FUNCTIONAL CHARACTERIZATION OF ADP RIBOSYL CYCLASE SIGNALING PATHWAYS IN THE RENAL MICROCIRCULATION

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ABSTRACT

TIFFANY L THAI: Functional Characterization of ADP Ribosyl Cyclase Signaling Pathways in the Renal Microcirculation
(Under the direction of William J. Arendshorst, PhD)

Due to its role in the regulation of salt and water balance, the kidney is thought to be the primary organ involved in arterial blood pressure regulation. The mechanisms governing contraction of renal resistance arteries/arterioles are incompletely understood, however. The purpose of my dissertation was to investigate the importance of a novel signaling pathway involving the enzyme ADP ribosyl (ADPR) cyclase in the regulation of calcium (Ca^{2+}) signaling in afferent arterioles and regulation of renal vascular resistance.

Physiological measurements of renal blood flow (RBF) were performed on anesthetized rats before, during, and after pharmacological blockade of ADPR cyclase and its downstream target, the ryanodine receptors (RyR). Inhibition of ADPR cyclase under basal conditions resulted in a significant increase in RBF, and impairment of ADPR cyclase or RyR function attenuated RBF responses to the vasoconstrictors angiotensin II (Ang II), norepinephrine (NE), and endothelin-1 (ET-1) by 30-50%. Further analysis revealed that ADPR cyclase and RyR contribute to vasoconstrictor responses induced by either ET receptor, ET_A or ET_B.

Measurements of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated rat afferent arterioles revealed that the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) participated in the actions of NE and ET-1. $[Ca^{2+}]_i$ transients produced by either agonist were

diminished by at least 50% in the presence of disruption of lysosomal Ca^{2+} or NAADP receptor inhibition.

Real time quantitative RT-PCR studies on isolated mouse preglomerular resistance arterioles determined mRNA expression of ADPR cyclase family members CD38 and CD157. Genetic knockout of CD38 resulted in attenuated RBF responses to Ang II, NE, and ET-1. Pharmacological blockade of ADPR in CD38-/- animals resulted in no significant attenuation of Ang II-induced renal vasoconstriction, suggesting that CD38 functions as the predominant ADPR cyclase.

Overall, these studies provide evidence for the physiological function of ADPR cyclase, its second messengers, and downstream effectors in the regulation of renal hemodynamics. Further investigation of this pathway in the renal vasculature will result in a deeper understanding of renal hemodynamics.

To Richard, Alyssa, and Isaac.

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ADPR	ADP ribosyl
ANG II	angiotensin II
BAF A1	bafilomycin A1
$[Ca^{2+}]_i$	cytosolic calcium concentration
cADPR	cyclic ADP ribose
CICR	calcium-induced calcium release
CCA	concanamycin A
DAG	diacylglycerol
EC	endothelial cell
ER	endoplasmic reticulum
ET-1	endothelin-1
FKBP	FK506 binding protein
GFR	glomerular filtration rate
GPCR	G-protein coupled receptor
IP_3	inositol 1,4,5-trisphosphate
MLCK	myosin light chain kinase
mRNA	messenger ribonucleic acid
NAADP	nicotinic acid adenine dinucleotide phosphate
NE	norepinephrine
PGA	preglomerular resistance arteriole
PKC	protein kinase C
PLC	phospholipase C
RBF	renal blood flow
ROC	receptor-operated channel

RVR	renal vascular resistance
RYR	ryanodine receptor
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
SHR	spontaneously hypertensive rat
SOC	store-operated channel
SR	sarcoplasmic reticulum
TRP	transient receptor potential
VSMC	vascular smooth muscle cell

CHAPTER I

Introduction

A. ROLE OF THE RENAL VASCULATURE IN BLOOD PRESSURE REGULATION.

Inside the kidney lies a vast network of tubules and blood vessels designed to efficiently regulate the volume and composition of plasma and extracellular fluid, thus playing a crucial role in determining arterial pressure. Blood flows into the kidney through the renal artery and travels through the preglomerular vasculature consisting of interlobar, arcuate, and interlobular arteries before entering the afferent arteriole and glomerulus. In the glomerulus, ultrafiltration occurs as fluid passes through hypothetical pores of glomerular endothelial cells, collagen and glycoproteins of the basement membrane, and slit-pores of podocytes of Bowman's capsule (which may have a filtration function in addition to laying down basement membrane) (133; 244). Post-glomerular efferent arterioles hold blood containing high concentrations of blood cells, proteins, and other un-filterable substances. Efferent arterioles branch to form the cortical peritubular capillaries and medullary vasa recta where blood becomes de-oxygenated before returning to the venous circulation. The state of contraction and relaxation imparted by the pre- and post-glomerular resistance vessels determine glomerular capillary hydrostatic pressure and the rate of fluid flow through the glomerulus, into Bowman's space, and into the proximal tubule where the reabsorption and secretion of electrolytes is initiated to maintain plasma volume and electrolyte composition.

Fluid flows through the proximal tubule where cells covered in luminal microvilli reabsorb 67% of the filtered salt and water and virtually all glucose and amino acids, past the “leaky” cells of the descending limb of the loop of Henle that allow free passage of water and solute and the “tight” cells of the ascending limb that prevent water reabsorption. Reabsorption of solute without water sets the gradient for “free” water reabsorption at more

distal nephron sites that are sensitive to anti-diuretic hormone (vasopressin). Tubular water permeability regulated by hormone-sensitive aquaporin-2 favors the formation of concentrated or dilute urine depending on hydration state and the plasma level of anti-diuretic hormone. Fluid leaving the thick ascending limb of Henle is presented to the macula densa, part of the juxtaglomerular apparatus important as a signal sensor for tubuloglomerular feedback and control of preglomerular vascular resistance. After the distal tubule fluid enters the collecting duct where aldosterone dictates the amount of salt reabsorbed by principal cells and intercalated cells determine pH. Atrial natriuretic hormone favors sodium excretion by inhibiting epithelial sodium channels (ENaC) along the medullary collecting duct. Fluid leaving the collecting ducts passes out through the papilla and into the pelvis, ureter and bladder for excretion.

Whereas the loop of Henle and vasa recta in the renal medulla are primarily responsible for fluid concentration, the afferent arteriole is primarily responsible for determining the glomerular filtration rate and fluid flow entering through the tubules. Regulatory factors affecting afferent arteriolar resistance include neural and hormonal systems and paracrine / autacrine substances (180). In addition, this action of the afferent arteriole occurs as a myogenic response to the hydrostatic pressure in the afferent arteriole and as a tubuloglomerular feedback response to the composition in the distal tubule and is termed autoregulation (180).

Autoregulation was first observed in the kidney in the 1930s upon the observation that blood flow remains constant despite changes in perfusion pressure over a defined pressure range (125; 258; 102). Currently, two mechanisms of autoregulation are generally accepted: a rapid myogenic response and more delayed tubuloglomerular feedback (TGF)

(43). The myogenic response is an inherent property of VSMC of resistance arterioles that causes the vessels to constrict in response to increases in pressure/distension/stretch since isolated perfused afferent arterioles respond to mechanical stretch by constricting (158). Since myogenic response is flow- independent, it has been presumed that wall tension or hoop stress is the primary signal (92), however this has yet to be determined. Whatever the initiating factor, VSMC in the wall of the preglomerular arteries/arterioles depolarize in response to an increase in luminal pressure to activate L-type Ca^{2+} channels and produce vasoconstriction (see section C).

A second, slower autoregulatory mechanism also exists. The anatomy of the nephron is such that the macula densa region at the end of the thick ascending limb of Henle's loop (and also cortical collecting duct) touches the parent afferent arteriole. As a result of this anatomy, TGF can occur to regulate blood flow and glomerular function. Modified epithelial cells called macula densa sense the composition of tubular fluid (44). Signals of tubular origin are transduced to the afferent arteriole to alter vessel tone and thus regulate RBF and GFR. It is generally accepted that Na^+, K^+, and Cl^- concentration or load delivered in the tubular fluid are sensed by the $Na^+/K^+/2Cl^-$ transporter (NKCC2) located in the luminal membrane of macula densa cells (98). In response to increased electrolyte transport, macula densa cells produce varying levels of adenosine and/or ATP (29). VSMC of afferent and to a lesser extent, efferent arterioles possess a family of purinergic receptors (A1 receptors for adenosine or P2X1 receptors for ATP) that respond to ATP and adenosine to produce vasoconstriction (245).

Recent data suggest that third and fourth autoregulatory mechanisms may also exist. What is currently known as the third autoregulatory mechanism has been identified by three

groups of investigators and is slower than the myogenic response and TGF (128; 130) and has been characterized as very low frequency (224). Very recently animals lacking A1 adenosine receptors were given the NKCC2 inhibitor furosemide to reveal a fourth possible autoregulatory mechanism (131). Since the putative fourth mechanism shares a time course with TGF, however, it may simply represent a previously unidentified component of TGF that is independent of both A1 receptors and NKCC2.

The volume and composition of blood entering the kidney directly impacts sodium excretion and blood volume. Changes in GFR imparted by alterations in afferent and efferent arteriolar tone determine the amount of sodium passing into the proximal tubule and thus the amount of sodium reabsorbed. In addition, afferent arteriolar contraction and relaxation increases and decreases cortical blood flow. Since proximal and distal tubular sodium reabsorption occurs largely by diffusion, changes in cortical blood flow impact reabsorption in these segments (138). Efferent arteriolar tone determines medullary blood flow. This impacts reabsorption by proximal tubules of deep nephrons. In addition, it is thought that equilibration of the fluid in the descending limb of Henle's loop with the blood in the vasa recta of juxtamedullary nephrons is a necessary step in urine concentration. Thus, efferent arteriolar tone directly influences urine concentration 166; 203).

B. REGULATION OF VASCULAR TONE.

Due to the importance of renal resistance arteriolar diameter in governing (GFR and systemic AP, the mechanisms dictating the contractile state of these vessels has been the subject of a great deal of research. The basic cellular mechanisms of VSMC contraction are similar in systemic arteries and renal microvessels, with the bulk of the contractile response

relying on the regulation of cytosolic calcium concentration ($[Ca^{2+}]_i$). VSMC work together with underlying endothelial cells (EC) to dictate arteriolar tone, and $[Ca^{2+}]_i$ is a key regulatory factor in both cell types. In VSMC, $[Ca^{2+}]_i$ binds to calmodulin (CaM), leading to the activation of myosin light chain kinase and phosphorylation of myosin light chain. This alters the conformation of myosin such that the heads bind actin filaments and cause cellular contraction. Myosin light chain kinase is also regulated by myosin light chain phospohrylase, an enzyme that dephosphorylates and inactivates the kinase. Myosin light chain phosphorylase is, in turn, inhibited by phosphorylation by Rho kinase(43). In EC, on the other hand, $[Ca^{2+}]_i$ activates endothelial nitric oxide synthase, leading to production of nitric oxide (NO), a potent vasodilator(76). NO diffuses rapidly into nearby VSMC, activating guanylyl cyclase and increasing production of cyclic GMP. Cyclic GMP then produces VSMC relaxation by three main mechanisms: 1) directly inhibiting Ca^{2+} entry through store-operated Ca^{2+} channels, 2) stimulating large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) and K^+ efflux to produce plasma membrane hyperpolarization, thus inactivating voltage gated Ca^{2+} channels, and 3) increasing activity of cGMP-dependent protein kinase, an enzyme that phosphorylates and activates myosin light chain phosphatase leading to the dephosphorylation of MLCK (28).

Due to the importance of Ca^{2+} in vasomotor tone, VSMC have evolved a variety of different signaling pathways regulating a plethora of Ca^{2+} channels. These channels can cause global increases in $[Ca^{2+}]_i$ to produce vasoconstriction or a small subset of channels can act locally to produce small increases in localized Ca^{2+} called “sparks” that influence a variety of signals in subcellular domains. $[Ca^{2+}]_i$ is increased primarily via two general mechanisms: entry from outside via plasma membrane channels and release or mobilization

from sarcoplasmic reticular (SR) stores maintained by the sarcoplasmic/endoplasmic reticulum ATPase (SERCA).

Three families of Ca^{2+} channels exist in the plasma membrane: voltage-gated Ca^{2+} channels whose activities depend on the plasma membrane potential, and two classes of voltage-independent channels: receptor-operated Ca^{2+} channels (ROC) that are directly activated by G protein-coupled receptors (GPCR), and store-operated Ca^{2+} channels (SOC) that open in response to depletion of SR Ca^{2+} stores. Of the voltage-gated Ca^{2+} channels, three are known to be present in renal microvessels. L-type and T-type channels exist on both afferent and efferent arterioles (101) whereas P/Q type vessels are found only on afferent arterioles (104).

L-type channels have been implicated in the regulation of basal afferent arteriolar tone and responses to ATP, Ang II, norepinephrine (NE), vasopressin, and endothelin-1 (ET-1) (118; 141; 193). Inhibitors of L-type channels preferentially dilate the afferent arteriole, suggesting a minor role for L type channels in the regulation of basal efferent arteriolar tone (26). L-type channels in the efferent arteriole may be activated by aldosterone (7) or by inhibition of nitric oxide synthases (71). In the afferent arteriole, depolarization caused by cell stretching, nerve stimulation, or activation of GPCR on VSMC increases the probability of opening of the L-type channel to allow Ca^{2+} influx (85; 13).

More recently, T-type voltage gated Ca^{2+} channels have been shown to participate in the regulation of basal tone and in Ang II-induced constriction of both afferent and efferent arterioles. Inhibitors of T-type channels produce efferent and afferent arteriolar dilation (103; 70) and attenuate constrictor responses to Ang II (72) in an isolated perfused

hydronephrotic kidney model. The constrictor effect of T-channel activation is thought to be mediated by release of Ca^{2+} from SR stores since T channel inhibitors have no effect on Ang II responses in the hydronephrotic kidney model in the presence of inhibition of SERCA by thapsigargin (103).

P/Q type voltage-gated Ca^{2+} channels have also been identified in afferent arterioles, but the exact function and importance of these channels remain a mystery (104). One study has shown that inhibition of a subunit of P/Q type voltage gated Ca^{2+} channels attenuates $[Ca^{2+}]_i$ and contractile responses to depolarization in isolated afferent arterioles (100).

A family of plasma membrane transient receptor potential channels (TRP) are currently emerging as important regulators of $[Ca^{2+}]_i$ in renal microvessels. TRPC 1,3,4,5, and 6 have been identified in preglomerular resistance arterioles (56), with 3 and 6 being most abundantly expressed. TRPV4, TRPV5, and TRPM7 are expressed at very high levels in whole kidney homogenate, but the exact location of these channels is unknown (142). In addition, TRPC6 stimulation results in increases in $[Ca^{2+}]_i$ in renal afferent arterioles, suggesting a possible physiological function for this channel(67). TRP channels are voltage-independent and are therefore classified as ROCs (though it has been argued that some TRPs can be store-operated). In particular, TRPC6 has been shown to be receptor-operated in VSMC, opening in response to Ang II and vasopressin (207; 149). Activation of a GPCR stimulates TRP channels via phospholipase C (PLC)-mediated cleavage of phosphatidylinositol bisphosphate (PIP_2) to form diacylglycerol (DAG). DAG then activates a signaling pathway independent of protein kinase C (PKC) to stimulate TRP channels to allow Ca^{2+} entry (3).

Besides the channels listed above, it has long been thought that another channel exists in the plasma membrane that opens to allow Ca^{2+}entry into the cell after depletion of SR or ER stores. This process was termed "capacitative calcium entry" or "store-operated calcium entry" and the putative channels involved were called "Ca^{2+} release activated Ca^{2+}current channels" (CRAC) or store-operated Ca^{2+} channels (SOC). Early attempts at the identification of SOCs centered around the TRP channels, in particular canonical TRP channels (TRPCs) (263; 243; 154; 200; 155; 249; 264). Fault was seen in most of these experiments, however, and TRPC1 is the only TRP channel currently thought to be involved in store-operated Ca^{2+} entry (5; 115). The main argument against the role of TRP channels as SOC is that the large, non-selective currents generated by TRP channels differ from the currents generated upon store-depletion (5; 225). Recently, however, a channel system has been identified whose current looks very similar to the traditional CRAC currents. This system consists of two proteins: Stim1 and Orai1 or Orai2 (169; 205). Stim1 is thought to reside in the membrane of the SR/ER and contain an EF hand domain that senses Ca^{2+} levels inside SR/ER stores. Conformational changes in Stim are thought to cause changes in the conformation of Orai whereupon Orai allows Ca^{2+} to enter the cell through its central pore (199). These new data does not exclude the possibility that TRP channels may also function as SOC channels with different currents from the traditional CRAC currents. Whether or not TRP channels use Stim1 as a sensor is currently being debated (199; 5).

In the afferent arteriole, Ca^{2+} entry can be almost obliterated by the L-type voltage-gated Ca^{2+} channel antagonist, nifedipine (27). This is not the case in the efferent arteriole, however, and it has been suggested that SOCs are responsible for much of the Ca^{2+} entry (157). Although less important in Ca^{2+} entry in the afferent arteriole, SOCs are also present

in this vascular segment (58). Interestingly, Ca^{2+} entry via L-type channels and SOC is enhanced in preglomerular VSMC of the spontaneously hypertensive rat (SHR) (60; 124; 69). The roles of Stim1, Orai1 and 2, and TRPC1 store-operated Ca^{2+} entry in renal VSMC remain to be determined.

In the SR, ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate receptors (IP_3R) regulate efflux of Ca^{2+}stores maintained by SERCA. IP_3R are present in the membranes of the SR and are activated by IP_3. GPCRs activate PLC leading to cleavage of PIP2 into IP_3 and DAG. As mentioned previously, DAG goes on to activate TRP channels in the plasma membrane. IP_3 on the other hand, diffuses into the cytoplasm to activate IP_3R on the SR. Once activated, a conformational change causes IP_3R to release Ca^{2+} from SR stores (218; 182).

Type 1 IP_3R are expressed in renal afferent arterioles and glomeruli (106) and play important roles in vasoconstriction of renal vessels. IP_3 is required for autoregulation in a blood perfused juxtamedullary nephron preparation (119). Ang II-induced Ca^{2+} transients in afferent arterioles(61) and isolated preglomerular VSMC(81), and RBF responses in vivo(206) are significantly attenuated during IP_3R inhibition. The actions of norepinephrine(208), vasopressin(68), and ghrelin(52) on VSMC $[Ca^{2+}]_i$ regulation in the renal vasculature are also mediated by IP_3.

RyR are also present in SR membranes where they function to amplify any small increase in $[Ca^{2+}]_i$. Ca^{2+} binds directly to the RyR causing a conformational change that allows Ca^{2+} to pass from the SR, through the pore between the four subunits of the RyR, and into the cytosol (117). Recent research indicates that RyR are present in the renal vasculature

and participate in renal vascular reactivity to a variety of GPCR agonists (60; 59; 240; 277; 212). RyR signaling is discussed at length below.

In addition to SR and extracellular stores, a small amount of Ca^{2+} is maintained in other organelles and contributes to overall $[Ca^{2+}]_i$ in some cell types. Late endosomal and lysosomal vesicles possess a Ca^{2+}/H^+ exchanger that maintains a high concentration of Ca^{2+} in these organelles. The contribution of these vesicles to renal vasoconstriction has not been studied, however both pulmonary and coronary artery VSMC show attenuated Ca^{2+} signaling in the absence of acidified vesicles, indicating participation of lysosomal Ca^{2+} stores in global VSMC Ca^{2+} responses(274; 135). It is important to note that the amount of Ca^{2+} inside these vesicles is not large enough to trigger a global Ca^{2+} response and that RyR coupled to lysosomal vesicles most likely amplify small Ca^{2+} effluxes from acidic vesicles (135).

Although their function has not been studied specifically in renal microvessel VSMC mitochondria also participate in the regulation of $[Ca^{2+}]_i$ in some VSMC. Mitochondria take up large quantities of Ca^{2+} from the cytoplasm. As a result of this property, mitochondria influence $[Ca^{2+}]_i$ in two ways: 1) decreases in $[Ca^{2+}]_i$ modulate SOC and 2) positioning of mitochondria near Ca^{2+} channels can alter the influence of that channel on $[Ca^{2+}]_i$. Mitochondria positioned near the SR have also been shown to directly mediate the activity of IP_3R in the SR(30).

Many of the same Ca^{2+} channels in VSMC are also present in endothelial cells with Ca^{2+} increases activating eNOS and releasing NO to cause vasodilatation(163) as well as regulation of PLA2 and production of prostaglandins. Voltage-gated Ca^{2+} channels (23; 246),

store-operated Ca^{2+} channels(186; 40), IP_3R(187; 96), receptor-operated channels(192; 126), and RyR(153) are all present in endothelial cells. Vasodilators such as bradykinin activate these channels to enhance NO production (25).

C. RYANODINE RECEPTORS--REGULATORS OF $[Ca^{2+}]_i$

Half a century ago, the observation was made that ryanodine, an insecticide isolated from the South American plant *Ryania speciosa* could contract skeletal (196) and cardiac muscle (109) by excitation-contraction coupling. At that time it was widely believed that Ca^{2+} release from the SR/ER was due to reversal of SERCA (42). Studies using $^{45}Ca^{2+}$ then led to the discovery that release occurred in the cisternae of the SR/ER, areas in which SERCA was absent (174). The search for the Ca^{2+} release channel soon focused on identification of the target of ryanodine and the RyR was characterized (190). RyR binding assays resulted in the discovery that ryanodine colocalized with the bursts of Ca^{2+} observed in cisternae (75). The primary function of RyR is Ca^{2+} mobilization and excitation-contraction coupling. In non-skeletal muscle, this largely occurs by amplification of $[Ca^{2+}]_i$ by Ca^{2+}-induced Ca^{2+} release (CICR).

Early on, scientists determined that skeletal muscle RyR differed structurally and functionally from cardiac RyR (116; 171). Presently, three RyR subtypes have been identified and termed RyR1 (the typical skeletal muscle RyR), RyR2 (the primary cardiac muscle RyR), and RyR3, a more ubiquitously expressed RyR originally characterized from brain tissue (185). Skeletal muscle RyR are coupled to dihydropyridine receptors in the skeletal muscle plasma membrane so that opening of these channels directly triggers release of Ca^{2+} from the SR by conformational coupling(270). Ca^{2+} entry is not a prerequisite for

RyR opening in skeletal muscle. Rather, a conformational change of the dihydropyridine receptor leads to RyR opening (16). Non-skeletal RyR do not physically interact with voltage-gated Ca^{2+} channels, but rather amplify Ca^{2+} by CICR secondary to Ca^{2+} entry. The magnitude of Ca^{2+} release elicited by RyRs may vary according to subtype. Whereas RyR1 and RyR2 are thought to primarily mediate cellular-wide global Ca^{2+} responses, stimulation of RyR3 releases only small amounts of Ca^{2+} from the SR/ER in highly localized regions (251; 195). These small Ca^{2+} "sparks" may exist in microdomains near Ca^{2+}-activated K^+ channels to produce membrane hyperpolarization (156; 137).

Gating of RyRs and/or sensitivity to Ca^{2+} can be altered by a variety of factors including protein binding, ion binding, oxidation, nitrosylation, and phosphorylation. Calmodulin binds to RyR in both Ca^{2+} bound and unbound states. In skeletal muscle, Ca^{2+} bound calmodulin impairs RyR gating, whereas Ca^{2+} free calmodulin enhances activity (80). In cardiac muscle, calmodulin inhibits RyR opening independent of whether it is bound to Ca^{2+} (168) and decreases RyR2 sensitivity to Ca^{2+}(15). Calmodulin also alters RyR sensitivity by activating calcium/calmodulin-dependent protein kinase II (CaMII), leading to phosphorylation and activation of RyR(259; 99). RyR in cardiac muscle can also be phosphorylated by protein kinase A (PKA). PKA is anchored to RyRs by the A kinase anchoring protein mAKAP (15). The effect of serine phosphorylation by PKA is controversial. Some labs suggest that phosphorylation impairs binding of inhibitory FK506 binding proteins (FKBP) leading to activation(164; 165). These results have been contested, however (226; 262). In renal VSMC, activation of PKA by prostaglandin PGI_2 may lead to vasodilation by inhibiting IP_3-mediated Ca^{2+} release (198).

Several other proteins have been shown to regulate RyR activity. Most notably, the FKBP, known for their affinity for the immunosuppressant FK506 (tacrolimus) are potent inhibitors of RyR. FKBP stabilize the RyR tetramer and decrease sensitivity to Ca^{2+}(15). RyR1 and RyR3 specifically associate with FKBP12. RyR2 can associate with both FKBP12 and FKBP12.6, with much higher affinity for FKBP12.6 (15). Sorcin is also inhibitory and may help turn off RyR responses since it binds to RyRs only in the presence of high $[Ca^{2+}]_i$ (15). Calsequestrin, a SR/ER Ca^{2+} binding protein, may inhibit RyR by forming a complex with junction and triadin and binding to RyR when $[Ca^{2+}]$SR/ER is low(97). The adapter protein homer inhibits RyR2 and activates RyR(15).

Ca^{2+} can activate RyR from both the cytosol and the SR/ER lumen. Cytosolic Ca^{2+} can inhibit RyR1 at high concentrations (1 mM), and RyR2 at even higher concentrations (10 mM) (144). In addition to Ca^{2+}, 2 mM Mg^{2+} can also bind RyR1 and 2 to competitively inhibit RyR responses to Ca^{2+}. In the presence of physiological ATP (5 mM), free $[Mg^{2+}]$ is low (0.5-1 mM) since most Mg^{2+} is bound by ATP. In this way, ATP enhances RyR activity through decreasing free Mg^{2+}. Under certain physiological conditions such as ischemia, however, ATP levels decrease and Mg^{2+} levels increase, causing Mg^{2+}-induced inactivation of RyRs(15). RyR3s are not inhibited by Ca^{2+} or Mg^{2+} (144).

All 3 RyR exist in VSMC (107; 181) and RyR have been implicated in VSMC responses to constrictor responses to Ang II(61), norepinephrine(173), acetylcholine(87), and endothelin-1(62; 135) and dilatory responses to agmatine, bradykinin, and NO(276).

D. IDENTIFICATION AND CHARACTERIZATION OF ADP RIBOSYL CYCLASE

IP_3 was identified in the early 1980s as a second messenger responsible for Ca^{2+} mobilization (230). Subsequent studies showed incidences where Ca^{2+} mobilization in several cell types such as airway epithelium, pancreatic beta cells, and sea urchin oocytes can occur in the presence of little or no IP_3 production (167; 233). Cyclic ADP ribose (cADPR) was one of several compounds identified in the search for Ca^{2+} mobilizing metabolites along with sphingosine derivatives, cyclic IP_3, arachadonic acid, and inositol tetrakisphosphate (256). While there were little data ever produced indicating the importance of the other identified compounds in vivo, cADPR is equally potent at mobilizing Ca^{2+} as IP_3 (45) and is present in a variety of mammalian tissues including brain, heart, and liver (247). Furthermore, an endogenous producer of cADPR, ADP ribosyl cyclase (ADPR cyclase) was isolated from the ovotestes of the sea hare *Aplysia californica* (145) and subsequently found to share homology with two mammalian enzymes: CD38 and CD157 (231; 112). It is possible that other mammalian isoforms exist including a form specific to aortic VSMC (49) and one unique to mesangial cells(178; 134).

Further characterization revealed that ADPR cyclases catalyze a reaction transforming NAD^+ to cADPR and nicotinamide(93). In dimeric form, ADPR cyclases residing in the plasma membrane produce cADPR, functioning optimally at a pH of 6.7. In addition plasma membrane cyclases act as ADPR hydrolases, converting cADPR to ADPR. Dimeric ADPR may enhance the Ca^{2+} mobilizing actions of cADPR(46). Studies using erythrocyte ghosts showed that the catalytic activity of ADPR cyclases occurs primarily in the extracellular domain (147). As a result of a large pore formed between monomers in the dimeric form, it was hypothesized that cADPR is formed extracellularly and transported through the pore into the cell where it acts to mobilize Ca^{2+} (78). 3T3 fibroblasts lacking

ADPR cyclase also transport cADPR, however, so other nucleoside transporters responsible for importing extracellularly-formed cADPR must exist (78). One conundrum remained, however. Extracellular concentrations of NAD^+ are low except during cases of tissue necrosis, so how does the extracellular enzymatic portion of ADPR cyclase access regulated levels of its substrate? To answer this question, experiments were performed in which connexin 43 hemichannels were shown to colocalize with ADPR cyclase in the plasma membrane and transport NAD^+ (78). It is currently thought that the hemichannels provide a high enough local concentration of NAD^+ near the extracellular domain of the cyclase to drive the forward reaction, producing cADPR. It has also been proposed that uncharacterized channels exist that link adjacent cells to transport cADPR from one cell to another. This has been demonstrated in tracheal smooth muscle strips whose $[Ca^{2+}]_i$ increases in response to cADPR production by neighboring mucosal cells.

It is clear that cyclase dimerization is required for cADPR production. The remaining question is what signal produces dimerization. Like many molecules, dimers of ADPR cyclase form via disulphide bonding, a likely consequence of oxidation of thiol groups by superoxide. Oxidative stress enhances cADPR production by ADPR cyclases of bovine coronary artery VSMC (271). In isolated afferent arterioles, Ang II increases superoxide production and Ca^{2+} responses are inhibited by the superoxide dismutase mimetic tempol (64). In the same study, Ca^{2+} responses to Ang II were also shown to be attenuated in the presence of the NADPH oxidase inhibitor apocynin (64). The already attenuated responses show no further inhibition by subsequent addition of the ADPR cyclase inhibitor nicotinamide, suggesting a common signaling pathway. Collectively, these experiments indicate that ADPR cyclase is likely activated by NADPH oxidase-induced production of

superoxide in the plasma membrane. Recent research indicates that ADPR cyclase may also activate NADPH oxidase in the SR membrane of bovine coronary arterial myocytes(275). Application of a cell permeable indicator of reactive oxygen species showed local generation of superoxide near the SR membrane that was blocked by inhibitors of NADPH oxidase, ADPR cyclase, or RyR. Since redox status may alter RyR activity, it was suggested that superoxide from SR NADPH oxidase may directly increase the probability of opening of RyR. This hypothesis remains to be tested, however.

It was obvious from early studies that IP_3 and cADPR signaling overlapped based on the characteristics of Ca^{2+} release induced by cADPR and IP_3 in sea urchin egg homogenates (45). For this reason, it was first thought that cADPR may activate IP_3Rs in the ER membrane. This was proven not to be the case as inhibitors of IP_3R had no effect on cADPR-induced Ca^{2+} release (45) and since IP_3 did not competitively displace radiolabelled cADPR (146). The mechanism of cADPR-induced Ca^{2+} mobilization was discovered to occur via binding to RyR in the ER membrane (83). When ryanodine was given at an inhibitory dose of 100μM, cADPR-induced Ca^{2+} mobilization from rat brain microsomes was attenuated (254). Other studies showed that cADPR increases the frequency of cardiac RyR channel opening (170). Photoaffinity labeling revealed that cADPR binds to two proteins, both much smaller than RyR, indicating that cADPR most likely did not directly bind to RyR (248).

It was soon determined that a family of inhibitory FK506 binding proteins including FKBP12 and FKBP12.6 bind to RyR to prevent Ca^{2+} activation(242). Pharmacologically, FK506 removes FKBP from RyR, increasing open probability of RyR (239). Experiments in pancreatic cell microsomes show that cADPR removes FKBP12.6 from RyR and that

microsomes lacking FKBP12.6 show no Ca^{2+} release in response to cADPR. These results suggest that cADPR activates RyR by removing inhibitory FKBP (184). Further evidence for this hypothesis comes from experiments on reconstituted RyR from bovine aortic VSMC. An antibody against FKBP12 prevents cADPR-induced increases in the probability of opening of RyRs (239). Tracheal myocytes lacking FKBP12.6 do not respond to cADPR (250).

While knowledge of the specific intracellular actions of cADPR was expanding, a key discovery solidified the significance of ADPR cyclase as an important regulator of $[Ca^{2+}]_i$. In addition to IP_3 and NAD^+, NADP could also cause Ca^{2+} release from sea urchin microsomes (41). This Ca^{2+} release was insensitive to the IP_3R inhibitor heparin and the cADPR competitive antagonist 8-amino-cADPR and occurs by production of nicotinic acid adenine dinucleotide phosphate (NAADP) (148). Furthermore, thionicotinamide-NADP, an analogue of NADP which does not inhibit IP_3- or cADPR-induced Ca^{2+} release, inhibited the actions of NAADP injected into sea urchin eggs (35). Endogenous NAADP releases Ca^{2+} from internal stores of a variety of mammalian tissues as well (34).

The search for the mechanism by which NAADP is generated in intact cells was soon underway. ADPR cyclase isolated from *Aplysia californica* and human CD38 could synthesize NAADP from NADP in an *in vitro* setting at a pH of 4.0(1). This requirement for a low pH was a welcome surprise since both *Aplysia* ADPR cyclase and CD38 were known to internalize into endocytic vesicles (1; 221; 47). After it was determined that ADPR cyclases produced NAADP, the question remained as to how NAADP was degraded. An efficient second messenger must be degraded to ensure sensitivity of the system. Recent experiments show that, like cADPR, NAADP can be converted to ADP-ribose 2'-phosphate

by CD38 (94), thus reversing the actions of NAADP production. Unlike synthesis of NAADP, which only occurs at low pH, breakdown of NAADP can occur at physiological or low pH.

NAADP induces colocalization of lysosomal vesicles with RyR (135; 136). This idea spurred the "trigger zone" hypothesis proposed by Kinnear et al. These investigators thought that NAADP receptors inside of lysosomal vesicles allowed small amounts of Ca^{2+} to exit the vesicle upon production of NAADP by lysosomal ADPR cyclase. It was later shown that NAADP binds to TRP-ML1 channels in lysosomal membranes to cause this Ca^{2+} release (2; 14). This small amount of Ca^{2+} activates nearby RyR on the SR/ ER to amplify the signal and cause global Ca^{2+} increases. Another recent advance in thinking about NAADP signaling is based on the observation that NAADP (isolated from sea urchin eggs) requires a phospholipid environment to potentiate binding to its receptor (37). NAADP might also be produced by ADPR cyclases in lipid rafts or calveole in addition to production in lysosomal vesicles. Cell membranes of pulmonary VSMC fractionated based on cholesterol content reveal that regions enriched in cholesterol (lipid rafts/calveolae) contain high levels of CD38. Furthermore, cholesterol depletion attenuates Ca^{2+} responses to endothelin ET_B receptor stimulation and ET_B -mediated enhancement of ADPR cyclase activity was abolished in the absence of calveolae. More research is needed to explore this exciting new aspect of NAADP signaling.

E. EVIDENCE SUGGESTING A ROLE FOR ADPR CYCLASE IN NON-RENAL VASCULAR SMOOTH MUSCLE

Due to the virtually ubiquitous role of ADPR cyclase in Ca^{2+} signaling and the importance of Ca^{2+} signaling in regulation of vascular tone, it is not surprising that the

hypothesis appeared shortly after the discovery of cADPR and NAADP that these second messengers are important in hemodynamics. Indeed, eight years after the initial characterization of cADPR from sea urchin eggs, the first study was published examining the effects of this second messenger on Ca^{2+} mobilization from the SR of coronary arterial myocytes (132). In this study, porcine VSMC were permeabilized and injected with cADPR. cADPR increased $[Ca^{2+}]_i$ in these cells, an effect that was not blocked by the IP_3R inhibitor heparin. Depletion of SR Ca^{2+} stores resulted in no effect of cADPR. Not only is ADPR cyclase present in VSMC, but activity is at least 20 times higher than that seen in HL-60 cells, an immune cell type containing the ADPR cyclase/antigen CD38(50). cADPR was subsequently shown to increase Ca^{2+} in aortic VSMC microsomes(269). Inhibition of ADPR cyclase produced relaxation of pre-contracted coronary artery rings, indicating a role for ADPR cyclase in the maintenance of vascular tone (88). Although prior to the work presented herein, therewas no current evidence implicating ADPR cyclase in regulation of vascular tone in an animal in vivo, pulmonary arteries had been shown to be prevented from constricting under hypoxic conditions in rat lung in situ (53).

Characterization of VSMC ADPR cyclase revealed the enzyme to be similar to previously characterized ADPR cyclases. A selective antagonist of cADPR, 8Br-cADPR attenuated KCl-induced Ca^{2+} and contractile responses in isolated small coronary arteries (273), indicating involvement of cADPR in CICR. Like other cell types, cADPR increases the open probability of RyRs reconstituted from coronary artery VSMC (151). cADPR in VSMC also likely works by removal of FKBP12.6 from RyRs since FKBP12.6 and not FKBP12 is expressed in coronary arterial VSMC and since both FK506 and a nonselective FKBP antibody attenuate the increase in open probability of reconstituted RyRs observed

after treatment with cADPR(239). The similarities of ADPR cyclase signaling between cell types is not surprising considering the ubiquitous nature of CD38, an ADPR cyclase family member expressed in VSMC(50; 173). Another ADPR cyclase family member may exist with properties different from CD38. Unlike CD38 from HL-60 cells, aortic ADPR cyclase is inhibited by Zn^{2+} and Cu^{2+} and activated by retinoic acid and 3,5,3'-triiodothyronine, a property different from HL-60 cell CD38(50). Furthermore, unlike ADPR cyclase in endothelial cells (276) and sea urchin egg homogenates(255), VSMC ADPR cyclase is inhibited by NO(266), suggesting cell type specific properties of ADPR cyclases.

Although the data are sparse, ADPR cyclase is likely also present in endothelial cells. ADPR cyclase activity is present in coronary artery endothelial cells under resting conditions. This activity is increased by the vasodilator bradykinin and 8Br-cADPR impairs bradykinin-induced Ca^{2+} transients in intact coronary artery endothelium (276) indicating a possible role for ADPR cyclase in bradykinin-mediated dilation secondary to Ca^{2+} activation of eNOS.

Of physiological relevance, ADPR cyclase is likely a downstream target for a variety of G protein coupled receptors (GPCRs) important in vascular health and disease. Small mesenteric arteriolar contractile responses (90) and shark anterior mesenteric arterial Ca^{2+} responses (62) to endothelin-1(ET-1) are attenuated in during ADPR cyclase and cADPR inhibiton. Contractile responses to norepinephrine are smaller in aortas of CD38-/- mice than in wild type control animals(173) and coronary artery SMC responses to acetylcholine are attenuated by inhibitors of ADPR cyclase and cADPR(87), further proof of the important role of ADPR cyclase in agonist-induced responses in VSMC. NAADP may be particularly important in VSMC responses to endothelin-1. Coronary arterial SMCs produce NAADP in

response to ET-1 (274) and both pulmonary (135) and coronary (274) arterial VSMC Ca^{2+} responses are significantly blunted by disrupters of NAADP signaling.

F. EVIDENCE SUGGESTING A ROLE FOR ADPR CYCLASE IN THE KIDNEY

CD38 and CD157 are both expressed at high levels in kidney tissue (122; 234). In addition, there is evidence suggesting that the kidney may express an alternative form of ADPR cyclase since the inhibitor 4,4-dihydroxyazobenzene is reported to inhibit ADPR cyclase in kidney but not human CD38 or ADPR cyclase from other tissues(178). The kidney is an extremely heterogeneous tissue, however, where many specialized cell types work together to achieve the overall goal of capillary fluid exchange and epithelial transport. In vitro studies indicate that ADPR cyclase likely mediates key functions of many kidney tissue types. The concerted effort of VSMCs and endothelial cells in small arterioles, including the afferent arterioles controls vascular resistance and the volume of blood passing through the glomerulus and ultrafiltrate into the tubules. ADPR cyclase activity is present in VSMC of small renal arterioles where it mediates increases in $[Ca^{2+}]_i$ as shown by cADPR injection into permeabilized single renal VSMC (150), and the cADPR inhibitor 8Br-cADPR attenuates responses to $CaCl_2$ in small renal VSMC, indicating participation of cADPR in CICR. The strength of this hypothesis is solidified by findings that Ca^{2+} increases by KCl-induced hyperpolarization in isolated renal afferent arterioles are attenuated in the presence of 8Br-cADPR (66).

Much recent work has been done indicating that isolated renal afferent arterioles rely heavily on ADPR cyclase and cADPR to mediate signaling pathways downstream of GPCR. Ang II-induced Ca^{2+} responses in afferent arterioles were significantly attenuated in the

presence of ADPR cyclase inhibitors nicotinamide and Zn^{2+} or 8Br-cADPR (61). Addition of superoxide inhibitors did not enhance this effect of ADPR cyclase inhibitors, but did attenuate Ang II-induced Ca^{2+} transients on their own, indicating a dependence of ADPR cyclase signaling responses to Ang II on superoxide production(64). ET_A receptor signaling is also inhibited by nicotinamide and 8Br-cADPR more than signaling mediated by ET_B receptors, suggesting heterogeneity amongst GPCR signaling pathways in the renal vasculature (65).

Although very little work has been done to confirm the fact, endothelial cells of preglomerular resistance arterioles may also possess ADPR cyclase signaling pathways downstream of vasodilators. One study showed that agmatine, a vasodilator that works by increasing NO production in endothelial cells produces its effects by activation of ADPR cyclase. Single nephron GFR is increased by agmatine, indicative of vasodilatation of preglomerular resistance arterioles, an effect attenuated in the presence of 8Br-cADPR (212).

Glomerular mesangial cells have very high ADPR cyclase activity (36) when compared to whole kidney homogenates. The expression and activity of CD38 in these cells is regulated by TNF-α, indicating a likely immune function (268). cADPR may function to regulate mesangial cell proliferation and protein synthesis via alterations in $[Ca^{2+}]_i$ (134). NAADP also elicits Ca^{2+} release from isolated mesangial cell microsomes, but the effect of this release is unknown (267). Glomeruli also have very high levels of ADPR cyclase activity (36), though little is known about the precise function of ADPR cyclase in glomeruli.

Juxtaglomerular cells at the end of the afferent arteriole produce renin to regulate levels of local and circulating Ang II for long-term regulation of GFR and tubular salt

reabsorption as well as aldosterone production. Cultured As4.1 juxtglomerular cells produce cADPR for the regulation of $[Ca^{2+}]_i$ (265). Increased $[Ca^{2+}]_i$ commonly inhibits renin synthesis and release (216) . Further study is required to determine the physiological importance of cADPR or ADPR cyclase in renin release.

G. STATEMENT OF PURPOSE

I have tested the hypothesis that the ADPR cyclase signaling cascade participates importantly to the regulation of renal hemodynamics. To this end, I have used a combination of $[Ca^{2+}]_i$ measurements and molecular biological analysis of ADPR cyclase mRNA levels in isolated preglomerular resistance arterioles and in vivo RBF and MAP measurements in anesthetized rats and mice. These studies provide the first evidence indicating renal vascular expression of ADPR cyclases as well as a physiological role for the enzyme and its downstream targets in the regulation of renal afferent arteriolar $[Ca^{2+}]_i$, baseline RBF regulation, and integrated RBF responses to GPCR agonist-induced renal vasoconstriction. Characterization of the physiological effects of this pathway will further our knowledge of the regulation of renal hemodynamics in health and disease.

CHAPTER II

ADP-ribosyl cyclase and ryanodine receptor activity contribute to basal renal vasomotor tone and agonist-induced renal vasoconstriction in vivo

A. ABSTRACT

An important role for the enzyme ADP-ribosylcyclase (ADPR cyclase) and its downstream targets, the ryanodine receptors (RyR), is emerging for a variety of vascular systems. We hypothesized that the ADPR cyclase/RyR pathway contributes to regulation of renal vasomotor tone in vivo. To test this, we continuously measured renal blood flow (RBF) in anesthetized Sprague-Dawley rats. Infusion of the ADPR cyclase inhibitor nicotinamide intrarenally at low doses inhibits angiotensin II (Ang II)- and norepinephrine (NE)-induced vasoconstriction by 72% and 67% ($P<0.001$). RBF studies in rats were extended to mice lacking the predominant form of ADPR cyclase (CD38). Acute renal vasoconstrictor responses to Ang II and NE are impaired by 59% and 52%, respectively in anesthetized CD38-/- mice compared to wild type controls ($P<0.05$). Intrarenal injection of the RyR activator FK506 decreases RBF by 22% ($P<0.03$). Furthermore, RyR inhibition with ruthenium red attenuates Ang II and NE responses by 50% and 59%, respectively ($P\leq0.01$). Given at higher doses, nicotinamide increases basal RBF by 22% ($P<0.001$). Non-receptor-mediated renal vasoconstriction by L-type voltage-gated Ca^{2+} channels is also dependent on ADPR cyclase and RyRs. Nicotinamide and ruthenium red inhibit constriction by the L-type channel agonist Bay-K8644 by 59% ($P<0.02$) and 63% ($P<0.001$). We conclude that: 1) ADPR cyclase activity contributes to regulation of renal vasomotor tone under resting conditions, 2) renal vasoconstriction induced by G-protein coupled receptor agonists Ang II and NE is mediated in part by ADPR cyclase and RyRs, and 3) ADPR cyclase and RyRs participate in L-type channel-mediated renal vasoconstriction in vivo.

B. INTRODUCTION

Regulation of renal hemodynamics is essential for the maintenance of fluid and electrolyte balance and arterial blood pressure. Recent rises in morbidity and mortality rates associated with obesity and hypertension have made apparent the urgency of understanding the molecular mechanisms of blood pressure regulation, resulting in discovery of complex, integrated Ca^{2+} signaling pathways for regulation of renal vascular resistance (RVR) (6; 210). Stimulation of these pathways leads to increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) via activation of plasma membrane Ca^{2+} channels as well as Ca^{2+} release channels in the sarcoplasmic reticulum (SR). The latter include inositol trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs). The wide variety of mechanisms involved in $[Ca^{2+}]_i$ regulation make it possible for vascular smooth muscle cells (VSMCs) to generate individualized responses to different vasoconstrictor stimuli. For example, it has been suggested that greater than 50% of angiotensin II (Ang II)-induced changes in $[Ca^{2+}]_i$ in afferent arterioles occurs by Ca^{2+} entry, whereas the actions of norepinephrine (NE) are more dependent on Ca^{2+} mobilization from internal stores (210).

The role of IP_3Rs in agonist-induced regulation of vascular resistance has been extensively studied (73; 119; 206; 208). Considerably less is known about the role of RyRs in agonist-induced constriction in renal and other vascular beds (21; 51). One means of RyR activation involves the enzyme ADP ribosyl cyclase (ADPR cyclase) and generation of the second messengers cyclic ADP ribose (cADPR) and nicotinic acid ADP (NAADP) (61; 272). Evidence indicates ADPR cyclase is important in Ca^{2+} signaling in the renal vasculature. Cyclase activity is high in lysates from VSMCs in renal microvessels and glomeruli(36; 150) and cADPR applied extracellularly to isolated permeabilized VSMCs from rat renal artery

increases $[Ca^{2+}]_i$ (150). Furthermore, cADPR contracts isolated rat interlobular arteries (150), and inhibition of ADPR cyclase, cADPR, or RyRs attenuates Ca^{2+} responses to Ang II or endothelin-1 (ET-1) in isolated rat afferent arterioles (61; 65). These in vitro studies demonstrate a potential physiological role for the enzyme in the renal microcirculation.

Whereas in vitro stimulation of ADPR cyclase activity in VSMCs leads to activation of pathways implicated in vasoconstriction, the actions of this enzyme in endothelial cells are predicted to cause vasodilatation. Inhibition of ADPR cyclase, cADPR, or RyRs is reported to prevent production of nitric oxide (NO), a potent vasodilator, in response to bradykinin or the Ca^{2+} ionophore A-23187 in bovine coronary artery endothelium (272). In some cases, ADPR cyclase in VSMCs may lead to dilation rather than constriction. This is suggested by the fact that inhibition of cADPR in isolated rat renal arteries attenuates relaxation produced by urocortin (211).

The importance of the ADPR cyclase/RyR signaling pathway has not been determined in any vascular network in vivo. Although experiments in isolated cells and vessels provide useful information regarding the nature of ADPR cyclase, the seemingly opposite results of ADPR cyclase activation in VSMCs and endothelial cells in vitro do not integrate possible interactions between endothelial cells and VSMCs that occur in vivo. To address this deficiency, we tested the hypothesis that the ADPR cyclase pathway contributes to both basal renal vascular tone and vasoconstriction produced acutely by Ang II, and norepinephrine (NE), and the L type voltage-gated Ca^{2+} channel agonist, Bay-K8644. The effects of pharmacological inhibitors of ADPR cyclase and its downstream effectors, RyRs, were determined in acute RBF studies conducted on anesthetized rats. The effects of genetic

deletion of ADPR cyclase were determined in acute RBF studies comparing mice lacking the predominant form of ADPR cyclase (215), CD38 (CD38-/-) with wild type animals.

C. MATERIALS AND METHODS

Sprague-Dawley rats were obtained from our local breeding facility. CD38-/- mice on a C57 black 6 background were obtained as breeding pairs from Dr. Fran Lund (Trudeau Institute, (188)) and bred locally. Wild type mice of a similar background were obtained from Jackson Labs as breeder pairs. All animals were cared for and used for research in accordance with institutional guidelines. Animals were anesthetized using sodium pentobarbital (Nembutal, 50-60 mg/kg body wt ip for rats, 80-90 mg/kg body wt ip for mice, Abbott, Chicago, IL).

The surgical preparation for acute RBF studies in rats was performed as is standard for our laboratory (129). Briefly, the right femoral artery was catheterized for continuous measurement of MAP. The right femoral vein was catheterized for infusion of bovine serum albumin (4.75% at 50 µl/min for a length of time equal to body wt/4, then reduced to 10µl/min for the remainder of the experiment) and administration of subsequent doses of pentobarbital as needed. A tracheotomy was performed and a curved catheter was inserted into the aorta and positioned at the opening of the left renal artery for direct intrarenal infusion of pharmacological agents. RBF was measured in the left renal artery by a flow probe (model 1RB, Transonic, Ithaca, NY).

The surgical preparation for RBF studies in mice was previously established in our laboratory (19) and modified for the purposes of this study. Briefly, a pulled PE100 catheter was inserted into the right femoral artery and attached to a pressure transducer (Statham P23

DB) for measurement of MAP. Two pulled PE10 catheters were inserted into the femoral vein for bolus injections of Ang II and NE and continuous infusion of 2.4% bovine serum albumin (10 µl/min) throughout the experiment. A tracheotomy was performed using a PE100 catheter. A flow probe (Transonic system TS420, Ithaca, NY; 0.5-V probe) was placed around the left renal artery for measurement of RBF. Animals were allowed to stabilize for 1 hr prior to the start of an experiment. Hematocrit, urine flow, kidney wt, and body wt were measured to ensure consistency of animal conditions.

Bolus Injections- For rats, bolus injections (10 µl) were given intrarenally in the manner standard in our laboratory (129). The following concentrations were used: Ang II (0.38 µM = 4 ng), NE (9.75 µM = 20 ng), FK506 (31 mM = 250 µg), Bay-K8644 (0.70 mM = 2.5 µg). The same doses of Ang II and NE were used for 10 µl venous injection in mice.

Pharmacological Inhibitors- We used nicotinamide to inhibit ADPR cyclase (24; 88; 152; 172). Nicotinamide is a byproduct of the ADPR cyclase reaction(145), and shifts the reaction to produce NAD+ rather than cADPR or NAADP. Nicotinamide may also inhibit Poly (ADP-ribose) polymerase-1 (PARP-1) (179; 260). PARP-1 is found in both endothelial cells (232) and VSMCs (91) and is activated under situations of extreme stress (8). Since the animals used in our study were closely monitored for stable hemodynamic conditions, activation of PARP-1 is unlikely.

Ruthenium red was used to inhibit RyRs. The effects of ruthenium red on RyRs are well documented (39; 161). Ruthenium red may also inhibit Ca^{2+}-activated K+ channels (BK_{Ca}) (261). Although these channels have been shown to be present in renal microvessels (57), BK_{Ca} channel inhibition would result in vessel contraction and our studies show

ruthenium red inhibits agonist-induced contraction, arguing that the predominant effect of ruthenium red is on RyRs.

Nicotinamide or ruthenium red was infused into the renal artery at 140 µl/min for 3 min before vasoconstrictor injection and continued for an additional 2 min. Multiple doses of these agonists were tested in the same animal. Concentrations of ruthenium red and nicotinamide were based upon in vitro published concentrations (61; 63) and calculated as estimated final renal arterial concentration. Concentrations that altered MAP or RBF were not used to inhibit vasoconstrictive agents. The target plasma concentrations of nicotinamide and ruthenium red are 3 mM (6 mg/kg/min) and 5 µM (126 µg/kg/min) in the renal artery, respectively.

Inhibitors were given at the highest dose that did not significantly alter basal RBF or MAP. The estimated plasma concentration of nicotinamide (3 mM) is higher than the IC_{50} of nicotinamide on cyclase activities of sea urchin egg homogenates (1.5 mM) and ADPR cyclase isolated from *Aplysia californica* (0.04 mM) (219). Concentrations from 3-5 mM have also been shown to inhibit Ca^{2+} responses to β-NAD, the substrate for ADPR cyclase in sea urchin egg homogenates after 2 min (219) and inhibit Ca^{2+} responses to Ang II in isolated afferent arterioles shortly after application (61). Infusion of nicotinamide at 6 mg/kg/min is therefore likely sufficient to inhibit ADPR cyclase activity. Similarly, the estimated plasma concentration of ruthenium red (5 µM) is much higher than the IC_{50} published for ruthenium red on isolated RyR from rabbit skeletal muscle (117).

Inhibition of Basal Vascular Tone- To evaluate activity of ADPR cyclase under resting conditions, a high dose of nicotinamide (12 mg/kg/min) was infused into the renal

artery for 20 min at 140 µl/min. The animal was then allowed to rest for 20 min, during which time RBF returned to normal. Multiple doses were given to the same animal. Concentrations after which RBF did not return to normal or MAP changed were not used.

Pharmacological Agents- Nicotinamide, ruthenium red, Bay-K8644, and Ang II were obtained from Sigma (St Louis, MO), FK506 was from Cayman Chemical (Ann Arbor, MI), and NE was from Abbott Labs (Chicago, IL). NE, Ang II, ruthenium red, and nicotinamide were dissolved in 0.9% NaCl. Bay-K8644 and FK506 were dissolved in 10% DMSO and 0.9% NaCl.

Data Analysis- Data were collected using Labtech Notebook software and graphs were created using Sigma Plot software. Statistics were performed by one way ANOVA test using Sigma Stat software.

D. RESULTS

Results are reported on 36 Sprague-Dawley rats whose age averaged 7.6 ± 0.1 weeks. Under basal conditions, mean MAP, RBF, hematocrit, and urine flow were 112 ± 3 mmHg, 4.47 ± 0.23 ml/min/g kidney wt, 43 ± 1%, and 32 ± 2 µl/min, respectively. Due to the reversible nature of Bay-K8644, FK506, Ang II, and NE, bolus injections of multiple agents could be given within the same animal. Only one inhibitor was used in an animal; each rat received either nicotinamide or ruthenium red.

ADPR cyclase activity mediates vasoconstriction produced by Ang II and NE. To determine the physiological importance of ADPR cyclase activity in agonist-induced renal vasoconstriction, we assessed the effect of the ADPR cyclase inhibitor nicotinamide on the acute renal response to Ang II. Ang II injected into the renal artery decreased RBF by 25 ±

3% in the control period (Fig. 2.1A and B). Intrarenal infusion of nicotinamide (≤6 mg/kg/min) did not significantly alter baseline MAP or RBF, but attenuated RBF responses to Ang II to a 7 ± 2% decrease in RBF ($P<0.001$). This effect of nicotinamide was rapidly reversible; the RBF response to Ang II was completely restored after 10 min (Fig. 2.1B). These data demonstrate that ADPR cyclase activity strongly influences Ang II-mediated renal vasoconstriction in vivo.

We also tested nicotinamide's ability to inhibit NE-induced renal vasoconstriction. NE injected into the renal artery produced an average 24 ± 3% decrease in RBF (Fig. 2.2A and B). Short-term nicotinamide infusion inhibited the effect of NE by ~70%, as NE decreased RBF to 8 ± 1% of normal ($P<0.001$). These data indicate that ADPR cyclase activation contributes to a significant percentage of NE-induced renal vasoconstriction. In the recovery period, the response to NE was greater than control.

ADPR cyclase activity contributes to renal vasoconstriction induced by L-type voltage-gated Ca^{2+} channels. Since nicotinamide similarly inhibited Ang II- and NE-induced contraction despite reported differences in the degree of Ca^{2+} mobilization (210), we tested whether basal activity of ADPR cyclase contributes to vasoconstriction produced independently of GPCRs. For this purpose, we evaluated renal vasoconstriction triggered by directly activating L-type voltage-gated Ca^{2+} channels using the agonist Bay-K8644. Intrarenal BayK-8644 injection produced a 78 ± 7% decrease in RBF (Fig. 2.3A and B). This response was inhibited by 59% with nicotinamide ($P=0.01$), attenuating constriction to 33 ± 12% of baseline RBF. Again, the effects of nicotinamide were reversible; the response to Bay-K8644 returned to normal after 10 min. These data establish a physiological role of

basal ADPR cyclase activity in constriction induced by Ca^{2+} entry via voltage-gated Ca^{2+} channels.

Basal renal vasomotor tone involves ADPR cyclase activity Doses of nicotinamide used to inhibit Ang II, NE, and Bay-K8644 did not significantly alter RBF or MAP when infused into the renal artery for 3 min (Fig. 2.4A) prior to vasoconstrictor injection. To determine the contribution of ADPR cyclase activity to basal renal vascular tone in vivo, we infused nicotinamide at a higher dose (12 mg/kg/min for 20 min). At this dose, localized relaxation of the renal vasculature was observed (Fig. 2.4B). RBF increased by 22 ± 4% ($P<0.001$) without changing MAP. Doses higher than 12 mg/kg/min reduced MAP, indicating systemic effects, and were therefore not used. Our results demonstrate that nicotinamide inhibits the tonic renal actions of endogenous stimuli signaling through ADPR cyclase to maintain basal renal vascular resistance.

Stimulation of RyRs causes renal vasoconstriction. To determine whether RyRs function in the renal vasculature in vivo, we used FK506 to stimulate RyRs. FK506 activates RyRs in the same manner as cADPR, by binding and removing the inhibitory molecule FKBP12 or FKBP12.6 from the RyR (33). Injection of FK506 into the renal artery caused a 22 ± 6% constriction, compared with 5 ± 1% constriction due to vehicle alone ($P=0.02$; Fig. 2.5). These results demonstrate the presence of functional RyRs, capable of contracting the renal vasculature upon activation.

RyRs mediate Ang II- and NE-induced renal vasoconstriction. To further assess the physiological importance of RyRs in the renal vasculature, we determined the extent to which RyRs are involved in acute vasoconstriction produced by Ang II and NE. Ruthenium

red was used to inhibit RyRs. Ang II produced a 30 ± 5% decrease in RBF during control conditions (Fig. 2.6). Intrarenal infusion of ruthenium red did not alter basal RBF or MAP after 3 min, but inhibited RBF responses to Ang II in a dose-dependent manner. The highest dose attenuated Ang II- mediated renal vasoconstriction to a 15 ± 2% decrease in RBF ($P<0.01$). The inhibitory effect of ruthenium red was reversible after 10 min.

Similarly, ruthenium red attenuated NE-induced renal vasoconstriction. NE produced a 27 ± 4% decrease in RBF in the control period (Fig. 2.7). The highest dose of ruthenium red tested decreased the renal vascular response to 11 ± 2% ($P<0.01$). Responses to NE returned to normal after 10 min. We conclude that RyRs contribute to GPCR-mediated renal vasoconstriction in vivo.

RyRs mediate voltage-gated Ca^{2+} channel-induced renal vasoconstriction. Due to the apparent similarity of RyR involvement in the vascular effects of Ang II and NE, we tested whether RyRs contribute to L-type voltage-gated Ca^{2+} channel-induced renal vasoconstriction. Intrarenal injection of the L-type channel agonist BayK-8644 produced a 78 ± 7% decrease in RBF (Fig. 2.8). This response was inhibited 63% by ruthenium red ($P<0.001$), resulting in an attenuated RBF response of 29 ± 4%. Bay-K8644-induced RBF responses returned to normal after 10 min. These results demonstrate involvement of RyRs in renal vascular responses elicited by stimulating Ca^{2+} entry.

Genetic disruption of ADPR cyclase in mice leads to impaired renal vascular responses to Ang II and NE. To determine the effect of chronic inhibition of ADPR cyclase on Ang II- and NE-induced renal vasoconstriction, we compared RBF responses to Ang II and NE injected iv in wild type and CD38-/- mice. CD38-/- mice showed impaired renal

vascular reactivity to both Ang II and NE. Whereas Ang II and NE produced 30 ± 8% and 37 ± 6% decreases in RBF in wild type animals, mice lacking CD38 showed 8 ± 1% and 19 ± 4% decreases in RBF, respectively (Fig. 2.9, $P<0.05$ for both). These data indicate that mutation of the ADPR cyclase, CD38, results in attenuated renal vascular responses to Ang II and NE in vivo. Furthermore, our results demonstrate the requirement for ADPR cyclase in renal vasoconstriction is not specific to rats, but exists in multiple species.

E. DISCUSSION

Our study is the first to provide information about the functional importance of the ADPR cyclase/RyR signaling pathway in the regulation of renal vascular resistance in vivo. Collectively, our results support the notion that ADPR cyclase and its intermediates are linked to renal vasoconstriction through activation of RyR and enhancement of Ca^{2+}-induced Ca^{2+} release (CICR). Results obtained using pharmacological inhibitors and a knockout mouse model indicate that ADPR cyclase and RyRs mediate the renal vascular actions of Ang II and NE. In addition, the ADPR cyclase/RyR system contributes to renal vasoconstriction elicited by activation of Ca^{2+} entry through L-type channels independent of GPCR associated second messengers, providing additional insight into the importance of this signaling system in Ca^{2+} metabolism and contraction of resistance arterioles in vivo. Our study reinforces our previous results showing that the ADPR cyclase/RyR pathway contributes significantly to the regulation of $[Ca^{2+}]_i$ in individual afferent arterioles (64; 61) and extends these findings to the regulation of vascular resistance in the intact renal microcirculation during basal conditions as well as during GPCR agonist-induced renal vasoconstriction.

Basal levels of ADPR cyclase contribute to the maintenance of tonic renal vasomotor tone under resting conditions. The vasodepressor effects of nicotinamide have long been recognized (113; 238). More recent work shows that the mechanism is related to ADPR cyclase inhibition (12; 88). In the present study, infusion of high dose nicotinamide into the renal artery produced a significant increase in RBF while MAP is stable, indicative of renal vasodilatation. The dilation observed is rapid in onset, increases over the observation period, and is readily reversible. One possible explanation for this biphasic response is that the large immediate increase in RBF reflects inhibition of ADPR cyclase and associated attenuation of downstream Ca^{2+} signaling. The secondary, slower progressive increase in RBF may represent a component of Ca^{2+} signaling affected by prolonged period of low $[Ca^{2+}]_i$ caused by depletion of SR stores, associated with vasodilatation.

Basal activities of ADPR cyclase and RyRs are functionally important in the renal vasculature as evidenced by their contribution to the acute renal constrictor response to L-type Ca^{2+} channel activation. Nicotinamide and ruthenium red inhibited a significant portion of the renal vasoconstriction induced by the L-type Ca^{2+} channel agonist Bay-K8644 which was not dependent on GPCR activation of ADPR cyclase. It is not clear whether the Bay-K8644 response involves direct ADPR cyclase activation or the presence of basal tonic levels of cADPR and/or NAADP, which are sufficient to enhance RyR receptor activation by Ca^{2+} entry initiated by Bay-K8644. The latter seems more plausible; participation of ADPR cyclase activity in vasoconstriction induced by activation of L-type Ca^{2+} channels is most likely due to the impact of cADPR on CICR. Biochemical studies show that cADPR greatly sensitizes RyR to $[Ca^{2+}]_i$ (175; 223) and acts predominately, if not exclusively, by this mechanism (159) and that tonic ADPR cyclase activity is present in coronary artery

homogenates (87; 88). Tonic activity of ADPR cyclase seems sufficient to provide cADPR to sensitize RyRs to respond to small changes in $[Ca^{2+}]_i$, resulting in CICR and amplification leading to increased RVR. This idea is supported by recent data showing that specific inhibition of cADPR with 8Br-cADPR attenuates KCl-induced increases in $[Ca^{2+}]_i$ in isolated afferent arterioles (66).

RyRs are generally considered downstream targets for the products of ADPR cyclase in other vascular beds (4; 63; 87). cADPR activates RyRs by removing FK506 binding proteins (FKBPs) that associate with the receptor (184; 239). When used clinically as an immunosuppressant, FK506 often produces a side-effect of hypertension (191; 235). In the present study, FK506 caused pronounced renal vasoconstriction when injected into the renal artery, demonstrating the functional importance of RyRs in acute regulation of the renal circulation. Aside from effects on RyRs, FK506 also inhibits the actions of the calcium activated protein phosphatase calcineurin. Calcineurin is present in VSMC and functions in gene expression (183). Effects of calcineurin in VSMC and other cell types, however, are primarily elicited through activation of transcription factors including nucleated factor of activated T-cells (NFAT) and changes in protein levels (108; 229). Such changes mediate long-term renal effects of FK506 and likely contribute to FK506-induced hypertension. The nearly instantaneous effect of FK506 on RBF seen in our study, however, is too rapid to be dependent on changes in gene expression and is therefore not likely due to calcineurin inhibition.

Our in vivo data verify previously reported effects of FK506 on blood flow in isolated preparations. FK506 is reported to decrease RBF by 23% in an in situ autoperfused rat kidney(10) and contract isolated rat and human renal arteries (217). To our knowledge, only

one other study has investigated the effects of acute FK506 infusion on RBF in vivo (252). In this study, FK506 was continuously administered iv in the presence of L-NAME for 1 hr and resulted in a 47% decrease in RBF. Our study shows that effects of FK506 on RBF are localized to the kidney and occur when nitric oxide is present.

The actions of ADPR cyclase and RyRs are responsible for a majority of acute renal vasoconstriction elicited by Ang II and NE. We found that either nicotinamide or ruthenium red blocked up to 70% of agonist-induced renal vasoconstriction without changing baseline MAP. Furthermore, we show that CD38-/- mice have significantly attenuated renal vascular responses to Ang II and NE. It is unclear whether ADPR cyclase is directly activated by Ang II and NE, or if basal levels of cADPR and/or NAADP enhance renal vascular responses to Ang II and NE. The previously reported literature has suggested that ADPR cyclase contributes to agonist-induced vasoconstriction. ADPR cyclase, cADPR, and RyRs participate in Ang II-mediated increases in $[Ca^{2+}]_i$ in preglomerular resistance arterioles (61), those triggered by KCl-induced depolarization (66) and constrictor responses to NE are attenuated in aortic rings of CD38-/- mice (173). RyRs have also been implicated in NE signaling in vascular myocytes (21). Our studies add to this pool of knowledge by demonstrating a functional role of ADPR cyclase and RyRs in the renal vasculature in vivo. Further studies are required to clarify the relative importance of agonist-induced activation of this signaling pathway as compared to basal levels sufficient to accommodate CICR.

It is interesting to note that we observed a trend towards greater renal vascular responses to Ang II and NE during recovery after acute dose-dependent inhibition of the ADPR cyclase/RyR pathway. Sustained inhibition of Ca^{2+} mobilization may have resulted in accumulation of Ca^{2+} in SR stores that were unmasked as exaggerated Ca^{2+} release and

agonist-induced contraction during the recovery period. This exaggerated response was particularly prominent in NE experiments involving nicotinamide.

Our results suggest that ADPR cyclase and RyRs may function importantly in both afferent and efferent arterioles. Activation of L-type Ca^{2+} channels is likely to increase renal vascular resistance by a primary action of Ca^{2+} entry in the preglomerular vasculature, predominantly afferent arterioles (26; 77). In contrast, efferent arterioles appear to have few, if any, L-type Ca^{2+} channels that are activated by Bay-K8644 or KCl-induced depolarization (26; 77; 157). Our results indicate that nicotinamide and ruthenium red inhibit more than 50% of the renal vascular response to Bay-K8644. In this regard, our results highlight the functional role of ADPR cyclase/RyR signaling in afferent arteriolar-mediated renal vasoconstriction initiated by Ca^{2+} entry.

ADPR cyclase may contribute to efferent arteriolar constriction as well. Earlier work on isolated rat afferent arterioles indicates an important role of the ADPR cyclase/RyR system in Ang II- and ET-1-induced increases in $[Ca^{2+}]_i$ (65; 61). The importance of this system in Ca^{2+} signaling in the efferent arteriole is unknown. It is well accepted that the major resistance sites responsible for regulation of RBF are the small diameter afferent and efferent arterioles and that Ang II and NE constrict both sets of glomerular arterioles. The relative strength of contraction is reported to be equal (17; 227; 228), or with predominant effects on efferent arterioles (176). Since these studies suggest that at least 50% of renal vasoconstriction takes place in the efferent arteriole, our findings that nicotinamide and ruthenium red inhibit 50-70% of Ang II- and NE-induced contraction raise the question that the ADPR cyclase/RyR signaling pathway may contribute to efferent arteriolar constriction as well.

In summary, we present RBF evidence that the ADPR cyclase/RyR pathway plays an important physiological role in the regulation of basal renal vascular resistance during resting conditions and in acute renal vasoconstrictor responses elicited by Ang II, NE and Bay-K8644 injection into the renal artery. This is the first study to document the functional importance of the ADPR cyclase/RyR pathway in the vasculature in vivo. Intrarenal infusion of high-dose nicotinamide to inhibit ADPR cyclase activity produces renal vasodilatation as evidenced by increased RBF and reduced RVR in the absence of a change in MAP. Lower doses of nicotinamide that did not affect basal RBF markedly attenuate the acute renal vasoconstriction produced by intrarenal injection of Ang II, NE or Bay-K8644. In all three cases, the constriction appeared to be mediated by RyR as ruthenium red reduced the renal microcirculatory response to each agonist. Renal vascular reactivity to Ang II and NE was markedly attenuated in mice lacking the ADPR cyclase CD38, solidifying conclusions of vascular signaling based on nicotinamide inhibition of ADPR cyclase in rats. The Bay-K8644 studies provide insight into the functional importance of the ADPR cyclase/RyR pathway in Ca^{2+} signaling and CICR in renal vasoconstriction that occurs independent of GPCRs.

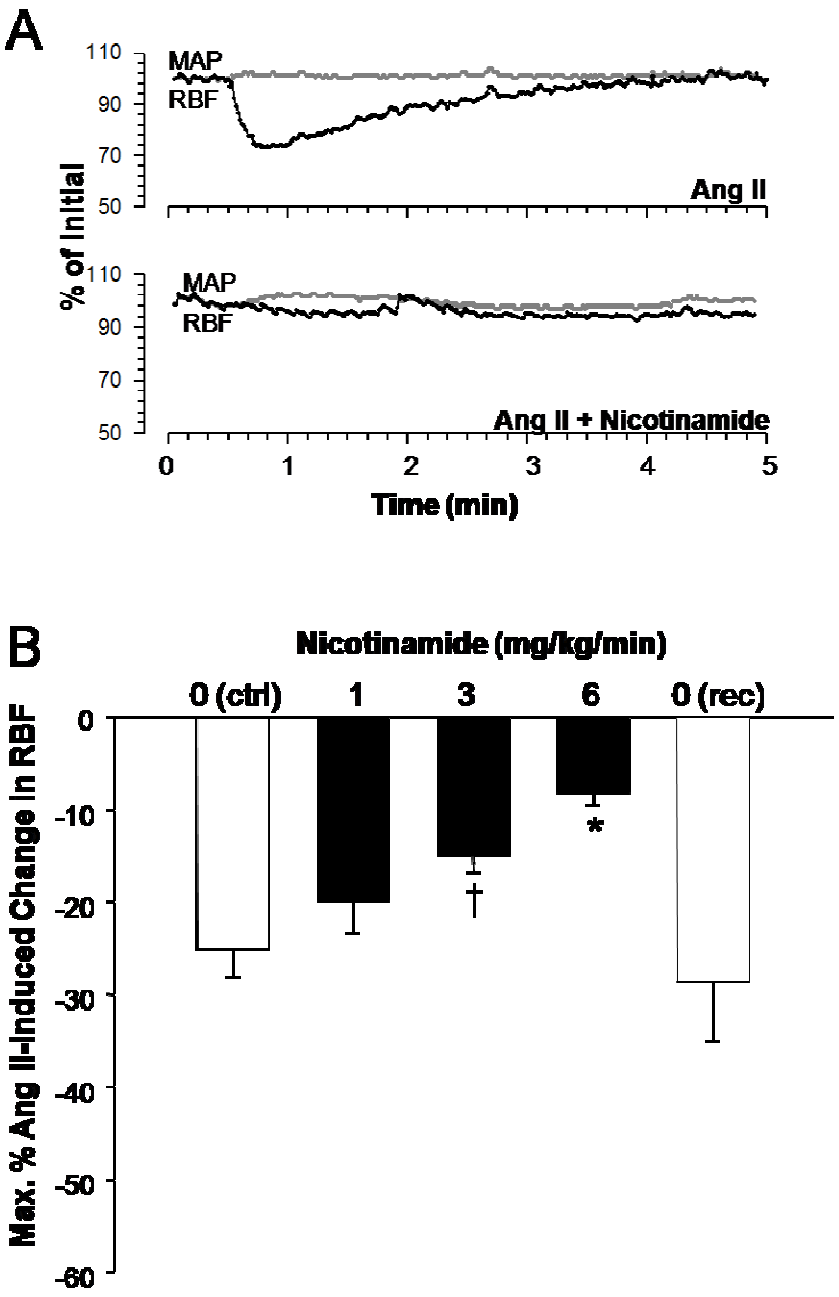

Figure 2.1. A: Representative recording of RBF (black) and MAP (gray) after Ang II injection at time zero during infusion of saline (top) or nicotinamide (bottom). **B:** Average maximum decrease in RBF in response to Ang II during control (ctrl), increasing doses of nicotinamide, or recovery period (rec). n=8; *: $P<0.001$, †: $P<0.01$ vs. control.

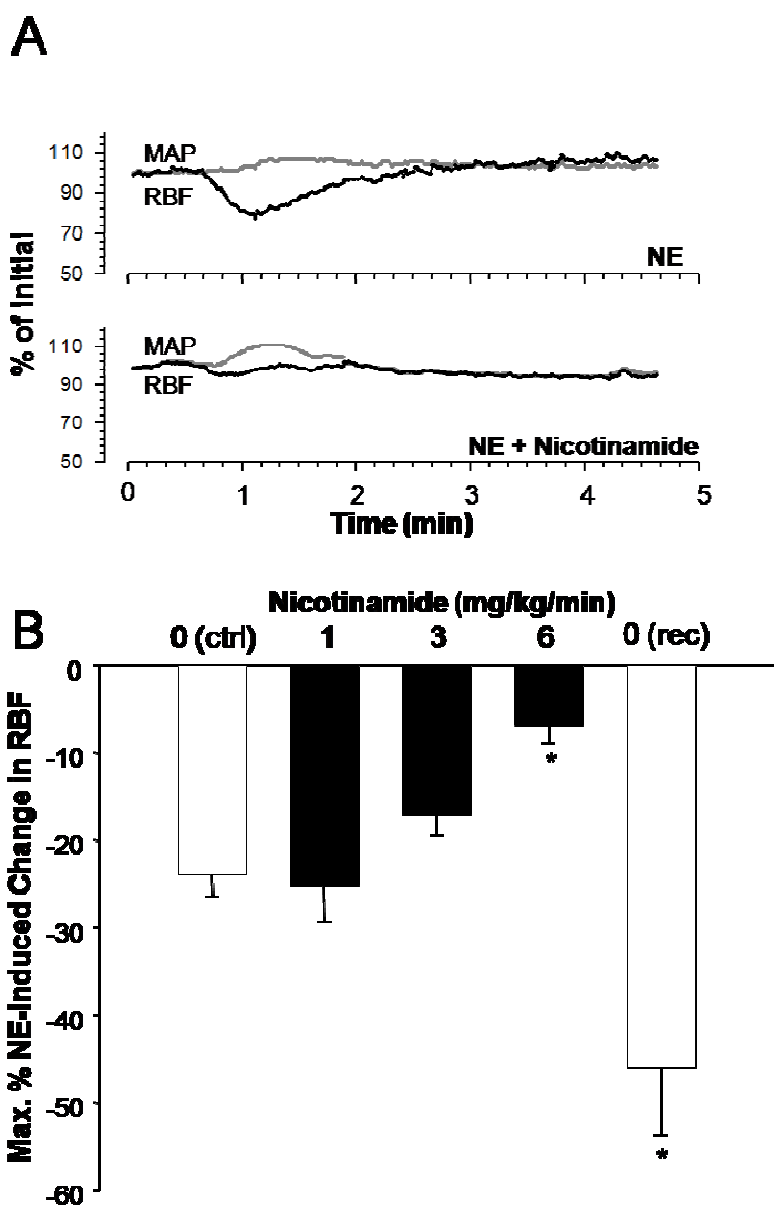

Figure 2.2. A: Recording of RBF (black) and MAP (gray) responses to NE injection at time zero during infusion of saline (top) or nicotinamide to inhibit ADPR cyclase (bottom). **B:** Average maximum percent change in RBF in response to NE during the control period (ctrl), in the presence of increasing doses of nicotinamide, or during recovery period (rec). n=8. *: $P<0.05$ vs. control.

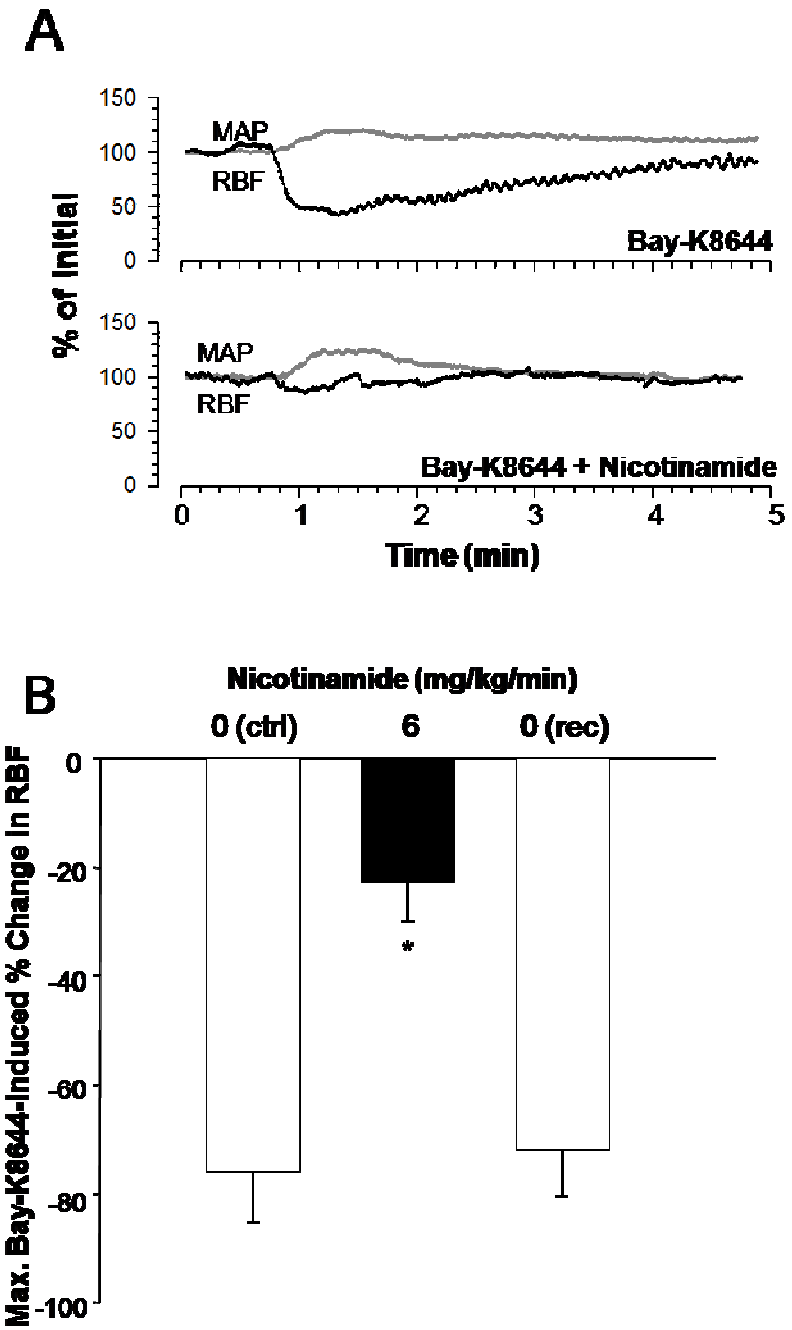

Figure 2.3. A: Typical tracing of RBF (black) and MAP (gray) in response to intrarenal injection of the L-type voltage-gated Ca^{2+} channel agonist, Bay-K8644 (2.5 µg) during infusion of saline (top) or nicotinamide (6 mg/kg/min, bottom). **B:** Average maximum decrease in RBF in response to BayK-8644 before (ctrl), during (6), or after (rec) nicotinamide infusion, n=5. *: P<0.02 vs. control.

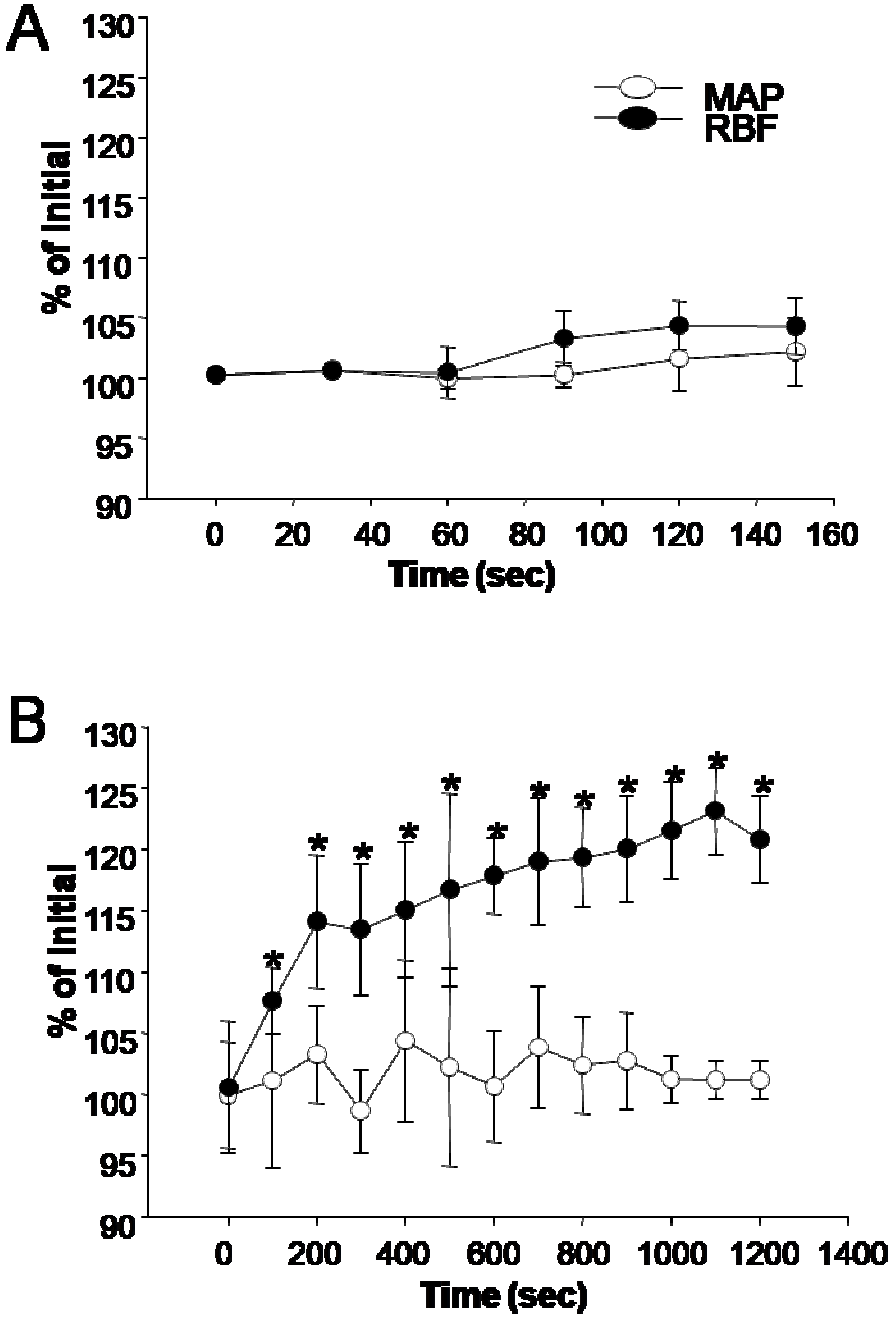

Figure 2.4. A: Percent of initial RBF (closed circles) and MAP (open circles) during 3 min infusion of nicotinamide at 6 mg/kg/min (n=5). **B:** Percent of initial RBF (closed circles) and MAP (open circles) during 20 min infusion of nicotinamide at 12 mg/kg/min (n=6). *P<0.005 vs. initial.

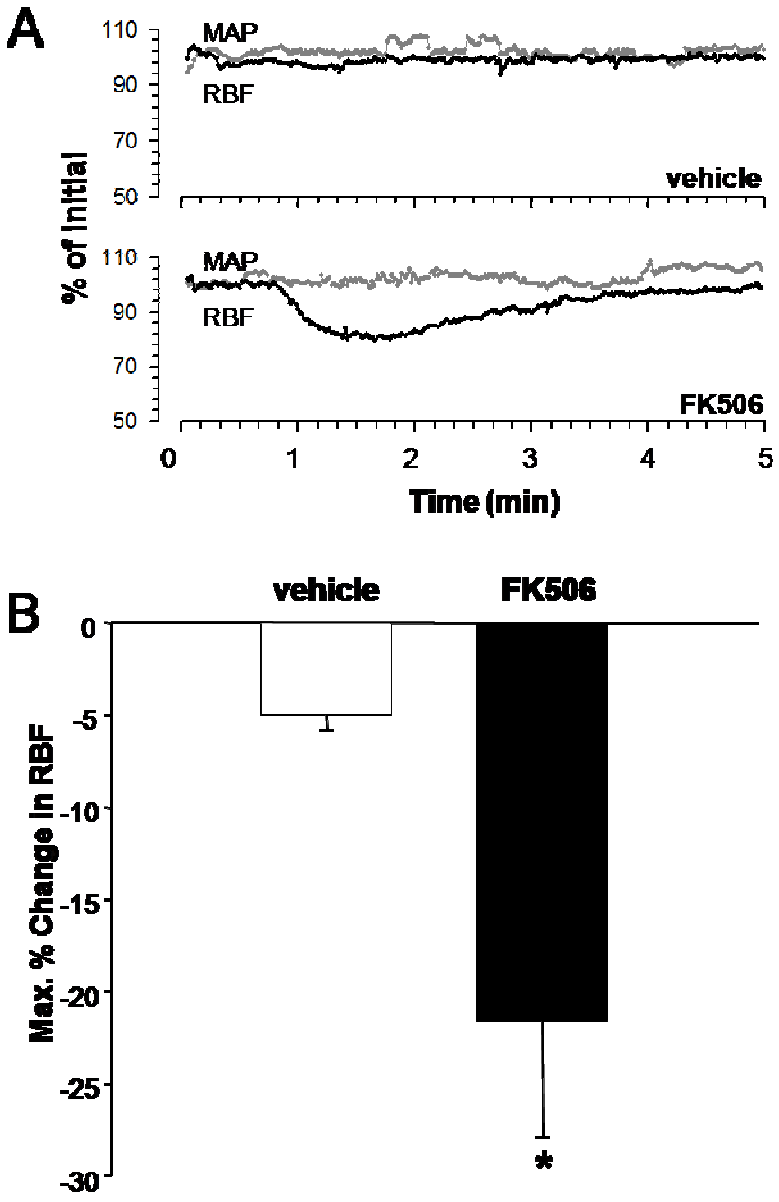

Figure 2.5. A: Original recording of changes in RBF (black) and MAP (gray) in response to injection of FK506 to stimulate RyRs (250 µg, bottom) or vehicle (top). **B:** Average maximum percent change in RBF in response to FK506 or vehicle (n=8). *: P=0.021 vs. vehicle alone.

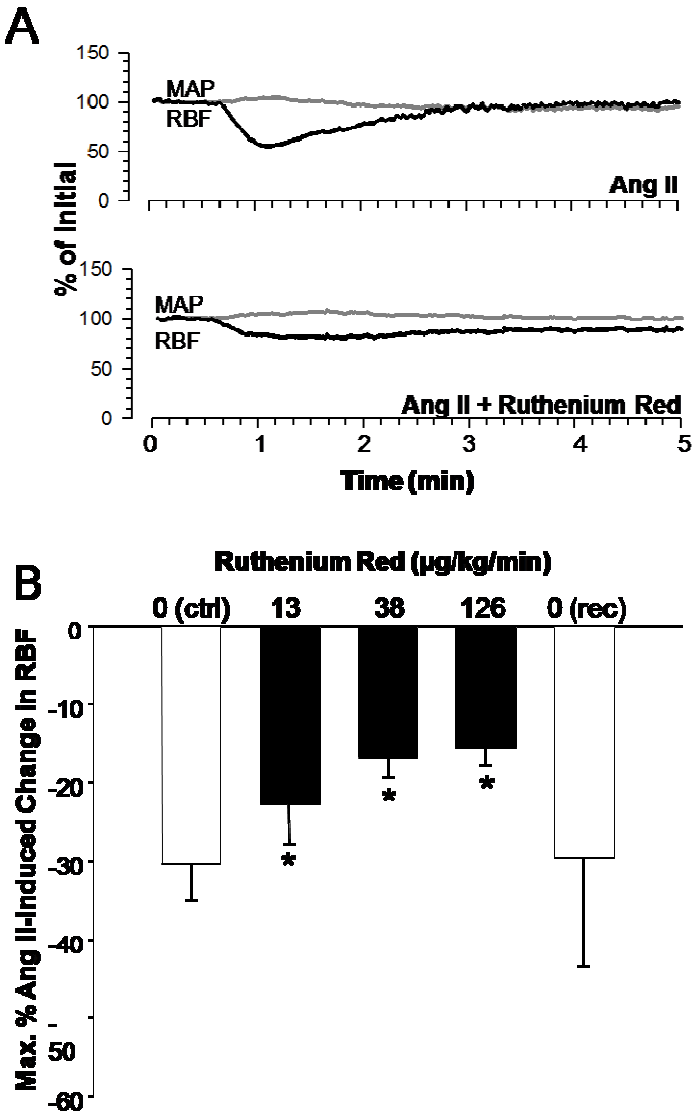

Figure 2.6. **A:** Tracing of changes in RBF (black) and MAP (gray) after Ang II injection given at time zero during infusion of saline (top) or ruthenium red (bottom). **B:** Average maximum decrease in RBF in response to Ang II in the presence of increasing doses of ruthenium red or during recovery period (rec). n=8. *: P<0.01 vs control.

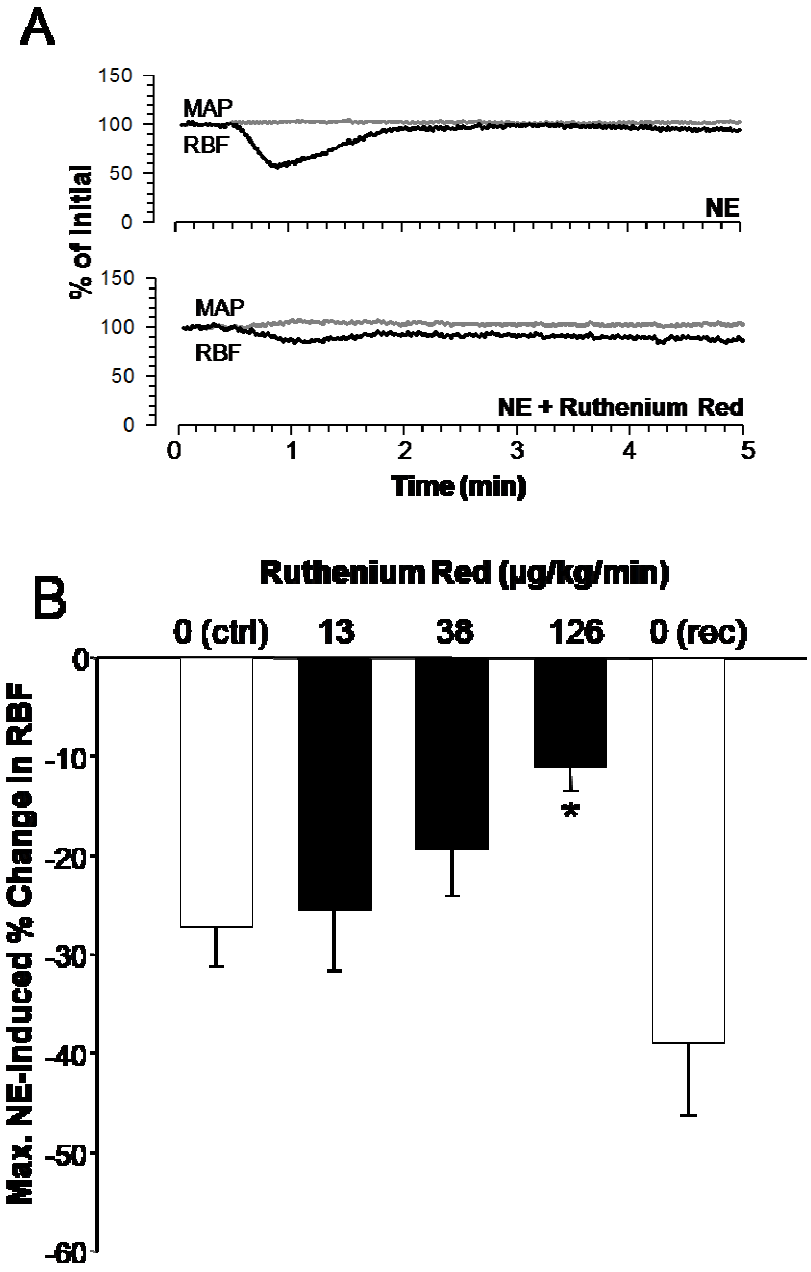

Figure 2.7. A: Recording of changes in RBF (black) and MAP (gray) after NE injection at time zero during infusion of saline (top) or ruthenium red to inhibit RyRs (126 µg/kg/min, bottom). **B:** Average maximum percent change in RBF in response to NE in the presence of increasing doses of ruthenium red or during recovery period (rec). n=8. *: $P<0.01$ vs. control.

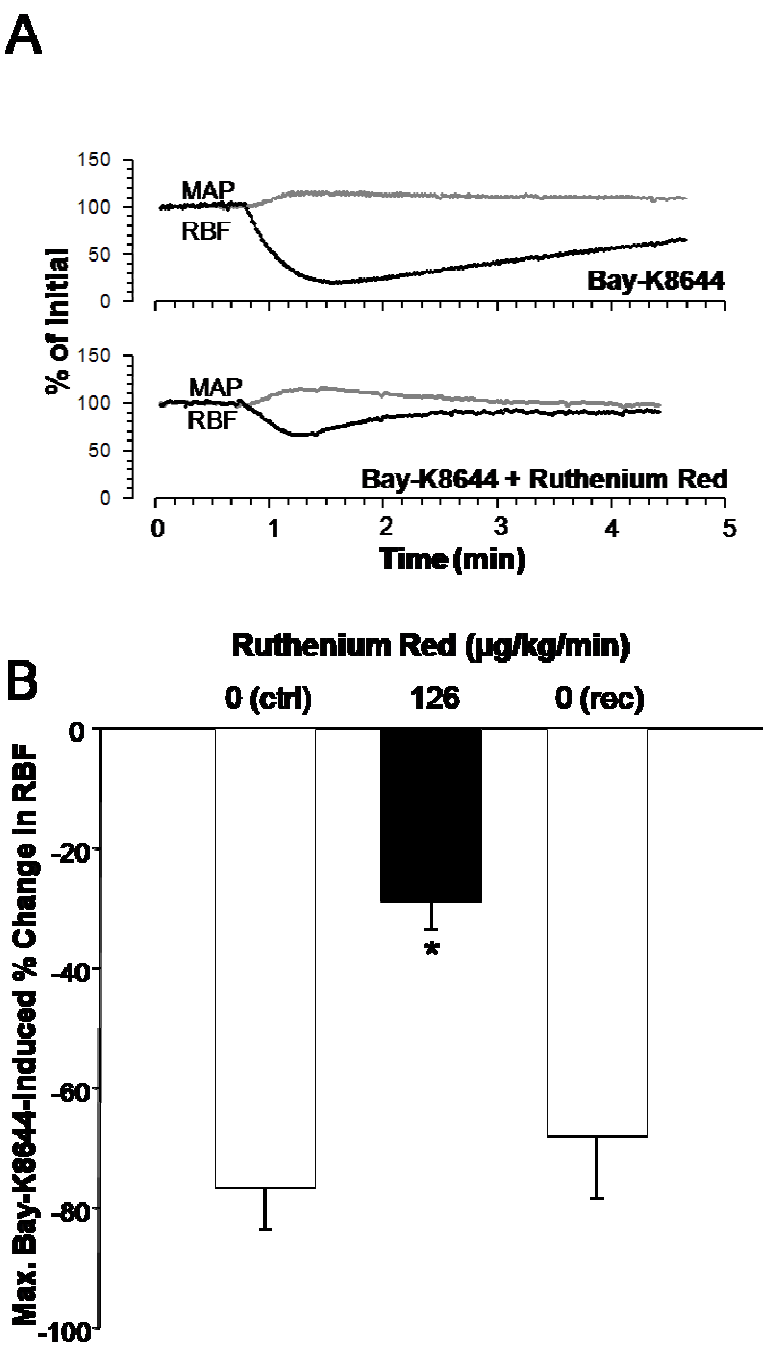

Figure 2.8. A: Representative recording of RBF (black) and MAP (gray) after injection of Bay-K8644 during infusion of saline (top) or ruthenium red (bottom). **B:** Average maximum decrease in RBF in response to BayK-8644 before (ctrl), during (126), or after (rec) ruthenium red infusion, n=7. *: $P<0.001$ vs. control.

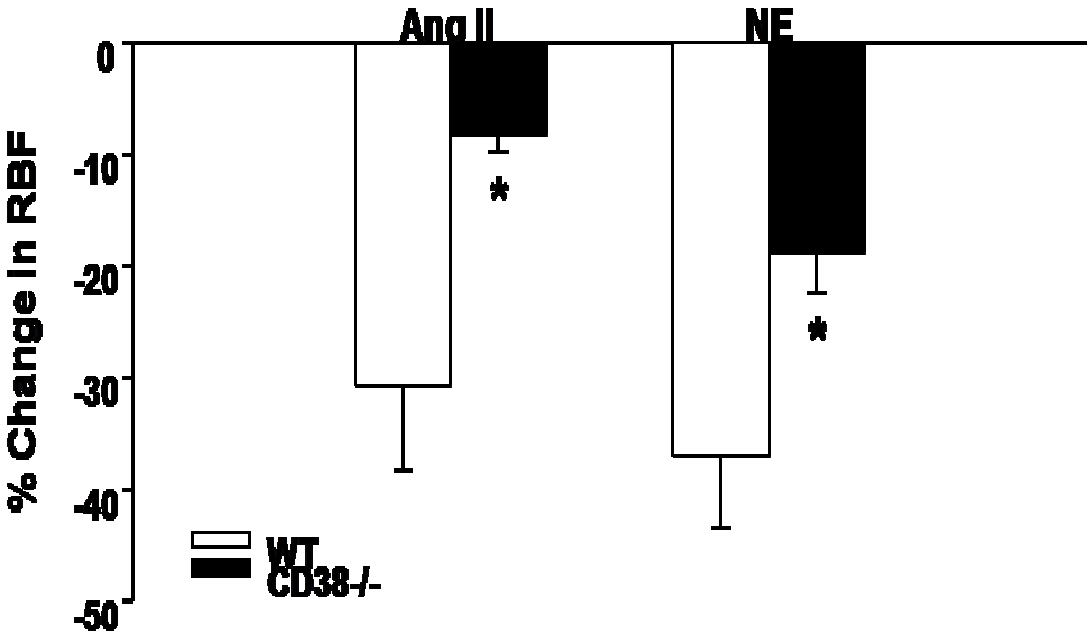

Figure 2.9. Average decrease in RBF in response to systemically injected Ang II and NE in wild type mice (white bars, WT) and mice lacking CD38 (black bars), n=6 for each group. *: $P<0.05$ vs. wild type.

CHAPTER III

ADP-ribosyl cyclase and ryanodine receptors mediate endothelin ET_A and ET_B receptor-induced renal vasoconstriction in vivo

A. ABSTRACT

ADP ribosyl cyclase (ADPR cyclase) and ryanodine receptors (RyR) participate in calcium transduction in isolated afferent arterioles. We hypothesized that this signaling pathway is activated by ET_A and ET_B receptors in the renal vasculature to mediate vasoconstriction in vivo. To test this, we measured acute renal blood flow (RBF) responses to ET-1 in anesthetized rats and mice in the presence and absence of functional ADPR cyclase and/or RyR. Inhibitors of ADPR cyclase (nicotinamide) or RyR (ruthenium red) reduced RBF responses to ET-1 by 44% ($P<0.04$ for both) in Sprague-Dawley rats. Mice lacking the predominant form of ADPR cyclase (CD38-/-) had RBF responses to ET-1 that were 47% weaker than those seen in wild type mice ($P=0.01$). Selective ET_A receptor stimulation (ET-1 + BQ788) produced decreases in RBF that were attenuated by 43% and 56% by nicotinamide or ruthenium red, respectively ($P<0.02$ for both). ADPR cyclase or RyR inhibition also reduced vasoconstrictor effects of the ET_B receptor agonist sarafotoxin S6c (77% and 54%, respectively, $P<0.02$ for both). ET_B receptor stimulation by ET-1 + the ET_A receptor antagonist BQ123 elicited responses that were attenuated by 59% and 60% by nicotinamide and ruthenium red, respectively ($P<0.01$ for both). Nicotinamide attenuated RBF responses to S6c by 54% during inhibition of nitric oxide synthesis ($P=0.001$). We conclude that in the renal microcirculation in vivo: 1) ET-1-induced vasoconstriction is mediated by ADPR cyclase and RyR, 8) both ET_A and ET_B receptors activate this pathway, and 3) ADPR cyclase participates in ET_B receptor signaling independent of NO.

B. INTRODUCTION

Endothelin-1 (ET-1) is one of the most potent vasoconstrictors identified to date. Dysfunction in ET-1 regulation or receptor signaling has been implicated in several cardiovascular diseases including atherosclerosis, coronary artery disease, congestive heart failure, cerebrovascular disease, and systemic and pulmonary hypertension and in acute and chronic renal disease (82; 139; 160; 204; 213; 214). ET-1 is thought to act primarily in a local paracrine fashion in the vasculature, being secreted from endothelial cells abluminally to act on nearby vascular smooth muscle cells (VSMC). Circulating levels of ET-1 appear to have a relatively minor influence on vascular tone. In this regard, it has been proposed that ETB receptors bind circulating ET-1 and provide a clearance function (214).

The renal vasculature is particularly responsive to ET-1 (140; 201). Acute intravenous administration of ET-1 decreases in renal blood flow (RBF) in animals (110; 140) and increases renal vascular resistance in humans without affecting arterial pressure (202). The effects of ET-1 on RBF are due to contraction of preglomerular arteries and afferent and efferent arterioles as has been shown in specialized isolated vascular preparations (55; 120; 143) and in vivo (140; 201). Although ET-1 does not affect steady-state RBF autoregulation, ET-1 stimulation of NO production alters the dynamics of the preglomerular myogenic response (220).

ET-1 signals via two G protein coupled receptors(GPCR): ET_A and ET_B (160; 194; 214). Stimulation of ET_A or ET_B receptors results in elevation of cytosolic calcium concentration $[Ca^{2+}]_i$. ET-1 stimulates the production of inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 can bind to IP_3Rs on the sarcoplasmic reticulum (SR) to

release Ca^{2+} while DAG activates PKC, resulting in downstream activation of myosin light chain kinase and cell contraction.

Most VSMC have SR with Ca^{2+} sensitive ryanodine receptors (RyR) that participate in Ca^{2+} mobilization. RyR can be activated by second messengers produced by the enzyme ADP ribosyl cyclase (ADPR cyclase) (1; 272). We have previously observed that this ADPR cyclase/RyR signaling pathway plays a significant role in mediating responses to Ang II in Ca^{2+} signaling in isolated afferent arterioles (64) and renal vasoconstriction in vivo (241). However, little is known about which GPCRs activate this particular second messenger system in specific vascular beds. Participation may depend on vessel size and function and vascular bed. For example, it appears that thromboxane A_2 (TxA_2) signaling through the TP receptor does not involve ADPR cyclase and RyR in isolated renal arteries (240), whereas ADPR cyclase inhibition dilates TxA_2 preconstricted coronary arteries (88). Interestingly, isolated aortas from mice genetically lacking the predominant form of ADPR cyclase (CD38-/-) show normal contractile responses to ET-1 but contraction to the α-adrenergic receptor agonist phenylephrine is weaker than that seen in wild type mice (173). We find that α-adrenergic receptor-induced renal vasoconstriction is dependent on ADPR cyclase and RyR in vivo (241).

Evidence indicates that ET-1 activates ADPR cyclase in VSMC of coronary, renal, mesenteric, and pulmonary beds. The ADPR cyclase inhibitor nicotinamide attenuates contraction of mesenteric arteries elicited by ET-1 (90). Evidence implicates both second messengers produced by ADPR cyclase in VSMC responses to ET-1. Incubation of coronary arterial myocytes with ET-1 stimulates production of the ADPR cyclase metabolite nicotinic acid ADP (NAADP) (274). In pulmonary arterial VSMC, Ca^{2+} responses to ET-1 are

attenuated by bafilomycin A1, an inhibitor of NAADP-induced Ca^{2+} release (135). Selective inhibition of cyclic ADP ribose, a second metabolite formed by ADPR cyclase with 8Br-Cadpr attenuates Ca^{2+} responses to ET-1 in rat renal afferent arterioles (65).

To our knowledge, the importance of ADPR cyclase/RyR signaling in mediating ET-1-induced vasoconstriction has not been studied in vivo in any vascular bed. Due to the emerging importance of ADPR cyclase in GPCR signaling, we hypothesized that ADPR cyclase and RyR contribute to renal vascular responses to ET-1 in vivo. We determined the importance of this signaling pathway in responses to ET_A and ET_B receptor stimulation in the renal microcirculation.

C. MATERIALS AND METHODS

Sprague Dawley rats, wild type C57BL6 mice, and CD38-/- mice on a C57BL6 background were obtained from our Chapel Hill breeding facility. CD38-/- mice and wild type control breeder pairs were originally obtained from Dr. Frances Lund (Trudeau Institute, Saranac Lake, NY) and Jackson Labs (Bar Harbor, ME), respectively. All animals were cared for and used in accordance with institutional guidelines. Protocols were approved by the local Institutional Animal Care and Use Committee. Animals were anesthetized using sodium pentobarbital (Nembutal, 50-60 mg/kg body wt ip for rats, 80-90 mg/kg body wt ip for mice, Abbott, Chicago, IL).

Surgical Procedures in Rats- The surgical preparation for measurement of RBF in rats was performed as is standard in our laboratory (129; 127; 241). The right femoral artery was catheterized using a PE50 catheter for continuous measurement of mean arterial pressure (MAP) via a pressure transducer (Statham P23 dB). The right femoral vein was catheterized

using three PE10 catheters for infusion of bovine serum albumin (4.75% at 50 µl/min for min=body wt (g)/4, then reduced to 10 µl/min for the remainder of the experiment), maintenance of anesthesia using sodium pentobarbital as required, and injection of N_{ω}-nitro-L-arginine methyl ester (L-NAME) when applicable. The bladder was catheterized and tracheotomy was performed. A curved PE50 catheter was inserted into the left common iliac, passed up the aorta, and positioned such that the tip of the catheter was facing but not obstructing the left renal artery for administration of pharmacological agents. An ultrasonic flow transducer was placed around the left renal artery to measure RBF (model 1RB, Transonic, Ithaca, NY).

Surgical Procedures in Mice- Procedures for measuring RBF in mice were modified from those previously developed in our laboratory (20). A pulled PE50 catheter was inserted into the right femoral artery for continuous measurement of MAP via a pressure transducer (Statham P23 dB). Pulled PE10 catheters were inserted into a femoral vein for iv administration of bovine serum albumin (2.4%, 10 µl/min for the duration of the experiment) and ET-1. A tracheotomy was performed and the bladder catheterized. The left renal artery was freed from the renal vein, and RBF was measured by an ultrasonic flow transducer (0.5V, Transonic, Ithaca, NY).

Assessment of Renal Vascular Reactivity- In rats, 10 µl bolus injections of ET-1 (7.5 ng) and S6c (7.5 ng) were given directly into the renal artery in the manner previously described (129; 127). Previous studies show that RBF and MAP responses to ET-1 and S6c recover, albeit slowly, over 30 min (129). As a result, multiple doses of ET-1 and/or S6c were given to the same animal. In mice, a 10 µl bolus of ET-1 (7.5 ng) was injected into a

femoral vein. L-NAME (25 mg/kg in 1 ml/kg 0.9% NaCl) was injected into a femoral vein of rats 30 min prior to the start of an experiment.

Ruthenium red (126 µg/kg/min), an inhibitor of RyR, nicotinamide (6 mg/kg/min), an ADPR cyclase inhibitor, BQ123 (18.64 µg/kg/min), an ET_A receptor antagonist, and BQ788 (18.64 µg/kg/min), an ET_B receptor antagonist, were infused into the renal artery 3 min prior and 5 min following ET-1 or S6c injection. These doses of nicotinamide and ruthenium red were based on an earlier study in which we established effective inhibition of Ang II- and NE- induced renal vasoconstriction while neither agent affected basal RBF or MAP (241). It is important to note that complete inhibition was not reached at the doses used in the present study as higher amounts are known to produce more pronounced inhibition of ADPR cyclase as evidenced by frank renal vasodilatation (241). Following the inhibitory period, the animal was allowed to recover for 10 min prior to a final assessment of reactivity. In all cases, the response during the recovery period returned to the control level indicating rapid reversibility of inhibitors and stability of the preparation. The doses of ET receptor antagonists are known to effectively antagonize ET_A and ET_B receptors selectively (129). To avoid possible overlapping effects, each rat received only one inhibitor (nicotinamide or ruthenium red).

Pharmacological Agents- ET-1, L-NAME, nicotinamide, and ruthenium red were purchased from Sigma Aldrich (St. Louis, MO). S6c, BQ123, and BQ788 were obtained from American Peptide Company (Sunnyvale, CA). All pharmacological agents were dissolved in 0.9% NaCl.

Data Analysis- Data were collected using Labtech Notebook software and graphs were created using SigmaPlot software**.** Statistical analyses of differences in RBF responses

between control and experimental periods were performed by a paired student's t-test using SigmaStat software.

D. RESULTS

Results are reported for a total of 58 male Sprague-Dawley rats averaging 7.3 ± 0.1 weeks of age. In rats not treated with L-NAME, RBF and MAP were 3.8 ± 0.2 ml/min/g kidney wt and 100 ± 4 mmHg, respectively. L-NAME treated animals showed an increased MAP (145 ± 3 mmHg, $P<0.001$) and decreased RBF (3.0 ± 0.3 ml/min/g, $P<0.05$).

ET-1-induced renal vasoconstriction is dependent on ADPR cyclase activation and RyR. To determine whether ADPR cyclase mediates renal vascular responses to ET-1, we gave intrarenal bolus injections of ET-1 to rats before and during intrarenal infusion of the ADPR cyclase inhibitor nicotinamide. ET-1 injection into the renal artery decreased RBF by 31 ± 3% (Fig. 3.1). This response was impaired in the presence of nicotinamide such that only a 17 ± 3% decrease in RBF was produced by the same amount of ET-1, a response that was decreased 45% from that observed in the control period ($P<0.01$).

To test the importance of RyR, we compared renal vascular responses to ET-1 in the presence or absence of the RyR inhibitor ruthenium red in other animals. The 29 ± 4% decrease in RBF was induced by ET-1 under control conditions was attenuated by ruthenium red. ET-1 produced a 16 ± 3% decrease in RBF in the experimental period (Fig. 3.2). Thus, both ADPR cyclase and RyR appear to play a role in acute ET-1 responses in the renal microcirculation of normotensive rats.

Renal vascular responses to ET_A receptor stimulation are mediated by ADPR cyclase and RyR. ET_A and ET_B receptors are both present in the renal microvasculature and mediate

total RBF responses to ET-1 (48; 89; 129). As a result, we asked whether the importance of ADPR cyclase in the renal hemodynamic actions of ET-1 is selectively dependent on one ET receptor subtype over the other.

To specifically stimulate ET_A receptors, we injected ET-1 into the renal artery of rats in the presence of the selective ET_B receptor antagonist BQ788. In the absence of nicotinamide, the combination of ET-1 + BQ788 decreased RBF by 50 ± 5% (Fig. 3.3). When nicotinamide was infused into the renal artery to inhibit ADPR cyclase, the constrictor response to ET_A receptor stimulation was markedly attenuated. During ADPR cyclase inhibition, ET-1 + BQ788 decreased RBF by 28 ± 3%, an attenuated response compared to ET-1 + BQ788 given without nicotinamide ($P<0.01$). Similarly, RyR inhibition with ruthenium red attenuated the acute RBF response to ET-1 + BQ788 from 56 ± 10% to 25 ± 4% ($P<0.02$, Fig. 3.4). Together, these data demonstrate a significant role for ADPR cyclase and RyR in ET_A receptor-mediated renal vasoconstriction.

Vasoconstrictor responses to ET_B receptor stimulation are dependent on activation of ADPR cyclase and RyR. To test the importance of ADPR cyclase and RyR in ET_B receptor signaling, we injected the specific ET_B receptor agonist sarafotoxin S6c into the renal artery. In other animals, a combination of the ET_A antagonist BQ123 and ET-1 was tested. When given into the renal artery, S6c and ET-1 + BQ123 reduced RBF by 22 ± 4% (Fig. 3.5) and 13 ± 2% (Fig. 3.6), respectively. Thus, as has been previously demonstrated (129), we found that ET_B receptor stimulation elicits net renal vasoconstriction. Nicotinamide markedly inhibited the vascular effects of both S6c and ET-1 + BQ123 such that only a 5 ± 2% decrease in RBF was observed in both groups ($P<0.01$ for both). These findings suggest that

ADPR cyclase participates in acute renal vasoconstrictor responses to selective ET_B receptor stimulation in a healthy rat kidney.

Other experiments indicate that RyR participate in ET_B receptor responses in the renal vasculature in vivo. Prior to administration of ruthenium red, S6c and ET-1 + BQ123 decreased RBF by 16 ± 3 (Fig. 3.7) and 9 ± 1% (Fig. 3.8), respectively. In the same animals, ruthenium red attenuated RBF changes during ET_B receptor stimulation. During RyR inhibition in these groups, S6c and ET-1 + BQ123 decreased RBF by 7 ± 2% and 4 ± 1%, respectively ($P<0.02$ for both), reductions of approximately 50%.

ADPR cyclase activation by ET_B receptors is independent of nitric oxide. In addition to vasoconstrictor properties of ET_B receptors on VSMC, ET_B receptors are present on endothelial cells of renal vessels (114; 253). When stimulated, these receptors produce NO and other dilator agents that buffer ET-1-induced renal vasoconstriction (111; 127). Since interactions between NO and ADPR cyclase have been reported in both VSMC and endothelial cells of non-renal arteries (266; 276), we asked whether the effects of nicotinamide on ET_B receptor-induced renal vasoconstriction persist in the absence of NO production. To test this, we evaluated the effect of ADPR cyclase inhibition on S6c responses during inhibition of NO synthase using L-NAME. During L-NAME infusion, S6c decreased RBF by 28 ± 3% (Fig. 3.9). Nicotinamide attenuated the S6c response such that a 13 ± 2 % decrease in RBF was observed in the experimental period ($P<0.005$). These results suggest that ADPR cyclase activation by ET_B receptor stimulation occurs in the absence as well as the presence of NO.

RBF responses to ET-1 are impaired in mice with decreased ADPR cyclase activity. The importance of ADPR cyclase in renal vascular responses to ET-1 was assessed in mice lacking the predominant form of ADPR cyclase (CD38). ET-1 injected iv produced a 19 ± 3% decrease in RBF in wild type mice, compared with a 10 ± 1% decrease in CD38-/- mice ($P<0.02$, Fig. 3.10). This finding in mice reinforces our pharmacological studies on rats demonstrating the importance of ADPR cyclase in renal vascular responses to ET-1 in the rat.

E. DISCUSSION

Our study is the first to demonstrate a dependence of ET-1 signaling on ADPR cyclase activation and RyR in the microcirculation of any organ in rats and mice in vivo. Acute ET-1-induced vasoconstriction in the kidney is critically dependent on the activity of ADPR cyclase and RyR. This conclusion is based on results obtained using pharmacological inhibitors and a knockout mouse. We find that both ET_A and ET_B receptors utilize the ADPR cyclase/RyR signaling pathway to produce vasoconstriction of renal resistance arterioles in rats. Experiments in mice demonstrate the importance of one ADPR cyclase family member in particular, CD38, in ET-1-induced renal vasoconstriction. Our study extends previous work establishing the importance of ADPR cyclase and RyR in mediating $[Ca^{2+}]_i$ responses to ET-1 receptor stimulation in isolated renal afferent arterioles (65) and provides new information regarding the importance of this pathway in an integrated, natural environment.

We found that ADPR cyclase and RyR mediate a significant portion of acute renal vasoconstriction produced by ET-1. In our rat studies, pharmacological agents used to inhibit both ADPR cyclase and RyR attenuated renal vasoconstrictor responses to ET-1 by

~45%. Nicotinamide and ruthenium red are widely used to inhibit ADPR cyclase and RyR, respectively. Nicotinamide is a byproduct of ADPR cyclase conversion of NAD+ to cADPR and NADP+ to NAADP (1; 145) and nicotinamide shifts the ADPR cyclase reactions to produce relatively small amounts of NAD+ and NADP+ rather than adding significantly to the pool of cADPR and NAADP. Although nicotinamide may also inhibit poly (ADP-ribose) polymerase-1 (PARP-1) (260), this is unlikely to be a major factor in our studies since PARP-1 is generally activated only under incidences of extreme stress (8) and our animals were closely monitored to ensure the most physiological conditions possible. As a commonly used inhibitor of RyR, ruthenium red binds directly to RyR, causing a conformational change that renders the channel inactive (161). Ruthenium red may also inhibit Ca^{2+}-activated K^+ channels (BK_{Ca}) (22; 161), known to be present in renal microvessels (162). If this were the primary action, however, ruthenium red would be predicted to enhance vasoconstriction rather than oppose it as is seen in our study.

Our rat results were reinforced by studies on mice lacking the predominant form of ADPR cyclase (CD38-/-). Three forms of ADPR cyclase are known to exist: the non-mammalian ADPR cyclase of the sea hare *Aplysia californica* and the two mammalian ADPR cyclases lymphocyte antigen CD38, and bone marrow stromal cell surface antigen BST-1 (CD157) (215). Whereas CD157 is present primarily on immune cell types and is upregulated during periods of stress, CD38 is more constitutively expressed and is expressed in several tissues (74). Isolated aortas without intact endothelium from CD38-/- mice have impaired responses to phenylephrine, suggesting a vasoconstrictor role of CD38 linked to α-adrenoceptors (173). It is noteworthy that another form of ADPR cyclase sensitive to retinoic acid may exist in VSMC, although this form is poorly characterized (50). In an

earlier study, we reported weaker than normal acute renal vascular responses to Ang II and norepinephrine in CD38-/- mice, suggesting that CD38 exists in resistance arterioles and mediates vasoconstriction in the kidney triggered by AT_1 and adrenoceptors (241). Presently, we found that mice deficient in CD38 show attenuated renal vascular responses to iv injection of ET-1, supporting the notion that ADPR cyclase mediates acute ET-1-induced renal vasoconstriction in the mouse.

ET-1 responses were attenuated by 45% in the presence of either nicotinamide or ruthenium red. Similarly, responses to ET-1 in CD38-/- mice were 47% less than in wild type controls. Collectively, these results indicate that the ADPR cyclase/RyR signaling pathway accounts for roughly one-half of acute vasoconstriction of resistance arterioles in the kidney in response to ET-1. Previous in vitro results indicate that IP_3 and its receptor play an important role in mediating $[Ca^{2+}]_i$ responses to ET-1 in renal microvessels (9; 105). Although one cannot discern from the current data whether the contribution of the ADPR cyclase and IP_3 pathways to ET-1-induced renal vasoconstriction work independently, it is probable that a synergistic effect exists between these two signaling mechanisms. RyR are Ca^{2+} sensitive receptors that can amplify $[Ca^{2+}]_i$ from any source. Ca^{2+} release from IP_3Rs on the SR may activate nearby RyR by Ca^{2+}-induced Ca^{2+} release (CICR). cADPR may also contribute to the amplification of IP_3R signaling since cADPR sensitizes RyR to CICR. In addition to cADPR, ET-1 stimulation of ADPR cyclase may produce NAADP in VSMC. Ca^{2+} exiting from lysosomal stores via stimulation of NAADP receptors may also activate RyR to cause CICR (135). It is noteworthy that we used concentrations of pharmacological agents that produced less than maximal inhibition of their intended targets, so the observed

degree of inhibition in rats represents a conservative estimate of the involvement of ADP ribosyl cyclase and RyR in the integrated renal responses.

Large systemic arteries predominantly express ET_A receptors on VSMC and ET_B receptors on endothelial cells (214). In many vascular beds, ET_B receptors are also expressed on venous VSMC (194). In the kidney, ET_A receptor stimulation on VSMC produces vasoconstriction (129; 127). ET_B receptor stimulation on endothelial cells leads to production of the vasodilator NO (127; 214) whereas ET_B receptors on VSMC elicit vasoconstriction (55; 120; 129). This occurs both in vitro and in vivo (54; 55; 120; 140; 201). Since both ET_A and ET_B receptors mediate renal vascular responses to ET-1, we analyzed the contribution of the ADPR cyclase/RyR pathway to signaling by each receptor. Our experimental design included selective stimulation of each receptor subtype as well as both together. Antagonist specificity and dosage have been established previously in our laboratory (129; 127). Both BQ123 and BQ788 effectively produce near-complete inhibition of ET_A and ET_B receptor subtypes, respectively, at the doses employed herein. Our present results together with previous studies by Just et al. (129; 127) suggest that interactions must exist between ET_A and ET_B receptors since ET_B receptor stimulation produces a net dilator effect when ET_A receptors are co-stimulated, but selective stimulation of ET_B receptors alone results in vasoconstriction.

Nicotinamide attenuated renal vasoconstriction produced by ET_A receptor stimulation by ET-1 + BQ788, indicating a role for ADPR cyclase signaling in ET_A receptor-induced renal vasoconstriction in vivo. Our previous study demonstrated a dilator effect on basal renal vascular resistance when a high dose of nicotinamide was administered (241). Importantly, the dose used in the present study was low enough to not affect resting RBF or

MAP. Our in vivo results for vasomotor tone extend those for $[Ca^{2+}]_i$ seen in isolated afferent arterioles (65) and testicular peritubular smooth muscle cells (11) and establish the importance of the ADPR cyclase signaling pathway in renal vascular responses in anesthetized rodents. In individual afferent arterioles, both nicotinamide and the cADPR selective blocker 8Br-cADPR attenuated $[Ca^{2+}]_i$ responses to ET_A receptor stimulation (65). Similarly, the cADPR selective antagonist $8NH_2$-cADPR attenuated Ca^{2+} increases in response to ET_A receptor stimulation in testicular peritubular smooth muscle cells (11). To our knowledge, only one study has examined the link between ET_A receptor activation and RyR. This study of isolated peritubular smooth muscle cells showed that antagonist concentrations of ryanodine inhibited $[Ca^{2+}]_i$ responses to ET_A receptor stimulation (11). We provide new information that RyR are important mediators of ET_A receptor signaling in the renal vasculature in vivo. By design we focused on the role of ADPR cyclase and RyRs in mediating ET-1-induced renal vasoconstriction and did not assess participation by other Ca^{2+} signaling pathways. Interactions among multiple pathways are probable since cADPR has been shown to sensitize the RyRs to $[Ca^{2+}]_i$ to favor CICR (84; 269; 272). For example, the $[Ca^{2+}]_i$ and renal vasoconstrictor response initiated by Ca^{2+} entry through L-type Ca^{2+} channels is potentiated by basal RyR activity (66; 241).

We provide new evidence that the magnitude of constriction elicited by ET_B receptors is dependent upon the activity of the ADPR cyclase/RyR signaling pathway. Our results are consistent with those for $[Ca^{2+}]_i$ experiments in peritubular smooth muscle cells in vitro(11) and rat cerebellum where ET_B receptor signaling is dependent on cADPR and/or RyR, but differ from previous findings in isolated afferent arterioles showing that $[Ca^{2+}]_i$ responses to S6c were not significantly diminished by nicotinamide (65). The contrast between results

likely reflects differences between in vitro and in vivo preparations. Importantly, our study indicates that, whereas ET_B receptor responses may not be mediated by ADPR cyclase in afferent arterioles without intact endothelium in vitro, in vivo renal constrictor responses to ET_B receptor activation are dependent on the enzyme.

Unlike ET_A receptors that are only found on VSMCs, ET_B receptors are present on both VSMCs and endothelial cells and contribute to both constrictor and dilator responses (214). Likewise, ADPR cyclase contributes to signaling pathways in both cell types (65; 212), a fact that may have an impact on interpretation of our results. Since we have previously seen that high concentrations of nicotinamide can increase basal RBF (241) and since the vasoconstrictor response to ET_B is reduced rather than enhanced by nicotinamide in the current study as would be expected if ET_B receptor-induced dilation were inhibited, we conclude that the bulk of the activity of ADPR cyclase in response to ET-1 is likely in the VSMCs rather than endothelial cells. ADPR cyclase signaling may occur in renal endothelial cells. However, inhibition of ADPR cyclase suggests a predominant action in renal VSMC. The role of ADPR cyclase and RyRs in ET_B receptor-induced vasodilatation in the renal microcirculation requires futher investigation.

Interestingly, the attenuation of the ET_B receptor agonist S6c-induced renal vasoconstriction by nicotinamide was significantly greater that of BQ123 + ET-1 or BQ788 + ET-1 ($P<0.05$, data not shown). Synergy may exist between ET_A and ET_B receptor signaling pathways in the renal vasculature (120; 129). Our data likely reflects this idea, suggesting that ADPR cyclase is more important in ET_B receptor signaling in the presence of active ET_A receptors.

Since endothelial cells possess ET_B receptors, we tested whether in vivo responses depend on ET_B receptor-mediated NO production by investigating the contribution of ADPR cyclase to S6c-induced renal vasoconstriction during inhibition of nitric oxide synthase (NOS) activity. We found that inhibition of S6c responses by nicotinamide occurred in the presence of L-NAME, indicating involvement of ADPR cyclase independent of functioning endothelium. As mentioned previously, the interactions between ADPR cyclase and NO appear to be complex and cell-type specific. Our data clearly show that renal vasoconstriction elicited by ET_B receptors occurs via ADPR cyclase activation whether or not NO is present and add to the aforementioned suggestion that the bulk of the effect of ADPR cyclase inhibition in the renal vasculature in vivo is dependent on VSMCs and not endothelial cells. In this regard, our results suggest that NO is probably not exerting a major inhibitory effect on ADPR cyclase activity in renal VSMC in vivo as has been reported for coronary artery VSMC in vitro(266).

In summary, we present RBF evidence showing that ADPR cyclase and RyR mediate acute renal vascular responses to ET-1 in vivo and extend our previous study of the renal microcirculation demonstrating the importance of the ADPR cyclase/RyR signaling pathway in G protein coupled receptor stimulation by Ang II and norepinephrine (241). Ours is the first animal study to investigate this area in any vascular bed in vivo where complex interactions between cell and tissue types occur naturally. Intrarenal infusion of nicotinamide or ruthenium red at doses that did not alter MAP or basal RBF attenuated RBF responses to ET-1. Importantly, this was the case for responses to selective ET_A and ET_B receptor stimulation as well. Moreover, CD38-/- mice showed attenuated RBF responses to ET-1. ET_B receptor dependence on ADPR cyclase was seen even in the absence as well as

the presence of NO production, suggesting direct effects on renal VSMC. Our results provide new insight into ET-1's regulation of renal hemodynamics under normal conditions and have implications for regulation of renal hemodynamics in the hypertensive state when ET receptors are upregulated and more tonically activated (213; 214).

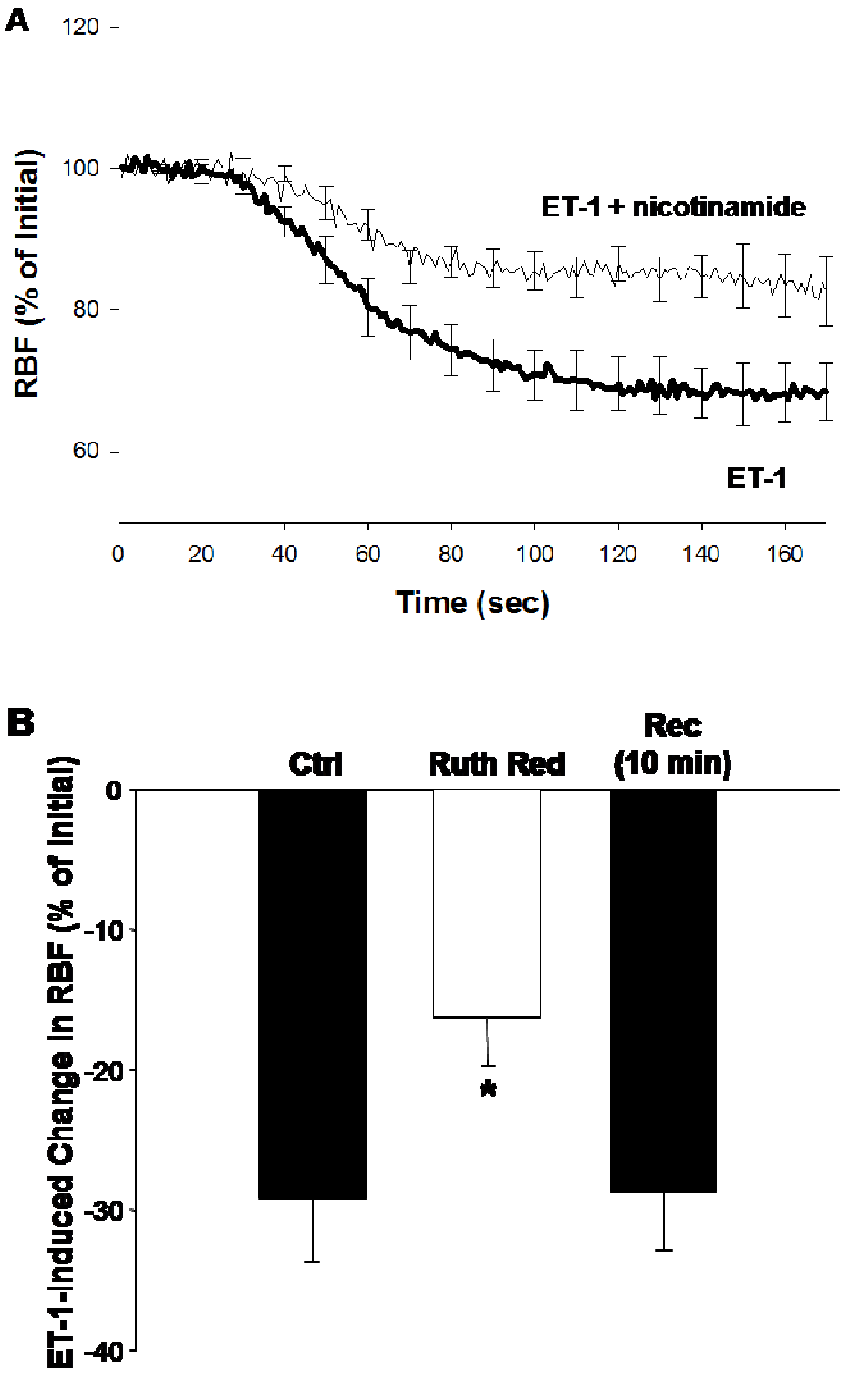

Figure 3.1. A: Average RBF responses to intrarenal ET-1 injection under control conditions before (bold line) and during nicotinamide infusion into the renal artery (6 mg/kg/min, fine line). **B:** Average change in RBF in response to ET-1 before (ctrl), during (nic), and after (rec) nicotinamide, n=8. *: P<0.01.

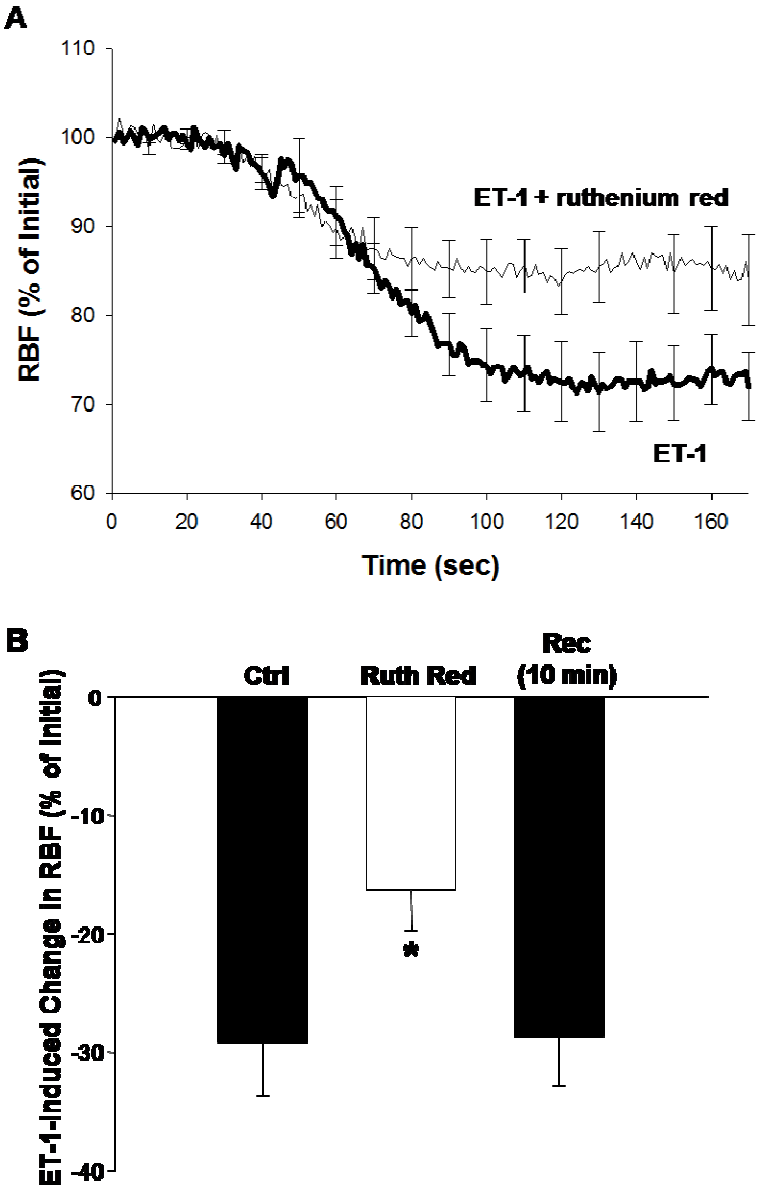

Figure 3.2. A: Average RBF responses to intrarenal ET-1 during infusion of vehicle (bold line) and during ruthenium red infusion into the renal artery (126 µg/kg/min, fine line). **B:** Average RBF response to ET-1 before (ctrl), during (ruth red), and after (rec) ruthenium red, n=8. *: $P<0.05$.

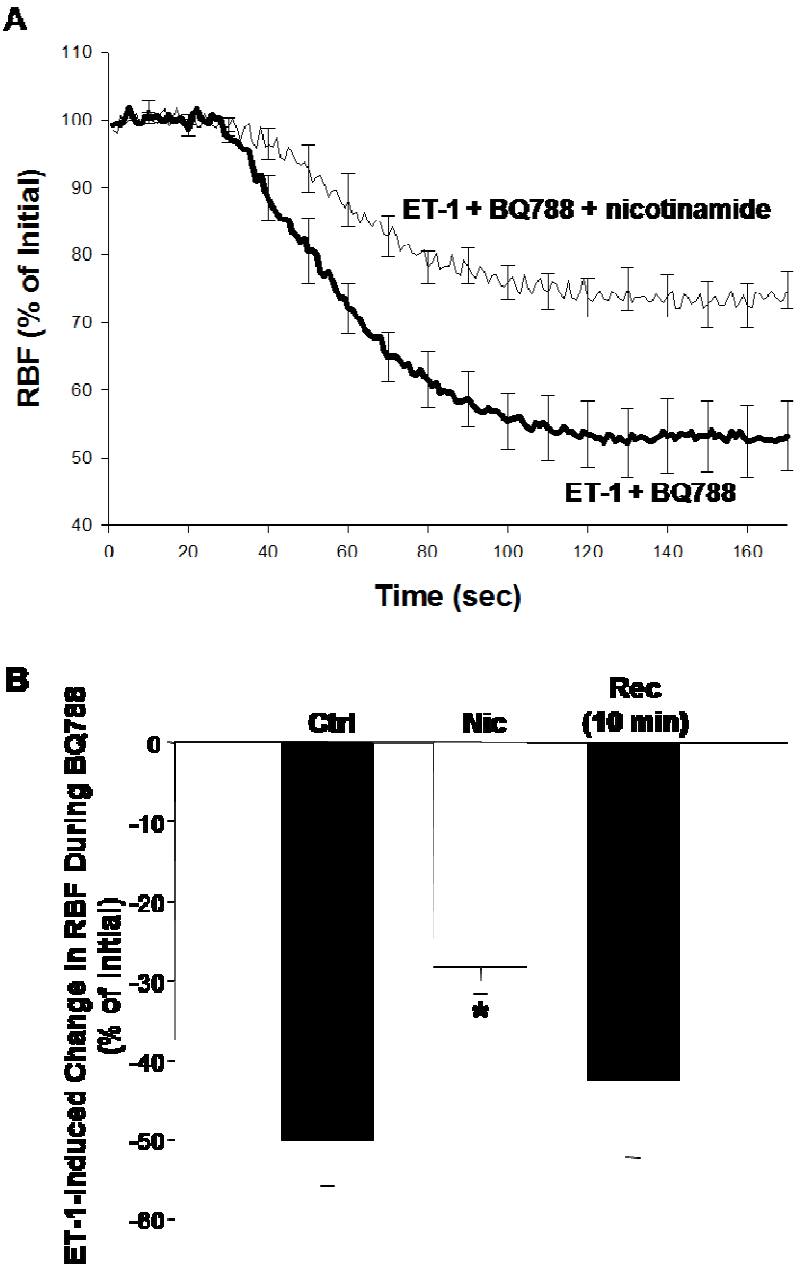

Figure 3.3. A: Average RBF responses to intrarenal ET-1 injection during infusion of BQ788 before (bold line) and during nicotinamide infusion into the renal artery (6 mg/kg/min, fine line). **B:** Average change in RBF in response to intrarenal ET-1 injection during infusion of BQ788 before (ctrl), during (nic), and after (rec) nicotinamide, n=9. *: $P<0.01$.

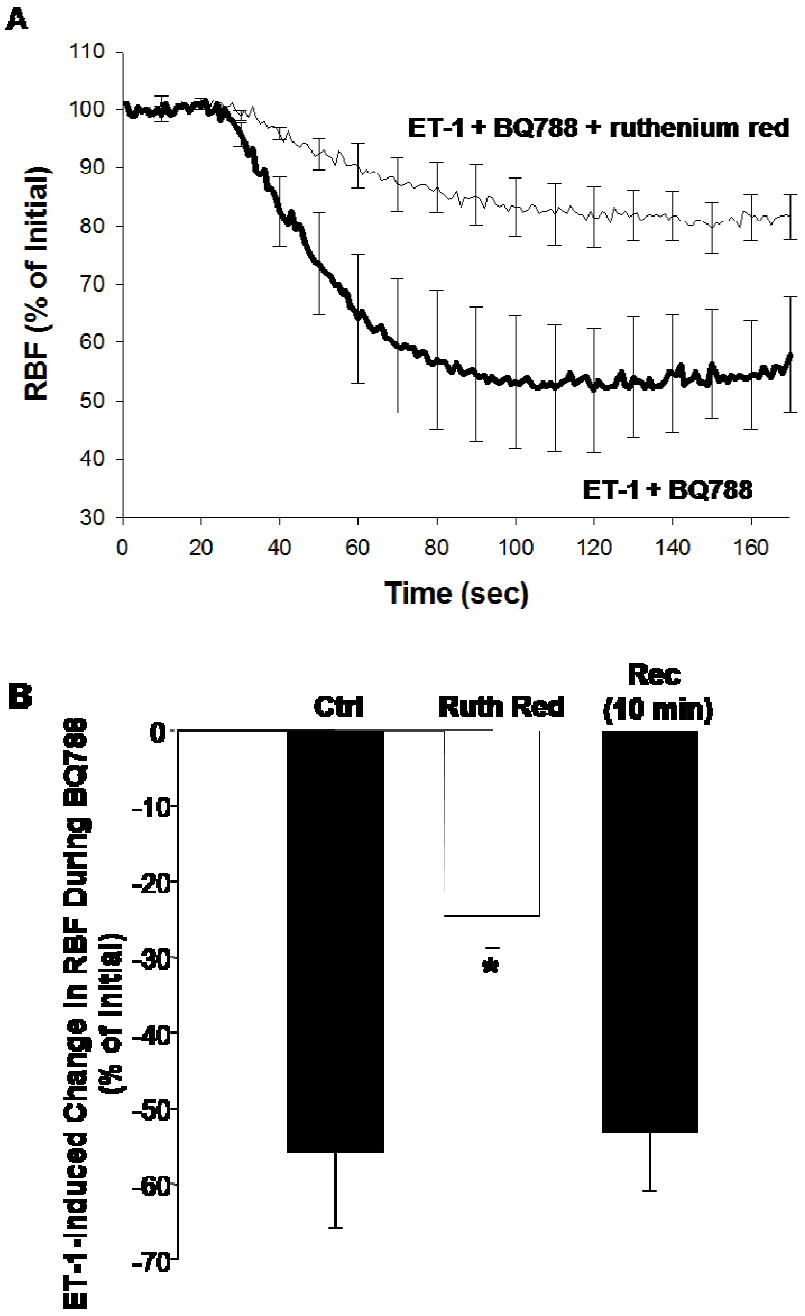

Figure 3.4. A: Average change in RBF following intrarenal ET-1 injection in the presence of BQ788 before (bold line) or during intrarenal ruthenium red infusion (126 µg/kg/min, fine line). **B:** Average RBF responses to ET-1 during BQ788 infusion before (ctrl), during (ruth red), or after (rec) ruthenium red, n=7. *: P<0.02.

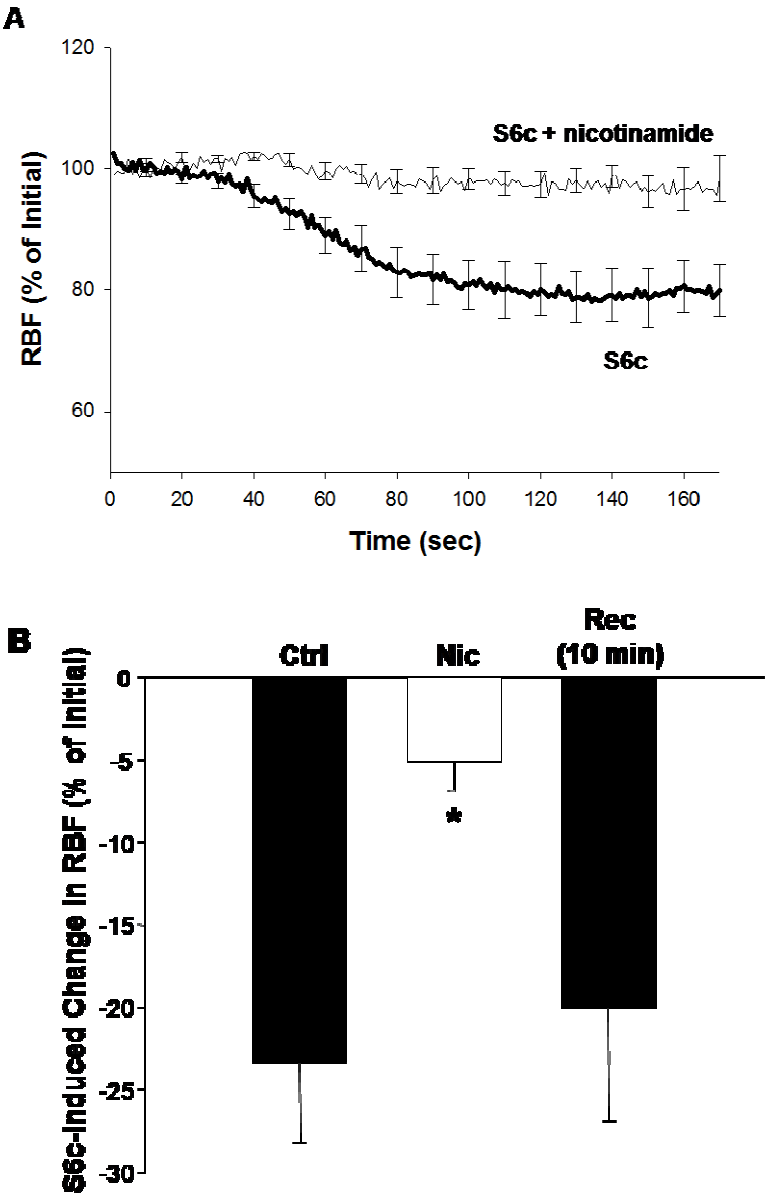

Figure 3.5. A: Average RBF changes over time in response to intrarenal sarafotoxin S6c injection before (bold line) and during nicotinamide infusion into the renal artery (6 mg/kg/min, fine line). **B:** Average percent S6c-induced changes in RBF before (ctrl), during (nic), and after (rec) nicotinamide, n=8. *: $P<0.005$.

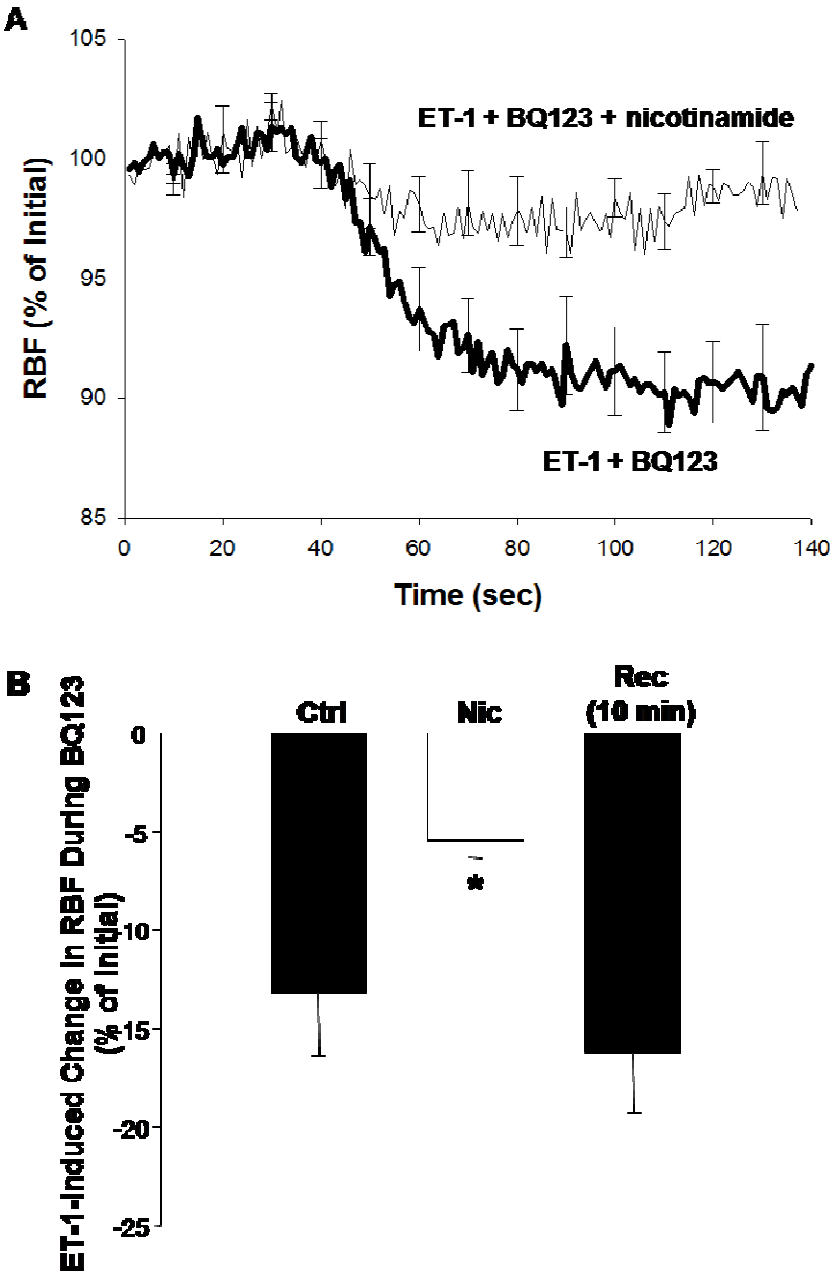

Figure 3.6. A: Average RBF changes produced by ET-1 injection in the presence of the ET_A receptor antagonist BQ123 prior (bold line) or during intrarenal infusion of nicotinamide (6 mg/kg/min, fine line). **B:** Average maximum RBF responses to ET-1 in the presence of BQ123 before (ctrl), during (nic), and after (rec) nicotinamide, n=12. *: $P<0.01$.

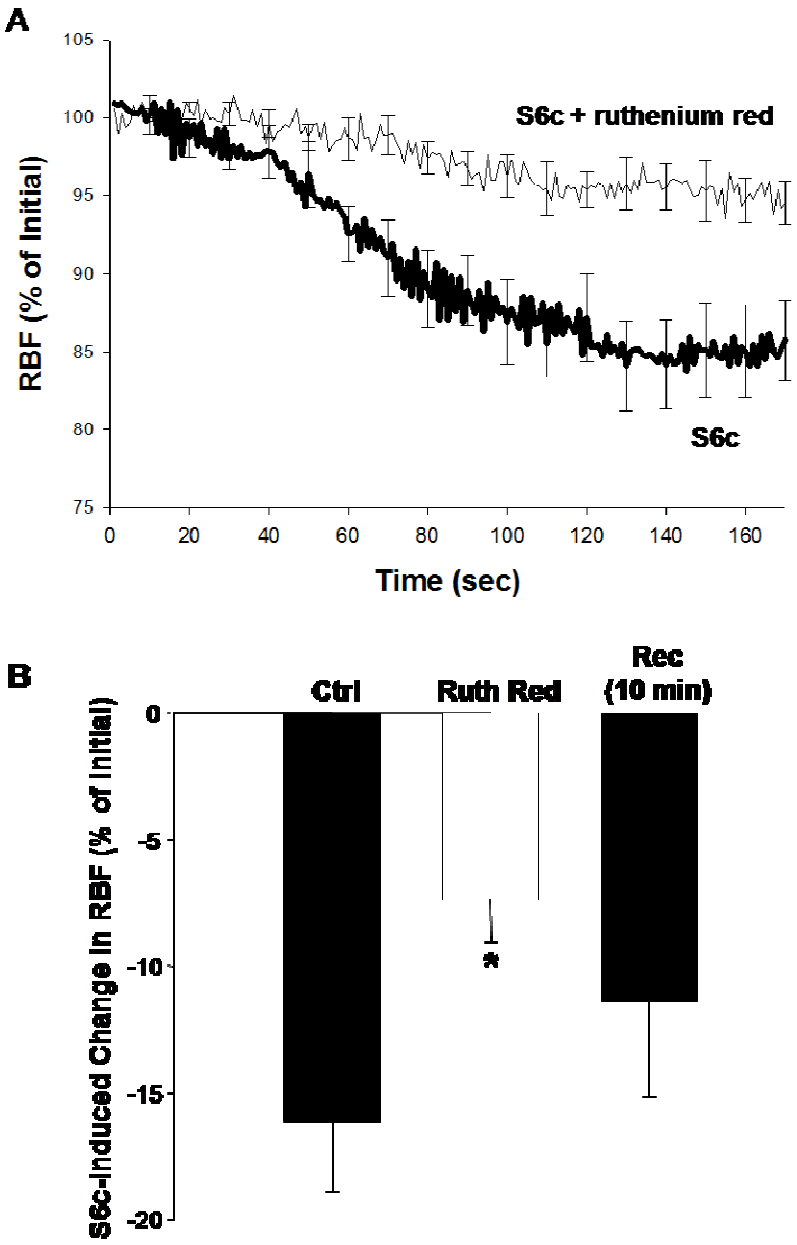

Figure 3.7. A: Average RBF change in response to intrarenal injection of sarafotoxin S6c under control conditions (bold line) and in the presence of ruthenium red infusion into the renal artery (fine line, 126 µg/kg/min). **B:** Average RBF responses to S6c before (ctrl), during (ruth red), and after (rec) ruthenium red, n=8. *: P<0.02.

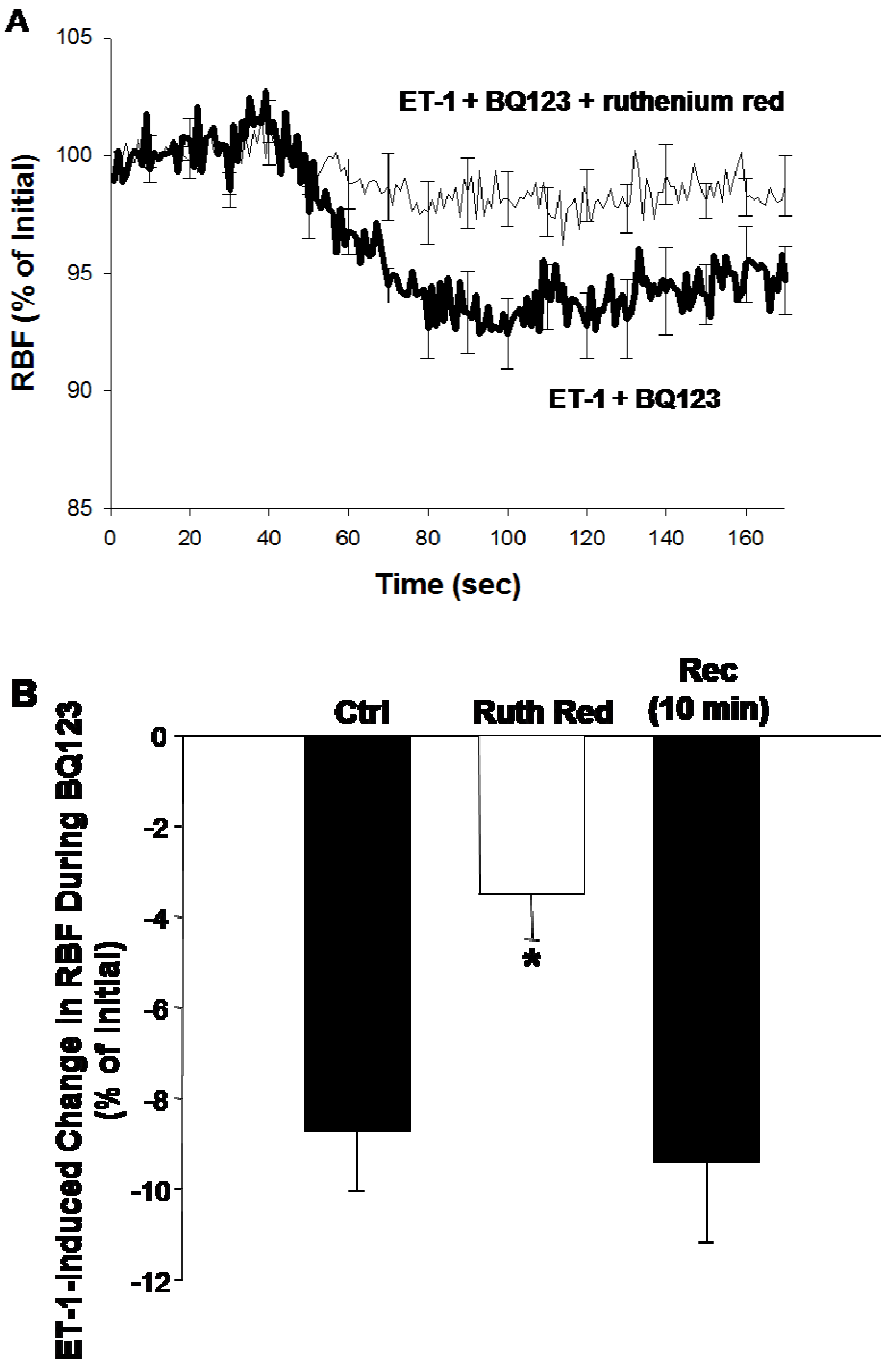

Figure 3.8. A: Average RBF response to ET-1 injected intrarenally in the presence of BQ123 before (bold line) and during intrarenal nicotinamide infusion (6 mg/kg/min, fine line). **B:** Average change in RBF in response to ET-1 in the presence of BQ123 before (ctrl), during (nic), and after (rec) nicotinamide, n=9. *: P<0.01.

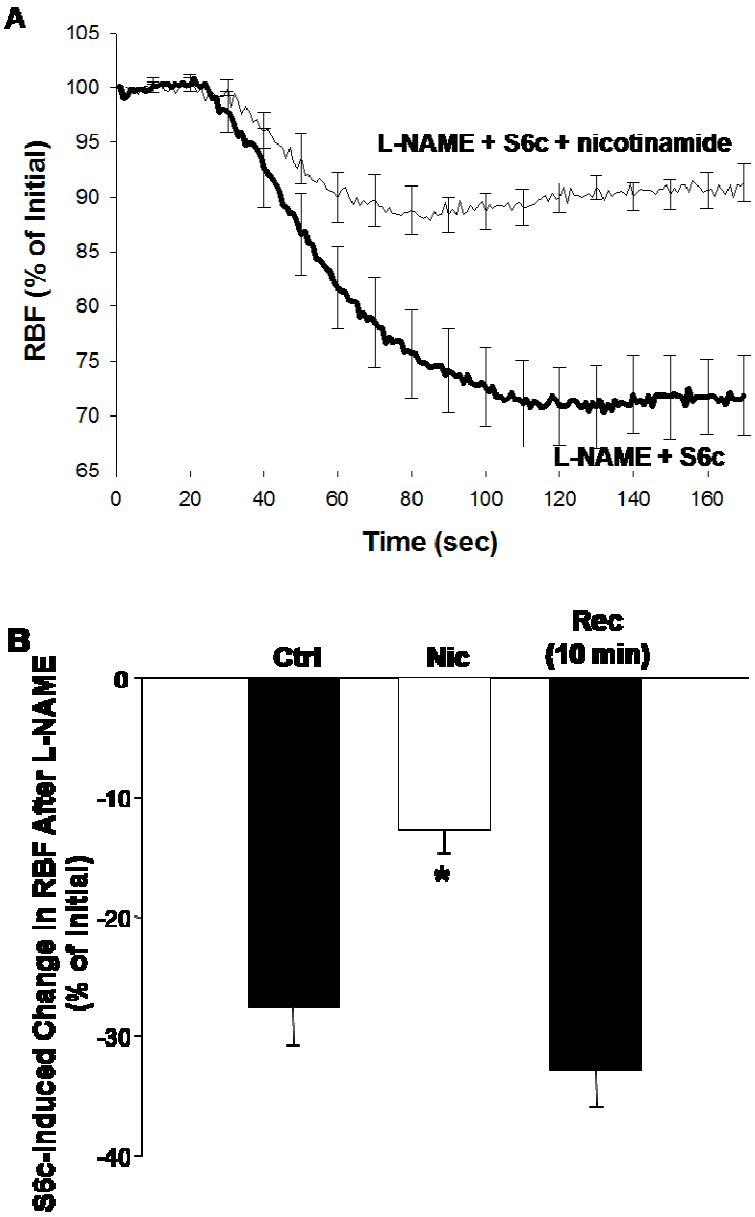

Figure 3.9. A: RBF response to sarafotoxin S6c during L-NAME infusion before (bold line) and during nicotinamide infusion into the renal artery (6 mg/kg/min, fine line). **B:** Change in RBF in response to S6c during $_{L}$-NAME infusion before (ctrl), during (nic) and after (rec) nicotinamide, n=8. *: P<0.005.

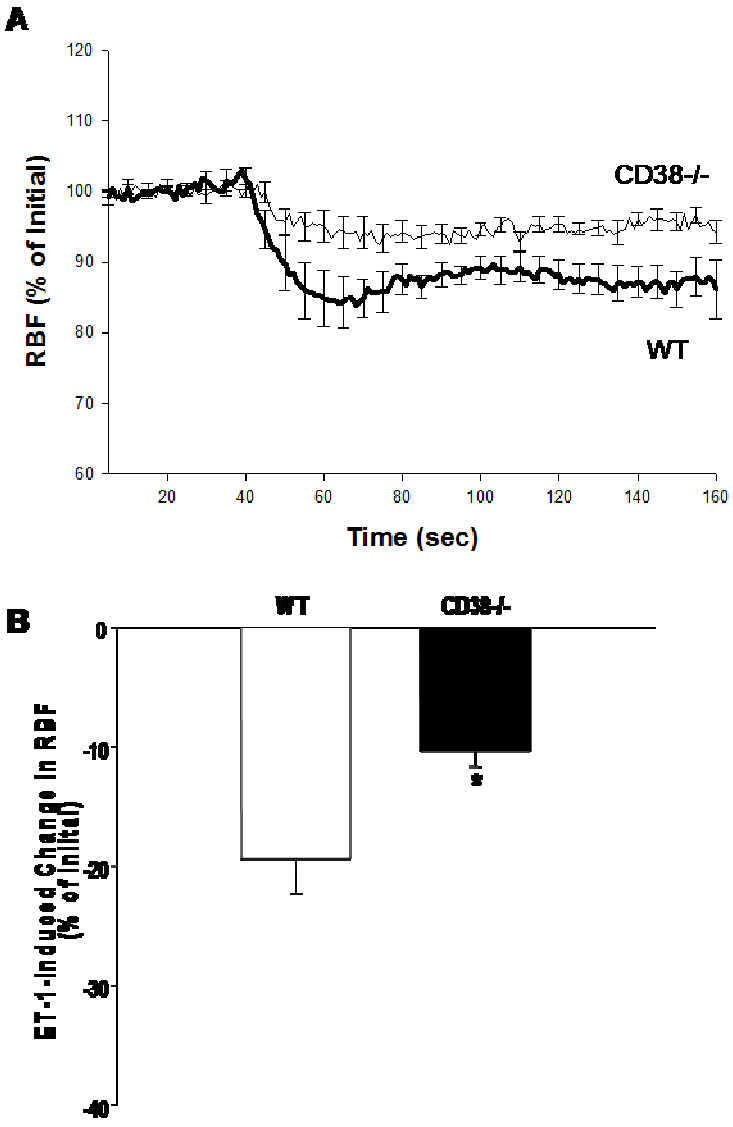

Figure 3.10. **A**: RBF response to ET-1 in wild type (WT, bold line) and CD38-/- (fine line) animals. **B:** Maximum RBF responses to iv injection of ET-1 in wild type control mice (WT, open bar) and mice lacking CD38 (CD38-/-, closed bar), n≥9. *: P<0.02.

CHAPTER IV

NAADP receptors mediate calcium signaling stimulated by endothelin-1 and norepinephrine in renal afferent arterioles

Submitted to the American Journal of Physiology—Renal Physiology

CHAPTER V

Mice Lacking the ADP Ribosyl Cyclase CD38 Exhibit Attenuated Renal Vasoconstriction to Angiotensin II, Endothelin-1, and Norepinephrine

Submitted to the American Journal of Physiology—Renal Physiology

CHAPTER VI

General Discussion

A. OVERVIEW OF RESULTS

The overall purpose of this dissertation was to determine the role, if any, of the ADPR cyclase signaling pathway in the regulation of renal hemodynamics. To this end, I employed a variety of in vivo and in vitro techniques to demonstrate expression and physiological relevance of ADPR cyclase signaling in the renal vasculature. I found that ADPR cyclase regulates baseline RBF and ADPR cyclase and RyR mediate in vivo vasoconstrictor responses to Ang II, NE, and ET-1. Furthermore, I showed expression of two ADPR cyclase family members in renal preglomerular resistance arterioles and determined CD38 to be the family member primarily responsible for the physiological effects of the enzyme on RBF. Finally, I determined a functional role for the second messenger NAADP in mediating NE- and ET-1-induced $[Ca^{2+}]_i$ responses in renal afferent arterioles. These findings contribute greatly to the current understanding of RBF regulation and provide the groundwork for future studies characterizing the physiological effects of this pathway in health and disease and are summarized in figure 6.1.

$[Ca^{2+}]_i$ regulation in VSMC and endothelial cells contributes importantly to overall vessel tone and regulation of local tissue perfusion. In the renal vasculature, alterations in $[Ca^{2+}]_i$ of these cells and concurrent changes in contractile state of pre and postglomerular resistance arterioles influence RBF and GFR, leading to alterations in salt and water excretion and ultimately blood volume and blood pressure. Thus, afferent and efferent arteriolar $[Ca^{2+}]_i$ signaling can produce systemic hypertension and kidney disease. The purpose of this dissertation was to investigate a novel Ca^{2+} signaling pathway involved in the regulation of renal vascular tone. While previous work has lead to the discovery of a plethora of plasma membrane Ca^{2+} channels in VSMC and endothelial cells, I chose to

investigate a newly discovered enzyme system, ADPR cyclase, capable of generating two second messengers involved in mobilization of Ca^{2+} from intracellular stores in VSMC.

Prior to the start of this dissertation, little was known about the presence of ADPR cyclase in renal resistance arterioles. In fact, only two publications existed: one demonstrating production of the second messenger cADPR in renal microvessels and its ability to increase $[Ca^{2+}]_i$ in VSMC (150) and a second showing attenuated $[Ca^{2+}]_i$ responses to the vasoconstrictor Ang II in isolated renal afferent arterioles in the presence of inhibitors of ADPR cyclase, cADPR, and RyR (61). Based on this information, I began to address the question of the significance of ADPR cyclase in the renal vasculature by performing in vivo studies aimed at testing whether ADPR cyclase and the downstream target of both second messengers produced by the enzyme, RyR perform a physiological role in the renal vasculature to regulate RBF. Measurements of RBF in anesthetized Sprague-Dawley rats showed functional RyR as evidenced by vasoconstrictor responses to Ca^{2+} mobilization secondary to RyR opening by FK506, an agent that removes inhibitory FK506 binding proteins. Vasodilatory responses to ADPR cyclase inhibition by nicotinamide led to the conclusion that ADPR cyclase activity contributes to the regulation of basal renal vascular tone under resting conditions.

Renal vascular tone in vivo is largely determined by local paracrine / autacrine factors and GPCR agonists. As a result, my subsequent studies asked whether ADPR cyclase and RyR participate in downstream in vivo renal vasoconstriction by three common vasoconstrictors: Ang II, NE, and ET-1. Renal vasoconstrictor responses to Ang II and NE were attenuated during ADPR cyclase or RyR inhibition in anesthetized rats, implicating cADPR and/or NAADP production or activity downstream of AT_1 and α_1 adrenergic

receptors. While AT_1 is the primary contractile Ang II receptor in the renal vasculature (222) and NE likely works almost exclusively by α_1 adrenergic receptor activation (209), ET-1 produces renal vasoconstriction by acting on both ET_A and ET_B receptor subtypes (129). For this reason, I used a combination of ET_A and ET_B receptor agonists and antagonists to analyze individual effects of ADPR cyclase and RyR inhibition on ET receptor subtypes. Interestingly, I found inhibition of both ET_A and ET_B receptor-induced renal vasoconstriction during ADPR cyclase or RyR inhibition, suggesting involvement of ADPR cyclase signaling pathways downstream of both receptor subtypes in renal VSMC.

While the cardiovascular importance of ADPR cyclase is only beginning to be elucidated, biochemical studies of the enzyme led to extensive characterization of two mammalian ADPR cyclase family members: CD38 and CD157 (BST-1) (224), both well-known antigens presented on a variety of immune cell types. As a result, while immunological and biochemical studies often differentiate between the two family members, cardiovascular studies tend to focus on ADPR cyclases in general. CD38 often has greater expression and activity levels than CD157. However, few studies have determined the expression or action of specific ADPR cyclases in any blood vessel. Thus, I next sought to examine expression levels of both ADPR cyclases in renal microvessels. To accomplish this, I used an iron oxide and sieving preparation to isolate preglomerular resistance arterioles from wild type C57black6 mice and expression levels of CD38 and CD157 mRNA were measured by real time quantitative RT-PCR. mRNA of both ADPR cyclases was expressed in wild type tissues. To determine whether knockout of CD38 increased CD157, I compared expression between wild type and CD38-/- strains. Surprisingly, CD157 mRNA was

decreased, rather than increased, indicating a lack of upregulation of CD157 in the absence of CD38.

To ask whether CD38 is the primary ADPR cyclase regulating renal vascular reactivity to Ang II, NE, and ET-1, responses to these agonists were compared between wild type and CD38-/- mice. Animals lacking CD38 produced consistently weaker RBF responses to GPCR stimulation. The ADPR cyclase inhibitor nicotinamide, which almost completely abolished Ang II-induced renal vasoconstriction in wild type mice, had no significant effect on Ang II responses in CD38-/- animals. Nicotinamide is a byproduct produced by all ADPR cyclases and therefore should equally inhibit all members of this family. The fact that nicotinamide exerted no inhibitory action in CD38-/- mice strongly suggests that, while CD157 is detectable in renal preglomerular resistance arterioles, it is unlikely that this ADPR cyclase contributes significantly to Ang II-induced renal vasoconstriction.

While the bulk of my dissertation research has focused on ADPR cyclase and RyR with little emphasis on the second messengers produced in between, my final experiments addressed the question of which metabolites mediate $[Ca^{2+}]_i$ responses to the vasoconstrictors tested in previous studies. Considerable work has focused on production of one metabolite, cADPR, both in renal and extrarenal vascular beds as well as the in vitro importance of this second messenger in mediating $[Ca^{2+}]_i$ responses to a variety of GPCR agonists. Very little effort, on the other hand, has focused on the role of NAADP in such processes and no single study existed examining the effects of NAADP in the renal vasculature. Therefore, my final experiments were designed to determine the possible involvement of NAADP in renal afferent arteriolar $[Ca^{2+}]_i$ signaling. While all of my

previous experiments were performed in vivo to gain understanding of the overall coordination of the integrated physiological processes opposing and cooperating with each other to produce renal vasoconstrictor responses, the lack of knowledge in this specific area led me to choose to investigate $[Ca^{2+}]_i$ responses in afferent arteriolar VSMC using intact isolated afferent arterioles without functioning endothelium. Biochemical studies as well as studies in other isolated cells have shown that ADPR cyclase produces NAADP only at very low pH, such as that found in a lysosome (1) and that NAADP releases Ca^{2+} from lysosomes near RyR on the SR (135). As a result, my study asked two questions: whether lysosomal vesicle Ca^{2+} stores participate in GPCR-induced afferent arteriolar $[Ca^{2+}]_i$ responses and whether NAADP acts downstream of these vasoconstrictors. I found attenuated $[Ca^{2+}]_i$ responses to NE and ET-1 in the presence of either lysosomal disruption with Concanamycin A and Bafilomycin A1 or inhibition of NAADP receptor with Ned-19. These results indicate involvement of both lysosomes and NAADP in GPCR signaling pathways of afferent arterioles. NAADP therefore likely contributes to NE and ET-1 signaling in renal afferent arterioles, an observation that opens the door for future investigation.

B. INTRACELLULAR MECHANISMS REGULATING Ca^{2+} MOBILIZATION IN AFFERENT ARTERIOLAR VSMC

My dissertation focused almost exclusively on Ca^{2+} mobilization from intracellular stores. Nevertheless, extracellular Ca^{2+} has long been known to contribute importantly to changes in renal afferent and efferent arteriolar VSMC (236). A wide variety of channels exist on VSMC plasma membranes to allow for Ca^{2+} entry. L-type, T-type, and P/Q-type voltage-gated Ca^{2+} entry channels have all been identified in renal VSMC (70; 123; 58). Depolarization stimulated by GPCR agonists leads to activation of these channels. In

addition, receptor-operated channels lie directly downstream of GPCR agonists (237; 56) and store-operated channels open in response to depletion of SR stores by GPCR agonists (58; 257).

Despite the prevalence of these channels, early research on Ca^{2+} release from internal stores suggested an alternative pathway dependent on phospholipase C (236) and IP_3. SR mobilization in renal VSMC was first thought to occur solely on IP_3 production and stimulation of IP_3R on the SR. While ET-1 and NE induced such mobilization, it was thought that Ang II acted preferentially through Ca^{2+} entry and almost independent of mobilization pathways (236; 208). We now know that IP_3-mediated Ca^{2+} mobilization actually contributes importantly to Ca^{2+} responses elicited by Ang II in renal afferent arterioles (61).

In addition to IP_3R, RyR exist on SR membranes and contribute importantly to the amplification of Ca^{2+} responses by CICR in cardiac and skeletal muscle. Only just before I began my dissertation did evidence emerge for function of RyR in renal VSMC including renal resistance arterioles (61; 150). These studies showed ADPR cyclase and RyR participating in Ca^{2+} responses in isolated afferent arterioles. I expanded this knowledge by demonstrating that Ang II and ET-1 work by actions on ADPR cyclase and RyR in vivo. In addition, my in vivo animal experiments helped clarify functional importance and related issues beyond those addressed in isolated vessels. I observed increases in basal RBF in response to ADPR cyclase inhibition, implicating, for the first time, regulation of basal Ca^{2+} levels by ADPR cyclase and RyR in renal VSMC under resting conditions. One can speculate that these effects are due largely to basal production of cADPR known to sensitize

RyR, however, this hypothesis has not been tested in a systematic manner and thus awaits future experimentation.

My data also are novel in that they are the first to indicate that CD38 is the key ADPR cyclase participating in Ca^{2+} responses to Ang II, NE, and ET-1 in the renal microcirculation. The experiments were all done in vivo, and future experiments examining Ca^{2+} responses in isolated afferent arterioles of CD38-/- mice would document effects of CD38 deletion on afferent arteriolar $[Ca^{2+}]_i$.

In addition to SR stores, high $[Ca^{2+}]$ is maintained in mitochondria and lysosomes (135; 95). Although $[Ca^{2+}]$ is high in these organelles, its pool is significantly smaller than that in extracellular or SR stores and, for this reason, it has long been assumed that these stores do not significantly contribute to global $[Ca^{2+}]_i$ responses in VSMC. At present, my data are the only information available showing a contribution of lysosomal vesicle stores to global $[Ca^{2+}]_i$ responses to any agonist in any resistance arteriole. Specifically, my findings implicate that this Ca^{2+} exits lysosomes through NAADP receptors sensitive to Ned-19. Publications on pulmonary arterial VSMC suggest that NAADP receptors are coupled to RyR, but experiments are needed to show whether this is the case in afferent arterioles. Indeed, future studies should focus on determining whether this is the only path for lysosomal vesicle Ca^{2+} in afferent arterioles and whether this path is similar or different from that observed in VSMC from other vascular beds.

My studies indicate an extremely important effect of ADPR cyclase and NAADP on in vivo and in vitro afferent arteriolar $[Ca^{2+}]_i$ responses to a variety of GPCR agonists. It is noteworthy that other studies have seen large effects of inhibition of Ca^{2+} entry (193) and IP_3

receptors (65) on ET-1-induced afferent arteriolar $[Ca^{2+}]_i$ responses. It is therefore likely that a considerable amount of crosstalk exists between the various pathways. My data suggest at least one type of crosstalk: that L-type channel responses are amplified by the actions of ADPR cyclase and RyR. However, other types of intereactions must be involved to explain the large degree of inhibition seen during blockade of NAADP receptors, IP_3 receptors, RyR, and various plasma membrane Ca^{2+} channels independent of one another. An extensive set of studies is required to investigate crosstalk among pathways in VSMC in general and specifically in afferent arterioles.

C. PHYSIOLOGICAL IMPACT OF VSMC Ca^{2+} MOBILIZATION ON RENAL HEMODYNAMICS

Ca^{2+} plays a fundamental role in regulating in vivo renal vascular tone both through VSMC and endothelial cells. Increases in $[Ca^{2+}]_i$ in EC lead to eNOS activation and vessel dilation whereas activation of similar pathways (i.e. mobilization by IP_3R and RyR) in VSMC produce vessel contraction. As a result, it is difficult to predict overall physiological effects of pathways that increase $[Ca^{2+}]_i$ in both cell types, and the study of integrated renal vascular responses is extremely important. On the other hand, the complexity of integrated responses means that physiological studies may not always reveal the intricacies of Ca^{2+} signaling pathways. As a result, it is useful to consider in vitro preparations as well. The simplest preparation (in terms of data interperetation) is isolation of a single cell type, cultured VSMC of preglomerular resistance arterioles. These arterioles are isolated using the iron oxide/sieving method, and VSMC grown in culture conditions that provide overwhelmingly slower growth of endothelial cells (278; 197). The result is a pure, stable, primary culture that does not require the sacrifice of many animals. However, there are

limitations. For example, expression of some proteins (189) changes over time in culture. Certain receptors and signaling pathways may change in these artificial conditions. For this reason, we believe it is advantageous to study freshly isolated over cultured cells. Even closer to the in vivo setting, but still in a controllable environment are intact vessel segments where isolated afferent arterioles can be studied. The benefits of this VSMC preparation include the absence of functioning endothelial cells (65) and selective identification of afferent arterioles in a reproducible controllable environment. In addition to relevance, freshly isolated arteriolar segments and their VSMC are attractive for initial studies for these reasons as well as the low concentration of reagents required. Thus, I chose this preparation for my in vitro studies of Ca^{2+} signaling and NAADP receptors. Future studies are required to determine the physiological role of NAADP receptors and lysosmal vesicles in the regulation of integrated renal vascular responses in vivo.

For the majority of my studies of vascular contraction and reactivity in vivo, I performed whole animal surgeries on either rats or mice. My initial RBF studies investigating the use of ADPR cyclase and RyR inhibitors in vivo were conducted in parallel to in vitro Ca^{2+} signaling studies on isolated afferent arterioles (65; 61; 66). In almost all cases, the in vivo and in vitro data agree. An exception of my in vivo studies is the finding of the importance of ADPR cyclase and RyR in both ET_A and ET_B receptor subtype signaling in RBF experiments, as compared to primary mediation of ET_B receptor actions in isolated afferent arterioles. It is not known whether the ET_B receptor is more labile and may have been disrupted during isolation or whether this difference reflects interactions between ET_A and ET_B receptor subtypes not present in isolated vessels. The exact nature of ET receptor interactions in vivo is only beginning to be investigated and it is therefore difficult to guess

as to the specific action present in vivo vs. in vitro (129; 18). Future experiments should explore ET receptor subtype interactions in the renal vasculature and the effect of these interactions on ADPR cyclase activation.

My in vivo studies provide new information demonstrating basal and agonist-induced physiological actions of ADPR cyclase and RyR in the renal microcirculation. I also show that the bulk of the actions of ADPR cyclase inhibition occur through effects on the ADPR cyclase CD38. It is important to note that biochemical and genetic characterization of ADPR cyclase family members is in its infancy. It has been suggested that subpopulations of ADPR cyclases exist such as cytosolic ADPR cyclase (38) and a specific vascular ADPR cyclase (50; 64). Characterization of these enzymes as CD38, CD157, or a novel family member remains to be seen. My studies were by design therefore limited to CD38 and CD157 and, due to the unavailability of CD157 knockout animals, were largely focused on CD38. My experiments convincingly demonstrate the absence of effect of ADPR cyclase inhibition in CD38-/- animals and suggest predominance of CD38 in integrated renal vascular responses, an effect of other family members cannot be excluded. As new data emerge further characterizing this family, the role of various members in renal vasoconstrictor responses in vivo should/could be investigated.

D. IMPLICATIONS FOR HEALTH AND DISEASE

Renal afferent arterioles are known to participate in the pathogenesis of several diseases including hypertension and kidney disease. The contractile state of these vessels determines RBF, GFR, glomerular capillary pressure and post-glomerular capillary pressures. Decreases in RBF and GFR due to enhanced afferent arteriolar contraction can lead to salt

and water retention and an increase in blood volume. This increase in blood volume can, over time, produce hypertension. High arterial pressure can, in turn, depending on the efficiency of preglomerular autoregulatory mechanisms, lead to glomerular injury and kidney disease. Although the exact mechanisms underlying the increase in total peripheral vascular resistance associated with hypertension are not completely known, our current understanding suggests that alterations in downstream pathways of common renal vasoconstrictors contribute. This idea is evidenced by the fact that spontaneously hypertensive rats (SHR), a strain selectively bred for high blood pressure as a model of essential hypertension in humans, have increased renal vascular reactivity to many vasoconstrictors including Ang II (31), NE (177), and ET-1 (86), AVP (68), thromboxane (31) and reduced responses to vasodilators such a prostaglandins and dopamine (32). It is for this reason that the constrictors Ang II, ET-1 and NE were chosen for use in this dissertation.

In summary, my dissertation research sheds new light on signaling pathways downstream of GPCR of common renal vasoconstrictors and provides compelling evidence for an important role of the ADPR cyclase/RyR signaling pathway in mediating acute constrictor effects of Ang II, NE, and ET-1 in normotensive animals. While my research provides essential basic knowledge regarding these pathways in health, it remains to be seen whether exaggerated activity of the ADPR cyclase/RyR pathway participates in the development or maintenance of cardiovascular diseases such as hypertension. Future experiments could compare the effects of acute and chronic pharmacological ADPR cyclase inhibition in SHR and normotensive rats. Also of interest would be the ability of Ang II infusion and/or high salt diet to produce hypertension in CD38-/- mice vs. wild type controls. Such experiments should help determine the contribution of this newly discovered signaling

pathway to cardiovascular disease and hypertension and may help pave the way for the discovery of novel antihypertensive therapies.

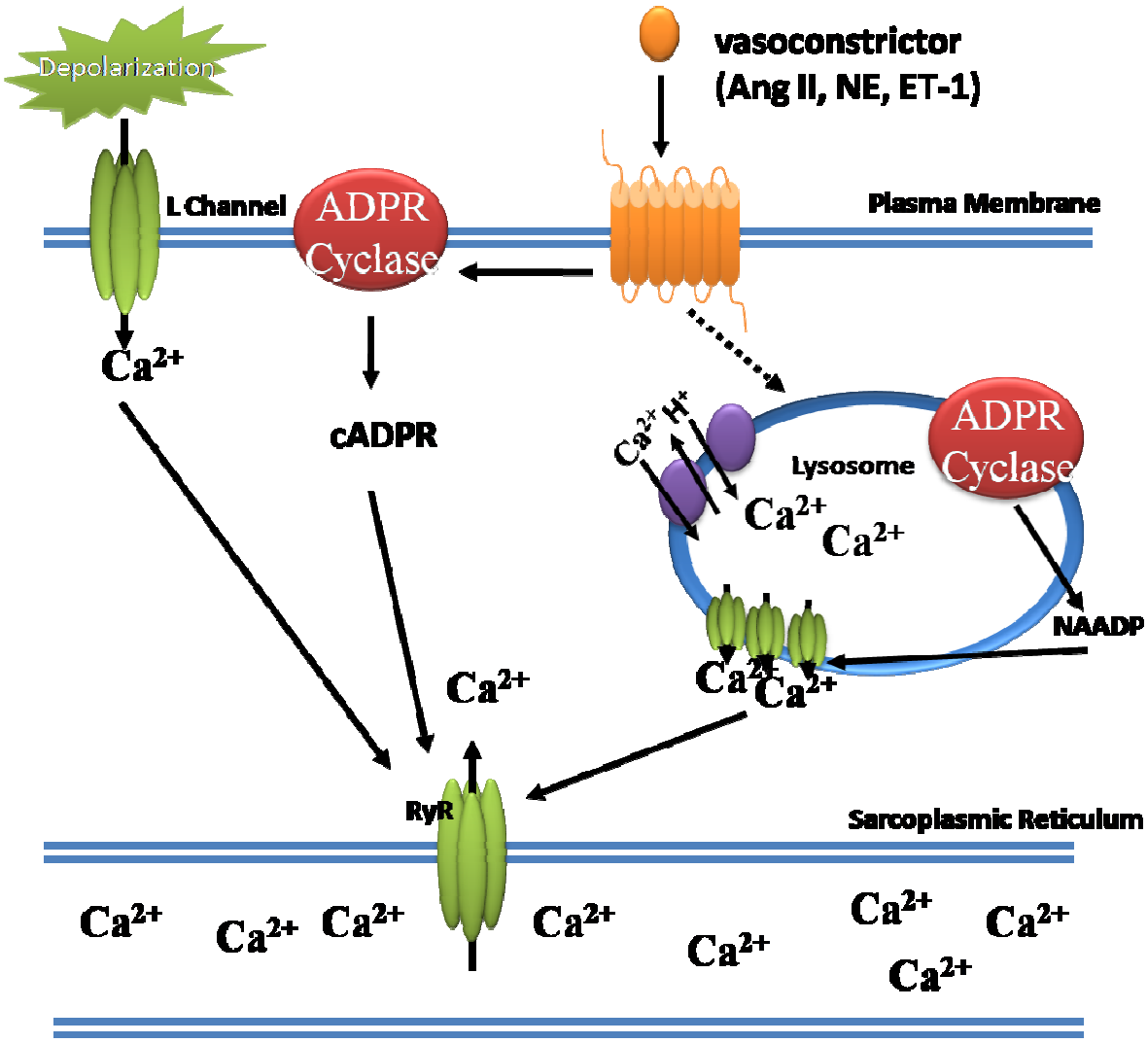

Figure 6.1. ADPR cyclase activation leads to stimulation and/or sensitization of RyR via production of cADPR and/or NAADP. In renal microvessels, ADPR cyclase functions basally to enhance Ca^{2+} entry by L-type voltage-gated Ca^{2+} channels or can be directly activated downstream of GPCR agonists.

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