THE UNIVERSITY OF CALGARY

Modulation of Renal Microvascular Reactivity by ATP-sensitive K-channels

by

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Abstract

The role of K_{ATP} in the regulation of afferent (AA) and efferent arteriolar (EA) reactivity was examined using the *in vitro* perfused hydronephrotic rat kidney model. Activation of K_{ATP} by pinacidil preferentially inhibited AA reactivity, reflecting the predominant role of L-type calcium channels in AA vasoconstriction. EA reactivity was only observed at relatively high concentrations of pinacidil and this effect did not involve modulation of L-type calcium channel activity. In the AA, calcitonin gene-related peptide (CGRP) and adenosine elicit vasodilations that involve K_{ATP}. The adenosine effects on K_{ATP} appear to be mediated by activation of high-affinity adenosine A_{2a} receptors, as this response is observed at adenosine concentrations of 100-300 nM, and is mimicked by the adenosine A_{2a} agonist CV1808. Pinacidil, adenosine and CGRP preferentially attenuated angiotensin II- versus pressure-induced AA vasoconstriction and this appeared to reflect the influence of underlying vasoconstrictor tone on the ability of vasodilators to activate K_{ATP}. We propose that this phenomenon is due to the inhibitory effects of angiotensin II on K_{ATP}. In the EA, CGRP and adenosine elicit vasodilations that are independent of K_{ATP}. The differing vasodilatory mechanisms evoked by these agents in the AA and EA illustrate the distinct regulatory processes in these two vessel types. As the actions of both adenosine and CGRP were shown to be mediated by cAMP in other vascular tissues, we next examined whether elevations of cAMP activate K_{ATP} in the AA. We compared the vasodilatory actions of forskolin, db-cAMP and isoproterenol on AA myogenic vasoconstriction. Elevation of cAMP by forskolin or administration of cell-permeable cAMP analogue db-cAMP elicited AA vasodilations that were insensitive to glibenclamide and therefore did not involve K_{ATP}. In contrast, the AA vasodilations in response to isoproterenol were found to be K_{ATP}-dependent. Since isoproterenol acts through a G_s protein-mediated activation of adenyl cyclase, but cAMP itself failed to activate K_{ATP}, we interpret these findings as evidence of direct receptor-mediated activation of K_{ATP}, presumably via G_s.

In conclusion, these results characterize the regulation of renal microvascular reactivity by K_{ATP} and provide evidence for a role of K_{ATP} in the differential activating mechanisms regulating pre- (AA) versus postglomerular (EA) reactivity.

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Some people, like Robert Fulghum, claim that all they need to know they learned in kindergarten. To convince you, they write a book about it and call it "All I really need to know I learned in kindergarten". If I was Mr. Fulghum, I would have to call this dissertation "All I really need to know about research I learned in SMRG."

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Bobince, Pepíkovi & Jani.

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AA	afferent arteriole
ADO	adenosine
ADP	adenosine diphosphate
Ang II	angiotensin II
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	cyclic adenosine 3',5'-monophosphate
CCPA	2-chloro-N^6-cyclopentyladenosine
CGRP	calcitonin gene-related peptide
CGS 21680	2-p-(2-carboxyethyl)-phenylethylamino-5'-N-ethylcarboxyamidoadenosine hydrochloride
CGS 15493	9-chloro-2-(2-furyl) [1,2,4]-triazolo[1,5] quinazolin-5-amine
CV1808	2-phenylaminoadenosine
DAG	diacylglycerol
db-cAMP	dibutyryl - cyclic adenosine 3',5'-monophosphate
DOG	1,2-dioctanoyl-sn-glycerol
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
EA	efferent arteriole
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
E_K	potassium equlibrium potential
E_m	membrane potential
GFR	glomerular filtration rate
HEPES	N-2-hydroxyethypiperazine-N-2-ethanesulfonic acid
HETE	hydroxyeicosatetraenoic acid
IAA-94	indanyloxyacetic acid
IBMX	3-isobutyl-1-methylxanthine
IP_3	inositol 1,4,5 trisphosphate

JMN	juxtamedullary nephron
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	calcium activated potassium channel
K_{dr}	delayed rectifier potassium channel
K_{ir}	inward rectifier potassium channel
K_{NDP}	NDP-sensitive potassium channel
L-NAME	N^{ω}-nitro-L-arginine methyl ester
ml/min	millilitre per minute
NDP	nucleotide diphosphate
NOS	nitric oxide synthase
PCO	potassium channel opener
PDBu	phorbol 12,13-dibutyrate
P_{GC}	glomerular capillary pressure
PIN	pinacidil
PIP_2	phosphatidylinositol 4,5-bisphosphate
PMA	phorbol 12-myristate, 13-acetate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
RBF	renal blood flow
SAC	stretch-activated channel
SHR	spontaneously hypertensive rat
SR	sarcoplasmic reticulum
SEM	standard error of the mean
SUR	sulfonylurea
TEA	tetraethylammonium
TGF	tubuloglomerular feedback
VIP	vasoactive intestinal peptide
VOCC	voltage-operated calcium channel
VSM	vascular smooth muscle

Chapter 1: INTRODUCTION

The resistance microvasculature in the kidney ensures a precise control of renal function under a wide variety of conditions. This control is executed via changes in afferent (AA) and efferent (EA) arteriolar diameters and consequent changes in pressure within glomerular capillaries (P_{GC}). P_{GC}, in turn, is a main determinant of glomerular filtration rate (GFR).

Several mechanisms are in place to ensure relatively independent regulation of the AA and EA reactivity. Elevations in renal perfusion pressure elicit "myogenic" vasoconstriction of the AA, protecting the glomerulus from the effects of increased arterial pressure and maintaining P_{GC} and GFR within normal levels. The failure of this mechanism leads to glomerular hypertension and ultimately renal failure in long-standing systemic hypertension. The EA lacks the myogenic vasoconstriction, but selective EA vasoconstriction by vasoconstrictor agonists becomes prominent in pathophysiological settings where glomerular filtration is insufficient, such as renal arterial stenosis or congestive heart failure. Here, by increasing P_{GC}, the EA vasoconstriction preserves GFR. These two examples illustrate the vital importance of regulation of renal microvascular reactivity in both physiologic and pathophysiologic settings in preserving the glomerular integrity and renal function. Our knowledge of the differing regulatory mechanisms of AA and EA is rather limited. The aim of this work was to examine the role that K-channels could play in the differential regulation of afferent and efferent arteriolar reactivity.

1.1 Structural and functional organization of the kidney

The kidney plays an important role in the maintenance of body fluid volume and solute composition. The process of urine formation begins at the level of the glomerulus with the formation of ultrafiltrate of plasma. This glomerular filtrate is further handled by the tubular system of the kidney to ensure preservation of essential components of plasma (reabsorption), as well as excretion of unwanted products of metabolism into the tubular fluid and consequently urine.

The nephron, the structural and functional unit of the kidney, consists of both vascular and tubular components. The vascular component of the glomerulus is formed by a capillary network enclosed in the Bowman's capsule. Intraglomerular capillaries are surrounded by mesangial cells and matrix. Mesangial cells, modified smooth muscle cells, not only support the glomerular capillary network, but are thought to influence glomerular hemodynamics via their close interaction with the capillaries. On the vascular pole, the afferent and efferent arterioles enter and leave the glomerulus, respectively. The barrier through which the ultrafiltrate has to pass to reach the tubular structures of the nephron consists of three layers: capillary endothelium, basal lamina and specialized epithelial cells termed podocytes. Opposite to the vascular pole of the glomerulus, the ultrafiltrate leaves the Bowman's capsule via the first portion of the tubular component of the nephron - pars convoluta of the proximal tubule. This tubular segment absorbs approximately 2/3 of the glomerular filtrate. The second portion of the proximal tubule, termed pars recta, constitutes the first part of the loop of Henle. The remaining portions of the loop of Henle are the thin descending and ascending limbs and the thick ascending limb. The thick ascending limb is vital for the formation of dilute urine and contributes to medullary hypertonicity and the countercurrent multiplication mechanism. Based on the length of the loop of Henle, the nephrons are generally classified into two groups (see Figure 1.1, panel A): cortical and juxtamedullary, the later possessing long loops of Henle that reach down to the level of medullary pyramids. The terminal portion of the thick ascending limb of the loop of Henle returns to the close proximity of the vascular pole of the glomerulus, forming the juxtaglomerular apparatus, a specialized structure consisting of modified afferent arteriolar cells secreting renin, macula densa cells of tubular epithelial origin, and extraglomerular as well as glomerular mesangial cells (see Figure 1.1, panel B). This structure is thought to be involved in the regulation of renin release and tubuloglomerular feedback (TGF) mechanism, as discussed bellow. Following the loop of Henle structures, the tubular fluid passes through the distal convoluted tubule into the connecting tubule to the cortical and medullary collecting duct. Along the tubule, the ultrafiltrate of plasma undergoes a series of modifications to eventually leave the renal papilla as urine. From here, the final urine is transported via the renal pelvis and ureter into the urinary bladder.

Figure 1.1: Panel A illustrates the arrangement of renal microcirculation. The blood supply of a cortical and a juxtamedullary nephron is depicted separately on the left. The tubular components of the nephrons are added in the diagram on the right.
Panel B illustrates the spatial relationship of individual components of the juxtaglomerular apparatus, as described in the text.
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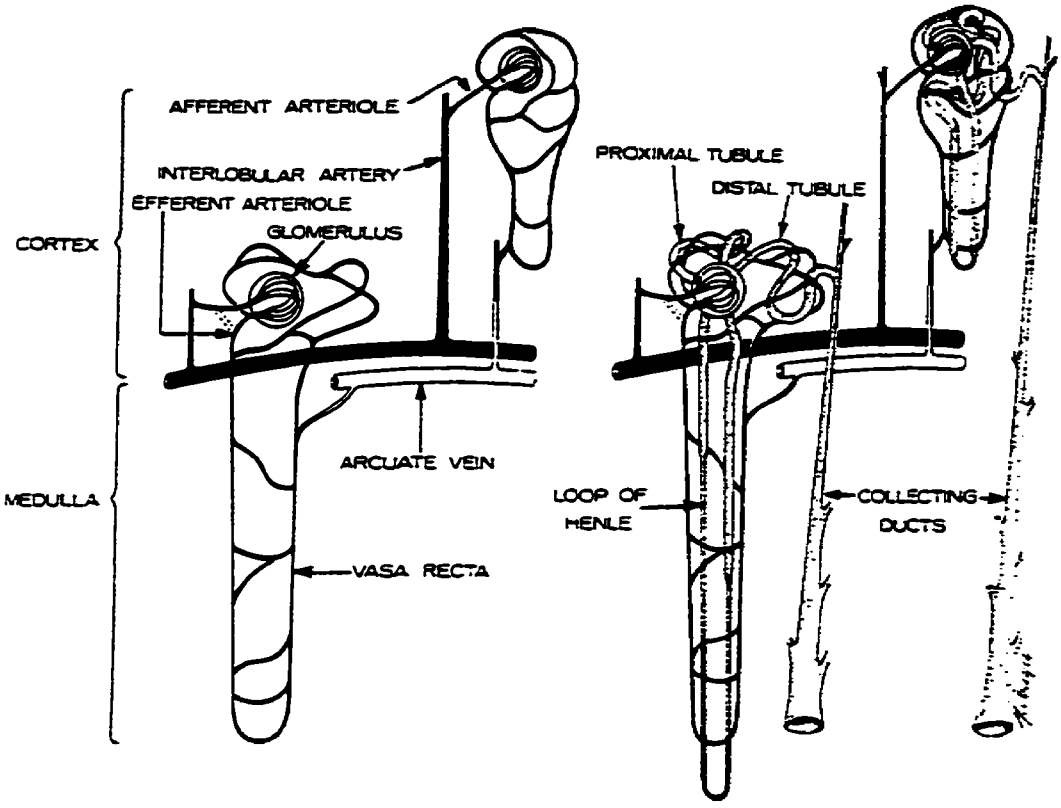
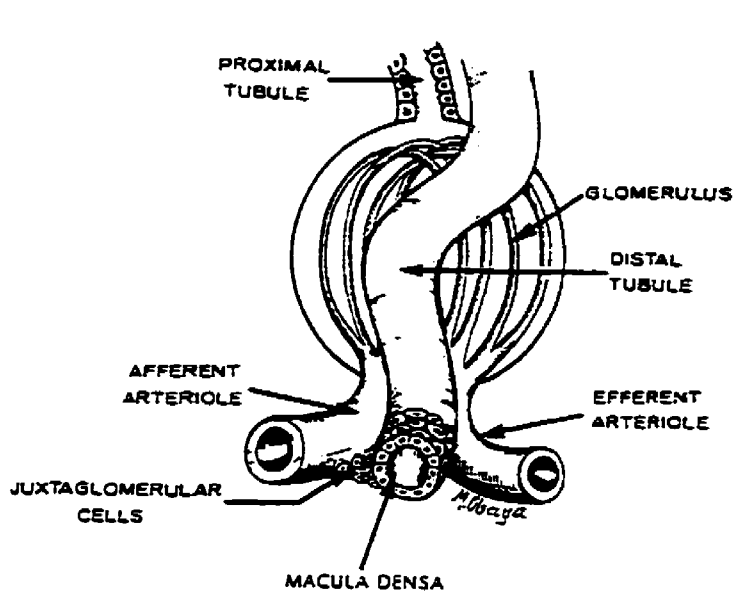
A


AFFERENT ARTERIOLE
INTERLOBULAR ARTERY
EFFERENT ARTERIOLE
GLOMERULUS
CORTEX
MEDULLA
ARCUATE VEIN
VASA RECTA
PROXIMAL TUBULE
DISTAL TUBULE
LOOP OF HENLE
COLLECTING DUCTS

B


PROXIMAL TUBULE
GLOMERULUS
DISTAL TUBULE
AFFERENT ARTERIOLE
EFFERENT ARTERIOLE
JUXTAGLOMERULAR CELLS
MACULA DENSA

Renal circulation

The left and right renal arteries leave the abdominal aorta to reach the hilus of each kidney, where they branch to give rise to the interlobar arteries. These intraparenchymal arteries further branch to form arcuate arteries which run along the border of the renal cortex and the medulla. Perpendicular to arcuate arteries, the interlobular (cortical radial) arteries ascend through the cortex to supply individual glomeruli via afferent arterioles. The glomerular capillaries coalesce at the vascular pole of the glomerulus to form the efferent arterioles. Depending on the localization of the glomerulus, the efferent arterioles give rise to two type of capillary networks. The efferent arterioles of the superficial, cortical nephrons supply the corresponding cortical areas of the kidney. In addition to the peritubular capillaries, the efferent arterioles of the juxtamedullary nephrons branch into vasa recta which descend deep into the medulla with the loops of Henle (see Figue 1.1, panel A). The intralobular, afferent and efferent arterioles are considered the resistance vessels in the kidney.

Given the fact that approximately 25% of cardiac output reaches the renal circulation and 20% of this amount is eventually filtered, one understands the evolutionary pressure to provide precise yet flexible regulatory mechanisms for the rate of glomerular filtration. GFR represents an amount of plasma filtered per unit time (ml/min) and is a function of the glomerular capillary membrane permeability and the net ultrafiltration pressure. The permeability characteristics of the capillary membrane (also termed the filtration coefficient) are determined by the permeability and surface area of the glomerulus. The net ultrafiltration pressure is determined by pressure within the Bowman's capsule and glomerular capillary pressure (P_{GC}). P_{GC} is determined by the changes in afferent and efferent vascular resistances. An increase in the preglomerular resistance (afferent arteriolar vasoconstriction) decreases P_{GC} and consequently GFR. In contrast, increasing the postglomerular resistance (efferent arteriolar vasoconstriction) elevates P_{GC} and GFR. Regulation of renal blood flow at the level of the resistance microvasculature, therefore, represents one of the most important determinants of renal function.

1.2 Autoregulation of renal blood flow

Autoregulation refers to the intrinsic ability of any organ to regulate its own perfusion. In the kidney, the autoregulatory mechanisms ensure constant RBF over a wide range of arterial blood pressures (80 to 180 mm Hg). Changes in renal vascular resistance as a result of autoregulation tend to stabilize the GFR, ensuring the renal functional integrity. Two mechanisms are in place to mediate the autoregulatory responses of the kidney: tubuloglomerular feedback and the myogenic mechanism. These two mechanism process distinct signals (see below), yet act at the same effector site, the afferent arteriole, to adjust preglomerular resistance, and consequently, glomerular hemodynamics.

Tubuloglomerular feedback

As evident from its name, the tubuloglomerular feedback mechanism relays signals from the tubule to the glomerulus, more specifically to the afferent arteriole. The anatomical basis for this mechanism is the juxtaglomerular apparatus (see Figure 1.1, panel B). An increased solute load at the level of macula densa is assessed and relayed via an unknown signal to the effector, the afferent arteriole, which constricts and adjusts the GFR, forming a classical negative feedback loop. The primary purpose of this mechanism therefore appears to be coordination of GFR and distal salt load. Although the existence of this mechanism is well established, a number of crucial questions regarding individual components of TGF remains unanswered. First, how does the macula densa sense and signal changes in solute load? It has been suggested that it is the Cl rather than Na concentration that is probably sensed by the macula densa cells (Schnermann & Briggs, 1992). The furosemide-sensitive Na-K-2Cl cotransport in the apical membrane of the macula densa cells was traditionally thought to be crucial for the generation of TGF signal, as application of furosemide, a blocker of Na-K-2Cl cotransport, blocks TGF (Persson et al., 1991). This view was challenged by recent evidence localizing additional Na-K-2Cl cotransporter proteins at the juxtaglomerular afferent arteriole and mesangium (Kaplan et al., 1996). It will be of interest to identify which of these sites is the target for furosemide actions and therefore, involved in the signalling mechanism for TGF. The nature of the

mediator that modulates afferent arteriolar reactivity is also currently unknown. Of a number of mediators proposed, adenosine, ATP and arachidonic acid metabolites seem to be the most promising ones. Adenosine is formed from ATP in a variety of tissues under conditions of high metabolic demand. It has been proposed that, in the kidney, the increased salt load represents a metabolic challenge for the tubular transport mechanisms. The subsequent breakdown of ATP serves as a source of adenosine, which then acts on the afferent arteriole (Osswald et al., 1982), eliciting an adenosine A_1 receptor-mediated vasoconstriction (Schnermann et al., 1990). Inscho and co-workers have proposed a role for ATP as a mediator of TGF, based on the selective preglomerular vasoconstrictor actions of ATP (Inscho et al, 1992), as well as sensitivity of autoregulatory responses of the juxtamedullary nephron (JMN) preparation to blockade of purinoceptor activation (Inscho et al., 1996). The P-450 metabolites of the arachidonic acid pathway, namely 20 - hydroxyeicosatetraenoic acid (20-HETE) have recently been implicated to play a role in TGF-mediated autoregulatory responses. The evidence for the role of 20-HETE comes from an observed sensitivity of renal autoregulatory responses to the inhibition of P-450 pathway (Zou et al., 1994 a,b).

Studies using either the blood-perfused JMN or microperfused, microdissected nephron preparations have shown that the effector site of TGF is limited to the juxtaglomerular region of the afferent arteriole (Casellas & Moore, 1990, Ito & Carretero, 1990). Based on these observations, it was proposed that TGF-mediated distal afferent vasoconstriction increases upstream intravascular pressure and elicits an "ascending" myogenic response in the more proximal regions of the afferent arteriole (Moore et al., 1994). In fact, mathematical models of RBF regulation by myogenic and TGF mechanisms have confirmed that operational "ascending" myogenic response is essential for autoregulatory effects of TGF (Feldberg et al., 1995).

Myogenic mechanism

The intrinsic ability of arterioles to sense and respond to increases in intraluminal pressure is termed the myogenic response. Myogenic vasoconstriction increases in magnitude with

decreasing vessel diameter and is generally most pronounced (and physiologically relevant) in the resistance vasculature (D'Angelo & Meininger, 1994). Despite the fact that this phenomenon was first described over 90 years ago (Bayliss, 1902), fundamental questions regarding this unique mechanism remain unanswered. It is unclear how the vascular wall senses changes in intramural pressure and how this signal is translated into vasoconstriction.

Renal resistance vessels exhibit myogenic reactivity in response to elevations in perfusion pressure in a variety of renal microvascular models, including embryonic renal vascular tissue transplanted into the hamster cheek pouch (Gilmore et al., 1980), the perfused juxtamedullary nephron preparation and the hydronephrotic kidney model (reviewed by Roman et al., 1991). Myogenic vasoconstriction can be observed in some (Yip & Marsh, 1996), but not all (Ito et al., 1992) microdissected renal microvessels. In addition, the myogenic vasoconstriction of isolated vessels has been described by some investigators as "sluggish" (Yip & Marsh, 1996), i.e. slower than predicted by *in vivo* studies (Young & Marsh, 1981). In general, the myogenic responsiveness is best preserved in vessels minimally exposed to mechanical trauma, hypoxia or other perturbations.

Sparse information is currently available regarding the signal transduction mechanism of myogenic vasoconstriction in the renal microcirculation. Evidence from renal (Harder et al., 1987) as well as other microvascular beds (Harder, 1984, Knot & Nelson, 1995) links myogenic vasoconstriction with membrane depolarization. In the afferent arteriole, $[Ca^{2+}]_i$ rises in response to elevated perfusion pressure (Yip & Marsh, 1996). The sensitivity of afferent arteriolar myogenic reactivity to L-type calcium channel blockers suggests voltage-gated calcium entry is the primary source for this rise in $[Ca^{2+}]_i$ (Harder et al., 1987, Hayashi et al., 1989).

Assuming that depolarization of the membrane potential initiates the myogenic vasoconstriction, how does the vascular wall translate changes in perfusion pressure into changes in membrane potential? In vascular preparations where myogenic vasoconstriction in response to elevated luminal pressure is preceded by an initial distension of the vessel,

such as in the coronary circulation, stretch has been implicated as a stimulus initiating the myogenic response. Indeed, stretching the isolated coronary VSM cells by about 10% of their length elicits increases in $[Ca^{2+}]_i$ and constricts the myocyte (Davis et al., 1992b). This type of myogenic vasoconstriction is thought to be mediated by mechanosensitive ion channels. Single channel measurements confirm the presence of nonselective, stretch-activated cation channels (SACs) in isolated VSM (Davis et al., 1992a). The function of SACs is to contribute to myogenic vasoconstriction by depolarizing the membrane and by allowing calcium to enter the cell (Meininger & Davis, 1992). The depolarization of the membrane allows additional calcium entry via voltage-operated calcium channels (VOCCs). Thus, the stretch-induced myogenic responses are only partially attenuated by blockers of VOCCs (Hwa & Bevan, 1986). The myogenic responsiveness of renal arterioles appears to be distinct from the stretch-induced vasoconstriction described above in at least two aspects: first, there is no evidence of a distension preceding the vasoconstriction of renal arterioles (Loutzenhiser & Parker, 1994) and secondly, this response is exquisitely sensitive to blockers of VOCCs, suggesting a crucial role for voltage-gated calcium entry through these channels, rather than through SACs.

Alternatively, activation of PLC could be involved in myogenic response, as stimulation of IP_3 and DAG formation following pressurization was reported in isolated renal arteries (Narayanan et al., 1994). This would suggest that the rise in $[Ca^{2+}]_i$ is due to IP_3-mediated release from the SR. This is not likely to be a primary mechanism for afferent arteriolar myogenic vasoconstriction, given the sensitivity of this response to blockers of voltage-gated calcium entry. DAG in turn activates PKC, which could contribute to myogenic vasoconstriction by acting on its multiple intracellular targets (reviewed by Meininger & Davis, 1992).

The observation that the myogenic vasoconstriction in the renal microvasculature can be inhibited by blockers of cytochrome P-450 metabolism has led to a speculation that endogenously produced metabolites of this pathway mediate myogenic vasoconstriction (reviewed by Harder et al., 1995). These authors have shown that 20-HETE, a vasoconstricting metabolite of arachidonic acid, can be formed by afferent arterioles via a

cytochrome P-450 pathway (Imig et al., 1996) and elicits afferent arteriolar vasoconstriction by inhibiting K_{Ca} (Zou et al., 1996). The authors propose that following the initial increase in $[Ca^{2+}]_i$, 20-HETE elicits further vasoconstriction by preventing the activation of K_{Ca} that would have normally occurred in response to the $[Ca^{2+}]_i$ increase (Brayden & Nelson, 1992). This is apparently not the case, as the myogenic responsiveness of the afferent arteriole is potentiated during blockade of K_{Ca} with TEA (Loutzenhiser & Parker, 1994), indicating that 20-HETE potentially modulates, but does not mediate, the myogenic vasoconstriction in this vessel.

Even though the term myogenic vasoconstriction implies an event originating in the muscular layer of a vessel, the endothelial layer of the afferent arterioles acts as an important modulator of underlying VSM activity. Evidence from hamster cheek pouch has clearly shown that the VSM and endothelial cells are connected by means of gap junctions to act as a single functional unit (Little et al., 1995), allowing communication of chemical or electrical signals between the two cell types. Thus, myogenic vasoconstrictions of afferent arterioles are accompanied by elevations in endothelial $[Ca^{2+}]_i$ (Wagner et al., 1996), which are probably secondary to increases in VSM $[Ca^{2+}]_i$. The increase in endothelial $[Ca^{2+}]_i$ could trigger release of endothelium-derived factors, which could then act to provide fine adjustments of the myogenic response. For example, recent studies have indeed described augmentation of myogenic responsiveness following the blockade of NOS in perfused afferent arterioles of rat (Hayashi et al., 1995, Yip & Marsh, 1996) or rabbit (Juncos et al., 1995), suggesting a modulatory role of endothelium-derived NO on the afferent arteriolar responses to pressure. Further investigations addressing this issue will undoubtedly take advantage of methods for selective removal of endothelium using antibodies raised against endothelium-specific antigens (Juncos et al., 1994) over previously used mechanical or chemical means that are likely to damage the underlying smooth muscle as well.

Evidence suggests that both myogenic and TGF mechanisms are necessary for the autoregulation of RBF. Mathematical models combined with frequency domain analysis studies of RBF and pressure oscillations in anaesthetized rats (Holstein-Rathlou et al., 1994) revealed two components of renal autoregulatory response. A slow frequency

component (30-50 mHz) represents TGF, while the faster frequency component (100 - 200 mHz) is thought to reflect an intrinsic vascular, i.e. myogenic, mechanism. Similarly, both mechanisms were responsible for the autoregulation of blood flow in the juxtamedullary nephron preparation (Takenaka et al., 1994). Clearly, the two mechanisms interact in a complex manner to regulate glomerular hemodynamics. For example, the TGF response was reported to be dependent on the level of afferent arteriolar pressure - induced tone (Schnermann & Briggs, 1989), suggesting an important role for the myogenic component in pre-setting the resistance of the effector of the TGF response. The observed sensitivity of both myogenic and TGF-mediated afferent arteriolar vasoconstriction to calcium channel blockers (Carmines et al., 1992) is not exclusive to autoregulatory responses, but presumably reflects the general dependency of the reactivity of this vessel on voltage gated calcium entry, as described in the next section.

In addition to the above described autoregulatory responses, the reactivity of the renal vasculature is physiologically regulated by a wide array of modulators, ranging from systemic hormones to more localized systems such as pO_2 levels or autocrine or paracrine regulators (reviewed by Navar et al., 1996).

1.3 Characteristic properties of the renal microcirculation

Several lines of evidence suggest distinct regulatory mechanisms for renal afferent and efferent arterioles. Angiotensin II and norepinephrine constrict both the afferent and efferent arterioles , but only the afferent arteriolar responsiveness to these agonists is reversed by blockers of L-type calcium channels (Fleming et al., 1987, Carmines & Navar, 1989, Loutzenhiser & Epstein, 1990, Conger & Falk, 1993, Hayashi et al., 1996). In the hydronephrotic kidney model, activation of L-type calcium channels by KCl-induced depolarization predominantly constricts the afferent arteriole with negligible effect on the efferent arteriole (Loutzenhiser et al., 1989), further confirming the dependency of the afferent arteriolar reactivity on voltage-gated calcium entry. In isolated renal microvessels, however, KCl-induced depolarization constricted both afferent and efferent arterioles (by 60% and 40%, respectively) (Conger & Falk, 1993). The reason for these contrasting

observations is unknown. Depolarization-induced changes in $[Ca^{2+}]i$ were compared in isolated rabbit afferent and efferent arterioles (Carmines et al., 1993). In the afferent arteriole, depolarization with 100 mM extracellular K^+ increased $[Ca^{2+}]i$, while the same manipulation decreased $[Ca^{2+}]i$ in the efferent arteriole. It has been postulated that in cells lacking voltage-gated calcium entry, depolarization would in fact reduce the $[Ca^{2+}]i$ by reducing the calcium "leak" into the cell. The finding observed in the efferent arteriole is therefore consistent with the premise that this vessel lacks voltage gated calcium entry pathway. The afferent arteriolar changes in $[Ca^{2+}]i$ were reversed either by either removing the extracellular calcium or by pretreatment with nifedipine, confirming that calcium entry via L-type calcium channels is responsible for this response (Carmines et al., 1993). Collectively, these data provide direct evidence that L-type calcium channels are either absent or functionally silent in the efferent arteriole.

Certain vasoconstrictor agonists, such as angiotensin II or norepinephrine, are capable of constricting both the afferent and efferent arterioles. Unlike the afferent arteriolar vasoconstrictions of these agents, the efferent arteriolar actions of these agonists are not affected by L-type calcium channels blockers (see above). This would suggest that the efferent arteriole utilizes other activating pathways, independent of voltage-gated calcium entry, such as mobilization of intracellular calcium stores. Indirect evidence supporting this premise comes from studies by Conger and Falk (1993), who demonstrated that afferent, but not efferent, angiotensin II-induced arteriolar vasoconstriction was attenuated by either L-type calcium channel blocker or by low extracellular Ca^{2+}. Using fura-2 to determine changes in $[Ca^{2+}]i$ in pressurized isolated renal arterioles, the same authors demonstrated distinct patterns of $[Ca^{2+}]i$ increase in response to angiotensin II in afferent and efferent arterioles (Conger et al., 1993). In the afferent arteriole, angiotensin II caused a slow increase in $[Ca^{2+}]i$, typical for voltage-gated calcium entry. The pattern observed in the EA, an initial peak followed by a decline in $[Ca^{2+}]i$, is indicative of agonist-induced mobilization of intracellular stores. In further support of this premise, the efferent arteriolar vasoconstriction to angiotensin II in the absence of extracellular Ca^{2+} was abolished by pretreatment with the SR calcium release blocker, dantrolene (Conger & Falk, 1993). More recently, Inscho et al. (1997) reported that the afferent arteriolar vasoconstrictions to

angiotensin II or norepinephrine were only partially attenuated by thapsigargin, while the efferent responses were completely prevented by this agent (Inscho et al., 1997). The efferent arteriole thus relies solely on agonist-induced calcium release during angiotensin II- or norepinephrine-induced vasoconstriction. This evidence further confirms the existence of differing activating mechanisms in renal afferent and efferent arterioles.

Accumulating evidence has provided some insights into the differing signal transduction pathways following activation of angiotensin II AT_1 receptors (Loutzenhiser et al., 1991) in afferent and efferent arterioles. *In situ* measurements of membrane potential of afferent and efferent arterioles (Loutzenhiser et al., 1997) revealed a close correlation between membrane depolarization and vasoconstriction in response to angiotensin II in the afferent arteriole. This pattern was not observed in the efferent arteriole, where the vasoconstrictor effects of angiotensin II were not accompanied by significant membrane depolarization. These findings suggest important differences in electro-mechanical coupling between the afferent and efferent arterioles. Calcium-activated chloride channels were identified in the renal microvasculature (Gordienko et al., 1994) and several investigators have reported that activation of these channels plays an important role in afferent arteriolar actions of angiotensin II. The afferent arteriolar vasoconstrictions to angiotensin II were blocked by the chloride channel blocker IAA-94 (Carmines, 1995, Takenaka et al., 1996) or DIDS (Jensen & Skott, 1996). Thus, membrane depolarization elicited by angiotensin II could result from opening of chloride channels by this agonist and lead to subsequent activation of L-type calcium channels. In contrast, the efferent arteriolar reactivity to angiotensin II is independent of chloride channel activation (Carmines, 1995), providing further evidence for distinct activating mechanisms in the two vessel types. Similarly, endothelin constricts both afferent and efferent arterioles, but only the afferent arteriolar actions are sensitive to L-type calcium channel blockers (Loutzenshiser et al., 1990, Takenaka et al., 1993) and appear to be mediated by activation of calcium-activated chloride channels (Takenaka et al., 1992, Gordienko et al., 1994). It is currently unknown whether afferent arteriolar reactivity to vasoconstrictory agonists other than angiotensin II or endothelin involves activation of chloride channels.

Taken together, the available evidence indicates important differences on the dependence of afferent versus efferent arteriolar reactivity on changes in membrane potential. Since the regulation of membrane potential is determined chiefly by potassium conductance (reviewed by Nelson & Quayle, 1994), it could be argued that differential reactivity of pre- and postglomerular arterioles reflects differences in potassium channel activity. The work presented in this dissertation characterizes the role of one subtype of potassium channels, ATP-sensitive potassium channel (K_{ATP}), in the regulation of afferent and efferent arteriolar reactivity.

1.4 ATP-sensitive potassium channels (K_{ATP})

First described in cardiac myocytes (Noma, 1983), K_{ATP} channels are now documented in other cell types - pancreatic β cells (regulation of insulin release), peripheral and central neurons, skeletal and smooth muscle cells (Edwards & Weston, 1993). These channels are generally closed at physiological levels of intracellular ATP and open when intracellular ATP concentrations drop, such as under conditions of metabolic stress (i.e. hypoxia or ischemia). K_{ATP} channels, therefore, represent a unique class of potassium channels that form an important link between the energetic state of the cell and membrane excitability. Opening of the channel results in an outward K^+ current that is voltage- and calcium- independent. Although primarily regulated by intracellular levels of ATP, K_{ATP} channels are also modulated by other parameters such as ATP/ADP ratio, nucleotide diphosphates, intracellular pH, divalent cation concentration, G-proteins and possibly phosphorylation (reviewed by Ashcroft & Ashcroft, 1990). Pharmacologically, the channels can be blocked by sulfonylureas, such as glibenclamide (Meisheri et al., 1993), or activated by a structurally heterogeneous group of vasodilators, known as potassium channel openers (PCOs) (Edwards & Weston, 1990).

The functional properties of K_{ATP} channels have been studied extensively over the past decade, using electrophysiological techniques and pharmacological tools (i.e. blockers and openers of the channel). Attempts at cloning and expression of K_{ATP} have only recently succeeded in revealing the channel structure. The channel molecule consists of a pore-

forming α-subunit of the inward rectifier family (Kir6.2) and a sulfonylurea receptor, β-subunit (SUR). The expression of the functional channels requires both Kir6.2 and SUR (Inagaki et al., 1995, Sakura et al., 1995), but the exact stoichiometry of the channel subunits is currently unknown (Krapiwinski et al., 1995). The sulfonylurea receptor, first cloned from a pancreatic cell line (Aguilar-Bryan et al., 1995), belongs to the family of ATP-binding cassette proteins. Multiple isoforms of sulfonylurea receptor cloned to date appear to confer distinct properties of KATP channels in different cell types (Inagaki et al., 1996, Isomoto et al., 1996). Interestingly, in preparations that possess multiple Kir isoforms, the sulfonylurea receptor associates exclusively with the appropriate KATP-forming subunit (Wellman et al., 1996).

K_{ATP} channels in vascular smooth muscle

Since 1983, when Noma described KATP channels in cardiac muscle, research activities have concentrated on the role of KATP in the heart. It wasn't until 1989 that the activity of these channels was first described in vascular smooth muscle (Standen et al., 1989). The role and regulation of KATP has been extensively investigated in certain vascular beds, namely the coronary and mesenteric circulation (reviewed by Nelson & Quayle, 1995). The unitary conductance of the vascular KATP channel varies considerably, ranging from 20 to 260 pS, reflecting a highly heterogenous population of KATP in vascular tissues (reviewed by Quayle & Standen, 1994). The vascular KATP shares similar pharmacological characteristics with its pancreatic or cardiac counterpart, i.e. activation by PCOs and inhibition by glibenclamide. There are, however, important differences in the affinities of both openers and blockers of this channels in vascular tissues. By definition, a "classical" KATP should be activated by ATP-free solution in electrophysiological studies. In addition, recent evidence suggests that nucleotide diphosphates (NDPs), but not decreases in intracellular ATP concentration, activate potassium currents with characteristics similar to that of KATP (Zhang & Bolton, 1995). The observed differences in regulation of KATP by either ATP or NDPs (reviewed by Terzic et al., 1994) could be explained by the existence of more than one type of vascular KATP. Indeed, in smooth muscle cells isolated from portal vein, two types of K-currents were identified, one activated by ATP-free solutions (i.e. KATP)

and a second channel, activated by NDPs (i.e. NDP-sensitive K-channel, KNDP) (Zhang & Bolton, 1996). Both channel types were blocked by glibenclamide, but differed in their sensitivity to PCOs. Clearly, the identification of channel protein(s) responsible for KATP and KNDP will be needed to fully resolve this issue.

In the kidney, the KATP channels are relatively well characterized in the tubular system, where they play an important role in the regulation of epithelial transport (reviewed by Quast, 1996). In contrast, the evidence for either existence or physiological role of KATP in the renal microcirculation is very limited. K^+ channels that share some characteristics of KATP, such as the ATP- and glibenclamide sensitivity, were reported in isolated afferent arteriolar cells (Lorenz et al., 1992). The same study demonstrated that decreasing intracellular ATP levels (by inhibition of glycolysis by 2-deoxy-glucose) reversed phenylephrine-induced vasoconstriction in isolated rabbit afferent arterioles via glibenclamide-sensitive mechanism. Similarly, hypoxia was found to inhibit afferent arteriolar myogenic vasoconstriction via a glibenclamide-sensitive mechanism in the rat hydronephrotic kidney (Loutzenhiser & Parker, 1994). These studies have, therefore, provided evidence for a potential functional relevance of KATP in the renal microcirculation. In addition, binding studies in rat glomerular preparations revealed specific binding sites for P1075, a PCO, in rat preglomerular vessels (Metzger & Quast, 1996), indirectly confirming the presence of KATP in rat afferent arterioles.

1.5 Pharmacology of vascular K_{ATP}

Sulfonylureas - K_{ATP} blocking agents

Traditionally, the evidence for involvement of KATP has been based solely on the sensitivity of the observed effects to sulfonylureas. The most frequently used agent in this group, glibenclamide, relatively specifically blocks KATP at concentrations of 10 μM and below in vascular tissues (Post & Jones, 1991, Quayle et al., 1995). There are, however, some important considerations regarding the specificity of sulfonylureas that need to be taken into account when interpreting experimental results. For example, in hypoxic or ischemic

cardiac muscle, increasing concentrations of intracellular ADP have been shown to limit the efficacy of the sulfonylurea block (Venkatesh et al., 1991). The efficacy of sulphonylureas might be also influenced by factors other than ADP, such as pH (Meisheri et al., 1993).

Potassium channel openers

The K_{ATP} channel serves as a target for exogenous activators, a pharmacologically diverse group of vasodilators classified as potassium channels openers (PCOs). The mechanism of action of these drugs is not clearly established. It is generally agreed that PCOs exert their vasodilatory effects through activation of K_{ATP} and hyperpolarization, but there is also evidence suggesting that the action of these drugs is at least partially due to mechanisms independent of K_{ATP} opening (Quast, 1993). As summarized by Quast (1993), in order to be classified as a PCO, a drug must meet the following criteria: 1/ induction of an outward K^+ current and hyperpolarization of the membrane potential, 2/ a vasodilatory effect must be abolished in the presence of high (i.e. >50 mM) extracellular K^+ (which results in the Em approaching the value for E_K, eliminating the influence of the opening of a K^+ channel on the Em), and 3/ inhibition of the effect of a PCO by sulphonylureas.

Hyperpolarization induced by PCOs can lead to vasodilation through diverse pathways (Quast et al., 1994). The generally accepted mechanism by which hyperpolarization (induced by opening of K_{ATP}) elicits vasodilation is by preventing the activation of voltage-dependent L-type calcium channels. Based on this mechanism, PCOs can be viewed as indirect blockers of L-type calcium channels. If this was the only pathway by which hyperpolarization elicits vasodilation, the vasodilatory profile of PCOs should correspond to that of L-type calcium channel blockers (dihydropyridines etc.). A number of studies (see below) have revealed that PCOs are in fact more efficacious than calcium entry blockers, suggesting additional mechanism(s) of action.

Changes in $[Ca^{2+}]i$ are essential for the contraction or relaxation of vascular smooth muscle. Increases in $[Ca^{2+}]i$ from either the extracellular space (entry through voltage- or receptor-mediated calcium channels) or from intracellular stores (SR) leads to vasoconstriction. A

subsequent decrease in $[Ca^{2+}]_i$ is necessary for vasodilation. Contractile agonists activate phospholipase C (PLC) and stimulate hydrolysis of PIP_2 to inositoltrisphosphate (IP_3) and diacylgycerol (DAG). IP_3, in turn, releases Ca^{2+} from IP_3-sensitive intracellular stores. This mechanism is responsible for agonist-induced contractions in Ca^{2+}-free medium. In the absence of external Ca^{2+} (which precludes Ca^{2+} entry), PCOs inhibited agonist-induced contractions in canine coronary arteries (Yamagishi et al., 1992), rat mesenteric arteries (Quast & Baumlin, 1991), rabbit aorta (Bray et al., 1991) and mesenteric artery (Ito et al, 1991, Itoh et al., 1992). Similarly, the PCO cromakalim inhibited dihydropyridine-insensitive contractile responses of rabbit aorta (Cook et al., 1988, Bray et al., 1991) and in the perfused mesenteric bed (Quast & Baumlin, 1992). These findings suggest that PCOs interfere with Ca^{2+} release from intracellular stores. This action of PCOs is specific for agonist-induced Ca^{2+} release, since release of Ca^{2+} from caffeine-sensitive stores is not affected by PCOs (Ito et al., 1991, Quast & Baumlin, 1992, Yamagishi et al., 1992).

Several studies have examined the hypothesis that PCO-induced hyperpolarization can influence phospholipase C activation. PCOs have been demonstrated to inhibit agonist-induced formation of IP_3 in coronary arterial rings (Yamagishi et al., 1992). The inhibitory effects of PCOs on agonist-induced IP_3 synthesis were observed in intact, but not in chemically skinned rabbit mesenteric arteries, suggesting that these effects are dependent upon membrane potential hyperpolarization by PCOs (Ito et al., 1991, Itoh et al., 1992). Direct evidence for modulation of PLC by membrane potential was provided by Ganitkevitch and Isenberg (1993). In this study, depolarization stimulated and hyperpolarization attenuated IP_3-dependent Ca^{2+} transients in voltage-clamped coronary myocytes.

DAG, the second product of PIP_2 hydrolysis, activates protein kinase C (PKC). Among its multiple effects on vascular smooth muscle, PKC is known to increase the sensitivity of contractile elements to Ca^{2+} (Mori et al., 1990). Okada et al. (1993) used canine coronary arterial rings to determine whether PCOs affect the Ca^{2+} sensitivity of contractile elements. By simultaneous measurements of force of contraction and $[Ca^{2+}]_i$ (using fura-2) these studies demonstrated that levcromakalim altered the relationship between $[Ca^{2+}]_i$ and tension, reducing the Ca^{2+} sensitivity of contractile elements.

In addition, PCOs have been shown to lower resting $[Ca^{2+}]_i$ in both Ca^{2+}-free and Ca^{2+}-containing media (Ito et al., 1991). This effect also seems to be related to hyperpolarization. As proposed by Quast (1993), hyperpolarization may either enhance the extrusion of extracellular Ca^{2+} through Na/Ca exchange or it may increase the negative charge of the membrane and augment the binding of intracellular Ca^{2+} to the internal side of the membrane.

The list of proposed vasodilatory mechanisms of PCOs would not be complete without mentioning possible hyperpolarization-independent mechanisms. Some investigators (Bray et al., 1991, Videbaek et al., 1988) have noted that vasorelaxation is induced by low concentrations of PCOs that do not affect the membrane potential. These findings suggest an as yet unidentified pathway which is presumably independent of K_{ATP} activation.

The relative contribution of the above mentioned mechanisms appears to vary for different PCOs and tissues. In the renal microcirculation, the differing dependence of renal afferent and efferent arteriolar reactivity on L-type calcium channels (as described in section 1.3) provides a convenient means for distinguishing L-type calcium channel-dependent and -independent actions of PCOs at the level of a resistance vessel.

1.6 Physiological roles of vascular K_{ATP}

K^+-channels are thought to play an important role in the pharmacological and physiological modulation of vascular tone (Quayle & Standen, 1994). The metabolic coupling of ATP sensitive K^+-channels suggests that these channels may be particularly important in the autoregulation of vascular tone under conditions of metabolic compromise, such as hypoxia or ischemia. Under these conditions, K_{ATP} channels can be activated either by effects of hypoxia on intracellular ATP levels or by adenosine released during hypoxia (reviewed by Quayle & Standen, 1994). K_{ATP} was shown to play a role in maintenance of basal tone in some vascular beds, such as coronary (Samaha et al., 1992), mesenteric (Nelson et al., 1990) or hamster cheek pouch circulation (Jackson, 1993). In addition, K_{ATP} channels have

been shown to be involved in a variety of both physiological and pathophysiological phenomena, such as acidosis-induced vasodilation (Ishizaka & Kuo, 1996), reactive hyperemia or altered vascular reactivity in endotoxic shock and diabetes (reviewed by Nelson & Quayle, 1995).

Endogenous modulators of K_{ATP} in vascular smooth muscle

Several endogenous substances are proposed to modulate K_{ATP} in vascular smooth muscle. The channels are subject to dual modulation by vasodilators (which activate K_{ATP}) and vasoconstrictors (which inhibit K_{ATP}), as summarized below.

Vasoconstrictors

Nakaya and coworkers have used cultured porcine coronary arterial myocytes to investigate the effects of several vasoconstrictors on the activity of K_{ATP}-like channels that were inhibited by intracellular ATP and activated by the PCO nicorandil. These channels were inhibited by extracellular application of angiotensin II (Miyoshi & Nakaya, 1991), endothelin (Miyoshi et al., 1992) and vasopressin (Wakatsuki et al., 1992) in both outside-out and cell-attached patches. These authors have proposed that inhibition of K_{ATP} contributes to membrane depolarization and vasoconstrictions by these agonists. More recently, the agonist-induced inhibition of K_{ATP} was documented for histamine and serotonin in freshly isolated cerebral arteriolar VSM (Kleppisch & Nelson, 1995a) and histamine was reported to inhibit K_{ATP} currents in cultured mesenteric arteriolar VSM (Kleppisch et al., 1996). These actions of vasoconstrictors could be mimicked by a protein kinase C activator, phorbol ester (Kleppisch & Nelson, 1995a), suggesting that PKC mediates the inhibitory actions of vasoconstrictors on K_{ATP}. The inhibitory actions of vasoconstrictors on K_{ATP}-mediated currents were further characterized by Bonev & Nelson (1996). In their study, neuropeptide Y, phenylephrine, serotonin and histamine all inhibited K_{ATP} currents in isolated rabbit mesenteric VSM cells. Similar inhibitions were observed following addition of the membrane permeable DAG analogue ,DOG, or the PKC activator, PMA. Furthermore, the inhibitory effects of vasoconstrictor agonists on K_{ATP} used in this study

were ameliorated by inhibitors of PLC (D609) or PKC (GF109203X). The results of this study confirm that vasoconstrictors inhibit K_{ATP} by a mechanism involving PKC in isolated VSM cells. These authors also proposed that this mechanism may be physiologically relevant in intact tissues that have intrinsically high levels of receptor-mediated PKC activity. As a result of the inhibitory effects of PKC on K_{ATP} in these tissues, PCOs would be expected to be less effective as vasodilators (Bonev & Nelson, 1996).

Vasodilators

A number of endogenous vasodilators have been demonstrated to act at least in part by activation of K_{ATP}. In general, the vasodilators that have been reported to activate K_{ATP} in vascular smooth muscle share a common signal transduction mechanism - activation of adenylyl cyclase. The vasodilators proposed to act via K_{ATP} include vasoactive intestinal peptide (VIP) (Standen et al., 1989), prostacyclin (Jackson et al., 1993), β adrenergic agonists, endothelium-derived relaxing factor (EDRF or NO) and endothelium-derived hyperpolarizing factor (EDHF) (reviewed by Nelson & Quayle, 1995). The involvement of K_{ATP} in the vasodilatory actions of calcitonin gene-related peptide (CGRP) and adenosine have been investigated most extensively and will be discussed separately.

Calcitonin gene-related peptide (CGRP)

The signal transduction mechanism for CGRP-induced vasodilation is an issue of great controversy. It appears, however, that the majority of findings seem to agree on one conclusion, which is the involvement of cAMP (reviewed by Bell & McDermott, 1996). The involvement of K_{ATP} channels in the vasodilatory actions of CGRP has been proposed for certain vascular beds, but the findings are inconsistent, presumably due to tissue and species heterogeneity and possibly due to multiple vasodilatory mechanisms of CGRP (see Maggi et al., 1994). To illustrate this point, one can use mesenteric vasculature as an example. In rabbit mesenteric arteries, CGRP elicited membrane hyperpolarization that was completely reversed by glibenclamide (Nelson et al., 1990). In the same study, however, glibenclamide only partially reversed the CGRP-induced vasodilation, suggesting a

component of CGRP-induced vasodilation that is independent of K_{ATP}. This K_{ATP}-independent component may be responsible for CGRP-induced vasodilations of mesenteric arteries preconstricted with 50 mM K^+ (Nelson et al., 1990). In isolated VSM cells from rabbit mesenteric artery, CGRP activated a K^+ current that was inhibited by glibenclamide (Quayle et al., 1994). Application of agents that elevate intracellular cAMP levels or the catalytic subunit of cAMP-dependent protein kinase (PKA) elicited a K^+ current that was inhibited by glibenclamide. Most importantly, in the presence of a specific inhibitor of PKA, CGRP failed to activate K_{ATP} (Quayle et al., 1994). Based on these observations, the authors have proposed that CGRP activates K_{ATP} via a PKA-mediated phosphorylation. In contrast, in the mesenteric arteries from rats, glibenclamide did not alter the vasodilatory actions of CGRP, suggesting a lack of involvement of K_{ATP} (Lei et al., 1994). Similar discrepancies are reported for almost every vascular bed studied.

CGRP and renal hemodynamics

Nerve endings containing CGRP have been identified on the renal vasculature (Knight et al., 1991), especially in the vicinity of the glomerulus (Reinecke et al., 1987, Kurtz et al., 1988), suggesting a physiological role for locally released CGRP. *In vivo* studies confirm a potential role of CGRP-induced vasodilation in the regulation of renal blood flow (RBF) and renal function (reviewed by Villareal et al., 1994). The receptor mediating the renal vasodilatory actions of CGRP has been identified as the $CGRP_1$ receptor subtype, based on the sensitivity of observed effects to a specific antagonist CGRP-(8-27) both *in vitro* (Castellucci et al., 1993, Chin et al., 1994) and *in vivo* studies (Haynes & Cooper, 1995, Elhawary et al., 1995). Intravenous administration of lower doses of CGRP, which cause only moderate hypotension, increased both RBF (Villareal et al., 1988, Amuchastegui et al., 1994) and GFR (Amuchastegui et al., 1994). At high concentrations, systemic (intravenous) administration of CGRP caused a significant hypotension accompanied by increase in heart rate (Villareal et al., 1988, Gardiner et al., 1989, 1991, Abdelrahman, 1992, Amuchastegui et al., 1994). The hypotensive doses of CGRP lead to reflex activation of the renin-angiotensin system and an indirect reduction in RBF (Villareal et al., 1988, Gardiner 1989, 1991). In addition to the reflex-mediated stimulation of renin release, there is also evidence

for direct stimulation of renin release by CGRP (Kurtz et al., 1988). Nevertheless, despite decreased RBF with higher doses of CGRP, the renal vascular resistance remains decreased, suggesting a direct vasodilatory effect on the renal resistance vasculature (Villareal et al., 1988, Gardiner et al., 1989).

To avoid the indirect, reflex-mediated, actions of CGRP on the renal function, CGRP was administered intra-arterially in an *in vivo* rat model. Here, CGRP elicited a biphasic change in RBF and GFR, as well as in renal vascular resistance: low doses reduced both RBF and GFR and induced renal vasodilation, whereas the higher doses decreased RBF and caused renal vasoconstriction, with no effect on GFR (Elhawary & Pang, 1995, Elhawary et al., 1995). The vasoconstrictory response is probably due to the above mentioned direct effects of CGRP on renin release. Similarly, evidence for direct vasodilatory actions of CGRP was obtained in studies in isolated *in vitro* perfused rat kidneys, where CGRP was shown to block the vasoconstrictory effects of norepinephrine (Geppetti et al., 1989, Castellucci et al., 1993), phenylephrine (Chin et al. 1994, Haynes & Cooper, 1995) or angiotensin II (Kurtz et al., 1989). The first study to determine direct renal microvascular effects of CGRP was performed on isolated pressurized non-perfused microdissected rabbit afferent and efferent arterioles. In this preparation, CGRP reversed the afferent arteriolar vasoconstriction to norepinephrine, but failed to cause any significant vasodilation in efferent arterioles preconstricted with either norepinephrine or angiotensin II (Edwards & Trizna, 1990).

Similar to other vascular tissues (see above), application of CGRP increased cAMP in isolated glomeruli (Edwards & Trizna 1990, Edwards et al., 1996), as well as mesangial cells (Kurtz et al., 1989). Only a few studies have studied the involvement of K_{ATP} in renal hemodynamic effects of CGRP. In rat small renal arteries (<200 µm outer diameter), glibenclamide failed to affect CGRP-induced vasodilations in arterial rings preconstricted with norepinephrine (Gao et al., 1994). Similarly, the hypotensive effects of intravenously administered CGRP in conscious rats were not affected by glibenclamide (Abdelrahman et al., 1992). The ability of CGRP to reverse vasoconstriction elicited by 60 mM KCl in isolated perfused rat kidney further supports the notion that changes in K^+ conductance do not

mediate the renal vasodilatory actions of CGRP (Castelluci et al., 1993). The involvement of K_{ATP} in CGRP-induced vasodilations of renal resistance vessels, however, has not been established.

Adenosine

The metabolic gating of K_{ATP} channel suggests that these channels could play a substantial role in hypoxic vasodilation, a well recognized phenomenon with an, as yet, unknown mechanism. Opening of K_{ATP} through either a drop in intracellular ATP ("ATP hypothesis") or through activation of adenosine receptor ("adenosine hypothesis") or a combination of both mechanisms (Nichols & Lederer, 1991) could lead to vasodilation under hypoxic conditions. In support for the ATP hypothesis, K_{ATP}-mediated hypoxic vasodilation (independent of adenosine) was observed in coronary (Daut et al., 1990), cerebral (Taguchi et al., 1994) and renal (Loutzenhiser & Parker, 1994) circulations. Exogenous adenosine elicited coronary vasodilation in the perfused heart preparation that could be inhibited by glibenclamide (Daut et al., 1990, Nakhostine & Lamontagne, 1993). The K_{ATP}-mediated component of adenosine-induced coronary vasodilation was shown to be mediated via adenosine A_1 receptor subtype (Merkel et al., 1992, Nakhostine & Lamontagne, 1993). In isolated porcine coronary myocytes, activation of adenosine A_1, but not A_2, receptors elicited glibenclamide-sensitive potassium current, confirming data obtained from whole tissues (Dart & Standen, 1993). Since adenosine A_1 receptors are coupled to Gi and, therefore, inhibit adenylyl cyclase activity which typically leads to vasoconstriction (Fredholm et al., 1994), the vasodilatory effects of adenosine in the coronary circulation could be explained by a direct (PTX-sensitive) G protein mediated coupling to K_{ATP}, as reported in ventricular myocytes (Kirsch et al., 1990). In vascular beds other than the coronary circulation, adenosine-induced vasodilations are mediated via A_2 receptors, which stimulate adenylyl cyclase activity via G_s proteins (Fredholm et al., 1994). In pithed rats, intravenous administration of either adenosine or adenosine A_2 agonist (YT-146) elicited hypotension that was antagonized by glibenclamide, but not PTX (Furukawa et al., 1993). The results of this study suggest that adenosine elicits vasodilations in resistance vasculature predominantly via its A_2 receptor activating K_{ATP}. Examples of results

supporting this premise obtained in individual vascular beds are described below. In isolated vascular myocytes from rabbit mesenteric arteries, adenosine or a selective adenosine A_2 agonist, CGS-21680, but not an adenosine A_1 agonist, CCPA, activated glibenclamide-sensitive potassium currents (Kleppisch & Nelson, 1995b). In addition, blockers of PKA reduced adenosine-activated currents, confirming that adenosine acting at its A_2 receptors activates K_{ATP} via an adenylyl cyclase - PKA pathway (Kleppisch & Nelson, 1995b). Adenosine also activated K_{ATP} in isolated cells from rat basilary artery (Kleppisch et al., 1995a) or dilated hamster cheek pouch arterioles via a glibenclamide-sensitive mechanism (Jackson, 1993). In summary, vasodilations elicited by adenosine can be mediated via both A_1 and A_2 receptor subtypes in coronary and other vascular beds respectively. The signal transduction mechanism mediating adenosine A_2-induced vasodilations appears to involve cAMP-dependent activation of K_{ATP}.

Adenosine and renal hemodynamics

Adenosine is proposed to play a role in a variety of physiological and pathophysiological settings in the kidney, ranging from tubuloglomerular feedback (see section 1.2) to radiographic contrast media-induced nephropathy (Arakawa et al., 1996). In the renal microcirculation, adenosine acts via both A_1 and A_2 adenosine receptors. Activation of adenosine A_1 receptor elicits transient preglomerular vasoconstriction, whereas activation of A_2 receptors mediates a slower and more sustained (postglomerular) vasodilation (reviewed by Spielman & Arend, 1991, Navar et al., 1996). As a result of these actions, adenosine decreases GFR. Unlike the adenosine A_1-mediated vasoconstriction, which is relatively well characterized and consistently reported in both *in vitro* and *in vivo* models, the vasodilatory actions of adenosine remain to be fully characterized. The A_1 receptors exhibit a much higher affinity (approximately 100-fold) for adenosine compared to the A_2 receptors (Spielman & Arend, 1991). For example, in the JMN preparation, a non-selective adenosine agonist, 2-chloroadenosine, elicited afferent arteriolar vasoconstriction at 1 μM and vasodilation at 100 μM, illustrating the biphasic dose-dependent effect of adenosine on the renal microcirculation (Inscho et al., 1991). Given the recent estimates of interstitial adenosine levels that averaged 200 nM (Baranowski & Westenfelder, 1994), it is likely that

the vasoconstrictor (A_1-mediated) response is readily observed under physiological conditions, while the vasodilatory (A_2-mediated) response is probably more relevant under pathophysiological conditions that tend to increase intra-renal levels of adenosine, such as hypoxia. Furthermore, there appears to be a regional difference in the vasodilatory actions of adenosine between renal cortex and medulla. In anaesthetized rats, interstitial administration of adenosine or adenosine A_2 agonist CGS-21680C increased medullary, but not cortical, blood flow (Agmon et al., 1993), suggesting possible differences in A_2 receptor density.

Adenosine receptors were originally classified based on their ability to modulate adenylyl cyclase activity: the A_1 subclass inhibiting cAMP formation via a PTX-sensitive G_i protein and the A_2 subclass increasing cAMP formation via G_s protein. The A_2 receptor subclass can be further subdivided into the high-affinity, A_{2a}, and the low-affinity A_{2b} adenosine receptor subclasses (Fredholm et al., 1994). The A_{2a} versus A_{2b} adenosine receptor subclass can be distinguished functionally by rank orders of agonist potency (Gurden et al., 1993). Despite the availability of relatively selective agonists and antagonists, the adenosine A_2 receptor subtype involved in the renal vasodilatory actions of adenosine has not been characterized.

In addition to the classical adenosine A_1-mediated transient afferent arteriolar vasoconstriction, adenosine elicited a biphasic vasodilation of pressure-induced afferent arteriolar vasoconstriction in the hydronephrotic rat kidney preparation (Loutzenhiser et al., 1994). The high-affinity vasodilatory response was observed at 10-100 nM concentration of adenosine, while the low-affinity vasodilatory response was elicited by 1-30 μM adenosine. The low-affinity adenosine response reversed a KCl-induced vasoconstriction of the afferent arteriole, suggesting a lack of involvement of K^+-channels, and appeared to be mediated by cAMP, as suggested by the potentiation of this response in the presence of IBMX. The novel, high-affinity vasodilatory response to adenosine was prevented by pretreatment with glibenclamide or CGS15493, suggesting an involvement of K_{ATP} and the adenosine A_{2a} receptor subtype, respectively. The adenosine A_2 receptor agonist, CV1808, also elicited glibenclamide-sensitive afferent arteriolar vasodilation over a concentration

range typical for an adenosine A_{2a} receptor subtype. These findings indicate that the afferent arteriole expresses adenosine A_{2a} vasodilatory responses that are mediated via K_{ATP} (Loutzenhiser et al., 1994). Whether these responses are unique to the hydronephrotic kidney remains to be determined.

Activation of K_{ATP} by cAMP

It is well established that the vasodilatory actions of β adrenergic agonists are mediated via G_s-mediated stimulation of adenylyl cyclase, increase in intracellular cAMP level and subsequent activation of PKA. As described above, this signal transduction pathway is activated by CGRP and adenosine as well and, in some preparations, leads to activation of K_{ATP}. In order to establish unequivocally whether the cAMP-PKA is a common signal transduction pathway for activation of K_{ATP}, several studies have examined the ability of β adrenergic agonists to activate K_{ATP}.

Glibenclamide-sensitive vasodilations elicited by the nonselective β adrenergic agonist isoproterenol were reported in hamster cheek pouch microcirculation (Jackson, 1993), rat isolated mesenteric arterial bed (Randall & Mc Culloch, 1995), rat aortic rings (Husken et al., 1994) and canine coronary circulation (Narishige et al, 1994). In canine saphenous vein, isoproterenol elicited endothelium-independent hyperpolarizations of membrane potential, acting at the β_2 receptor subtype (Nakashima et al., 1995). Similarly, forskolin, a direct activator of adenylyl cyclase, elicited glibenclamide-sensitive hyperpolarizations, suggesting a cAMP-mediated activation of K_{ATP} in this vascular bed (Nakashima et al., 1995). Similar findings were reported in rat basilary artery (Kitazono et al., 1993), where β_1-mediated vasodilatory actions of norepinephrine were partially inhibited by glibenclamide. Glibenclamide also partially inhibited forskolin-induced vasodilations in this preparation (Kitazono et al., 1993). In cultured porcine coronary myocytes, isoproterenol or forskolin activated K_{ATP} in cell-attached patches (Miyoshi & Nakaya, 1993). Application of PKA to excised inside-out patches activated K_{ATP} channels as well (Miyoshi & Nakaya, 1993). In concert, these findings support the premise that agents elevating intracellular cAMP levels activate K_{ATP} in a variety of vascular beds.

1.7 Objectives

Given the apparent heterogeneity of the K_{ATP} population and regulatory mechanism in different tissues, it is crucial that this channel be investigated separately in individual vascular beds, since only limited information can be generalized for all vascular smooth muscle. Figure 1.2 summarizes the regulatory pathways proposed for K_{ATP} in other vascular tissues.

The aim of this project was to examine the role of K_{ATP} in the regulation of renal microcirculation. The general hypotheses to be tested are that K_{ATP} is an important modulator of renal microvascular reactivity, and that K_{ATP} contributes to the actions of endogenous renal vasodilators by preferentially modulating afferent versus efferent arteriolar reactivity. The following questions were addressed:

1. Does activation of K_{ATP} by pinacidil selectively alter afferent versus efferent arteriolar reactivity?
2. Is K_{ATP} involved in the afferent and/or efferent vasodilatory actions of CGRP and adenosine?
3. Does cAMP activate K_{ATP} in the afferent arteriole?
4. Is the inhibition of K_{ATP} by vasoconstrictors physiologically relevant in the afferent arteriole, i.e. does the nature of the underlying tone influence the magnitude of K_{ATP}-mediated vasodilations?

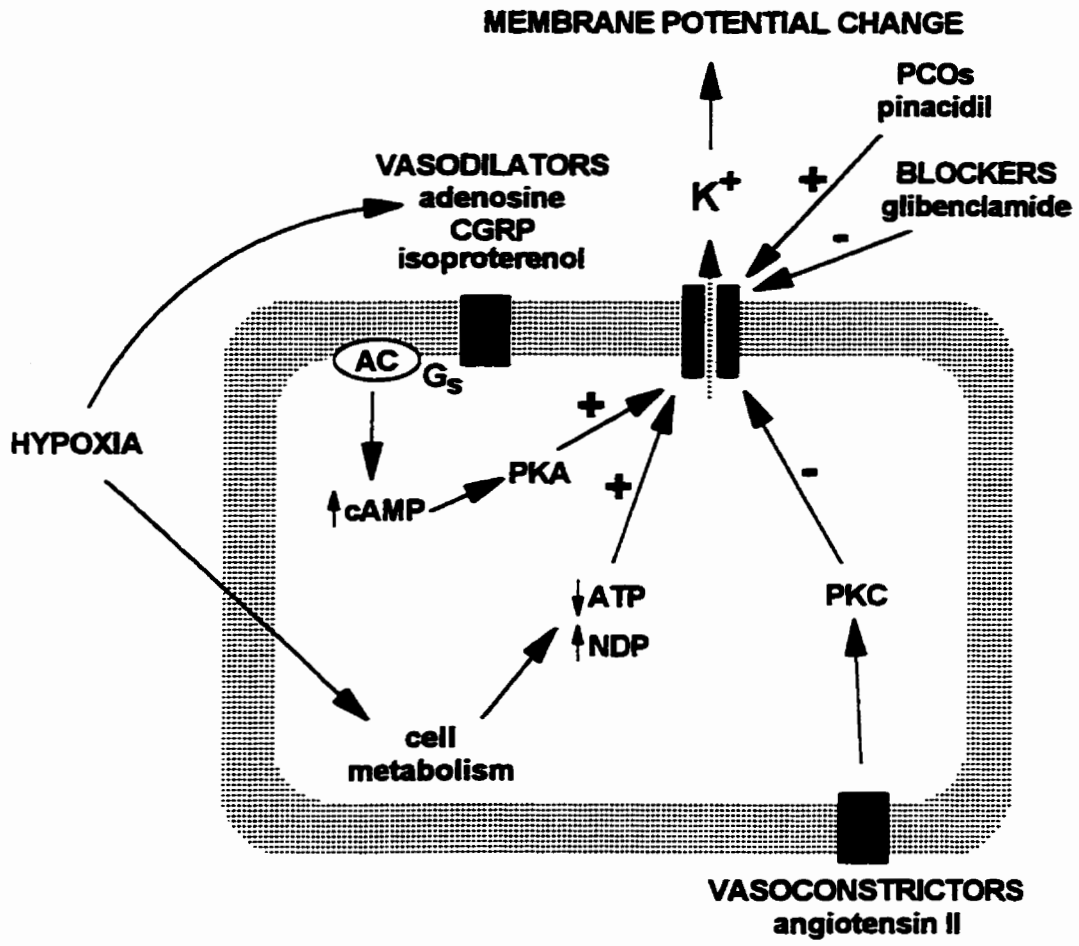


Figure 1.2: Proposed regulation of K_{ATP} in VSM. The channel can be opened as a result of metabolic compromise (hypoxia), action of endogenous vasodilators (adenosine, CGRP) or by potassium channel openers (pinacidil). The signal transduction pathway for activation of the channel by vasodilators involves cAMP-dependent protein kinase (PKA). Vasoconstrictors (angiotensin II) inhibit the channel via protein kinase C (PKC). The channel can be blocked by sulfonylureas (glibenclamide).

The result of opening/closing the channel is a change in the membrane potential and regulation of VSM reactivity.

(adapted from Quayle & Standen, 1994).

Chapter 2: METHODS

2.1 The isolated perfused hydronephrotic kidney model

The hydronephrotic kidney model is one of the few experimental models currently available to study renal microvascular reactivity. The other models are the juxtamedullary nephron preparation (Casellas & Navar, 1984) and isolated renal microvessels with (Ito & Carretero, 1990) or without (Edwards, 1983) attached glomeruli. All experiments presented in this work were performed using the *in vitro* perfused hydronephrotic rat kidney model (Loutzenhiser, 1996). Chronic hydronephrosis results in a complete atrophy of the tubular elements with minimal effects on the renal microvasculature (Steinhausen et al., 1983). The individual afferent and efferent arterioles can thus be visualized in this model without exposure to surgical trauma, ischemia, hypoperfusion or hypoxia. Since the tubular components are destroyed by the hydronephrosis, the TGF mechanism is absent in this model. This preparation therefore allows a direct assessment of vascular reactivity, without the need to account for possible indirect effects mediated via TGF.

Characteristics of the model

Experimental hydronephrosis appears to have minimal effects on the microvasculature of the kidney. The ultrastructure of glomerular arterioles, the distribution of renin (Nobiling et al., 1986) and the effects of renin secretagogues (Buhrle et al., 1986) are not affected by hydronephrosis. Morphological characteristics of the endothelium, mesangial structures and sympathetic nerve terminals are preserved (Nobiling et al., 1986). Functionally, stimulation of renal nerves elicits similar responses in the hydronephrotic and contralateral normal kidney (Fleming et al., 1992). The macula densa and tubular components are destroyed in the course of hydronephrosis. In addition, the basal lamina thickens and podocyte structure is altered in this non-filtering kidney (Nobiling et al., 1986). Despite the obvious tubular pathology, the microvascular reactivity of the hydronephrotic versus normal kidney does not exhibit any significant differences. *In vitro* studies demonstrate comparable

responses to ANP (Loutzenhiser et al., 1988), endothelin (Loutzenhiser et al., 1990), KCl (Loutzenhiser et al., 1987, Loutzenhiser et al., 1990) and calcium antagonists (Loutzenhiser & Epstein, 1990) in hydronephrotic and normal isolated kidneys. Hydronephrotic kidneys exhibit autoregulation of glomerular blood flow *in vivo* (Steinhausen et al., 1989). In the *in vitro* hydronephrotic kidney model, the afferent arterioles exhibit a graded, pressure-induced vasoconstriction in response to increases in perfusion pressure from 80 to 180 mm Hg, i.e. within the normal autoregulatory range (Loutzenhiser, 1996). Since the TGF-mediated autoregulatory component is absent, the autoregulation of RPF must, therefore, be mediated by the myogenic component in this model. The frequency domain analysis of RPF of the *in vitro* perfused hydronephrotic kidney reveals an autoregulatory system operating at 200 mHz (Cupples & Loutzenhiser, 1997), which is identical to that observed in the normal kidney or predicted by simulation studies (Feldberg et al., 1995). The hydronephrotic kidney model thus retains several characteristics of "normal" renal microcirculation.

In disease states, the microvasculature of the hydronephrotic kidney exhibits pathophysiological changes similar to those observed *in vivo*. The early stages of diabetes mellitus are characterized by hyperfiltration, i.e. increased GFR, (Mogensen et al. 1983) and impairment of autoregulation (Hashimoto et al., 1989). Similarly, in hydronephrotic kidneys isolated from diabetic rats, the afferent arteriolar myogenic vasoconstriction is severely impaired (Hayashi et al., 1992). In the SHR model of hypertension, the renal autoregulatory responses are shifted to higher values of blood pressure (Iversen et al., 1987). Similar changes are manifested by shift of the threshold for afferent arteriolar myogenic vasoconstrictions to higher pressure in hydronephrotic kidneys from spontaneously hypertensive rats (Hayashi et al., 1989). The salt-dependent changes in autoregulation in the Dahl rat correspond to the salt-dependent changes in myogenic reactivity in the hydronephrotic kidney model (Takenaka et al., 1992).

Based on these observations, it can be concluded that the intrinsic properties of renal microvascular elements remain intact in the hydronephrotic kidney in both physiological and pathophysiological states. Due to the absence of TGF-mediated responses, this model

provides a unique approach to explore the reactivity of the renal microvasculature.

Surgical procedures

Hydronephrosis is induced in young male Sprague Dawley rats (100 - 150 g) by ligating the left ureter under general halothane-induced anaesthesia. The midline abdominal incision (ca 1 cm long) allows localization of the left ureter which is ligated surgically with two 4-0 sutures. One ml of physiological saline solution is then instilled into the abdominal cavity to prevent adhesion formation. The muscular abdominal wall is closed with 2-3 atraumatic sutures and the skin incision is sealed with tissue glue (Vet-Seal). The procedure typically takes 5-10 minutes and is concluded by administering a single dose of prophylactic antibiotic (0.1 ml of Penlong XL i.m.) to minimize postoperative infectious complications. Throughout the surgery, care is taken to maintain a sterile operative field. The rats are then kept in the University of Calgary Animal Care facility and given standard rat diet and water *ad libitum*. Following the artificial ureter obstruction, the tubular apparatus of the left kidney undergoes atrophy. After 4-8 weeks the kidneys develop full hydronephrosis and are then harvested for the *in vitro* perfusion.

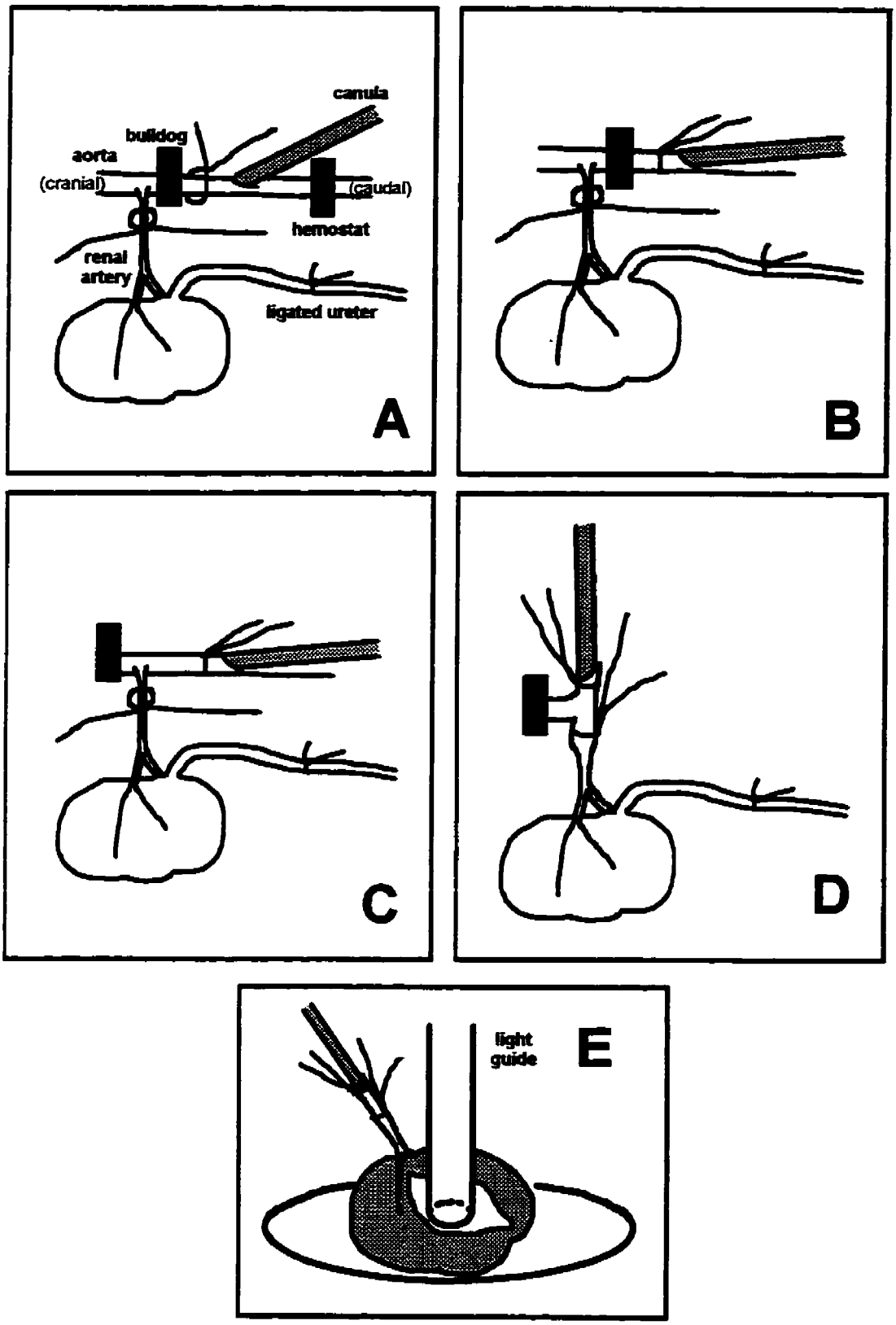
When hydronephrosis is fully developed, the hydronephrotic kidney is exposed via a wide abdominal incision under general methoxyflurane-induced anaesthesia. The connective tissue surrounding the left renal artery and abdominal aorta is cleaned and sutures are placed under the origin of the renal artery and under the abdominal aorta just caudal to the origin of the renal artery (see panel A, Figure 2.1). Heparin (1000 IU, i.e. 0.1 ml of Hepalean, Organon Teknika) is administered intravenously into the mesenteric vein to prevent thrombosis and facilitate the subsequent "washout" of blood elements. At this stage, the anaesthetized rat is moved near the perfusion apparatus to perform the cannulation procedure. A small vascular clamp (bulldog) is placed immediately distal to the origin of the renal artery (see panel A, Figure 2.1). A second clamp, a small hemostat, is placed about 0.5 cm distal to the first clamp (see panel A, Figure 2.1). A cannula (Cathlon IV catheter with custom-made pressure sensing steel inner canula) is then inserted through a small cut into the occluded section of the abdominal aorta (see panel A, Figure 2.1). Note

that throughout these manipulations, the kidney is continuously perfused via the abdominal aorta proximal to the occlusion site. The next step involves tying the suture around the cannula (see panel B, Figure 2.1) to prevent leakage of the perfusion media. At this point, the "bulldog" is removed from its original site and positioned just cranial to the origin of the renal artery (see panel C, Figure 2.1) while the perfusion through the cannula is started. Thus, without any disruption of flow, the kidney is now perfused with medium. The kidney is then carefully excised. The final step of the cannulation procedure consists of advancing the cannula into the renal artery and tying the second suture around the tip of the cannula (see panel D, Figure 2.1). The hilus of the kidney is cleaned of fat and connective tissue. The renal vein is transected and the renal artery carefully inspected for leaks. Then the renal capsule is gently removed. Before the kidney is transferred onto the microscope stage, an opening is made through one side of the kidney to allow the insertion of the fibre light guide (see panel E, Figure 2.1).

Experimental apparatus

The perfusion apparatus, depicted in Figure 2.2, provides a single-pass presentation of medium to the kidney. The perfusate consists of modified Dulbecco's Eagle Medium (GIBCO, Grand Island, NY) containing 30 mM bicarbonate, 1 mM sodium pyruvate, 5mM glucose and 5mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid). The medium is equilibrated with 95% air/5%CO_2 and pumped on demand through a pressurized, heated reservoir. The pH, pO_2 and temperature are maintained at 7.4, 150 mm Hg and 37°C, respectively. The perfusion pressure is measured at the level of the renal artery and adjusted manually by a back-pressure regulator (Fairchild, Model 10BP). Flow is determined by an in-line electromagnetic flow probe (Carolina Medical Electronics, Inc.). Both perfusion pressure and flow are recorded by a Grass polygraph and the tracings stored for analysis. The kidney itself is perfused on a heated stage of an inverted microscope (Leica, model Leitz DM IL) and a fibre light guide (about 5 mm in diameter) serves to stabilize an area for viewing and facilitates the trans-illumination of this area (see panel E, Figure 2.1).

Figure 2.1: Cannulation of the renal artery of the hydronephrotic kidney. Panels A to E illustrate the sequence of events during the cannulation procedure. See text for detailed explanation.


canula
bulldog
aorta
(cranial)
(caudal)
hemostat
renal
artery
ligated ureter
A
B
C
D
light
guide
E

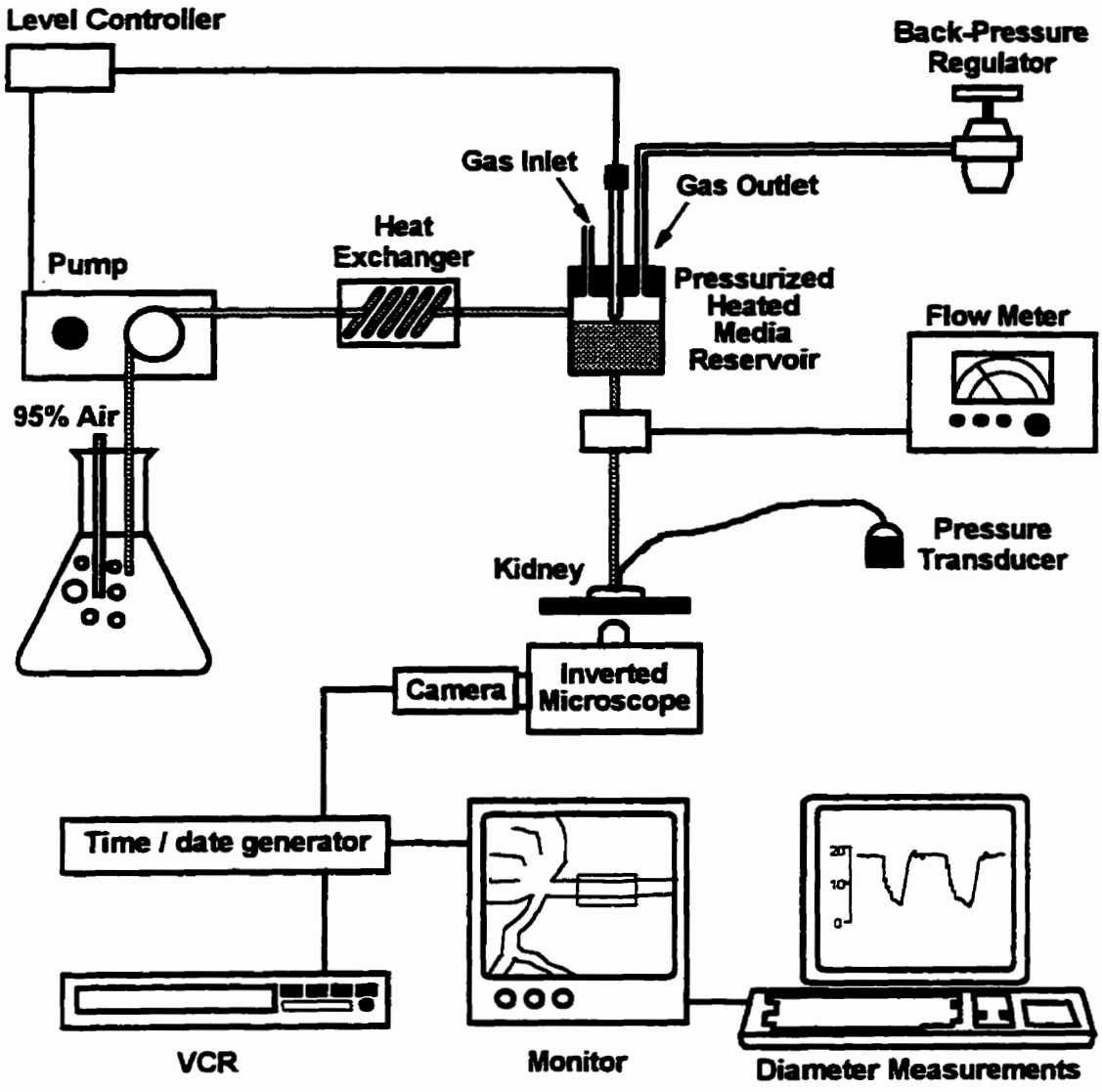


Figure 2.2: Experimental apparatus. Pressurized, heated media reservoir supplies the hydronephrotic kidney with oxygenated medium. Perfusion pressure is adjusted with a back-pressure regulator. Vessel diameter, renal perfusate flow and perfusion pressure are measured and recorded. See text for detailed description.

The microscope is equipped with a camera and the image is displayed on a monitor (Panasonic). To determine vessel diameter, the image is digitized, using a video imaging board (Model IVG-128, Datacube, Peabody, MA). Custom-made software is used to determine the mean diameter between parallel edges of the arteriole. A segment of the vessel is selected (usually 10-20 μm in length) and scanned at 0.5 second intervals. The diameter measurements are then obtained by averaging the individual diameter measurements along this segment per each scan.

At the end of each experiment, the diameter tracings are analysed by custom software. For each response, the mean diameter values are determined by averaging all measurements obtained during the plateau of the response and usually represent 60 to 100 measurements.

2.2 Experimental protocols

Kidneys were allowed to equilibrate for at least 30 minutes following the isolation to facilitate the recovery from the surgical manipulations. The perfusion pressure was kept at 80 mm Hg. The renal perfusate flow usually stabilized at a value between 10 to 20 ml/min during this equilibration period. At the end of equilibration, ibuprofen (10 μM) was added to the perfusate to eliminate effects of renal prostaglandins.

Depending on the experimental protocol, a suitable area for viewing was chosen. For the experiments involving determination of a myogenic response, an afferent arteriole with clearly distinguishable arteriolar walls was sufficient. The protocols determining the effects on both afferent and efferent arteriolar reactivity required a glomerulus with afferent and efferent arterioles clearly visible at the same focal plane. The afferent arteriolar diameters were typically determined at the branching point from their respective intralobular arteries while the efferent arteriolar diameters were measured at the level of the origin at the vascular pole of the glomerulus. The afferent arteriolar responses to pressure ramps were then determined to assess the reactivity of the preparation. Kidneys that lacked normal myogenic responsiveness were discarded.

Afferent arteriolar myogenic vasoconstrictions

The myogenic vasoconstriction of afferent arterioles was determined by performing "pressure ramps". The protocol is illustrated in Figure 2.3. To quantify the myogenic vasoconstriction, the perfusion pressure was first decreased to 60 mm Hg and then increased in 20 mm Hg increments to 180 mm Hg. Each pressure step was held for one minute. The accompanying changes in afferent arteriolar diameter were recorded. As depicted in Figure 2.3, the pressure ramps elicits a rapid and graded vasoconstriction of the afferent arteriole. Replicates of 2 to 3 pressure ramps were generally required to establish a steady-state control myogenic response prior to addition of the drug to be studied. The perfusion pressure was kept at 60 or 80 mm Hg between ramps, depending on the reactivity of the vessel. Afferent arteriolar diameter at this pressure was considered basal. To assess the effects of a compound on the myogenic response, the drug was added directly to the perfusate. The kidney was perfused with each concentration of the drug for a period of 10-20 minutes at 80 mm Hg. At the end of this pretreatment period, both the basal diameter as well as myogenic response to a pressure ramp were reassessed. This protocol was repeated for each concentration of the drug. In experiments which required addition of glibenclamide, the myogenic responses were first established in the absence of glibenclamide and then compared to responses obtained after addition of glibenclamide. Under the experimental conditions described, addition of glibenclamide does not alter the myogenic responses, suggesting that K_{ATP} is quiescent in this preparation (Loutzenhiser & Parker, 1994).

To establish the threshold for the myogenic response during a pressure ramp, the mean diameter at 60 mm Hg was compared versus the diameter values at 80 to 180 mm Hg. The perfusion pressure at which the decrease in mean arteriolar diameter reached statistical significance was considered the threshold for myogenic vasoconstriction.

Simultaneous determination of afferent and efferent arteriolar reactivity

In the protocols where both afferent and efferent responses were examined, angiotensin II

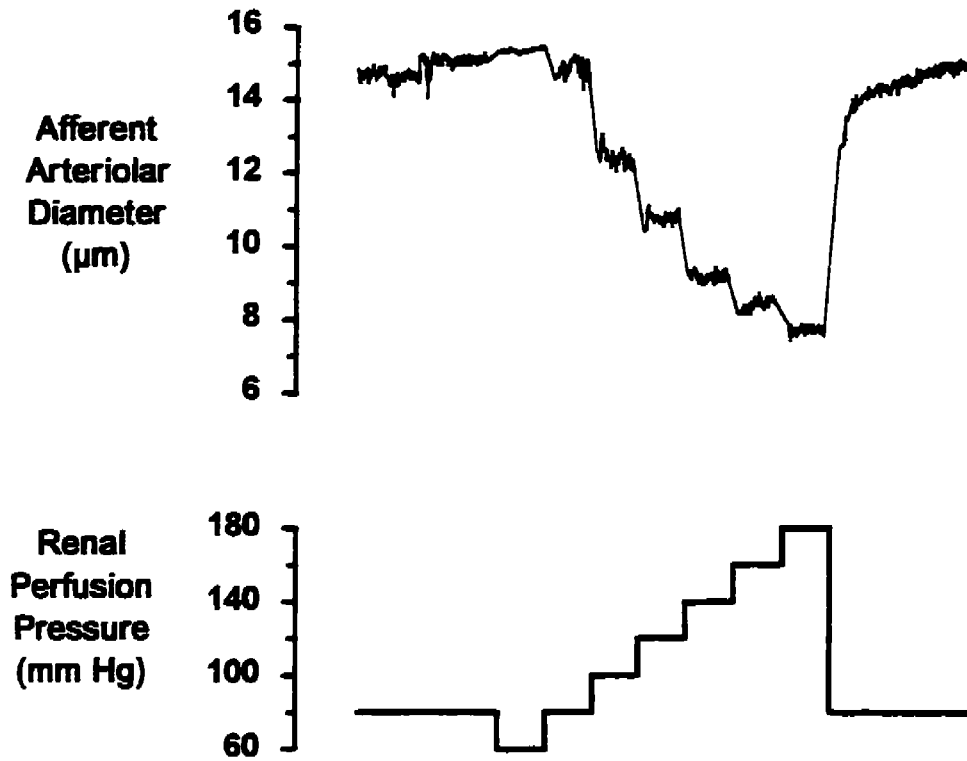


Figure 2.3: Original tracing depicting myogenic vasoconstriction of the afferent arteriole during a "pressure ramp". The basal diameter is determined at 80 mm Hg perfusion pressure. The afferent arteriolar diameter is measured during each 20 mm Hg pressure step (from 60 to 180 mm Hg). The pressure is held at each value for 1 minute. Note that the afferent arteriolar diameter decreases in a graded fashion with each pressure step, starting at 100 mm Hg. The myogenic response of this vessel is fast and reaches a steady-state without any preceding distension. The diameter returns to basal value following a decrease in pressure back to 80 mm Hg.

was used as a vasoconstrictor stimulus. As mentioned above, this protocol requires that afferent and efferent arterioles from the same glomerulus are visible in the same focal plane. The basal diameters at 80 mm Hg were first determined for each vessel. The kidney was then perfused for at least 5 minutes with 0.1 nM angiotensin II. During this period, the vasoconstrictor responses of afferent and efferent arterioles were determined. The angiotensin II was then washed out and the basal diameter recovered to the pre-angiotensin value. The kidney was then treated with increasing concentrations of each vasodilator studied (about 10-20 minutes with each concentration) and the basal diameters and angiotensin II responses were again determined for each respective dose.

Some experiments were performed in the presence of glibenclamide. In these experiments, the afferent and efferent arteriolar vasoconstriction by angiotensin II was first determined prior to addition of glibenclamide and then compared to the vasoconstriction in the presence of glibenclamide. As depicted in Figure 2.4, addition of glibenclamide does not alter the afferent or efferent arteriolar reactivity to angiotensin II under these experimental conditions.

2.3 Materials

All drugs were added directly to the perfusate. Where possible, the stock solutions were prepared fresh on the day of the experiment. With the exception of ibuprofen, pinacidil, glibenclamide and forskolin, which were dissolved in ethanol and CV1808, which was dissolved in 50% ethanol/50% distilled water, distilled water was used as a solvent. For some drugs, such as CGRP, forskolin and db-cAMP, the stock solutions were prepared in advance and stored at -80°C. The concentrations of the stock solutions for individual agents were as follows: adenosine, 10 mM; angiotensin II, 10 µM; Bay K 8644, 10 mM; CV1808, 10 mM; CGRP, 100 µM; db-cAMP, 1 mM; diltiazem, 10 mM; forskolin, 10 mM; glibenclamide, 10 mM; ibuprofen, 100 mM; isoproterenol, 10 mM; pinacidil, 10 mM. In experiments with isoproterenol, 1 mM ascorbic acid was added to the perfusate to prevent oxidation of this drug. Ibuprofen, glibenclamide, pinacidil, diltiazem, Bay K 8644, CV1808 and forskolin were obtained from Research Biochemical International, angiotensin II, rat CGRP, adenosine, isoproterenol and db-cAMP from Sigma Chemical Co. All other reagents

were obtained from GIBCO.

2.4 Statistical analysis of data

Throughout the text, the data are expressed as mean ± SEM as an index of dispersion. The number of replicates refers to the number of afferent or efferent arterioles examined. Only one vessel (afferent arteriole) or a pair of vessels (afferent and efferent arteriole) were studied in each kidney preparation.

The data were analysed with Student's t test (paired on unpaired). The Newman-Keuls' test was used for multiple comparisons. The p values <0.05 were considered significant.

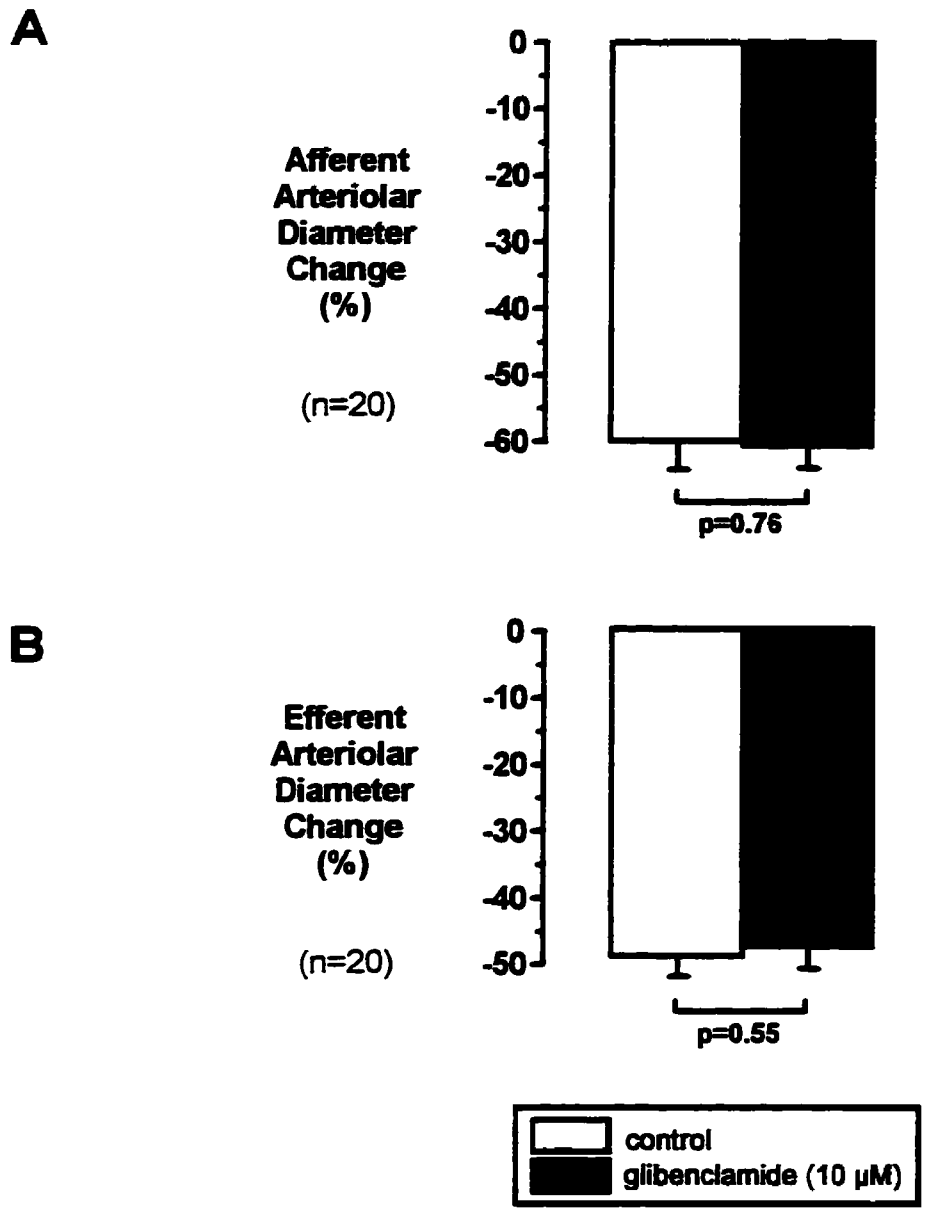


Figure 2.4: Angiotensin II-induced changes in afferent (panel A) and efferent (panel B) arteriolar diameter. Open bars represent decrease in diameter elicited by 0.1 nM angiotensin II (control). The corresponding values obtained in the presence of 10 µM glibenclamide are depicted as closed bars. Values are mean ± SEM, data obtained from 20 kidneys.

Chapter 3: RESULTS

3.1 Pinacidil

Hypothesis: *The K_{ATP} channel opener pinacidil preferentially alters the afferent (versus efferent) arteriolar vasoconstriction, reflecting the greater dependence of the afferent arteriolar reactivity on L-type calcium channels.*

Effects of pinacidil on afferent arteriolar vasoconstrictions induced by Bay K 8644 or KCl

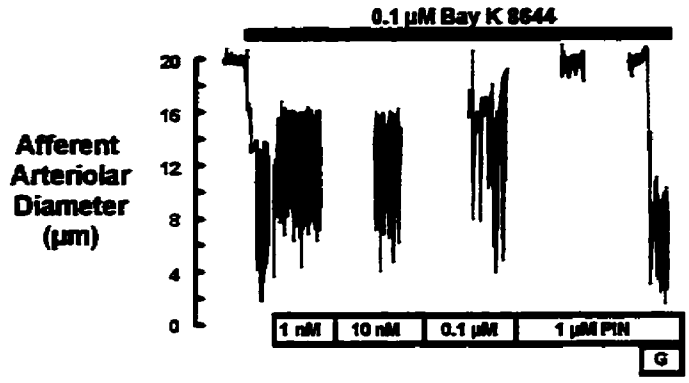
To directly test the hypothesis that activation of K_{ATP} attenuates L-type calcium channel-dependent vasoconstriction of the afferent arteriole, the effects of pinacidil on Bay K 8644-induced vasoconstrictions were determined first. Treatment with 0.1 μM Bay K 8644 elicited an oscillating vasoconstriction in all afferent arterioles studied (see original diameter tracing in Figure 3.1, panel A). Mean afferent arteriolar diameters were reduced from 17.7±1.0 μm (basal) to 12.0±1.0 μm ($p<0.005$, n=5). Pinacidil at concentrations of 0.001, 0.01, 0.1, 1.0 and 10 μM increased afferent arteriolar diameter to 12.2±1.0 μm, 12.8±1.3 μm, 16.6±1.0 μm, 16.9±1.0 μm and 17.0±0.8 μm, respectively, significantly inhibiting the Bay K 8644-induced vasoconstriction at 0.1, 1 and 10 μM ($p<0.05$ versus Bay K alone, n=5). Subsequent treatment with 10 μM glibenclamide completely restored the Bay K 8644-induced vasoconstriction (9.8±1.4 μm; $p>0.2$ versus Bay K 8644 alone, n=5), as illustrated in Figure 3.1, panel A. The pinacidil-induced inhibition of Bay K-induced afferent arteriolar vasoconstriction is summarized in Figure 3.1, panel C. The approximate IC_{50} (extrapolated from the concentration-response curve) was 54 nM.

In contrast to its effects on Bay K 8644-induced vasoconstriction, pinacidil had no significant effect on afferent arteriolar vasoconstriction when L-type calcium channels were activated by KCl-induced depolarization. A typical tracing is shown in Figure 3.1, panel B. KCl (20 to 30 mM) reduced mean afferent arteriolar diameter from 16.7±1.0 μm (basal) to 8.7±0.9

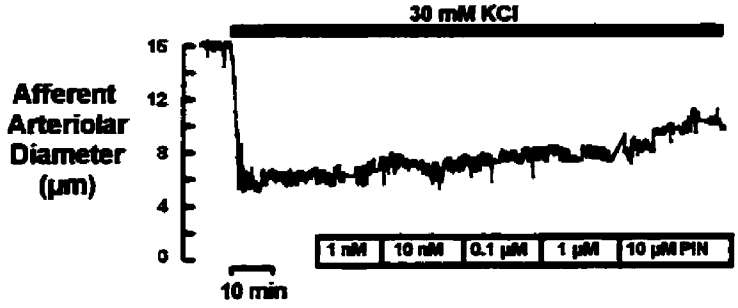
Figure 3.1: Effects of pinacidil on afferent arteriolar vasoconstrictions induced by Bay K 8644 or KCl. Original tracings depict typical responses of afferent arterioles to Bay K 8644 (panel A) and KCl (panel B). Bay K 8644 elicited oscillating vasoconstrictions in all afferent arterioles. Pinacidil (PIN) inhibited this response in a concentration-dependent manner. Subsequent administration of 10 μM glibenclamide (G) eliminated inhibitory actions of pinacidil, restoring the oscillatory vasoconstriction (panel A). In contrast, the KCl-induced vasoconstriction was relatively insensitive to the actions of pinacidil (PIN) over the same concentration range (panel B).

Panel C summarizes the inhibitory effects of increasing concentrations of pinacidil on afferent arteriolar vasoconstrictions induced by Bay K 8644 (closed circles) or KCl (open circles). Values are mean ± SEM, n=5 and n=4 for Bay K 8644 and KCl, respectively.

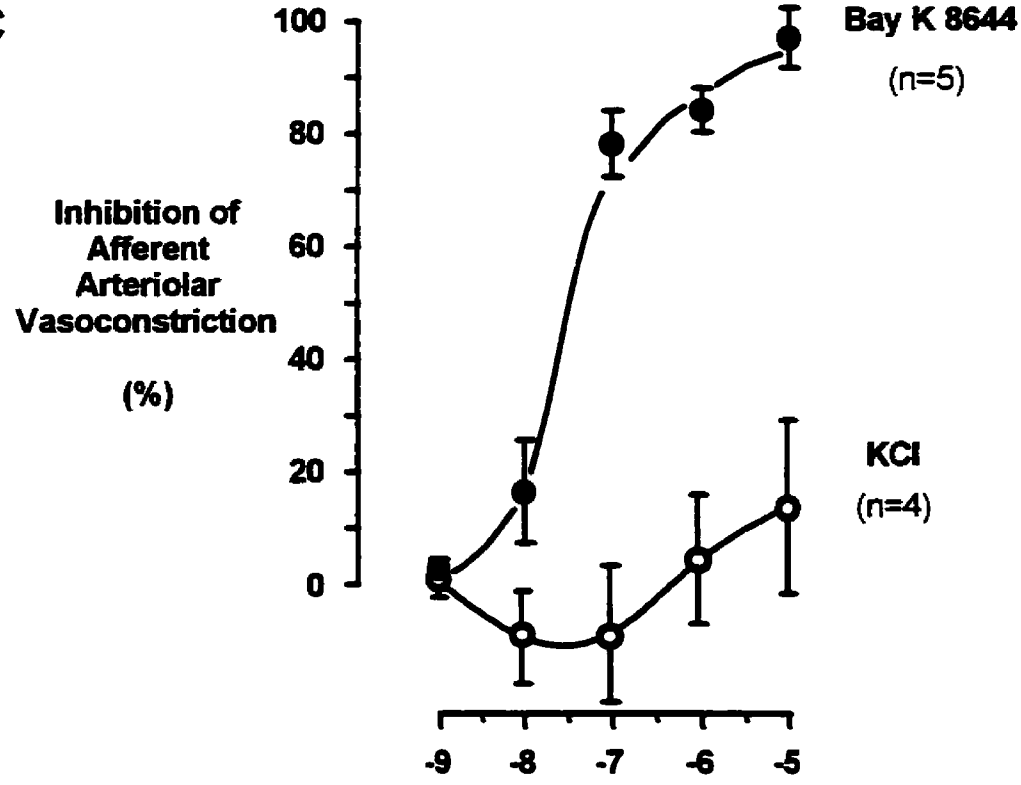
A


0.1 μM Bay K 8644
Afferent Arteriolar Diameter (μm)
20
16
12
8
4
0
1 nM
10 nM
0.1 μM
1 μM PIN
G

B


30 mM KCl
Afferent Arteriolar Diameter (μm)
16
12
8
4
0
1 nM
10 nM
0.1 μM
1 μM
10 μM PIN
10 min

C


Inhibition of Afferent Arteriolar Vasoconstriction (%)
100
80
60
40
20
0
-9
-8
-7
-6
-5
Bay K 8644
(n=5)
KCl
(n=4)

μm ($p<0.01$, n=4). The subsequent addition of 0.001, 0.01, 0.1, 1.0 and 10 μM pinacidil did not significantly alter the mean arteriolar diameter (i.e. 8.8±0.7 μm, 8.0±0.5 μm, 8.1±0.5 μm, 8.9±0.7 μm, 9.6±0.9 μm, respectively, $p>0.05$ versus KCl alone, n=4). The data are plotted in Figure 3.1, panel C, as the percent inhibition of KCl-induced vasoconstriction.

Effects of pinacidil on afferent and efferent arteriolar vasoconstrictions induced by angiotensin II

In order to investigate the actions of pinacidil on both afferent and efferent vasoconstriction, angiotensin II was used as the vasoconstrictory stimulus. In contrast to Bay K 8644 and KCl, which act exclusively on the afferent arteriole, angiotensin II constricts both vessels, but does so through different mechanisms. Figure 3.2 depicts original tracings illustrating the response of an afferent (panel A) and efferent (panel B) arteriole to 0.1 nM angiotensin II. In these tracings, angiotensin II decreased afferent arteriolar diameter from 15.7 μm to 4.2 μm and efferent arteriolar diameter from 10.5 μm to 3.0 μm. The addition of 1.0 μM pinacidil caused a prompt vasodilation of the afferent arteriole (panel A), but had little effect on the efferent arteriole (panel B). In contrast, 10 μM pinacidil dilated both vessels to pre-angiotensin levels. The subsequent addition of 10 μM glibenclamide restored the angiotensin II-induced vasoconstriction in the continued presence of pinacidil in both vessels (see Figure 3.2).

To further examine the effects of pinacidil on the afferent and efferent arterioles, paired vessels from the same glomerulus were exposed to repeated applications of 0.1 nM angiotensin II. The effects of increasing concentrations of pinacidil (0.001 to 10 μM) on basal diameters and on angiotensin II-induced vasoconstriction were assessed for each vessel. Table 3.1 and Figure 3.3 summarize these data. Over this concentration range, pinacidil had no effect on basal diameter of either vessel ($p>0.05$, n=8, Table 3.1). Pinacidil produced a concentration-dependent inhibition of the angiotensin II-induced vasoconstriction of the afferent arteriole, significantly increasing the diameters of angiotensin II-treated vessels at both 1 μM and 10 μM ($p<0.05$ versus 0 pinacidil, n=8, Table 3.1). In the efferent arteriole, pinacidil significantly increased diameters during

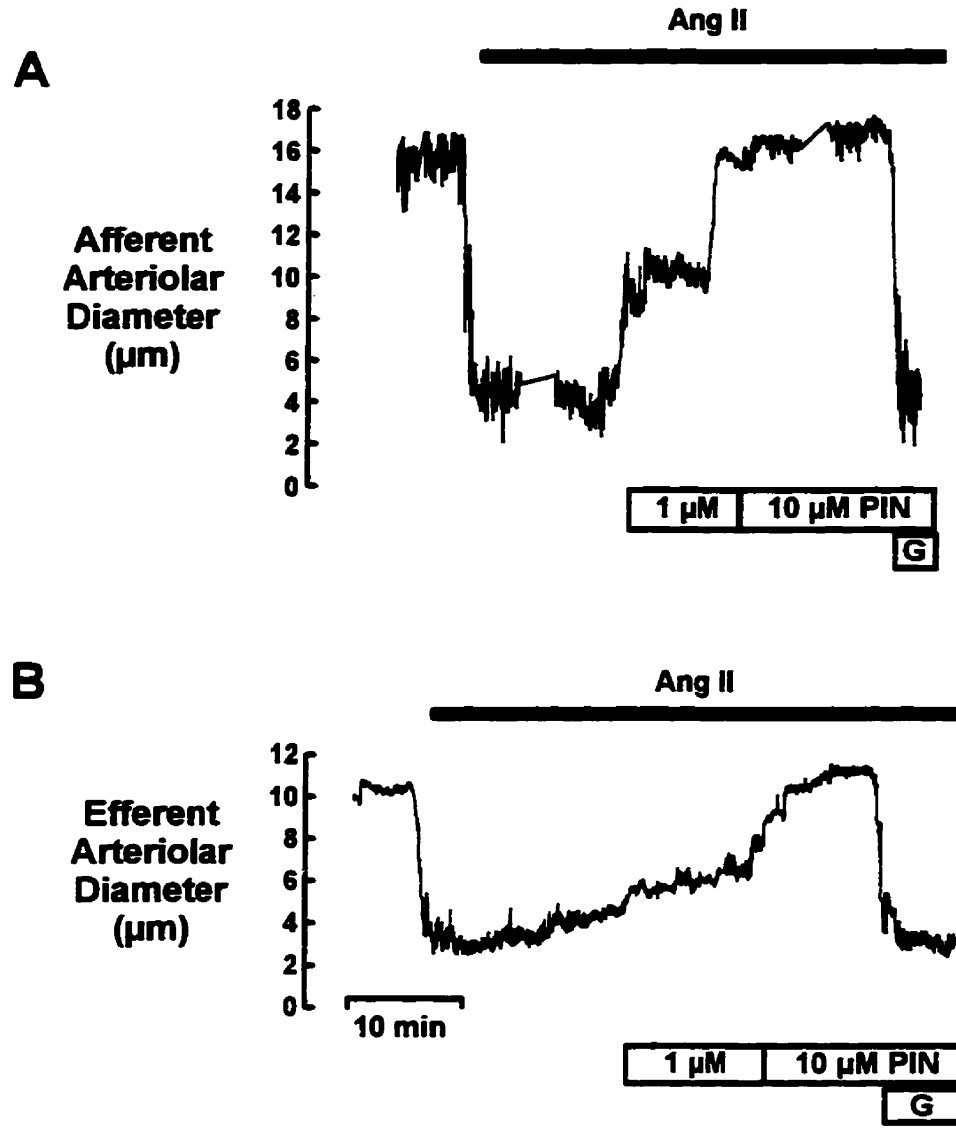


Figure 3.2: Original tracings illustrating the vasodilatory actions of pinacidil (PIN) on afferent (panel A) and efferent (panel B) arterioles preconstricted with 0.1 nM angiotensin II (Ang II). In both vessels, pinacidil-induced vasodilation was reversed by 10 µM glibenclamide (G).

Table 3.1: Changes in afferent (top) and efferent (bottom) arteriolar diameters induced by 0.1 nM angiotensin II (Ang II) in the absence (0) and presence of pinacidil (0.001 to 10 μM).

Afferent Arteriolar Diameter (μm)

pinacidil, M	control	Ang II (10^{-10} M)
0	15.2±0.7	6.1±1.1*
10^{-9}	14.9±0.6	6.3±1.0*
10^{-8}	14.7±0.5	6.7±1.0*
10^{-7}	14.7±0.4	8.6±0.7*
10^{-6}	15.1±0.4	11.1±1.5*†
10^{-5}	15.5±0.3	13.8±1.3*†

Efferent Arteriolar Diameter (μm)

pinacidil, M	control	Ang II (10^{-10} M)
0	11.1±1.3	4.4±0.3*
10^{-9}	11.2±1.3	4.5±0.3*
10^{-8}	11.1±1.2	4.6±0.4*
10^{-7}	11.0±1.1	5.0±0.6*
10^{-6}	11.5±1.3	6.0±1.0*
10^{-5}	11.4±1.2	9.6±1.3*†

Values are mean ± SEM, n=8

* $p<0.05$ vs control, † $p<0.05$ vs 0 pinacidil

angiotensin II-treatment only at 10 μM ($p<0.05$, n=8, Table 3.1). These data are plotted as the percent inhibition of the angiotensin II-induced change in diameter in Figure 3.3. Pinacidil produced a greater inhibition of the afferent arteriolar response at concentrations of 0.1 μM (31±6%, afferent and -3±14%, efferent, $p<0.05$, n=8) and 1.0 μM (59±13%, afferent and 15±9%, efferent, $p<0.05$, n=8). The IC_{50}'s (extrapolated from Figure 3.3) were 0.5 μM and 4.6 μM for the afferent and efferent arteriole, respectively. Nevertheless, the maximal inhibition produced by 10 μM pinacidil was similar in both vessels (84±10% and 71±9% for afferent and efferent arterioles, respectively, $p=0.35$).

Glibenclamide reversed the effects of pinacidil on both afferent and efferent arterioles, suggesting that each of these actions involved activation of K_{ATP} (Figure 3.4). Basal diameters were not affected by 10 μM pinacidil or 10 μM pinacidil plus 10 μM glibenclamide in either afferent or efferent arterioles ($p>0.05$, n=5, Figure 3.4, panel A or B, respectively). In the absence of pinacidil (control), angiotensin II reduced afferent arteriolar diameter from 15.9±0.7 μm to 6.9±1.5 μm ($p=0.002$). 10 μM pinacidil completely inhibited the response to angiotensin II (basal diameter 15.4±0.5 μm, angiotensin II 13.5±2.1 μm, $p=0.35$); whereas glibenclamide restored normal afferent arteriolar responsiveness (basal diameter 14.0±1.1 μm, angiotensin II 6.9±1.2 μm, $p<0.0001$). In the efferent arteriole, the corresponding angiotensin II-induced changes were, for controls, from 10.7±2.1 μm to 4.3±0.3 μm ($p=0.03$); for pinacidil from 10.5±1.8 μm to 8.8±1.9 μm ($p=0.16$); and for pinacidil plus glibenclamide, from 10.4±1.8 μm to 5.2±0.6 μm ($p=0.04$).

Further characterization of efferent arteriolar actions of pinacidil

Additional studies were conducted to further define the mechanisms underlying the actions of pinacidil on the reactivity of the efferent arteriole. In agreement with the premise that efferent arteriolar reactivity is not dependent on L-type calcium channels, 10 μM diltiazem had no effect on the vasoconstrictor response of this vessel to angiotensin II. In five efferent arterioles, angiotensin II reduced the mean diameter from 12.3±1.3 μm to 6.3±1.0 μm ($p=0.006$) in the absence and from 12.0±1.1 μm to 6.9±1.2 μm ($p=0.003$) in the presence of diltiazem (Figure 3.5, panel B). The effects of pinacidil on angiotensin II-

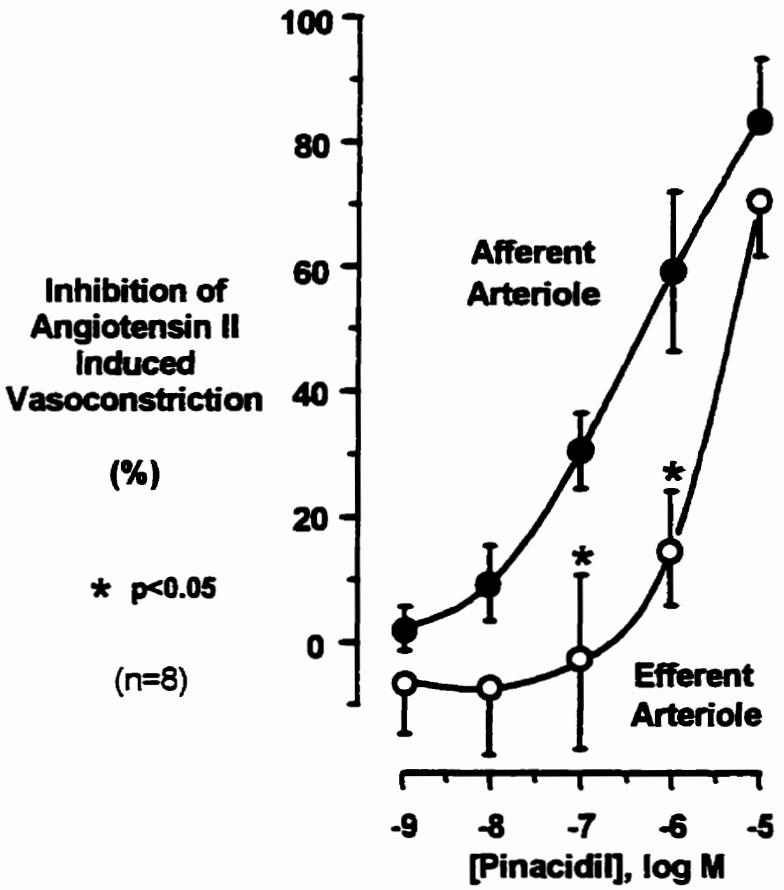


Figure 3.3: Graph summarizing the inhibitory effects of increasing concentrations of pinacidil on the angiotensin II-induced vasoconstriction of afferent (closed circles) and efferent (open circles) arterioles. Pinacidil at 0.1 and 1 μM preferentially inhibited the afferent vasoconstriction ($p<0.05$). At 10 μM, pinacidil inhibited afferent and efferent arteriolar responses to similar extents ($p=0.35$). Values are mean ± SEM, n=8.

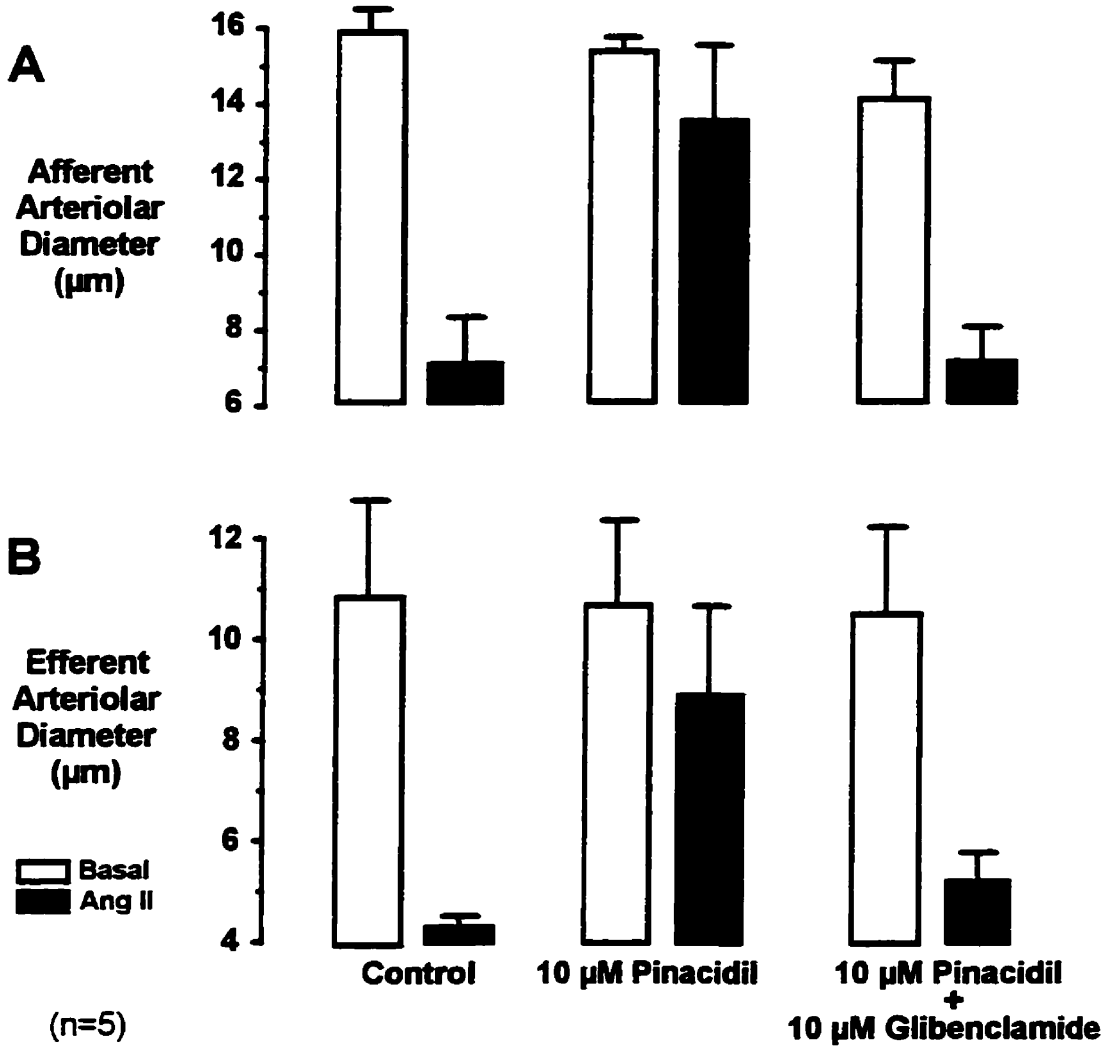


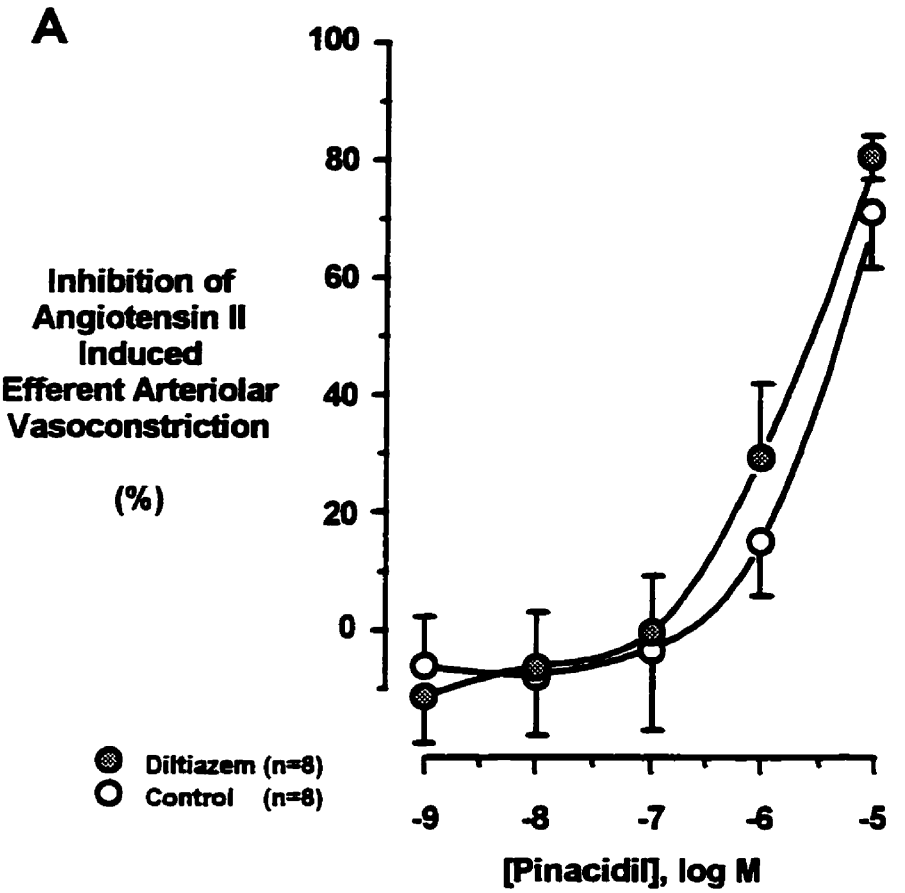
Figure 3.4: Graph summarizing the effects of pinacidil and glibenclamide on angiotensin II-induced changes in afferent (panel A) and efferent (panel B) arteriolar diameters. Open bars represent basal (pre-angiotensin II) diameters. Closed bars represent diameters after the administration of 0.1 nM angiotensin II. The inhibitory effects of 10 µM pinacidil (middle bars) on both afferent and efferent arterioles were reversed by 10 µM glibenclamide (right bars). Values are mean ± SEM, data obtained from five kidneys (n=5).

induced changes in efferent arteriolar diameters in vessels pretreated with diltiazem are summarized in Table 3.2. Figure 3.5, panel A compares the percent inhibition of angiotensin II responses by pinacidil in the presence and absence of 10 μM diltiazem (data from Tables 3.1 and 3.2). Identical concentration-response curves to pinacidil were obtained under these two conditions ($p>0.15$ for all points).

Since the above results suggested that the efferent arteriolar actions of pinacidil may involve K_{ATP} (reversed by glibenclamide), but did not involve modulation of L-type calcium channels (not affected by diltiazem), we next examined the dependence of pinacidil's actions on the potassium gradient. If the effects of pinacidil on the efferent arteriole were mediated exclusively by K-channel activation, elevation of external K^+ would be anticipated to eliminate the inhibition. Therefore, we determined if 45 mM KCl reversed and/or prevented the inhibitory actions of pinacidil. In order to eliminate effects of KCl on renal perfusate flow and afferent arteriolar tone, these studies were conducted in the presence of 10 μM diltiazem. The protocol used in this series of experiments is illustrated by the representative tracing in Figure 3.6, panel A. As depicted, the inhibitory effects of pinacidil were completely reversed by the elevation of external K^+ (from 5 mM to 45 mM KCl). Figure 3.6, panel B summarizes the results obtained using this protocol in ten efferent arterioles. Neither pinacidil nor KCl altered basal diameter in the presence of diltiazem (12.4±0.9 μm, 12.8±0.8 μm, 12.5±0.7 μm, diltiazem alone, plus pinacidil, plus pinacidil and KCl, respectively, $p>0.05$ versus diltiazem alone, $n=10$). In the absence of pinacidil, angiotensin II reduced efferent arteriolar diameter from 12.4±0.9 μm to 8.1±0.7 μm ($p<0.0001$). Pinacidil attenuated the angiotensin II-induced vasoconstriction (from 12.8±0.8 μm to 11.4±0.8 μm, $p=0.0001$ versus diltiazem alone). Subsequent treatment with 45 mM KCl restored original reactivity to angiotensin II in the continued presence of pinacidil (from 12.5±0.7 μm to 9.0±0.8 μm, $p=0.001$ versus pinacidil/diltiazem and $p=0.15$ versus diltiazem alone).

To further examine the effects of KCl on the inhibitory actions of pinacidil and to assure that KCl had no potentiating action on angiotensin II responsiveness on its own, additional studies were conducted in which the order of KCl and pinacidil addition were reversed. As depicted by the tracing in Figure 3.7, when the KCl was added prior to treatment with

Figure 3.5: Graph summarizing the inhibitory effects of increasing doses of pinacidil on the angiotensin II-induced vasoconstriction of efferent arterioles in the absence (open circles) and presence (closed circles) of 10 μM diltiazem (panel A).
The lack of effect of 10 μM diltiazem on the efferent arteriolar responses to angiotensin II is depicted in panel B. Open bars indicate basal diameters, closed bars represent diameters after the administration of 0.1 nM angiotensin II (Ang II).


A
Inhibition of Angiotensin II Induced Efferent Arteriolar Vasoconstriction
(%)
100
80
60
40
20
0
Diltiazem (n=8)
Control (n=8)
-9
-8
-7
-6
-5
[Pinacidil], log M

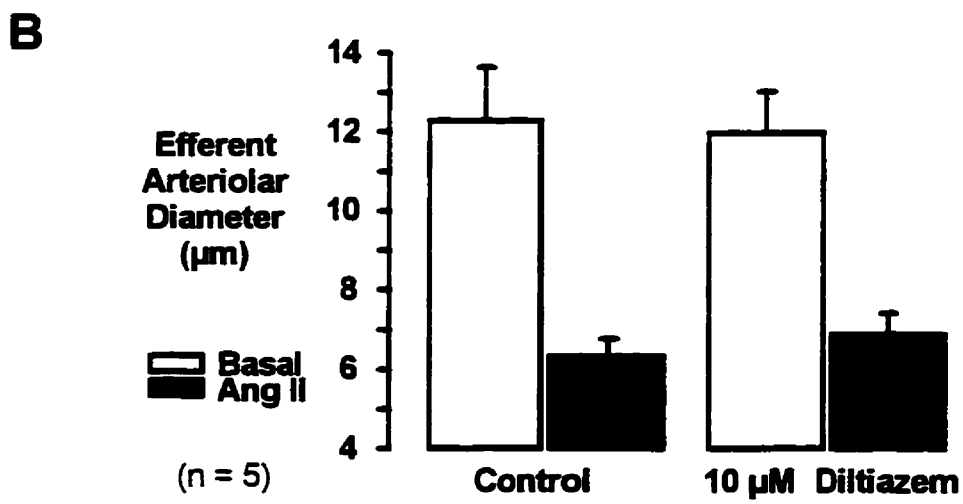

B
Efferent Arteriolar Diameter (µm)
Basal
Ang II
(n = 5)
14
12
10
8
6
4
Control
10 µM Diltiazem

Table 3.2: Changes in efferent arteriolar diameters induced by 0.1 nM angiotensin II (Ang II) in the presence of increasing concentration of pinacidil (0.001 to 10 μM) in the continuous presence of 10 μM diltiazem.

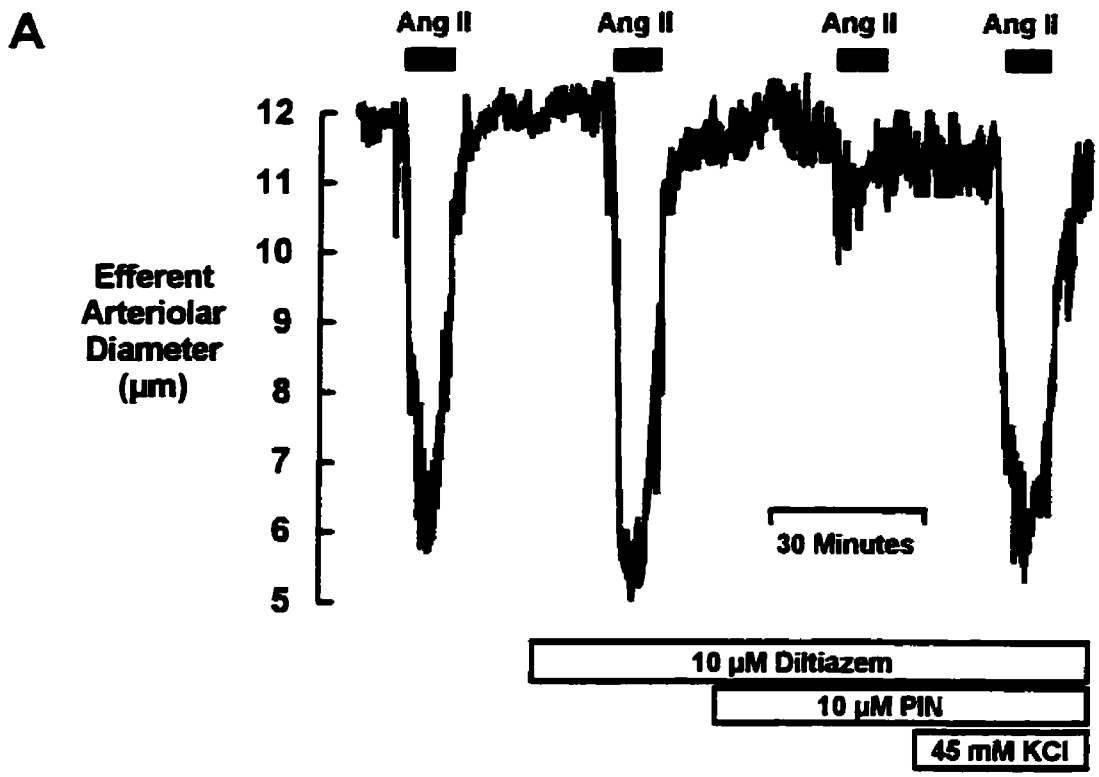
	Efferent Arteriolar Diameter (μm)	
pinacidil, M	**control**	**Ang II (10^{-10} M)**
0	14.4±1.0	8.7±0.8*
10^{-9}	13.6±1.0	8.4±0.9*
10^{-8}	14.1±1.2	9.0±1.0*
10^{-7}	14.5±1.1	9.6±0.9*
10^{-6}	14.5±1.2	11.0±1.1*
10^{-5}	14.9±1.1	13.8±1.1*†

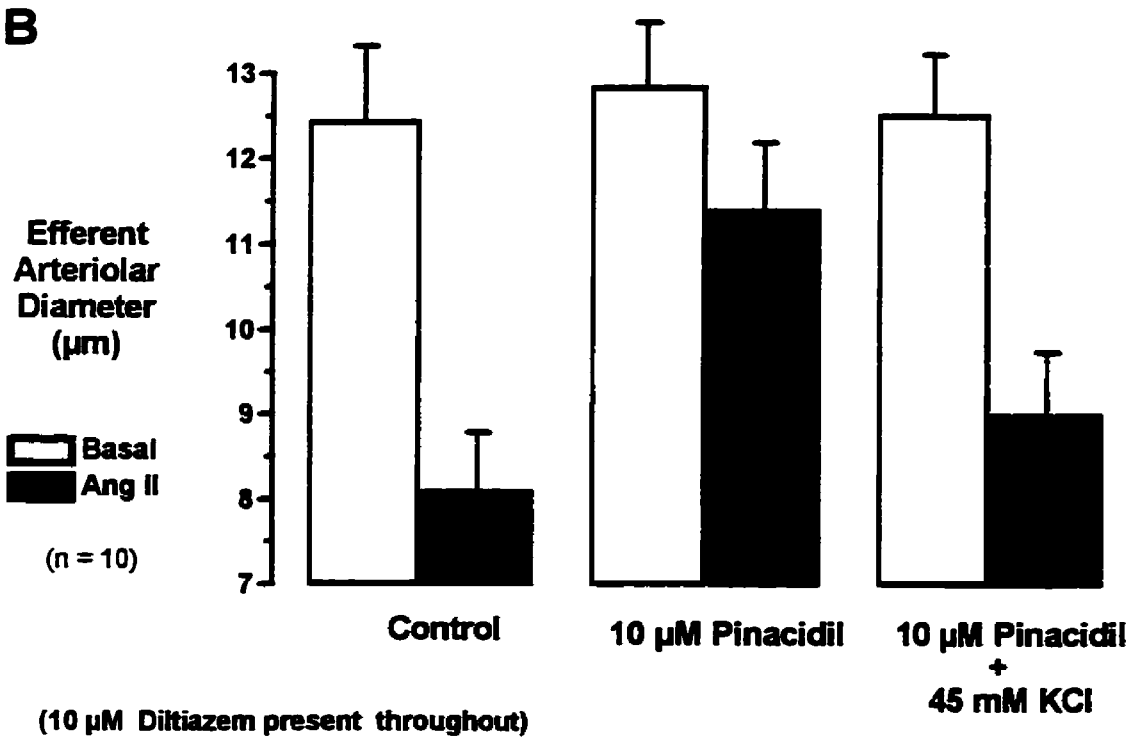
Values are mean ± SEM, n=8, 10 μM diltiazem present throughout the duration of experiments.

* $p<0.05$ vs control, † $p<0.05$ vs 0 pinacidil

Figure 3.6: Original tracing illustrating the inhibitory effects of 10 μM pinacidil (PIN) on the angiotensin II-induced (Ang II) efferent arteriolar vasoconstriction and the reversal of this effect with 45 mM KCl (panel A).

Data obtained from ten efferent arterioles using the above experimental protocol are summarized in panel B. Values are mean ± SEM, n=10.


A
Ang II
Ang II
Ang II
Ang II
Efferent Arteriolar Diameter (µm)
12
11
10
9
8
7
6
5
30 Minutes
10 µM Diltiazem
10 µM PIN
45 mM KCl


B
Efferent Arteriolar Diameter (µm)
13
12
11
10
9
8
7
Basal
Ang II
(n = 10)
Control
10 µM Pinacidil
10 µM Pinacidil + 45 mM KCl
(10 µM Diltiazem present throughout)

pinacidil, the pinacidil-induced inhibition of angiotensin II vasoconstriction was prevented. Mean results obtained from 3 replicate studies were as follows: under control conditions angiotensin II reduced diameters from 11.8±1.7 μm to 7.5±1.5 μm; in the presence of 10 μM diltiazem from 11.1±1.8 μm to 6.4±1.6 μm; in the presence of 45 mM KCl (plus diltiazem) from 11.0±1.7 μm to 6.1±1.7 μm; and in the presence of 10 μM pinacidil (plus 45 mM KCl and 10 μM diltiazem) from 11.1±1.6 μm to 6.4±1.8 μm.

Effects of pinacidil on pressure-induced afferent arteriolar vasoconstriction

Elevations in perfusion pressure in 20 mm Hg increments from 60 to 180 mm Hg elicited graded afferent arteriolar vasoconstriction (dashed line, control, Figure 3.8). The afferent arteriolar diameter changed from 14.5±0.6 μm at 60 mm Hg to 14.5±0.6 μm, 11.7±1.2 μm, 9.3±1.2 μm, 7.4±1.4 μm, 6.7±1.4 μm and 6.3±1.4 μm at 80, 100, 120, 140, 160 and 180 mm Hg, respectively. The afferent arteriolar diameter decreased significantly at pressures of 120 mm Hg and above ($p<0.05$, $n=6$). As evident from Figure 3.8, pretreatment with 0.001 to 1 μM pinacidil inhibited afferent arteriolar myogenic vasoconstriction in a dose-dependent manner. 0.1 μM pinacidil thus shifted the threshold for the myogenic vasoconstriction to 140 mm Hg, while 1 μM pinacidil abolished the myogenic response completely (15.1±0.5 μm, 15.4±0.4 μm, 15.5±0.5 μm, 15.5±0.4 μm, 15.5±0.5 μm, 15.6±0.5 μm and 15.1±0.5 μm at 60, 80, 100, 120, 140, 160 and 180 mm Hg, respectively, $p<0.05$ versus corresponding control values at 100 mm Hg and above). At all concentrations, pinacidil did not alter the afferent arteriolar diameters at either 60 or 80 mm Hg ($p>0.05$ versus control, $n=6$). Addition of 10 μM glibenclamide completely restored the myogenic reactivity in the continued presence of 10 μM pinacidil (14.1±0.7 μm, 12.7±0.8 μm, 8.3±0.4 μm, 5.6±0.6 μm, 5.1±0.7 μm, 4.8±0.7 μm and 4.4±1.0 μm at 60, 80, 100, 120, 140, 160 and 180 mm Hg, respectively, $p>0.05$ versus corresponding control values).

The original tracing in Figure 3.9 illustrates the effects of increasing doses of glibenclamide on pinacidil-induced inhibition of myogenic reactivity. In this experiment, afferent arteriolar myogenic vasoconstriction was completely inhibited by 1 μM pinacidil. Pretreatment with 0.1 μM glibenclamide failed to affect the inhibitory actions of pinacidil. At 1 and 10 μM,

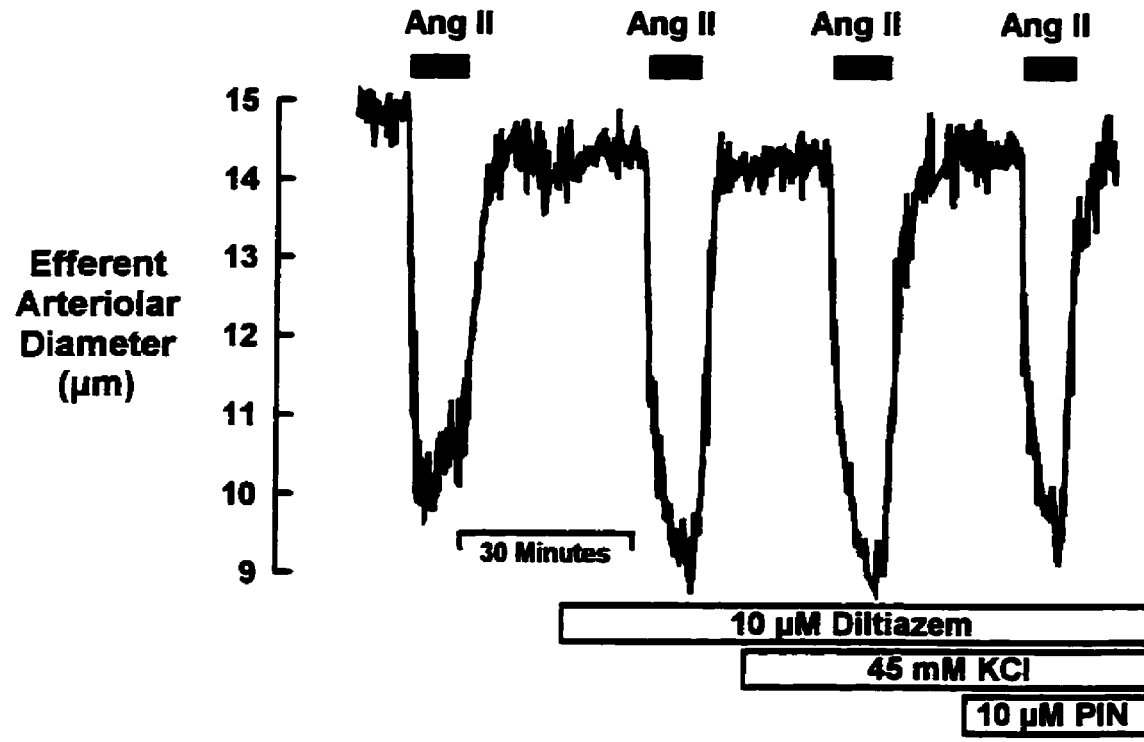


Figure 3.7: Original tracing illustrating that pretreatment with 45 mM KCl prevents the inhibitory effects of 10 µM pinacidil (PIN) on the angiotensin II-induced (Ang II) efferent arteriolar vasoconstriction. Note that KCl on its own has no effect on the angiotensin II actions in the efferent arteriole.

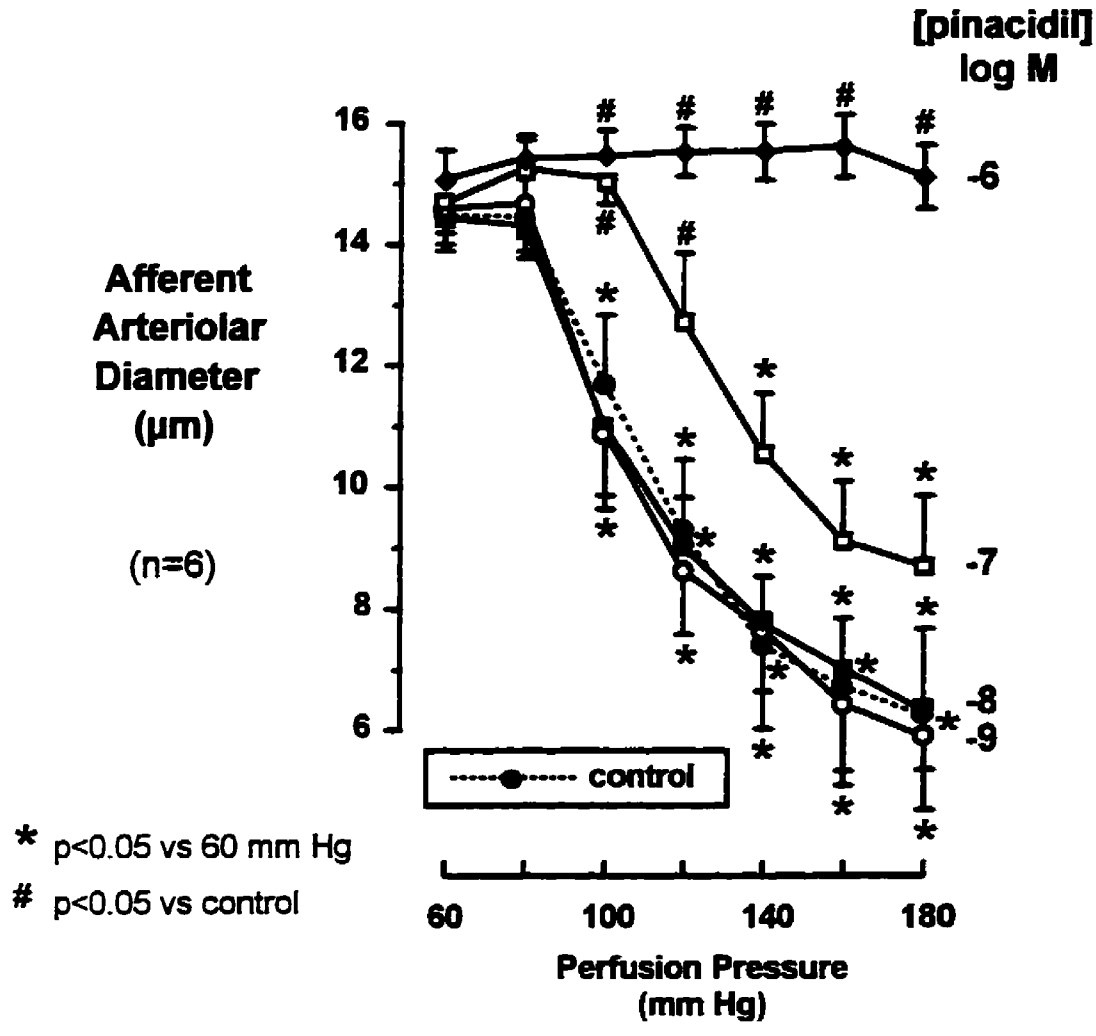


Figure 3.8: Graph summarizing the inhibitory effects of pinacidil on afferent arteriolar myogenic vasoconstriction. Pinacidil (0.001 to 1 µM) inhibited pressure-induced vasoconstriction (control, dashed line) in a dose dependent manner. Values are mean ± SEM, n=6.

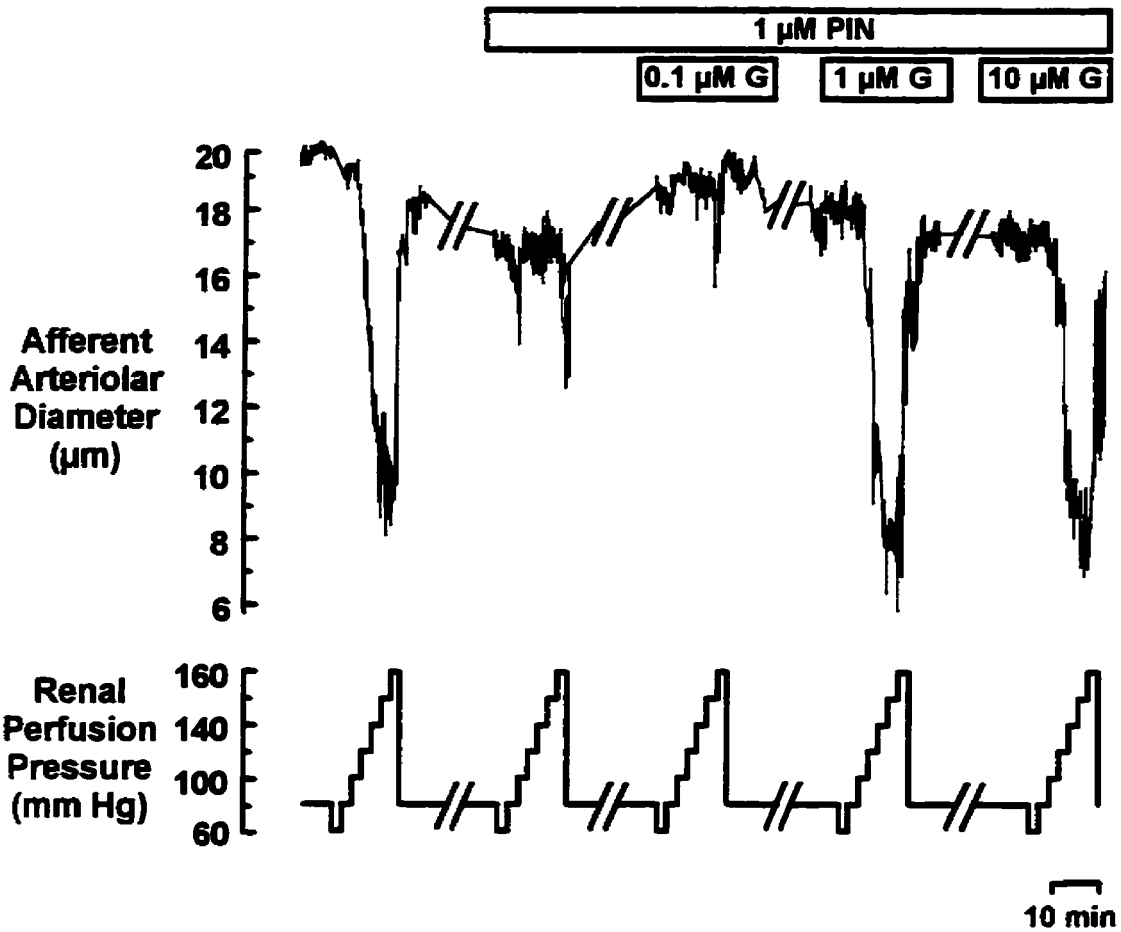


Figure 3.9: Original tracing illustrating the inhibitory effects of 1 µM pinacidil (PIN) on the myogenic vasoconstriction of the afferent arteriole. In the absence of pinacidil, stepwise elevations in perfusion pressure from 60 to 180 mm Hg elicited myogenic vasoconstriction of the afferent arteriole, decreasing the diameter from 20 µm at 80 mm Hg (basal) to approximately 10 µm at 180 mm Hg. In the presence of 1 µM pinacidil, the diameter decreased only minimally (to 16 µm at 180 mm Hg) in response to elevations in perfusion pressure. 1 and 10 µM glibenclamide (G) restored the myogenic reactivity to control levels, while 0.1 µM glibenclamide failed to alter the actions of pinacidil.

glibenclamide completely restored the myogenic response in the continued presence of 1 μM pinacidil. In all experiments throughout this thesis, the higher effective dose (i.e. 10 μM) of glibenclamide was used to determine the involvement of K_{ATP}.

Comparison of vasodilatory effects of pinacidil on myogenic and angiotensin II-induced afferent arteriolar vasoconstriction

As illustrated in Figure 3.10, panel B, 0.1 nM angiotensin II and elevation in perfusion pressure from 80 to 180 mm Hg elicited similar changes in afferent arteriolar diameters (61±6% and 59±8% decrease in diameter with angiotensin II and elevated pressure, respectively, p=0.81). Figure 3.10, panel A, depicts the inhibitory actions of pinacidil on angiotensin II-induced (data from Figure 3.3) and myogenic (data calculated from Figure 3.8) vasoconstriction of the afferent arteriole. At 1 μM, pinacidil preferentially inhibited the myogenic vasoconstriction (97±5% vs 59±13%, p=0.034). At 10 μM, pinacidil inhibited the angiotensin II-induced vasoconstriction by 84±10%.

Interpretation of results obtained with pinacidil

In the afferent arteriole, pinacidil inhibited vasoconstriction elicited by Bay K 8644, a direct activator of L-type calcium channels. Pinacidil also inhibited pressure- and angiotensin II-induced afferent arteriolar vasoconstrictions, which are sensitive to L-type calcium channel blockade. Interestingly, the pressure-induced vasoconstriction was found to be more sensitive to the vasodilatory actions of pinacidil than angiotensin II-induced vasoconstriction, although both stimuli elicited vasoconstrictor responses of similar magnitude. In all cases, the afferent arteriolar actions of pinacidil were inhibited by glibenclamide. The failure of pinacidil to dilate afferent arterioles preconstricted with KCl reflects a general dependence of its actions on the K^+ gradient. Taken together, these findings support the premise that pinacidil elicits the afferent arteriolar vasodilation by inhibiting the activation of L-type calcium channels. In contrast, the efferent arteriolar reactivity is not dependent on L-type calcium channels and should, therefore, have been resistant to vasodilatory actions of pinacidil, if this agent acted exclusively by inhibiting of L-type calcium channel activation.

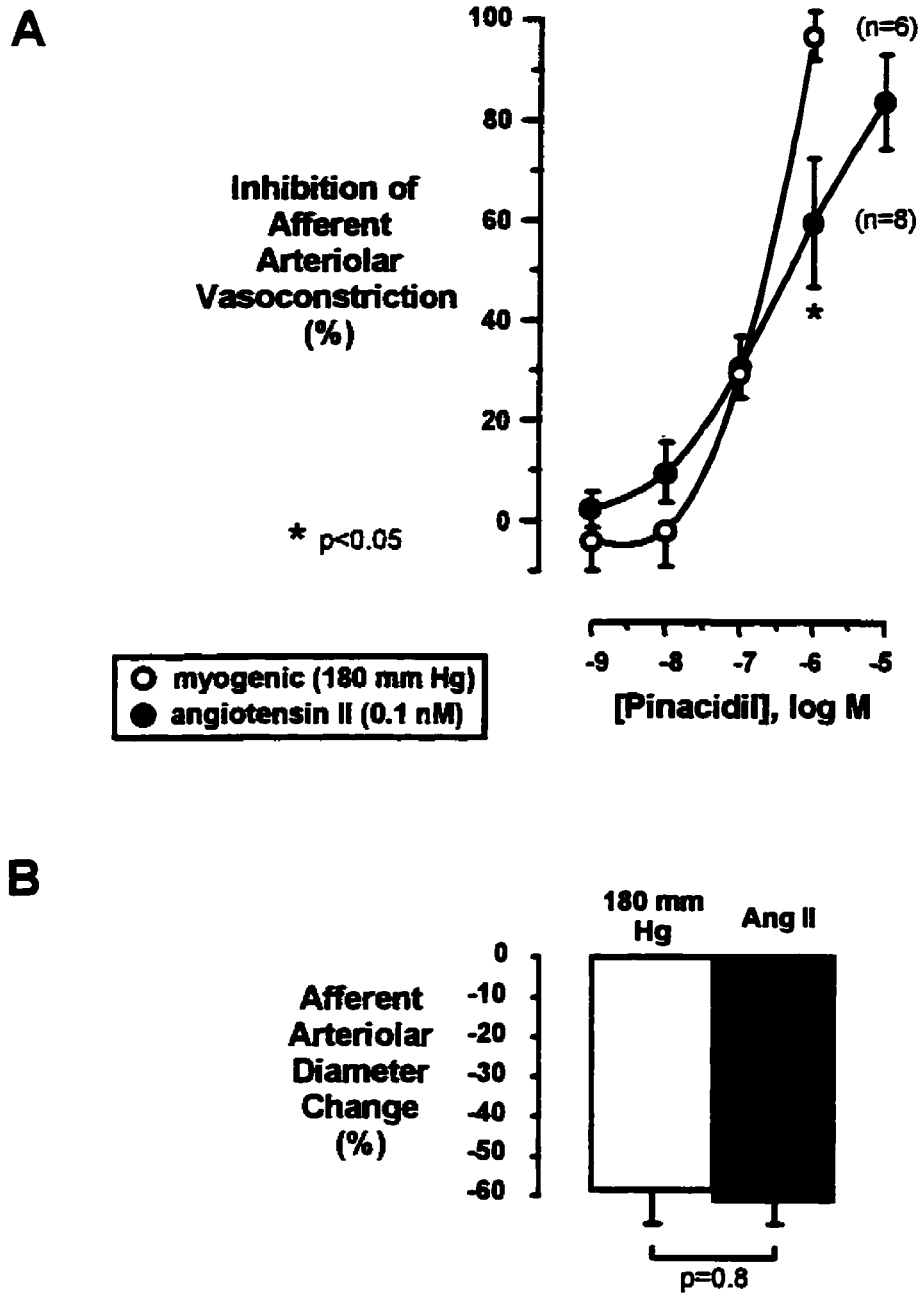


Figure 3.10: Inhibitory effects of pinacidil on angiotensin II-induced (closed circles) and myogenic (open circles) vasoconstriction of the afferent arterioles (panel A). In the absence of pinacidil, 0.1 nM angiotensin II (Ang II, closed bar) and elevation of perfusion pressure from 80 to 180 mm Hg (open bar) decreased afferent arteriolar diameter to similar extent, as shown in panel B. Values are mean ± SEM, n= 6 and n=8 for myogenic and angiotensin II-induced vasoconstriction, respectively.

Pinacidil indeed preferentially dilated afferent versus efferent arterioles over a concentration range 0.001 to 1μM. At 10 μM, however, pinacidil inhibited the efferent arteriolar vasoconstriction by angiotensin II as well. This action of pinacidil was found to be resistant to L-type calcium channel blockade, but was blocked by glibenclamide or elevations of extracellular K^+, suggesting a K_{ATP}-mediated event.

As described in the section 1.6, the actions of several endogenous vasodilators, such as CGRP and adenosine have been linked to activation of K_{ATP} in other vascular beds. The next section examined whether CGRP and adenosine activate K_{ATP} in the renal microcirculation.

3.2 Calcitonin gene-related peptide (CGRP)

Hypothesis: *CGRP-induced afferent and efferent arteriolar vasodilations are mediated via a glibenclamide-sensitive mechanism.*

Effects of CGRP on afferent and efferent arteriolar vasoconstrictions induced by angiotensin II

Afferent and efferent arterioles from the same glomerulus were pretreated with increasing concentrations of CGRP (0.01 to 10 nM). The vasoconstrictory responses to angiotensin II were determined for controls (0 CGRP) and following each concentration of CGRP (0.01, 0.1 and 1 nM CGRP). The data obtained from eight kidneys are summarized in Table 3.3. As depicted, CGRP had no effect on basal diameters of either afferent or efferent arterioles ($p>0.05$ versus 0 CGRP, Table 3.3). In the absence of CGRP, angiotensin II reduced afferent arteriolar diameters from 16.3±0.5 μm to 6.1±0.7 μm ($p<0.0001$, $n=8$) and efferent arteriolar diameters from 11.8±1.1 μm to 4.6±0.5 μm ($p<0.0001$, $n=8$). In both the afferent and efferent arterioles, increasing concentrations of CGRP progressively attenuated the angiotensin II responses. At 10 nM CGRP elicited a statistically significant attenuation of the angiotensin II responses in both afferent and efferent arterioles ($p<0.05$ versus 0 CGRP, Table 3.3). However, even though angiotensin II responses were attenuated, angiotensin

II continued to elicit a significant vasoconstriction in both afferent and efferent arterioles.

To determine the involvement of K_{ATP}, the actions of CGRP were re-assessed in a separate group of kidneys in the presence of 10 μM glibenclamide. The responses to angiotensin II were determined in the absence and presence of glibenclamide for each vessel. Angiotensin II decreased afferent arteriolar diameters by 62±6% and 63±7% in the absence and presence of glibenclamide, respectively ($p=0.88$, $n=8$) and by 47±3% and 39±3%, respectively in the efferent arterioles ($p=0.09$, $n=8$). Table 3.4 summarizes the effects of increasing concentrations of CGRP on angiotensin II-induced vasoconstrictions in the presence of glibenclamide. As in the control (0 glibenclamide) group, CGRP (0.1 to 10 nM) had no effect on basal diameters of either afferent and efferent arterioles ($p>0.05$, Table 3.4). In the afferent arteriole, 10 nM CGRP significantly attenuated the angiotensin II response ($p<0.05$ versus 0 CGRP). In the efferent arteriole, the attenuation of the angiotensin II vasoconstriction failed to reach statistical significance ($p>0.05$ versus 0 CGRP).

The data from Tables 3.3 and 3.4 were then expressed as percent inhibition of angiotensin II-induced vasoconstriction and compared to assess the effect of glibenclamide. Thus in the afferent arteriole, 10 nM CGRP inhibited the angiotensin II responses by 53±11% in the absence and by 41±11% in the presence of glibenclamide ($p=0.42$, $n=8$). The corresponding values for the efferent arteriole were 33±13% and 39±14% ($p=0.77$, $n=8$). CGRP therefore attenuated the angiotensin II-induced vasoconstriction in a very similar manner in either absence or presence of 10 μM glibenclamide in both afferent and efferent arterioles, suggesting that activation of K_{ATP} is not involved in the actions of CGRP during angiotensin II-induced vasoconstriction.

Effects of CGRP on pressure-induced afferent arteriolar vasoconstriction

Figure 3.11, panel A illustrates the effects of CGRP on the pressure-induced afferent arteriolar vasoconstriction. In the absence of CGRP (dashed line, control), the afferent arteriolar diameter was 16.7±0.9 μm at 60 mm Hg. Threshold vasoconstrictor responses

Table 3.3: Changes in afferent (top) and efferent (bottom) arteriolar diameters induced by 0.1 nM angiotensin II (Ang II) in the absence (0) and presence of CGRP (0.1 to 10 nM).

Afferent Arteriolar Diameter (μm)

CGRP, M	control	Ang II (10^{-10} M)
0	16.3±0.5	6.1±0.6*
10^{-10}	16.0±0.5	7.0±0.7*
10^{-9}	16.4±0.6	9.6±1.5*
10^{-8}	16.3±0.8	11.5±1.4*†

Efferent Arteriolar Diameter (μm)

CGRP, M	control	Ang II (10^{-10} M)
0	11.8±1.1	4.6±0.5*
10^{-10}	11.5±1.0	4.5±0.6*
10^{-9}	12.0±1.1	5.2±0.5*
10^{-8}	11.7±1.1	7.1±1.0*†

Values are mean ± SEM, n=8

* $p<0.005$ vs control, † $p<0.05$ vs 0 CGRP

Table 3.4: Changes in afferent (top) and efferent (bottom) arteriolar diameters induced by 0.1 nM angiotensin II (Ang II) in the absence (0) and presence of CGRP (0.1 to 10 nM). In this set of kidneys, 10 μM glibenclamide was present throughout.

Afferent Arteriolar Diameter (μm)

CGRP, M	control	Ang II (10^{-10} M)
0	12.5±0.8	4.7±0.8*
10^{-10}	13.0±0.6	5.0±1.0*
10^{-9}	12.9±0.7	5.2±1.0*
10^{-8}	14.7±0.6	9.0±1.1*†

Efferent Arteriolar Diameter (μm)

CGRP, M	control	Ang II (10^{-10} M)
0	9.2±0.8	5.5±0.5*
10^{-10}	9.2±0.7	5.1±0.6*
10^{-9}	9.1±0.6	5.2±0.6*
10^{-8}	9.0±0.7	6.7±0.7*

Values are mean ± SEM, n=8

* $p<0.05$ vs control, † $p<0.05$ vs 0 CGRP

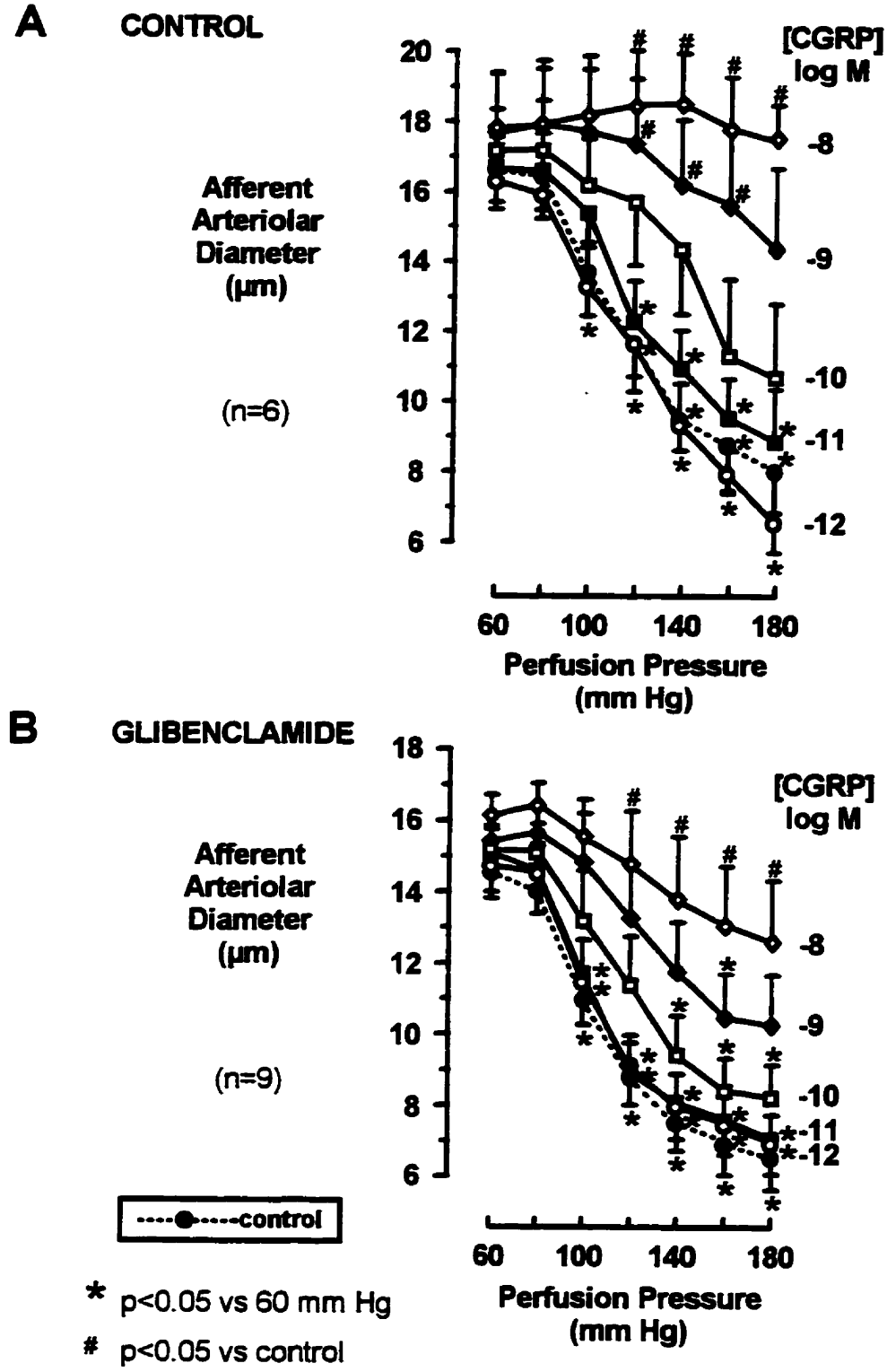
were elicited at perfusion pressures of 120 mm Hg and higher (diameters decreased to 11.8±0.9 µm, 9.6±1.0 µm, 8.9±1.3 µm and 8.2±1.2 µm at 120, 140, 160 and 180 mm Hg, respectively, $p<0.05$ versus 60 mm Hg for all values). Pretreatment with increasing doses of CGRP (0.001 to 10 nM) did not alter the afferent arteriolar diameter at 60 or 80 mm Hg ($p>0.05$ versus control, n=6). As depicted in Figure 3.11, panel A, CGRP inhibited the pressure-induced vasoconstriction of the afferent arteriole at concentrations of 0.1 nM and higher.

To determine the role of K_{ATP} in the actions of CGRP in this setting, the protocol was repeated in the presence of 10 µm glibenclamide in a separate group of kidneys. These data are summarized in Figure 3.11, panel B. In the absence of CGRP, the mean afferent arteriolar diameter decreased significantly from 15.2±0.7 µm at 60 mm Hg to 11.7± 1.0 µm, 9.9±1.1 µm, 8.6±0.9 µm, 7.5±0.9 µm and 7.0±0.8 µm at 100, 120, 140, 160 and 180 mm Hg, respectively ($p<0.05$ versus 60 mm Hg, n=9). Pretreatment with 0.001 to 10 nM CGRP did not alter basal diameters at either 60 or 80 mm Hg ($p>0.05$ versus control, n=9). At 0.1 nM and 1 nM, CGRP shifted the threshold for pressure-induced vasoconstriction to 140 and 160 mm Hg, respectively (from 100 mm Hg in the absence of CGRP). Only at 10 nM did CGRP inhibit the myogenic vasoconstriction of the afferent arteriole at all pressures. Glibenclamide thus partially attenuated the inhibitory actions of CGRP on myogenic vasoconstriction of the afferent arteriole, suggesting both K_{ATP}-dependent and -independent actions of CGRP in this setting.

Comparison of vasodilatory effects of CGRP on myogenic and angiotensin II-induced afferent arteriolar vasoconstriction

The inhibitory actions of 10 nM CGRP on pressure- and angiotensin II-induced responses are plotted as percent inhibition of afferent arteriolar vasoconstriction in Figure 3.12, panel A. Panel B illustrates that both in the control and glibenclamide pretreated groups, 0.1 nM angiotensin II and elevated perfusion pressure (from 80 to 180 mm Hg) constricted afferent arterioles to a similar degree. In the absence of glibenclamide (control, Figure 3.12, panel A), 10 nM CGRP elicited a significantly greater inhibition of myogenic versus angiotensin

Figure 3.11: Graph summarizing the effects of CGRP on the afferent arteriolar myogenic vasoconstriction. In the absence of glibenclamide (panel A, n=6), CGRP abolished the pressure-induced vasoconstriction at concentrations of 0.1 nM (open squares), 1 nM (closed diamonds) and 10 nM (open diamonds). In the presence of glibenclamide (panel B, n=9), only 10 nM CGRP (open diamonds) prevented the pressure-induced afferent arteriolar vasoconstriction. Values are mean ± SEM.


A
CONTROL
Afferent Arteriolar Diameter (µm)
(n=6)
[CGRP] log M
-8
-9
-10
-11
-12
20
18
16
14
12
10
8
6
60
100
140
180
Perfusion Pressure (mm Hg)
B
GLIBENCLAMIDE
Afferent Arteriolar Diameter (µm)
(n=9)
[CGRP] log M
-8
-9
-10
-11
-12
18
16
14
12
10
8
6
60
100
140
180
Perfusion Pressure (mm Hg)
control
* p<0.05 vs 60 mm Hg
# p<0.05 vs control

II-induced vasoconstriction (95±10% versus 53±11%, respectively, p=0.025). Pretreatment with glibenclamide (glibenclamide, Figure 3.12, panel A) eliminated this difference (62±16% and 41±11% inhibition of myogenic and angiotensin II-induced responses, respectively, p=0.31). These data suggest that the differing effects of CGRP on pressure- versus angiotensin II-induced vasoconstrictions are due to glibenclamide-sensitive (i.e. K_{ATP}) component of CGRP vasodilations that is observed during pressure-induced vasoconstriction, but is not seen when vasoconstriction is induced by angiotensin II.

Interpretation of results obtained with CGRP

In the angiotensin II-constricted afferent and efferent arterioles, CGRP elicited vasodilations that were insensitive to glibenclamide and therefore not mediated by K_{ATP}. During the myogenic vasoconstriction of the afferent arteriole however, the vasodilatory actions of CGRP were partially attenuated by glibenclamide, suggesting a role of K_{ATP} in this response. The glibenclamide-sensitive, i.e. K_{ATP}-mediated, component of the CGRP actions appears to depend on the nature of the underlying vasoconstrictor tone, as it manifests during pressure- but not angiotensin II-induced afferent arteriolar vasoconstriction.

3.3 Adenosine

Hypothesis: *High-affinity, adenosine A_2-induced afferent and efferent arteriolar vasodilations are mediated via a glibenclamide-sensitive mechanism.*

As discussed in the Introduction, the actions of adenosine on the renal microvasculature are dose-dependent. The vasoconstrictor response to micromolar levels of adenosine is mediated via adenosine A_1 receptor subtype. At higher concentrations (10-30 μM), adenosine elicits vasodilation by low-affinity adenosine A_{2b} receptors. In addition, the hydronephrotic kidney preparation exhibits a high-affinity adenosine vasodilatory response, seen at submicromolar concentrations of adenosine.

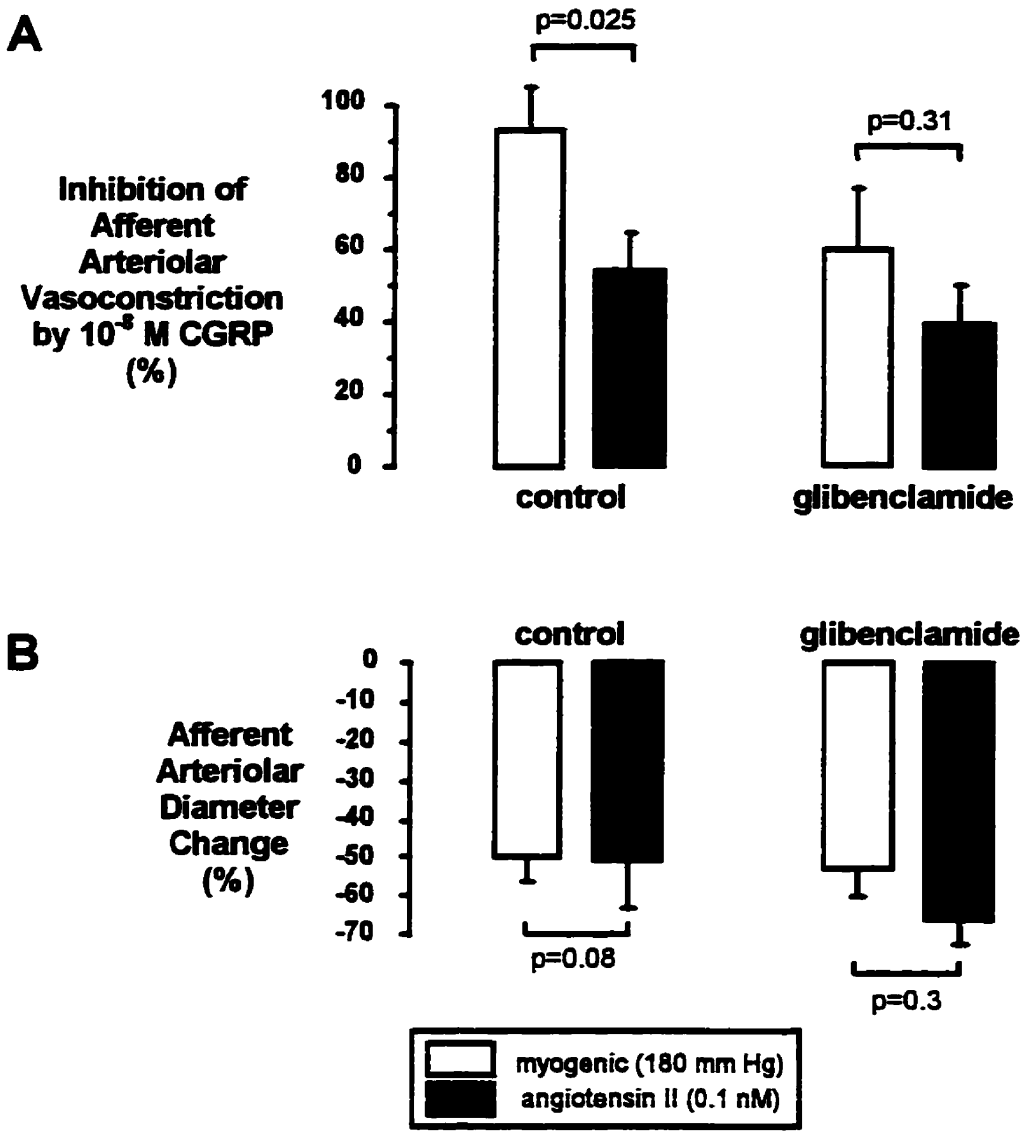


Figure 3.12: Panel A illustrates the inhibitory actions of 10 nM CGRP on afferent arteriolar vasoconstriction elicited by 0.1 nM angiotensin II (closed bars) or elevated perfusion pressure (open bars). In the absence of glibenclamide (control), 10 nM CGRP preferentially inhibited the myogenic vasoconstriction (left). In the presence of glibenclamide (glibenclamide), the myogenic and angiotensin II-induced vasoconstrictions were inhibited to similar extent (right). Panel B compares the levels of vasoconstrictions elicited by angiotensin II or pressure in control (right) or glibenclamide (left) groups. Values are mean ± SEM, n=6/n=9 for myogenic and n=8/n=8 for angiotensin II vasoconstrictions in the control/glibenclamide group, respectively.

Effects of adenosine on afferent and efferent arteriolar vasoconstriction induced by angiotensin II

Original tracings of afferent and efferent arteriolar responses to submicromolar concentrations of adenosine are depicted in Figure 3.13. In these tracings, 0.1 nM angiotensin II reduced afferent arteriolar diameter from 17.9 to 9.1 μm (panel A) and efferent arteriolar diameter from 16.9 to 4.2 μm (panel B). Application of 0.1 and 0.3 μM adenosine caused vasodilation in the continued presence of angiotensin II in both vessels. Addition of glibenclamide (10 μM) reversed adenosine-induced vasodilation in the afferent arteriole (Figure 13.3, panel A), while the same manipulation had no effect on the vasodilatory actions of adenosine in the efferent arteriole.

A different protocol from the one illustrated in Figure 3.13 was used to examine the vasodilatory effects of adenosine on angiotensin II-induced vasoconstriction of afferent and efferent arterioles from the same glomerulus. Vessels were first exposed to 0.1 nM angiotensin II in the absence of adenosine (control). Following the washout of angiotensin II, the vessels were pretreated with adenosine (0.1 and 0.3 μM) and the responses to angiotensin II were reassessed. In the absence of adenosine, angiotensin II constricted the afferent arterioles from 14.6±0.7 μm (basal) to 4.9±0.6 μm ($p<0.0001$, $n=9$), decreasing the diameter by 67±3%. The efferent arterioles constricted by 48±5%, diameters being reduced from 11.5±1.3 μm to 5.8±0.8 μm ($p=0.0004$, $n=9$), upon application of angiotensin II. Pretreatment with 0.1 and 0.3 μM adenosine did not alter basal diameters of either vessel ($p>0.05$, $n=9$). Figure 3.14 depicts the effects of adenosine on angiotensin II-induced changes in afferent (panel A, left) and efferent (panel B, left) arteriolar diameters. Following pretreatment with 0.1 and 0.3 μM adenosine, the afferent arteriolar diameters decreased by 65±5% ($p>0.05$ versus control) and 49±5% ($p<0.05$ versus control), respectively. The corresponding values for the efferent arterioles were 37±6% ($p>0.05$ versus control) and 26±7% ($p<0.05$ versus control), respectively. 0.3 μM adenosine thus caused significant inhibition of angiotensin II-induced vasoconstriction in both vessels (26±8% and 49±12%inhibition of control angiotensin II responses for afferent and efferent arterioles, respectively).

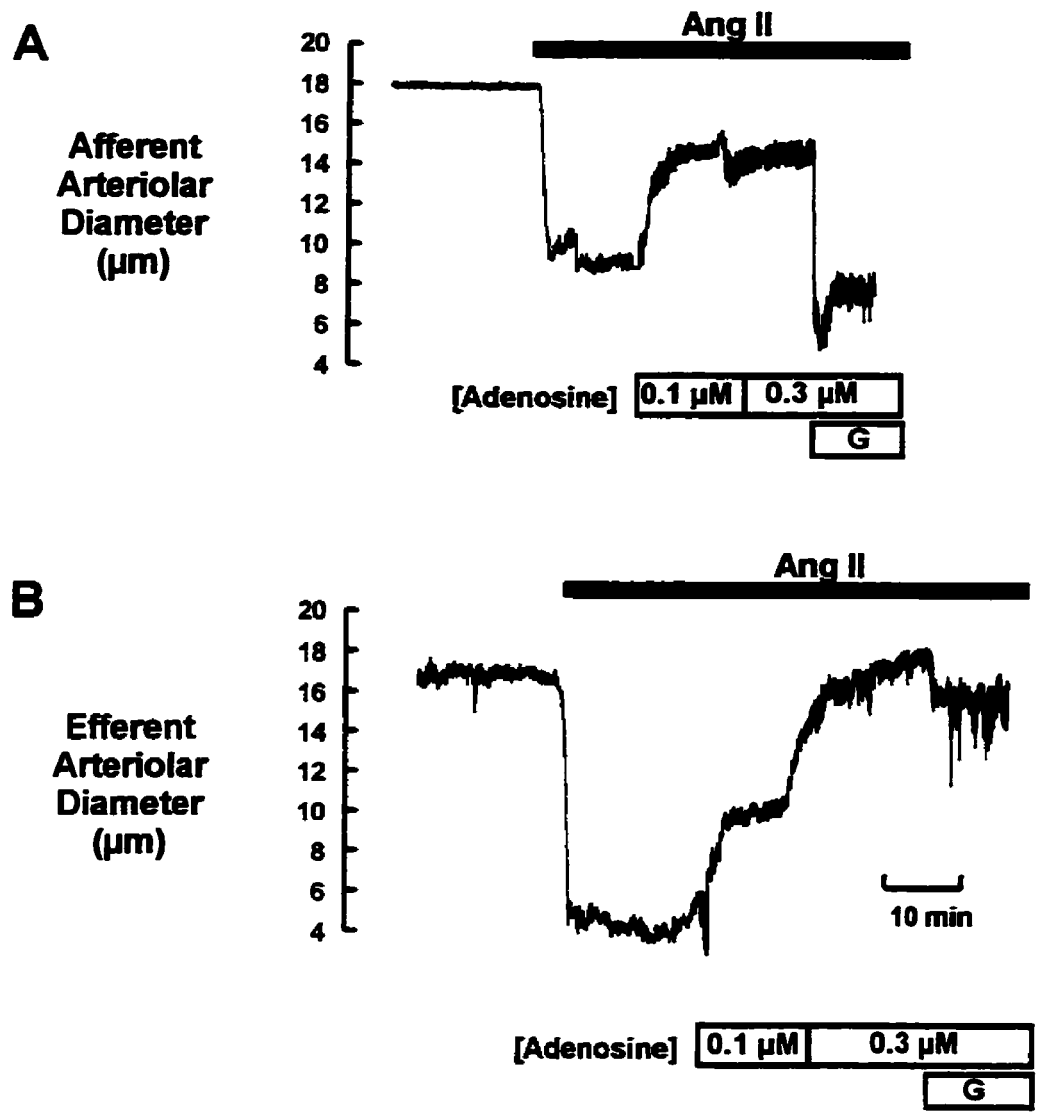


Figure 3.13: Original tracings illustrating the vasodilatory action of adenosine on afferent (panel A) and efferent (panel B) arterioles constricted with 0.1 nM angiotensin II (Ang II). In the afferent arteriole, 10 µM glibenclamide (G) reversed the vasodilation by adenosine, while it failed to alter the efferent arteriolar vasodilations by adenosine.

To determine the involvement of K_{ATP} in the vasodilatory actions of adenosine, the identical experimental protocol was repeated in the presence of 10 μM glibenclamide in a separate group of vessels. In the presence of glibenclamide, 0.1 nM angiotensin II decreased afferent arteriolar diameters by 55±7%, from 15.1±1.2 μm to 7.0±1.3 μm ($p=0.001$, $n=5$), and the efferent arteriolar diameters by 52±3%, from 11.7±1.1 μm to 5.7±0.8 μm ($p=0.0001$, $n=5$). The effects of 0.1 and 0.3 μM adenosine in this setting are depicted in Figure 3.14, right, for afferent (panel A) and efferent (panel B) arterioles. As in the controls, adenosine did not alter the basal diameter of either vessel ($p>0.05$, $n=5$). In the presence of glibenclamide, adenosine failed to inhibit the angiotensin II-induced vasoconstrictions in the afferent arterioles (70±7% and 63±11% decreases in diameter in 0.1 and 0.3 μM adenosine, respectively, $p>0.05$ versus 0 adenosine, $n=5$). As illustrated in Figure 3.16, panel B, rather than inhibiting the angiotensin II responses in the afferent arterioles, as observed in the absence of glibenclamide, adenosine potentiated angiotensin II responses (by 27±5% and 16±20% at 0.1 and 0.3 μM). A similar phenomenon is suggested in Figure 3.13, panel A, where addition of glibenclamide in the presence of adenosine tended to decrease the afferent arteriolar diameter more than angiotensin II alone. The efferent arteriolar actions of adenosine were not prevented by glibenclamide, as both concentrations of adenosine continued to attenuate the angiotensin II-induced decreases in diameter (to 32±5% and 23±8% at 0.1 and 0.3 μM adenosine, respectively, $p<0.05$ vs 0 adenosine, $n=5$). Adenosine thus inhibited the efferent arteriolar vasoconstriction in the presence of glibenclamide by 37±12% and 58±14% (0.1 and 0.3 μM adenosine, respectively). The vasodilatory actions of submicromolar concentrations adenosine on the efferent arteriole were therefore independent of K_{ATP}, in contrast to the afferent arteriole, where glibenclamide prevented the adenosine-induced vasodilations, suggesting a K_{ATP}-mediated mechanism.

Effects of adenosine on pressure-induced afferent arteriolar vasoconstriction

In order to further examine the vasodilatory actions of submicromolar adenosine on the afferent arteriole, the ability of adenosine to inhibit the pressure-induced vasoconstriction of this vessel was investigated. In ten afferent arterioles, mean diameters decreased from 17.7±0.7 μm at 60 mm Hg in response to stepwise increases in perfusion pressure to 180

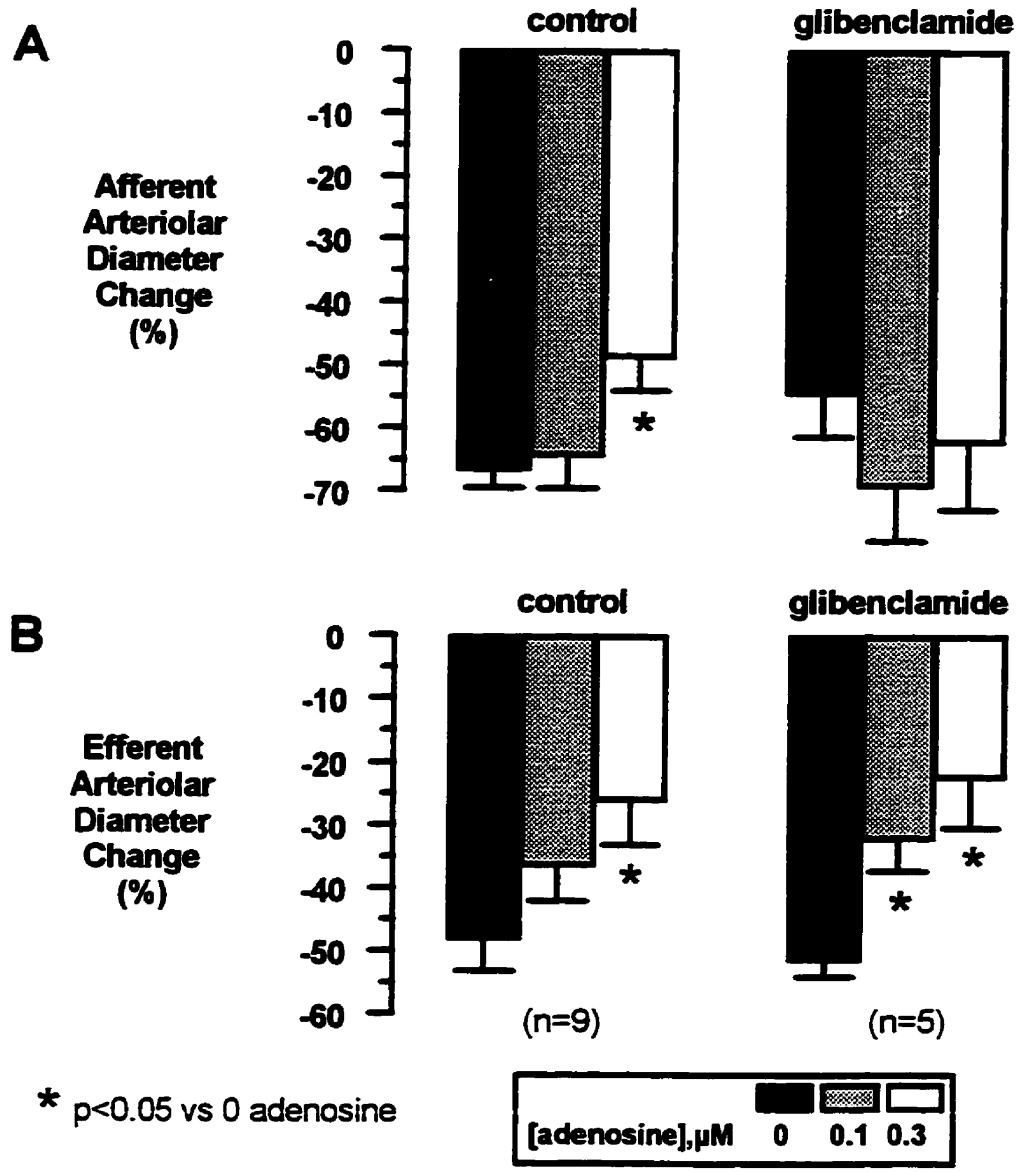


Figure 3.14: Bar graph summarizing the vasodilatory actions of submicromolar concentrations of adenosine on angiotensin II-induced vasoconstrictions (expressed as percent change in diameter) of afferent (panel A) and efferent (panel B) arterioles. The bar graphs on the left represent responses observed in the absence of glibenclamide (control). The responses obtained in the presence of 10 μM glibenclamide are depicted on the right (glibenclamide). Open bars represent angiotensin II responses in the absence of adenosine, shaded and closed bars responses in the presence of 0.1 and 0.3 μM adenosine, respectively. Values are mean ± SEM, n=9 and n=5 for control and glibenclamide groups, respectively.

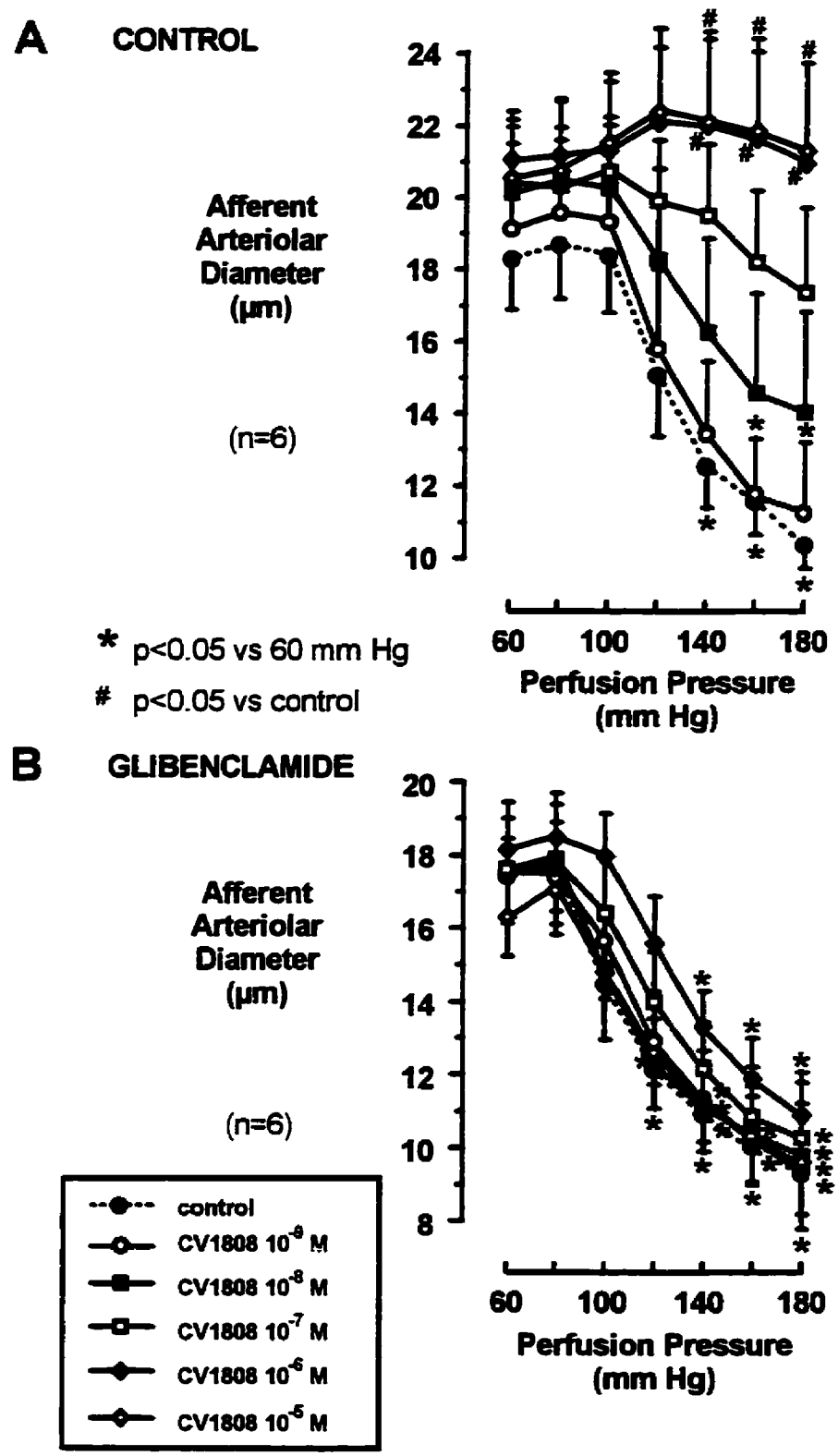
mm Hg. Threshold myogenic vasoconstrictions were observed at 100 mm Hg (12.0±1.1 µm, 10.0±1.4 µm, 8.8±1.1 µm, 7.4±0.7 µm and 6.9±0.7 µm at 100, 120, 140, 160 and 180 mm Hg, respectively, $p<0.05$ versus control, n=10). Pretreatment with 0.1 and 0.3 µM adenosine did not alter the afferent arteriolar diameter at either 60 or 80 mm Hg ($p>0.05$, n=10). Nevertheless, both concentrations of adenosine shifted the threshold for myogenic vasoconstriction from 100 mm Hg to 160 mm Hg. Furthermore, at both 0.1 and 0.3 µM adenosine, arteriolar diameters at pressures of 100 mm Hg and higher were significantly greater than the corresponding diameters in the controls ($p<0.05$). These data are summarized in Figure 3.15, panel A.

In a separate group of eleven afferent arterioles, the same protocol was repeated in the continued presence of 10 µM glibenclamide. In this group, mean afferent arteriolar diameter was 20.1±0.7 µm at 60 mm Hg and decreased significantly to 14.3±1.3 µm, 11.3±1.2 µm, 10.3±1.2 µm and 9.4±1.1 µm at 120, 140, 160 and 180 mm Hg, respectively ($p<0.05$ versus control, n=11). As seen in the absence of glibenclamide, adenosine did not alter the afferent arteriolar diameter at either 60 or 80 mm Hg ($p>0.05$ versus control). Although adenosine shifted the threshold for myogenic vasoconstriction to 160 mm Hg at both concentrations, the afferent arteriolar diameters did not differ significantly from the control values at all pressures ($p>0.05$ versus control). These data suggest that submicromolar concentrations of adenosine inhibit the afferent arteriolar myogenic reactivity at least in part by a glibenclamide-sensitive (i.e. K_{ATP}-mediated) mechanism.

Comparison of vasodilatory effects of adenosine on myogenic and angiotensin II-induced afferent arteriolar vasoconstriction

Since 0.1 nM angiotensin II and elevation of perfusion pressure from 80 to 180 mm Hg elicited afferent arteriolar vasoconstrictory responses that were of similar magnitude (Figure 3.16, panel C), the vasodilatory potency of adenosine could be compared in vessels constricted with either stimulus. Figure 3.16, panels A and B depict the calculated inhibition of afferent arteriolar vasoconstriction induced by 0.1 and 0.3 µM adenosine in the absence (panel A) or presence (panel B) of 10 µM glibenclamide. As seen with CGRP, 0.1 µM

Figure 3.15: Graph summarizing the inhibition of afferent arteriolar myogenic vasoconstriction (control, dashed line) by 0.1 μM (open circles) and 0.3 μM (closed squares) adenosine (ADO) in the absence (panel A) or presence (panel B) of 10 μM glibenclamide. Values are mean ± SEM, n=10 and n=11 for the control (0 glibenclamide) and glibenclamide group, respectively.


A
CONTROL
Afferent Arteriolar Diameter (µm)
(n=6)
24
22
20
18
16
14
12
10
60
100
140
180
Perfusion Pressure (mm Hg)
* p<0.05 vs 60 mm Hg
# p<0.05 vs control
B
GLIBENCLAMIDE
Afferent Arteriolar Diameter (µm)
(n=6)
20
18
16
14
12
10
8
60
100
140
180
Perfusion Pressure (mm Hg)
control
CV1808 10-9 M
CV1808 10-8 M
CV1808 10-7 M
CV1808 10-6 M
CV1808 10-5 M

adenosine preferentially inhibited myogenic versus angiotensin II-induced vasoconstriction of the afferent arteriole (49±9% versus 5±5 % inhibition, p=0.0004). This preferential effect failed to reach statistical significance at 0.3 μM adenosine (52±9% and 26±8% inhibition during myogenic and angiotensin II vasoconstriction, respectively, p=0.054). In the glibenclamide group (Figure 3.16, panel B), the interpretation of data was complicated by what appeared to be a potentiation of angiotensin II responses in the continued presence of adenosine and angiotensin II, as described above. This phenomenon was unique to adenosine and angiotensin II, as it was not observed during myogenic vasoconstriction, where in the presence of glibenclamide adenosine continued to inhibit the myogenic response (26±5% and 30±8% inhibition at 0.1 and 0.3 μM adenosine, respectively). We postulated that this potentiation is mediated by a synergistic interaction between adenosine A_1 receptors and angiotensin II and that this synergism is unmasked by glibenclamide. To circumvent this adenosine A_1/angiotensin II interaction, additional studies were conducted using the adenosine A_2 agonist CV1808.

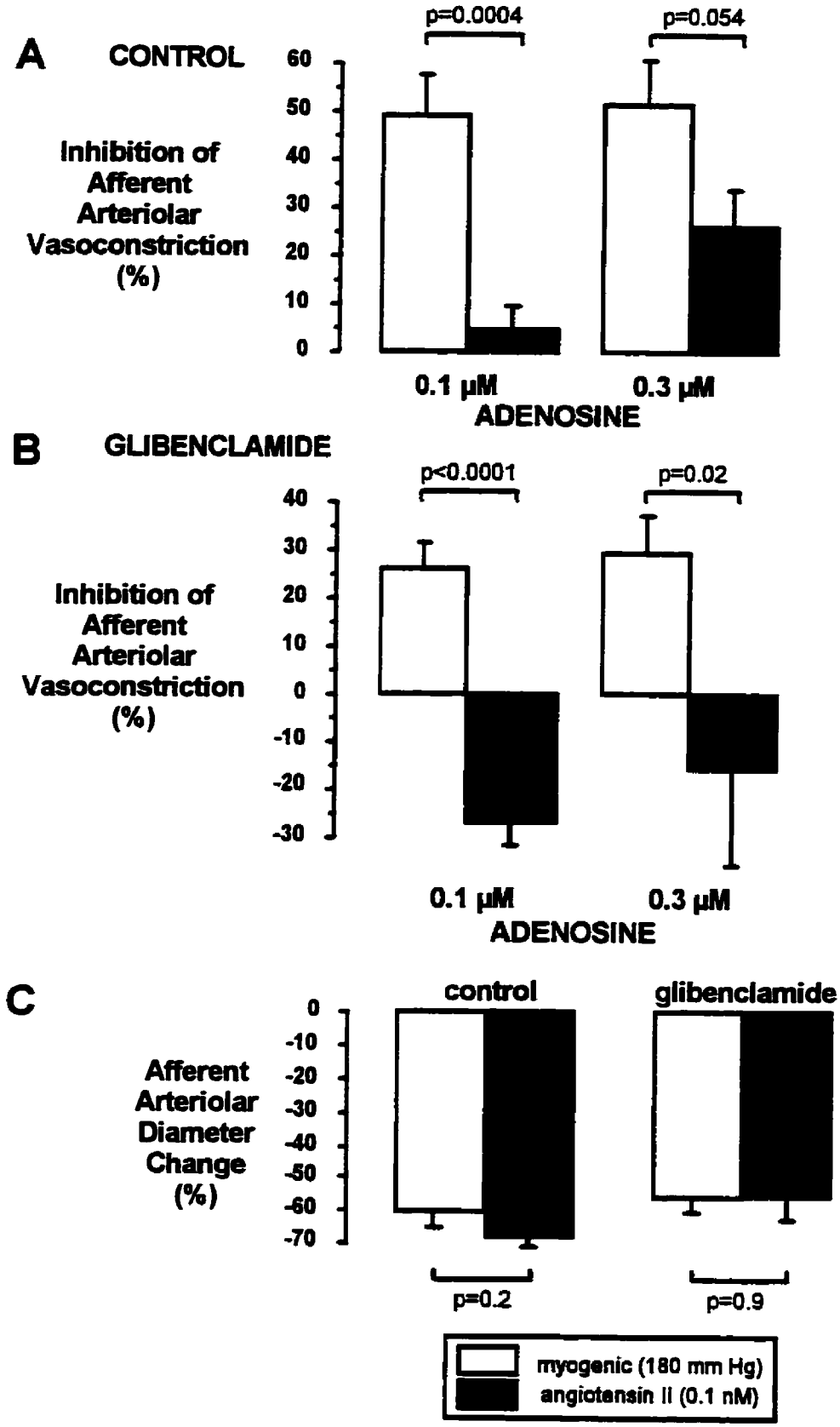
Effects of CV1808 on afferent and efferent arteriolar vasoconstriction induced by angiotensin II

Table 3.5 summarizes the effects of 0.001 to 10 μM CV1808 on afferent and efferent arterioles (from the same glomerulus) exposed to repeated applications of 0.1 nM angiotensin II. Pretreatment with CV1808 did not alter the basal diameters of either arteriole ($p>0.05$ versus 0 CV1808, Table 3.5). The angiotensin II-induced vasoconstrictions were inhibited by 1 and 10 μM CV1808 in both the afferent and efferent arterioles ($p<0.05$ versus 0 CV1808, Table 3.5).

In order to determine the involvement of K_{ATP} in the vasodilatory actions of CV1808, an identical protocol was repeated in a separate group of afferent and efferent arterioles in the presence of 10 μM glibenclamide. Table 3.6 summarizes the responses to CV1808 in the presence of glibenclamide. As in the control (0 glibenclamide) group, CV1808 had no effect on basal afferent or efferent arteriolar diameters ($p>0.05$, Table 3.6). In the afferent arteriole, CV1808 did not alter the angiotensin II-induced vasoconstrictions over the

Figure 3.16: Comparison of vasodilatory actions of adenosine on afferent arterioles constricted with either angiotensin II or elevated pressure. Panel A illustrates the preferential inhibition of myogenic (open bars) versus angiotensin II-induced (closed bars) vasoconstriction by 0.1 μM and 0.3 μM adenosine in the absence of glibenclamide. In the presence of glibenclamide (panel B), the adenosine-induced inhibition of myogenic vasoconstriction was attenuated (open bars), but not completely prevented. However, the angiotensin II-induced vasoconstriction appeared to be potentiated by adenosine in the presence of glibenclamide (closed bars).

Panel C compares the levels of vasoconstriction elicited by angiotensin II (closed bars) or elevated perfusion pressure (open bars) in control (right) or glibenclamide (left) groups. Values are mean ± SEM, n=10/n=11 for myogenic and n=9/n=5 for angiotensin II vasoconstriction in the control/glibenclamide group, respectively.


A
CONTROL
Inhibition of Afferent Arteriolar Vasoconstriction (%)
60
50
40
30
20
10
0
p=0.0004
p=0.054
0.1 µM
0.3 µM
ADENOSINE
B
GLIBENCLAMIDE
Inhibition of Afferent Arteriolar Vasoconstriction (%)
40
30
20
10
0
-10
-20
-30
p<0.0001
p=0.02
0.1 µM
0.3 µM
ADENOSINE
C
Afferent Arteriolar Diameter Change (%)
0
-10
-20
-30
-40
-50
-60
-70
control
glibenclamide
p=0.2
p=0.9
myogenic (180 mm Hg)
angiotensin II (0.1 nM)

concentration range 0.001 to 10 μM ($p>0.05$ versus 0 CV1808). In the efferent arteriole, CV1808 tended to increase the diameters of angiotensin II treated vessels in a concentration-dependent manner (see Table 3.6). These effects of CV1808, however, failed to reach statistical significance ($p>0.05$ versus 0 CV1808, Table 3.6).

Effects of CV1808 on pressure-induced afferent arteriolar vasoconstriction

The ability of CV1808 to block the myogenic responsiveness of the afferent arteriole is illustrated in Figure 3.17. Pretreatment with increasing doses of CV1808 (0.001 to 10 μM) did not alter afferent arteriolar diameters at either 60 or 80 mm Hg ($p>0.05$ versus control). In the absence of CV 1808 (control, Figure 3.17, panel A), elevations in perfusion pressure elicited a myogenic vasoconstriction at perfusion pressures of 140 mm Hg and above. Thus the diameter decreased from 18.3±1.4 μm at 60 mm Hg to 12.6±1.2 μm, 14.3±1.3 μm 11.6±0.9 μm and 10.4±0.7 μm at 140, 160 and 180 mm Hg, respectively ($p<0.05$ versus 60 mm Hg, $n=6$). Pretreatment with CV1808 inhibited this response in concentration-dependent manner, shifting the threshold for myogenic vasoconstriction to 160 mm Hg at 0.001 μM, and completely preventing the vasoconstriction at concentrations of 0.01 μM CV1808 and higher (see Figure 3.17, panel A).

In a separate set of six kidneys, the vasodilatory actions of CV1808 were examined in the presence of 10 μM glibenclamide. These data are summarized in Figure 3.17, panel B. As evident, CV1808 failed to elicit significant vasodilation over a concentration range of 0.001 to 10 μM, in this setting. Pretreatment with CV1808 failed to alter the afferent arteriolar diameter at all pressures ($p>0.05$ versus 0 CV1808, $n=6$). The threshold for the myogenic vasoconstriction varied from 120 mm Hg in the absence of CV1808 (control) to 140, 120, 180, 140 and 140 mm Hg in the presence of 0.001, 0.01, 0.1, 1 and 10 μM CV1808, respectively.

Table 3.5: Changes in afferent (top) and efferent (bottom) arteriolar diameters induced by 0.1 nM angiotensin II (Ang II) in the absence (0) and presence of CV1808 (0.001 to 10 μM).

Afferent Arteriolar Diameter (μm)

CV1808, M	control	Ang II (10^{-10} M)
0	17.0±3.6	3.6±0.7*
10^{-9}	16.6±0.9	3.8±0.7*
10^{-8}	17.8±1.5	3.6±0.5*
10^{-7}	16.9±1.2	6.6±1.9*
10^{-6}	17.6±1.6	13.9±2.1†
10^{-5}	16.9±1.1	14.1±1.8†

Efferent Arteriolar Diameter (μm)

CV1808, M	control	Ang II (10^{-10} M)
0	11.9±1.8	4.2±0.5*
10^{-9}	11.9±1.7	4.2±0.5*
10^{-8}	11.9±1.8	4.4±0.8*
10^{-7}	12.3±1.7	6.7±1.1*
10^{-6}	12.1±1.5	9.3±1.7*†
10^{-5}	11.7±1.6	10.0±1.7†

Values are mean ± SEM, n=5

* $p<0.05$ vs control, † $p<0.05$ vs 0 CV1808

Table 3.6: Changes in afferent (top) and efferent (bottom) arteriolar diameters induced by 0.1 nM angiotensin II (Ang II) in the absence (0) and presence of CV1808 (0.001 to 10 μM) in the continuous presence of 10 μM glibenclamide.

Afferent Arteriolar Diameter (μm)

CV1808, M	control	Ang II (10^{-10} M)
0	13.6±1.6	4.9±0.7*
10^{-9}	13.4±1.2	5.1±0.7*
10^{-8}	13.7±1.4	4.7±0.6*
10^{-7}	14.1±1.3	5.4±0.5*
10^{-6}	14.1±1.2	6.3±1.0*
10^{-5}	13.3±1.2	6.2±0.9*

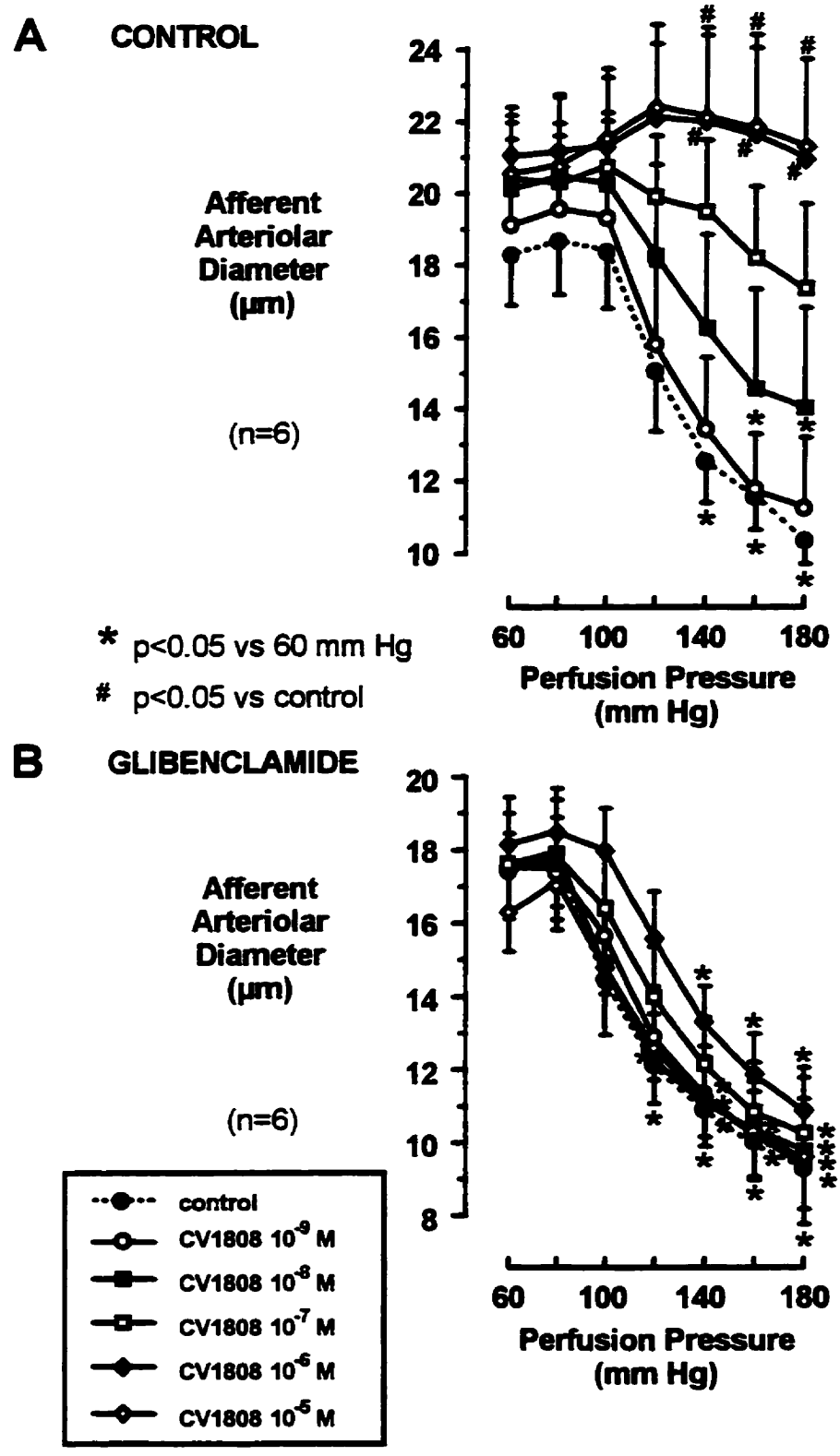
Efferent Arteriolar Diameter (μm)

CV1808, M	control	Ang II (10^{-10} M)
0	12.1±1.4	5.4±1.0*
10^{-9}	12.0±1.4	5.4±0.9*
10^{-8}	12.4±1.4	5.9±0.9*
10^{-7}	12.2±1.5	7.5±1.5*
10^{-6}	12.6±1.4	8.7±1.6*
10^{-5}	12.6±1.6	9.2±1.6*

Values are mean ± SEM, n=7

* $p<0.05$ vs control

Figure 3.17: Inhibition of afferent arteriolar myogenic vasoconstriction (control, dashed line) by CV1808 in the absence (panel A) and presence (panel B) of 10 μM glibenclamide. In the absence of glibenclamide (panel A), CV 1808 elicited a dose-dependent inhibition of myogenic response, preventing statistically significant myogenic vasoconstriction at concentrations of 0.1 μM and above. In the glibenclamide group (panel B), identical doses of CV 1808 (0.001 to 10 μM) failed to elicit any significant inhibition of afferent arteriolar myogenic vasoconstriction. Values are mean ± SEM, n=6 and n=6 for control (0 glibenclamide) and glibenclamide group, respectively.


A
CONTROL
Afferent Arteriolar Diameter (µm)
(n=6)
24
22
20
18
16
14
12
10
60
100
140
180
Perfusion Pressure (mm Hg)
* p<0.05 vs 60 mm Hg
# p<0.05 vs control
B
GLIBENCLAMIDE
Afferent Arteriolar Diameter (µm)
(n=6)
20
18
16
14
12
10
8
60
100
140
180
Perfusion Pressure (mm Hg)
control
CV1808 10-9 M
CV1808 10-8 M
CV1808 10-7 M
CV1808 10-6 M
CV1808 10-5 M

Glibenclamide-sensitivity of CV1808-induced vasodilations

The sensitivity of CV1808-induced vasodilations to glibenclamide is depicted in Figure 3.18. The inhibition of maximal myogenic vasoconstriction (elicited by elevation of perfusion pressure from 80 to 180 mm Hg) is plotted in Figure 3.18, panel A. The vasodilatory actions of CV1808 in this setting were completely prevented by glibenclamide.

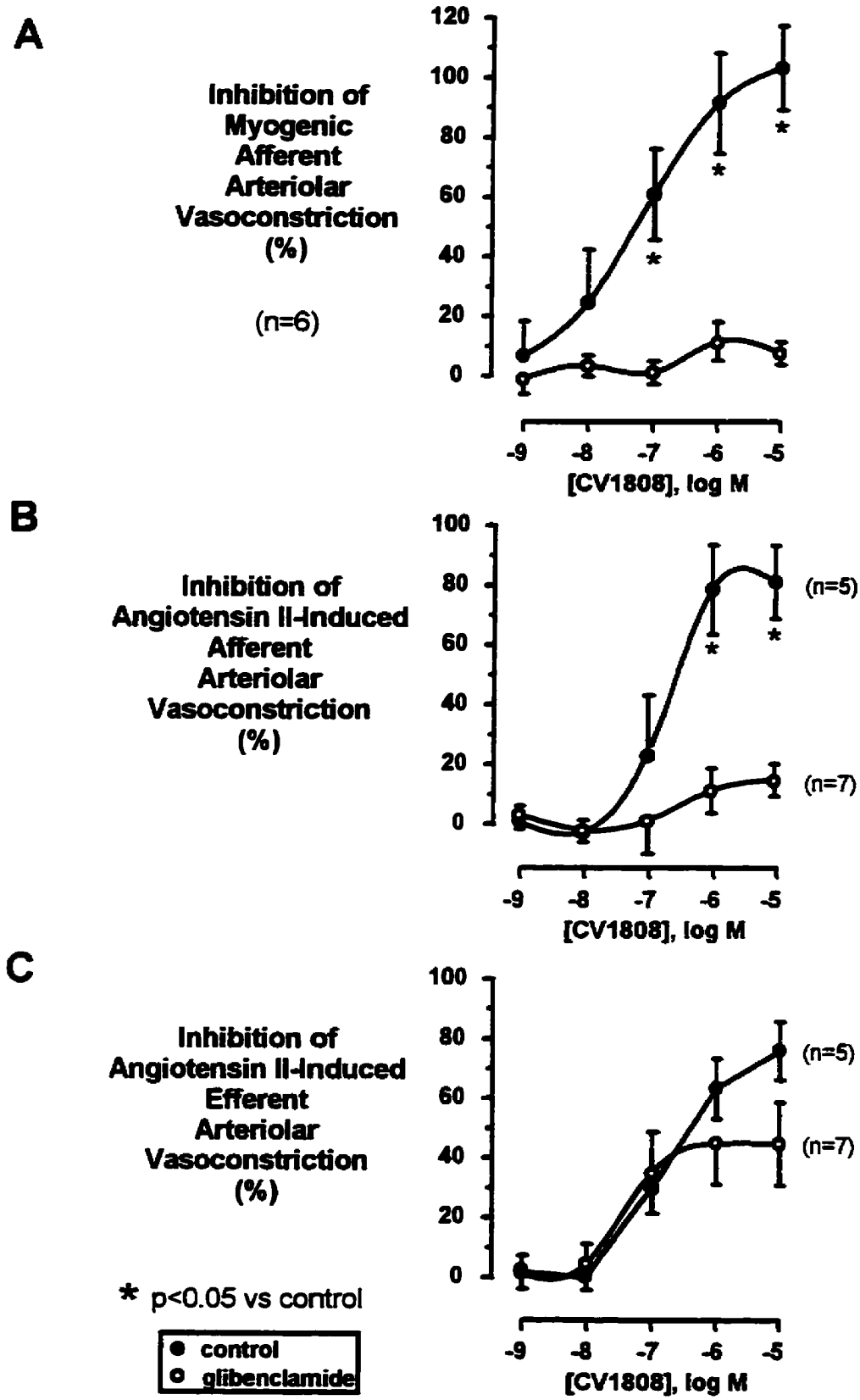
The percent inhibition of angiotensin II-induced changes in diameter was calculated and plotted in Figure 3.18 for afferent (panel B) and efferent (panel C) arterioles. In the afferent arteriole, the inhibition of angiotensin II responses was 79±15% and 81±12% by 1 and 10 μM CV1808, respectively. In the glibenclamide group, this inhibition was significantly attenuated (to 11±8% at 1 μM CV1808, p=0.001 and 15±6% at 10 μM CV1808, p=0.0003). In contrast, the efferent arteriolar actions of CV1808 were not statistically different in the control versus glibenclamide group, although the inhibitory effects of higher concentrations of CV1808 tended to be attenuated by glibenclamide. In the efferent arteriole, 1 μM CV1808 inhibited the angiotensin II-induced vasoconstriction by 64±10% and 45±14% in the absence and presence of glibenclamide, respectively (p=0.34). The corresponding values in 10 μM CV1808 were 76±10% and 45±14% in the absence and presence of glibenclamide, respectively (p=0.12).

Activation of adenosine A_2 receptor subtype by CV1808 thus mimics the vasodilatory actions of low concentrations of adenosine on afferent and efferent arterioles. The afferent arteriolar vasodilations by adenosine and CV1808 were both inhibited by glibenclamide, while the efferent arteriolar actions of adenosine and CV1808 were less sensitive to glibenclamide, suggesting differing roles of K_{ATP} in afferent and efferent arteriolar responses to adenosine.

Interpretation of results obtained with adenosine and CV1808

Both adenosine and adenosine A_2 agonist CV1808 inhibited the pressure- and angiotensin II-induced afferent arteriolar vasoconstriction. Adenosine, but not CV1808, preferentially

Figure 3.18: Graphs summarizing the inhibitory actions of CV1808 and the sensitivity of these actions to glibenclamide. In panel A, the afferent arteriolar myogenic response was inhibited by CV1808 in a dose-dependent manner. The same effect was observed in afferent arterioles constricted with angiotensin II (panel B). In both cases, the afferent arteriolar actions of CV1808 were prevented by 10 μM glibenclamide (open circles). In the efferent arterioles (panel C), angiotensin II-induced vasoconstriction was inhibited by CV1808, but unlike in the afferent arterioles, glibenclamide did not prevent this response. Closed circles represent control values, open circles corresponding values obtained in the presence of glibenclamide. Values are mean ± SEM, see figure for individual n values.


A
Inhibition of Myogenic Afferent Arteriolar Vasoconstriction (%)
(n=6)
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
[CV1808], log M
B
Inhibition of Angiotensin II-Induced Afferent Arteriolar Vasoconstriction (%)
(n=5)
(n=7)
C
Inhibition of Angiotensin II-Induced Efferent Arteriolar Vasoconstriction (%)
* p<0.05 vs control
control
glibenclamide

inhibited the myogenic versus angiotensin II-induced responses in this vessel. Pretreatment with glibenclamide prevented the inhibition of myogenic vasoconstriction by adenosine and CV1808. Similarly, the inhibitory actions of adenosine and CV1808 during the angiotensin II-induced vasoconstriction were sensitive to glibenclamide. In addition, application of glibenclamide unmasked an apparent interaction between adenosine and angiotensin II. Since this phenomenon was not present with CV1808, we speculate that it was mediated by adenosine acting at its A_1 receptor subtype.

The efferent arteriolar vasoconstrictions elicited by angiotensin II were inhibited by adenosine as well as CV1808. In contrast to the afferent arteriolar actions of these agents, glibenclamide failed to prevent the efferent arteriolar vasodilatory action of adenosine and CV1808. These findings suggest that adenosine A_2-mediated vasodilatory responses are mediated via K_{ATP} in the afferent, but not the efferent arteriole.

3.4: cAMP

Hypothesis: *Agents increasing intracellular cAMP concentration elicit afferent arteriolar vasodilation via a glibenclamide-sensitive mechanism.*

Effects of isoproterenol on pressure-induced afferent arteriolar vasoconstriction

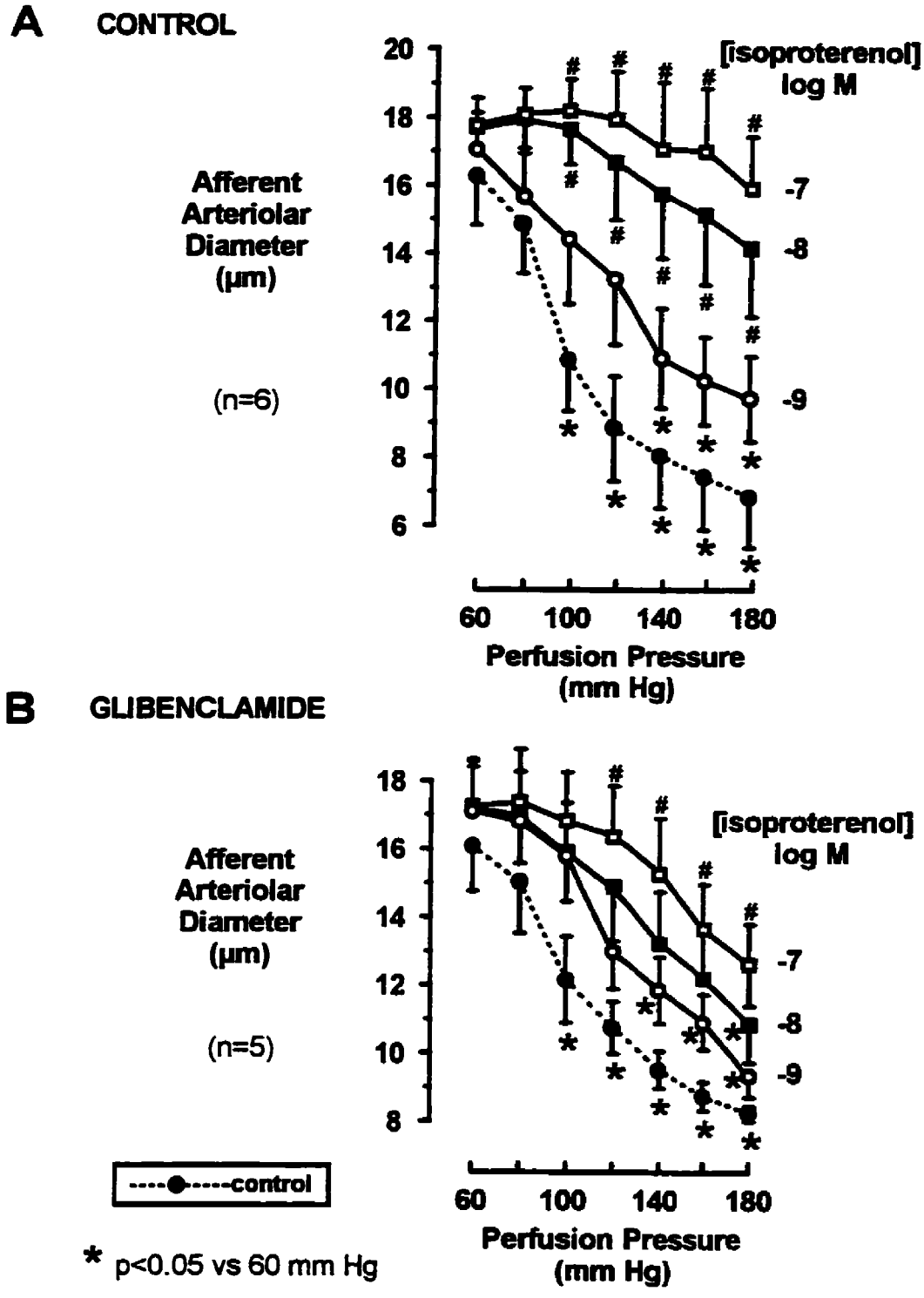
As discussed in the Introduction, the vasodilatory actions of both CGRP and adenosine have been linked to elevated cAMP and it was suggested that K_{ATP} is activated by cAMP-dependent protein kinase. In this set of experiments, isoproterenol, a non-selective β adrenergic agonist, was used to examine whether elevations in intracellular cAMP elicited by this agent elicit glibenclamide-sensitive vasodilations, i.e. activate K_{ATP}. The myogenic rather than angiotensin II-induced afferent arteriolar vasoconstrictions were studied, since previous observations with pinacidil, CGRP and adenosine suggested that K_{ATP}-mediated vasodilations are observed more readily in afferent arterioles constricted by elevations in perfusion pressure.

As depicted in Figure 3.19, panel A, pretreatment with 0.001 to 0.1 μM isoproterenol did not change the mean afferent arteriolar diameter at perfusion pressure of either 60 or 80 mm Hg ($p>0.05$ versus control, n=6). In the absence of isoproterenol, the mean diameter at 60 mm Hg (16.2±1.3 μm) decreased significantly in response to elevating perfusion pressure to 100 mm Hg and higher (10.8±1.5 μm, 8.8±1.5 μm, 8.0±1.5 μm, 7.4±1.6 μm and 6.8±1.5 μm at 100, 120, 140, 160 and 180 mm Hg, respectively, $p<0.05$ versus 60 mm Hg, n=6). The threshold for myogenic vasoconstriction was shifted to 140 mm Hg by 0.001 μM isoproterenol. Following pretreatment with 0.01 and 0.1 μM isoproterenol, pressure-induced vasoconstrictory responses no longer reached statistical significance. Figure 3.19, panel A, summarizes these findings.

To determine an involvement of K_{ATP} in these actions of isoproterenol, identical experiments were performed in five kidneys in the presence of 10 μM glibenclamide. As in the control group, isoproterenol did not alter the mean afferent arteriolar diameter at 60 or 80 mm Hg at any concentration used ($p>0.05$ versus control, n=5). In this group, threshold myogenic vasoconstrictions were observed at 100 mm Hg and above (see Figure 3.19, panel B). This threshold was shifted to 140 mm Hg and 180 mm Hg by 0.001 and 0.01 μM isoproterenol, respectively. At 0.1 μM isoproterenol, elevations in perfusion pressure failed to elicit statistically significant myogenic vasoconstriction. As evident from comparing graphs in panels A and B in Figure 3.19, pretreatment with glibenclamide partially attenuated isoproterenol-induced inhibition of myogenic vasoconstriction.

Since the magnitude of glibenclamide-sensitive component of isoproterenol-induced vasodilations could not be unequivocally established using the above described experimental approach, a different protocol was employed to allow a clearer distinction between glibenclamide-sensitive and -insensitive components of this response. This protocol is illustrated by the tracing depicted in Figure 3.20, panel A. Rather than performing multiple pressure steps, as described above, the pressure was elevated from a basal value of 80 mm Hg to 180 mm Hg in a single step and held at this level. Isoproterenol (0.001 to 0.1 μM) was then added in a cumulative manner. As the effects of isoproterenol could be washed out (not shown), the protocol was then repeated in the

Figure 3.19: Inhibition of afferent arteriolar myogenic vasoconstriction by isoproterenol in the absence (panel A) and presence (panel B) of 10 μM glibenclamide. Control afferent arteriolar myogenic vasoconstriction (dashed line) was completely prevented by 0.01 (closed squares) and 0.1 μM (open squares) isoproterenol in the absence of glibenclamide (panel A). In the glibenclamide group (panel B), only 0.1 μM isoproterenol (open squares) significantly attenuated the myogenic vasoconstriction. Values are mean ± SEM, n=6 and n=5 for the control (0 glibenclamide) and glibenclamide group, respectively.


A
CONTROL
Afferent Arteriolar Diameter (µm)
(n=6)
[isoproterenol] log M
-7
-8
-9
20
18
16
14
12
10
8
6
60
100
140
180
Perfusion Pressure (mm Hg)
B
GLIBENCLAMIDE
Afferent Arteriolar Diameter (µm)
(n=5)
[isoproterenol] log M
-7
-8
-9
18
16
14
12
10
8
----●----control
60
100
140
180
Perfusion Pressure (mm Hg)
* p<0.05 vs 60 mm Hg
# p<0.05 vs control

presence of 10 μM glibenclamide in the same vessel. Data obtained from five afferent arterioles are summarized in Figure 3.20, panels B and C. In the absence of glibenclamide, the control diameters (at 80 mm Hg) decreased from 13.9±0.9 μm to 6.8±0.7 μm upon elevation of perfusion pressure to 180 mm Hg ($p=0.005$, $n=5$). Following treatment with 0.001, 0.01 and 0.1 μM isoproterenol, the mean diameters increased to 10.3±1.7 μm, 14.6±1.2 μm and 15.6±0.8 μm, respectively ($p<0.05$ versus elevated pressure alone for all three concentrations of isoproterenol), while the pressure was maintained at 180 mm Hg (see panel B, Figure 3.20). The addition of glibenclamide did not affect the control diameter at 80 mm Hg (14.2±0.8 μm, $p=0.56$ versus 0 glibenclamide) or 180 mm Hg (6.8±0.8 μm, $p=1.00$ versus 0 glibenclamide). The mean diameter values following treatment with 0.001, 0.01 and 0.1 μM isoproterenol were 7.8±0.8 μm, 8.6±0.9 μm and 9.3±0.9 μm, respectively ($p=0.14$, $p=0.0002$ and $p=0.0004$, respectively versus corresponding values in the absence of glibenclamide). As depicted in panel C of Figure 3.20, in the continuous presence of glibenclamide, isoproterenol failed to significantly inhibit the pressure-induced afferent arteriolar vasoconstriction. These results suggest that vasodilatory actions of isoproterenol are in part mediated by activation of K_{ATP}.

Effects of forskolin on pressure-induced afferent arteriolar vasoconstriction

Unlike isoproterenol, which increases intracellular cAMP concentration via a receptor-mediated, G_s-dependent, mechanism, forskolin elevates cAMP levels by direct activation of adenylyl cyclase (Seamon & Daly, 1981). Like isoproterenol, forskolin elicited a dose-dependent inhibition of afferent arteriolar myogenic vasoconstriction, as illustrated in Figure 3.21, panel A. Prior to the addition of forskolin, elevating the perfusion pressure from 60 to 140 mm Hg elicited myogenic vasoconstriction of the afferent arteriole. The mean diameter decreased from 15.4±1.3 μm at 60 mm Hg to 9.9±0.8 μm, 8.3±0.6 μm and 7.9±0.5 μm at 140, 160 and 180 mm Hg ($p<0.05$ versus 60 mm Hg). Following pretreatment with 0.01 to 1 μM forskolin, the mean diameter at 60 or 80 mm Hg did not change ($p>0.05$ versus control). The threshold for myogenic vasoconstriction was shifted to 160 mm Hg by 0.01 μM forskolin. Higher concentrations of forskolin (0.1 to 1 μM) prevented statistically significant pressure-induced afferent arteriolar vasoconstrictions.

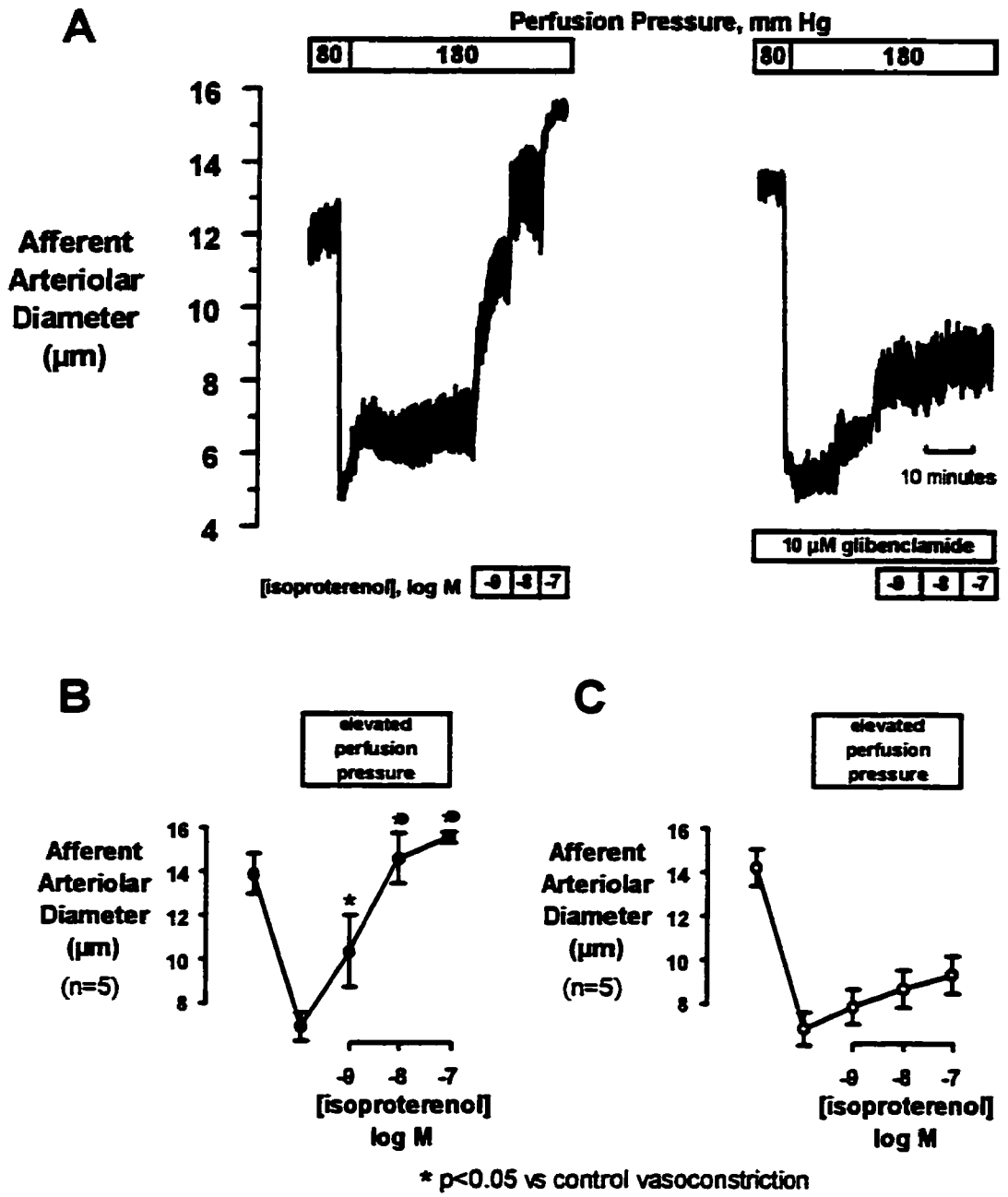


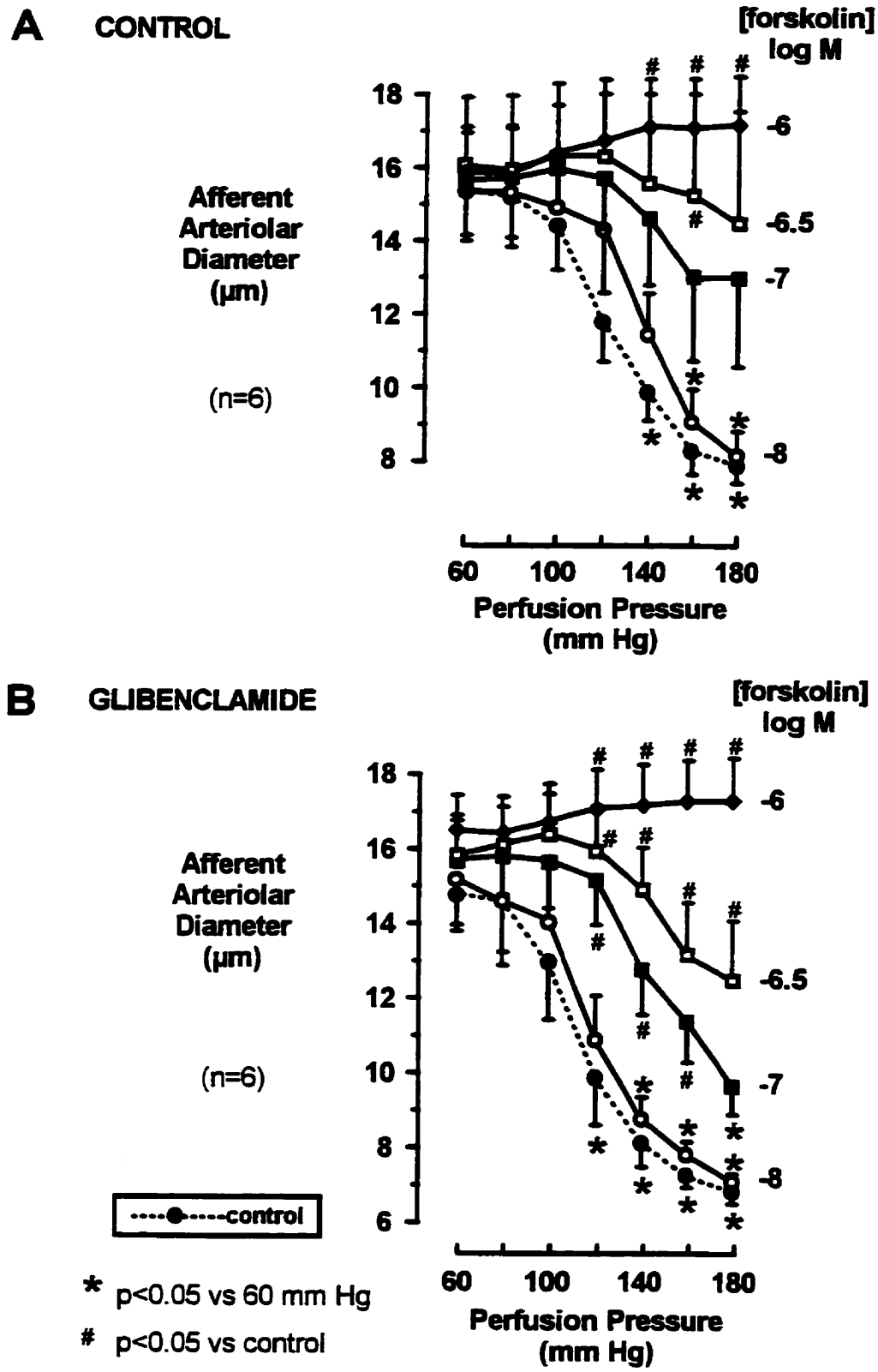
Figure 3.20: Original tracing in panel A depicts the effects of isoproterenol on the afferent arteriolar myogenic vasoconstriction elicited by stepping perfusion pressure from 80 to 180 mm Hg. The responses to isoproterenol were examined first in the absence (left) and then in the presence (right) of 10 µM glibenclamide. Mean data obtained from five afferent arterioles using the above protocol are summarized in panel B and C for control and glibenclamide-pretreated groups, respectively. Values are mean ± SEM, n=5.

In the second group of afferent arterioles, the protocol was repeated in the continuous presence of glibenclamide. Panel B, Figure 3.21 summarizes the myogenic responses of six afferent arterioles and the effects of forskolin in the presence of glibenclamide. As in the absence of glibenclamide, forskolin failed to change mean afferent arteriolar diameters at either 60 or 80 mm Hg ($p>0.05$ versus control). The vasodilatory responses to forskolin appeared to be only minimally affected by glibenclamide. At 0.01 and 0.1 μM forskolin shifted the threshold for myogenic vasoconstriction to 140 and 180 mm Hg, respectively (from 120 mm Hg in control) and higher concentrations of forskolin abolished the myogenic vasoconstrictions completely.

To allow comparison with results obtained with isoproterenol, the actions of forskolin were re-examined using the pressure step protocol described above for isoproterenol (see original tracing in Figure 3.22, panel A). In the control group (without glibenclamide), the diameter decreased from 15.4±0.9 μm at 80 mm Hg to 8.9±0.8 μm at 180 mm Hg ($p=0.0005$, $n=6$). Subsequent addition of increasing concentration of forskolin resulted in a progressive inhibition of this vasoconstriction. The diameters were 9.3±0.9 μm, 9.8±0.7 μm, 10.7±0.8 μm, 12.9±0.7 μm, 16.2±1.4 μm and 18.4±1.4 μm at 0.001, 0.003, 0.01, 0.03, 0.1 and 0.3 μM forskolin. As illustrated in Figure 3.22, panel B, the control myogenic vasoconstriction was inhibited by concentrations of forskolin of 0.03 μM and higher ($p<0.05$ versus elevated pressure alone).

Since forskolin could not be completely washed out in some vessels, data in the presence of glibenclamide (Figure 3.22, panel C) were obtained from different afferent arterioles in separate experiments. In the presence of glibenclamide, neither basal diameters at 80 mm Hg nor the diameters at 180 mm Hg were found to be significantly different from the control group (14.9±1.4 μm at 80 mm Hg and 8.8±0.6 μm at 180 mm Hg, $p=0.77$ and $p=0.96$, respectively). The mean diameter values following the addition of 0.001, 0.003, 0.01, 0.03, 0.1 and 0.3 μM forskolin were 9.8±0.7 μm,10.3±0.7 μm, 10.5±0.8 μm, 11.6±1.0 μm, 13.6±0.9 μm and 17.7±1.1 μm, respectively ($p=0.86$, $p=0.62$, $p=0.89$, $p=0.33$, $p=0.18$ and $p=0.73$, respectively, versus corresponding values in the absence of glibenclamide). As depicted in Figure 3.22, panel C, the afferent arteriolar myogenic vasoconstriction was

Figure 3.21: Inhibition of afferent arteriolar myogenic vasoconstriction by forskolin in the absence (panel A) and presence (panel B) of 10 μM glibenclamide. Control afferent arteriolar vasoconstriction was completely prevented by forskolin at concentration of 0.1 μM (closed boxes) and higher in the absence of glibenclamide (panel A). In the glibenclamide pretreated vessels (panel B), forskolin abolished myogenic vasoconstriction at 0.3 and 1 μM. In both groups in the presence of 1 μM forskolin, elevations in perfusion pressure caused increased, rather than decreased, afferent arteriolar diameters (closed diamonds, panels A and B). Values are mean ± SEM, n=6 for each group.


A
CONTROL
[forskolin]
log M
Afferent
Arteriolar
Diameter
(µm)
(n=6)
18
16
14
12
10
8
-6
-6.5
-7
-8
60
100
140
180
Perfusion Pressure
(mm Hg)
B
GLIBENCLAMIDE
[forskolin]
log M
Afferent
Arteriolar
Diameter
(µm)
(n=6)
18
16
14
12
10
8
6
-6
-6.5
-7
-8
control
* p<0.05 vs 60 mm Hg
# p<0.05 vs control
60
100
140
180
Perfusion Pressure
(mm Hg)

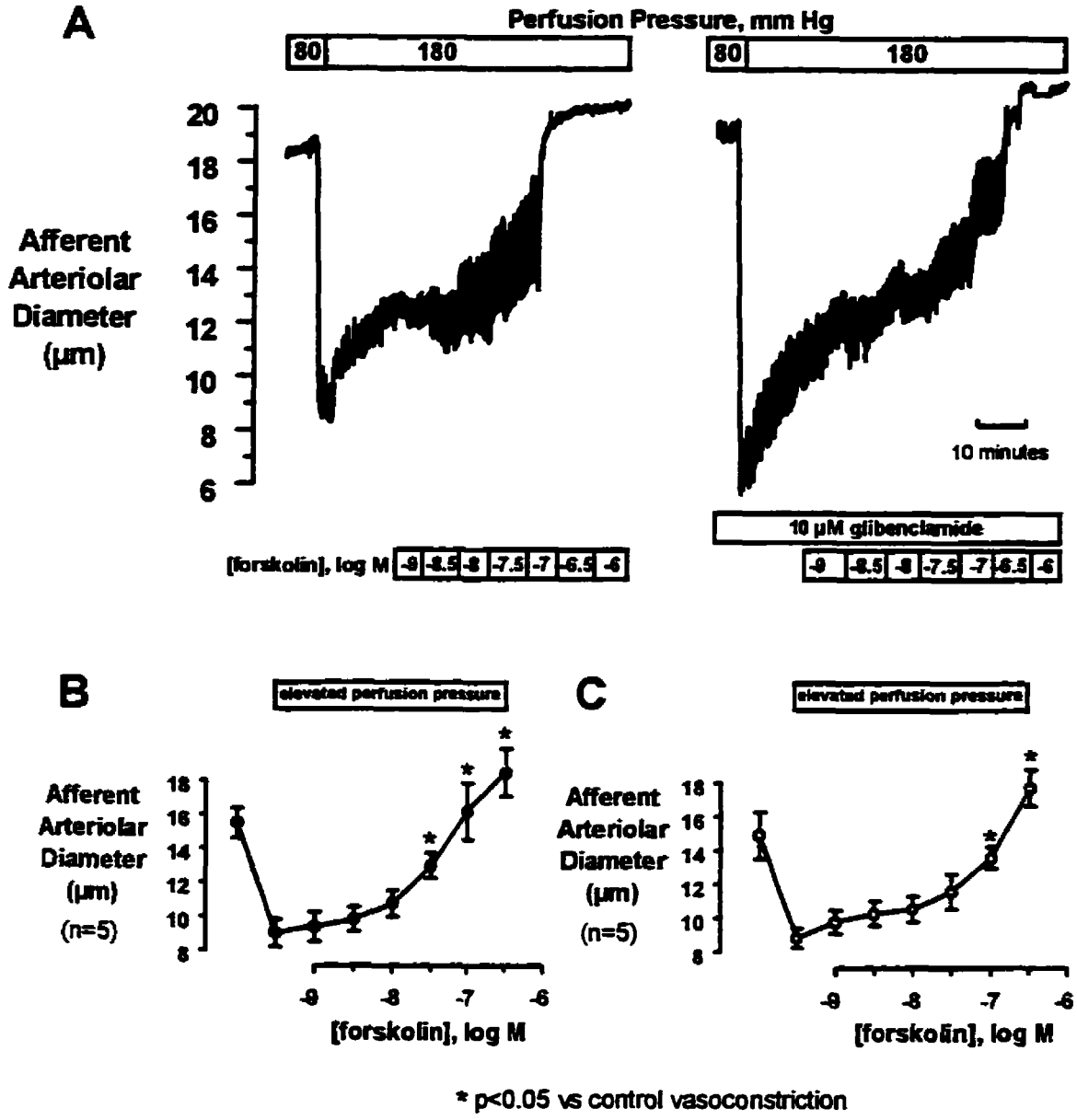


Figure 3.22: Original tracing (panel A) and mean diameter values illustrating the effects of forskolin on afferent arteriolar myogenic vasoconstriction in the absence (panel B) and presence (panel C) of 10 µM glibenclamide. Values are mean ± SEM, n=5.

inhibited by 0.1 and 0.3 μM glibenclamide ($p<0.05$ versus elevated perfusion pressure alone). In contrast to isoproterenol, the vasodilatory actions of forskolin were found to be relatively insensitive to glibenclamide and therefore not mediated by K_{ATP}.

Effects of db-cAMP on pressure-induced afferent arteriolar vasoconstriction

To further investigate the conflicting results regarding the glibenclamide-sensitivity of actions of isoproterenol and forskolin, a membrane permeable analogue of cAMP, dibutyryl-cAMP, was used to increase intracellular cAMP levels. The pressure step protocol was also used in this study (illustrated by the original tracing in Figure 3.23, panel A). Dibutyryl-cAMP inhibited afferent arteriolar myogenic vasoconstriction in a dose-dependent manner (see Figure 3.23, panel C). Increasing the perfusion pressure from 80 to 180 mm Hg decreased afferent arteriolar diameter from 16.3±0.6 μm to 8.2±1.1 μm ($p=0.002$, $n=5$). Subsequent addition of 1, 3, 10, 30 and 100 μM db-cAMP caused a progressive inhibition of this vasoconstriction (the corresponding diameters were 8.2±1.2 μm, 8.8±1.4 μm, 9.6±1.3 μm, 12.6±1.7 μm and 15.4±1.0 μm, respectively). The effect of db-cAMP reached statistical significance at 100 μM ($p<0.05$ versus elevated pressure alone).

Following the washout of db-cAMP, the protocols were repeated in 10 μM glibenclamide in the same vessels (see Figure 3.23, panel A for original tracing). As summarized in Figure 3.23, panel C, the afferent arterioles constricted from 16.2±0.9 μm at 80 mm Hg to 9.4±0.9 μm at 180 mm Hg ($p=0.007$, $n=5$). As in the absence of glibenclamide, 100 μM db-cAMP inhibited this vasoconstriction (15.6±1.1 μm, $p<0.05$ versus elevated pressure alone). The vasodilatory actions of db-cAMP thus appeared to be mediated by a mechanism that does not involve K_{ATP}, as evidenced by the lack of sensitivity of these effects to glibenclamide.

Figure 3.24 summarizes the results obtained with isoproterenol, forskolin and db-cAMP using the pressure step protocol. As depicted, the inhibition of afferent arteriolar myogenic vasoconstrictions by both forskolin (panel B) and db-cAMP (panel C) were not altered by glibenclamide ($p>0.05$ versus control for all data points). The inhibition of afferent arteriolar myogenic vasoconstriction by isoproterenol is depicted in panel A, Figure 3.24.

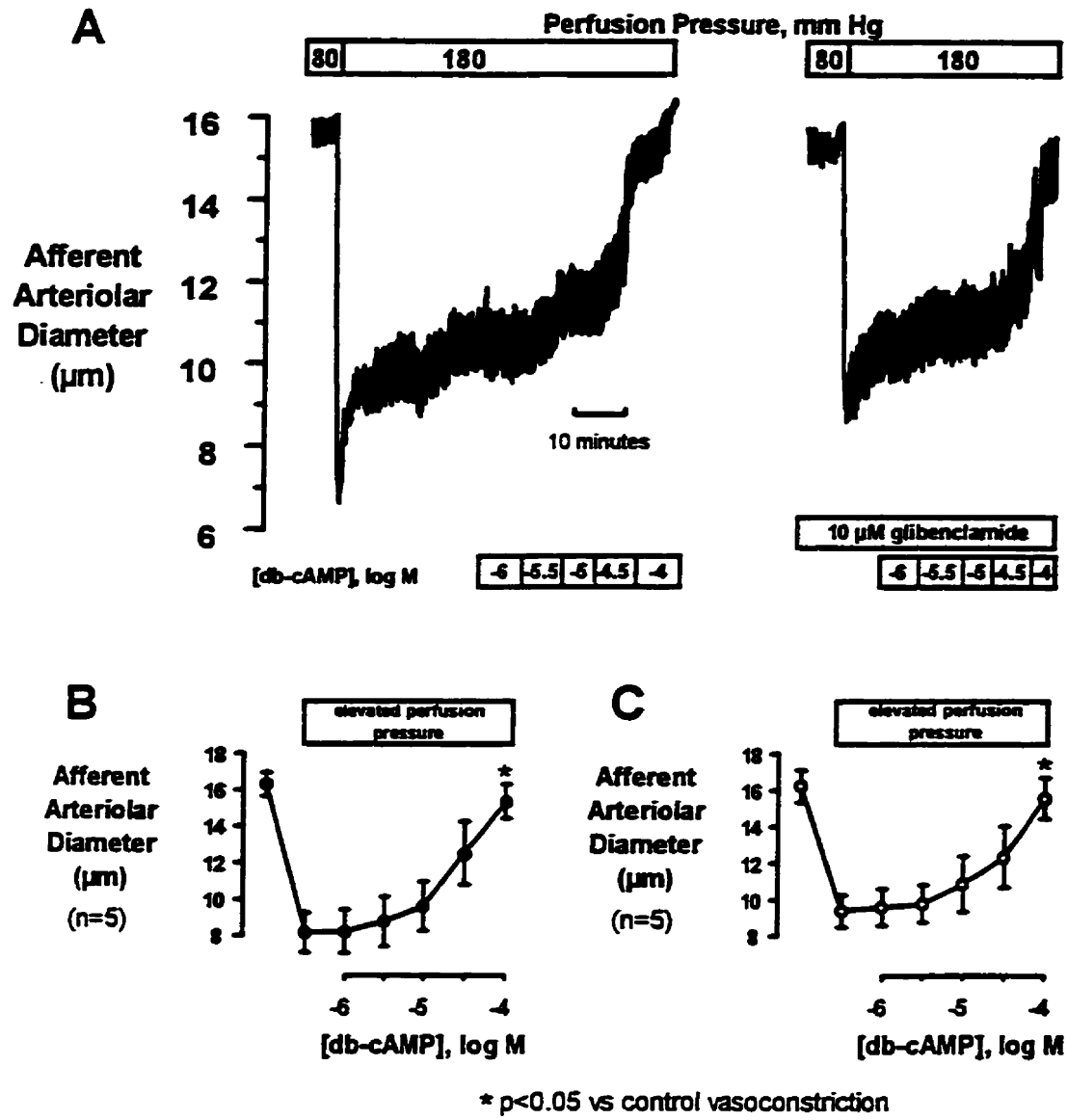


Figure 3.23: Original tracing (panel A) and mean diameter values illustrating the effects of db-cAMP on afferent arteriolar myogenic vasoconstriction in the absence (panel B) and presence (panel C) of 10 µM glibenclamide. Values are mean±SEM, n=5.

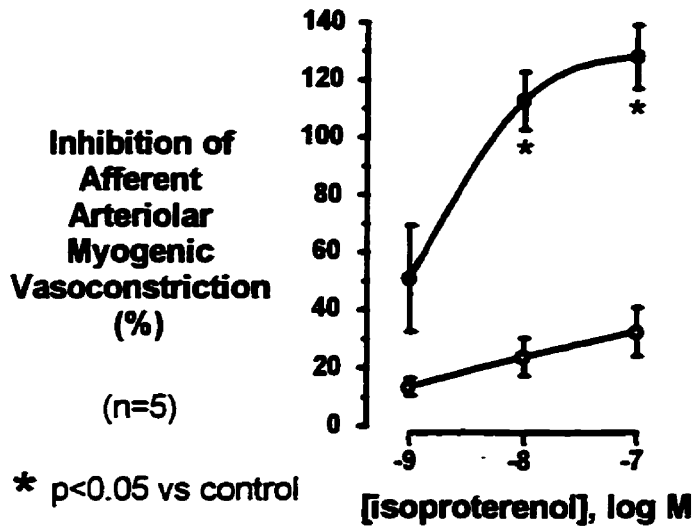
Isoproterenol inhibited the myogenic vasoconstriction by 51±19%, 113±10% and 128±11% at 0.001, 0.01 and 0.1 μM, respectively. Following pretreatment with glibenclamide, the same concentrations of isoproterenol inhibited the myogenic vasoconstriction by 14±3%, 24±6 and 33±8%, respectively ($p=0.07$, $p=0.0004$ and $p=0.0014$, respectively versus corresponding control values). Thus even though all three of these agents are known to elicit vasodilations via elevations of intracellular cAMP levels, only isoproterenol elicited vasodilations via a glibenclamide-sensitive mechanism.

Interpretation of results obtained with agents elevating intracellular cAMP

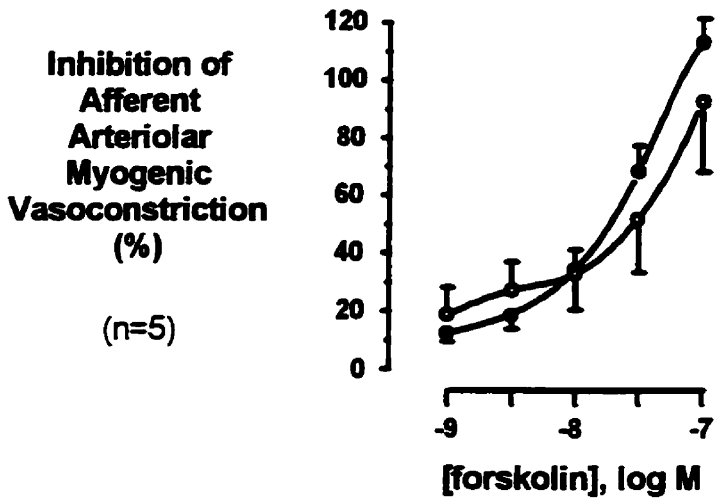
The afferent arteriolar myogenic vasoconstriction was inhibited by isoproterenol, forskolin or db-cAMP. The data suggest that elevations of intracellular cAMP levels *per se* by either direct stimulation of adenylyl cyclase (i.e. forskolin) or by addition of cAMP analogue (i.e. db-cAMP) do not activate K_{ATP}, since vasodilations elicited by forskolin or db-cAMP were found to be insensitive to glibenclamide. The sensitivity of isoproterenol-induced vasodilation to glibenclamide must therefore reflect the activation of K_{ATP} by a receptor-mediated, but not cAMP-dependent, mechanism.

Figure 3.24: Isoproterenol- (panel A), forskolin- (panel B) and db-cAMP- (panel C) induced inhibition of afferent arteriolar myogenic vasoconstriction in the absence (closed circles) or presence (open circles) of glibenclamide. In the glibenclamide pretreated groups, forskolin (panel B) and db-cAMP (panel C) continued to inhibit the myogenic response, while the actions of isoproterenol (panel A) were prevented. These data are calculated from data obtained using the pressure step protocol for each drug. Values are mean ± SEM, n=5 for each group.

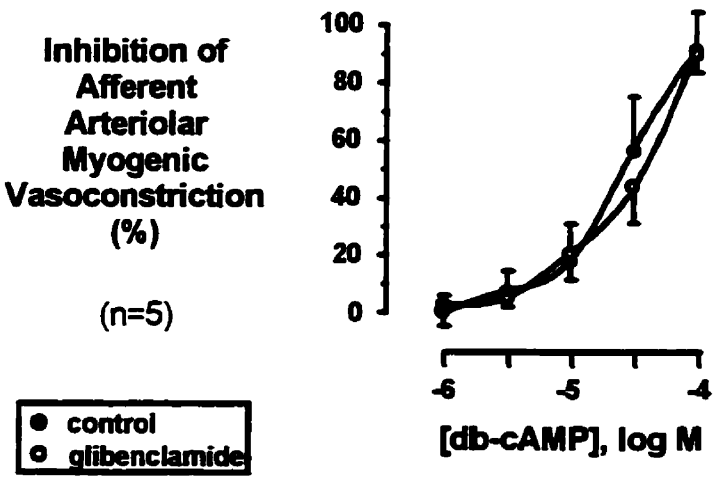
A


Inhibition of Afferent Arteriolar Myogenic Vasoconstriction (%)
(n=5)
* p<0.05 vs control
140
120
100
80
60
40
20
0
-9
-8
-7
[isoproterenol], log M

B


Inhibition of Afferent Arteriolar Myogenic Vasoconstriction (%)
(n=5)
120
100
80
60
40
20
0
-9
-8
-7
[forskolin], log M

C


Inhibition of Afferent Arteriolar Myogenic Vasoconstriction (%)
(n=5)
control
glibenclamide
100
80
60
40
20
0
-6
-5
-4
[db-cAMP], log M

Chapter 4 DISCUSSION

4.1 Actions of pinacidil on afferent and efferent arterioles

The experiments described in section 3.1 compared the effects of K_{ATP} activation on renal afferent and efferent arterioles. The findings indicate that K_{ATP}-induced hyperpolarization preferentially attenuates afferent arteriolar reactivity, in that pinacidil was approximately ninefold more potent in inhibiting angiotensin II responses of afferent versus efferent arterioles. This preferential action most likely reflects the greater dependence of afferent arteriolar vasoconstriction on L-type calcium channel activation, as described in section 1.3. Consistent with this interpretation, pinacidil potently inhibited the afferent arteriolar responses to Bay K 8644, a direct activator of L-type calcium channels. Pinacidil also inhibited the afferent arteriolar myogenic reactivity, which is mediated by activation of L-type calcium channels. In contrast, pinacidil had no effect on KCl-induced activation of L-type calcium channels, presumably reflecting the dependence of K^+-channel induced hyperpolarization on a physiological plasmalemmal K^+ gradient and K^+ equilibrium potential.

In the afferent arteriole, the vasodilatory potency of pinacidil appears to depend on the nature of underlying tone. Angiotensin II and elevated pressure elicited comparable levels of afferent arteriolar vasoconstriction. Despite the similar magnitude of vasoconstrictor response to both stimuli, pinacidil was more potent against the myogenic versus angiotensin II-induced vasoconstriction (Figure 3.10). The possible explanations for this difference in vasodilatory potency of pinacidil are discussed separately in section 4.5.

Although pinacidil exhibited a greater potency on the afferent arteriole, maximal concentrations of pinacidil produced similar inhibitory effects on both afferent and efferent arteriolar responses to angiotensin II. Both the afferent and efferent arteriolar actions of pinacidil were reversed by glibenclamide and blocked by KCl, suggesting that pinacidil acts via K_{ATP}-induced membrane hyperpolarization in both vessels. These findings were not anticipated, since it is well documented that the renal efferent arteriole, in contrast to the

afferent arteriole, is insensitive to vasodilators acting exclusively on L-type calcium channels (Loutzenhiser & Epstein, 1990). In agreement with this premise, diltiazem had no effect on the efferent arteriolar response to angiotensin II. More importantly, diltiazem did not alter the pinacidil-induced inhibition of this response. The possibility that hyperpolarization alters voltage-sensitive calcium entry through non-L-type calcium channels is unlikely, as 45 mM KCl was found to have no effect on efferent arteriolar diameters in the presence of diltiazem (see Figure 3.7). Thus these findings indicate that the efferent arteriolar actions of pinacidil are mediated via K_{ATP}-induced hyperpolarization, but clearly do not involve alternation in activity of VOCCs (L-type or non-L-type). This is the first demonstration of PCO-induced vasodilation of a resistance arteriole under physiological conditions through a mechanism that is completely independent of modulation of voltage-gated calcium entry.

Possible mechanisms for efferent arteriolar actions of pinacidil

In addition to inhibiting the activity of L-type calcium channels, alternative mechanisms for hyperpolarization-induced vasodilation have been proposed. In rabbit aorta, cromakalim has been reported to inhibit dihydropyridine-insensitive contractile responses (Cook et al., 1988, Bray et al., 1991), suggesting that, in addition to its indirect effects on L-type calcium channel activity, this agent specifically affects receptor-mediated Ca^{2+} entry or some other mechanism specific to agonist-induced vasoconstriction. Okada et al. (1993) reported that levcromakalim reduced the Ca^{2+} sensitivity of the contractile apparatus in canine coronary artery. In this study, the relationship between membrane potential, tension and $[Ca^{2+}]i$ (fura-2) were assessed, under conditions in which tension was altered, but membrane potential either remained constant (changing external Ca^{2+}) or was altered (levcromakalim or changing external K^+). The authors observed a membrane potential-dependent shift in Ca^{2+} sensitivity and suggested that this mechanism could contribute to vasodilatory actions of this PCO. While we cannot, at the present time, rule out such a mechanism in our studies, it is important to note this alteration of Ca^{2+} sensitivity by levcromakalim was observed in the presence of 30 mM KCl. Since, unlike levcromakalim, pinacidil does not elicit vasodilation in the presence of 30 mM KCl in our preparation (Figure 3.1, panel B), we consider this mechanism to be unlikely.

A number of studies suggest that PCO-induced hyperpolarization influences phospholipase C activation. Quast and Baumlin (1991) reported that cromakalim inhibits isradipine-insensitive phasic responses to norepinephrine in the perfused rat mesenteric bed. Since cromakalim had no effect on the phasic vasoconstriction induced by caffeine, these authors suggested that cromakalim interferes with norepinephrine-induced Ca^{2+} mobilization. Similarly, Ito et al. (1991) found that lemakalim blocked norepinephrine-induced contraction and Ca^{2+} signalling in calcium-free medium and inhibited norepinephrine-stimulated IP_3 formation in isolated mesenteric arteries. These actions of lemakalim were abolished in chemically skinned arteries, which obviously lack the ability to generate a membrane potential, indicating that the ability of PCOs to interfere with agonist-induced IP_3 synthesis and Ca^{2+} release is dependent upon hyperpolarization of the membrane potential (Ito et al., 1991). Pinacidil also inhibited norepinephrine-induced Ca^{2+} signalling and IP_3 formation in intact, but not chemically skinned smooth muscle (Itoh et al., 1992). These actions of pinacidil were accompanied by membrane hyperpolarization and were prevented by glibenclamide. Similarly, in isolated canine coronary arteries, PCOs were shown to inhibit thromboxane-induced intracellular Ca^{2+} release and IP_3 formation (Yamagishi et al., 1992). This action is specific for agonist-induced Ca^{2+} release, as caffeine-induced Ca^{2+} release was not affected by PCOs in this study. Finally, Ganitkevich and Isenberg (1993) directly demonstrated that IP_3-mediated Ca^{2+} signals are modulated by membrane potential. In voltage-clamped coronary myocytes, depolarization stimulated and hyperpolarization attenuated Ca^{2+} transients monitored simultaneously by indo 1-fluorescence (Ganitkevich & Isenberg, 1993).

Hyperpolarization-induced inhibition of phosholipase C could contribute to actions of pinacidil observed in the efferent arteriole, especially since the efferent arteriolar reactivity to angiotensin II is suggested to rely on the calcium release from intracellular stores. This postulate is based on studies in which blocking SR calcium release by dantrolene (Conger & Falk, 1993) or depleting the SR by thapsigargin (Inscho et al., 1997) prevented efferent arteriolar reactivity to angiotensin II. Future studies that investigating the effects of pinacidil on membrane potential, Ca^{2+} signalling or possibly IP_3 formation in the efferent arteriole are needed to resolve this issue.

In addition to interfering with agonist-induced Ca^{2+} signalling, PCOs also lower resting $[Ca^{2+}]i$ (Ito et al., 1991, Itoh et al., 1991). Quast (1993) proposed that hyperpolarization could decrease $[Ca^{2+}]i$ by two possible mechanisms: by stimulating calcium extrusion via Na-Ca exchanger or by decreasing the free $[Ca^{2+}]i$ concentration by increasing the binding of Ca^{2+} to plasmalemma. Stimulation of the Na-Ca exchanger by pinacidil-induced hyperpolarization could potentially explain the efferent arteriolar actions of this PCO. Recent evidence (Fowler et al., 1996) demonstrated that both afferent and efferent arterioles express the Na-Ca exchanger. In smooth muscle, this antiport mechanism is thought to extrude 1 Ca ion in exchange for 3 Na ions, but the exact stoichiometry has not been unequivocally established. The activity of Na-Ca exchanger in cardiac muscle was shown to be dependent on membrane potential (Bridge et al., 1991). Assuming that the Na-Ca exchanger in VSM is regulated by membrane potential as well, it can be speculated that hyperpolarization would stimulate its activity, leading to enhanced extrusion of Ca^{2+} through this mechanism. To test this possibility, Na^+ in the perfusate medium could be replaced with choline or sucrose to reverse Na^+ gradient. This would reverse the mode of the Na-Ca exchanger and thereby eliminate any actions of pinacidil on the efferent arteriole that involve Na-Ca exchange.

In conclusion, the results obtained with pinacidil demonstrate that activation of K_{ATP} elicits vasodilation of both afferent and efferent arterioles but does so through different mechanisms. Pinacidil potently inhibits L-type calcium channel activity in the afferent arteriole and this mechanism likely accounts for the preferential afferent arteriolar actions of K_{ATP} activation. The ability of high concentrations of pinacidil to reduce the reactivity of the efferent arteriole clearly does not involve modulation of voltage-gated calcium entry but appears to be mediated by K_{ATP}-induced hyperpolarization. This is the first demonstration of physiological relevance of previously described L-type calcium channel independent actions of a PCO. Future studies will be required to further characterize the nature of mechanism responsible for the actions of pinacidil in the efferent arteriole.

Having established the responses of renal afferent and efferent arterioles to pinacidil, we proceeded to examine the effects of endogenous vasodilators that have been shown to act

via K_{ATP} in other vascular tissues. CGRP and adenosine were studied in respect to their ability to modulate renal microvascular reactivity via activation of K_{ATP}. Based on the preferential actions of pinacidil on the afferent arteriole, we anticipated that CGRP and/or adenosine would also preferentially attenuate the reactivity of this vessel via a glibenclamide-sensitive mechanism.

4.2 Actions of CGRP on afferent and efferent arterioles

The data described in section 3.2 demonstrate that renal microvascular actions of CGRP involve both K_{ATP}-dependent and K_{ATP}-independent mechanisms. During angiotensin II-induced vasoconstriction, CGRP attenuates both afferent and efferent arteriolar vasoconstrictions. However, K_{ATP} does not appear to contribute to the actions of CGRP in this setting. In contrast, the vasodilatory actions of CGRP on pressure-induced afferent arteriolar vasoconstriction are partially attenuated by blockade of K_{ATP}. The potency of CGRP and the involvement of K_{ATP} in its actions differ during myogenic versus angiotensin II-induced vasoconstriction. Specifically, CGRP is more potent in inhibiting myogenic vasoconstriction and this difference in potency is abolished by the K_{ATP} blocker glibenclamide (Figure 3.12). These observations indicate that the involvement of K_{ATP} in CGRP-induced afferent arteriolar vasodilations is influenced by the nature of the underlying tone. Again, section 4.5 discusses possible mechanisms responsible for this phenomenon.

CGRP-induced renal vasodilation has been documented in numerous *in vivo* studies (reviewed by Villareal et al., 1994), where CGRP was found to increase both RBF and GFR. As discussed in section 1.6, the interpretations of effects of CGRP on RBF are complicated by reflex activation of renin-angiotensin axis elicited by hypotensive doses of CGRP. In isolated *in vitro* perfused rat kidneys (i.e. in the absence of indirect, reflex-mediated effects on RBF), CGRP inhibited the vasoconstrictory actions of angiotensin II (Kurtz et al., 1989), norepinephrine (Gepetti et al., 1989, Castellucci et al., 1993) and phenylephrine (Chin et al., 1994, Haynes & Cooper, 1995). To examine the vasodilatory actions of CGRP on the renal microvasculature, Edwards & Trizna (1990) studied isolated rabbit renal arterioles. In this preparation, CGRP reversed norepinephrine-induced afferent arteriolar vasodilation but

failed to elicit a significant vasodilation in efferent arterioles constricted by either norepinephrine or angiotensin II. In our experiments, CGRP exerted more prominent vasodilatory actions on the afferent arteriole, attenuating the afferent arteriolar response to angiotensin II by about 50%. In contrast to the observations by Edwards & Trizna (1990), we observed significant attenuation (ca 30%) of angiotensin II-induced efferent arteriolar vasoconstriction. The discrepancies regarding the efferent arteriolar actions of CGRP between the two studies could reflect species difference or differences inherent to the experimental models.

Only a limited number of studies have investigated the involvement of K_{ATP} in renal hemodynamic actions of CGRP. In conscious rats, glibenclamide did not alter the hypotensive actions of intravenously administered CGRP (Abdelrahman et al., 1992). In the *in vitro* perfused rat kidney, CGRP reversed the vasoconstriction elicited by 60 mM KCl (Castelucci et al., 1993). In the presence of elevated extracellular K^+, CGRP would be expected to elicit vasodilation only via mechanisms not involving K^+ conductance. Furthermore, CGRP relaxed norepinephrine-preconstricted arteriolar rings from small renal arteries (Gao et al., 1994) and these actions were insensitive to glibenclamide. In agreement with the above findings, in our experiments glibenclamide failed to alter the effects of CGRP on angiotensin II-induced vasodilation. Thus, CGRP clearly exhibits K_{ATP}-independent vasodilatory actions. However, we found that the actions of CGRP on pressure-induced vasoconstriction were partially attenuated by blockade of K_{ATP}.

It is generally accepted that, in most vascular beds, CGRP elicits vasodilation via accumulation of cAMP (reviewed by Bell & McDermott, 1996). In some vascular preparations, such as rabbit mesenteric arteries, CGRP activated K_{ATP} via cAMP-dependent protein kinase (Quayle et al., 1994). In the kidney, CGRP was found to increase cAMP levels in glomeruli (Edwards & Trizna, 1990, Edwards et al., 1996) and mesangial cells (Kurtz et al., 1989), implying a role for cAMP in renal vasodilatory actions of CGRP. If we assume a causal relationship between increases in cAMP levels and vasodilation in response to CGRP in the renal microcirculation, then the K_{ATP}-mediated component of CGRP-induced vasodilation would be consistent with cAMP-mediated activation of K_{ATP}.

However, a substantial component of vasodilatory actions of CGRP is insensitive to glibenclamide, suggesting that K_{ATP} is not a crucial target for the CGRP-activated cAMP signal transduction cascade.

Future experimental approaches

Our present findings demonstrate that multiple mechanisms, including activation of K_{ATP}, contribute to the vasodilatory actions of CGRP in the renal microvasculature. Future studies could be directed towards identifying the signal transduction mechanism(s) underlying the K_{ATP}-independent component of CGRP-induced vasodilations.

It has been proposed that the vasodilatory actions of CGRP are endothelium-dependent, but the evidence regarding the involvement of endothelium in renal vascular actions of CGRP remains controversial. In small renal arteries, the vasodilatory actions of CGRP are not affected by removal of the endothelium (Gao et al., 1994). Since the endothelium-dependent component is thought to be mediated by NO, an insensitivity of CGRP actions to blockers of NO synthesis would provide evidence against and endothelium-dependence of CGRP actions. Consistent with the data from isolated renal arteries, renal vasodilation can be elicited by CGRP in the presence of L-NAME in conscious rats (Gardiner et al., 1991), suggesting that endothelium-derived NO is not required for CGRP-mediated responses in renal circulation. In contrast, in a study by Amuchstegui et al. (1994), L-NAME completely prevented the renal hemodynamic changes in response to systemic administration of CGRP. Similarly, renal vasodilatory responses to intraarterially administered CGRP were abolished by L-NAME in anaesthetized rats in a recent study by Elhawary & Pang (1995). It should be noted that in the studies which reported sensitivity of CGRP actions to L-NAME, administration of L-NAME significantly increased basal values of mean arterial pressure (Amuchastegui et al., 1994) and decrease basal RBF and GFR (Elhawary & Pang, 1995). These changes in basal parameters preclude unequivocal interpretation of these data. In an attempt to resolve this controversy regarding the involvement of NO in the actions of CGRP, the effects of L-NAME on CGRP-induced afferent and efferent arteriolar vasodilations could be assessed in our *in vitro* model.

In summary, we characterized the renal microvascular actions of CGRP. Based on observations from other vascular beds, we speculated that CGRP would act as an endogenous activator of K_{ATP}. When afferent and efferent arterioles were constricted with angiotensin II, CGRP elicited vasodilation via a glibenclamide-insensitive (and therefore K_{ATP}-independent) mechanism. However, CGRP reversed pressure-induced afferent arteriolar vasoconstriction via mechanism that was partially attenuated by blockade of K_{ATP}. We conclude that K_{ATP} contributes to the vasodilatory actions of CGRP during myogenic, but not angiotensin II-induced, vasoconstriction. Thus, the nature of the underlying tone K_{ATP} appears to be an important determinant of the involvement of K_{ATP} in the vasodilations evoked by CGRP in the renal microcirculation.

4.3 Actions of adenosine on afferent and efferent arterioles

The ability of adenosine to elicit renal vasoconstriction or vasodilation reflects actions of adenosine via the adenosine A_1 or A_2 receptors, respectively. Both receptor subtypes are coupled to adenylyl cyclase. Activation of the adenosine A_1 receptor inhibits the activity of this enzyme via PTX-sensitive G_i protein while activation of the adenosine A_2 receptor stimulates adenylyl cyclase activity via G_s (Fredholm et al., 1994). The adenosine A_2 receptors are further classified as high-affinity A_{2a} subclass or low-affinity A_{2b} subclass. Both adenosine A_2 receptor subtypes have been cloned from a variety of tissues. The overall sequence homology of two adenosine A_2 receptor subtypes averages about 80%. The A_{2a} receptor differs from the A_{2b} receptor by its larger C-terminal domain (Linden et al., 1994). These two subclasses of adenosine A_2 receptors can be distinguished experimentally by their affinity for adenosine (i.e. high versus low) and by the use of selective agonists and antagonists (Fredholm et al., 1994). In the renal circulation, activation of the adenosine A_1 receptor subtype elicits transient afferent arteriolar vasoconstriction. Activation of the adenosine A_2 receptor elicits vasodilation that is thought to be preferentially postglomerular (Spielman & Arend, 1991).

The renal microvascular adenosine A_2-vasodilatory response is not fully characterized. In our model, adenosine elicits a biphasic vasodilation of afferent arterioles when these

vessels are constricted with elevated pressure (Loutzenhiser et al., 1994). At concentrations of 10-100 nM, adenosine elicits afferent arteriolar vasodilation that is blocked by glibenclamide. This response is suggested to be mediated by the high-affinity adenosine A_{2a} receptor. At high concentrations (1-30 μM), adenosine elicited afferent arteriolar vasodilation that was not sensitive to glibenclamide, and therefore did not involve K_{ATP}. The ability of this concentration of adenosine to inhibit KCl-induced afferent arteriolar vasoconstriction provides further support for the lack of involvement K-channels in this vasodilatory response to adenosine. This response may be mediated by the low-affinity adenosine A_{2b} receptor subtype. It therefore appeared that, at submicromolar concentrations, adenosine acts as an endogenous activator of K_{ATP}. We proceeded to determine the effect of submicromolar concentrations of adenosine on afferent and efferent arteriolar reactivity to angiotensin II.

The data presented in section 3.3 characterize the high-affinity adenosine vasodilatory responses in afferent and efferent arterioles. Submicromolar concentrations of adenosine attenuate both afferent and efferent arteriolar responses to angiotensin II. The afferent, but not the efferent, arteriolar actions of adenosine in this setting are inhibited by glibenclamide. Similarly, afferent arteriolar myogenic vasoconstriction is also inhibited by submicromolar concentrations of adenosine via a glibenclamide-sensitive mechanism. Thus, the high-affinity adenosine-induced afferent arteriolar vasodilations appear to involve K_{ATP}, whereas the efferent arteriolar vasodilatory actions of adenosine appear to be independent of K_{ATP}.

Adenosine - angiotensin II interactions in the afferent arteriole

During angiotensin II-induced afferent arteriolar vasoconstriction, glibenclamide not only prevents the vasodilatory actions of adenosine, but unmasks what appears to be a potentiating effect of adenosine on angiotensin II-induced vasoconstriction. This phenomenon is illustrated in figure 3.16. In the absence of glibenclamide, submicromolar concentrations of adenosine attenuated the angiotensin II-induced afferent arteriolar vasodilation. Following the addition of glibenclamide, we no longer observed any inhibitory

effect of adenosine on the afferent arteriole, but rather a potentiation of afferent arteriolar vasoconstrictor response to angiotensin II. Note that this effect is observed during angiotensin II- but not pressure-induced vasoconstriction (Figure 3.16), suggesting a specific interaction between adenosine and angiotensin II. We speculate that, at submicromolar concentrations, adenosine acts as a mixed A_1/A_{2a} receptor agonist. Following the application of glibenclamide, which presumably inhibits the vasodilatory (A_{2a}) component of this response, adenosine acts via its A_1 receptor and appears to potentiate the angiotensin II-induced vasoconstriction. Thus our data provide evidence for a synergistic interaction of adenosine and angiotensin II that is unmasked by blocking the vasodilatory actions of adenosine.

The interaction between renal vascular actions of adenosine (A_1 receptor-mediated) and angiotensin II has been previously suggested, but not uniformly confirmed, by whole kidney functional studies (reviewed by Navar et al., 1996). Conflicting results have also been obtained in renal microvascular preparations, which allow direct assessment of afferent arteriolar reactivity. In agreement with our observations, Weihprecht et al. (1994) reported a synergistic afferent arteriolar vasoconstrictor response when angiotensin II and adenosine were applied simultaneously in isolated rabbit afferent arterioles. In the rat hydronephrotic kidney model, adenosine A_1-mediated afferent arteriolar vasoconstriction was attenuated by the angiotensin II receptor blocker saralasin, suggesting that adenosine interacts with the angiotensin II receptor system to induce vasoconstriction (Dietrich et al., 1991). In contrast, saralasin was found to have no effect on adenosine-induced afferent arteriolar vasoconstriction in renal tissue transplanted to the hamster cheek pouch (Joyner et al., 1988). In the JMN nephron preparation, adenosine failed to potentiate the angiotensin II-induced vasoconstriction (Carmines & Inscho, 1994). It is possible that these conflicting observations reflect specific properties of individual renal microvascular models. Alternatively, our findings suggest that a functional antagonism between adenosine A_1 and A_2 receptor activation may mask this angiotensin II - adenosine interaction in some experimental settings.

CV1808

In an attempt to avoid the interaction between angiotensin II and adenosine A_1 receptor activation, we utilized the adenosine receptor agonist CV1808. CV1808 specifically activates the adenosine A_{2a} receptor subtype at concentrations of 20-200 nM. At higher concentrations (above 100 μM) CV1808 activates the adenosine A_{2b} receptor subtype (Fredholm et al., 1994). Thus the use of this compound allowed us to further characterize the involvement of K_{ATP} in afferent and efferent arteriolar adenosine A_{2a}-mediated vasodilations.

Like adenosine, CV1808 attenuates afferent and efferent arteriolar vasoconstriction by angiotensin II as well as afferent arteriolar myogenic vasoconstriction. During both angiotensin II- and pressure-induced vasoconstrictions, the vasodilatory actions of CV 1808 are prevented by glibenclamide, suggesting an involvement of K_{ATP} in both settings. The vasodilatory actions of CV 1808 on the efferent arteriole are not affected by blockade of K_{ATP}. In addition, CV 1808 does not potentiate the angiotensin II-induced afferent arteriolar vasodilation in the presence of glibenclamide (see figure 3.18, panel B). This finding provides indirect evidence that the interactions of adenosine and angiotensin II observed in the afferent arteriole in the presence of glibenclamide were most likely due to adenosine acting as an A_1 agonist.

The efferent arteriolar actions of adenosine were not significantly altered by glibenclamide (see Figure 3.14). Glibenclamide, however, attenuated the efferent arteriolar vasodilatory actions of higher concentrations of CV 1808 (i.e. 1 and 10 μM) (see Figure 3.18, panel C). Although this effect of glibenclamide did not attain statistical significance, we cannot exclude the possibility of a K_{ATP}-mediated component in efferent arteriolar actions of higher concentrations of CV 1808. Further studies directly assessing the effects of CV 1808 on membrane potentials of afferent and efferent arterioles should clarify the involvement of K_{ATP} in CV 1808-induced vasodilations.

In conclusion, both adenosine and CV 1808 elicit afferent and efferent arteriolar

vasodilations that are mediated via the adenosine A_{2a} receptor subtype. The afferent arteriolar adenosine A_{2a} responses appear to be mediated via K_{ATP}. The mechanism underlying the efferent arteriolar adenosine A_{2a}-mediated vasodilation is K_{ATP}-independent at least at lower levels of adenosine A_{2a} receptor activation. These results indicate that K_{ATP} is a potentially important modulator of afferent, but not efferent, arteriolar reactivity. The situation whereby the adenosine A_{2a} receptor-coupling mechanisms differ within the same vascular bed is quite unique and provides further support for the distinct regulatory mechanisms of the preglomerular and postglomerular resistance vasculature.

4.4 Does cAMP activate K_{ATP} in the afferent arteriole?

Since the majority of endogenous vasodilators that activate K_{ATP} have been shown to increase intracellular levels of cAMP, it has been proposed that K_{ATP} is activated via a cAMP-dependent mechanism. Electrophysiological studies have provided compelling evidence for activation of K_{ATP} channels by cAMP via cAMP-dependent protein kinase (PKA). In myocytes isolated from rabbit mesenteric arteries, CGRP activated a K^+ current that was identified as K_{ATP} based on its sensitivity to glibenclamide, inhibition by ATP and voltage-independence (Quayle et al., 1994). Similar glibenclamide-sensitive currents were observed in the presence of forskolin (a direct activator of adenylyl cyclase), Sp-cAMPS (a cell-permeable cAMP analogue) and the catalytic subunit of PKA. Furthermore, in cells pretreated with inhibitors of PKA (PKI or H-8), the activation of K_{ATP} by CGRP was prevented (Quayle et al., 1994). These authors proposed that activation of K_{ATP} via cAMP-PKA pathway could be a general mechanism by which endogenous vasodilators elicit hyperpolarization. In the same vascular preparation (i.e. rabbit mesenteric arteriolar myocytes), adenosine and the adenosine A_2 agonist CGS-21680 activated K_{ATP} (Kleppisch & Nelson, 1995b). This adenosine-activated current was inhibited by blockers of PKA (Rp-cAMPS, H-89, PKA inhibitor peptide), further supporting the premise that activation of K_{ATP} by endogenous vasodilators is mediated via PKA (Kleppisch & Nelson, 1995b). Activation of K_{ATP} by PKA was also demonstrated in porcine coronary arterial myocytes (Miyoshi & Nakaya, 1993). In this study, application of the catalytic subunit of PKA activated K_{ATP} in inside-out patch configurations (Miyoshi & Nakaya, 1993). In isolated canine saphenous

vein, isoproterenol, as well as forskolin, elicited glibenclamide-sensitive hyperpolarization (Nakashima & Vanhoute, 1995). In concert, these data provide evidence that vasodilatory agonists activate K_{ATP} via cAMP-dependent signal transduction mechanism.

In our microvascular preparation, adenosine and, under certain conditions, CGRP elicit afferent arteriolar vasodilations that are inhibited by glibenclamide. To determine whether cAMP is a common signal transduction mechanism for activation of K_{ATP} in the afferent arteriole, we examined whether elevating intracellular cAMP levels elicits glibenclamide-sensitive vasodilations. We compared the effects of β adrenoceptor-mediated activation of adenylyl cyclase (isoproterenol) and direct activation of this enzyme by forskolin or the membrane permeable cAMP analogue db-cAMP.

We found that isoproterenol, forskolin and db-cAMP each inhibited afferent arteriolar myogenic vasoconstriction. Glibenclamide significantly attenuated the vasodilatory actions of isoproterenol, but did not affect the actions of either forskolin or db-cAMP (see Figure 3.24). Since we anticipated that elevation of cAMP in response to each of these agents would activate PKA and consequently K_{ATP}, these results were rather perplexing. The only possible interpretations are that the cAMP/PKA-mediated activation of K_{ATP} observed in isolated myocytes (Miyoshi & Nakaya, 1993, Quayle et al., 1994, Kleppisch & Nelson, 1995b) does not occur in the afferent arteriole and that isoproterenol activates K_{ATP} via mechanism(s) not involving cAMP.

Studies in other vascular beds support our observations that elevation of cAMP *per se* does not activate K_{ATP}, but activation of the β adrenergic receptor does. Jackson (1993) compared the vasodilatory actions of isoproterenol, adenosine, forskolin and db-cAMP in hamster cheek pouch arterioles with respect to the sensitivity of these vasodilations to glibenclamide. Similar to our results, glibenclamide attenuated the actions of isoproterenol and adenosine, but did not affect the vasodilation induced by forskolin or db-cAMP (Jackson, 1993). Further evidence for cAMP-independent actions of isoproterenol was obtained in the isolated perfused mesenteric vascular bed (Randall & McCulloch, 1995). In this preparation, the vasodilatory responses to db-cAMP were independent of K_{ATP}.

However, the isoproterenol-induced vasodilations were sensitive to K_{ATP} blockade (Randall & McCulloch, 1995).

If cAMP does not mediate the effects of isoproterenol, how could isoproterenol activate K_{ATP}? One possibility is a direct β adrenoceptor effect on K_{ATP}, via membrane delimited actions of a G protein on K_{ATP}. The regulation of K_{ATP} channels by G proteins is well characterized in the heart, where the cardiac K_{ATP} is regulated by PTX-sensitive G proteins (Kirsch et al., 1990). Interestingly, the stimulatory effect of G proteins on K_{ATP} channel activity is only observed when these channels are closed by intracellular ATP. In the absence of ATP, G proteins have no effect on channel activity (Terzic et al., 1994, Ito et al., 1994). Thus, it has been proposed that the mechanism by which G proteins activate the cardiac K_{ATP} channel involves regulation of ATP gating of this channel (Terzic et al., 1994). In vascular smooth muscle, the regulation of K_{ATP} by G proteins has not been reported. Only indirect evidence is available to support a possible role of a G_i protein in regulation of K_{ATP} in coronary vasculature. In the coronary circulation, adenosine elicits vasodilation that is mediated via the adenosine A_1 receptor subtype and involves activation of K_{ATP} (Dart & Standen, 1993, Merkel et al., 1992, Nakhostine & Lamontagne, 1993). Since it is well established that adenosine A_1 receptors are coupled to adenylyl cyclase via a G_i protein, the resulting decrease in intracellular cAMP levels would be expected to inhibit rather than activate K_{ATP}, if indeed K_{ATP} was regulated by cAMP/PKA. The observation that this G_i-coupled receptor activates K_{ATP} suggests a cAMP-independent mechanism of action. Since G_i is directly coupled to K_{ATP} in the cardiac muscle, it is possible these adenosine A_1-mediated vasodilations in the coronary vasculature a direct, G_i-mediated gating of K_{ATP}.

In contrast to adenosine A_1 receptors, β adrenoceptors are coupled to G_s, rather than G_i, proteins. Thus, we cannot explain the actions of isoproterenol in our study by the mechanism described in cardiomyocytes. There is currently no evidence available to suggest that G_s proteins directly activate K_{ATP}. However, such a mechanism would provide a plausible explanation for actions of isoproterenol in the afferent arteriole.

4.5 Inhibition of K_{ATP} by angiotensin II?

Our findings with pinacidil and CGRP suggested that the nature of the underlying tone influences the mechanisms involved in the actions of vasodilators. We found pinacidil to be more potent against myogenic versus angiotensin II-induced afferent arteriolar vasoconstriction. CGRP was also more potent against myogenic versus angiotensin II-induced afferent arteriolar vasoconstriction. Moreover, glibenclamide attenuated the actions of CGRP during myogenic, but not angiotensin II-induced vasoconstriction, suggesting that the K_{ATP}-mediated component of vasodilatory actions of CGRP is only observed during myogenic tone. Thus, the myogenic vasoconstriction appears to be more sensitive to activation of K_{ATP} than the angiotensin II-induced vasoconstriction. Even though angiotensin II and elevated pressure elicited afferent arteriolar vasoconstriction of similar magnitude, the signal transduction mechanisms through which these stimuli elicit vasoconstriction are different in many aspects. We consider two of these aspects as possible explanations for our observations with pinacidil and CGRP:

First, the level of membrane potential depolarization could differ during angiotensin II- and pressure-induced vasoconstriction. In the afferent arteriole, angiotensin II (0.1 nM) elicits a 11 mV depolarization (Loutzenhiser et al., 1997). The level of depolarization during afferent arteriolar myogenic response has not been determined. If angiotensin II elicits a a greater depolarization, one might anticipate K_{ATP}-mediated hyperpolarization to be less effective in this setting. There are at least two mechanisms that could lead to a greater depolarization in response to angiotensin II versus elevated pressure. The vasoconstrictor responses to both angiotensin II and elevated pressure are blocked by dihydropyridines, indicating an obligate role of depolarization and L-type calcium channels in the underlying activation mechanism. Recent evidence suggests that activation of L-type calcium channels is preceded by activation of chloride channels in response to angiotensin II (Carmines 1995, Jensen & Skott, 1996, Takenaka et al., 1996). Opening of chloride channels in response to angiotensin II-induced calcium release is thought to cause initial membrane depolarization that leads to opening of L-type calcium channels. The afferent arteriolar myogenic vasoconstriction, however, is not affected by blockers of chloride channels

(Takenaka et al., 1996). It is therefore possible that angiotensin II-induced vasoconstriction is associated with a greater degree of depolarization due to opening of chloride channels. The other possible mechanism by which angiotensin II elicits greater depolarization relates to the possibility that PKC may be activated to a greater extent during agonist- versus pressure-induced vasoconstriction. Studies in other vascular preparations suggest that angiotensin II-induced activation of PKC inhibits the delayed rectifier K^+ current (Clement-Chomienne et al., 1996). This channel is activated upon depolarization and serves to repolarize the membrane potential. Thus, unless K_{dr} is inhibited, voltage-dependent activation of this channel would attenuate membrane depolarization. It is not known whether angiotensin II or PKC inhibit this channel in the afferent arteriole. Obviously, studies directly comparing the magnitude of depolarization in response to angiotensin II or elevated pressure will be required to investigate this issue further.

Alternatively, it is possible that activation of K_{ATP} may be directly affected by the nature of the vasoconstrictory stimulus. Miyoshi and Nakaya (1991) were the first to report that extracellular application of angiotensin II inhibits the activity of ATP-sensitive K^+ channels in cultured coronary VSM. They proposed that inhibition of K_{ATP} by angiotensin II would cause depolarization and vasoconstriction. The same authors demonstrated that other vasoconstrictors, such as endothelin (Miyoshi et al., 1992) and vasopressin (Wakatsuki, 1992) also inhibit the K_{ATP} channel and this inhibition can be reversed by a PCO nicorandil. Since in these studies the K_{ATP} channel activity was observed in intact cell-attached patches, the authors suggested that in the coronary artery, K_{ATP} contributes to the resting membrane potential and that the inhibition of this channel contributes to the vasoconstriction induced by endothelin (Miyoshi et al., 1992) and vasopressin (Wakatsuki et al., 1992). In freshly isolated cerebral arterial myocytes, histamine and serotonin inhibited pinacidil-activated whole cell currents (Kleppisch & Nelson, 1995a). The inhibitory actions of vasoconstrictors were mimicked by the PKC activator PDBu, suggesting that vasoconstrictors may act via PKC to inhibit K_{ATP} (Kleppisch & Nelson, 1995a). Histamine also inhibited pinacidil activated whole cell currents in cultured mesenteric arteriolar VSM (Kleppisch et al., 1996). The authors proposed that inhibition of K_{ATP} contributes to membrane depolarization and vasoconstriction by histamine or serotonin (Kleppisch &

Nelson, 1995a). A recent study by Bonev and Nelson (1996) explored signal transduction pathways through which vasoconstrictors inhibit K_{ATP}. In myocytes isolated from mesenteric artery, neuropeptide Y, phenylephrine, serotonin and histamine attenuated the whole-cell current activated by pinacidil. The inhibitory effects of vasoconstrictors on pinacidil-induced current were attenuated by pretreatment with the PLC inhibitor (D609) or the PKC inhibitor (GF 109203X), suggesting an involvement of PKC in this response. Furthermore, activators of PKC (DOG and PMA) mimicked the effect of vasoconstrictors on the pinacidil-induced current. This study thus provided convincing evidence that vasoconstrictors inhibit K_{ATP} via PKC. Furthermore, the authors suggested that inhibition of K_{ATP} by PKC may play a physiologically important role. Accordingly, in tissues with high levels of receptor-mediated PKC activation, PCOs would be less effective as vasodilators as K_{ATP} channels are already "heavily inhibited" (Bonev & Nelson, 1996).

Our findings with pinacidil, CV1808 and CGRP are consistent with this postulate. Thus the lesser potency of pinacidil during angiotensin II-induced vasoconstriction may reflect an inhibition of K_{ATP} by angiotensin II. A careful inspection of Figure 3.18 reveals that CV1808 is also more potent against myogenic versus angiotensin II-induced vasoconstriction. The results obtained with CGRP may be viewed as an extreme example of this phenomenon. Unlike pinacidil or CV1808, the effects of which are presumably mediated solely by K_{ATP}, CGRP possesses multiple vasodilatory mechanisms. The primary mechanism clearly does not depend on K_{ATP} in the afferent arteriole. The glibenclamide-sensitive, i.e. K_{ATP}-mediated, component of the CGRP-induced vasodilation is a relatively minor determinant and is only observed during myogenic vasoconstriction. This minor K_{ATP}-mediated component is not present during angiotensin II-induced vasoconstriction, possibly due to the inhibition of K_{ATP} by angiotensin II. Our data are thus consistent with the postulate that the nature of the underlying vasoconstrictor stimulus influences the potency of K_{ATP}-dependent vasodilators and provide evidence for the physiological relevance of inhibition of K_{ATP} by vasoconstrictory agonists.

4.6 Conclusions

In the renal microvasculature, activation of K_{ATP} by pinacidil preferentially attenuates afferent arteriolar reactivity. At relatively high concentrations, pinacidil attenuates efferent arteriolar reactivity as well, but does so through a mechanism that is independent of L-type calcium channels. Thus, activation of K_{ATP} is a potential mechanism for differential regulation of afferent versus efferent arteriolar reactivity. CGRP and adenosine both activate K_{ATP} in the renal microcirculation. CGRP dilates both afferent and efferent arterioles, but the involvement of K_{ATP} in these vasodilations is variable. The K_{ATP}-mediated component of CGRP-induced vasodilations is observed only in afferent arterioles activated by pressure and is not seen during angiotensin II-induced vasoconstriction (in either vessel). The major component of CGRP-induced vasodilation does not depend on K_{ATP}. Adenosine also elicits K_{ATP}-dependent and K_{ATP}-independent vasodilation via adenosine A_{2a} and A_{2b} receptors, respectively. Activation of adenosine A_{2a} receptors by CV1808 (0.001 to 10 μM) induces afferent arteriolar vasodilations that are mediated by K_{ATP}. Over the same concentration range, however, CV1808 elicits a K_{ATP}-independent vasodilation of the efferent arteriole. Thus, adenosine A_{2a} receptors are coupled to different mechanisms in renal afferent and efferent arterioles, providing yet another example of the disparate regulatory mechanisms governing pre- and postglomerular vascular reactivity.

Although cAMP has been implicated in the activation of K_{ATP} and in the vasodilatory actions of adenosine and CGRP, we found that elevation of cAMP per se does not result in activation of K_{ATP} in the renal afferent arteriole. Nevertheless, isoproterenol does activate K_{ATP}, presumably by a cAMP-independent mechanism. We speculate that this effect involves membrane delimited activation of K_{ATP} via G proteins (G_s).

The level of involvement of K_{ATP} in afferent arteriolar vasodilation varies depending on the nature of the underlying vasoconstrictory stimulus. In general, during angiotensin II-induced vasoconstriction, activation of K_{ATP} appears to be attenuated. Consequently, K_{ATP}-dependent vasodilators appear to be more potent during myogenic versus angiotensin II-induced vasoconstriction. We suggest that this is due to inhibition of K_{ATP} by angiotensin

II, as previously suggested.

These investigations addressed important questions related to the differential regulation of pre- and postglomerular resistances. Our results suggest that there are important differences in the modulation of afferent and efferent arteriolar reactivity by K_{ATP}.

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