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K^+- Induced Dilation of Cremasteric Arterioles Involves Na^+/K^+Atpase and Inward Rectifier K^+ Channels

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K^+-INDUCED DILATION OF CREMASTERIC ARTERIOLES INVOLVES NA^+/K^+ ATPASE AND INWARD RECTIFIER K^+ CHANNELS

by

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The purpose of this study was to evaluate the possible mechanisms involved in cremasteric arteriolar dilation as a result of increased concentrations of ($[K^+]_o$) extracellular potassium. Many other studies suggested that Na^+/K^+ ATPase or K_{IR} channels are responsible for potassium-induced vasodilation in other systems (2, 12, 22, 40, 46, 52, 16, 17, 25, 33, 34, 85, 99, 151). However, these studies show only partial inhibition of dilation as a result of independent blockage of each mechanism. The hypothesis of this study was that both mechanisms contribute to potassium-induced dilation seen in cremasteric arterioles.

First or second order arterioles were isolated (n=81), cannulated and pressurized with physiological salt solution (PSS) to 70 cm H_20 at 34° C with no flow through their lumens. The vessels developed spontaneous tone in PSS containing 5 mM K^+. Elevation of $[K^+]_o$ from 5 mM to 8.75, 12.5 or 20 mM was studied to determine if K^+ caused concentration-dependent dilation. Arteriolar diameter was measured using a computer-based diameter tracking system. To elucidate the mechanism(s) responsible for the dilation, vessels were exposed to known blockers of K_{IR} and Na^+/K^+ ATPase. Barium (50

μM or 100 μM), which blocks K_{IR} channels, was applied to vessels do determine if blockage of K_{IR} channels would inhibit potassium-induced vasodilation. Ouabain (0.1 mM or 1.0 mM), an inhibitor of the Na^+/K^+ ATPase, was applied to vessels in order to determine if blockage of Na^+/K^+ ATPase would result in inhibition of potassium-induced vasodilation.

Increased $[K^+]_o$ caused transient vasodilation. The arterioles dilated and then returned to, or below baseline despite the maintenance of the elevated $[K^+]_o$. Concentrations of barium (50 μM and 100 μM) (n=8 for both concentrations $p< 0.05$) resulted in a reduction in the peak dilation to 20 mM K^+. Ouabain, 0.1 mM (n=9, $p<0.05$) or 1 mM (n=14, $p<0.05$), resulted in a reduction in the peak dilation to 20 mM K^+. However, the dilation was not consistently eliminated by either blocker. A combination of barium (100 μM) and ouabain (1 mM) was required to dependably abolish K^+-induced arteriolar dilation.

The effects of both barium and ouabain appeared to be specific because dilation induced by acetylcholine was not inhibited by either agent.

These results suggest that skeletal muscle arterioles are intrinsically sensitive to changes in $[K^+]_o$, and that the transient nature of the response to elevated $[K^+]_o$ is an innate property of the arteriolar muscle cells. Increases in $[K^+]_o$ appear to dilate skeletal muscle arterioles by a mechanism that involves both K_{IR} channels and the Na^+/K^+ ATPase.

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CHAPTER 1

INTRODUCTION

Background and Purpose of the Study

Skeletal muscle represents 40% of body mass and contributes substantially in the regulation of blood pressure and flow, whole body metabolism, and cardiovascular homeostasis, in addition to its vital role in locomotion and other contraction-related functions (13, 47, 50, 95, 122, 146).

Skeletal muscle contraction leads to large increases in blood flow to the active muscle through dilation of resistance arteries and arterioles (27, 95, 126, 127, 133, 141). However, the mechanisms underlying this active or functional hyperemia remain poorly understood. The release of potassium ions from active skeletal muscle has been implicated in functional hyperemia for nearly 40 years. Numerous studies have shown that elevated extra-cellular concentration of K^+ ($[K^+]_o$) can cause vasodilatation, and that the K^+ released from skeletal muscle fibers during the re-polarization phase of muscle action potentials can accumulate in the interstitial space at concentrations sufficient to cause vasodilatation (68, 88, 122, 151). However, the cellular mechanisms by which the vascular smooth muscle cells in the walls of skeletal muscle arterioles relax to cause vasodilatation in response to elevated $[K^+]_o$ remains unclear. Early studies suggested that elevated $[K^+]_o$ caused vasodilatation by activation of the electrogenic Na^+/K^+ ATPase and transient membrane hyperpolarization (25). Recent in vivo studies of rat skeletal muscle

microcirculation support this hypothesis (99). However, other recent in vitro studies, performed in small cerebral and coronary arteries, suggest that in those vascular beds, elevated $[K^+]_o$ causes membrane hyper-polarization and relaxation of vascular smooth muscle cells by activating inward rectifying K^+ (K_{IR}) channels (16, 18, 33). The purpose of the present study was to: 1.) determine the effects of elevated $[K^+]_o$ on the diameter of cannulated arterioles isolated from hamster cremaster muscles as an in vitro model system to study the mechanism of action of elevated $[K^+]_o$ on skeletal muscle arterioles and 2.) evaluate the roles played by the Na^+/K^+ ATPase and K_{IR} channels in dilation of skeletal muscle arterioles in response to elevated $[K^+]_o$. This second aim was accomplished by assessing the effects of Ba^{2+}, an inhibitor of K_{IR} channels, and ouabain, an inhibitor of Na^+/K^+ ATPase , on dilation of skeletal muscle arterioles induced by elevated $[K^+]_o$.

Regulation of Skeletal Muscle Blood Flow

Proper function of skeletal muscle requires continuous, regulated blood flow to deliver oxygen, hormones, and nutrients to the tissue and for removal of CO_2 and other wastes produced during muscle contraction (13, 47, 50, 95, 122). Arterioles in the microcirculation of skeletal muscle play an important role in regulating muscle blood flow, as they are major sites of blood flow regulation within and to skeletal muscle (95, 96). Skeletal muscle arterioles also importantly determine total systemic vascular resistance and hence blood pressure regulation and cardiovascular homeostasis (13, 47, 50, 95, 122).

Regulation of blood flow and pressure is accomplished by changes in the diameter of arterioles resulting from contraction or relaxation of vascular smooth muscle cells in their walls (13, 57, 82). Under resting conditions, skeletal muscle arterioles have substantial tone (13, 57, 82). That is, the vascular smooth muscle in the wall of these microvessels is active and in a state of partial contraction resisting the distending force of the blood pressure in the vessel lumen. Increases in tone lead to decreases in diameter (vasoconstriction) and decreases in tone lead to increases in diameter (vasodilatation) (13, 57, 58, 82, 76).

Factors Influencing Arteriolar Tone

Arteriolar tone *in vivo* is a complex, dynamic variable that is determined by both vasoconstrictor and vasodilator influences on vascular smooth muscle cells. The primary vasoconstrictor influences that contribute to skeletal muscle arteriolar tone are the myogenic response of the smooth muscle cells to the blood pressure (myogenic tone), sympathetic nerve activity, circulating hormones (angiotensin II, vasopressin and epinephrine) and endothelin released from the vascular endothelium (23, 42). Vasodilator influences include endothelium-derived vasodilators such as nitric oxide (NO), prostacyclin (PGI2) and endothelium-derived hyperpolarizing factor (EDHF); vasodilator substances released from skeletal muscle cells such as adenosine, CO_2, H+ and K+; reduced PO2 and elevated interstitial osmolarity resulting from skeletal muscle metabolism; and substances released from sensory nerves such as substance P and calcitonin gene-related peptide (7, 14, 17, 29, 121). In vivo, all of these factors act on the

vascular smooth muscle cells in the wall of arterioles to determine arteriolar tone and blood flow.

The factors outlined above modulate arteriolar tone, by altering intracellular Ca^{2+} concentration $[Ca^{2+}]_I$ and/or the Ca^{2+}-sensitivity of the contractile machinery (85, 140). Like all muscle, vascular smooth muscle contraction is triggered by an increase in $[Ca^{2+}]_I$. Calcium binds to the regulatory protein, calmodulin, and the Ca^{2+}-calmodulin complex then activates myosin-light-chain kinase (MLCK). This kinase phosphorylates the 20kD myosin regulatory light chains, a process that then allows myosin to interact with actin in the crossbridge cycle resulting in force production and/or muscle cell shortening (140). The myosin light chains are dephosphorylated by myosin-light-chain phosphatase (MLCP) terminating the contraction sequence. The level of activation (i.e., tone) of the smooth muscle is determined, to a large extent, by the degree of phosphorylation of the myosin light chains which is determined by the ratio of activity of MLCK and MLCP: tone can be modulated by changes in the activity of either enzyme. As noted above, the intracellular concentration of Ca^{2+} determines the activity of the MLCK (140). The activity of MLCP, and hence the Ca^{2+} sensitivity of the system, can also be modulated. Vasoconstrictors that act through G-protein-coupled receptors such as norepinephrine, angiotensin II, and vasopressin tend to lead to inhibition of the MLCP and hence an increase in Ca^{2+} sensitivity (140). In contrast, vasodilators such as NO and PGI_2 that act through the cGMP and cAMP second messenger pathways appear to increase the activity of the MLCP and decrease the Ca^{2+} sensitivity of the contractile machinery (29, 42, 140).

Intracellular Ca^{2+} concentration in vascular smooth muscle depends on the influx of Ca^{2+} through sarcolemmal ion channels, release of Ca^{2+} from intracellular stores and the activity of various pumps and exchangers that remove Ca^{2+} from the cytosol (20, 23, 76, 78, 82). In the microcirculation, Ca^{2+} influx through voltage-gated Ca^{2+} channels appears to play a major role as a source of activator Ca^{2+} used by the vascular smooth muscle (78). Thus, the membrane potential of vascular smooth muscle cells, by controlling the open-state probability of voltage-gated Ca^{2+} channels, importantly affects vascular smooth muscle tone (78, 82). Many of the factors listed above that modulate arteriolar tone do so by influencing the membrane potential of vascular smooth muscle cells with depolarization leading to vasoconstriction (increased tone) and hyperpolarization leading to vasodilatation (decreased tone) (4). As will be discussed below, elevated $[K^+]_o$ appears to cause dilation by hyperpolarizing the membrane of vascular smooth muscle cells leading to closure of voltage-gated Ca^{2+} channels, a reduction in intracellular Ca^{2+}, and decreased tone.

Membrane Potential and K^+ Channels

Membrane potential of vascular smooth muscle is determined by concentration gradients of ions across the plasma membrane, the permeability of the membrane to these ions (determined by the open state probability and the number of ion channels present), and the activity of ion pumps and exchangers moving these charged molecules across the membrane (78). In the steady state, with a constant membrane potential, the movement of charge across the membrane is balanced. That is, the net movement of charge into the cell is exactly balanced by the net movement out so that the cell is in a dynamic equilibrium

(48, 49, 76, 78). Disruption of this balance by altering the influx or efflux of any ionic component will result in a change in membrane potential, and a subsequent change in smooth muscle tone.

In smooth muscle, as in most cells, K^+ ions play a dominant role in determining resting membrane potential because of the large number of K^+ channels expressed and their activity (18, 30). Opening of additional K^+ channels allows K^+ ions to diffuse out of the cells, down their electrochemical gradient. This loss of positive charge from the cells hyperpolarizes the membrane from its resting potential of –30 to –40 mV (in arteriolar smooth muscles) and moves the membrane potential closer to the K^+ equilibrium potential (-83 mV). Similarly, closure of K^+ channels leads to membrane depolarization.

There are at least four different types of potassium channels that contribute to the regulation of membrane potential in vascular smooth muscle cells: 1) ATP-sensitive K^+ (K_{ATP}) channels, 2) Calcium-activated K^+ (K_{Ca}) channels, 3) Voltage gated K^+ (K_V) channels and 4) Inward rectifier K+ (K_{IR}) channels (18, 76, 77, 80). Of these, K_{IR} channels have been implicated in K^+-induced vasodilation (18, 33, 34). Therefore, the remainder of the discussion of K^+ channels and regulation of membrane potential and arteriolar tone will focus on these channels.

Inward Rectifier K^+ Channels in Vascular Smooth Muscle

Inward rectifier potassium channels were first found in skeletal muscle by Katz (85). These channels were first proposed to be in vascular smooth muscle by Edwards and Hirst (33, 34). Rectification refers to a change in channel conductance with voltage such that ions pass more easily through the channel in one direction than in the other

direction. (18, 33, 34, 118) Inward rectification means that at a given electrochemical gradient (driving force), the inward flow of K^+ ions is greater than the outward flow for the opposite driving force. (33, 34) The mechanism of inward rectification in K_{IR} channels involves voltage dependent block of the channel pore by cytoplasmic magnesium and polyamines (16, 33, 34, 125). Inward rectifier potassium channels mainly conduct inward current below the equilibrium potential for K^+ and do not allow much outward flow of current (33, 34, 156).

Structure

Inward rectifier potassium (K_{IR}) channels that are expressed in blood vessels are from a gene family called Kir, specifically Kir 2.X (2, 16, 80, 84, 91, 97, 118-120, 157). The channels in this family are considered strong rectifiers (see below). These channels are tetramers made of proteins that are approximately 370-500 amino acids in length, and have a molecular mass of approximately 40 KDa (120). K_{IR} channels have two transmembrane domains (M1 and M2) and between them is an H5 or P region, which dips into the membrane from the outside, and is considered to form the ion conductive pore (120, 123). (Figure) 1. A functional K_{IR} channel is composed of four of these subunits. The amino (N) and carboxy (C) terminals are both located within the cytoplasm. The N and C terminals are variable, but the central portion is more conserved allowing for subtypes of K_{IR} channels (120). Subtypes include weak (Kir 1.1 and 6.1 and 6.2 subfamilies) and strong inward rectifiers (Kir 2.1-2.3, 3.1-3.5 and 4.1) (120). Weak inward rectifiers are those whose rectification is attributed to block by internal divalent

metal cations, such as Mg^{2+}. Strong inward rectifiers have an additional intrinsic gating mechanism that can be attributed to cytoplasmic polyamines. (66, 112) The Kir 2.X family of channels are considered to be "classical" strong inward rectifiers (120).

Distribution

Genes encoding different subtypes of inward rectifying potassium channels have been found in several species and organ systems. Those similar to channels thought to be activated in this study, Kir 2.X, have been observed in skeletal muscle, heart, brain, bronchial smooth muscle, and vascular smooth muscle (66, 120). The distribution of K_{IR} channels varies from cell to cell, even within a particular tissue (118, 120). In vascular smooth muscle the classical Kir 2.1, strong rectifiers, as well as some weak rectifiers from the Kir 6.1 and 6.2 subfamilies (more closely associated with K_{ATP} channels) (66, 120) are found. The strong rectifiers (Kir 2.1) are those believed to be involved in potassium-induced arteriolar dilation (66, 120). Studies have suggested that within the vasculature, the density of K_{IR} channels is greater in vessels with smaller diameters, such as resistance-sized arteries and arterioles (118). K_{IR} channels have been estimated to have a density of approximately 100-500 per smooth muscle cell (120). Open potassium channels do not have to be great in number in order to significantly contribute to membrane potential and tone (66).

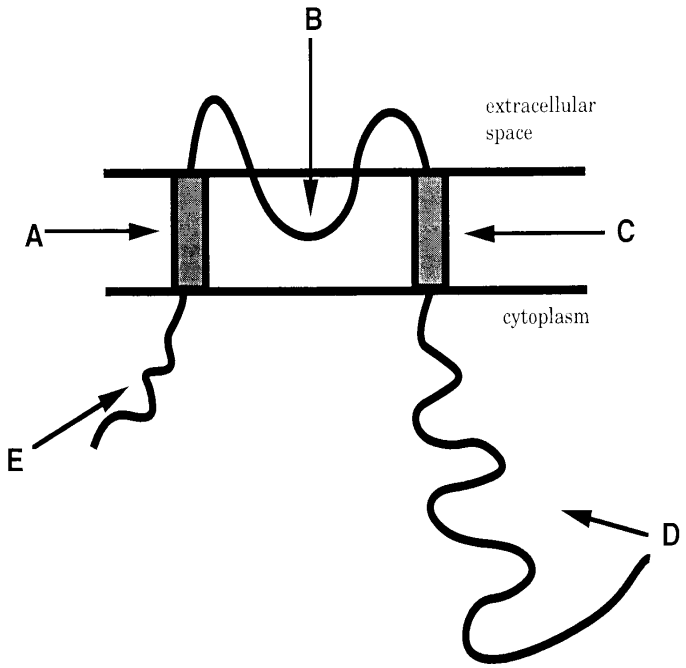

Fig. 1 – Schematic of Molecular Structure.

Figure 1 illustrates the typical molecular structure of inward rectifier channels. Arrow A represents the M1 trans-membrane domain. Arrow B represents the H5 or P pore region, which is within the membrane. Arrow C represents the M2 trans-membrane domain. Arrow D represents the C-terminus. Arrow E represents the N terminus. An entire functional channel is composed of four peptide protein subunits.

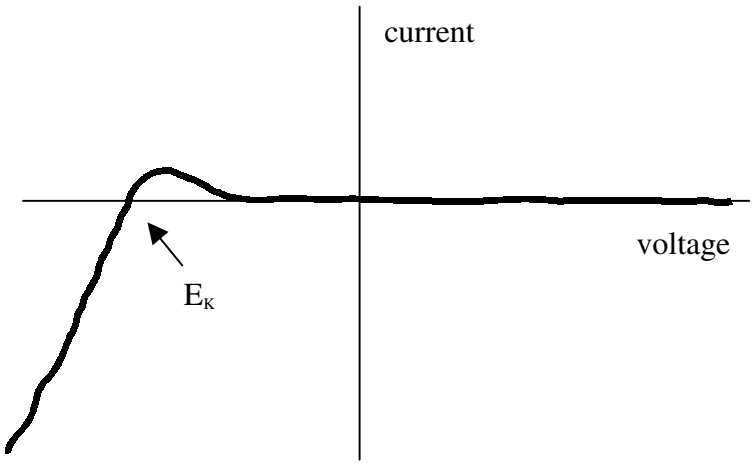

Fig 2 - Current-Voltage Relationship for K_{IR} Channels.

Figure 2 shows a theoretical I-V plot for K_{IR} channels showing the inward rectification at potentials positive to the K^+ equilibrium potential (E_K), and the outward "hump" just positive to E_K. By convention, negative current reflects positive charge moving into cells (inward current) and positive current reflects positive charge moving out of cells (outward current). See text for more information.

Function

In cells such as cardiac muscle, skeletal muscle, and neurons, K_{IR} channels primarily function to stabilize the resting membrane potential of these cells at, or very near, the K^+ equilibrium potential (E_K = -83 mV) (120). The current-voltage relationship of these channels shows strong inward rectification at potentials positive to the E_K (Figure 2). That is, these channels conduct K^+ ions into the cells much more easily than out of the cells. At membrane potentials slightly more positive (5-15 mV) than E_K, there is an outward "hump" in the I-V relationship such that in this region the channels can conduct K^+ ions out of cells. At potentials >15 mV positive to E_K these channels are relatively impermeable to K^+. This "N" shaped I-V relationship provides the stabilization function noted above. Any process (unrelated to K_{IR} channels) that would tend to hyperpolarize cells beyond E_K would lead to inward current flow through K_{IR} channels, which would return the membrane potential towards E_K. Similarly, anything that would tend to depolarize the cells and push membrane potential more positive than E_K would result in outward current flow through K_{IR} channels which would counteract the depolarizing stimulus and return the membrane potential towards E_K.

K_{IR} channels may act as sensors for small changes in $[K^+]_o$ (76, 77, 118, 120). Elevated $[K^+]_o$ increases the conductance of these channels to K^+ ions, and shifts E_K toward more positive potentials. As can be seen in Figure 3, this shifts the outward "hump" of the K_{IR} I-V relationship such that outward K^+ ion current can flow through the channels at more positive potentials. This outward current would tend to hyperpolarize the cells and push the membrane potential towards the new E_K .

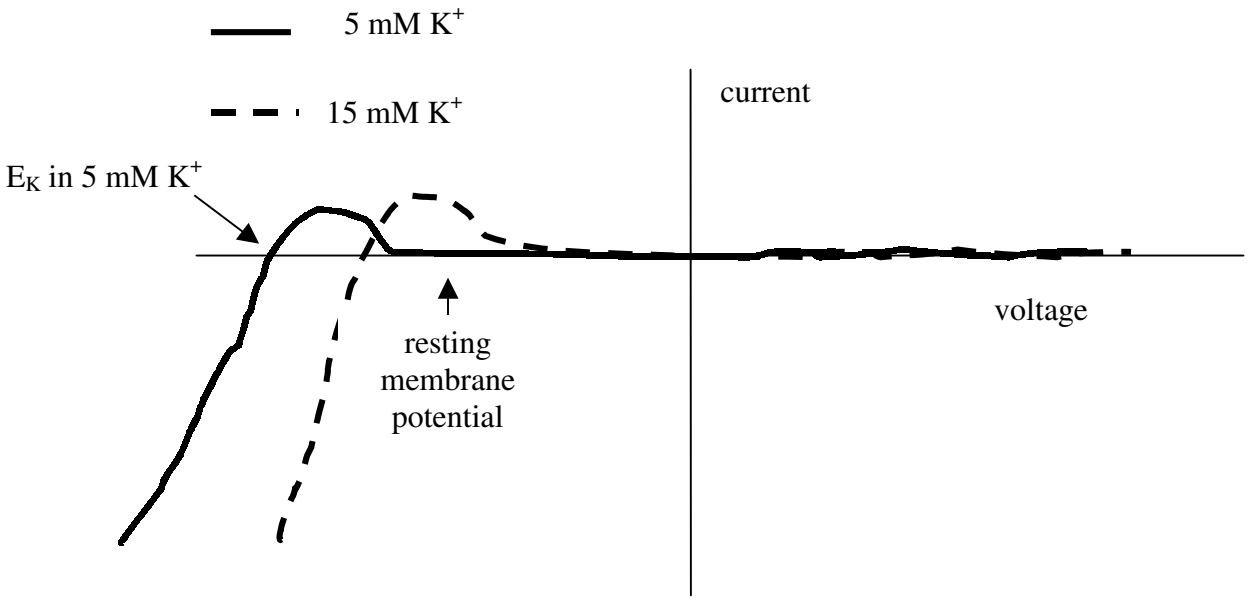

Fig. 3- Effects of Increased $[K^+]_o$ on K_{IR} Channel I-V Relationship.

Figure 3 shows theoretical I-V relationships for K_{IR} channels in normal (5 mM) and elevated (15 mM) $[K^+]_o$. Elevated $[K^+]_o$ increases the conductance of the K_{IR} channels as noted by the increase in the slope of the inward portion of the I-V curve in 15 mM K^+. In addition, E_K, the reversal potential in the I-V plot, shifts to more positive values. This allows K^+ ions to flow out of the cell at resting membrane potential. See text for more information.

Studies in submucosal arterioles and in cerebral arterioles and small arteries have clearly demonstrated K^+ currents that display inward rectification that varies with the $[K^+]_o$ (33-35, 84, 91, 97, 118, 119). Others have shown that outward current at –50 mV can be inhibited by 100 μM Ba^{2+} in smooth muscle cells isolated from coronary arteries of the rat (123, 125). These data are consistent with the idea that vascular K_{IR} channels can conduct K^+ in the outward direction and potentially could be involved in dilation induced by elevated K^+.

Pharmacology

Barium ions block K_{IR} channels at concentrations lower than 100 μM. Concentrations of Ba^{2+} greater than 100 μM also block K_{ATP} (76, 112, 120). Inhibition of currents through K_{IR} channels by Ba^{2+} is greater at more negative membrane potentials (76, 112). Cesium (Cs^+) 100 μM, has also been shown to block K_{IR} channels in a variety of tissues (104, 119, 125). Barium block of K_{IR} channels is voltage dependent and affects currents through the channel(120, 125). Barium block occurs when the molecule attaches to the P loop, which is the K^+ selectivity filter (118, 119). Barium block is reversible when the molecule is removed. Currents through K_{IR} channels have also been reduced by elevation of external calcium and magnesium concentrations, up to 10 mM. This type of inhibition has been shown to occur at low-affinity block sites, is superficial and lacks voltage dependence. (16, 33, 34)

Other potassium channel blockers such as glibenclamide (K_{ATP} channel blocker), low concentrations of TEA^+ (K_{Ca} channel blocker) and charybdotoxin (K_{Ca} channel blocker) have had little effect on K_{IR} channel currents (21, 76, 77, 101). Alterations in the

extra-cellular pH have been shown to have little effect on the inward currents through K_{IR} channels (118, 119).

Na^+/K^+ ATPase in Vascular Smooth Muscle

In addition to ion channels, membrane potential of vascular smooth muscle cells can be influenced by other processes that move ions across the cell membrane such as exchangers and pumps. In particular, the Na^+/K^+ ATPase has received considerable attention as a potential mechanism by which elevated $[K^+]_o$ might cause membrane hyperpolarization and vasodilation.

The Na^+/K^+ ATPase is a transmembrane enzyme complex that couples ATP hydrolysis with the transport of sodium and potassium against their electrochemical gradients (9, 65). Dean (1941) was the first to propose the existence of this enzyme. The Na^+/K^+ ATPase has been found in nearly all mammalian cells (9). The Na^+/K^+ ATPase was first shown to be in vascular tissues by Bonting (1961). There have been numerous studies of this enzyme in many cell types including vascular smooth muscle (21). This section of the paper will discuss the structure, distribution and function of the Na^+/K^+ ATPase within vascular smooth muscle.

Structure

The Na^+/K^+ ATPase is a heterodimer consisting of two subunits, an alpha (α) subunit and a beta (β) subunit. The alpha subunit is known to have three possible isoforms and the beta subunit is known to have two possible isoforms (9, 12, 40, 65, 100). The alpha subunit has cytoplasmic N and C terminals, with a span of ten

transmembrane domains (9, 12, 100). This subunit has a molecular weight of approximately 100,000 KDa. The alpha subunit contains the ATP binding site as well as the cardiac glycoside (Na^+/K^+ ATPase blocker) binding site (9, 12, 46, 100). The beta subunit has a cytoplasmic N terminus and a C terminus that lies on the outside of the cell, with only one transmembrane domain. The beta subunit is glycosylated and can range is molecular weight from 35,000 to 65,000 (when fully glycosylated) (9, 12. 46, 100). The specific function of this subunit has yet to be clearly elucidated. The α and β isoforms are thought to be tissue specific (12, 65, 100). Figure 4 illustrates this ATPase.

Distribution

The Na^+/K^+ ATPase has been shown to be expressed in many species and in many tissues. The sodium-potassium ATPases are a part of the large family known as P-ATPases, along with Ca-ATPase, H-ATPases (seen in lower organisms) and hydrogen-potassium ATPase (seen along with sodium-potassium ATPases in higher eukaryotes). (12) In mammals, the α and β isoforms are expressed and are distributed in a tissue specific manner (40, 46). This enzyme is known to be present in large amounts in the heart, kidney, brain and skeletal muscle (12, 40, 46). The Na^+/K^+ ATPase is also expressed in the vascular system (52, 55). The $\alpha_1\beta_1$ form is ubiquitous, and is seen in vascular smooth muscle along with a truncated α_1 subunit (12, 46). However, the electrogenic contribution of this ATPase differs in smooth muscle cells of different vascular beds (12, 52).

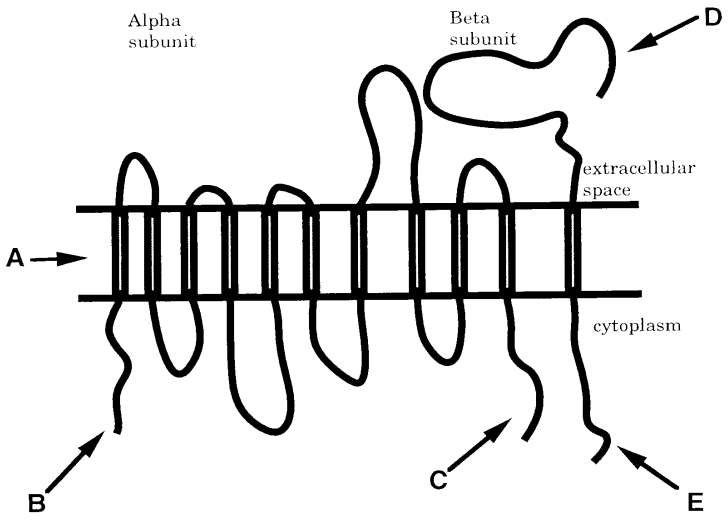

Fig. 4- Typical Molecular Structure of the Na^+/K^+ ATPase.

The Na^+/K^+ ATPase has alpha and beta subunits. The A arrow represents the transmembrane domains, the alpha subunit having ten and the beta subunit having one. Arrow B represents the N terminus of the alpha subunit. Arrow C represents the C terminus of the alpha subunit. Arrow E represents the C-terminus of the beta subunit. Arrow D represents the N terminus of the beta subunit.

Function

The Na^+/K^+ ATP pump moves three Na^+ ions out of the cell for each two K+ ion moved into the cell per ATP hydrolyzed. The activity of the Na^+/K^+ ATPase is stimulated by elevation in intracellular sodium or extra-cellular potassium, and pumps more sodium out than it moves potassium in, so it is said to be electrogenic (12, 22, 52). Conversely, the pump is inhibited by decreases in intracellular Na^+ or extra-cellular K^+. The exchange of ions occurs as sodium attaches to the first intracellular loop of the molecule (12). In the presence of potassium, this loop is hidden and instead a large cytoplasmic loop is exposed (12). Both loops are involved in the exchange and make a conformational change during activation (12). Denervation does not inhibit the activity of the Na^+/K^+ ATPase (14). This enzyme plays an important role in the maintenance of the electrochemical gradients for Na^+ and K^+. It also contributes to the control of membrane potential, thus contributing to the regulation of vascular tone and may be important in vascular smooth muscle reactivity (11, 28, 35, 64). The Na^+/K^+ ATPase has been proposed to contribute to vascular tone because inhibition of the enzyme results in vasoconstriction in some systems (21, 55). As noted above, vasodilation as a result of Na^+/K^+ ATPase activation through an increase in extra-cellular K^+, has also lead to the idea that this enzyme contributes to the regulation of vascular tone (11, 35).

Pharmacology

The Na^+/K^+ ATPase is inhibited by cardiac glycosides such as ouabain (12). Ouabain has been used extensively as an indicator of activity of this enzyme. Ouabain, in

concentrations ranging from 10 μM to 1 mM, has been used to inhibit the activity of the Na^+/K^+ ATPase (14, 19, 22, 35). Ouabain is found in mammals and is considered an endogenous digitalis-like factor (12, 40). Ouabain is believed to act by inhibiting Na^+/K^+ ATPase pumping causing a more positively charged intracellular environment, which, in turn, causes depolarization and vasoconstriction (12). The α subunit contains the ouabain binding site (12). Ouabain binds to the pump when it is loaded with sodium (12). The H1 transmembrane segment has a high affinity for ouabain (12, 112), and the binding of ouabain is stabilized by a conformational change of the molecule (12). The mechanism is complex and little is understood about the binding site and process of inhibition.

Removal of K^+ from the extra-cellular space or Na^+ from the intracellular fluid also inhibits the activity of the Na^+/K^+ ATPase. (14, 21, 22, 52). The pump is also inactivated by reduced temperatures (28). Studies have shown that as temperatures change from 26° to 36° C there is an increase in the pump activity (28).

Functional Hyperemia

Blood flow to resting skeletal muscle is quite low, approximately 3-5 ml 100 g $tissue^{-1}$ min^{-1} . During maximal activity blood flow can increase to 10-15 times this level . Studies in isolated, perfused muscles demonstrated that this functional hyperemia, the increased blood flow associated with muscle contraction, results from local factors intrinsic to the muscle and its microcirculation. Despite intensive research over the past 40 years, the mechanisms responsible for functional hyperemia remain unclear probably because there are a number of complimentary, overlapping and redundant processes

involved in this response. To maintain focus the remainder of this section will concentrate on the role played by elevated $[K^+]_o$ in this process.

Early studies demonstrated that contracting skeletal muscle releases K^+ and that infusion of solutions with elevated K^+ into resting muscle causes vasodilatation supporting a role for K^+ in functional hyperemia. Subsequent studies showed that interstitial K^+ is indeed increased during muscle contraction gracilis muscle exercise in dogs, and this maximum concentration was reached quickly and slowly declined to a steady state level (8). In dog gastrocnemius, the maximum potassium concentration reached was 7.4 mM and also gradually decreased to a steady state (67). It was also shown that the dilation associated with infusion of elevated K^+ was transient and could be involved with the initial phase of functional hyperemia, but not the sustained increase in blood flow associated with maintained exercise (27).

It is not known if the transient nature of the response to elevated extracellular K^+ observed in intact skeletal muscles reflects an intrinsic property of arteriolar smooth muscle, or of the entire system. However, early studies of a number of preparations from large vessels suggest that some vascular smooth muscle may relax in a transient fashion to elevated K^+ (55). In contrast recent studies of isolated small cerebral and coronary arteries suggest that elevated K^+ produces a sustained vasodilation in those vessels. (52)

Mechanism of Action of Elevated K^+

The mechanism by which elevated K^+ causes dilation of skeletal muscle arterioles remains unclear. Studies in other systems showed that when extra-cellular K^+ concentration is raised from 5 mM to 8-20 mM (dependent on the blood vessel studied),

that the smooth muscle membrane potential hyperpolarized leading to vasodilation , a finding supported by recent studies in small cerebral and coronary arteries (33, 34, 91, 104). In vitro studies of vessels from a variety of sources have implicated the Na^+/K^+ ATPase in this hyperpolarization and subsequent vasodilation for two reasons. First, the hyperpolarization that is observed in many of these preparations is transient, consistent with activation of the Na^+/K^+ ATPase, loss of intracellular Na^+ and subsequent inhibition of the pump (30). Second, in a number of cases the hyper-polarization or vascular smooth muscle relaxation could be inhibited by ouabain (14, 19, 22, 35, 109). Consistent with this hypothesis, Lombard and Stekiel have shown that a high concentration of ouabain (1 mM) inhibits K^+-induced dilation of arterioles in rat cremaster muscle, in vivo. (99)

In contrast, studies by Edwards and Hirst in cerebral arterioles and recent studies in small cerebral and coronary arteries have suggested that elevated K^+ dilates these vessels by activating K_{IR} channels (33, 34, 84, 91, 104). It was shown that elevated K^+ caused sustained hyperpolarization and dilation of these vessels that could be abolished by a low concentration of Ba^{2+} (50 μM), and that this concentration of Ba^{2+} was selective for K_{IR} channels (33, 97, 125). In addition, Nelson's group recently showed that there was no K^+-induced dilation of cerebral arteries in mice lacking the gene for Kir 2.1 (112). Loeb and co-worker have suggested a similar mechanism in the in vivo response of cremaster muscle arterioles to elevated K^+ (97). However, these latter studies are difficult to interpret because of changes in baseline diameter, data normalization and lack of concentration-dependence in the responses that they recorded. Thus, the mechanism by which elevated K^+ dilates skeletal muscle arterioles remains to be established and forms the central focus of this dissertation.

CHAPTER 2

MATERIALS AND METHODS

Techniques

Animal Handling and Euthanasia

Male golden Syrian hamsters (80-150 g): Charles River Laboratories, Wilmington, MA) were euthanised with sodium pentobarbital (>150 mg/kg body weight, i.p.). The right testicle was exposed by scrotal incision and connective tissue surrounding the cremaster muscle was carefully dissected away. The testicle was removed and placed in a water-jacketed dissection bath maintained at 4° C, filled with calcium-free physiological salt solution (PSS; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 1.8 mM $CaCl_2$ pH = 7.4), containing sodium nitroprusside (10 μM) and diltiazem (10 μM) to eliminate vasospasm during dissection.

Removal and Dissection of the Cremaster Muscle and Arterioles

The cremaster muscle was carefully separated from the testicle by cutting away the connective tissue between them. An incision was made in the cremaster to expose the serosal side of the muscle, allowing visualization of all of the blood vessels. The muscle was spread out and pinned (#000 insect pins) on a silicon (Sylgard 184) pad for further dissection. Either first or second order arterioles,

devoid of smaller side branches, then were isolated by hand dissection. The muscle layers surrounding the desired arteriole were dissected away, one layer at a time, by cutting individual muscle fibers on either side of the vessel. This was repeated until the desired length (1-2 mm) of arteriole was completely exposed. The vessel segment was excised and transferred to the cannulation chamber using a Wiretrol pipettor (50-100 µl) pretreated with albumin to reduce adhesion of the isolated vessels to the glass barrel of the pipettor. Microdissections were performed with the aid of a Wild Heerbrugg stereomicroscope (80X).

Cannulation

Cannulation was performed in a chamber filled with calcium-free PSS, kept at room temperature. Arterioles were cannulated using glass micropipettes with tip outer diameters of 40-50 µm, filled with PSS containing calcium (1.8 mM). Micropipettes were formed from glass tubing (borosilicate capillary tubing, Warner Instruments) using a pipette-puller and a microforge. The ends of the pipette were fire polished and bent at a 45° angle. Free ends of arterioles were gently pulled onto glass pipette tips and secured with 11-0 nylon monofilament opthalmic suture (16-18 µm outside diameter), using sharpened #5 Dumont forceps. The suture was prepared as loops prior to cannulation, so that they would rest on the end of the pipette tip and easily slide over the cannulated vessel and be easily tightened. Cannulation of arterioles was performed with the aid of a Wild Heerbrugg stereomicroscope (40-80X magnification).

Collecting and Recording Data

Arterioles were pressurized to 70 cm H_2O and further exposed to a room temperature flow of calcium-free PSS for ten minutes and the distance between the two pipette tips adjusted to approximate the in vivo length of the arterioles. The cannulated vessels were tested for leaks by closing the stopcock (Discofix 4-way) between the vessel and the fluid column and monitoring diameter for several minutes. If a diameter change was noted (indicating a leak in the vessel), the experiment was terminated and a new vessel selected. If no leaks were detected, then the bath was slowly warmed to a temperature of 34° C with a continuous flow of PSS containing calcium (1.8 mM $CaCl_2$). The solution flowed from a heated bath through heated tubing into the chamber. Heated solution continually flowed in one end of the chamber and was aspirated from the other end by suction.

Vessels were allowed to reach physiological temperature and equilibrate under no-luminal flow conditions for 30-45 min. During this time the arterioles usually developed tone, indicated by a reduction in diameter at constant pressure and a thickening of the vessel walls. Once vessels developed tone, experimental protocols were performed (see below). Cannulated vessels remained physiologically viable for at least 4-5 hours under these conditions. All diameter changes were visualized and read continuously using Diamtrak software, an automated diameter recording system. All data were graphically recorded using a MacLab data acquisition system running Chart software. Figure 5 illustrates this experimental set up. At the end of each experiment, arterioles were exposed to calcium-free PSS for at least 30 minutes to determine the maximum passive diameter of each arteriole.

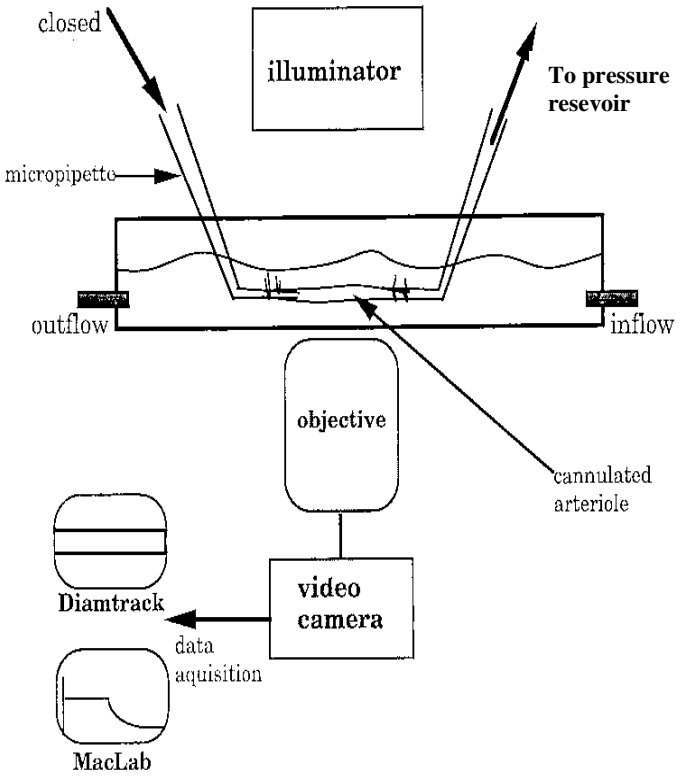

Fig. 5- Cannulated Arteriole Set Up for Experimental Protocols.

Figure 5 shows the arteriole is cannulated and put in a chamber with continuous flow and the diameter changes are visualized using Diamtrak and recorded by MacLab data acquisition system.

Experiment Protocols

Effects of a Calcium Channel Blocker: Diltiazem

The initial experiments looked at the vascular response to 10 μM diltiazem (DTZM). Diltiazem causes vasodilation by blocking L-type calcium channels (4, 37, 76, 138,155). Elevated $[K^+]_o$ has been proposed to cause vasodilation by membrane hyper-polarization and closing these channels (33, 63, 64). The presence and function of these channels had to be established before any other experiments could be carried out regarding a response to extra-cellular K^+. Arterioles were exposed to PSS containing 10 μM DTZM for approximately five minutes. The DTZM was washed off with PSS and the vessels were allowed to regain tone.

Does Elevated $[K^+]_o$ Dilate Cannulated Cremasteric Arterioles?

To evaluate cannulated cremasteric arterioles as a model to study the mechanism by which elevated $[K^+]_o$ causes vasodilation in skeletal muscle, the effects of raising $[K^+]_o$ from 5 mM to 8.75 mM, 12.5 mM or 20 mM was assessed. This was accomplished by measuring the diameter of arterioles bathed in PSS containing 5 mM K^+ and then rapidly switching to solutions containing, 8.75 mM, 12.5 mM or 20 mM while measuring diameter for at least 10 minutes. The various K^+ concentrations were applied in random order, each followed by a 15-minute wash with normal PSS (5 mM K^+). This procedure was used to determine the stability of the K^+ -induced dilations (transient or sustained). At the end of this series, the vessels were washed for at least 30 minutes in PSS containing calcium and allowed to regain tone.

Does Dilation Induced by Elevated $[K^+]_o$ Require a Functional Endothelium?

Endothelial cells can influence arteriolar tone by releasing vasoactive substances such as NO and PGI_2 (1, 42). Therefore, experiments were performed to determine if the endothelium was involved in the response of cremasteric arterioles to elevated $[K^+]_o$. This was done by comparing the dilation of arterioles when $[K^+]_o$ was increased from 5 to 20 mM before and after removal of, or damage to, the endothelium. The vessels were initially exposed to 20 mM K^+ to determine the control response, followed by a 10 minute PSS wash and reestablishment of tone. Next, the vessels were exposed to acetylcholine (Ach) (10 μM) very briefly to initiate a maximal dilatory response to this agent, then washed for 10 minutes with PSS. Acetylcholine is known to elicit vasodilation that is absolutely dependent on a functional endothelium (7, 11, 17, 49, 42) and served as a functional test of the presence or absence of endothelial cells. A bubble of air (approximately 0.5cc) then was allowed to perfuse through the vessel lumen. This was accomplished by injecting a bolus of air into the line connected to the upstream pipette and then opening the stopcock connected to the down stream pipette to allow flow through the lumen of the arteriole. After the bubble had passed through the vessel, the stopcock was closed and flow through the vessel stopped. The vessel then was allowed to regain tone. If the vessels did not spontaneously regain tone, it was briefly exposed to 10 μM norepinephrine and then washed in PSS for at least 10 minutes. Once the arterioles achieved a steady state of resting tone, they were re-exposed to 20 mM K^+ and ACh to determine their dilatory effects. If, however, tone was not reestablished, the experiment was terminated.

Does Ba^{2+} Inhibit Dilation Induced by Elevated $[K^+]_o$?

To test the hypothesis that K_{IR} channels mediate K^+ -induced dilation, arterioles were exposed to 20 mM K^+ in the absence or presence of the K_{IR} channel blocker, $BaCl_2$ (50 μM and 100 μM). The initial concentration of 50 μM was chosen because it has been shown to abolish K^+-induced dilation in cerebral and coronary arteries (119). The higher concentration was employed because the 50 μM concentration did not produce consistent abolition of the dilatory response in the present study. An initial control response was determined using 20 mM K^+ followed by a PSS wash. The arterioles were then exposed to a pretreatment of barium in PSS containing 5 mM K^+ at either of the concentrations for 10 minutes, directly followed by 20 mM K^+ containing the same barium concentration.

Are K_{ATP} Channels Involved in Dilation Induced by Elevated $[K^+]_o$?

Barium ions have been reported to block K_{ATP} channels as well as K_{IR} channels (119). Therefore, to exclude the possibility that K_{ATP} channels were involved in the response to elevated K^+, the effects of K_{ATP} channel blocker glibenclamide (1 μM) were assessed. The efficacy of glibenclamide was verified by assessing its effect on dilation induced by cromakalim, a K_{ATP} channel agonist. An initial 20 mM K^+ response was determined, followed by a 10 minute wash in PSS. Next, the vessels were exposed to a pretreatment of glibenclamide in PSS containing 5 mM K^+ for 15 minutes, after which time, the vessels were exposed to the antagonist in the presence of 20 mM K^+. The vessels were then washed for at least 10 minutes in PSS. Next, cromakalim was applied for 10 minutes. This exposure was followed by a very long PSS wash, as this K_{ATP} channel agonist was difficult to wash off. Finally, as a control, the vessels were

prewashed again with glibenclamide for 15 min, this time followed by an exposure to glibenclamide in the presence of cromakalim.

Are Effects of Ba^{2+} Selective?

The specificity of barium was also assessed by testing its effects on dilation induced by acetylcholine (ACh) (5 μM). Acetylcholine is well known to elicit arteriolar dilation associated with membrane hyperpolarization (7, 17, 121). An initial control potassium response was determined by exposing the vessels to 20 mM K^+. The vessels were then washed with normal PSS for approximately 10 minutes, followed by exposure to ACh (10 μM). The vessels were again washed with normal PSS for 10 minutes. A 10 minute prewash of 50 μM of Ba^{2+} was performed, directly followed by ACh in the presence of Ba^{2+}, for a duration of at least 5 minutes. The vessels were again washed with PSS.

Does Ouabain Inhibit Dilation Induced by Elevated $[K^+]_o$?

In order to determine the role of the Na^+/K^+ ATPase, experiments were conducted, using the same protocol used when determining the effects of $BaCl_2$, in the absence or presence of the Na^+/K^+ ATPase inhibitor, ouabain (0.1 mM and 1 mM). The initial concentration of 0.1 mM was chosen because it has been used in other studies to block Na^+/K^+ ATPase. (52, 65, 81, 98) A higher concentration was employed because consistent abolition of the dilation, as a result of Na^+/K^+ ATPase block, was not seen in this skeletal muscle arteriole model, and it has been used in other studies of skeletal muscle microcirculation (14, 35, 52).

Are Effects of Ouabain Specific?

In an effort to insure that the ouabain block was specific, acetylcholine (ACh) (5 μM) was used in place of the potassium, as a vasodilator. An initial control potassium response was determined by exposing the vessel to 20 mM K^+. The vessels were then washed with PSS for approximately 10 minutes, followed by exposure to ACh. The vessels were again washed with PSS for 10 minutes. This was followed by ACh in the presence of ouabain, for a duration of at least 5 minutes. The vessels were again washed with PSS. The ten minute prewash of 0.1 mM of ouabain was not performed because recovery from ouabain exposure was not reliable. To ensure that all aspects of the experiment could be accomplished, the number of ouabain exposures, per protocol was limited.

Does a Combination of Ba^{2+} & Ouabain Abolish Dilation Induced by Elevated $[K^+]_o$

The previous experiments implicated both K_{IR} channels and the Na^+/K^+ ATPase in K^+ -induced dilation. An initial control response was determined using 20 mM K^+ followed by a PSS wash. The vessels were allowed to regain tone. Then, to determine if both mechanisms contribute to K^+ -induced dilation, a cocktail of barium and ouabain, with the optimal concentrations, 100 μM and 1 mM respectively, were used in an effort to totally inhibit the response. Arterioles were exposed to 20 mM K^+ in the absence or presence of the cocktail of barium and ouabain.

Statistics

Summary data are presented as means ± SE. Data were analyzed using t tests and ANOVA (single factor, two factor without replication or factorial) followed by a Student-Newman-Keuls test to compare mean values, if appropriate. Differences were considered to be of statistical significance if $P < 0.05$.

CHAPTER 3

RESULTS

Arteriole Responses

First and second order arterioles were isolated from hamster cremaster muscles. A total of 81 viable vessels were studied. Their average maximal diameter was 107 ± 2 μm and their average diameter with tone was 59 ± 1 μm.

Effects of Diltiazem

These initial experiments were designed to determine the presence and activity of L-type calcium channels. Exposure of cremasteric arterioles to 10 μM diltiazem caused dilation (Fig. 6). Arterioles exposed to calcium-free PSS also dilated (Fig. 6). Both diltiazem and calcium-free PSS caused similar maximal dilation. Arterioles with a resting diameter (tone) of 71 ± 5 μm dilated to a diameter of 105 ± 7 μm in the presence of diltiazem and to 113 ± 6 μm in the presence of calcium-free PSS, both being significantly different from the control diameter ($n = 6$, $P < 0.05$), but not significantly different from one another ($P > 0.05$).

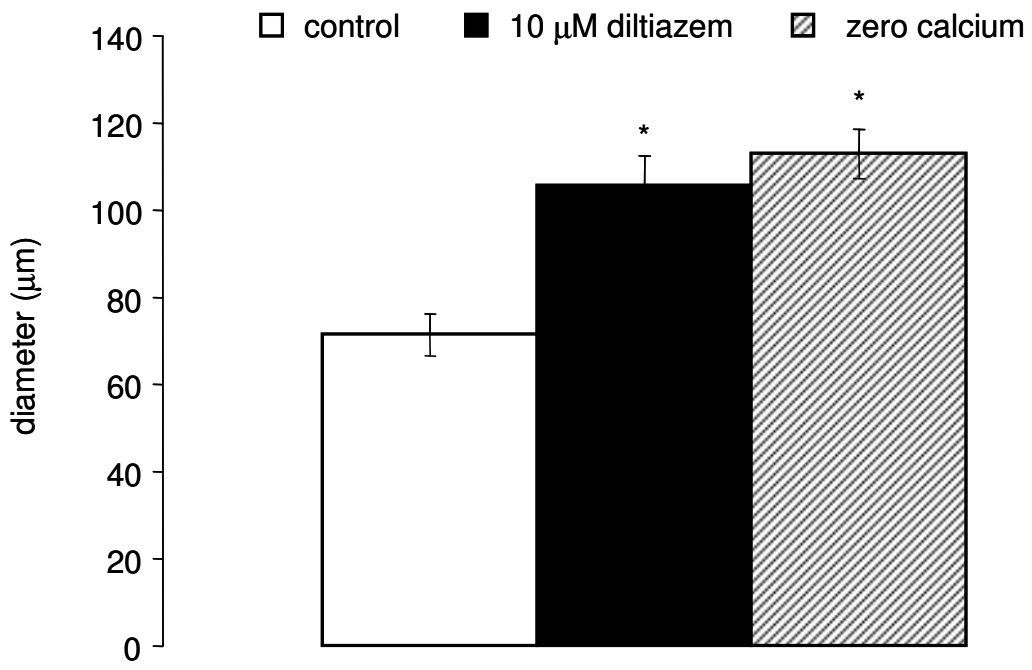

Fig. 6-Diltiazem and Arteriolar Dilation

Figure 6 provides an analysis of variance corrected for repeated measures indicated a significant effect of DTZM and Ca^{2+} free PSS on diameter. * = significantly different from control diameter (at 0 μM DTZM and 1.8 mM Ca^{2+}), $P < 0.05$, but not significantly different from each other, $P > 0.05$.

Elevated $[K^+]_o$ Results in Dilation of Cannulated Cremasteric Arterioles

Elevation of bath potassium concentration from 5 mM to 8.75 mM, 12.5 mM or 20 mM resulted in concentration-dependent arteriolar dilation from a resting diameter of 66 ± 6 μm to 72 ± 4 μm ($P > 0.05$), 81 ± 5 μm ($P < 0.05$), and 85 ± 5 μm ($P < 0.05$) respectively ($n = 8$, Fig. 7). Six out of eight times, the peak diameter occurred in the presence of 20 mM K^+. Dilations at all concentrations were transient as seen Figure 7.

Peak potassium-dilation, in nearly all of the 75 control cases, was reached in less than two minutes and the vessels returned to, or below resting diameter after 7.5 minutes of K^+ exposure.

There appeared to be a weak, negative correlation between the dilation induced by 20 mM K^+ (relative to the maximum possible dilation) and the maximum diameter of the arterioles studied ($r = -0.34$, $n = 75$, $P < 0.05$). That is, larger vessels tended to dilate less than smaller vessels.

Dilation Induced by Elevated $[K^+]_o$ does not Require a Functional Endothelium

Endothelial cells release a number of vasodilator substances in response to agonist and physical stimulation that strongly impact the contractile activity of the overlying smooth muscle cells (1). The role played by endothelial cells in the response of skeletal muscle arterioles to elevated extracellular potassium is not known. Therefore, the effects of removal of endothelial cells on K^+-induced dilation of cremasteric arterioles were examined. Perfusion of cannulated arterioles with an air bubble to remove the

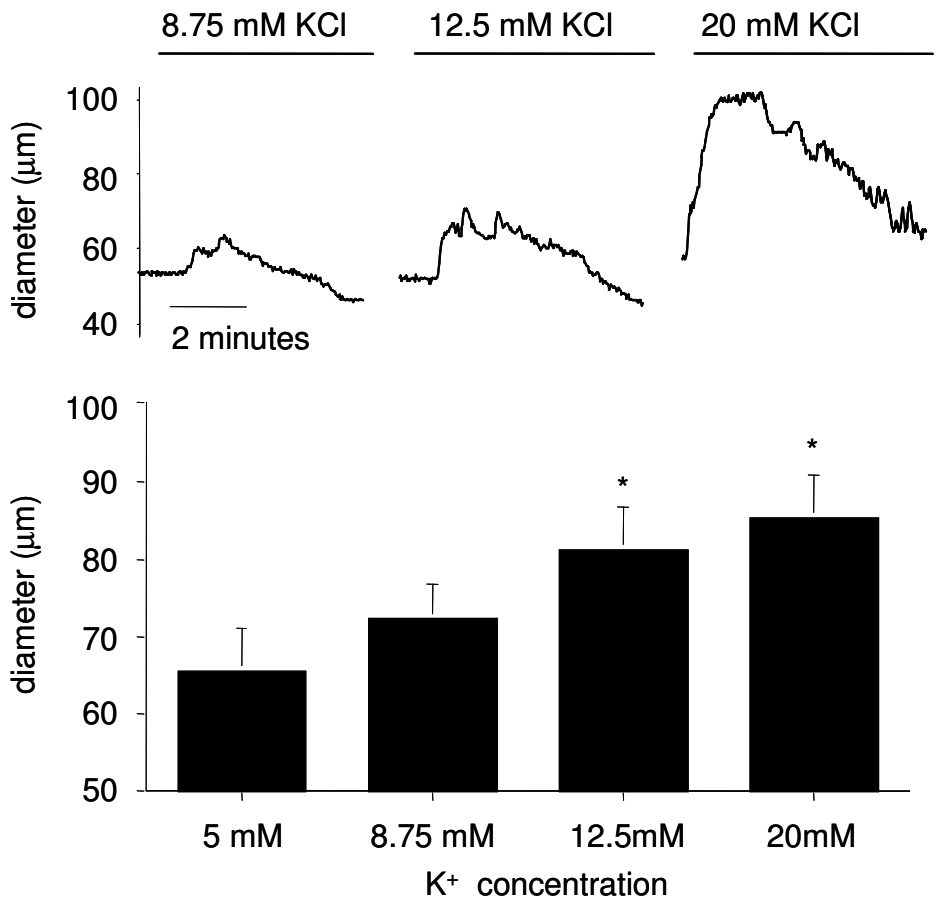

Fig. 7- Concentration-dependent Potassium Dilation

Figure 7 top panel digitized diameter demonstrates the transient and concentration-dependent nature of K^+-induced dilation in isolated hamster cremasteric arterioles. Bottom panel: Peak arteriolar diameters (mean ± SE, n = 8) in response to the K^+ concentrations indicated. Analysis of variance corrected for repeated measures.

endothelium caused no significant change in resting diameter (control diameter = 54 ± 3 μm; diameter after air bubble perfusion = 61 ± 2 μm, n=5; $P > 0.05$). However, acetylcholine-induced dilation of the arterioles, which depends on a functional endothelium (7, 11, 17, 39), was abolished. (Fig 8). These data verify that air bubble perfusion eliminated endothelium-dependent dilation of cremasteric arterioles. Despite the loss of endothelium-dependent reactivity, the arterioles continued to respond to elevated $[K^+]_0$. (Fig. 9), and attained the same peak diameter as prior to air bubble perfusion.

Removal of the arteriolar endothelial lining was important in determining its role in the K+-induced dilation, but perfusion of the vessels with an air bubble proved to be a somewhat difficult technique. Although much care was taken, it was often difficult to control the speed at which bubbles passed through the arteriole lumen. If a large bubble moved through a vessel too quickly, there appeared to be significant damage to the vessel. The damage may have been across the full length or partial length of the arteriole; the vessels appeared dilated and there was no recovery even after long washes with PSS. The damaged vessels also would not respond to vasoactive substances.

The passage of small, slower air bubbles proved to be most the effective in the removal of the endothelium while maintaining the integrity of the smooth muscles. These vessels recovered after a PSS wash and were responsive.

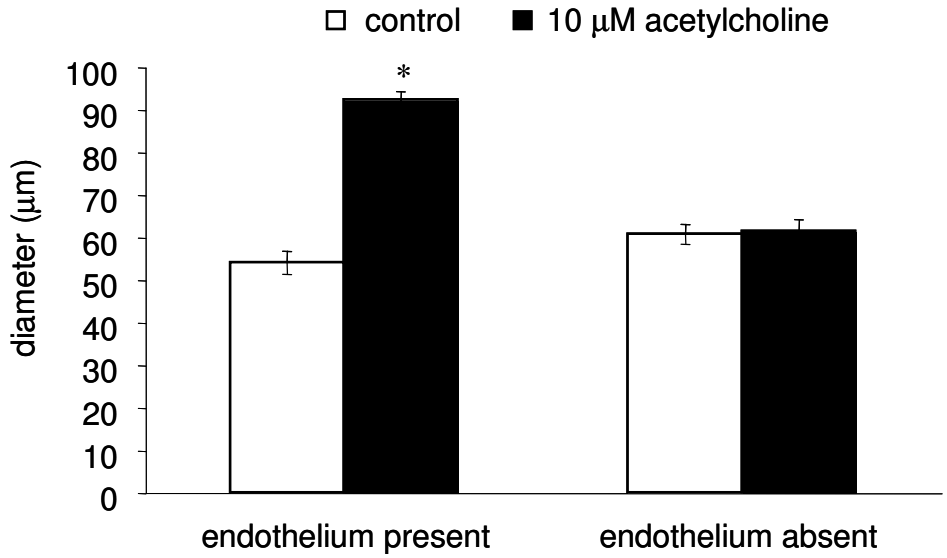

Fig. 8- Removal of Endothelium

Figure 8 shows peak arteriolar diameters (mean ± SE, n = 5). Control diameters represent resting diameters in the presence and absence of endothelium. Acetylcholine diameters represent the peak diameters in response to 10 μM acetylcholine, both in the presence and absence of endothelium. Factorial ANOVA indicated significant acetylcholine effects with endothelium present, $P < 0.05$, but there was no significant acetylcholine effect with endothelium absent. * = significantly different from control diameter.

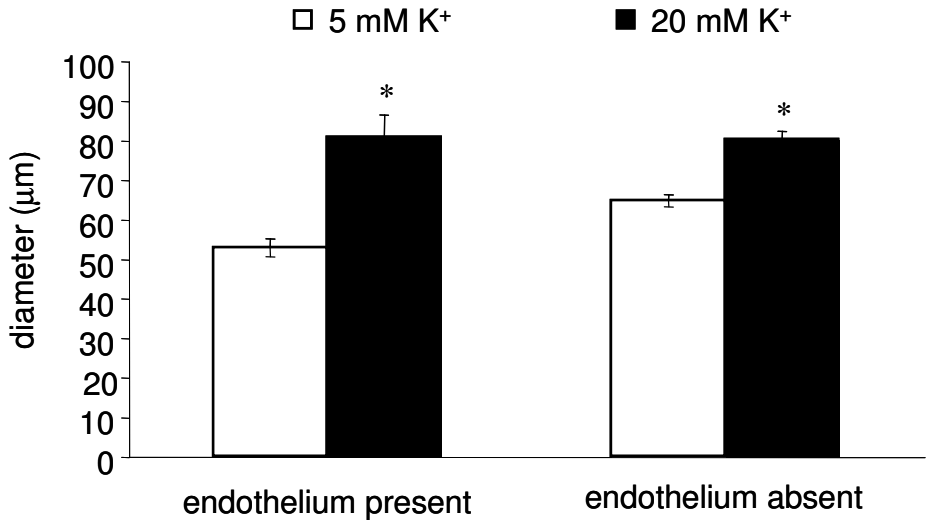

Fig. 9- Uninhibited K+ Induced Dilation

Figure 9 shows peak arteriolar diameters (mean ± SE, n = 5). Control diameters represent resting diameters in the presence and absence of endothelium. KCl diameters represent peak diameters during exposure to 20 mM KCl in the presence and absence of endothelium. Factorial ANOVA indicated significant KCl effects both in the presence and absence of endothelium. * = significantly different from control diameter, $P < 0.05$.

Role of K_{IR} Channels

Studies in other blood vessels have implicated K_{IR} channels in K^+-induced dilation (33-35, 84, 91, 97, 118, 119). However, the role played by these channels in K^+-induced dilation of skeletal muscle arterioles remains unclear. Therefore, we examine the effects of Ba^{2+}, a known K_{IR} channel blocker (118-120) on dilation of cremasteric arterioles to elevated $[K^+]_o$. We found that exposure of cannulated arterioles to Ba^{2+} (50 or 100 μM) caused significant constriction (Fig. 10) and inhibition of the peak dilation induced by raising $[K^+]_o$ from 5 to 20 mM (Fig. 10). Although 100 μM Ba^{2+} tended to cause a slightly greater increase in tone, the degree of inhibition of the K^+-induced dilation was not significantly different for the two concentrations of Ba^{2+} tested: 50 μM Ba^{2+} caused 48 ± 14% inhibition and 100 μM Ba^{2+} caused 41 ± 16% inhibition of 20 mM K^+-induced dilation ($P > 0.05$). These data suggest that their effects were near maximal. Dilation was abolished in only one experiment at each of the two Ba^{2+} concentrations tested.

Role of K_{ATP} Channels

In the previous section barium was used to assess the role played by K_{IR} channels in potassium-induced vasodilation. However, barium has also been demonstrated to block currents through K_{ATP} channels (118-120, 125). Therefore, the effects of glibenclamide (1 μM), a selective K_{ATP} channel inhibitor, on potassium induced dilation were examined. Superfusion of cannulated cremasteric arterioles with 1 μM glibenclamide had no

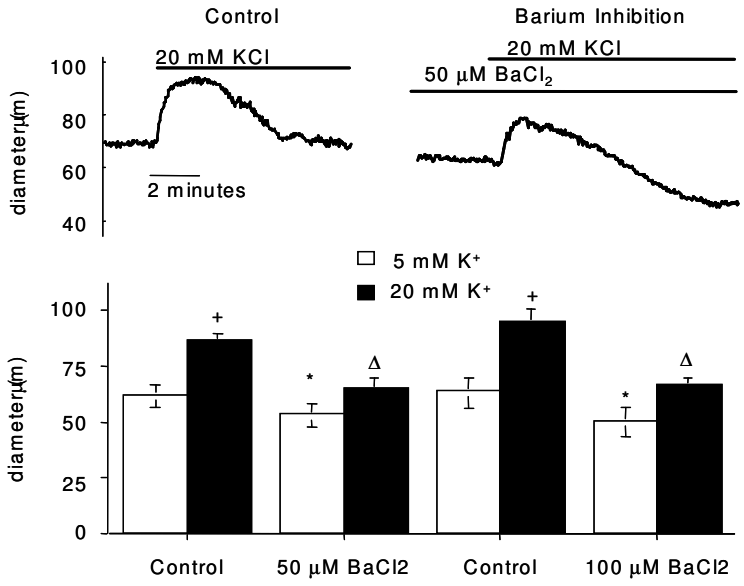

Fig. 10- Effects of Barium on Potassium-induced Dilation

The top panel of Figure 10 shows partial inhibition of K^+-induced dilation by Barium (50 µM). Bottom panel: Peak arteriolar diameters (mean ± SE, n = 8 for both concentrations). Control diameters represent diameters before and after exposure to K^+ in the absence of barium and with barium pre-wash, respectively. 5 mM K^+ diameters represent resting diameters prior to 20 mM K^+ exposure. 20 mM K^+ diameters represent the peak diameter during exposure to 20 mM K^+, both in the absence and presence of 50 or 100 µM barium. Factorial ANOVA indicated significant K^+ and barium effects. + = significantly different from diameter in 5 mM K^+ in the same group, * = significantly different from control diameter, same K^+ concentration, Δ = significantly different from 5 mM K^+ same group and different from control 20 mM K^+ diameter, $P < 0.05$.

significant effect on resting diameter and did not inhibit dilation of arterioles when extracellular potassium concentration was elevated from 5 to 20 mM (Fig. 11). The efficacy of K_{ATP} channel block by glibenclamide was verified using the K_{ATP} channel agonist, cromakalim. As seen in Fig. 12, 1 μM glibenclamide eliminated arteriolar dilation induced by 10 μM cromakalim. Thus, 1 μM glibenclamide effectively inhibited K_{ATP} channels in cremasteric arterioles but had no effect on K^+-induced dilation.

Determination of Selectivity of Barium Inhibition for K_{IR}

As a control for selectivity, the effects of barium on acetylcholine-induced dilation were determined. The magnitudes of arteriolar dilation due to increased potassium or exposure to 5 μM acetylcholine are similar (Fig. 13). As previously shown, Ba^{2+} (50 μM) inhibited potassium-induced dilation (Fig. 13). However, dilation induced by 5 μM acetylcholine was unaffected (Fig. 13). These data suggest that the effects of Ba^+ are selective for K^+-induced dilation.

Role of Na^+/K^+ ATPase

The Na^+/K^+ ATPase has also been proposed to mediate K^+-induced vasodilation (12, 22, 52). However, the role played by this transporter in skeletal muscle arterioles has not been firmly established. Therefore, the effects of ouabain, an inhibitor of the pump, on dilation of cremasteric arterioles in response to elevation of $[K^+]_o$ from 5 to 20 mM

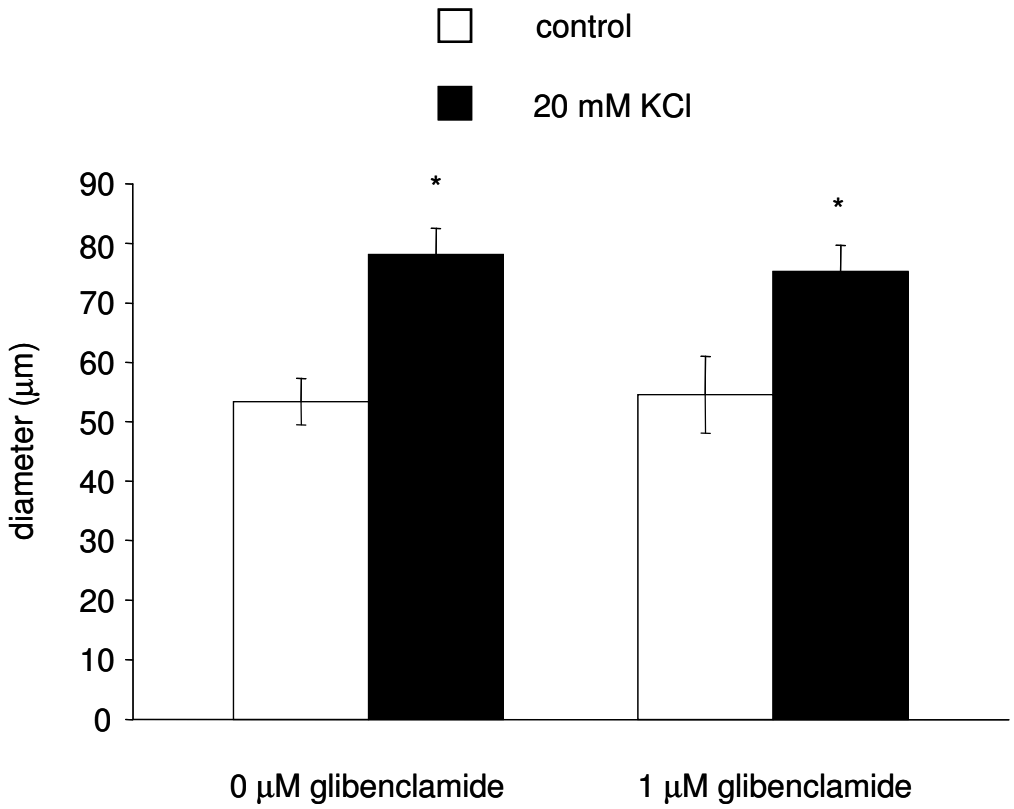

Fig. 11- K+ Induced Dilation Uninhibited by Glibenclamide

Figure 11 shows peak arteriolar diameters (mean ± SE, n = 6). Control diameters represent resting diameters prior to Glibenclamide (1 µM) exposure and prior to KCl (20 mM) exposure. KCl diameters represent the peak diameter during exposure to KCl, both in the absence and presence of Glibenclamide. Factorial ANOVA indicated significant KCl effects in the absence and presence of Glibenclamide. * = significantly different from control diameter, $P < 0.05$.

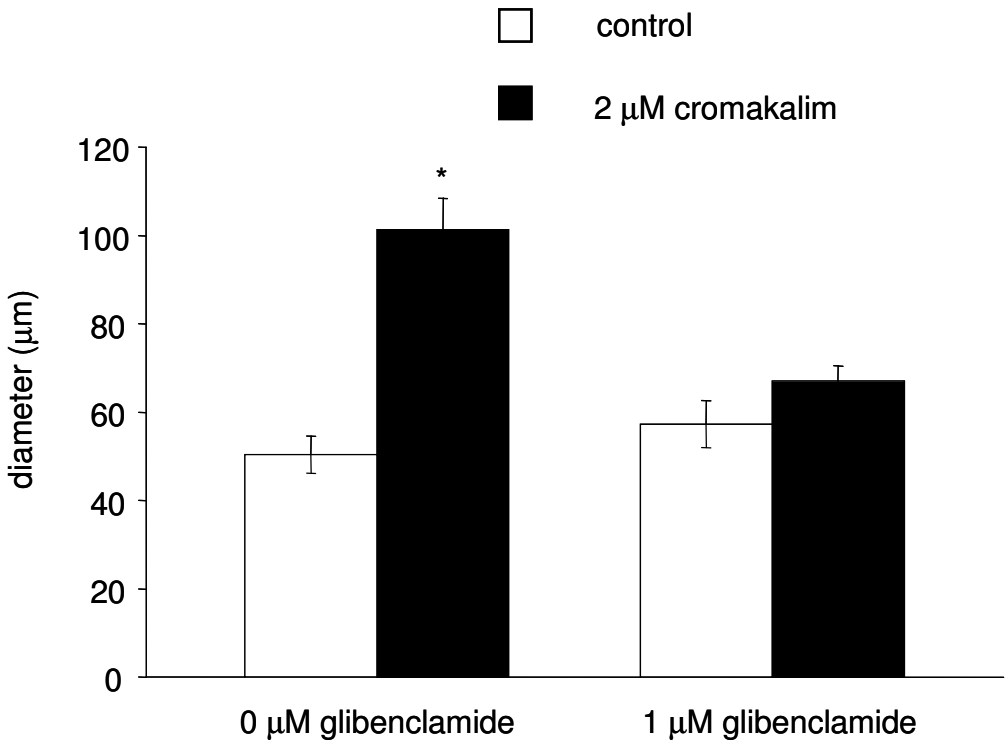

Fig. 12- Cromakalim-induced Dilation Inhibited by Glibenclamide

Figure 12 shows peak arteriolar diameters (mean ± SE, n = 6). Control diameters represent resting diameters prior to glibenclamide (1 μM) exposure and prior to cromakalim (2 μM) exposure. cromakalim diameters represent the peak diameter during exposure to cromakalim, both in the absence and presence of glibenclamide. Factorial ANOVA indicated significant glibenclamide effects, * = significantly different from control diameter, $P < 0.05$.

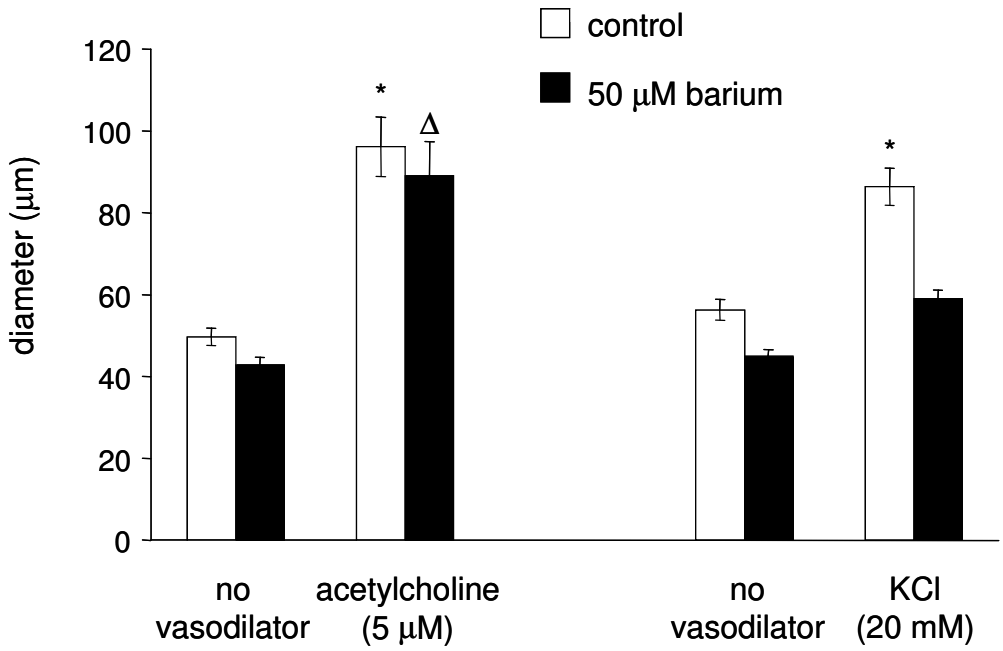

Fig. 13- Acetylcholine-induced Dilation Uninhibited by Barium

Figure 13 shows peak arteriolar diameters (mean ± SE, n = 4). Control diameters represent resting diameters in the absence of Ba^{2+} (50 µM) and peak diameters seen with indicated vasodilators (5 µM Acetylcholine, 20 mM KCl), in the absence of Ba^{2+}. Barium diameters represent diameters in the absence and presence of indicated dilators. Factorial ANOVA indicated significant acetylcholine and KCl response in the absence of Ba^{2+}, $P < 0.05$. Significant acetylcholine response in the presence of Ba^{2+} was also indicated, $P < 0.05$. * = significantly different than control with no vasodilator. Δ = significantly different than control with Ba^{2+} present.

were assessed. As shown in Figure 14, superfusion of arterioles either concentration of ouabain (0.1 or 1.0 mM) had no effect on the diameter of arterioles in 5 mM K^+. However, both concentrations of this cardiac glycoside (0.1 and 1.0 mM) significantly inhibited K^+-induced dilation in a concentration-dependent manner: the mean dilations that remained were not significantly different from zero for either concentration of ouabain. At the 0.1 mM concentration, ouabain abolished dilator responses to elevated $[K^+]_o$ in 4 out of 9 experiments (44%) and inhibited by 91% in one additional experiment (mean %inhibition = 73 ± 15 %). In the remaining 4 experiments, dilation was inhibited by less than 58%. With 1 mM ouabain, dilation was eliminated in 7 of 14 experiments (50%) and severely inhibited by 75 to 93% in three other experiments (mean %inhibition = 80 ± 11 %). In the remaining four trials, dilation was inhibited by less than 54%.

Ouabain Effects on Acetylcholine-induced Dilation

As a control for selectivity, the effects of ouabain on acetylcholine–induced dilation were examined. As shown previously, dilation induced by 5 µM acetylcholine caused dilation similar to that observed when $[K^+]_o$ was elevated from 5 to 20 mM (Fig. 15) and ouabain (0.1 mM) inhibited K^+-induced dilation (Fig. 15). However, this concentration of ouabain had no significant effect on dilation induced by 5 µM acetylcholine (Fig. 15). These data suggest that this concentration of ouabain selectively inhibited K^+-induced dilation.

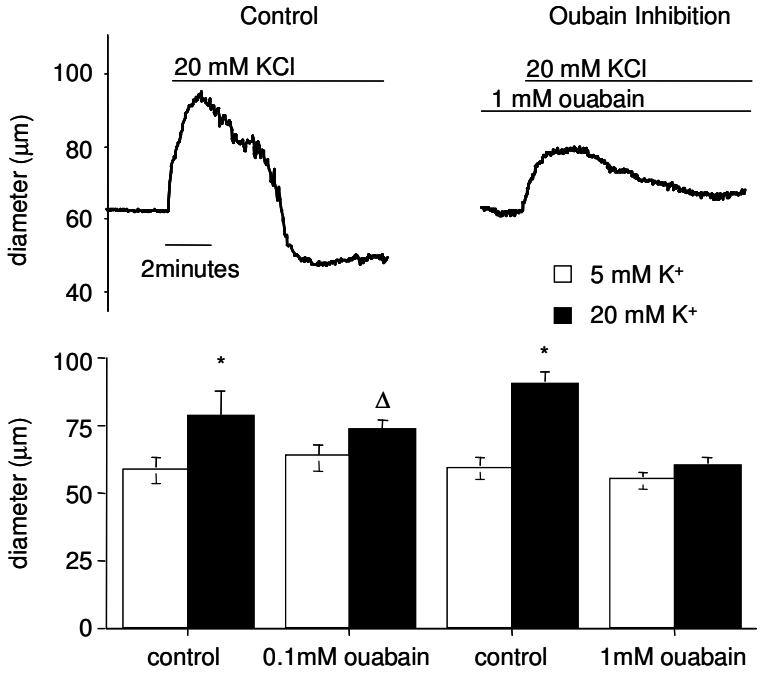

Fig. 14- Ouabain and Potassium-induced Dilation

The top panel of Figure 14 records partial inhibition of K^+-induced dilation by ouabain (1 mM). Bottom panel: Peak arteriolar diameters (mean ± SE, n = 9 at 0.1 mM, n = 14 at 1 mM). 5 mM K^+ diameters represent resting diameters prior to 20 mM K^+ exposure. 20 mM K^+ diameters represent the peak diameter during exposure to 20 mM K^+, both in the absence and presence of 0.1 or 1 mM ouabain. + = significantly different from diameter in 5 mM K^+ in the same group, * = significantly different from control diameter, same K^+ concentration, Δ = significantly different from 5 mM K^+ same group and different from control 20 mM K^+ diameter, $P < 0.05$.

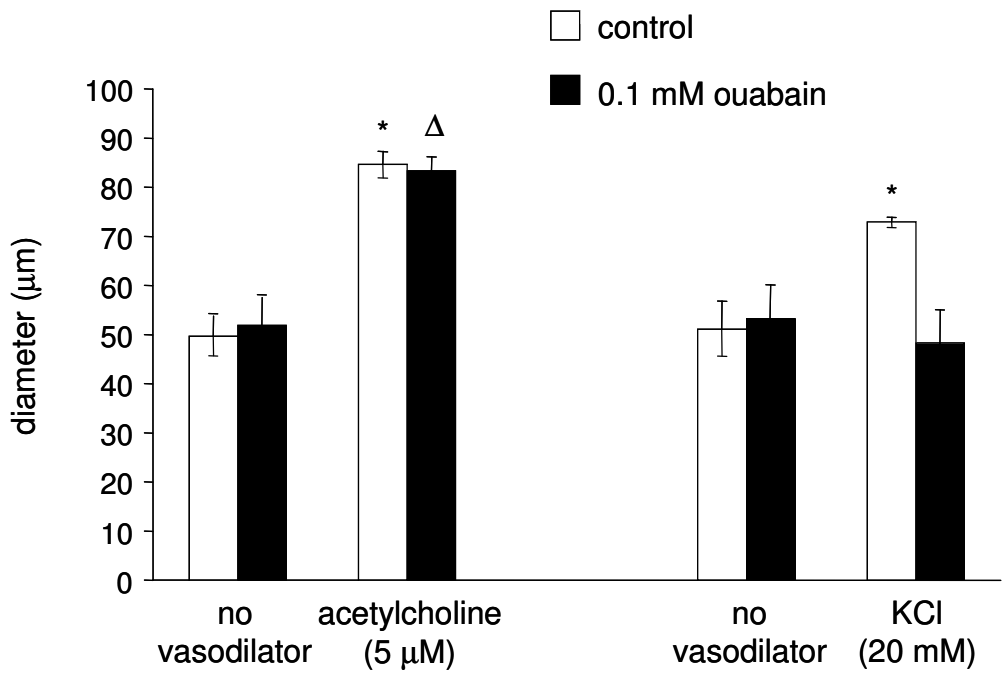

Fig. 15- Ouabain and Acetylcholine-induced Dilation

Figure 15 shows peak arteriolar diameters (mean ± SE, n = 4). Control diameters represent resting diameters without any vasodilator present and peak diameters seen with indicated dilators (5 μM Acetylcholine, 20 mM KCl), in the absence of Ouabain (0.1 mM) Ouabain diameters represent diameters in the absence and presence of indicated dilators. Factorial ANOVA indicated significant Acetylcholine and KCl response in the absence of Ouabain, $P < 0.05$. Significant Acetylcholine response in the presence of Ouabain was also indicated, $P < 0.05$. * = significantly different than control with no vasodilator. Δ = significantly different than control with Ouabain present.

Ouabain – Barium Cocktail Abolishes K^+-induced Dilation

Neither Ba^{+2} nor ouabain consistently eliminated K^+-induced dilation. Therefore, the effects of the combination of these two inhibitors were assessed. Superfusion of cremasteric arterioles with both Ba^{2+} (100 μM) and ouabain (1 mM) caused arteriolar constriction and consistent inhibition of dilation induced by elevation of $[K^+]_o$ from 5 to 20 mM (Fig. 16). In all seven experiments, this inhibitor combination inhibited dilation by more than 91% (mean % inhibition = 95 ± 1.5%).

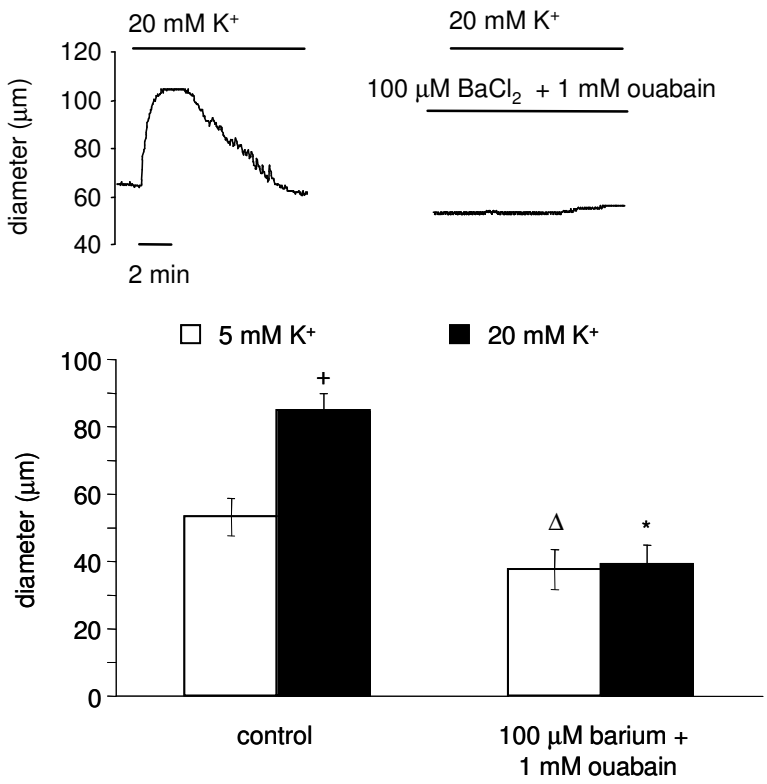

Fig. 16- Barium – Ouabain Cocktail

Top panel of Figure 16 shows an abolition of K^+-induced dilation by the combination of Ba^{2+} (100 µM) and ouabain (1 mM). Bottom panel shows peak arteriolar diameters (mean ± SE, n = 7). 5 mM K^+ diameters represent resting diameters prior to 20 mM K^+ exposure. 20 mM K^+ diameters represent the peak diameter during exposure to 20 mM K^+, both in the absence and presence of the combination of Ba^{2+} (100 µM) and ouabain (1 mM). + = significantly different from diameter in 5 mM K^+ in the same group, * = significantly different from control diameter, the same K^+ concentration, Δ = significantly different from 5 mM K^+ same group and different from control, 20 mM K^+ diameter, $P < 0.05$.

CHAPTER 4

DISCUSSION

The purpose of the present investigation was to evaluate the mechanism by which elevated $[K^+]_o$ causes dilation of skeletal muscle arterioles. This was accomplished by studying the responses of isolated, cannulated, pressurized segments of hamster cremasteric arterioles. As studies in the literature suggested that K_{IR} channels (18, 33, 34, 84, 85, 97) or the Na^+/K^+ ATPase (9, 12, 40, 46, 52, 55, 65, 100) were likely potential mechanisms, the focus was on these two pathways. The results reported in this study suggest that both mechanisms may contribute to the means by which elevated $[K^+]_o$ causes arteriolar dilation.

Elevated $[K^+]_o$ caused concentration-dependent dilation, with maximal effects observed at a concentration of 20 mM. These findings are similar to recent results reported in other systems (14, 30, 53, 94, 97, 104, 115). However, in contrast to findings in small cerebral and coronary arteries, K^+-induced dilation of cremasteric arterioles was transient (111, 112, 119). These data are consistent with in vivo studies of hamster cremasteric arterioles (30) and indicate that the transient nature of the response to elevated $[K^+]_o$ is an intrinsic property of skeletal muscle arterioles. These findings support the hypothesis that elevated $[K^+]_o$ can contribute to the initiation of functional hyperemia in skeletal muscle, but that it is not responsible for the maintained phase of

vasodilation that is associated with prolonged exercise (8, 27, 67).

There is cellular communication between vascular smooth muscle and endothelium (52). Endothelial cells have been shown to respond to different stimuli, and to cause relaxation of smooth muscle by releasing vasodilating factors such as prostacyclin and nitric oxide (1, 42, 52). In addition, endothelial cells may be electrically coupled to smooth muscle cells by myoendothelial gap junctions (52). Therefore, we assessed the role played by the endothelium in the dilatory response to elevated $[K^+]_o$. Removal of the endothelium by air-bubble perfusion, verified by loss of reactivity to acetylcholine, did not eliminate K^+-induced dilation in the present study. These data indicate that K^+-induced dilation of skeletal muscle arterioles does not require the presence of a functional endothelium and are consistent with observations in other systems (84). The possibility that a functional endothelium may contribute to, or modulate the arteriolar response to elevated $[K^+]_o$ cannot be excluded. However, these data indicate that arteriolar muscle cells can respond independently to this stimulus.

Elevated $[K^+]_o$ appears to relax vascular smooth muscle by hyperpolarizing the membrane of these cells (33, 34, 52, 55, 84, 91, 104]. This hyperpolarization then closes voltage-gated Ca^{2+} channels, decreasing the influx of activator Ca^{2+} and resulting in relaxation of the smooth muscle and vasodilatation (76). During this study, membrane potential was not measured. However, the presence of functioning L-type calcium channels that contribute to arteriolar tone was determined. Arterioles dilated near maximally when exposed to the L-type Ca^{2+} channel blocker, diltiazem. These data are consistent with numerous in vitro studies (4, 37, 76) and indicate that Ca^{2+} influx through L-type Ca^{2+} channels contribute substantially to arteriolar tone. These data also suggest

that hyperpolarization would be a viable mechanism by which to dilate these arterioles.

Edwards and Hirst first proposed that K_{ir} channels were involved in K^+-induced dilation in cerebral arterioles (33). This idea was later illustrated by Nelson and colleagues (112) who have provided compelling evidence that K_{ir} channels mediate K^+-induced dilation of small cerebral and coronary arteries (33, 34, 84, 91, 104). In those studies, they showed that low micromolar concentrations of Ba^{2+} could abolish the hyperpolarization and vasodilation induced by elevated $[K^+]_o$. In contrast, this study shows that Ba^{2+} concentrations up to 100 μM only attenuated K^+-induced dilation. It does not appear that the low of efficacy of Ba^{2+} in the present study resulted from the use of submaximal concentrations of this K_{ir} channel blocker, because the degree of inhibition observed was similar with both 50 and 100 μM concentrations.

Barium ions have been shown to block K_{ATP} channels as well as K_{IR} channels (112, 120). To eliminate the possibility that K_{ATP} channels were involved in K^+-induced dilation, the effects of glibenclamide, a K_{ATP} channel blocker, on the response to elevated $[K^+]_o$ were assessed. Glibenclamide (1 μM) eliminated vasodilation to the K_{ATP} channel agonist, cromakalim (10 μM). However, glibenclamide did not inhibit K^+-induced dilation of cremasteric arterioles. These data indicate that K_{ATP} channels are not involved in the arteriolar response to elevated $[K^+]_o$ in this tissue.

Exposure of cremasteric arterioles to Ba^{2+} caused a sustained increase in tone. Non-specific effects due to this constriction cannot be completely ruled out. However, dilation induced by acetylcholine was not inhibited by Ba^{2+} suggesting that the effects of the blocker were not totally non-specific. Therefore, we conclude that K_{ir} channels may contribute to a portion of the response to elevated $[K^+]_o$.

Early studies of the mechanism by which elevated $[K^+]_o$ causes vasodilation in skeletal muscle proposed that the Na^+/K^+ ATPase may be involved (55, 71, 82, 133, 140). More recently, Lombard and colleagues supported this hypothesis by showing that ouabain (1 mM) inhibits K^+-induced dilation of cremasteric arterioles in vivo. The findings in this study corroborate their conclusions. Ouabain (0.1 and 1.0 mM) substantially inhibited K^+-induced dilation of cremaster arterioles in vitro. Furthermore, this effect appeared to be specific because acetylcholine-induced dilation was not altered. These data also support a role for the Na^+/K^+ ATPase in K^+-induced dilation of skeletal muscle arterioles.

While ouabain was quite effective in dampening K^+-induced dilation, substantial dilation remained in a number of experiments even in the presence of a high concentration of this Na^+/K^+ ATPase inhibitor (1 mM ouabain). This observation, coupled with these findings with Ba^{2+}, suggested that both K_{ir} channels and the Na^+/K^+ ATPase may mediate K^+-induced dilation in skeletal muscle arterioles. Therefore, the effects of the combination of Ba^{2+} (0.1 mM) and ouabain (1 mM) were examined. This cocktail of inhibitors consistently produced greater than 91% inhibition of K^+-induced dilation in isolated cremasteric arterioles. These observations support the hypothesis that both K_{ir} channels and the Na^+/K^+ ATPase are involved in the mechanism of action of elevated $[K^+]_o$.

As was observed with Ba^{2+} alone, treatment of arterioles with both Ba^{2+} and ouabain caused arteriolar constriction. Furthermore, control experiments to assess the specificity of the combination of inhibitors were not performed as they were with Ba^{2+} and ouabain alone. Thus, the possiblility of non-specific effects of the inhibitor cocktail

cannot be completely dismissed. Future experiments should address this issue.

The data collected in this study support the hypothesis that K^+-induced dilation of cremasteric arterioles involves activation of both K_{IR} channels and the Na^+/K^+ ATPase. Similar conclusions have been drawn in rat cerebral and renal arteries (33, 44, 84, 91, 100, 104, 115, 119). These studies may also resolve the conflict between the findings of Lombard and Stekiel (99) supporting a role for the Na^+/K^+ ATPase in rat cremasteric arterioles, and Loeb et al. (97) who suggest that K_{IR} channels are involved in the same tissues. In both studies, inhibition of K^+-induced dilation by ouabain (Lombard and Stekiel (99)) or Ba^{2+} (Loeb, et al. (97)) was only partial. Thus, dual mechanisms, as we propose in the present study, may have been involved.

As noted above, K^+-induced dilations were consistently transient in all vessels studied and for all concentrations of K^+ that were tested. The mechanism responsible for the lack of a sustained response is not clear. Activation of the Na^+/K^+ ATPase by elevated $[K^+]_o$ should be transient and should cause as transitory hyperpolarization as Na^+ is pumped out of the cells and intracellular $[Na^+]$ decreases (12, 22, 52). This is consistent with the transient dilation that is observed. However, in contrast, activation of K_{IR} channels should lead to a sustained hyperpolarization and hence dilation as has been reported by others (112). Thus, if both mechanisms are involved one would predict that inhibition of the pump by ouabain should lead to a more sustained response. However, this was not observed. In the presence of ouabain, what responses remained also were transient (see RESULTS). It is possible that hyperpolarization activates depolarizing currents that overwhelm any hyperpolarization caused by small outward currents through K_{IR} channels such that maintained hyperpolarization (dilation) is prevented.

Electrophysiological studies will be required to resolve this dilemma and to determine exactly why K^+-induced dilations are transient in skeletal muscle arterioles.

This study showed that in cremasteric arterioles, K^+-induced dilation likely results from two mechanisms: activation of K_{ir} channels and the Na^+/K^+ ATPase. These mechanisms have been shown to result in potassium induced dilations seen in cerebral, coronary and renal arteries (33, 34, 44, 84, 91, 100, 104, 115, 119, 125). However, these studies show only partial inhibition of the dilation as a result of applying blockers for only one mechanism. This study shows that applying blockers for both mechanisms simultaneously resulted in abolition of potassium induced dilation. It appears that initiation of this dilation is dependent upon the Na^+/K^+ ATPase, while K_{IR} channels may modulate dilation seen during activity. These data in correlation with single cell patch clamp data may provide a more clear responsibility of each mechanism during potassium induced arteriolar dilation.

Finally, it was observed that large arterioles tended to dilate less (relative to their maximal diameters) than did small arterioles. This may indicate that there are significant functional differences in different size arterioles. The density of K_{ir} channels has been reported to increase as vessel diameter decreases in the coronary vasculature (118). Such a change might contribute to the apparent differences in reactivity to $[K^+]_o$ observed in the present study. Further research will be required to settle this issue.

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