiological Relevance of The Local Control Reflex

Autoregulation is the maintenance of constant blood flow despite marked changes in arterial pressure (Guyton, 1991). For nearly a century, two

theories have been proposed to explain the acute autoregulation mechanism: (1) the metabolic theory; and, (2) the myogenic theory.

The metabolic theory of autoregulation states that increase pressure increases blood flow into the tissue. The greater blood flow washes away vasodilatory products and returns the normal concentration of nutrients and oxygen to the tissue. The vessel will then constrict, which decreases blood flow to normal despite the increase pressure.

The myogenic theory suggests that autoregulation of blood flow is an endogenous property of the smooth muscle. When vascular smooth muscle is stretched it responds with contraction. The arterial wall attempts to maintain a constant tension in the face of changing pressure by altering the vessel radius (Law of Laplace). Therefore, it is believed that high arterial pressure is the stimulus which causes constriction that returns blood flow back to normal. The problem that exists with the myogenic hypothesis is that it lacks any identifiable feedback mechanisms. For instance, if arterial pressure were elevated it would cause the blood vessel to stretch, which would cause it to constrict. The increased constriction would increase peripheral resistance and raise blood pressure. Elevated blood pressure would cause another cycle of stretch followed by still more constriction and then still more increase in pressure. Thus, a vicious cycle would occur, and if this would occur throughout the body, arterial pressure would rise enough to

cause the heart to fail (Guyton, 1991). The inherent problem with the myogenic theory is that a negative feedback system regulating the degree of constriction has never been shown.

Our data suggest the following negative feedback circuit regulating the myogenic response. An increase in arterial pressure would stretch the blood vessel and increase the arterial tension. The muscle would respond to the elevation of arterial by increasing cytosolic Ca^{++} and constricting. However, an endogenous smooth muscle K^+ efflux mechanism (K_{ATP}, $K_{Ca^{++}}$) would hyperpolarize the smooth muscle membrane to modulate the degree of constriction. A greater arterial pressure would cause both greater stretch of the vessel wall and arterial tension. The magnitude of the cytosolic Ca^{++} concentration would increase with arterial tension causing an action potential in the vascular smooth muscle. Two consequences of the action potential are: 1) greater vascular smooth muscle contraction; and, 2) endothelial release of nitric oxide. Although the additional Ca^{++} influx would serve to increase the degree of constriction, it would also magnify the signal between smooth muscle and endothelium. Since the synthesis and release of endothelial NO is directly proportional to intensity of the electrochemical signal, the released NO would cause greater modulation of arterial tone.

Clinical Relevance of The Local Control Reflex

The etiology of essential hypertension is unknown. Characteristics of a late stage essential hypertension patient include: 1) the mean arterial pressure is increased 40 to 60 percent; 2) the renal blood flow is reduced by one-half; 3) the resistance to blood flow through the kidney is increased twofold to fourfold; 4) glomerular filtration rate is near normal; 5) cardiac output is normal; 6) total peripheral resistance is increased 40 to 60 percent, equal to the changes in arterial blood pressure; and 7) the kidney will not excrete adequate salt or water unless the arterial pressure is high. Guyton (1991) suggests that the cause of the decrease in salt and water excretion is the reduced renal plasma flow. A causative mechanism might include the hypothesized endothelial negative feedback mechanism. During the earliest stages of essential hypertension the endothelium's ability to sense adrenergic changes in arterial tension decreases. The dysfunction might occur at the level of the propagated signal through low impedance pathways (Figure 20). Without the signal transversion from medial to endothelial layer the synthesis and release of NO would decrease. This alteration in the normal feedback mechanism would cause renal peripheral resistance to increase and RPF and GFR to decrease. The kidney compensates for the decreased RPF by increasing reabsorption of salt and water to increase arterial pressure in an attempt to return RPF and GFR to normal. Although an increase in fluid volume

returns GFR to normal, RPF remains low. Support for endothelial pathology as a causative mechanism in essential hypertension was shown by Panza *et al.* (1990). They showed that essential hypertension in human patients was caused by a reduction in the endothelium's ability to release NO. Furthermore, it was shown that the smooth muscle's ability to respond to exogenous NO was not dysfunctional. They could not determine if the endothelial dysfunction was primary or secondary to the elevated arterial pressure. However, their results do show that an impaired endothelium is a characteristic of essential hypertension.

Future Research

The primary area of future research is to anatomically locate the low impedance pathway between the medial and endothelial layer. Evidence has been provided by Davies *et al.* (1988), Wehrmacher *et al.* (1988), and Asada and Lee (1992) that low impedance pathways exist. However, the majority of the research is inconclusive (Segal and Bény, 1992). Once the anatomical data is obtained, functional research must establish that low impedance pathways transmit an electrochemical signal from medial to endothelial layer. Equally important is the functional relationship between the electrochemical signal and NO synthesis. Does the electrochemical signal function as a direct stimulus for NO synthesis? Does the electrochemical signal cause the endothelial VOCC to open which allows extracellular Ca^{++} to enter the

endothelium? The extracellular Ca^{++} would then serve as the stimulus for NO synthesis. Alternatively, does the released NO act on the gap junction causing closure of the transduction pathway between medial to endothelial layer?

Finally, if the above mentioned areas are shown to exist and function as speculated in this research, the local reflex system can be applied to pathophysiological conditions. Two areas of immediate importance are essential hypertension and diabetes. Although the etiology of the vascular disease associated with these conditions is unknown, both clinical conditions have shown altered endothelial responsiveness to sympathetic input (Weber and MacLeod, 1994; Panza *et al.*, 1990).

Summary and Conclusions

The endothelium's role as a modulator of arterial tone is clearly evident (Hynes *et al.*, 1988; Vo *et al.*, 1991; Bucher *et al.*, 1992), and dependent on the degree of vascular tone (Vargus, Ignarro and Chaudhuri, 1990; Vo *et al.*, 1992). In our experiments, the level of tone created by PE-induced preconstriction is associated with depolarization of the smooth muscle membrane and an increase in the Ca^{++} influx through voltage operated Ca^{++} channels as suggested by Neild and Kotecha (1987), Nelson *et al.* (1990), and Nelson (1993). The subsequent EFS further depolarizes the vascular smooth muscle membrane causing additional Ca^{++} influx. At this point, the initial

tone created by PE-induced preconstriction is great enough to override any further increases in tension generated by EFS opening of voltage operated Ca^{++} channels. However, the Ca^{++} current generated by EFS causes an electrochemical signal that propagates through the smooth muscle directly to the endothelium. This electrical signal results in the influx of Ca^{++} into the endothelium with subsequent synthesis, release and diffusion of NO from the endothelium to the smooth muscle. In turn, the nitric oxide modulates the degree of tension within the smooth muscle (Figure 20). Although the mechanism of signal transmission between the smooth muscle and endothelium is unidentified, evidence for myoendothelial junctions exists (Davies *et al.*, 1988; Wehrmacher *et al.*, 1988; Asada and Lee, 1992). Furthermore, unidirectional communication, via electrotonic transmission, from the smooth muscle to endothelium has been indirectly shown in pig coronary arteries (Von Der Weid and Beny, 1993). Experiments with freshly isolated BAEC shows the capacity of endothelial cells to sense changes in voltage, and that these changes result in the synthesis and release of NO.

The endothelium also regulates the smooth muscle membrane potential through a hyperpolarization mechanism (either K_{ATP} and/or $K_{Ca^{++}}$ channel), perhaps by the hyperpolarizing factor identified by Chen *et al.* (1991). These data were presented exclusively in our ring preparations. An endothelium-independent, smooth muscle specific,

hyperpolarization mechanism regulates the membrane potential by a K_{ATP}-sensitive channel. The precise mechanism is not clear, however inhibition of voltage operated Ca^{++} influx and enhanced Na^{+}/Ca^{++} exchange has been shown to relax smooth muscle.

Our data suggest that the vascular smooth muscle in the RTA is peripherally controlled by a dual regulatory mechanism. The endothelium-independent component is endogenous to the smooth muscle layers and mediated by K^{+} efflux and hyperpolarization. These components may act initially to regulate moderate changes in arterial tone. The endothelium-dependent component requires a stronger neuronal stimulus, causing greater elevation of intracellular Ca^{++} and further depolarization of the smooth muscle. This electrochemical signal is rapidly propagated through the smooth muscle syncytium to the endothelium resulting in NO synthesis and modulation of tone.

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