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ENHANCED ADRENERGIC SENSITIVITY OF MESENTERIC VEINS COMPARED TO ARTERIES AND ITS RELATION TO CALCIUM UTILIZATION AND HANDLING

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ENHANCED ADRENERGIC SENSITIVITY OF MESENTERIC VEINS COMPARED TO ARTERIES AND ITS RELATION TO CALCIUM UTILIZATION AND HANDLING

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ABSTRACT

ENHANCED ADRENERGIC SENSITIVITY OF MESENTERIC VEINS COMPARED TO ARTERIES AND ITS RELATION TO CALCIUM UTILIZATION AND HANDLING

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Mesenteric venous (MV) capacitance contributes to blood pressure regulation. MV are more sensitive to the constrictor effects sympathetic nerve stimulation and exogenous norepinephrine (NE) than mesenteric arteries (MA). The focus of this dissertation was to clarify the mechanism responsible for enhanced venous adrenergic reactivity.

My studies showed that there are differences in the contribution of α-adrenergic receptor (αAR) subtypes in NE-induced constrictions of MA and MV. While α_1ARs alone mediate NE-induced constriction of MA, α_2AR and α_1AR functionally interact to potentiate NE-induced constrictions in MV. Previous studies showed that α_{1D}ARs are the main functional α_1AR subtype in MV. I showed that the α_{2C}AR subtype is the constrictor α_2AR in MV.

The mechanisms responsible for the interaction between α_{1D}- and α_{2C}AR were investigated using heterologous receptor expression techniques and HEK-293 cells. I found that α_2ARs do not co-localize with the α_{1D}AR nor do α_2ARs facilitate trafficking of α_{1D}ARs to the plasma membrane. A positive control showed that β_2AR increased α_{1D}AR trafficking to the membrane. Calcium imaging experiments were performed to test, whether functional interaction between α_{1D}- and α_2AR occurs in HEK-293 cells. While β_2AR increased calcium

responses to NE in α_{1D}AR – expressing HEK-293 cells, α_2ARs did not increase calcium signals caused by α_{1D}AR activation, suggesting that the functional interaction between α_{1D}AR and α_2AR is specific for veins. Furthermore, immunocytochemical analysis on arterial and venous myocytes revealed that MV and MA express α_2ARs. These results suggest that factors other than differential receptor expression are required for the α_1AR-α_2AR interaction.

Receptor interaction was investigated in pressurized MV *in vitro* by measuring calcium signaling. NE responses were inhibited by the calcium channel blockers, nifedipine and gadolinium. In the presence nifedipine of gadolinium the α_2AR antagonist yohimbine did not inhibit NE-induced constrictions, suggesting that α_2AR in MV couples to calcium influx. My data also indicate that MV have less efficient intracellular calcium buffering mechanisms than MA. Therefore, the additional calcium influx in MV contributes to enhanced venous reactivity to NE. Taken together, α_2AR contribute to increased MV reactivity to NE by promoting influx of extracellular calcium; this mechanism is not present in MA. In addition, MA but not MV efficiently buffer intracellular calcium and this further enhances venous NE reactivity. Overall, α_2AR in veins might prove to be a more selective venous target in hypertension, where increased sympathetic nerve activity increases the tone of veins and produces blood redistribution from veins to arteries. In addition, less efficient calcium buffering of veins might be very important in physiological situations such as postural changes, where abrupt venous filling could produce frequent syncopes.

To my parents, who taught me the value of perseverance and gave me the confidence to pursue my dreams.

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LIST OF ABBREVIATIONS

αAR	alpha adrenergic receptor
2-APB	2-aminoethoxydiphenyl borate
Ach	acetyl choline
ATP	adenosine 5' – triphosphate
βAR	beta adrenergic receptor
BP	blood pressure
Ca^{2+}	calcium
CaM	calmodulin
Cd^{2+}	cadmium
CICR	calcium – induced calcium release
CO	cardiac output
COMT	catechol-o-methyltransferase
CRC	concentration – response curve
DAG	diacyl glycerol
DOCA	deoxycorticosterone acetate
DOPA	L-dihydroxy – L – phenylalanine
EGTA	ethylene glycol tetraacetic acid
EPI	epinephrine
Gd^{3+}	gadolinium
HEK	human embryonic kidney
HR	heart rate

IP_3	inositol triphosphate
K^+	potassium
M3 receptors	muscarinic receptors 3
MA	mesenteric artery
MAO	monoamine oxidase
MAP	mean arterial pressure
MAP kinase	mitogen – activated protein kinase
MCFP	mean circulatory filling pressure
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MV	mesenteric vein
Na^+	sodium
NCX	sodium – calcium exchanger
NE	norepinephrine
NET	norepinephrine transporter
NKA	sodium – potassium ATP-ase
NLA	nitro – L - arginine
NO	nitric oxide
NSCC	non – slective cation current
PE	phenylephrine
PIP2	phosphatidylinositol bisphosphate
PKC	protein kinase C
PLC	phospholipase C

PM	plasma membrane
PNS	parasympathetic nervous system
PTX	pertussis toxin
RGS	regulators of G – protein signaling
ROC	receptor – operated channels
RyR	ryanodine receptor
SER	smooth endoplasmic reticulum
SHR	spontaneously hypertensive rat
SMC	smooth muscle cell
SNS	sympathetic nervous system
SOC	store – operated channels
TPR	total peripheral resistance
TRPC	transient receptor potential channel
VSMC	vascular smooth muscle cell
WT	wild type
KO	knock - out

CHAPTER 1

Introduction

1. Regulation of Blood Pressure (BP) by the Nervous System.

Many studies stress the importance of neurogenic mechanisms in the long-term regulation of BP (Morimoto et al. 2001,Kishi et al. 2001). Neural control of BP is accomplished through the activity of the autonomic nervous system and sensory nerves that supply blood vessels. Two divisions of the autonomic nervous system regulate BP: the sympathetic and parasympathetic divisions. Both systems originate in nuclei within the central nervous system (CNS) and give rise to preganglionic efferent nerve fibers that exit from the brain stem or spinal cord. Sympathetic preganglionic fibers leave the CNS at the thoracic and lumbar portions of spinal cord (thoracolumbar system), whereas parasympathetic preganglionic fibers leave the CNS at the cranial and sacral portions of the spinal cord (craniosacral system). Preganglionic fibers synapse with the postganglionic neurons in sympathetic and parasympathetic ganglia. Postganglionic nerves fibers innervate the target visceral organs. Although not exclusively, sympathetic and parasympathetic nervous systems exhibit opposing actions on the target visceral organs and the two systems work in a cooperative and integrated manner to control organ function.

There are major differences in the anatomical organization of the sympathetic and parasympathetic divisions of the autonomic nervous system. Preganglionic sympathetic nerve fibers synapse with multiple postganglionic neurons (with ratio up to 1:20) providing for more diffuse action of sympathetic nerves on target tissues. There are two groups of sympathetic ganglia, paravertebral chain ganglia pervertrabral ganglia. Sympathetic neurons in these

ganglia send their postganglionic axons to target tissues. Parasympathetic postganglionic neurons are in ganglia embedded within the organs innervated. In most organs, the ratio between preganglionic and postganglionic cells is 1:1.

Both SNS and PSN utilize acetylcholine (ACh) as the dominant neurotransmitter in the preganglionic nerve fibers. However in the PNS, ACh is released from postganglionic nerve fibers. Norepinephrine (NE) and epinephrine (EPI) are the major neurotransmitters released by the postganglionic nerve fibers in SNS with the exception of thermoregulatory sweat glands, where ACh mediates the action of SNS.

PNS in blood pressure regulation. Parasympathetic fibers innervate the heart and control heart rate and the strength of contraction. In the sinoatrial (SA) node, each cardiac impulse is initiated by a spontaneous depolarization of pacemaker cells. At threshold potential, this depolarization initiates an action potential that is conducted through the atrial muscle fibers to atriovenricular (AV) node. ACh decreases spontaneous depolarization by activating potassium (K^+) currents in the SA node, delaying the time to threshold potential and therefore heart rate. The strength of atrial contraction is also reduced by ACh via inhibition of Ca^{2+} channel activity and reduction of cAMP levels. In addition, at larger ACh concentration muscarinic receptors (M_2) can directly couple to K^+ channels, producing a direct inhibition of atrial contraction. Owing to these inhibitory actions together with systemic vasodilation described below, systemic administration of

small doses of ACh or cholinergic agonists produces a transient fall in BP and bradycardia.

Parasympathetic control of vascular tone. While most vascular beds lack parasympathetic innervation, but in those vessels receiving a parasympathetic supply, Ach released from parasympathetic nerves causes vasodilation. ACh activates muscarinic receptors, primarily the M_3 subtypes located on the endothelial cells of the vasculature. Activation of these receptors activates Ca^{2+}-calmodulin dependent activation of endothelial NO synthase (eNOS), triggering NO production that diffuses to adjacent SMCs and hyperpolarizes them. Vasodilatation may also ensue by the action of Ach on sympathetic nerve endings, where it inhibits norepinephrine release. In instances where endothelium is damaged, high concentrations of ACh can activate M_3 receptors in vascular smooth muscle cells (SMCs) and produce vasoconstriction.

SNS in blood pressure regulation. The SNS is continuously active and the degree of its activity is constantly adapting to changes in the environment. As described above, actions of SNS are more diffuse than actions of PNS. For example, during the "flight of fight" response, all sympathetically – innervated organs are affected simultaneously to cooperatively meet the demands of organism during increased stress levels. In addition, increased SNS promotes secretion of epinephrine (EPI) from adrenal medulla into the circulation, producing an even more wide-spread effect of SNS activation. Three types of

sympathetic fibers (barosensitive, thermosensitive and glucosensitive cardiovascular) innervate blood vessels, the heart, kidney and adrenal medulla and contribute to BP regulation.

The SNS increases heart rate and contractility by activating β_1AR and to lesser extent β_2ARs. β_3AR and αAR are also present in the heart, but role of these receptors is not as dominant in the regulation of cardiac contractility. βARs couple to Gs proteins and stimulation of the activity of adenylyl cyclase. This results into accumulation of cAMP and activation of PKA. PKA affects function of many cellular proteins, including ion channels by phosphorylation. Activation of βARs accelerates the slow depolarization of SA node leading to faster rates to reach threshold potential by the pacemaker cells, resulting into increased heart rate. In addition, stimulation of β_1AR increases contractile force; activation of Gs can also directly activate the voltage–sensitive Ca^{2+} channels in the plasma membrane of cardiac muscle, enhancing Ca^{2+} influx during contraction. Overall, SNS activation increases cardiac output due to its positive chronotropic and ionotropic effect on the heart. Together with the effects on the peripheral vasculature (see below), SNS activation elevates BP.

EPI released from adrenal medulla directly increases cardiac heart rate (HR) and contractility. EPI differentially regulates the tone of various vascular beds, producing vasoconstriction in most resistance vasculature and veins while producing vasodilatation of skeletal muscles.

Sympathetic control of vascular tone. NE and to lesser extent EPI released from the sympathetic nerve endings and adrenal medulla are the major

circulating hormones regulating the vascular tone. The biosynthetic pathways for catecholamine production are well understood and involve hydroxylation of the amino acid tyrosine to the precursor L-dihydroxy – L – phenylalanine (DOPA). This is the rate limiting step in the biosynthetic pathway. DOPA is decarboxylated to form dopaminie that is taken up by storage vesicles in the nerve terminals. Dopamine-β-hydroxylase then catalyses the production of NE in the vesicles. In the adrenal medulla where EPI is the dominant catecholamine being released, an additional step involves methylation of NE by phenylethanolamine-N-methyltransferase. EPI is also stored in the granules of chromaffin cells in adrenal medulla. Quantal release of neurotransmitters into synaptic cleft is facilitated by the arrival of action potential that triggers Ca^{2+} influx into the nerve terminal. Ca^{2+} facilitates the fusion of storage vesicles with axoplasmal membrane and content of the vesicles including enzymes other proteins are discharged to the synaptic cleft. However, NE is not the only transmitter released by sympathetic nerves and role of ATP as a co-transmitter in sympathetic regulation has been well established. The relative contribution of these two transmitters in different vascular beds might provide for differential regulation of their tone. While some studies suggest that sympathetic nerves supplying mesenteric veins (MV) release equal or greater amount of ATP than mesenteric arteries (MA) (Bobalova and Mutafova-Yambolieva 2001[1],Bobalova and Mutafova-Yambolieva 2001[2]), functional data indicate that MV constriction is exclusively mediated by NE (Park et al. 2007). In MA a two-phasic neurogenic constriction is observed in response to stimulation of sympathetic nerves. The

first phase is fast and involves activation of P2X receptors by ATP, whereas the second phase has slower onset and is mediated by NE (Sneddon and Westfall 1984, von Kugelgen and Starke 1991).

Termination of NE signaling occurs by the combination of simple diffusion and reuptake by the axonal terminals by norepinephrine transporter (NET). NE can be also taken up by extraneuronal tissue by the low affinity transporters OCT1 and OCT2. NE can be degraded by monoamine oxidase (MAO) and catechol-o-methyltransferase (COMT). However, metabolic transformation of NE plays a minor role in signal termination such as the powerful degradative pathway mediated by acetylcholine esterase in Ach signal degradation.

The structure and degree of sympathetic innervation influences the vessel responsiveness to sympathetic activation. SNS innervation varies with the vessel size and vascular bed. In general, large elastic arteries are sparsely innervated but as the vascular diameter decreases, the density of innervation increases. This nature of the innervation has important implication in the regulation of blood pressure. Because small arteries are the major contributor to peripheral resistance, their high innervation density allows for rapid rise of blood pressure under "flight or fight" conditions.

In addition to differences in the innervation between vessels of different sizes, vascular beds appear to be regionally regulated by SNS. For example, pharmacological treatment with the sympatholytic agent, clonidine, more effectively inhibits sympathetic outflow to hepatomesentery circulation than

systemic circulation (Esler et al. 1992). In obesity, sympathetic outflow to skeletal vasculature and kidney is increased, while it remains normal in the hepatomesentery circulation and skin and even attenuated in the heart (Vaz et al. 1997).

It is not known, whether MA and MV are also differentially regulated by SNS, but there is evidence that distinct sympathetic neurons project from inferior mesenteric ganglia to these two vessels (Browning et al. 1999). In addition, the nerve fibers of the perivenous plexus are less dense than of periarterial plexus and their orientation also differs. Individual axons are oriented circumferentially around veins, with larger density of vertical than horizontal fibers. In contrast, the arterial plexus consists of axons arranged in net – like manner, with similar density of horizontal and vertical fibers (Park et al. 2007). However, in contrast to arteries where sympathetic fibers rarely cross the media-adventitia border, sympathetic fibers penetrate deep into smooth muscle bundles of most veins (Pang 2001).

Besides the anatomical differences, the electrophysiological properties of the nerve fibers also differ between arteries and veins. Both arteries and veins exhibit frequency-dependent vascular contraction. However, stimulation at equal frequencies causes larger amount of NE release from nerve terminals in MV compared to MA (Bobalova and Mutafova-Yambolieva 2001, Park et al. 2007). This might be due to the fact that NE mediates all components of sympathetic neurotransmission in the MV, whereas in MA, NE, ATP and neuropeptide Y (NPY) mediate constriction (Westfall et al. 1996; Browning et al. 1999, Luo

2003). Furthermore, MV exhibit increased sensitivity compared to MA, with greater vasoconstrictor at any stimulation frequency (Karim and Hainsworth 1976; Hottenstein and Kreulen 1987; Luo et al. 2003). This can not be attributed solely to the fact that sympathetic nerves supplying MV release more NE during stimulation. As discussed below, veins are also more sensitive to exogenous application of adrenergic agonists, implicating the postsynaptic signaling differences in differential responsiveness of MA and MV to adrenergic stimulation (Perez-Rivera et al. 2004, Luo et al. 2003). Circulating hormones or NPY released form the sympathetic nerves and endothelial derived factors can modulate the concentration of Ca^{2+} in SMCs or Ca^{2+} sensitivity of SMCs.

2. Roles of veins and arteries in hemodynamics

Veins in hemodynamics. While a large amount of research has been performed on the regulation of resistance vasculature, comparable studies of the venous system are scarce. This deficit could be partly attributed to neglect on the side of scientists, who ascribed a purely conduit role to the venous system. In addition, monitoring venous pressures *in vivo* and venoconstriction *in vitro* poses significant technical challenges. Despite the prevailing focus of the scientific literature on the arterial side of the circulation, the role played by systemic veins in human physiology as well as in certain pathophysiological conditions is extremely important.

Veins and venules contain 60-75% of the total blood volume (Martin et al. 1998). About half of this amount is stored in the small veins and venules of a diameter less than 400 μm (Monos et al. 1995). Small veins from the splanchnic region are particularly critical in the regulation of blood distribution, because nearly 40% of blood volume resides in this region (Greenway and Lister 1974). The blood reservoir function of splanchnic bed is demonstrated by the fact that hemorrhage of significant amount (20%) of blood volume does not alter MAP, HR or CO. However, splanchnic blood volume is reduced (Greenway and Lister 1974, Pang 2001). This mechanism is also important during exercise, when blood is rapidly transferred from the splanchnic region into cardiopulmonary region in order to meet oxygen demand (Flamm et al. 1990). However, the most significant blood reservoir is not necessarily the area that contains a significant proportion of blood (area that regulates capacitance) but rather the area from which a significant blood volume can be shifted in a fast and controlled manner. Therefore the distribution of blood volume and the blood reservoir function are not necessarily the same (Greenway and Lister 1974). The greatest blood reservoirs are spleen, liver, large abdominal veins, subcutaneous venous plexus and heart and lungs (Guyton et al. 1972).

The capacitance function of veins is related to their much smaller wall to lumen ratio as well as much larger incremental distensibility at low pressure ranges than arteries, resulting in much greater compliance (~30X). Although there are studies investigating the contractile mechanisms of veins, the great majority of those focus on larger caliber vessels like the saphenous or femoral

veins (Crowley et al. 2002, MacLennan et al. 1997). These veins are not typical capacitance vessels but rather counduit vessels. The function of these large conduit veins greatly differs from the hemodynamic role of systemic and highly compliant small diameter veins. Larger conduit veins are thin-walled, sparsely innervated and exhibit differential sensitivity to baroreceptor reflex and vasoconstrictor agents. It is believed that veins in the lower extremities do not store significant amount of blood volume (Magder 1990) and veins from skeletal muscle likely do not play a significant capacitance role owing to their low responsiveness to sympathetic stimulation (Hainsworth et al. 1983). In contrast, MV play a key role in controlling changes of vascular capacity (MV contain ~25% of blood volume). In isolated canine MV preparation, elevation of intraluminal pressure from 0 to 30mm Hg increases the lumen volume by 360% (Gaehtgens and Uekermann 1971). Moreover, there are also differences in the distensibility of the different size veins in the mesentery: *in vivo* studies in anesthetized rats demonstrated that first-order venules are much more distensible than the larger collecting veins (Lang and Johns 1987). However, these studies could not differentiate between active tone and elastic properties of the vascular wall. Table 1 depicts typical capacitance veins as well as conduit veins.

The SNS is the major regulator of venous capacitance (Deschamps and Magder 1992). There are multiple control systems involved in SNS regulation of the venous blood flow: these include local and systemic volume receptors (Nijima 1977, Thrasher, et al. 1982) as well as arterial baroreceptors (Brunner et al. 1988). When blood pressure is reduced, the resulting increase of

Table 1. Not all veins are equal. Counduit veins including vena cava or femoral vein exhibit different properties in mediating responses to sympathetic nervous system than capacitance veins. Table depicts typical capacitance and conduit veins.

Capacitance Veins	Conduit Veins
splanchnic veins	femoral vein
mesenteric veins	saphenous vein
	vena cava
	skeletal muscle veins

sympathetic outflow induces venoconstriction, arterial constriction, increased HR and contractility. Most of the reflex change in the vascular capacitance is in the splanchnic bed (Brunner et al. 1981). As discussed below, another regulatory mechanism that does not involve neural reflex is myogenic activity of venous SMCs.

The capacity of the splanchnic bed to store blood has important implications not only under physiological conditions. When splanchnic storage capacity is chronically compromised, redistribution of blood from these vessels can lead to pathophysiological consequences in the long term. From a quantitative standpoint, capacitance of the systemic veins is the major determinant of venous return to the heart. Under any given conditions under equilibrium venous return equals CO; as a result of the increased preload in the right atria, more forceful contractions of the myocardium occur (Starling's law), leading to increased CO (Guyton et al. 1955) and MAP. Data from early studies suggest that even very small changes in venous capacitance mediated by baroreceptor reflex activation lead to significant increases in venous return and CO (Heymans et al., 1958).

The above mentioned studies emphasize the importance of proper regulation of venous tone in maintaining normal CO and MAP. To better understand the regulation of venous tone, we performed studies on mesenteric capacitance veins with the aim to identify the differences in αAR signaling between MA and MV that may contribute to enhanced adrenergic sensitivity of MV. This mechanism might be important in hypertension, where increased SNS

activity increases the tone of the vasculature. However, veins might be preferential target for this increased tone due to their sensitivity.

Arteries in hemodynamics. While veins contribute very little to TPR, arteries are the major resistor of the circulation. There are major differences in morphological properties of arteries and veins that are related to their differential role in the circulation. As opposed to veins, increase in pressure only modestly raises the radius of muscular arteries, maintaining the vascular resistance at more constant level. Based on the Poiseuille's law, the resistance of an individual arterial segment is proportional to the fourth power of the radius and therefore small arteries are the main contributor to vascular resistance. It has been estimated that more than 50% of total vascular resistance resides in small arteries of diameter <100 μm (Mulvany and Aalkjaer, 1990)

A large pressure drop has been detected in multiple vascular beds including MA, skeletal muscle, cremaster and hamster cheek pouch indicating the regulatory potential of these vascular beds (Mulvany and Aalkjaer 1990). These arteries exhibit an intrinsic tone, indicating that they are not just a passive resistor, but rather participate actively in the regulation of resistance (Stekiel et al. 1986). Besides the SNS control of arterial resistance, tone of small arteries is regulated by intravascular pressure (myogenic tone described in section below) and also blood flow. Similarly to the large conduit arteries, blood flow relaxes the tone of small arteries (Bevan and Joyce 1988).

TPR is increased in hypertension. This is accompanied by altered structural characteristics of small arteries, characterized by increased media to

lumen ratio and reduced lumen. Studies suggest that the altered structural characteristics involve autoregulatory mechanisms and can be perceived as an adaptation of vascular wall to increased intravascular pressure produced by hypertension (Guyton 1989). Neurohumoral factors that are altered in hypertension can also directly promote proliferation of vascular SMCs, leading to thickening of media in the resistance arteries.

Because regulation of arterial and venous tone determines TPR and CO and therefore BP, it is very important to understand the mechanisms involved in the regulation of the contractile state of both arteries and veins.

3. Adrenergic receptors are the main effectors of SNS.

There are two main families of adrenergic receptors that integrate the signals of NE released from the sympathetic nerves and EPI released from adrenal medulla: α- and β- adrenergic receptor families. ARs are 7-transmembrane domain G-protein coupled receptors (GPCR) that are expressed by different types of cells in the CNS and periphery (Guimaraes et al, 2001). αAR have been divided into two families: the α_1 and α_2ARs (McGrath et al, 1989).

Expression of αARs in blood vessels is not homogenous (Hrometz et al. 1999, Chotani et al. 2004, Hussain et al. 1997) and vasoconstriction may be mediated by a mixed population of αARs. The expression of αARs subtypes differs across vascular beds as well as across different species (Rudner et al. 1999). Furthermore, there is some evidence that different αAR subtypes mediate responses to nerve-released catecholamines (junctional receptors) as opposed

to circulating catecholamines (extrajunctional receptors) (Townsend et al. 2004; Yang and Chiba, 2001). Moreover, the expression of αAR subtypes in vascular beds is affected by aging (Rudner et al. 1999). In most blood vessels, the predominant receptors mediating constriction are the α_1AR subtypes.

α_1-Adrenergic Receptor Signaling: Three α_1AR subtypes have been cloned and named α_{1A}- α_{1B}-, α_{1D}AR. These subtypes differ in their efficiency to produce cellular responses; the α_{1A}AR and α_{1B}ARs are more efficient in activating PLC compared to α_{1D}AR (Schwinn and Johnston, 1995; Vazquez-Prado and Garcia-Sainz 1996). These intrinsic properties of the receptor subtypes might have important implications with regard to the differences in adrenergic responsiveness observed between MA and MV.

The best characterized actions in the cardiovascular system related to α_1AR activation include contraction, growth, proliferation of VSMCs and increased cardiac contractility (Rudner et al. 1999; Piascik and Perez, 2001; Lin et al. 2001). While α_1ARs can couple to several second messenger systems, the predominant pathway is via pertussis toxin insensitive $G_{q/11}$ to phospholipase C (PLC), leading to PI turnover with the production of inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG stimulates protein kinase C and IP_3 acts on the IP_3 receptor in endoplasmic reticulum to release intracellular [Ca^{2+}] (Minneman et al. 1988). It has been established that following the Ca^{2+} release from intracellular stores, α_1ARs also activate Ca^{2+} influx during receptor stimulation leading to a more sustained Ca^{2+} increase (Piascik and Perez 2001). Besides L-type calcium channels that may regulate this Ca^{2+} influx (Crowley et al. 2002; Villalba et al.

2007), α_1ARs activate non-selective cation channels (NSCC) via DAG (Helliwell and Large 1997). It is not yet clear whether Ca^{2+} influx through this pathway is triggered by store depletion of Ca^{2+} or whether it is activated by α_1AR via receptor-operated channels (ROC). Both (ROC) (Crowley et al. 2002; Facemire and Arendshorst 2005) and store-operated channels (SOC) (Arnon et al. 2000;Lee, Rahimian et al. 2002) have been implicated in vascular constriction mediated by α_1ARs. Recent studies suggest that at least some of the channels that mediate Ca^{2+} entry belong to the family of transient receptor potential canonical channels (TRPC).

NSCCs have been also detected in rat MA myocytes (Hill et al. 2006). Transient receptor potential channel homologue 6 (TRPC6) has been identified as the essential channel mediating the α_1AR-activated NSCC current in the vasculature (Inoue et al. 2001). A recent study has indicated, that a receptor-accessory protein named Snapin physically interacts with α_{1A}AR and TRPC6 upon receptor stimulation and enhances Ca^{2+} influx through TRPC6 (Suzuki et al. 2007). The authors suggested that the mechanism underlying this enhanced Ca^{2+} influx mediated by snapin is due to enhanced insertion of TRPC6 into plasma membrane upon α_1AR activation. This leads to increased Ca^{2+} influx.

While there is strong evidence linking activation of GPCR to stimulation of TRPC, it is not established whether these channels support constriction directly. It has been proposed that during adrenergic stimulation Na^+ entry through NSCC could drive Na^+-Ca^{2+}-exchanger (NCX) -mediated Ca^{2+} entry in small microdomains of the plasma membrane (PM) (Arnon et al. 2000). It has

been later found that TRPC6 can functionally couple to (NCX) by localized increases of Na^+ (Poburko et al. 2007). During resting membrane potentials, NCX mediates Ca^{2+} extrusion. However, during the opening of NSCC by αARs, the local increase of [Na^+] in small microdomains drives the NCX into its reverse mode of action, transporting Na^+ out of the cell and importing Ca^{2+}. NCX is indeed becoming recognized for its role in facilitating Ca^{2+} influx during vascular constriction (Yao et al. 1998). Studies have shown a great degree of co-localization between NCX, SERCA, Na+-K+-ATPase (NKA) and SOC in small microdomains of the cell (Amon et al. 2000; Ambudkar 2006; Lynch et al. 2008) allowing for efficient functional coupling between these Ca^{2+} handling proteins.

In addition to Ca^{2+} release and Ca^{2+} influx mediated by α_1AR, studies suggest that stimulation of this receptor also leads to Ca^{2+} sensitization that can contribute to tonic contractions. This sensitization is dependent on PKC, Rho kinase and tyrosine kinase (Crowley et al. 2002, Villalba et al. 2007, Dimopoulos et al. 2007). In rabbit mesenteric artery, enhancement of Ca^{2+} sensitivity by PE is dependent on tyrosine kinase activation while its is indepentent of PKC (Sasaki et al. 1998).

Regulation of α_1AR. Signaling of GPCR must be tightly regulated in order to maintain functional specificity. α_1ARs, like other GPCR are regulated by phosphorylation, transcription, trafficking and desensitization (Hawrylyshyn et al. 1980; Michelotti, 2000). While multiple mechanisms contribute to these regulatory processes, receptor phosphorylation by G protein receptor kinases

(GRK) and subsequent recruitment of β-arrestins plays a very important role in functional regulation of GPCR. Interaction of phosphorylated receptor with β-arrestin uncouples the receptor from activation of G proteins and promotes internalization of the receptor via clathrin-coated pits, resulting into removal of activated receptor from the plasma membrane. All three α_1AR have been shown to undergo receptor desensitization upon repetitive exposure to NE (Vazquez-Prado and Garcia-Sainz 1996).

There seems to be some functional specificity in receptor desensitization mediated by GRK; multiple GRK isoforms are expressed in cardiomyocytes that express αAR, βAR and ET receptors. While GRK2 seems to be selectively targeting β_1AR thereby modulating cardiac contractility, GRK3 seems to be more selective for α_1AR, modulating growth and hyperthrophic responses in the heart (Vinge et al. 2007).

The internalized receptor can be either dephosphorylated and recycled or trafficked to the lysosomes for receptor downregulation (Krupnick and Benovic 1998). This is many times dependent upon the length of exposure to the agonist; many receptors undergo significant downregulation only after hours of agonist exposure. For example, 24h exposure to NE (2 μM) suppresses α_{1B}- and α_{1D}AR while α_{1A}AR expression is enhanced. This regulation occurs at the level of gene expression and correlates with protein levels (Rokosh et al. 1996). These data suggest that in situations where SNS activity is chronically elevated like in hypertension, relative expression of individual subtypes might undergo dynamic changes.

In recent years it is becoming more accepted that structurally diverse ligands of a particular receptor induce ligand specific signaling. It is believed, that different chemical structures of these compounds produce differential conformation of the receptor, indirectly regulating downstream signaling of the receptor (Kobilka 2007). It has been shown that NE and EPI whose chemical structure is closely related can induce a differential conformation of β_2AR (Swaminath et al. 2004), resulting into activation of different signaling pathways in cardiomyocytes. EPI causes a rapid contraction as opposed to NE, where the onset of maximal response is slower. In addition, EPI, but not NE, induces signaling via Gi. It has been shown that following EPI stimulation, β_2AR undergoes a rapid phophorylation by GRK2 with subsequent dephosphorylation, that are necessary for receptor recycling and Gi activation (Wang et al. 2008). These data suggest that GRK can also function to fine tune receptor signaling. This might have great relevance not only in adrenergic receptor regulation, but also in modulating functional responses of other receptor families. Ligand-selective regulation of receptor conformation has also great implications for future drug development, where a specific conformation of receptor without any unwanted side effects could be exploited by selective ligands that stabilize this particular conformation.

Recently, a family of proteins named Regulators of G-protein Signaling (RGS) was identified that bind Gα-GTP and enhance the rate of GTP hydrolysis, thereby helping to terminate receptor-mediated responses (Watson et al. 1996). The importance of these regulatory proteins in Gq signaling is demonstrated in

the mice with RGS2 deletion that develop a marked and sustained hypertension with enhanced adrenergic contractility and attenuated responses to vasodilators (Tang et al. 2003). Even though the prevailing believ about the etiology of hypertension indicates that altered signaling of CNS and abnormal sodium handling by the kidney are required to maintain elevated BP, the author of this study suggests that the hypertension in RGS-/- mice likely ensues due to increased peripheral vasoconstriction that can alter the blood pressure set point (Tang et al. 2003).

RGS2 was shown to be activated by PKGI-α in nitric oxide (NO)-dependent manner (Tang et al. 2003) and the mechanism by which RGS2 attenuates Gq signaling likely involves inhibition of Ca^{2+} release from intracellular stores as well as inhibition of capacitative Ca^{2+} entry during agonist stimulation (Osei-Owusu et al. 2007). While more than 30 different RGS isoforms have been identified (Hollinger and Hepler 2002), RGS2 has been found to specifically interact with the third intracellular loop of α_{1A}AR, but not the closely related α_{1B}- and α_{1D}ARs, suggesting a functional specificity in RGS-mediated inhibition (Hague et al. 2005). Therefore, differences in the activity and expression of RGS proteins between MA and MV might also affect the functional responses mediated by GPCR agonists.

Physiological role of α_1ARs in cardiovascular system. Due to a lack of sufficiently sensitive subtype-selective ligands, the precise physiological function and therapeutic potential of the individual subtypes have not been

elucidated. However, gene-disruption strategies were successfully applied to generate strains with deficiencies of individual αAR subtypes. Acute administration of NE or PE induces rapid raise of BP due to vasoconstriction. However, in mice lacking individual α_1AR subtypes, this raise in BP is attenuated. This indicates that all three subtypes contribute to the regulation of vascular tone (Philipp and Hein 2004).

The α_{1A}AR has been shown to play an important functional role mainly in resistance arteries (Kong et al. 1994; Argyle and McGrath, 2000) and α_{1A}AR KO mice are hypotensive at rest (Rokosh and Simpson, 2002). The α_{1B}AR is expressed in the peripheral arteries (Hrometz et al. 1999). However its role in regulating vasoconstriction is controversial. Mesenteric arteries from the α_{1B}AR KO mice exhibit the same reactivity to phenylephrine as arteries from WT mice, suggesting that this receptor doesn't participate in agonist induced constriction (Zuscik et al. 2001). Mice overexpressing α_{1B}AR are characterized by hypotension, autonomic failure and cardiac hypertrophy, further contradicting a vasoconstrictor role of this receptor (Zuscik et al. 2001). However, this receptor subtype mediates the constrictor actions of nerve-released NE (Townsend et al. 2004). A key role has been detected for α_{1D}AR in blood pressure control. α_{1D}AR KO mice which have a lower basal blood pressure then their WT counterparts. Furthermore, this receptor plays a role in salt-induced hypertension, because α_{1D}AR KO mice have a significantly attenuated BP response to salt-loading (Hosoda et al. 2005). The role of α_{1D}AR in hypertension development might be also important with regard to MV. As discussed below, α_{1D}AR is the main

subtype regulating the tone of MV and therefore it may play a role in attenuating vascular capacitance that occurs in the developmental stages of salt-sensitive hypertension.

α_2-Adrenergic Receptors: Similarly to α_1ARs, three subtypes of α_2ARs have been cloned and characterized based on molecular, biochemical and pharmacological approaches (α_{2A}, α_{2B}, α_{2C}). These receptors share about 50-60% identity, but the data suggest that there is no functional redundancy of these three receptors. Initially, α_2ARs were believed to be expressed only prejunctionally to regulate neurotransmitter release. However, multiple studies have shown, that α_2ARs are expressed postjunctionally, where they are involved in a wide range of physiological processes. Aside from the regulation of sympathetic tone and cardiovascular function, α_2ARs are involved in lipid and carbohydrate metabolism, platelet aggregation, analgesia, gastrointestinal motility and maintenance of fluid and electrolyte balance by the kidneys (Ruffolo et al. 1993). All three receptors negatively couple to adenylate cyclase via pertussis toxin sensitive Gi. However, under certain conditions, α_2ARs mediate increases of cAMP, implicating the activation of Gs (Ullrich et al. 1984; Jones et al. 1991; Eason et al. 1992). In addition to inhibition of adenylate cyclase, α_2AR have been shown to activate receptor-operated K^+ channels, inhibit voltage-dependent Ca^{2+} channels and activate mitogen activated protein kinase cascade (Richman and Regan 1998).

After α_1ARs were established as the main mediators of vascular tone in response to NE, evidence started to accumulate that α_2AR are also expressed in vascular SMCs (Ruffolo, et al. 1986; Matthews et al. 1984; Daniel et al. 1991). Many studies have indicated that α_2AR plays a more prominent role in the regulation of venous than arterial tone (Flavahan et al. 1984; Patel et al. 1981). Even though arteries were found to express α_2ARs, their efficacy to produce constriction was much smaller than for α_1AR, indicating that they are functionally uncoupled in arteries (Daniel et al. 1991). In addition to studies utilizing isolated venous preparation, where a large α_2AR reserve has been detected (Ruffolo 1986), studies *in vivo* also implied that postjunctional α_2AR may play a more prominent role in venous capacitance vessels (Patel et al. 1981). In addition, activation of α_2AR *in vivo* results into increased mean circulatory filling pressure (MCFP), demonstrating its functional role in regulation of vascular capacitance (Appleton et al. 1986).

The main effect of α_2AR activation is inhibition of adenylate cyclase via Gi. However, whether this is the pathway producing vascular contractions remains to be elucidated. Several studies suggest, that decreased cAMP production is inadequate to explain all the actions elicited by α_2AR stimulation (Nakaki et al. 1983; Ulrich 1984). In porcine lateral vein, α_2AR produces strong constriction, but no change in the cAMP level is observed, indicating adenylate cyclase – independent mechanism of vascular constriction (Wright et al. 1995). Nonetheless, pressor responses mediated by α_2AR are sensitive to pertussis

toxin which will inactivate $G_{i/0}$ proteins (Boyer et al. 1983; Aburto, Jinsi et al. 1995).

Studies suggested that contraction mediated through α_2ARs is not dependent on intracellular Ca^{2+} release (Lepretre and Mironneau 1994), but rather involves a pertussis toxin sensitive G protein and extracellular Ca^{2+} influx, probably through voltage-gated calcium channels (VDCC) and (Ruffolo et al. 1984; Parkinson and Hughes). In the dog saphenous vein, stimulation of α_2AR leads to contraction and large increases in $[Ca^{2+}]_i$ and myosin light chain (MLC) phosphorylation (Aburto et al. 1993). The constriction is highly dependent on PKC and DAG (Aburto et al. 1995). The mechanism by which α_2AR activates PKC involves activation of phospholipase D (PLD) that is converted to phosphatidic acid followed by formation of DAG. DAG activates PKC and induces contraction. The link between α_2AR activation and PLD may be mediated by tyrosine kinase (Uings et al. 1992); in rat fibroblasts, α_2ARs activate tyrosine kinases via Gi (MacNulty et al. 1992). In addition, constriction mediated by α_2AR depends on activation of tyrosine kinase in saphenous vein or aorta (Jinsi and Deth 1995; Carter and Kanagy 2002). In porcine palmar lateral vein, Src- tyrosine kinase mediates constriction caused by α_2AR stimulation (Roberts 2001).

α_2AR-mediated constriction via tyrosine kinase might be also important in NO synthase (NOS)-inhibition induced hypertension where reactivity of vascular α_2AR is enhanced, while α_1AR-mediated constriction remains unchanged (Kanagy 1997); α_2AR-mediated constriction in these animals is more dependent on Ca^{2+} influx through VDCC and involves activation of mitogen

activated protein kinase extracellular-signal regulated kinase kinase (MAP/ERK tyrosine kinase) (Mukundan and Kanagy 2001; Carter and Kanagy, 2002). Several other receptors including α_1AR (Jinsi et al. 1996; Dessy et al. 1998), ET receptors (Liu, et al. 1992) and angiotensin II receptors(Lassegue et al. 1991), histamine receptors (Katoch and Moreland 1995) have been shown to constrict blood vessels via this mechanism.

The Erk pathway is also a possible downstream target for tyrosine kinase activation; MAP kinase can be activated by Src tyrosine kinase in Gi or Gq-dependent manner (Igishi and Gutkind, 1998; Richman and Regan 1998). In porcine palmar lateral vein, constriction caused by the α_2AR agonist, UK 14,304, is inhibited by 80% in the presence of inhibitor of mitogen activated protein kinase kinase (MEK), component of Erk transduction pathway. In addition, inhibition of Src tyrosine kinase by PP2 prevents activation of Erk and constriction to UK 14,304 in this vessel (Roberts 2001).

α_2AR regulation. Similarly to α_1AR, α_2ARs are also subject to regulation by phosphorylation, transcription and desensitization. Evidence suggests, that desensitization of α_2ARs is regulated in a subtype selective manner; the α_{2C}AR being more resistant to desensitization by continuous application of catecholamines compared to α_{2A} and α_{2B}ARs (Eason et al. 1992). This is likely related to the fact that α_{2A}- and α_{2B}AR are subject to more extensive phosphorylation by GRK after agonist exposure, compared to α_{2C}AR (Kurose and Lefkowitz 1994). Internalization of the three subtypes is dependent on the presence of β-arrestins. The interaction between β-arrestins and the receptor

subtypes is also selective; β-arrestin 2 promotes internalization of both α_{2B}- and α_{2C}AR whereas β-arrestin 1 only promotes α_{2B}AR internalization. However, neither of these β-arrestins significantly affected α_{2A}AR internalization (DeGraff et al. 1999).

The third intracellular loop of α_2AR has been shown to interact with β-arrestins and differences between the receptors in this sequence might provide a mechanism for selective regulation of desensitization (DeGraff et al. 1999). The subtype-specific differences in this sequence may also partially explain differences in trafficking. When the receptors are co-expressed in the same cells, they differ markedly in their agonist-regulated targeting to the endocytic pathway (Zastrow et al. 1992) and when the three receptors are expressed in HEK-293 cells, their cellular distribution also differs. While α_{2A}AR and α_{2B}AR are predominantly expressed in the membrane, α_{2C}AR seems to reside in the intracellular compartment (von Zastrow et al. 1993). However, the cellular distribution seems to be dependent also on the cellular context; while in rat 1 fibroblasts, COS7 and rat kidney cells α_{2C}AR accumulated in the endoplasmic reticulum, it was efficiently trafficked to the plasma membrane in the PC12 cells (Hurt et al. 2000).

Temperature also regulates α_2AR function. Even though same α_2AR subtypes might be expressed in the mesenteric vasculature, this regulatory mechanism might have greater implication in the cutaneous vasculature due to its role in thermoregulation. While reactivity of α_1AR is attenuated in cold conditions, cold temperatures have been shown to enhance reactivity of α_2AR

(Flavahan et al. 1985). Studies suggested that α_{2C}AR mediates the enhanced responses to adrenergic stimulation under cold conditions (Chotani et al. 2000). While α_{2C}AR are silent 37°C, studies in transfected HEK293 have indicated that cooling evokes redistribution of α_{2C}AR from Golgi compartment to the plasma membrane, enabling this receptor to mediate responses to α_2AR stimulation (Jeyaraj et al. 2001). The redistribution of this receptor to the membrane is dependent upon the activation of Rho kinase (Bailey et al. 2004).

The dependence of α_{2C}AR-mediated constriction on Rho kinase may have implications beyond the regulation of cutaneous blood vessels. As described in Chapter 2, α_{2C}AR appears to mediate NE responses in MV.

Physiological role of α_2ARs in cardiovascular system. In contrast to the monophasic vasopressor responses caused by systemic administration of α_1AR agonists like phenylephrine, systemic administration of α_2AR agonists causes a bi-phasic response. Clonidine, a partial α_2AR agonist, causes a transient rise in BP due to α_2AR mediated constriction of peripheral blood vessels. The transient vasopressor response is followed by a drop in BP to lower than basal levels due to the activation of α_2AR in the lower brainstem region that directly inhibit sympathetic outflow.

Experimental evidence indicates that α_{2A}ARs are responsible for the depressor effect mediated by clonidine. α_{2A}AR KO mice lacks this vasodepressor response (Link et al. 1996). This property makes α_{2A}AR a very suitable target in hypertension therapy, where activating this receptor by agonists

like clonidine inhibits sympathetic outflow (Ruffolo et al. 1993). In addition to the regulation of neurotransmitter release, α_{2A}AR are expressed by endothelial cells, where they mediate vasorelaxation by promoting NO production (Shafaroudi et al. 2005). Based on the data from the α_{2A}AR KO mice it is becoming more accepted, that this subtype doesn't play a functional role in vascular contraction. The α_{2B}AR KO mice exhibits some hemodynamic differences compared to WT mice. Clonidine doesn't cause the initial BP increase in these animals and a more pronounced fall in BP is observed compared to WT mice (Link et al. 1996). Furthermore, the α_{2B}AR KO mice have an attenuated BP response to salt loading, suggesting that this receptor plays a role in salt-induced hypertension (Makaritsis et al. 1999). However, it is still to be elucidated whether the effect is mediated by central, vascular or renal mechanisms. α_{2C}AR KO mice develop hypertension in response to salt loading comparable to WT mice (Makaritsis et al. 1999) and gross hemodynamic parameters are not affected by this receptor deletion.

Even though a great amount of progress has been achieved in characterizing the α_2AR subtypes due to the availability of KO models, it is difficult to specify which α_2AR acts in the periphery to mediate vasoconstrictor responses. Although α_{2B} and α_{2C}ARs are the most likely candidates for this function, similar binding profiles of these two subtypes make it very difficult to differentiate between them. α_{2B}AR mediate pressor responses in anesthetized rats (Docherty et al. 1998) and α_{2C}ARs play a functional role in human saphenous vein and also mediate cold-induced vasoconstriction (Gavin et al.

1997; Bailey et al. 2004). It has been suggested, that postjunctional α_{2A} and α_{2B}ARs play a more prominent role in arteries, while the α_{2C}AR might be expressed especially in venous SMC (Docherty et al. 1998). However, nobody has defined α_2ARs in small capacitance vessels.

αAR distribution in mesenteric vasculature. As described in the previous section, mesenteric vasculature is crucial in hemodynamic reglulation. Distribution and involvement of different αAR subtypes has significant impact on the regulation of this vasculature. In murine MA, only α_1AR seems to mediate the actions of NE (Perez-Rivera et al. 2004). Prazosin, an α_1AR antagonist, causes a parallel shift of the NE dose-response curve in this tissue. Studies with subtype-selective antagonists suggested that α_{1A}AR and to a lesser extend the α_{1B}AR, mediate the NE responses in the MA (Martinez-Salas et al. 2007;Perez-Rivera, dissertation). Furthermore, α_{1B}AR subtype seems to play a more prominent role in neurogenic constriction of MA (Perez-Rivera et al. 2005). Other studies have suggested the preferential role of α_{1B}AR in neurogenic constriction (Yang and Chiba 2000).

In contrast, prazosin does not behave as a competitive antagonist of NE mediated responses in the MV, suggesting the involvement of prazosin resistant mechanisms, possibly α_2ARs in venous constriction (Perez-Rivera et al. 2007). Studies with α_1AR-subtype selective antagonists in the MV indicated that α_{1D}AR plays the dominant role in PE-induced contraction, even though a slight effect of α_{1B}AR antagonist has also been observed (Perez-Rivera, dissertation).

The contractile role of α_{1D}AR has also been implied in the rat and mice aorta (Tanoue et al. 2002), canine MV (Daniel et al. 1997) as well as in mice femoral arteries (Zacharia et al. 2005).

It is interesting that despite differential functional involvement of the three α_1AR subtypes in MA and MV all three receptors were detected in MA and MV by western blot using whole blood vessel homogenate. Furthermore, analysis suggested that the three subtypes are expressed at similar level in MA and MV (Perez-Rivera, dissertation).

αAR-mediated responses of MA and MV: Several studies have implied, that isolated veins are more sensitive to exogenously applied NE (Abdelmawla, et al. 1996; Perez-Rivera et al. 2004). Also, stimulation of sympathetic nerves results into larger amplitude of depolarization with longer duration in venous SMCs compared to arterial SMCs.(Hottenstein and Kreulen 1987). At any given frequency, the contraction in the MV is always left-shifted relative to MA (Park et al. 2007; Luo et al. 2003).

Considering the differential function and embryonic origin of arteries and veins, multiple independent mechanisms may underlie the enhanced responsiveness of MV to adrenergic stimulation. Our preliminary data indicated that indirect contribution of α_2AR in MV and not in MA may enhance functional responses in MV. Taken together with data from other studies that indicate the role of α_2AR in the enhancement of responses mediated by α_1AR (Shepperson 1984; Haynes and Hill 1996; Daly et al. 1988; Wilson and Minneman 1991;

Reynen et al. 2000), we hypothesized that the cross-talk beween α_1- and α_2AR may be a significant contributor to differences in the adrenergic responses between MA and MV. While there is no evidence that the cross-talk between α_1- and α_2AR is based on heterodimer formation, the emerging concept of GPCR heterodimerization as an ubiquitous process required for proper receptor trafficking, signaling and enhancement of function provided an attractive target for our hypothesis.

4. Receptor cross-talk.

Early studies have suggested that the monomeric GPCR may not completely explain GPCR function; when a chimera receptor composed of α_{2C}AR and M3 muscarinic receptor, where the C-terminal portions were switched, was transfected to COS-7 cells, no detectable binding for muscarinic or adrenergic ligand was detected. However, when both chimeras were expressed together, specific binding sites for the two ligands were detected, suggesting that perhaps inter-molecular interaction between the two receptor chimeras alters the binding pocket for ligand (Maggio et al. 1993). These initial observations have revolutionized our perception about GPCR function and signaling, raising the possibilities of existence of additional receptor phenotypes that can not be simulated by expression of single receptor clones.

There is accumulating evidence, that signaling via a GPCR that couples preferentially to one pathway may be affected by inputs from GPCR

coupling to other pathways (Maggi et al. 1980). Such interactions may affect coupling specificity and efficacy, and therefore have important consequences for physiological and pathophysiological processes. Interaction between receptors can lead to a loss or enhancement of function (Werry et al. 2003).

Receptor heterodimerization. Interaction between GPCRs can lead to a formation of heterodimers with pharmacologically distinct properties from monomeric receptors. For example, physical interaction between μ and δ opioid receptors leads to a switch in receptor coupling from $G\alpha_{i/o}$ to $G\alpha_q$ mediated responses (Charles et al. 2003). In addition, heterodimerization may be required for proper receptor transport and folding in order for the receptor to exert a physiological function; for example the type R1 and type R2 GABA B receptors are inactive as monomers when expressed alone in mammalian cells; it has been observed that R1 is retained intracellularly as an immature glycoprotein when expressed alone, whereas R2 is transported to the membrane efficiently but can not bind GABA or mediate signaling. However, when co-expressed, the two proteins reached the cell surface and were able to participate in functional responses; in addition, co-immunoprecipitation experiments revealed that these two receptors physically interact (Jones et al. 1998; Kaupmann et al. 1998). The fact that these two receptors were found to co-localize in the same brain region and in addition the endogenous receptors could be co-immunoprecipitated from the membrane preparation of brain cortex (Kaupmann et al. 1998) indicates that heterodimerization is not a result of experimental artifact.

The significance of receptor heterodimerization in proper receptor folding and transport has been also found to bear a great relevance in human physiology. Coexpression of truncated CCR5 cytokine receptor that is not trafficked to the plasma membrane caused the wild type CCR5 receptor to be retained intracellularly, indicating that the wild type receptor heterodimerizes with the truncated receptor, leading to retention of the wilt type receptor in the endoplasmic reticulum (Benkirane et al., 1997). Since the wild type CCR5 receptor is the main co-receptor for CD4 required for HIV entry, it has been proposed that the dominant negative effect of the truncated receptor is responsible for the slow onset of AIDS in patients who are heterozygous for this mutation (Samson et al., 1996).

The rapidly accumulating data on receptor heterodimerization have raised the possibility that dimerization might be an ubiquitous process which is required for proper cellular expression of GPCR (Salahpour et al., 2004; Zhou et al., 2006). With the available data, however, it is hard to explain the existence of a ubiquitous domain that is involved in receptor dimerization. Available literature indicates that receptor dimers can be formed through the interaction in their N-terminal domains, C-terminal domains as well as through transmembrane domains. Some studies even suggest, that dimerization involves a combination of the three mechanisms.

As an example, N-terminal extracellular domain contributes to dimerization of C family of receptors. The dimers in this family are connected by disulfide bridges (mGluR, $GABA_BR$) (Tsuji et al., 2000; Romano et al., 1996).

Interactions through TM domains have also been observed, particularly TM1 and TMVI and TMVII have been implicated in dimer formation (Hebert et al. 1996). Even though interactions between TM domains might be harder to investigate, employing a peptide corresponding to the TM domain suspected in interaction may dissociate dimer formation. Using this approach, TMVI has been suggested as the interface for the dimerization of β_2AR (Hebert et al., 1996). Similarly, TMVI and VII have been suggested to be involved dopamine receptor dimerization (Ng et al. 1996). Heterodimerization can also be formed by interaction of the C-terminal domains; $GABA_BR1$ and R2 have been shown to dimerize through the C-terminus (Kaupmann et al., 1998).

Considering the implications of receptor dimerization in receptor trafficking, signaling and pharmacological properties, it is very important to understand its regulation. Studies suggest that heterodimerization can be either increased, decreased on remained unchanged by the action of agonists (Bouvier 2001). As described above, heterodimerization is constitutive and is even necessary for proper trafficking for certain receptors. These receptors usually form dimers before they reach the plasma membrane in the endoplasmic reticulum. In other cases agonists can increase the stability of receptor dimers. Activation of β_2AR by the agonist isoproterenol has been shown to stabilize receptor dimer, while inverse agonist timolol shifted the equilibrium towards monomeric receptor species (Hebert et al., 1996). In the case of δ-opioid receptors, dimerization is decreased after exposure to agonist. However, this process seems to be dependent upon receptor internalization; morphine that

does not induce receptor internalization has no effect on the balance between monomers and dimers (Cvejic and Devi 1997).

Because GPCR regulate multiple physiological functions of cardiovascular system, receptor heterodimerization might have great implications in modulating receptor responses in this system. Indeed, β_1– and β_2AR have been shown to heterodimerize in intact cardiomyocytes and this interaction resulted into increased ligand binding affinities for both subtype-selective ligands. The altered binding properties were also accompanied by enhanced efficiency of βAR agonist isoproterenol to stimulate intracellular response including cAMP production and contractility. In addition, the heterodimerization between these two receptors inhibited spontaneous activity of β_2AR (Zhu et al., 2005).

Receptor heterodimerization in cardiovascular system. There is also an example, how receptor heterodimerization may be involved in cardiovascular disease. In patients suffering from preeclampsia it has been noted that there is an overall increase of sensitivity to vasopressor angiotensin II. Because the increased sensitivity was exclusive for angiotensin II, altered signaling for angiotensin receptor (AT) has been proposed as the underlying mechanism in this disease (Haller et al. 1989). Later studies have shown that physical interaction between AT1 receptor and receptor for vasodepressor bradykinin (B_2) results into significantly enhanced responsiveness to angiotensin II (AbdAlla, et al., 2000). Because increased AT1-B_2 heterodimer density and angiotensin II signaling has been detected in the platelets and in omental vessels

from preeclamptic patients, it indicated that this heterodimer may at least partially contribute to the enhanced angiotensin II responsiveness observed in this disorder (AbdAlla et al., 2001). While the total amount of AT1 receptors did not change during preeclampsia, the increased amount of heterodimers was due to increased B_2 expression levels.

Dimerization of αAR. Receptor dimerization seems to be ubiquitous for αAR family members when these receptors are heterologously expressed. While there are many reports on αAR and their involvement in receptor dimerization, none of these reports relates this phenomenon to a physiological situation and therefore its relevance needs to be further explored. Data indicate that all three α_1AR subtypes are able to form homodimers and homooligomers when expressed in HEK293 cells (Vicentic et al., 2002; Uberti et al., 2003). In addition, αAR have been shown to heterodimerize with other members of the family and out of these, α_{1D}AR provides a great example of how receptor dimerization regulates its trafficking and pharmacology.

α_{1D}AR is known to be poorly trafficked to the plasma membrane when expressed in different types of cells; N-terminal of the receptor has been shown to contain a sequence that prevents it to be efficiently trafficked to the membrane while supporting its retention in the endoplasmic reticulum (Hague et al., 2004). α_{1B}AR physically interacts with α_{1D}AR and in addition to promoting membrane expression of α_{1D}AR, the interaction between α_{1B}- and α_{1D}AR leads to formation of a receptor entity with distinct pharmacological properties (Hague et al., 2005).

The functional responses of the dimer are stronger than responses of either receptor alone and even though the membrane expression of α_{1D}AR is significantly enhanced upon co-expression with α_{1B}AR as assessed by luminometer surface expression assay, no high affinity binding sites for BMY 7378, the α_{1D}AR selective antagonist, are detectable. This indicates that ligand binding properties of α_{1D}AR have been altered after it forms a heterodimer with α_{1B}AR (Hague et al. 2006). In addition to α_{1B}AR, α_{1D}AR has also been detected to heterodimerize with β_2AR. Similarly to previous example, this interaction affects receptor trafficking by promoting surface expression of α_{1D}AR. Stimulation of cells co-expressing these two receptors leads to increased functional responses to NE (Uberti et al., 2005). However, the pharmacological properties of α_{1D}AR are not altered by this heterodimerization as opposed to α_{1B}AR co-expression.

α_{1B}AR also forms heterodimers with α_{1A}AR, although the properties are of the receptors are not altered by this heterodimerization. The functional significance of this interaction remains to be elucidated (Uberti et al., 2003).

α_2AR have also been also shown to form homodimers and oligomers when expressed heterologously. α_{2B}AR forms homodimers and a truncated receptor mutant (α_{2B}ARm) that is retained in the endoplasmic reticulum confers negative effect on surface trafficking of WT receptor, indicating a crucial role for receptor dimerization in proper receptor trafficking to the plasma membrane. In addition, the α_{2B}ARm also inhibited membrane trafficking of α_{2A}- and α_{2C}AR, suggesting a role for heterodimerization between α_{2B}AR and the other α_2AR

subtypes (Zhou et al., 2006). While the functional consequence for this homo- and heterodimerization remains to be elucidated, if nothing else, this interaction might be an important tool for receptors to be properly trafficked in order to exert their function on the plasma membrane.

α_{2A}AR has been shown to heterodimerize with α_{2C}AR and this interaction results to altered receptor desensitization properties; α_{2A}AR undergoes a rapid agonist-induced desensitization mediated by GRK phosphorylation when expressed by itself (Eason and Liggett 1992). On the other hand, α_{2C}AR does not undergo receptor phosphorylation by GRK under similar conditions, suggesting differences in the regulation of these receptors (Jewell-Motz and Liggett 1996). However, formation of heterodimer between these two subtypes results into attenuated phosphorylation of α_{2A}AR by GRK and decreased β-arrestin activation, suggesting functional implications in this interaction (Small et al., 2006). This interaction might be important in the nerve terminals, where these two receptors are believed to be co-expressed. Considering the rapid desensitization of α_{2A}AR when exposed to agonist, its role in auto-inhibition would not be very effective on the sympathetic nerves that exhibit a basal tone of firing and NE release if the receptor desensitized very quickly upon NE exposure.

In addition to interactions occurring within the αAR family, several studies reported cross-talk between α_2- and βAR families. Since these receptor types exhibit opposing action of adenylate cyclase activity, it might be expected that if co-expressed in the tissue, they would antagonize each other when

activated by NE or EPI. However, several studies have shown that βAR and α_2AR can modulate each others pharmacology; in renal tissue stimulation of α_2AR results into decreased affinity of isoprenaline for βAR (Woodcock and Johnston 1980) and in cerebral cortical slices it has been shown that stimulation of βAR results into increased α_2AR binding (Maggi et al. 1980). In addition, α_2AR co-stimulation can increase the responsiveness of βAR (Northam and Mobley 1985). It has been recently shown that heterodimerization between β_1- and α_{2A}AR may underlie some of the initial observations (Xu et al., 2003). The β_1-α_2AR heterodimer has altered ligand binding properties compared to β_1AR alone and also exhibits a greater potency to stimulate adenylate cyclase.

Similarly to α_{1D}AR, α_{2C}AR has been shown to be localized intracellularly when expressed in HEK293 cells. Screening of 25 related GPCR revealed that only β_2AR is able to enhance the membrane expression and functionality of α_{2C}AR (Prinster et al., 2006). This finding is interesting in the light of data supporting heterodimerization between α_{1D}- and β_2AR. In particular, our studies (chapter 2) suggest that α_{2C} and α_{1D}AR are the main functional receptors in MV.

In the murine MV, α_2AR agonists such as clonidine and UK14304 do not cause venoconstriction. Nonetheless, our studies suggest that α_2AR is involved in mediating the actions of NE in murine MV, even though its actions might be indirect. While yohimbine, an α_2AR antagonist, does not affect arterial responses to NE, it produces a rightward shift of the dose-response curve to NE in MV (chapter 2).

The finding that α_2AR blockade shifts the NE dose-response curve to the right and α_2AR agonists do not cause a significant venous constriction suggests that α_2AR may indirectly contribute to venous but not arterial constriction. NE binds to α_1 and α_2AR and the α_2AR may synergistically interact with the α_1AR in veins to enhance constriction to NE. In fact, a very early study proposed a synergistic interaction between the two receptors in the saphenous vein (Daly et al., 1988). Because nobody investigated the role of receptor dimerization as a mechanism behind α_1-α_2 synergistic interaction, the purpose of a significant portion of this dissertation was to exclude/confirm the formation of receptor heterodimer as a basis for this many times reported receptor interaction.

Dimerization independent receptor cross-talk. Initially, we tested the hypothesis that synergistic interaction between α_1- and α_2AR is based on heterodimer formation. However, after evaluation of the data that tested this hypothesis, we have concluded that likely a different mechanism is responsible for this functional receptor interaction. In the section below, examples of interaction are presented that do not include formation of heterodimer.

Interactions between two receptors can occur via their intracellular signal transduction pathways to produce a distinct functional effect (Werry et al., 2003). While there are many examples in the literature describing synergistic effect produced by activation of two separate pathways, the molecular mechanism is many times not clarified. There are multiple points, where two transduction pathways can interact to produce a synergistic effect on mobilization

of Ca^{2+} that leads to enhanced response. Similarly to the interaction between AT1 and B2 receptor in preeclamptic patients that contributes to disease pathology, in many diseases including hypertension, interactions may contribute to enhanced signaling and disease pathology. Elucidation of a functional mechanism underlying receptor cross-talk may therefore reveal novel targets for therapeutic interventions.

Multiple studies have demonstrated a potentiating interaction on $[Ca^{2+}]i$ between $G_{\alpha i}$ and $G_{q/11}$ coupled receptors. CXCR2 is a chemokine receptor that preferentially couples to Gαi (Damaj et al., 1996). When expressed in HEK-293 cells, activation of this receptor by its ligand interleukin-8 (IL-8) did not cause Ca^{2+} elevation in the cells. However, following treatment with ATP that activates endogenous $P2Y_1$ and $P2Y_2$ receptors in these cells, IL-8 was able to produce a substantial Ca^{2+} elevation that did not seem to be dependent on influx, suggesting enhanced mobilization of Ca^{2+} from intracellular stores (Werry et al., 2002). While the molecular mechanism was not determined, the authors proposed that Gαq causes a reversible change on PLCβ that reveals a binding site for Gβγ. Another possible explanation for this finding is potentiation of PLC activity.

Potentiation of PLC activity. There are multiple examples of how a receptor cross-talk leads to potentiation of PLC activity. Treatment of HEK293 cells stably expressing M3 muscarinic receptors by carbachol leads to long-lasting sensitization of PLC activity. Responses to purinergic stimulation, carbachol and thrombin were significantly enhanced after carbachol pretreatment

for as long as 90min (Schmidt et al. 1996). Moreover, this potentiation was observed even upon direct stimulation of G proteins by AlF_4^- and GTPγS in permeabilized cells. The study also revealed, that carbachol pretreatment leads to increased substrate supply PtdIns(4,5)P_2 (PIP_2) for PLC in addition to increased PLC activity and this may be very important because limited amount of substrate for PLC may limit its activity to produce IP3 and DAG. These data indicate that PLC sensitization does not result from a direct coupling of receptors to PLC-stimulating G proteins, but rather occurs downstream of G protein activation.

While M3 receptors have been shown to activate both Gi and Gq_{11} proteins in transfected cells (Offermanns et al. 1994), the enhanced PLC activity by carbachol seems to be at least partially mediated by Gi proteins due to its sensitivity to PTX treatment. However, Gq is also likely involved in increased PLC activity observed after carbachol treatment because even in the presence of PTX, increased accumulation of PLC substrate PIP_2 was observed. Even though inhibition of PKC did not affect acute PLC stimulation, a subsequent study has shown that the long-term potentiation of PLC activity is also dependent on PKC (Schmidt et al., 1998). In addition, only a combined treatment of PTX and staurosporine (PKC inhibitor) was able to abolish increased PIP_2 substrate supply for PLC, suggesting multiple mechanisms involved.

Potentiation of PLC activity might be also relevant for the interaction between α_1-α_2AR; PTX-sensitive Gαi that are able to regulate PLC (Katz et al., 1992; Jiang et al., 1996) are known to be activated by α_2AR in the vascular

SMCs. PKC has also been suggested to be activated by α_2AR in the vasculature and play an important role in its mechanism of contraction (Aburto et al., 1995).

The increased PIP_2 supply seems to be an attractive target for receptor interactions, because continuous re-synthesis of this substrate seems to be required for acute and sustained inositol phosphate signaling (Willars et al., 1998). It is interesting that stimulation of Rho kinase may also increase the levels of PIP_2 by enhancing activity of PIP5- kinase (Chong et al., 1994) and Rho kinase is a downstream target of many contractile GPCR including α_1- and α_2ARs (Roberts et al., 2004; Tsai and Jiang 2006; Dimopoulos et al., 2007). Activation of PIP_2 by Rho kinase likely also involves activation of tyrosine kinases that work in the same pathway. In HEK293 cells, the levels of PtdIns(4,5)P_2 are reduced by inhibition of tyrosine kinase and this effect is similar to Rho kinase inhibition (Rumenapp et al., 1998). Since many studies point to functional coupling between α_2AR and tyrosine kinases as well as Rho kinases (Carter and Kanagy 2002; Jinsi and Deth, 1995), increased PIP_2 production as a mechanism underlying α_1-α_2AR synergistic interaction could also be considered.

While the enhanced PLC activity should lead to enhanced IP_3 production in receptor cross-talk, IP_3 levels are not necessarily elevated in a synergistic cross-talk (Yeo et al., 2001). In certain cases, the enhanced Ca^{2+} mobilization could be due to sensitization of Ins(1,4,5)P_3 receptors. PKA can sensitize these receptors through phosphorylation. This type of interaction has been described in many cell types including parotid acinar cells and hepatocytes (Bruce et al., 2002; Hajnoczky et al., 1993). This mechanism is likely relevant in

cells where Gs coupled receptors potentiate Gq-mediated responses, because activation of PKA is dependent on activity of adenylate cyclase.

Activation of Ca^{2+} influx. In the previous examples, enhanced mobilization of intracellular Ca^{2+} leading to enhanced responses seems to underlie receptor synergism. However, potentiating interaction can also be based on enhanced Ca^{2+} influx from extracellular space. For example, neuropeptide Y sensitizes rat MA to responses mediated by several contractile receptors including αAR; the tissue sensitization is partially mediated by increased Ca^{2+} influx through L-type calcium channels in the presence of neuropeptide Y due to the sensitivity of the potentiation to nitredipine, the L-type calcium channel antagonist (Andriantsitohaina and Stoclet 1988). In addition, some of the reported α_1-α_2AR synergistic interactions seem to be based on potentiation of Ca^{2+} influx (see below).

Pathways involved in α_1-α_2AR cross talk. While several studies documented α_1-α_2AR synergistic interaction, they do not seem to point to a single mechanism that would underlie this phenomenon. One study described α_1-α_2 receptor interaction in cell line overexpressing α_2ARs (Reynen et al., 2000), while other studies documented this interaction in tissue and native cells.

Chinese hamster lung fibroblasts (CHL) natively express α_1AR, although the specific subtype has not been characterized. When α_{2A}AR was co-expressed in these cells, neither PE nor UK14304 were able to increase $[Ca^{2+}]i$. However, NE was able to cause concentration-dependent elevation of Ca^{2+}. In

addition, when PE was co-applied with constant concentration of UK 14304, dose-dependent Ca^{2+} raise has been detected. This elevation of Ca^{2+} was inhibited by both prazosin and rauwolscine (Reynen et al., 2000). However, the mechanism behind this enhanced Ca^{2+} mobilization has not been investigated. Similar interaction has also been described in neuronal cells where instead of Ca^{2+} measurements the amount of 3H-inositol phosphates was quantified, perhaps providing better mechanistic information about this synergism; in primary glial cell cultures, PE caused only a fraction of inositol phosphates being produced compared to NE. However, upon addition of UK14304, PE became much more efficacious in inositol phosphates production (Wilson et al., 1991). The enhanced inositol phosphate production perhaps leads to increased Ca^{2+} mobilization, as described by previous study.

Another, slightly different mechanism of interaction has been suggested for α_1- α_2AR in the cauda epididymis of the guinea pig. In this tissue, xylazine (α_2AR agonist) did not produce a concentration-dependent constriction like PE did. Similarly to the previous examples, addition of xylazine significantly enhanced PE - mediated constriction. This enhanced response seems to be mediated by Gi due to its sensitivity to PTX and seems to be dependent on Ca^{2+} influx through L-type calcium channels, because pre-incubation of the tissue with L-type calcium channel blocker nifedipine attenuated the effect of xylazine. In contrast to the previously described studies, xylazine did not potentiate accumulation of inositol phosphates mediated by PE, suggesting that the synergism between the two receptors is based on Gi-dependent potentiation of

Ca^{2+} influx instead of increased Ca^{2+} mobilization from the stores (Haynes and Hill 1996). In fact, a similar mechanism has been described in rat tail artery; α_2AR did not affect Ca^{2+} mobilization from intracellular stores but was able to potentiate Ca^{2+} influx through activation of calcium channels (Xiao and Rand 1989).

5. Non-adrenergic non-cholinergic regulation of vascular tone.

Sensory innervation of blood vessels. While SNS is the major regulator of vascular tone, sensory nerves that also innervate blood vessels are very important in the regulation of vascular homeostasis. Sensory nerves function as afferent fibers collecting information about the environment and carrying it back to the CNS. In addition, these fibers also have efferent function; they may be activated locally to release vasoactive sensory neuropeptides including substance P, NO, vasoactive intestinal peptide (VIP), ATP and calcitonin gene-related peptide (CGRP) from the nerve endings (Holzer 1991; Lundberg 1996). NO and CGRP are very potent vasodilators that antagonize the effects of SNS; recently it has been noted that CGRP may play a greater role in reduction of MA than MV tone mediated by perivascular nerve stimulation; the arterial reduction of perfusion pressure in response to CGRP was at least partially dependent on intact endothelium(Legros et al. 2007).

Substance P and ATP are involved in neuromodulation of sympathetic and parasympathetic nervous system. Substance P can cause both vascular contraction and relaxation (Whittle et al., 1989); it is interesting to note, that

substance P relaxes MA but constricts MV (Galligan et al., 2006). This may be due to differential distribution of receptors between MA and MV, with contractile NK-3 receptors expressed in the venous SMCs and NK-1 receptors coupled to relaxation expressed in endothelial cells in arteries.

Substances modulating the vascular tone.

In addition to the above described mechanism regulating the vascular tone, a variety of relaxing and contracting factors are released by the endothelial cells located on the luminal side of the blood vessels. Vascular tone is determined by the balance between vasoconstrictor and vasodilator stimuli that directly affect $[Ca^{2+}]_i$ and Ca^{2+} sensitivity. The primary endogenous dilator is NO whose production is induced by Ca^{2+}- mobilizing agents and fluid shear stress (Govers and Rabelink 2001). Other relaxing factors derived from endothelium include prostaglandins and prostacyclins produced by the action of cyclooxygenase. Activation of receptors in the endothelium can increase production of these agents and α_2ARs mediate vasorelaxation through the release of NO (Nishina et al., 1999; Vanhoutte 2001).

The predominant vasoconstrictor agents released from the endothelium include endothelin-1, angiotensin II, tromboxane A_2 and prostaglandin H_2 (Ungvari and Koller 2000). Besides these well established endothelium-derived vasoactive substances, there is a great number of circulating hormones that contribute to short and long-term BP regulation.

Myogenic regulation of vascular tone

The contractile state of vascular smooth muscle primarily depends on intracellular calcium concentration $[Ca^{2+}]_i$ that is modulated by vasoactive agents and mechanical stimuli. In addition, the relationship between $[Ca^{2+}]_i$ and vascular tension can be modified by altering Ca^{2+} sensitivity of contractile proteins. This mechanism also significantly contributes to regulation of vascular tone.

Regulation of vascular tone is very dynamic and besides circulating vasoactive substances, intravascular pressure and flow also alter the local tone of the vasculature. Intraluminal pressure alters membrane potential of vascular SMS and this has a significant effect on vascular reactivity.

The signal transduction pathway mediating the effect of pressure on vascular tone is complex. Intravascular pressure acts as mechanical force on the vascular wall and distends it. The fact that tension of the SMCs inversely correlates with the membrane potential has been discovered long time ago. It has been found that stretching strips isolated from the guinea pig taenia coli produced progressive depolarization and tension development compared to unstretched preparations (Bulbring, 1954). Trachealis muscle cells progressively depolarized with step increases in cell length (Coburn 1987). However, because membrane potential came back to baseline despite maintained increase in cell length, author proposed that depolarization occurred in response to stretch as opposed to the maintained length itself. There is evidence that stretch-induced

non-selective cation channels play a role in depolarization of SMCs in response to stretch (Davis et al., 1992). Depolarizing the cell to lower potentials leads to activation of voltage-dependent calcium channels (Meininger and Davis 1992). The ensuing rise in intracellular Ca^{2+} activates contractile machinery and induces contraction. Increases in the intravascular pressures underlie the myogenic tone that is very important in the local regulation of blood flow. Increasing intravascular pressure leads to cell depolarization and development of the tone, limiting the blood flow and preventing tissue overperfusion. Myogenic tone plays a very important role in the resistance arteries and with decreasing lumen diameter, myogenic tone is more prevalent.

Data on the local regulation of venous myogenic activity are not very extensive compared to arteries. Early studies demonstrated the existence of myogenic tone in special veins like the portal vein in the rat and small veins of the bat wing (Johansson and Mellander 1975). One of the early studies detected spontaneous depolarization in MV of guinea pig, indicating the existence of myogenic tone in this tissue (Van Helden 1991). Some later studies suggested that myogenic tone correlates with chronic intraluminal pressures and veins that exhibit low pressures *in vivo* exhibit low degree of myogenic tone. For example, in the human cephalic vein, no myogenic tone has been detected whereas larger veins exposed to higher pressures *in vivo* like the saphenous veins were found to exhibit myogenic activity (Szentivanyi et al., 1997; Berczi et al., 1992). In rat MV, myogenic tone did not increase with stepped increases of intraluminal pressure like in MA (Li et al., 2005).

Even though the extent of myogenic activity in veins is smaller than in arteries, the significance of venous regulation by myogenic mechanism might be still important; even a small increase in muscle tension due to pressure may significantly attenuate the passive dilation of the highly distensible veins, significantly attenuating the vascular capacity. Increased reactivity of capacitance veins may contribute to arterial hypertension.

The myogenic activity of systemic veins is also very important from physiological standpoint: when the venous pressure in the lower limbs increases by the effect of gravitational force after standing up, activation of myogenic activity prevents orthostatic intolerance. Conversely, attenuated myogenic activity of systemic veins may contribute to hypotension and edema (Monos et al., 1995).

Because intravascular tone will alter the membrane potential and tone of vascular SMCs, the reactivity of blood vessels will also differ under pressurized and non-pressurized conditions. Physiologically, mice MA are exposed to high pressures *in vivo* (60 mm Hg) while MV belong to low pressure system. Therefore, the difference between the physiological intravascular pressures and pressures of isolated vascular preparation is not very large in the MV, but is large in MA. Therefore, studies of non-pressurized veins are likely to be physiologically more relevant than studies of non-pressurized arteries.

6. Biochemistry of the regulation of vascular tone

Regulation of vascular contractility.

Regulation of vascular tone centers on the regulation of $[Ca^{2+}]i$ in the vascular SMCs. Stimulation of blood vessels by vasoconstrictor agents ultimately raises $[Ca^{2+}]i$, leading to activation of Ca^{2+}-calmodulin dependent protein kinase. Ca^{2+}-calmodulin dependent protein kinase then stimulates myosin light chain-kinase (MLCK) which phoshorylates myosin light chains (Kamm and Stull 2001). Activation of myosin ATPase activity by MLCK enables actin and myosin to interact and generate tension.

Conversely, relaxation of SMCs is tightly coupled to dephoshorylation of myosin light chain by myosin light chain phosphatase (MLCP). The activities of both MLCK and MLCP are tightly regulated and while MLCK is activated by calcium/calmodulin that directly links intracellular $[Ca^{2+}]$ to SMC contractility, MLCP is activated by both vasodilator and vasoconstrictor stimuli and therefore contributes to the Ca^{2+}-independent regulation of vascular contractility. NO activates guanylate cyclase which synthesizes cGMP and the resulting activation of protein kinase G (PKG) directly activates MLCP (Surks et al., 1999). Regulation of MLCP activity is not only important during vascular relaxation, but it is considered the most important mechanism that influences Ca^{2+} sensitivity of the contractile apparatus.

Regulation of vascular tone by modifying Ca^{2+} sensitivity.

It has been established that the relationship between the extent Ca^{2+} elevation and tension development varies based on the alteration of Ca^{2+} sensitivity. Agonist–mediated elevation of Ca^{2+} produces greater tension at a given Ca^{2+} than depolarization (Karaki 1989; Somlyo and Himpens 1989; Hirano 2007). Agonist stimulation initially increases MLC phosphrylation through elevation of $[Ca^{2+}]i$, but this is immediately followed by decrease of MLCP activity, leading to increases Ca^{2+} sensitivity (Somlyo and Somlyo 1994). These two mechanisms cooperate in modulating vascular constriction.

Multiple second messengers mediate Ca^{2+} sensitivity. However, Rho A, a Ras-related small monomeric GTPase is believed to play the most important regulatory role (Hartshorne et al., 1998). Activation of many GPCR and the downstream stimulation of Rho – associated kinase has been shown to inhibit MLCP by phosphorylation of myosin phosphatase target subunit 1 (MYPT1) (Feng et al., 1999; Kitazawa et al., 2003). Multiple GPCRs regulate Ca^{2+} sensitivity by activation of Rho kinase; in vascular SMCs the receptor-dependent activation of Rho kinase relies on activation of $G_{12/13}$ and this pathway seems to be independent of Gq mediated phosphorylation of MLC (Gohla et al., 2000). Even though the role $G\alpha_{12/13}$ in activation of Rho A pathway has been well established, recent data indicate that Gq_{11} can also activate Rho kinase in PLC-independent manner (Vogt et al., 2003). However, similar studies in vascular

SMCs need to be performed to confirm that this mechanism is relevant to the regulation of vascular tone.

αAR induce large increases of $[Ca^{2+}]i$ and activation of Rho kinase (Roberts, 2004; Tsai and Jiang 2006; Dimopoulos et al., 2007). Activation of Rho kinase by NE and some other contractile agents is regulated by phosphoinositide 3- kinase (PI3K) that acts upstream of Rho kinase (Wang et al., 2006). In addition, src family of tyrosine kinases may be involved in activation of Rho kinase (Nagao et al., 1999).

In addition to Rho kinase, PKC can also regulate Ca^{2+} sensitivity of vascular SMCs. PKC is activated by DAG that is formed by activation of G proteins, including Gi and Gq. It has been suggested, that PKC also enhances Ca^{2+} sensitivity via its inhibitory action on MLCP (Masuo et al., 1994; Ikebe and Brozovich 1996). While PKC has not been shown to phosphorylate MYPT1 directly as has been demonstrated for the Rho associated kinase, studies have indicated that smooth muscle specific phosphoprotein CPI-17 acts downstream of both Rho A and PKC activation to directly and potently inhibit MLCP (Kitazawa et al., 2000). This suggests that CPI-17 might be a point of convergence for the Rho and PKC pathways. Expression of CPI-17 seems to correlate with Ca^{2+} sensitivity and PKC-dependent constriction; vascular smooth muscle that exhibit tonic activity also exhibit increased Ca^{2+} sensitivity and express larger amount of CPI-17 than smooth muscle that exhibits phasic constriction (Woodsome et al., 2001). Differences in the expression of CPI-17 between MA and MV could also contribute to differential sensitivity to contractile agonists. In addition, changes in

the expression of CPI-17 might be relevant in hypertension, where enhanced sensitivity to agonist stimulation has been noted. Increased vascular reactivity also occurs in diabetes and type 2 diabetic mice have been shown to have increased level of activated CPI-17 as well as Rho A (Xie, Su et al. 2006).

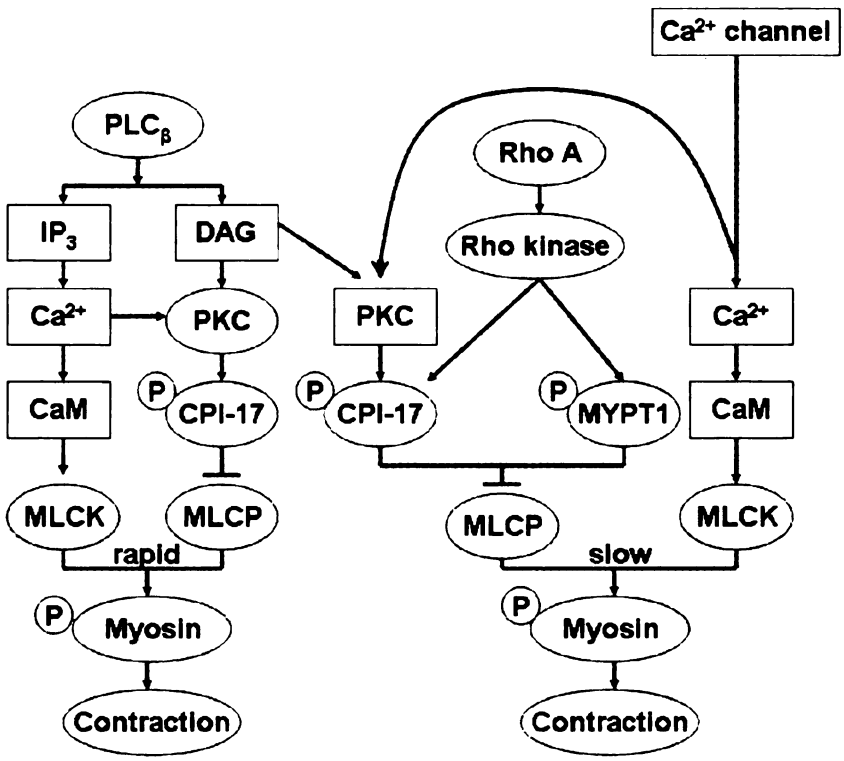


Fig. 1. Proposed dual mechanism for vascular sensitization. Circulation research, 2007 (100):121-129.

The signal transduction involving Ca^{2+} sensitization is well coordinated to maintain signal specificity. Under resting conditions, PKC, RhoA and ROK are distributed evenly in the cytosol. However, activation or receptor can induce translocation of these important regulatory proteins to the plasma membrane, contributing to the efficacy of agonist-induced constriction (Taggart et al. 1999).

A recent study has proposed a new mechanism of Ca^{2+} sensitization in SMCs that occurs within seconds of agonist stimulation. In rabbit femoral artery, PE induced phosphorylation of MLC occurred at very similar time points as phosphorylation of CPI-17, the main inhibitory protein of MLCP. This rapid

activation was dependent upon Ca^{2+} release from SR and also PKC. Activation of CPI-17 together with MLCK synergistically increased MLC phosphorylaton, resulting into constriction. This rapid Ca^{2+} sensitization was followed by slower onset, Rho/PKC-dependent CPI-17 phosphorylation (Dimopoulos, et al. 2007). Fig. 1 provides a diagram of pathways involved in the proposed dual inactivation of MLCP (Taken from Circulation research, 2007 (100):121-129).

Rho kinase in arteries and veins. It is well established that regulation of Rho kinase pathway plays an important role in agonist-induced constriction and the degree of its involvement affects vascular constriction. A study has found that inhibition of Rho kinase may produce greater reduction of agonist induced constriction in veins than arteries (Robertson et al. 2007) suggesting that perhaps Ca^{2+} sensitization mediated by agonist may play a greater role in regulation of venous than arterial tone. If this also applies for small MV, it would be also a possible contributor to their enhanced sensitivity to multiple vasoconstrictors.

Modulation of Ca^{2+} sensitivity by α_2AR. Besides directly stimulating MLC, another mechanism by which α_2AR can modulate vascular tone is independent of MLC phosphorylation and involves increase in Ca^{2+} sensitivity (Aburto et al. 1993). As discussed above, this can be mediated by PKC that is an upstream target of CPI-17.

However, Ca^{2+} sensitization can be also mediated by tyrosine kinases that play a role in α_2AR-mediated constriction. Although the precise mechanism

by which tyrosine kinase elicits Ca^{2+} sensitization in SMCs has not been clarified, studies suggest that src-family of tyrosine kinase are involved in the activation of Rho kinase, resulting into inhibition of protein phosphatase (Nakao et al. 2002). Even though Gi has been shown to activate tyrosine kinase and induce constriction, role of other G proteins is also possible. In transfected HEK-293 cells, G_{12} protein activates Src tyrosine kinase that acts upstream of Rho kinase (Nagao et al. 1999). While there is no report linking activation of α_2AR to G_{12}, role of this protein in Ca^{2+} sensitization would be also plausible due to the emerging role of this protein in Ca^{2+} sensitization.

The Ca^{2+} sensitization mediated by α_2AR might also prove be important in hypertension. In rats made hypertensive by NOS inhibition, α_2AR reactivity and Ca^{2+} sensitivity is enhanced. However, Ca^{2+} sensitization in these animals is independent of tyrosine kinase activation and but rather involves PKC-α (Carter and Kanagy 2003). Conversely, activation of PKC by non-adrenergic agonists can enhance Ca^{2+} sensitivity (Jiang et al 1987). In dog MV, activation of PKC ET-1 sensitizes the tissue to α_2AR stimulation by UK 14,304 (Shimamoto et al, 1995). Angiotensin II has also been shown to increase constrictions mediated by α_2AR in veins (Dunn et al. 1991). In addition, studies also indicate that α_1AR can also enhance the α_2AR function in the saphenous vein (Daly et al. 1988), cauda epididymis of the guinea pig (Haynes and Hill 1996) and rat tail artery (Xiao and Rand, 1989). As will be discussed later, this phenomenon might also occur in MV but not in MA, contributing to higher sensitivity of MV.

Regulation of Ca^{2+} desensitization. On the other hand, activation of PKG and PKA in some SMCs seems to oppose RhoA/ROK pathway and therefore involves Ca^{2+} desensitization. PKG is activated by cGMP that is produced by guanylate cyclase and nitric oxide (NO). The exact mechanism by which PKG inhibit Rho A has not been established but studies *in vitro* suggest direct inactivation of RhoA by PKA and PKG phosphorylation (Somlyo et al. 2003). In addition, PKG also directly interacts with myosin phosphatase and this interaction is required for cGMP-mediated activation of myosin phosphatase (Surks et al. 1999). As discussed below, differences between MA and MV in the functional GPCR and their modulation of Ca^{2+} sensitivity would also play an important role in the differential regulation of arterial and venous tone.

Focus of my studies. While it has been well established that veins are more sensitive to sympathetically – mediated stimulation than arteries, the mechanism behind this enhanced sensitivity has not been explained. These studies investigated the basic principles involved in the regulation of venous tone at the level of SMCs, with the emphasis on α_2AR contribution. Better understanding of the receptors and their signaling pathways in the veins may lead to increased awareness of processes than may be deregulated during disease process such as hypertension.

CHAPTER 2

Hypothesis and Specific Aims

Overall hypothesis

The sympathetic nervous system is the main regulator of venous tone and cardiac output. NE released from sympathetic nerves constricts MA and MV by acting on αAR. However, MV are significantly more sensitive to the contractile effects of NE compared to MA {Luo, 2003 #412}{Hottenstein, 1987 #433}{Perez-Rivera, 2004 #330}. While multiple factors could underlie this enhanced adrenergic sensitivity of MV, there are differences in the contribution of αAR subtypes to NE-induced constrictions of MA and MV. In MV both α_1- and α_2AR mediate NE-induced constriction while in MA α_1AR only mediates constriction to NE. α_2AR potentiates the action of α_1AR and receptor cross-talk could contribute to enhanced adrenergic sensitivity of MV.

Using an integrative approach, my goal was to characterize the mechanism of the functional interaction between α_1- and α_2AR that occurs in MV, but not in MA. I hypothesized, that the interaction between these receptors contributes to the increased calcium mobilization and adrenergic responsiveness of MV compared to MA. To accomplish this goal, four specific aims were addressed:

Specific aim #1: The overall goal of this aim was to test the hypothesis that there is differential expression of α_1- and α_2AR subtypes in arterial and venous myocytes obtained from murine mesenteric arteries and veins. Differential

receptor expression would contribute to differences in the sensitivity of MA and MV to adrenergic agonists.

Specific aim 1a: This aim tested the hypothesis that arterial and venous smooth muscle cells (SMCs) express different α_1AR subtypes. Immunocytochemical techniques were used to study receptor expression in freshly isolated murine mesenteric arterial and venous SMCs.

Specific aim 1b: This aim tested the hypothesis that murine veins but not arteries express α_2ARs. Co-expression of both α_1ARs and α_2ARs in veins, but not arteries, leads to receptor cross-talk in venous SMCs.

Specific aim #2: In this aim, I measured arterial and venous constriction *in vitro* to investigate pharmacological interaction between α_1 and α_2ARs in intact MV from mice. αAR subtype selective ligands were utilized to test the hypothesis that α_2AR synergistically interacts with α_1AR. In addition, studies were performed in MV of α_{2C}AR gene knockout (KO) mice to test for a functional role of α_{2C}AR in MV.

Specific aim #3: Interactions between α_{1D}- and α_2ARs was investigated at the molecular level, using heterologous receptor expression in HEK-293 cells. The first hypothesis I tested was that the interaction is based on direct physical interaction between the two receptors.

Specific aim 3a: α_2AR subtypes were transiently co-transfected into α_{1D}AR – expressing HEK-293 cells and receptor co-localization experiments were performed. In addition, α_1- and α_2AR co-expressing cells were treated with NE

and receptor trafficking was investigated. I also tested the hypothesis, that α_2ARs promote membrane trafficking of α_{1D}AR.

Specific aim 3b: To test whether the functional interaction between α_{1D}- and α_2AR is occurs in HEK-293 cells, I used calcium imaging of HEK-293 cells co-expressing α_{1D}- and α_2ARs.

Specific aim # 4: Calcium dependent signaling mechanisms activated by of αARs were investigated in intact MA and MV using a pressure myograph. Dependence of functional responses on calcium stores and calcium channels was assessed. Coupling of α_2AR to calcium influx and its role in the sensitivity of MV was investigated.

CHAPTER 3

Functional Interaction Between α_1- and α_2-Adrenoreceptors in Mesenteric Veins Does not Require Heterodimer Formation

Abstract

Mesenteric veins (MV) are more sensitive to the contractile effects of sympathetic nerve stimulation and α-adrenergic receptor (αAR) agonists than mesenteric arteries (MA). We tested the hypothesis that MV co-express α_1AR and α_2ARs and α_2ARs interact directly with α_1AR to increase adrenergic reactivity of MV. We studied neurogenic and agonist-induced constrictions of MA and MA *in vitro* and the subcellular distribution and function of αARs expressed in HEK-293 cells in an effort to mimic MV receptor expression. Frequency response curves (0.5-30 Hz) for neurogenic constriction and norepinephrine (NE) concentration response curves (CRCs) were left shifted in MV compared to MA. Yohimbine (α_2AR antagonist) and MK912 (α_{2C}AR antagonist) but not α_{2A}AR or α_{2B}AR antagonists, produced rightward shifts in NE CRCs in MV but not MA. UK 14,304 (0.01-1 μM)(α_2AR agonist) did not contract MA or MV but it potentiated constrictions mediated by α_1AR. NE constrictions in α_{2C}AR knock-out (KO) mouse MV were not different from wild type (WT) MV. Immunocytochemistry using dispersed MA and MV smooth muscle cells from WT mice revealed plasma membrane expression of α_{2A}AR and α_{2C}AR. MV expressed α_{1D}ARs predominantly in the intracellular compartment. In transfected HEK-293 cells, α_2ARs did not co-localize with α_{1D}ARs subcellularly and co-expression with α_2ARs did not alter α_{1D}AR distribution. Co-expression with β_2ARs increased plasma membrane localization of α_{1D}ARs and enhanced calcium signaling coupled to α_{1D}ARs. These data indicate that α_{1D}-α_2ARs do not interact directly to

enhance MV adrenergic reactivity. α_2AR-linked intracellular signaling specific for MV may account for enhanced adrenergic reactivity.

Introduction

Blood pressure regulation is complex and involves several interacting systems including the sympathetic nervous system (SNS) which contributes to regulation of body fluid volume and vascular tone (Guyenet, 2006). Sympathetic nerves innervate arteries and veins and regulate their tone (Anderson et al. 1989). Vascular tone and structure of small arteries are the main determinants of total peripheral resistance (TPR). Because veins store approximately 75% of total blood volume (Martin et al. 1998), they are the main capacitance vessels in the circulation. The storage capacity of veins is determined by structural characteristics, but the SNS is the major regulator of venous tone (Pang 2001). Since venous tone regulates capacitance, it directly affects blood return to the heart and cardiac output (CO) (Guyton 1955; Greenway and Lautt 1986). Because blood pressure is a product of TPR and CO, regulation of venous tone is critical for blood pressure control.

Despite the importance of venous tone in blood pressure control, veins have not been studied as extensively as arteries. In addition, most studies used large diameter veins (Gavin et al. 1997, Chen-Han Lee et al. 2002). The hemodynamic functions of large conduit veins (vena cava, for example) greatly differ from the functions of systemic, small diameter veins. Small veins, particularly splanchnic veins, are the most important venous bed due to their dense sympathetic innervation and high compliance (Pang 2001). These characteristics have important implications in the pathophysiology of hypertension, where reduction of venous capacitance occurs (Ferrario et al.

1970; Ricksten et al. 1981; London et al. 1985). When splanchnic storage capacity is reduced, redistribution of blood from these vessels increases venous return to the heart (Greenway and Lautt 1986). Reduced venous capacitance is accompanied by an increase in CO, which also occurs in pre-hypertensive humans (Drukteinis et al. 2007). These findings point to the relevance of studies investigating control of venous tone in blood pressure regulation.

Previous work revealed that MV are more sensitive to adrenergic stimulation than arteries (Hottenstein and Kreulen 1987, Perez-Rivera et al. 2004, Luo 2003). This might be due to artery-vein differences in αARs in vascular SMCs mediating contraction. Several studies indicated, that α_2ARs plays more prominent functional role in veins compared to arteries (Flavahan et al. 1984, Ruffolo 1986, Patel et al. 1981). Studies from our laboratory indicated, that α_2AR indirectly contributes to venous constriction, whereas only α_1AR mediates NE - induced constriction of arteries. Several studies indicated that α_2AR can exert a synergistic effect on α_1AR responses; functional interactions between α_1- and α_2AR have been detected in cauda epididymis (Haynes and Hill 1996), primary glial cell cultures (Wilson and Minneman 1991) and rat tail artery {Xiao, 1989 #660}. α_1-AR and α_2AR interactions also occur in heterologous expression systems (Reynen et al. 2000).

α-ARs are G-protein coupled receptors (GPCRs) and there is accumulating evidence, that signaling via a GPCR that couples preferentially to one pathway may be affected by inputs from GPCR coupling to other pathways (Milligan et al. 2006). The interaction between GPCR can lead to a loss or

enhancement of function (Werry and Wilkinson, 2003). In addition, GPCRs can form heterodimers or heteromultimers with pharmacologically distinct properties from monomeric receptors (Levac et al. 2002; Milligan et al. 2003). αARs can also form heterodimers (Hague et al. 2004; Uberti et al. 2005; Hague et al. 2006). Importantly, the α_{1D}AR that mediates constrictor responses caused by norepinephrine in MV (Perez-Rivera, 2007) is known to heterodimerize with other receptors (Hague et al. 2005; Uberti et al. 2005). Localization of the α_{1D}AR is predominantly intracellular when the receptor is expressed alone in HEK-293 cells. β_2AR as well as α_{1B}AR can form heterodimeric partners with α_{1D}ARs and as a result of this interaction, α_{1D}AR is targeted to the cell membrane more efficiently, resulting in enhanced signaling mediated by the α_{1D}AR (Uberti et al. 2004; Uberti et al. 2005).

In the present study we screened for the expression and functional involvement of different α_2AR subtypes in venous SMCs in order to identify the native α-AR subtypes relevant to this interaction. We also performed parallel immunocytochemical analysis of αARs expressed in arterial SMCs to asses the correlation of receptor expression with reactivity of MA and MV. In addition, we used tissue from α_{2C}AR KO mice to determine the effect of this receptor on adrenergic sensitivity of MV. We tested the hypothesis that α_2AR can increase membrane trafficking of α_{1D}AR by heterodimerizing with this receptor in MV and HEK-293 cells. This mechanism could be very important in MV due to the functional importance of α_{1D}AR in MV. In addition, we performed calcium imaging studies in transfected HEK-293 cells to test, whether the functional

interaction we observe in MV could be mimicked in HEK-293 cells that co-express α_{1D}AR and α_2ARs. We predicted that this heterodimer functions as an independent signaling unit with enhanced stimulus-response coupling compared to α_{1D}AR signaling alone.

Materials and Methods:

Animals. C57/Bl6 male mice (25-30g) were purchased from Charles River Breeding Laboratories (Portage, MI). Mice were maintained in the animal care facility, according to the standards approved by the Michigan State University Institutional Animal Care and Use Committee. Mice were individually housed in clear plastic cages with access to standard chow (Harlan/Teklad 8640 Rodent Diet) and tap water.

Heterozygotes for the neomycin-disrupted locus coding for α_{2C}AR were purchased from Jackson Laboratories, Bar Harbor, MA. These mice have been previously characterized (Link, Stevens et al. 1995). As the colony expanded, heterozygotes were bred and their offspring (9-12 weeks of age) WT and KO mice were utilized in experiments. A previously published PCR protocol (www.jacx.org) was followed to characterize the genotype of individual pups.

***In Vitro* Preparation of Mesenteric Arteries and Veins.** Mice were anesthetized, and the small intestine with its associated mesentery was removed and placed in oxygenated (95% oxygen, 5% carbon dioxide) Krebs' physiological saline solution of the following composition (millimolar): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 25 $NaHCO_3$, and 11 glucose. A piece of the intestine with associated vessels was removed and pinned flat in a silicone elastomer-lined (Sylgard; Dow Corning, Midland, MI) petri dish. A section of mesentery containing vessels close to the mesenteric border was cut out using fine scissors and forceps. The preparation was transferred to a smaller silicone

elastomer-lined recording bath and pinned flat. Second- or third-order mesenteric veins or arteries (100-200-μm diameter) were isolated for study by carefully clearing away the surrounding fat tissue. The recording bath containing the preparation was mounted on the stage of an inverted microscope (Olympus CK-2; Tokyo, Japan) and superfused with warm (37°C) Krebs' solution at a flow rate of 7 ml/min. All preparations were allowed a 20-min equilibration period during which the vessels relaxed to a stable resting diameter.

Video Monitoring of Vessel Diameter. The output of a black- and-white video camera (Hitachi model KP-111; Yokohama, Japan) attached to the microscope was fed to a framegrabber card (Picolo, Euresys Inc., TX, USA). mounted in a personal computer. The video images were analyzed in real-time using Diamtrak Edge-tracking software (version 3.5, Diamtrak, Adelaide, Australia), which tracks the distance between the outer edges of blood vessel in the observation field. Changes in vessel diameter as small as 1 μm could be resolved.

Transmural stimulation of perivascular nerves. Two Ag/AgCl electrodes connected to a Grass Instruments stimulator (S44) were placed parallel to the longitudinal axis of MA and MV. Parameters for nerve stimulation were the following: 60 stimuli, 0.5 ms duration of stimuli, frequency from 0.5, 1, 5, 10, 20 and 30 Hz and 150 V. Blood vessels were allowed to equilibrate for 15 min between each stimulus.

Antagonist Studies. After tissue equilibration, antagonists were applied for additional 20 min to attain equilibrium before NE concentration-response curves were generated. BRL44408 was used as an α_{2A}AR antagonist; BRL44408 pKi values for α_{2A}AR, α_{2B}AR and α_{2C}AR are 8.2, 6.2 and 6.8 respectively. Imiloxan was used as an α_{2B}AR antagonist: imiloxan pKi values for α_{2A}AR, α_{2B}AR and α_{2C}AR are 5.8, 6.9 and 6.0 respectively. MK912 was used as an α_{2C}AR antagonist; MK912 pKi values for α_{2A}AR, α_{2B}AR and α_{2C}AR are 8.9, 8.9 and 10.2 respectively. All drugs were purchased from Sigma-Aldrich (St. Louis, MO) and were added in known concentrations to the superfusing Krebs' solution. A single agonist concentration-response curve was obtained from each preparation. Because veins became sensitized to NE when two dose-response curves were generated using the same tissue, a fresh vein was used to construct control dose-response curves.

Data Analysis. Constrictions of blood vessels are expressed as a percentage of the resting diameter. Half-maximal effective agonist concentration (EC_{50}) and maximum response (E_{max}) were calculated from a least-squares fit of individual agonist concentration-response curves using the following logistic function from Origin 7.0 (Origin-Lab Corp., Northampton, MA):

$$Y = \{[E_{min} - E_{max}]/[1+(x/EC50)^n]+E_{max}$$

where E_{min} is the minimum response and was constrained to zero and n is the slope factor. All data are expressed as mean ± S.E.M. Statistical

differences between groups were assessed by Student's two-tailed unpaired *t* test.

HEK-293 cell culture and transfection. Tissue culture media and related reagents were purchased from Invitrogen (Carlsbad, CA). HEK-293 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum at 37°C, in a 5% CO_2 incubator. Cell passage 25 to 30 was used in experiments. For heterologous receptor expression cells were diluted to 10^5 cells/ml in complete medium and 800 µl of the cell suspension was transferred to disposable electroporation cuvettes (Bio-Rad). 2 µg of plasmid DNA was added to the cell suspension and electroporation was performed using a Gene Pulser (Bio-Rad), employing the following parameters: resistance= ∞, capacitance = 950 µFarads at 270 volts. After the procedure, cells were transferred to a 15 ml Falcon tube and spun down at 3000 rcf for 3min. The cell pellet was resuspended in tissue culture medium (see above). The transfection efficiency in our experiments was 15%.

Plasmids. Human α_{2A}, α_{2B} and β_2ARs were purchased from the cDNA Resource Center at the University of Missouri-Rolla (www.cdna.org). The Red express plasmid was purchased from Clontech (Mountain View, CA). Flag-tagged human α_{2C}AR was kindly provided from Dr. Maqsood A. Chotani (Ohio State University). Human α_{1D}AR and HA-tagged α_{1D}AR were kindly provided by Dr. Chris Hague (Emory University Medical School).

α_{1D}AR stable cell line. HEK-293 cells were transfected by electroporation as described. We used a hemagglutinin-tagged (HA) α_{1D}AR where the HA tag was attached to the N-terminal portion of the human α_{1D}AR. This plasmid contains the antibiotic resistance gene for geneticin, which was used for selection of transfected cells. Cells were grown for 4 weeks and geneticin-resistant colonies were isolated and grown in separate flasks. The individual colonies were than tested by Western blot and immunocytochemistry to screen for colonies with the highest receptor expression. To select the final colony, the colonies with highest receptor expression were further subjected to calcium imaging (see below) to identify the clones that yielded the largest NE-induced calcium signal.

Isolation of Mesenteric Vascular Smooth Muscle Cells (VSMC) and Immunocytochemistry. MA and MV veins from male C57B6 mice (25-30 g) were harvested and cells were isolated according to previously described protocols (Jackson and Huebner, 1997). The blood vessels were cleaned of surrounding fat and connective tissue in a cold dissociation buffer of the following composition (millimolar): 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES and 10 glucose (pH adjusted to 7.4). Tissues were than incubated with papain in the presence of dithioerythrol (1 mg/ml) for 35 minutes at 37°C and this incubation was followed by digestion in a collagenase (1.9 U/ml) /elastase (0.15 mg/ml) solution with soybean trypsin inhibitor. Cells were allowed to attach to poly-D-lysine – coated coverslips for 1 hour before they were fixed.

Table 2. Source and working dilutions of the primary and secondary antibodies used in αAR staining in venous SMCs and in HEK293 cells.

Primary antibodies against αAR subtypes and cellular organelles				
Antigen	Source	Host species	Dilution	
α_{1D}	Santa Cruz, CA	goat	1: 300	
α_{2A}	Santa Cruz, CA	rabbit	1:400	
α_{2B}	Santa Cruz, CA	rabbit	1:400	
α_{2C}	Neuromics, MN	rabbit	1:400	
HA	Sigma	Mouse	1:200	
Flag	Oncogene, MA	mouse	1:300	
Pan-cadherin	Abcam, MA	rabbit	1:400	
EEA1	Abcam, MA	mouse	1:400	
Calnexin	Abcam, MA	mouse	1:400	

Secondary Antibody				
	Source	Host species	Dilution	Fluorophore
goat IgG	Jackson Immunolaboratory	rabbit	1:500	Cy3
rabbit IgG	Jackson Immunolaboratory	goat	1:500	Cy3
mouse IgG	Jackson Immunolaboratory	rabbit	1:200	FITC
mouse IgG	Jackson Immunolaboratory	goat	1:400	Cy3

Immunocytochemical analysis. VSMCs were allowed 3-4 hours to attach to poly-D-lysine coated coverslips. Both HEK-293 and VSMCs were fixed using Zamboni fixative for 20 minutes. After fixation, cells were incubated

with a blocking serum (4% serum in PBS; pH 7.4) to diminish nonspecific binding. After overnight incubation with primary antibody (Table 2) at room temperature in PBS containing 0.01% triton, cells were incubated for 1 h with secondary antibodies (Table 1) at room temperature. In some experiments, the nucleus was stained with 4'-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO) during the last 10 min of incubation with secondary antibody. Cells were then washed in PBS and staining was visualized using a Nikon TE2000-U inverted microscope. Photographs were taken using a SPOT Insight Color Mosaic camera (Mager Scientific, Inc.) with MetaImaging Series software. Controls with no primary antibodies were used to ensure that binding is specific. In addition, antibodies were tested in transfected HEK-293 cells for their selectivity against other α_2AR subtypes.

Luminiscence surface expression assay. Transfected cells were grown overnight in a tissue culture flask. In 48 hours, cells were plated into 15-mm dishes at a concentration of 10^5 cells/ml. After 5 hours, cells were rinsed three times with PBS and subsequently fixed with 4% paraformaldehyde in PBS for 30 min. After washing out the fixative with PBS, cells were incubated with blocking buffer (2% non-fat dry milk in PBS). After 30 min incubation, primary antibody conjugated to horseradish peroxidase (HRP) was added to the blocking solution (Bethyl Laboratories, Montgomery, TX). The antibody was washed out after 1 hour by rinsing the cells three times with blocking buffer. 3,3',5,5'-tetramethylbenzidine (TMB) substrate kit (Vector Laboratories, Burlingame, CA) was used to quantify HRP-conjugated to primary antibodies. The reaction was

stopped after 1 hour following addition of 50 µl of 1 N sulfuric acid. Absorbance of yellow reaction product was measured at 450 nm using µ-Quant plate reader (Bio-Tek Instruments Inc.). The absorbance measured corresponds to the amount of cell surface receptor.

Calcium imaging. Transfected cells were plated on round coverslips at 5 x 10^4 cells/ml. 24 h post-transfection, coverslips were incubated in 1 ml of OPTI-MEM, containing 10 µl of pluronic acid and 1 µM Fluo-4-AM (Molecular Probes, Invitrogen). Cells were incubated at 37°C for 45 min. The coverslip was then mounted on the stage of confocal microscope (Leica TCS SL confocal microscope, Leica Microsystems, Bannockburn, IL). Cells were continuously superfused at room temperature with HEPES buffer of the following composition (millimolar): 147 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (pH = 7.4) and after 5 min, drug treatments were begun. After obtaining α-AR receptor mediated responses, a response to ionomycin (1 µM) was obtained and agonist responses were normalized to the ionomycin response. Fluo-4 was excited using an argon laser and a wavelength of 488 nm. Emissions were collected in the range of wavelengths between 480 and 520 nm. Images (512 x 512) were obtained every 3 seconds for 3 minutes. Each experiment consisted of 5 pre-drug (baseline) images followed by drug application for 60 s (20 images) and washout for 35 images (105 s). Drug responses were quantitated by measuring the area under delta fluorescence (response–baseline) curve. Responses of transfected cells from one coverslip (typically 5-10 co-transfected cells) were averaged and three

coverslips containing cells from the same transfection were used as one experimental sample. N values represent pooled data from one transfection.

Results

Neurogenic constrictions of MA and MV. MA and MV exhibited a frequency-dependent constriction in response to transmural electrical stimulation; however, MV exhibited greater sensitivity to stimulation than MA. The frequency response curve in MV was to the left of that in MA, EC_{50} of 2.7 ± 1.3 Hz in MV (n=4) and 7.5 ± 0.9 Hz (n=4) in the MA. Also, MV reached a higher maximal constriction than MA (32 ± 3% constriction in MV *vs.* 26 ± 2% constriction in MA)(Fig. 2A). Tetrodotoxin (TTX, 0.3 μM), an antagonist of voltage-gated sodium channels in neurons, abolished responses to electrical stimulation in MA and MV confirming that the constriction was neurogenic.

α_2AR do not mediate direct constriction of MV. Differences in the responses of MA and MV to nerve stimulation could be due to differences in transmitter release and/or and postjunctional signaling in VSMCs. We focused our attention on αAR signaling in the VSMCs that mediate the effect of NE released from sympathetic nerve endings. We found that NE, which activates α_1- and α_2ARs, was more potent in contracting MV than MA, with EC_{50} of 0.02 ±0.004 μM in MV and 4 ± 0.6 μM in MA (Fig. 2B), ($P < 0.05$), n=6. In contrast, the α_2AR agonist UK 14,304 did not contract MA or MV (Fig. 3A) (MA not shown). We tested the possibility whether the lack of effect of α_2AR agonist on vascular contraction could be potentially due to opposing effects of α_2ARs acting in the endothelium to stimulate synthesis and release of vasodilators including

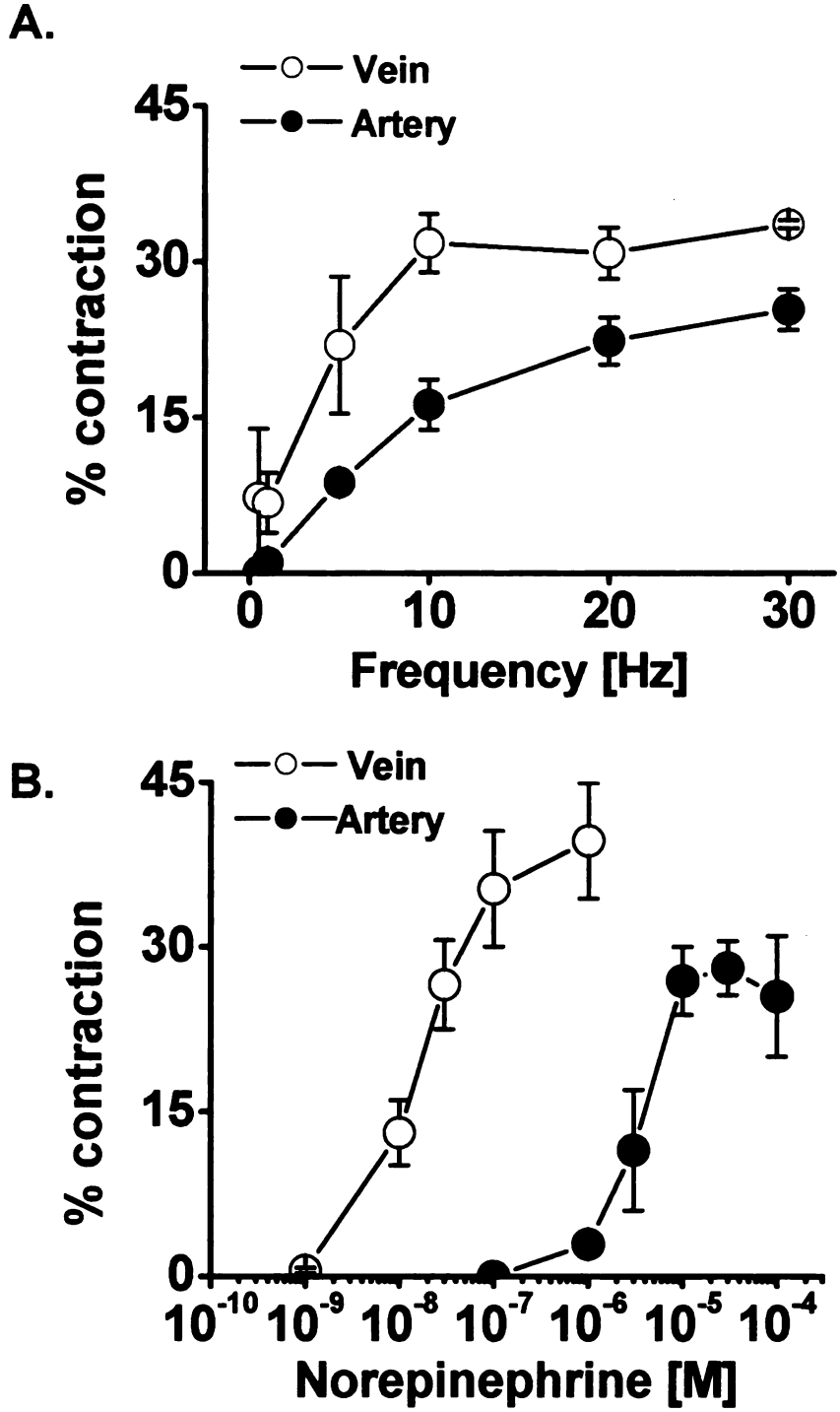


Fig. 2. Mesenteric veins are more sensitive to adrenergic stimulation then arteries. Transmural stimulation of perivascular nerves produced greater constriction in MV than in MA (A). Similarly, concentration-response curve for exogenously applied NE is shifted to the left in the MV (B).

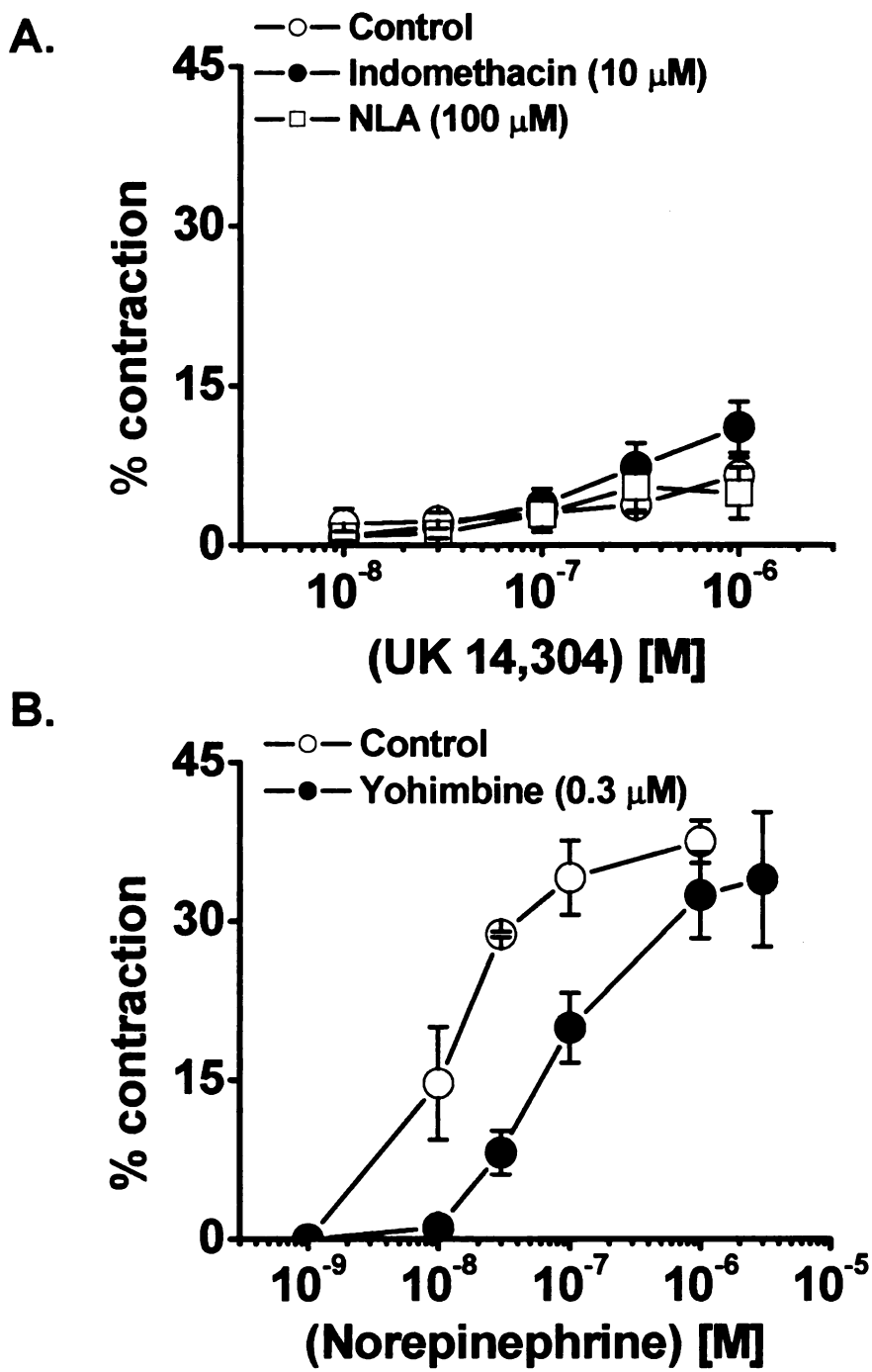


Fig. 3. Addition of indomethacin or nitro-L-arginine did not alter the responses mediated by α_2AR agonist UK 14,304 in MV (A). Despite the small effect produced by α_2AR agonist UK14,304 in the MV, α_2AR antagonist yohimbine produced a significant rightward shift in the NE concentration-response curve (B).

nitric oxide (NO) or prostacyclin. We used indomethacin (10 μM), an inhibitor of cyclooxygenase, to block prostacyclin production and nitro-L-arginine (100 μM), an inhibitor of nitric oxide synthase, to block NO production. Neither of these enzyme inhibitors revealed a constriction response caused by UK 14,304 (n = 7, Fig. 3A). However, yohimbine (0.3 μM), the α_2AR antagonist, caused a 7-fold rightward shift in the NE concentration dose-response curve in MV (Fig. 3B); the control NE EC_{50} was 12 ± 2 nM while in the presence of yohimbine this value was 80 ± 10 nM (n = 6, $P < 0.05$). These data confirmed previous findings that α_2ARs do not directly couple to NE-induced constriction of MV (Perez-Rivera et al. 2007). Yohimbine did not shift the NE CRC to the right in the MA (not shown).

Effect of α_2AR antagonists on NE responses in pressurized MA and MV. Previous studies were performed in the non-pressurized system. Because sensitivity of blood vessels is enhanced when they are pressurized, we wanted to test the effect of α_2AR antagonists in both MA and MV under physiological pressures. Because application of pressure will alter the functional responses of MA more than MV due to larger physiological pressures, by this experiment we also wanted to rule out the possibility that under these conditions, α_2AR antagonist would be able to inhibit NE responses in MA just like in the MV. Because studies indicated, that yohimbine can block α_1ARs we used idazoxan instead of yohimbine. Idazoxan is a α_2AR antagonist, that it is structurally unrelated to yohimbine and has higher selectivity for α_2ARs. Idazoxan did not produce a significant shift of the EC_{50} for NE in MA (Fig. 4A). However, it

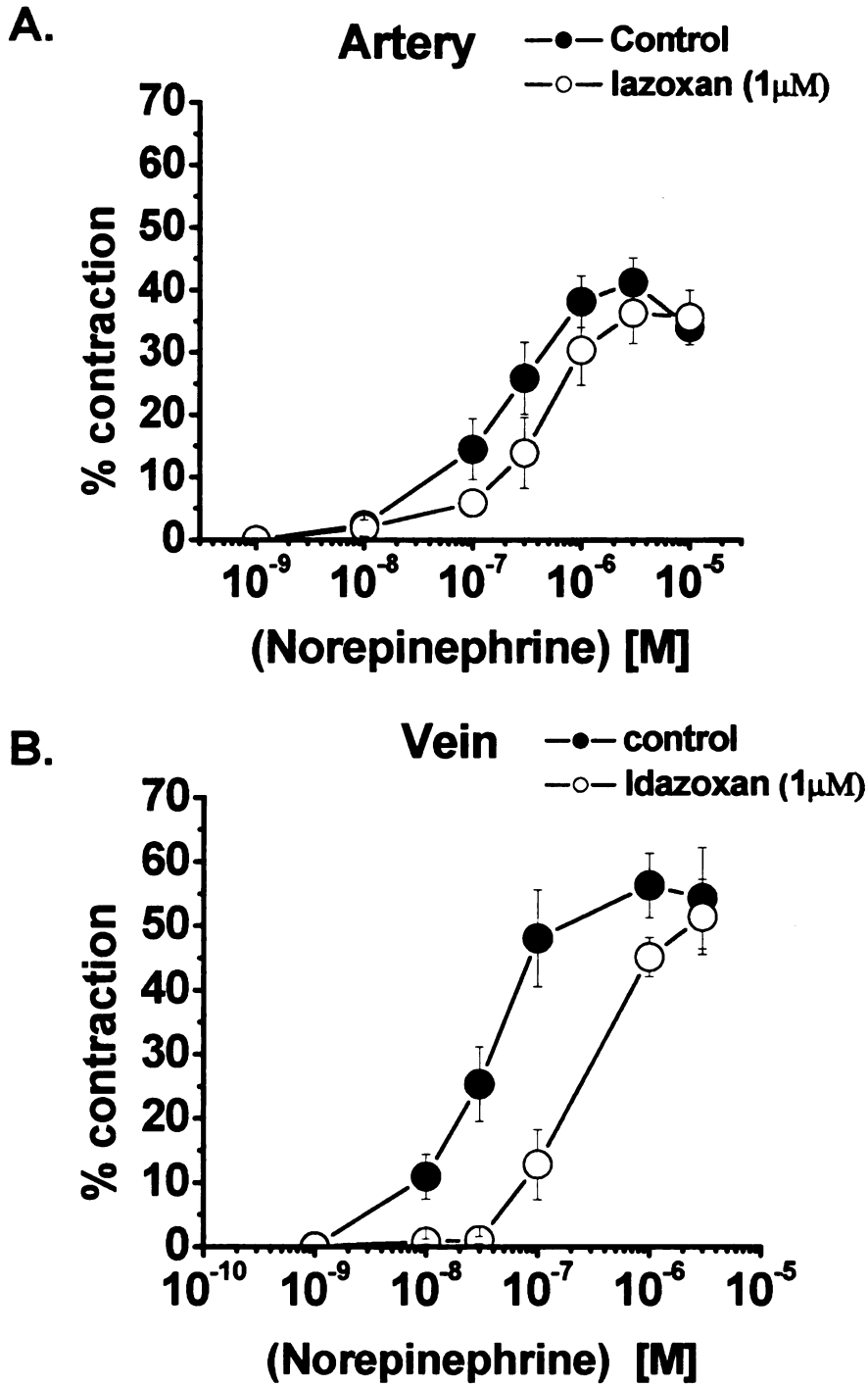


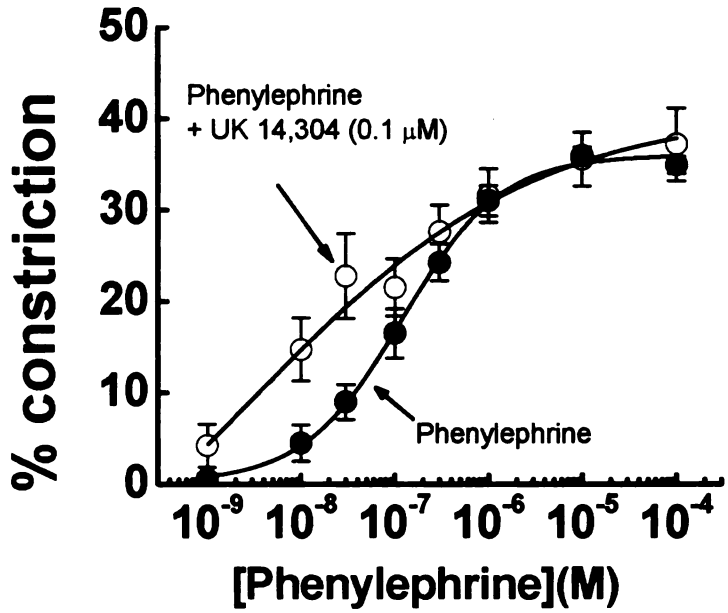
Fig. 4. Effect of α_2AR antagonist in pressurized vessels. Idazoxan, an α_2AR antagonist produced a significant rightward shift in the NE concentration response curve in pressurized MV (B) but not in pressurized MA (A).

produced a similar rightward shift in the MV than yohimbine (Fig. 4B) (0.03 vs 0.2 μM EC_{50} for NE).

α_2AR agonist potentiates responses mediated by α_1AR. Based on our data, we hypothesized that α_2AR enhances the action of α_1AR in the MV. We tested the possibility whether stimulation of α_2AR by UK 14,304 [0.1 μM] will affect the responses mediated by α_1AR in the MV. Concentration-response curves were generated for NE and PE in the presence of UK 14,304. Activation of α_2AR did not potentiate the maximal response of MV to α_1AR agonist PE (Fig. 5A). However, the results also indicated, that responses to lower concentration of PE were potentiated by α_2AR stimulation. This result further suggested that α_2AR might play a role in sensitizing the α_1AR in MV, at least in the lower agonist concentrations, enhancing the responsiveness of MV. Finally, we performed same experiment under pressurized conditions and UK 14,304 potentiated PE–induced constrictions at low PE concentrations (Fig. 5B).

In order to determine the mechanism by which α_2AR contribute to NE induced constriction of MV we next attempted to identify the α_2AR subtype that mediates this response using subtype-selective α_2AR antagonists. Neither the α_{2A}AR antagonist, BRL44408 (0.3 μM, Fig. 6A) or the α_{2B}AR antagonist, imiloxan (1 μM, Fig. 6B) changed the NE concentration-response curve in MV. In contrast, MK912, an α_{2C}AR antagonist (0.01 μM, Fig. 6C) caused a six-fold right-ward shift of the NE concentration-response curve. In these experiments the control NE EC_{50} was 7 $\pm$ 1 nM while in the presence of MK912 this value was 40 $\pm$ 9 nM ($n = 6$, $P < 0.05$).

A



B.

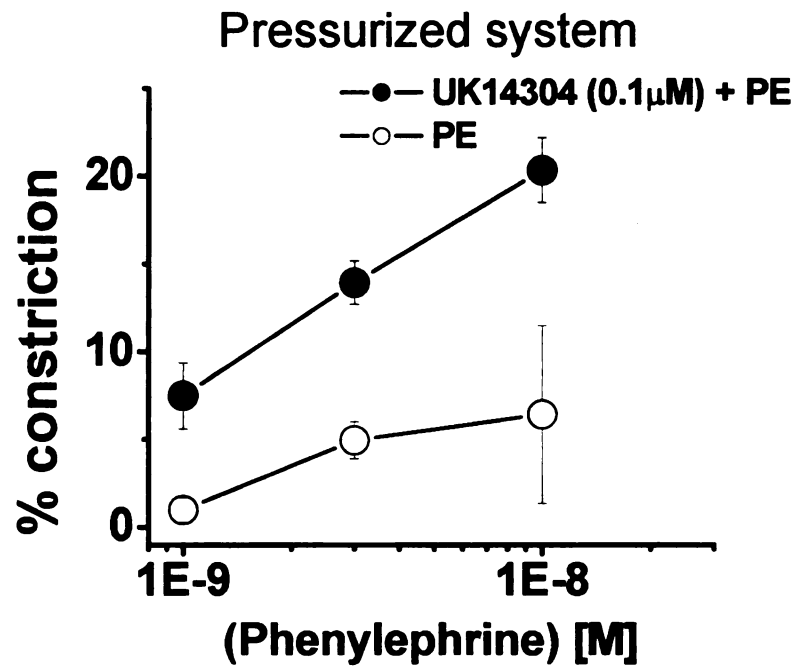


Fig. 5. α_2AR agonist potentiates α_1AR – mediated responses in MV. While UK14,304 did not affect the maximal response to PE in MV (A), it enhanced PE – mediated constriction at low PE concentrations. Similar effect was observed in the pressurized MV (B).

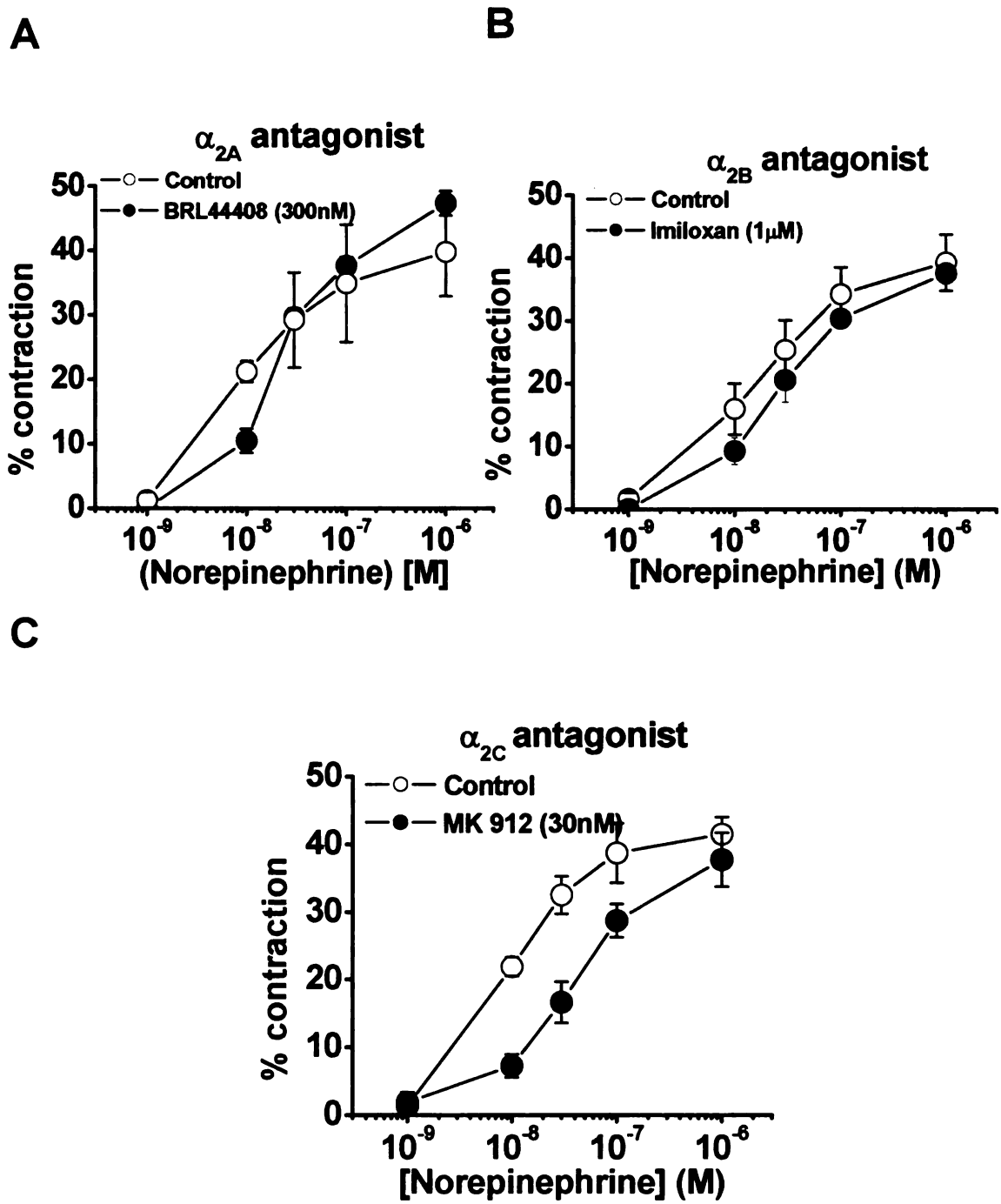
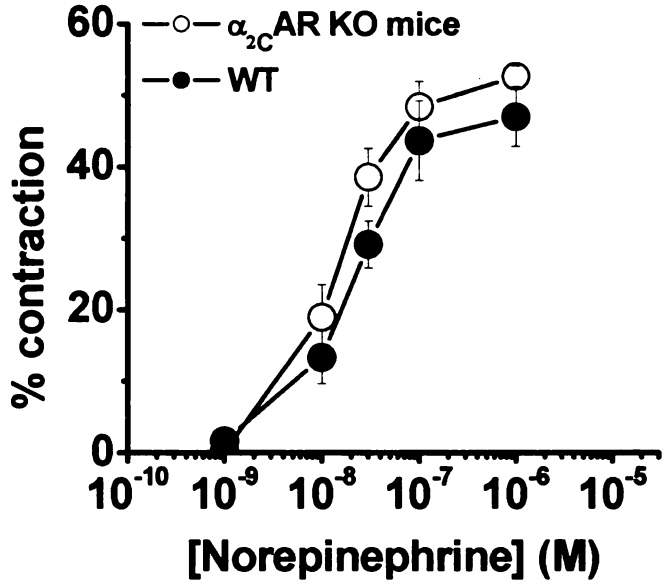


Fig. 6. Effect of subtype selective α_2AR antagonist on NE-induced constriction in MV. While no significant shift of the dose-response curve was observed with BRL44408 (α_{2A} antagonist) (A) and imiloxan (α_{2B} antagonist) (B). MK912 (C) (α_{2C} antagonist) produced a similar effect on NE concentration response curve as α_2AR antagonist yohimbine (Fig. 2B).

Functional responses of α_{2C}AR KO mice. To test the hypothesis that α_{2C}AR is indeed responsible for the enhanced adrenergic sensitivity of MV, we used tissues from α_{2C}AR KO mice in contractility experiments. We did not detect any differences in NE concentration-response curves in MV from WT and α_{2C}AR KO mice (Fig. 7A). In addition, yohimbine produced an 11-fold rightward in the NE concentration response curve in MV from KO mice (Fig. 7B). This was similar to the rightward shift caused by yohimbine in MV from WT mice (Fig. 3B). To test the possibility that other α_2AR subtypes increased their function as a result of physiological compensation to the loss of a functional α_{2C}AR, we utilized MV from the KO mice to test the effect of the previously employed α_2AR antagonists on NE constrictions. While imiloxan (α_{2B} antagonist) did not produce significant effect on NE concentration response curve, the α_{2A}AR antagonist BRL44408 inhibited NE-induced constrictions of MV from α_{2C}KO mice (n=4, Fig. 8). The maximal response to NE has also been suppressed ($P<0.05$) in these KO MV.

Immunocytochemical localization of αAR subtypes in VSMCs. To correlate functional data with receptor expression, we used immunocytochemical methods on freshly dispersed VSMCs from MA and MV to determine the distribution of individual α_1AR and α_2AR subtypes. Fig. 9 depicts pan-cadherin staining, a marker for the plasma membrane, in arterial (A) and venous (B) SMCs. α_{2A}ARs were detected in the plasma membrane of both arterial (9C) and venous SMCs (Fig. 9D). In contrast, α_{2B}AR did not localize to the plasma membrane of venous SMCs and only intracellularly-localized receptors were

A.



B.

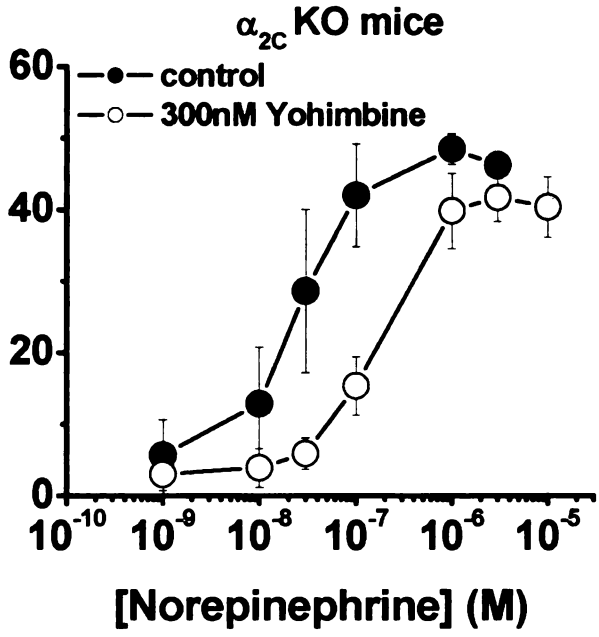


Fig. 7. Sensitivity to NE is not altered in α_{2C}AR KO mice. NE dose response curve of the α_{2C}AR KO mice MV does not differ from WT (A). Yohimbine still produces a rightward shift of the NE concentration response curve in the KO mesenteric veins (B).

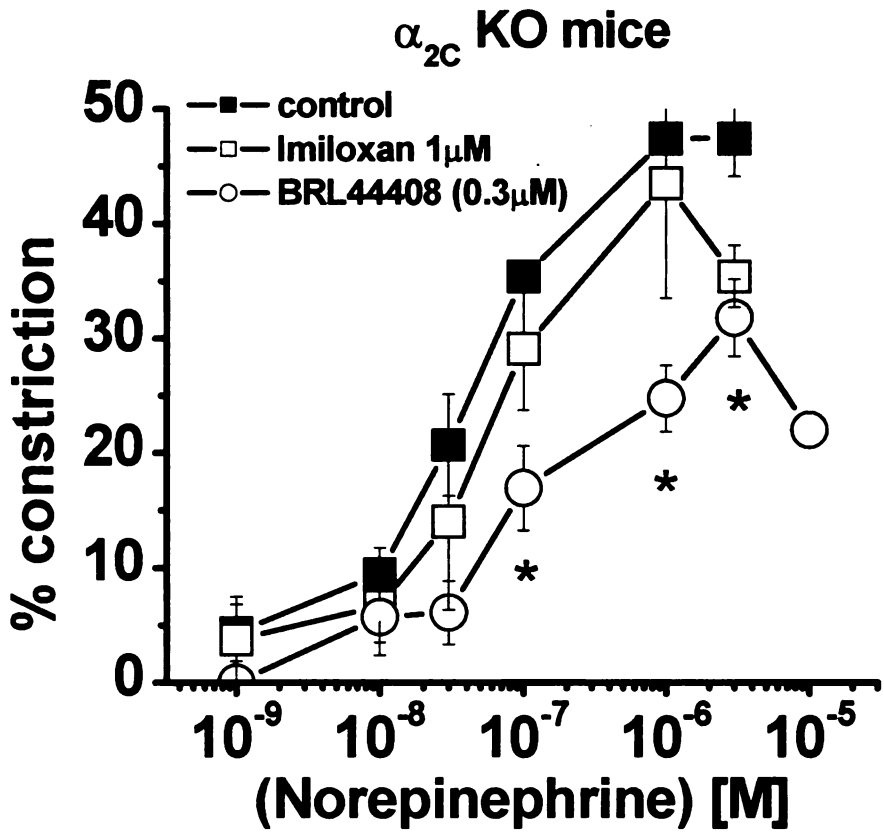


Fig. 8. Effect of subtype selective α_2AR antagonist on NE responses in the MV of α_{2C}AR KO mice. While imiloxan (α_{2B} antagonist) did not affect the concentration response curve to NE in these KO MV, the α_{2A}AR antagonist BRL44408 produced inhibition of NE responses, indicating enhanced function of this receptor in the KO mice

detected (Fig. 9F). We detected α_{2B}AR in intracellular compartment and in the membrane of arterial SMC (Fig. 9E). The α_{2C}AR was expressed both in the plasma membrane as well as in the intracellular compartment of venous SMC (Fig. 9H). These data are consistent with pharmacological data that suggest that α_{2C}ARs contribute to NE-induced constriction of MV from WT mice. α_{2C}AR was also detected in the membrane of arterial SMCs (Fig. 9G). With each experiment, we performed controls with no primary antibodies. No staining was detected in these controls. Contrary to what we expected, we detected expression of all three α_2AR subtypes in the arterial SMCs. When comparing the intensity of α_2AR staining between MA and MV, MV did not seem to express larger amount of receptors.

Expression of α_1ARs. α_{1A}ARs mediate NE-induced constriction of MA (Perez-Rivera) and immunocytochemistry revealed a strong expression of this subtype in the plasma membrane and nuclear membrane of arterial SMCs (Fig. 10A). In the MV, this subtype was not expressed in the membrane, but in the intracellular compartment (Fig. 10B). We also detected α_{1B}AR in the membrane of arterial SMCs (Fig. 10C). This subtype mediates neurogenic constriction of MA. α_{1D}ARs mediate NE-induced constrictions of MV and in venous SMCs, this subtype localized predominantly to intracellular compartment. We also detected strong immunostaining for α_{1D}AR subtype in MA in the membrane and in the sub-plasmalemmal compartment (Fig. 10F).

α_1AR and α_2ARs do not interact in HEK-293 cells. In order to begin to probe the mechanism of α_{1D}-α_2AR interaction, we used stably and transiently

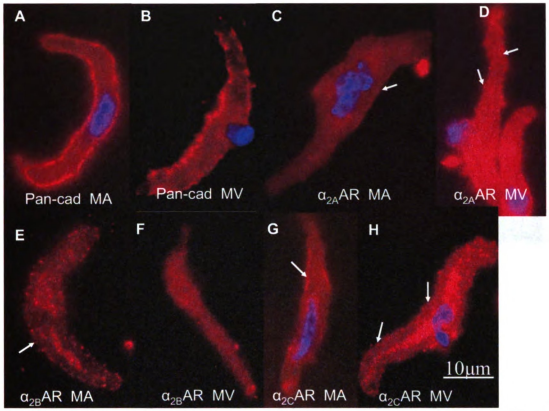


Fig. 9. Immunocytochemical analysis of α_2AR expression in arterial and venous SMCs. Pan-cadherin, the marker of plasma membrane in arterial SMC (A) and venous SMC (B). α_{2A}AR was detected in the membrane of arterial SMC (C), while in venous SMC α_{2A}AR staining was detected both in the intracellular compartment and in the membrane (D). α_{2B}AR localized in intracellular organelles, likely lysosomes in arterial SMC in addition to the membrane (E). α_{2B}AR localized only intracellularly in the venous SMC (F). α_{2C}AR was found in the membrane of arterial SMC (G) and in venous SMC, α_{2C}AR was expressed in the membrane with the majority of the receptor pool localized intracellularly (H). The magnitude of α_{2C}AR expression was larger than the expression of the other two α_2AR subtypes. The blue staining in most cells represents nucleus. White arrow points to receptor localized to the membrane.

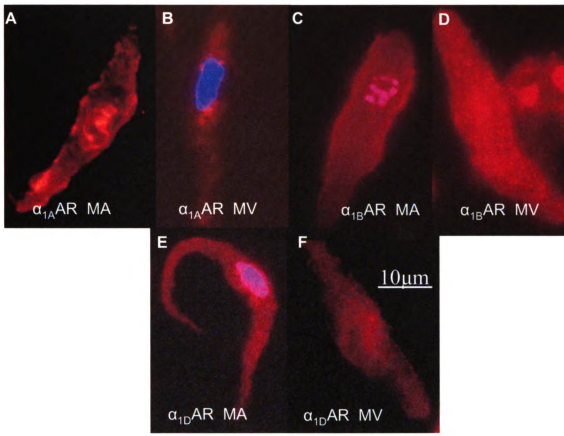


Fig. 10. Expression of α_1AR subtypes in MA and MV SMCs. α_{1A}AR was detected in plasma membrane and also in the nuclear membrane of arterial SMC (A). In venous SMCs, this receptor localized intracellularly. α_{1B}AR was detected in the membrane of arterial SMCs (C), while in the veins this receptor seemed to be expressed at low levels intracellularly (D). Lower panel represents immunocytochemical staining of α_{1D}AR in arterial (E) venous (F) SMCs. In the arterial SMC, this receptor localized to the plasma membrane and subplasmalemmal compartment, whereas in the venous SMC, it was mainly detected in the intracellular compartment. However, some membrane staining of this receptor was also detected in the venous SMC

transfected HEK-293 cells. To test the hypothesis that α_{1D}AR forms a heterodimer with α_{2C}AR, we first performed immunocytochemistry (ICC) experiments to test for co-localization of these receptors in HEK-293 cells. As has been previously reported, when α_{1D}AR was expressed alone in HEK-293 cells, its localization was predominantly intracellular. We employed organelle-specific markers to determine the cellular compartment where α_{1D}AR is localized. As there was little co-localization with the plasma membrane-specific marker pan-cadherin (Fig. 11A'), we used antibodies raised against the endosomal marker, EEA1, and endoplasmic reticulum marker, calnexin, to determine the intracellular compartment of α_{1D}AR expression. Co-localization with these markers suggested, that the α_{1D}AR localized to the endosomes (Fig. 11B') and endoplasmic reticulum (Fig. 11C'). The HA-tag did not alter the localization of α_{1D}AR. These data are similar to the immunocytochemistry results from MV SMCs where some α_{1D}AR has been detected in the plasma membrane, but a large fraction of total receptor pool has been also found intracellularly (Fig. 10F).

We next determined if co-expression with α_2ARs would alter the subcellular distribution of HA-α_{1D}ARs. α_{2A}ARs did not change the distribution of the HA-α_{1D}AR as the α_{2A}AR was predominantly expressed in the membrane whereas HA-α_{1D}AR remained in the intracellular compartment (Fig. 12A). Similarly, α_{2B}AR was efficiently trafficked to the plasma membrane and there was little subcellular localization with the HA-α_{1D}AR (Fig. 12B). When FLAG-tagged α_{2C}AR was co-expressed in the HA-α_{1D}AR stable cells, it localized to the same

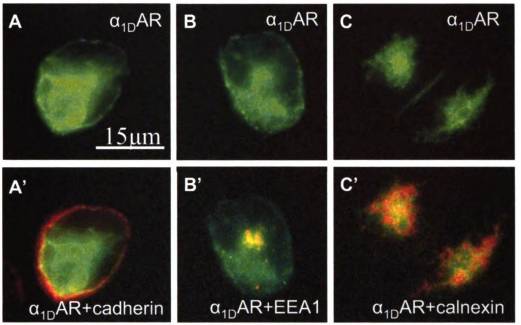


Fig. 11. Subcellular distribution of α_{1D}ARs in HEK-293 cells. A. α_{1D}AR was localized predominantly intracellularly, with little co-localization with the membrane marker pan-cadherin (A' lower panel). B. α_{1D}AR co-localized with the endosomal marker EAA1 (B'). C. α_{1D}AR also co-localized with calnexin (C'), a marker for endoplasmic reticulum.

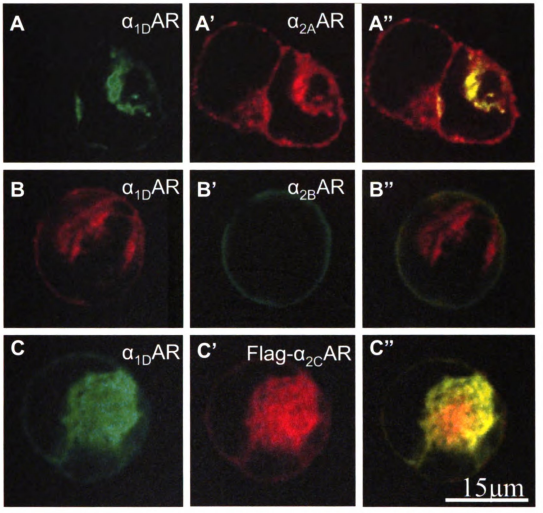


Fig. 12. Relative distribution of α_{1D}AR co-expressed with α_2AR subtypes in HEK293 cells. Each panel represents individual α_2AR subtypes expressed in HA-α_{1D}AR-expressing cell. Picture in the right panel shows the overlay of the two images. Only one cell is visible in fig. A as opposed to fig. A', because transient transfection did not result into 100% co-transfection. In this case, the second cell was transfected only by α_{2A}AR. Data suggested that none of the α_2AR produced redistribution of α_{1D}AR to the plasma membrane. Only α_{2C}AR appeared to localize to the same cellular compartment as α_{1D}AR.

cellular compartments as HA-α_{1D}AR (Fig. 12C) but HA-α_{1D}-AR distribution was not different from that seen in cells expressing only the HA-α_{1D}AR. Both α_{2A}AR and α_{2B}AR were co-localized with the membrane marker pan-cadherin when expressed in HA-α_{1D}AR stable cells (Fig. 13 A', B'). In contrast, there was little co-localization of Flag-α_{2C}AR with pan cadherin (Fig. 13C'). As reported previously, β_2AR physically interacts with α_{1D}AR in heterologous expression systems (Uberti, Hague et al. 2005) and as a consequence of this interaction, α_{1D}AR is trafficked to the cell membrane more efficiently. Therefore, we utilized the β_2AR as a positive control for our receptor trafficking experiments. We found co-localization of β_2AR with α_{1D}AR (Fig. 14C) and that the α_{1D}AR trafficked to the plasma membrane more efficiently when co-transfected with the β_2AR (Fig. 14A) as indicated by co-localization of these receptors with the plasma membrane marker pan-cadherin (Fig. 14E).

Quantitative assessment of cell surface expression of αARs. ICC provides only a qualitative assessment of the relative subcellular distribution of receptors. Therefore, we used a more quantitative assay to test the possibility that co-expression with α_2ARs enhances membrane expression of α_{1D}ARs. We used a luminescence-based surface expression assay to quantitatively evaluate the amount of HA-α_{1D}AR expressed on the cell membrane when receptor was expressed alone or when α_2AR subtypes were co-expressed. Because the HA tag was attached to the extracellular portion of the α_{1D}AR we were able to quantify membrane bound receptor. By this approach, we could use the highly specific monoclonal antibodies against HA-tag. Histogram in Fig. 15 shows

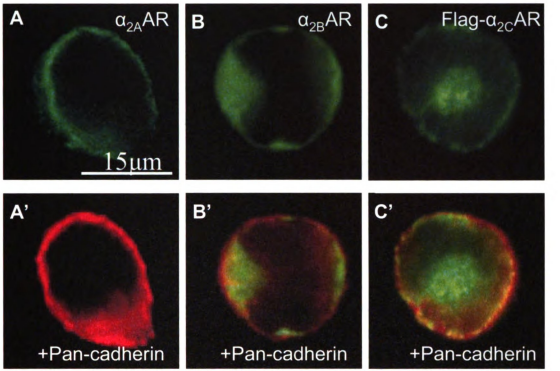


Fig. 13. α_{2A}AR expressed in α_{1D}AR-expressing cells localized predominantly to the cell membrane (A) and co-localized with the membrane marker pan-cadherin (A'). Similarly, α_{2B}AR was expressed mainly in the membrane (B), but also intracellularly. Fig. B' depicts colocalization of α_{2B}AR with the membrane marker pan-cadherin. Flag-α_{2C}AR was detected in the intracellular compartment (C') and little co-localization with pan-cadherin was detected (C').

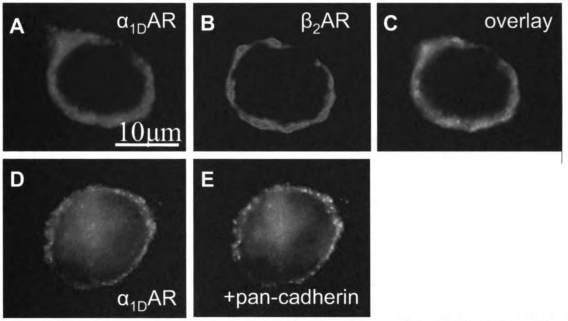


Fig. 14. β_2AR co-expression enhances the targeting of α_{1D}AR to the cellular membrane (A). The two receptors co-localize when expressed in the same cell (C). Figure E depicts co-localization of α_{1D}AR with pan-cadherin in a cell that also expresses β_2AR.

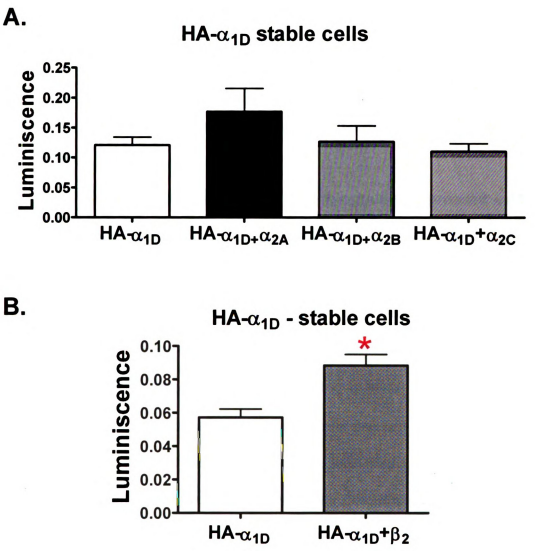


Fig. 15. β_2AR, but not α_2ARs, enhances the membrane distribution of HA – tagged α_{1D}AR. Histogram represents relative distribution of HA-α_{1D}AR in the plasma membrane that was measured by the luminescence surface expression assay. The effects of the three α_2AR subtypes on α_{1D}AR membrane expression are depicted in fig. A. Only β_2AR significantly enhanced the amount of α_{1D}AR localized in the membrane (B).

Luminescence data reflecting the surface expression of HA-α_{1D}AR when co-expressed with each α_2AR. None of the α_2AR subtypes enhanced the surface expression of HA-α_{1D}AR, n=6. However, membrane expression of HA-α_{1D}AR increased significantly when this receptor was co-expressed with β_2ARs, $p<0.05$ (Fig. 15B).

Functional assessment of heterologously expressed αARs. We next employed calcium imaging as a functional assay to test, whether the functional interaction we observe between α_{1D}AR and α_2AR in MV could be reproduced in HEK-293 cells stably expressing HA-α_{1D}AR and transiently co-transfected with individual α_2AR subtypes. The Red Express plasmid was used to mark the subset of cells transiently expressing α_2ARs. NE (1 μM) treatment of HA-α_{1D}AR expressing cells caused oscillatory calcium increases in individual cells (Fig. 16A) and the frequency of these oscillations varied from 1 to 4 per minute in different cells. We did not detect any difference between the functional responses of WT and HA-tagged α_{1D}AR. Because NE caused calcium oscillations, we quantified this response by measuring the area under the curve during the 1 minute NE application. After NE washout, a response to ionomycin was obtained. The calcium response to ionomycin (10 μM) was sustained and decayed only slowly (Fig. 16B). On average, NE calcium responses were approximately 15% of the ionomycin response. Fig. 17 depicts the magnitude of NE responses as a percentage of total ionomycin response of HA-α_{1D}AR-expressing cells co-tranfected with the α_2AR subtypes. The differences between the amplitude of the responses between HA-α_{1D}- and HA-α_{1D} – α_2AR co-

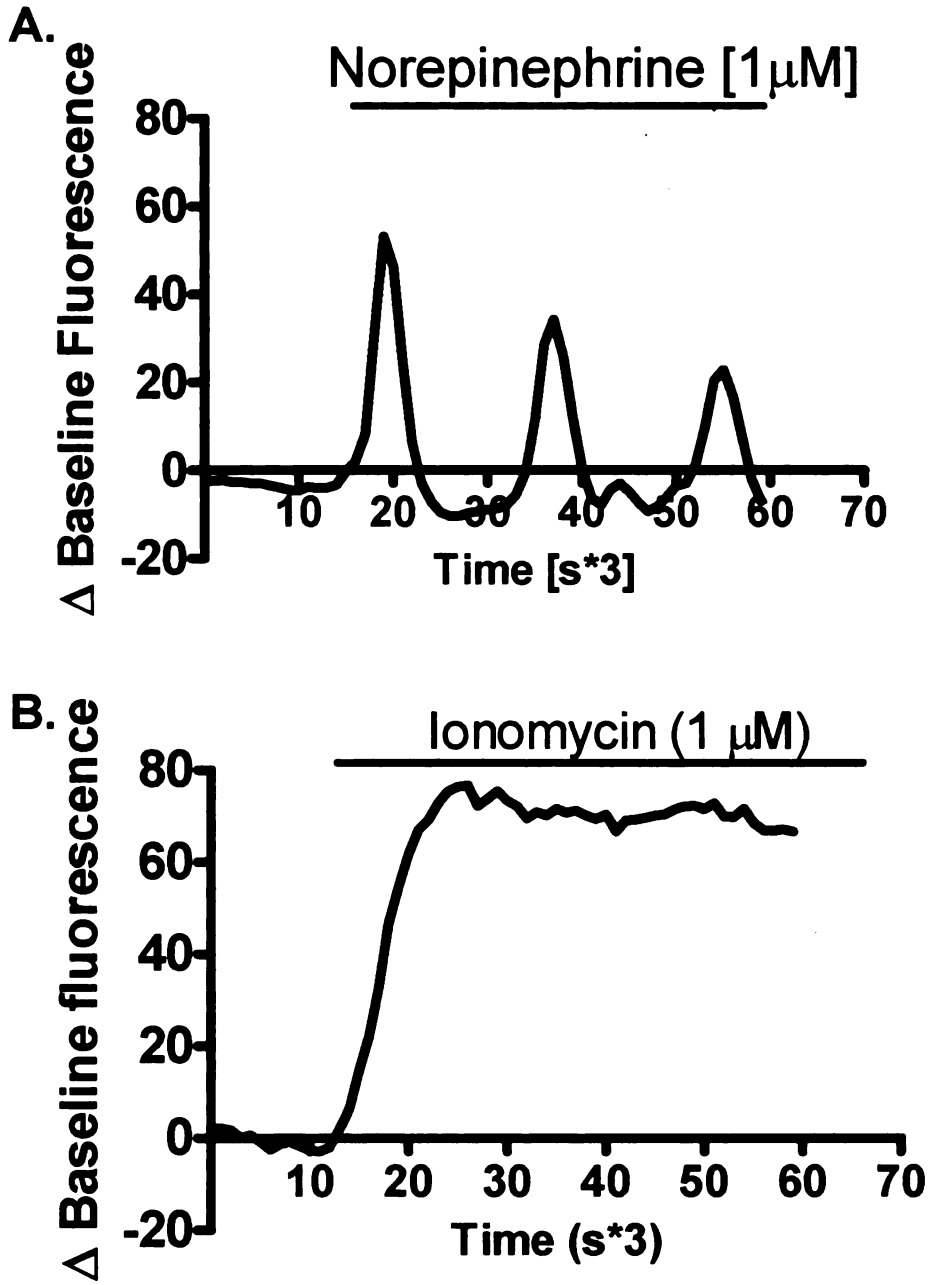


Fig. 16. NE treatment (1μM) of HA-α_{1D}AR stable cells produces oscillatory calcium response (A). Fig. B represents a typical cellular calcium rise induced by the Ca^{2+} ionophore ionomycin. Ionomycin was employed to normalize the data due to non-quantitative nature of our Ca^{2+} fluorophore Fluo-4. Data were normalized by calculating the area under the curve of NE response and expressed as % of area under the curve of ionomycin response.

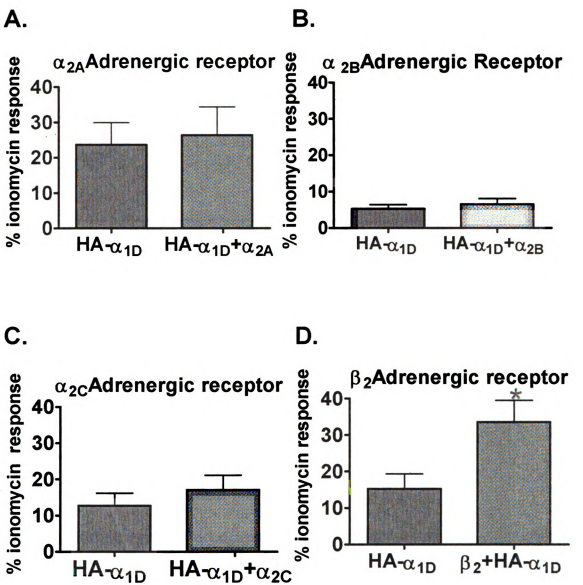


Fig. 17. α_2AR subtypes do not enhance calcium signaling mediated by α_{1D}AR. Calcium imaging of cells co-expressing α_2AR subtypes with α_{1D}AR. Response to NE was expressed as a percentage of ionomycin response for data normalization. None of the α_2AR subtypes significantly enhanced Ca^{2+} responses of α_{1D}AR – expressing cells. In contrast, β_2AR was able to enhance the Ca^{2+} responses to NE (D).

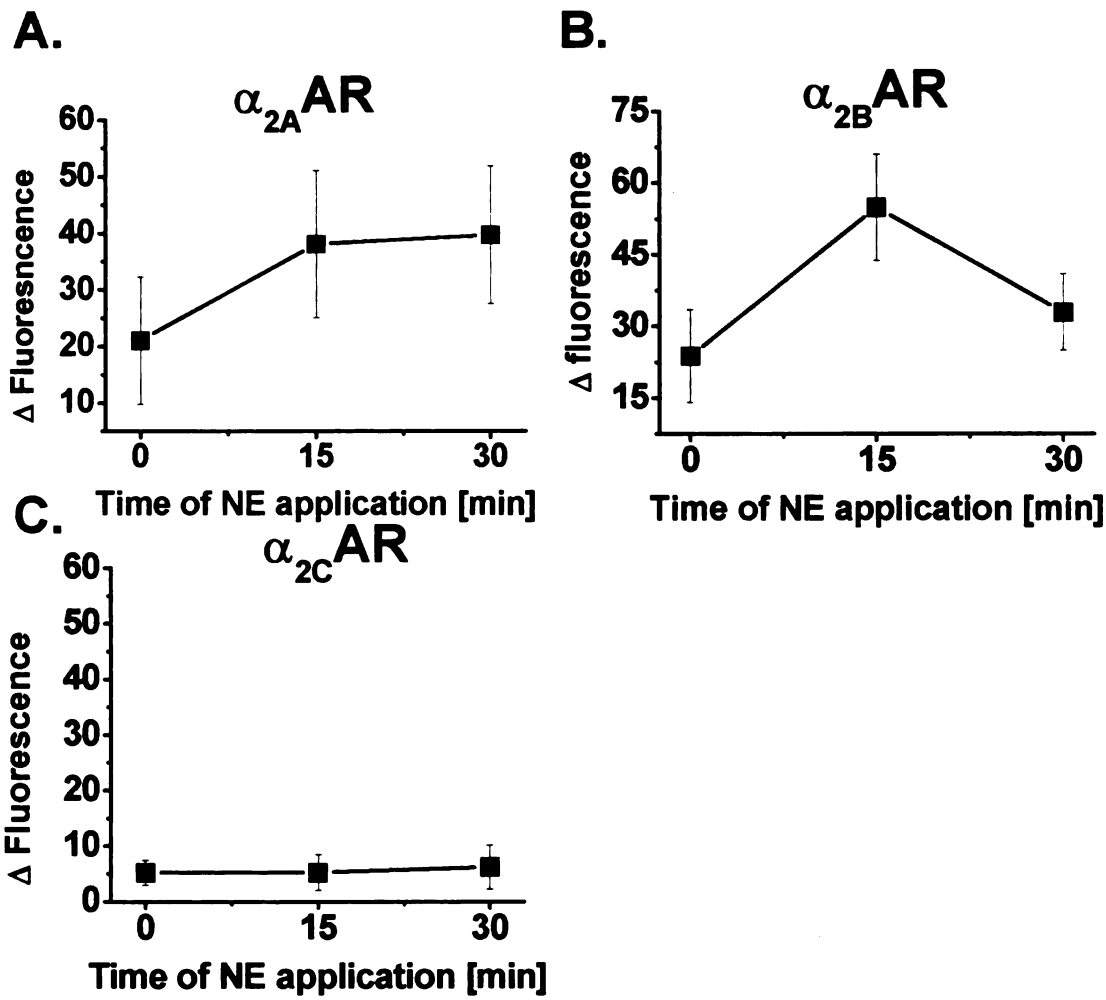


Fig. 18. Norepinephrine produces calcium elevation in α_2AR – transfected cells. Transiently transfected wild – type HEK293 cells with α_{2A}- (A), α_{2B}- (B) and α_{2C}AR (C) were treated with norepinephrine (1μM) at three different time points. Each data point represents average increase in fluorescence during NE treatment from 6 cells.

expressing cell were not statistically significant, n=6. β_2ARs co-expressed in HA-α_{1D}AR stable cells potentiated NE-induced calcium responses, $p<0.05$ (Fig. 17D). To confirm that the α_2AR subtypes are functional when hererologously expressed in HEK293 cells, I performed calcium imaging experiments on HEK293 cells transiently expressing individual α_2AR subtypes. NE (1μM) produced calcium elevation in both α_{2A} – and α_{2B}AR expressing cells (Fig. 18A and B). While NE produced calcium elevation in Flag-α_{2C}AR – expressing HEK293 cells, the response was much smaller than for the other two receptor subtypes, likely due to poor membrane expression of this receptor (Fig. 18C).

Implication of the interaction between α_{1D}- and β_2AR in the MV. Interaction between these two receptors occurs in heterologus expression system so it might be ubiquitous for tissues that co-express both receptors. In the MV, β_2AR might help with trafficking and function of α_{1D}AR. We employed immunocytochemistry on dispersed SMCs to test for potential co-localization of these two receptors in the SMCs. Indeed, we detected β_2AR in the MV and its localization was predominantly intracellular, with smaller amount of receptor localized to the membrane (Fig.19). It appeared to be co-localized with the α_{1D}AR (Fig. 19G).

We also detected β_2AR in the arterial SMCs and it localized predominantly to the plasma membrane and sub-plasmalemmal compartment (Fig. 19B). It strongly co-localized with the plasma membrane marker pan-cadherin (Fig. 19D). Even though we did not perform co-localization study of β_2- and α_{1D}AR in the arterial SMCs, these two receptors localize to the same compartments (Fig.19A and B).

We also performed a functional study in the intact MV to test the effect of potential α_{1D}-β_2AR interaction on adrenergic responses. We tested the effect of propranolol, the βAR antagonist on NE-mediated responses in the MV. This antagonist had no effect on the NE responses at 3 μM concentration in the pressurized MV (Fig. 20).

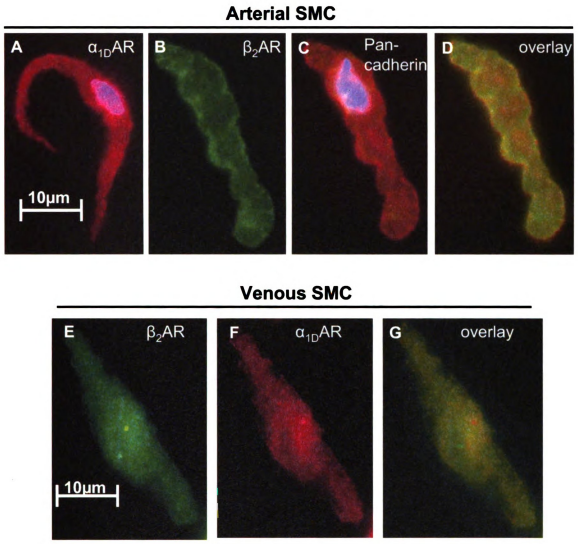


Fig. 19. Relative distribution of α_{1D}- and β_2AR in arterial and venous SMCs. In the arterial SMCs, α_{1D}- and β_2AR were found in the plasma membrane and sub-plasmalemmal compartment (A and B). While we did not perform an experiment to directly co-localize the two receptors, there is strong possibility that they co-localize due to their very similar distribution. β_2AR co-localized with pan-cadherin, suggesting membrane distribution of this receptor in the arterial SMC (D). In the venous SMC, α_{1D} - and β2AR seemed to be expressed mainly in the intracellular compartment, but also appeared on the membrane. The two receptors

A.

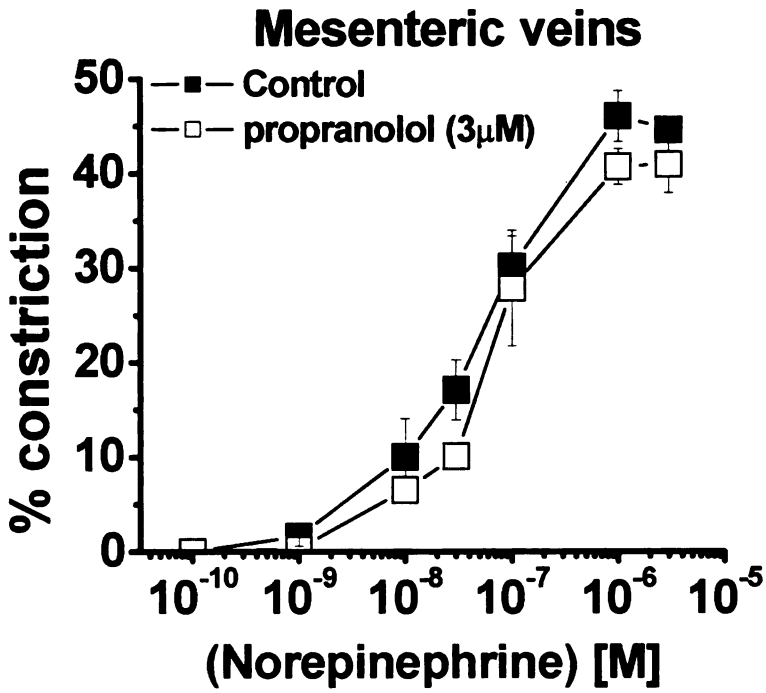


Fig. 20. Propranolol, β_2AR antagonist does not affect NE – mediated responses in MV. 3μM propranolol did not produce any effect on NE concentration – reponse curve (performed in pressurized system).

Discussion

MV play a major role in blood pressure regulation and I have shown that tone in MV and MA are regulated differently by sympathetic nerves. This conclusion is supported by data presented in this study where I showed enhanced sensitivity of MV to the constrictor effects of sympathetic nerve stimulation. Previous studies indicated, that nerve-mediated constriction is inhibited by the P2X receptor antagonist PPADS in the arteries, but not in the veins. In contrast, α_1AR antagonist prazosin blocks this constriction in the MV, but only inhibits responses in the MA (Park et al. 2007; Luo et al. 2003). These studies imply the role of αARs as dominant receptors mediating the effects of SNS in the MV, whereas P2X in addition to αAR mediate neurogenic responses in MA. However, since MV is also more sensitive to exogenous application of NE than MA, the present studies aimed to identify the postsynaptic adrenergic mechanisms that contribute to this enhanced reactivity of MV.

Because we were only able to detect the effect of α_2AR antagonist yohimbine on NE-mediated constriction and we did not see direct vasoconstrictor responses to α_2AR agonist UK14,304, I performed experiments in the presence of indomethacin and NLA to rule out the possibility that α_2AR-mediated constriction is concealed by the production of vasodilatory mediators NO and prostaglandins by the concomitant activation of α_2AR in the endothelium. Studies suggest that activation of α_2AR in the endothelium leads relaxation mediated by NO release (Vanhoutte et al. 2001; Nishina et al.1999) and combined inhibition of NO production and prostaglandings increases α-adrenergic reactivity (Dinenno et

al. 2004). However, in our experiments I did not detect any difference in UK14304 mediated responses in the presence of the enzyme blockers.

I also performed some pilot experiments in the pressurized vessels that more closely resemble the physiological conditions. Similarly to non-pressurized vessels, under these conditions, α_2AR antagonist still shifted the NE concentration – response curve to the right in MV but not MA. Experiment with structurally unrelated antagonist idazozan produced similar result than studies with yohimbine, suggesting that the data are not produced by an experimental artifact.

Even though α_2AR in MV does not directly couple to vascular constriction, my studies suggest that α_2AR in MV potentiates constrictions mediated by α_1AR and similar findings had not been observed in MA. I hypothetized that the indirect and potentiating contribution of α_2AR in MV might be based on a direct protein-protein interaction between α_1AR and α_2AR in the MV.

Previous studies established that the α_{1D}AR is the α_1AR subtype that mediates NE induced constriction of MV (Daniel, Low et al. 1997, Perez-Rivera 2007). In order to begin to understand a possible interaction between α_{1D}AR and α_2AR, I attempted to identify the α_2AR subtype expressed by MV. My first studies used α_2AR subtype-selective antagonists in an effort to inhibit NE constrictions of MV. Results from these pharmacological experiments suggested that α_{2C}AR subtype is the main α_2AR contributing to NE induced constriction of MV. This conclusion is supported by results from another study showing that the

α_{2C}AR mediates contractile responses to NE in veins (Gavin et al. 1997). BRL44408 has 25 and 100-fold higher affinity for the α_{2A}AR over the α_{2B}AR and α_{2C}AR respectively making this drug a useful tool for identifying responses mediated by the α_{2A}AR. However, antagonists for the α_{2B}AR (imiloxan) and the α_{2C}AR (MK912) are only 10-20-fold more selective over the other subtypes. This modest selectivity can limit their usefulness for receptor identification. Nonetheless, the right-ward shift of the NE concentration-response curve produced by MK912 very closely resembled the shift produced by yohimbine while BRL44408 and imiloxan did not change the NE concentration response curve at all. Based on these data, I concluded that the α_{2C}AR contributed to NE-induced constriction of MV.

Because of the limited selectivity of some of the α_2AR antagonists I sought alternative approaches to identification of the α_2AR subtype contributing to NE-induced constriction of MV. In these additional studies I used MV from α_{2C}AR KO mice. I predicted that the NE concentration response curve in MV from these mice would be shifted to the right (reduced sensitivity to the constrictor effects of NE) and that yohimbine would no longer change the NE concentration response curve. However, contrary to what I predicted, MV from the α_{2C}AR KO animals did not exhibit attenuated sensitivity to adrenergic stimulation. Furthermore, yohimbine still produced a rightward shift in the MV from the KO mice. This finding could be attributed to physiological compensation in the KO mice, where other α_2AR subtypes assume the function of the α_{2C}AR. Indeed, there are many examples where gene knockout leads to upregulation in

the expression and function of other related gene products. In studies using fermoral arteries from α_{1D}AR KO mice, it was found that chloroethylclonidine, an antagonist of α_{1B}AR, became much more effective in inhibiting NE-mediated contractile responses as it was in the WT. These authors concluded that there was an increased functional role for the α_{1B}AR in the α_{1D}AR KO mice (Zacharia et al. 2005). Another example of physiological compensation in the adrenergic receptor family has been reported for β_1/β_2AR KO mice that are characterized by significant reduction of muscarinic receptor density in the heart. In addition, these mice exhibit an exaggerated hypothensive response to stimulation by the β_3AR agonist CL316243, suggesting enhanced functionality of β_3AR in these mice (Rohrer et al. 1999). These studies demonstrate that disruption of one adrenergic receptor subtype can result into enhanced function of other adrenergic receptor subtype and therefore support the idea, that in the α_{2C}AR KO mice, other α_2AR subtypes may take on the function of α_{2C}AR.

My pharmacological studies suggest that the α_{2C}AR is the functional receptor α_2AR subtype expressed by MV SMCs. However, data from the α_{2C}AR KO mice suggest that other α_2AR subtypes can also be expressed by these cells. I next used immunocytochemical analysis on freshly dispersed venous SMC in an effort to determine what α_1AR and α_2AR are expressed by these cells. I confirmed that α_{1D}AR is expressed by MV SMCs and that most of this receptor is not localized to the plasma membrane but instead this receptor is found predominately in an intracellular compartment. If α_{1D}AR were to form a complex with α_{2C}ARs (heterodimer or heteromultimer), and this complex contributes to NE

induced constriction of MV, it would be predicted these two receptors would be localized together in the plasma membrane where they would be available to bind NE, and in subcellular compartments where the complex may be assembled or stored. To test this prediction I used immunocytochemical methods to localize α_2ARs with α_{1D}ARs in MV SMCs. I detected immunoreactivity for all three α_2ARs in MV SMCs and relatively little of this labeling was localized to the plasma membrane. The order of the magnitude of α_2AR subtypes expression was α_{2C}AR › α_{2A}AR › α_{2B}AR. Another study has also shown that α_{2C}AR mRNA is expressed at higher level than α_{2A}AR mRNA in venous SMCs (Chotani et al. 2004). This result is also consistent with our pharmacological data. The fact that we detected localization of all three receptors to venous SMCs supports our suggestion that another α_2AR subtype compensates for the loss of the α_{2C}AR in the KO mice by upregulating its expression and function. We also performed parallel studies in arterial SMCs. In contrast to what I predicted, we detected all three α_2AR subtypes in arterial myocytes. Although not exclusively, these receptors were detected in the plasma membrane. All three α_1AR subtypes were also detected in the membrane of arterial myocytes, supporting their functional role in these vessels; α_{1A}AR was found to mediate responses to exogenously applied adrenergic agonists, whereas α_{1B}AR appeared to mediate constriction to nerve stimulation in MA (Perez-Rivera et al. 2005) Even though no functional role for α_{1D}AR has been detected in MA, I detected this receptor in the membrane and subplasmalemmal compartment of MA SMCs.

There is possibility that the antibodies directed against α_2AR cross-react with epitopes of other αAR subtypes. Therefore, we tested these antibodies in transfected HEK293 cells against other αAR subtypes. Results from these control experiments suggested, that the individual antibodies are selective for the subtypes they are targeting, suggesting that they can be employed in detection of receptors in the native system.

Our data in MV maintained in vitro and in acutely isolated MV SMCs indicate that α_2ARs are localized to these cells and that one or more subtypes can contribute to NE induced constrictions. However, because of limitations of the pharmacological tools, antibody labeling and limited numbers of viable cells that can be isolated from MV we next used heterologous receptor expression in an effort to probe the mechanism by which α_2ARs can facilitate α_1AR function. Because previous studies in our laboratory suggested that α_{1D}AR is the main subtype mediating adrenergic contractility in mouse MV, we created a HEK-293 cell line that stably expressed a hemagglutinin (HA)-tagged human α_{1D}AR. The HA tag was localized to the extracellular N-terminus of the α_{1D}AR. Pilot studies revealed that this epitope tag did not alter the function (increases in intracellular calcium) and expression compared to the WT receptor. I was then able to transiently transfect α_2AR subtypes into this cell line.

I first tested the hypothesis that under appropriate conditions, the α_{1D}AR could form a heterodimer with α_2ARs that would facilitate trafficking of the α_{1D}AR to the plasma membrane. This might be indicative of receptor heterodimerization. When α_{1D}AR was expressed by itself in HEK-293 cells,

relatively little was localized to the plasma membrane as was found in MV SMCs. In HEK-293 cells, most of the α_{1D}AR was localized the endosomes (as revealed by co-localization with the endosomal marker, EEA1) and the endoplasmic reticulum (as revealed by co-loalization with the ER marker, calnexin). When the HA-α_{1D}AR cell line was transfected with α_2ARs we did not detect α_{1D}AR redistribution to the plasma membrane. This could also mean that only a small fraction of the receptors form a heterodimer and this might not be detectable using immunocytochemical methods. However, previous studies investigating receptor heterodimerization between α_{1D}- and β_2AR indicated, that immunocytochemical methods are sufficiently sensitive to detect changes in the α_{1D}AR distribution when co-expressed with β_2AR (Uberti et al. 2005). In these studies, visible overlap of expression between α_{1D}- and β_2AR has been noted. Indeed, by utilizing β_2AR as a positive control in my studies, I detected redistribution of α_{1D}AR to the plasma membrane and the α_{1D}AR and β_2AR were co-localized in the plasma membrane.

I used luminescence surface expression assay to more quantitatively asses the amount of HA-α_{1D}AR expressed in the membrane. By utilizing monoclonal antibodies against HA-tag, I was able to selectively measure the amount of membrane-bound receptor. This approached confirmed my findings that surface expression of HA-α_{1D}AR is not enhanced by co-expression with α_2ARs subtypes. These findings go along with previously reported data that did not support a role of α_2AR subtypes in promoting α_{1D}AR cell surface expression (Uberti et al. 2005).

Even though the receptor trafficking data did not support the hypothesis of receptor heterodimerization, we further speculated that functional interaction between the receptors might not be dependent on physical interaction between the two receptors and still might be detectable in HEK-293 cells. We performed calcium imaging studies in the HEK-293 cell system to screen for an enhancement of α_{1D}AR signaling during α_2AR co-expression. The magnitude of the response has not been affected by co-transfecting α_2AR subtypes to HA-α_{1D}AR cell line. In these experiments, we used a red plasmid to mark transfected cells and we found co-transfection efficiency of almost 90%. Therefore, it is unlikely that the results are due to measurements in cells not transfected with α_2ARs. Because Fluo-4 is not a ratiometric dye and therefore absolute magnitude of the response can not be quantified by this method, there is a possibility that the functional interaction has been overlooked in our experiments. In addition to the physiological calcium rise, calcium signal measured by Fluo-4 is affected by fluorescence bleaching and cell loading. Normalization of data to ionomycin can correct for some of these issues, even though there might also be slight differences between cellular responses to ionomycin. However, the fact that I detected the potentiating effect of the positive control β_2AR on α_{1D}AR function in our experimental setting minimizes the possibility that technical shortcomings prevented detection of a functional interaction between α_{1D}ARs and α_2ARs in HEK-293 cells.

Altogether, data from the heterologous system implied that differential receptor expression between arteries and veins alone likely does not account for

the higher adrenergic sensitivity of MV to exogenously applied NE than MA. Some additional factors, likely in the signaling pathway involving calcium regulation might differ in the veins, leading to a more efficient stimulus-response coupling. In fact, our findings from Western blot were similar to the current immunocytochemistry results; all 3 α_1AR subtypes were expressed in arteries and veins at a similar level even though functional data implied a larger receptor reserve in the veins (Perez-Rivera et al. 2004). Even though these data must be cautiously interpreted, they contribute to a bigger picture we see about the differential regulation of venous *versus* arterial tone; this view points us to the direction of differential receptor coupling to intracellular signaling pathways including differential calcium handling as opposed to differential receptor expression alone. Indeed, my studies suggest that there are differences in Ca^{2+} handling between MA and MV (chapter 3 and 4).

Even though I did not detect α_1-α_2AR interaction in HEK-293 cells, it does not contradict the original finding that α_2ARs sensitize α_1AR-mediated responses in MVs. Some additional mediators of the interaction not present in the arteries or HEK-293 cells likely contribute to this interaction. Previously reported studies with whole tissue and cell lines also detected functional interaction between α_1- and α_2AR (Fukui et al. 2005). Even though I could not detect it, cross-talk has also been described in heterologous expression system between α_1AR and α_{2A}AR (Reynen et al. 2000). In these studies, NE by itself did not raise Ca^{2+} in Chinese hamster lung fibroblasts that only express α_1ARs. After transfection with α_{2A}AR, robust increase in intracellular Ca^{2+} was observed, that

was antagonized by both α_1- and α_2AR antagonists. In our studies, both α_1- and α_2AR had to be transfected and perhaps the α_1AR subtype that the hamster lung fibroblasts natively express might not be the α_{1D}AR. However, in our case α_{1D}AR was the most relevant to study because of its prominent functional role in the MV.

Another possible mechanism that might play a role in this proposed functional interaction might be similar to the one described in cauda epididymis of guinea pig. In this tissue, α_2AR potentiated the influx of extracellular calcium through L-type calcium channels when α_1AR was co-activated (Haynes and Hill 1996), even though activation of α_2AR itself did not cause a concentration-dependent response. Similar mechanism has been detected in rat tail artery (Xiao et al., 1989). Indeed, such mechanism could be relevant to MV, because adrenergic responses of veins are very sensitive to Ca^{2+} channel blockade (chapter 4). However, taking this route in investigating the receptor interactions, it would not be pragmatic to use HEK293 cell as a model system. As opposed to the contractile VSMCs, these cells are non-excitable and lack the cellular architecture and biochemical properties of the native SMCs. Therefore, further functional studies on veins and venous SMCs are necessary to fully elucidate the cellular pathway underlying this interaction.

CHAPTER 4

Differential Calcium Utilization between Mesenteric Arteries and Veins during Alpha-Adrenergic Stimulation

ABSTRACT

Mesenteric veins (MV) are more sensitive to the constrictor effects of norepinephrine (NE) than mesenteric arteries (MA). In MV α_2ARs indirectly contribute to NE-induced constriction by enhancing constrictions caused by α_1AR activation. This interaction was not due to direct receptor interaction. Therefore, we tested the hypothesis that α_2ARs in MV couple to Ca^{2+} influx thereby enhancing the constrictions mediated by α_1AR. Agonist induced constrictions of MA and MV (200-300 mm outside diameter) were studied *in vitro* using a pressure myograph. $CdCl_2$ (100 μM), a subtype non-seletive Ca^{2+} channel blocker, inhibited NE–induced constriction in MA and MV. Nifedipine (1 μM, L-type Ca^{2+} channel blocker) blocked NE induced constriction in MA but had little effect in MV. $GdCl_3$ (10 μM), inhibitor of store–operated and stretch–activated channels reduced NE constrictions in MV but not MA. 2-APB (20 and 50 μM), an IP_3 receptor antagonist inhibited NE responses in MA but had little effect in MV. Thapsigargin, which depletes intracellular Ca^{2+} stores by inhibiting SERCA, constricted MV, but not MA, and inhibited NE responses in MA and MV. Co-application of nifedipine and $GdCl_3$ inhibited NE responses in MV. The α_2AR antagonist yohimbine produced no additional inhibition in the presence of nifedipine and $GdCl_3$ suggesting that α_2AR activate Ca^{2+} entry mechanisms. .NE responses were attenuated to larger degree in MA than MV by the Rho kinase inhibitor Y27632 (10 μM). Finally, Ca^{2+} response curves were obtained by incubating tissues in Ca^{2+} free media and then adding back Ca^{2+} in graded concentrations in the presence of NE (0.3 μM for MA and 0.03μM for MV). Ca^{2+} addition caused similar concentration-dependent constrictions in MA and MV.

These data indicate that differences in Ca^{2+} entry and buffering play a role in differential regulation of arterial and venous tone.

INTRODUCTION

MV are significantly more sensitive to the constrictor effects of norepinephrine (NE) than MA and this might be due to functional involvement of α_2AR in MV, but not in MA. α_2ARs in MV indirectly contribute to NE-mediated constriction. In addition, activation of α_2AR enhances constrictions mediated by α_1AR (Perez-Rivera et al., 2007). Our studies indicated that this functional interaction can not be reconstituted in HEK-293 cells co-expressing α_1AR and α_2ARs indicating that additional intracellular messengers present in MV that are activated by stimulation of α_2AR are required for this functional synergism.

αAR signaling. α_1ARs couple to stimulation of phospholipase C (PLC) activity and production of diacylglycerol (DAG) and inositol triphosphates (IP_3) that acts on IP_3 receptors in endoplasmic reticulum, leading to Ca^{2+} release. Inhibition of IP_3 receptors in small mesenteric arteries significantly attenuates phenylephrine (PE)– induced constriction (Lamont and Wier, 2004). Even though α_1AR can also activate Ca^{2+} influx in the vasculature through the activation of transient receptor potential channel 6 (TRPC6) and reverse mode Na^+-Ca^{2+}-exchanger (NCX), studies indicate that constriction mediated by α_2AR is even more dependent on Ca^{2+} influx from the extracellular space (Lepretre and Mironneau, 1994). L-type Ca^{2+} channels are activated by α_2AR in certain preparations (Ruffolo and Nichols, 1988; Mukundan and Kanagy, 2001). However, in rabbit saphenous vein α_2AR-mediated contraction is almost entirely dependent upon Ca^{2+} influx through non L-type Ca^{2+} channels (Aburto et al., 1995). Even though Ca^{2+} influx was necessary for α_2AR-mediated constriction in

this vessel, Ca^{2+} influx was not augmented during α_2AR-mediated constriction. Basal Ca^{2+} influx may be sufficient to induce constriction by α_2ARs. Although the specific channels mediating basal Ca^{2+} influx were not identified, it is possible that store-operated channels (SOC) contribute to Ca^{2+} store refilling and perhaps can play a functional role in α_2AR signaling.

In many blood vessels, pressor responses mediated by α_2AR are sensitive to pertussis toxin (PTX), implicating the involvement of $G_{i/o}$ proteins in mediating vascular constriction (Boyer et al. 1983; Aburto et al. 1993). However, inhibition of cAMP formation via a Gi dependent mechanism is not involved in α_2AR-mediated effects on vascular tone. While activation of adenylate cyclase by forskolin produces a rightward shift in concentration-response curve to NE in porcine palmar lateral vein, neither NE nor the α_2AR agonist, UK 14,304, affect basal levels of cAMP even though they constrict the veins (Wright et al., 1995). Several studies have reported involvement of protein kinase C (PKC) in α_2AR-mediated constriction (Aburto et al. 1995, Carter and Kanagy, 2003). In canine MV, PI3 kinase and PKC are involved in the membrane depolarization mediated by α_2AR (Yamboliev and Mutafova-Yambolieva, 2005). Depolarization of SMCs by α_2AR in this preparation is dependent on activation of non-selective cation channels (NSCC) and chloride channels, but not on L-type voltage – operated calcium channels (VOCC).

Mechanism of functional interaction between α_1 – and α_2ARs. Several reports have documented a synergistic functional interaction between α_1- and α_2ARs. Functional interaction between α_1- and α_2AR in MV could potentiate

NE-induced constriction, accounting for the enhanced adrenergic sensitivity of MV. While some studies suggest increased accumulation of IP_3 (Wilson and Minneman, 1991) when the two receptors are simultaneously activated, other studies indicate that α_2AR may potentiate Ca^{2+} influx from the extracellular space, leading to enhancement of α_1AR-mediated constriction (Haynes and Hill, 1996)(Xiao and Rand, 1989). In our studies, we were not able to detect enhanced Ca^{2+} mobilization in HEK-293 cells when co-stimulating the two receptors, suggesting that IP_3 production was not been enhanced by co-activation of α_1 – and α_2ARs in these cells. If stimulation of Ca^{2+} influx by α_2AR played a role in the interaction in MV, it is possible that we were not able to detect this effect in HEK-293 cells due to absence of Ca^{2+} channels. While HEK-293 cells express receptor operated channels (ROC) and SOC that are likely composed of multiple TRPC channels (TRPC1, TRPC3, TRPC4 and TRPC6 have been detected (Wu et al., 2002, Bugaj et al., 2005), the L-type voltage – operated Ca^{2+} channels have not been found in these cells (Berjukow et al., 1996). In addition, the different composition of SOC and ROC in HEK-293 cells may yield functional properties that differ from those in native venous SMC. Activation of Ca^{2+} channels by receptors and second messengers might also be differentially regulated in HEK-293 cells. Therefore, we used MV and venous SMC to study the role of Ca^{2+} channels and their potential involvement in the α_1 – α_2AR interaction.

Modulation of Ca^{2+} sensitivity by α_2AR. Another mechanism by which α_2AR can modulate vascular tone involves increases in the Ca^{2+} sensitivity

of contractile mechanisms in SMCs (Aburto et al. 1993, Carter and Kanagy, 2003). Increased Ca^{2+} sensitivity of the contractile proteins in MV could also enhance the responses mediated by α_1AR. It has been proposed that Ca^{2+} sensitization by α_2AR may be mediated by tyrosine kinases or PKC; Studies suggest that src-family of tyrosine kinase is involved in Rho kinase activation, resulting into direct inhibition of myosin light chain phosphatase (MLCP) (Nakao et al. 2002). MLCP dephosphorylates myosin light chain (MLC) and therefore inhibits the interaction between actin and myosin that generates the tension in SMCs. Indeed, in certain blood vessels like saphenous vein, palmar lateral vein or rat aorta constriction mediated by α_2AR is dependent on activation of tyrosine kinases (Jinsi and Deth, 1995, Roberts, 2001, Carter and Kanagy, 2002).

PKC, a downstream target of α_2AR activation, can also inhibit the action of MLCP (Masuo et al., 1994, Ikebe and Brozovich, 1996) via its phosphorylation of smooth muscle specific phosphoprotein CPI-17 (Kitazawa et al. 2000). CPI-17 is a downstream target of Rho kinase and PKC and it can directly and potently inhibit MLCP. Conversely, activation of PKC by non-α_2AR agonists can enhance Ca^{2+} sensitivity (Jiang et al. 1987). In dog MV, activation of PKC by endothelin-1 or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) sensitizes the tissue to α_2AR stimulation by UK14,304 (Shimamoto et al, 1995). Angiotensin II also increases the efficacy of agonists acting at α_2ARs in the veins (Dunn et al. 1991). Conversely, α_2AR stimulation potentiates vascular responses to angiotensin II and this interaction involves PLC/PKC/c-src/PI3K pathway (Jackson et al. 2005). NADPH oxidase and superoxide activate Rho A/Rho

kinase that is also involved in the interaction downstream of PLC/PKC/c-src/PI3K pathway (Jackson et al. 2008). Therefore, modulation of PKC activity is also a potential target for the functional α_1- α_2AR interaction. Activation of either Rho pathway or PKC by α_2AR may enhance Ca^{2+} sensitivity and constriction mediated by α_1ARs.

Because pressor responses mediated by α_2AR are dependent on $G_{i/o}$, I employed pertussis toxin (PTX) to investigate the involvement of this G protein in the functional interaction. To investigate whether α_2AR potentiates Ca^{2+} influx during NE-mediated constriction, I utilized $CdCl_2$ (Cd^{2+}) the non-selective Ca^{2+} channel blocker, nifedipine – the L-type Ca^{2+} channel blocker and $GdCl_3$ (Gd^{3+}) – the blocker of stretch activated cation channels and SOC. To determine the relative contribution of the IP_3 receptor and release of Ca^{2+} from the stores in arterial and venous constriction to NE, I used the IP_3 receptor antagonist 2-aminoethoxydiphenyl borate (2-APB). I hypothesized that if α_2AR enhances Ca^{2+} influx during NE constriction and thereby enhances contractile responses of α_1AR, constriction to NE would be inhibited to smaller degree after intracellular Ca^{2+} depletion by thapsigargin treatment that inhibits the smooth endoplasmic reticulum Ca^{2+} ATP-ase (SERCA), as compared to PE that activates only α_1AR. To test for the potential differences in Ca^{2+} sensitivity between MA and MV during NE – mediated responses, I employed the Rho kinase inhibitor Y272632 in both MA and MV to asses the relative contribution of Rho kinase pathway to Ca^{2+} sensitization mediated by NE. I also performed concentration response curves to $CaCl_2$ in MA and MV in the presence of constant NE concentrations to

determine, whether there are any differences in Ca^{2+} sensitivity during NE – mediated constriction.

Since Ca^{2+} signaling is highly dependent on membrane potential of vascular SMCs, we utilized pressurized system. While contractility experiments performed under non-pressurized conditions might not significantly affect the Ca^{2+} signaling of MV because they are only exposed to low pressures *in vivo*, the physiological pressures in mouse MA reach ~ 60mm Hg which would represents a substantial change compared to pressure levels in non-pressurized arteries. Therefore, the pressurized system was optimal for our experiments.

Materials and Methods

Animals. C57/Bl6 male mice (25-30g) were purchased from Charles River Breeding Laboratories (Portage, MI). In the animal care facility, mice were maintained according to the standards approved by the Michigan State University All-University Committee on Animal use and Care. Mice were individually housed in clear plastic cages with access to standard chow (Harlan/Teklad 8640 Rodent Diet) and tap water.

***In Vitro* Preparation of Mesenteric Arteries and Veins.** Mice were anesthetized, and the small intestine with its associated mesentery was removed and placed in oxygenated (95% oxygen, 5% carbon dioxide) Krebs' physiological saline solution of the following composition (millimolar): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 25 $NaHCO_3$, and 11 glucose. A piece of the intestine with associated vessels was removed and pinned flat in a silicone elastomer-lined (Sylgard; Dow Corning, Midland, MI) petri dish. A section of mesentery containing vessels close to the mesenteric border was cut out using fine scissors and forceps. The preparation was transferred to a smaller silicone elastomer-lined recording bath and pinned flat. Second- or third-order mesenteric veins or arteries (100-200-μm diameter) were isolated for study by carefully clearing away the surrounding fat tissue. The recording bath containing the preparation was mounted on the stage of an inverted microscope (Olympus CK-2; Tokyo, Japan) and superfused with warm (37°C) Krebs' solution at a flow rate

of 7 ml/min. All preparations were allowed a 20-min equilibration period during which the vessels relaxed to a stable resting diameter.

Video Monitoring of Vessel Diameter. The output of a black- and-white video camera (Hitachi model KP-111; Yokohama, Japan) attached to the microscope was fed to a framegrabber card (Picolo, Euresys Inc., TX, USA) mounted in a personal computer. The video images were analyzed real-time using Diamtrak Edge-tracking software (version 3.5, Diamtrak, Adelaide, Australia), which tracks the distance between the outer edges of blood vessel in the observation field. Changes in vessel diameter as small as 1 μm could be resolved.

Treatment with pertussis toxin. A piece of mesentery was placed in a tube with 2μg/ml PTX in Krebs buffer. Tube was placed into 37°C incubator and oxygenated. After two hours of incubation, tissue was pinned out and cleaned of connective tissue as described above. Controls were subjected to the same treatment without PTX.

Application of Y27632. Rho kinase inhibitor Y27632 was purchased from Sigma-Aldrich (St. Louis, MO). Solution with final concentration of Y27632 was applied to the superfusing Krebs' solution from a flow tube (0.3 mm tip diameter) positioned directly over the MA or MV. Flow rate of this application system was 0.5ml/min.

Data Analysis. Constrictions of blood vessels are expressed as percentage constriction from the resting diameter. Half-maximal effective agonist concentration (EC_{50}) and maximum response (E_{max}) were calculated from a least-squares fit of individual agonist concentration-response curves using a logistic function from Origin 7.0 (Origin-Lab Corp., Northampton, MA): $Y = \{[E_{min} - E_{max}]/[1+(x/EC50)^n]+E_{max}$, where E_{min} is the minimum response and was constrained to zero and n is the slope factor. All data are expressed as mean ± S.E.M. Statistical differences between groups were assessed by Student's two-tailed unpaired t test. In experiments testing for differences in Ca^{2+} sensitivity between MA and MV in NE–mediated constriction, we first established the basal vessel constriction to NE. Data are presented as a % of this control constriction for MA and MV.

RESULTS

Pressure affects adrenergic responses of MA and MV. Pressurization of MV to 7 mm Hg altered the NE concentration-response curve in MV. The EC50 of pressurized MV was shifted to the right; the EC_{50} in the pressurized MV was 0.05 ± 0.01 μM vs 0.02 ± 0.004 μM, ($P < 0.01$, n=9). However, the Emax was increased in the pressurized MV from 41± 5% to 59 ±1% constriction ($P<0.005$, Fig. 21A).

Even greater differences were observed in MA (Fig. 21B); when the intravascular pressure was raised from 0 to 60mm Hg, the EC_{50} for NE was significantly shifted to the left from 4 ±0.6 μM to 0.5 ±0.1 μM, ($P < 0.005$, n=6). In addition, the Emax was increased in pressurized MA from 25 ±2% to 44 ± 4% $P < 0.005$). Under pressurized conditions, MV were still significantly more sensitive ($P<0.005$) than MA to the constrictor effects of NE.

Pertussis toxin (PTX) does not change NE-induced constriction of MV. Our previous studies indicated that α_2AR indirectly participates in NE responses in MV and based on our results we hypothesized that involvement of second messengers and Ca^{2+} signaling pathways could underlie the observed receptor interaction as opposed to direct receptor heterodimerization. To test the involvement of $G_{i/o}$ protein in NE-mediated constriction of MV, the vessels were pre-incubated in 2 μg/ml PTX solution for 2 hours at 37°C. This treatment had no effect on NE–induced constriction of MV (n = 4, Fig. 22B).

Ca^{2+} channel blockade and NE responses in MA and MV. To test the dependence of NE responses on Ca^{2+} influx in MA and MV, we used the non-

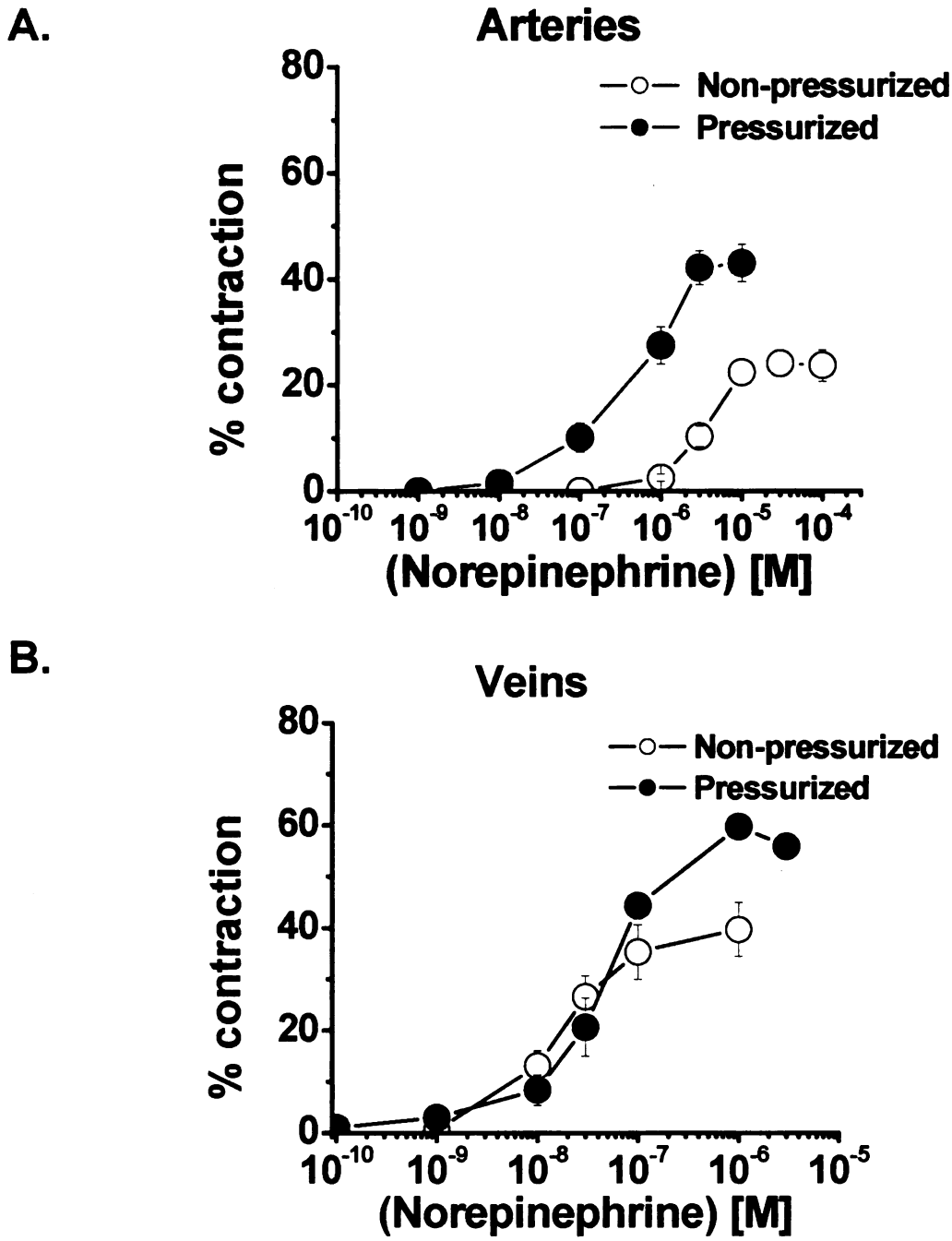


Fig. 21. Intraluminal pressure alters the reactivity of MA and MV. The EC50 for NE in the pressurized MV (6mm Hg) was shifted to the right compared to non-pressurized MV, while the maximal response has been significantly enhanced by application of pressure (A). More pronounced effect of pressure (60 mm Hg) has been observed in MA, where EC50 has been shifted 8-fold to the left. In addition, maximal response to NE almost doubled in MA (B).

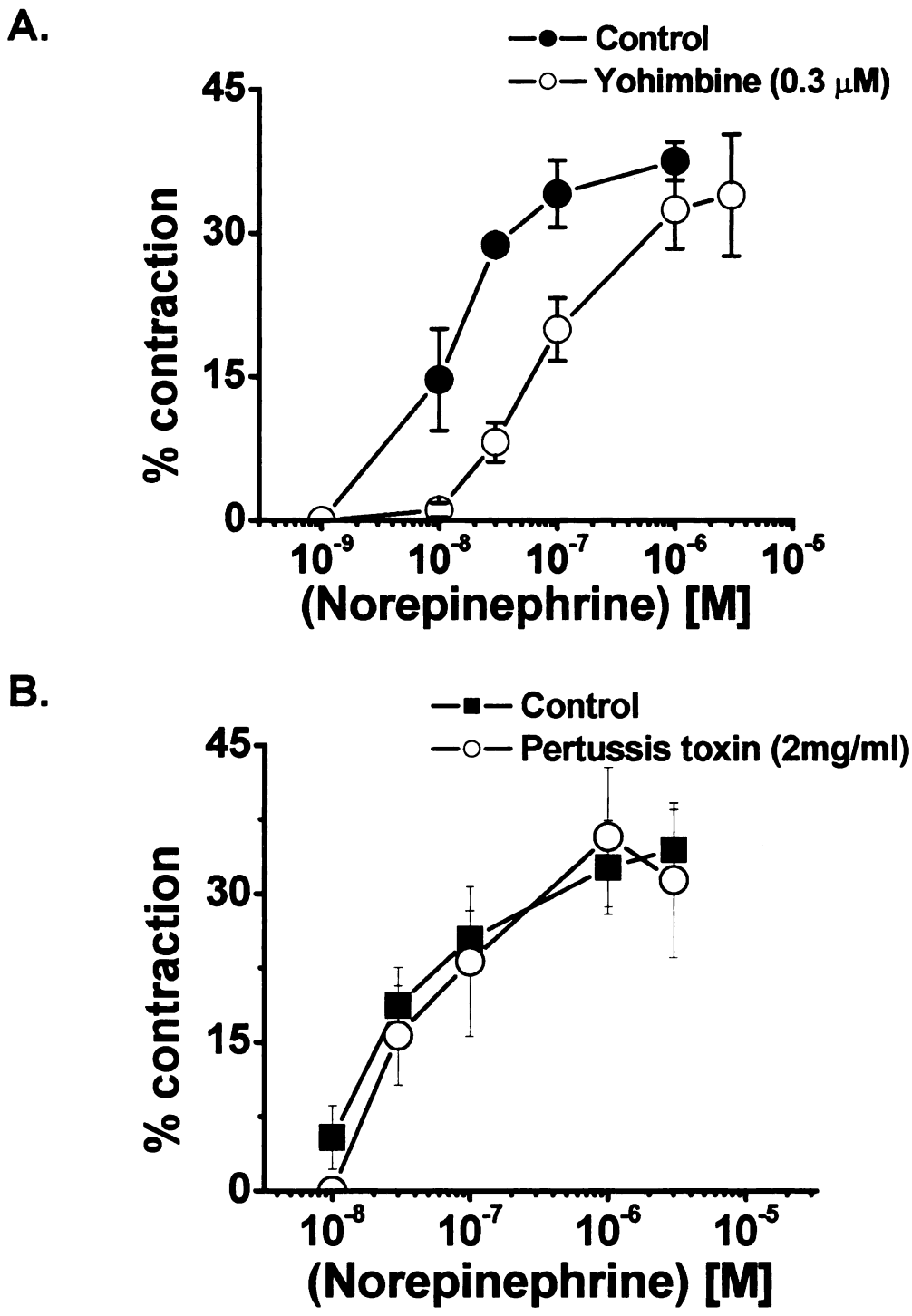


Fig. 22. $G_{i/o}$ proteins are not involved in NE – induced constriction of MV. While yohimbine, the α_2AR antagonist produced a right-ward shift in the NE CRC (A), pre-treatment of MV with PTX (2μg/ml) for 2 hours at 37°C in oxygenated Krebs buffer did not affect NE CRC in MV (B).

selective Ca^{2+} blocker Cd^{2+} (100 μM). Cd^{2+} inhibited constriction of both MA and MV, indicating the dependence of NE constriction on Ca^{2+} influx. The effect of Cd^{2+} was more pronounced in MV, where constriction was completely blocked at low NE concentrations. At higher agonist concentrations, NE produced a slight constriction that fell back again to 0 at 10 μM NE (n = 7, Fig. 23B). While Cd^{2+} inhibited NE–mediated constriction in MA, NE still caused a concentration–dependent constriction in these vessels (n=5, Fig. 23A).

In MA, nifedipine significantly attenuated the response to NE at 1 and 3μM NE ($P<0.05$), (Fig. 24A, n = 7). In MV; the Emax has not been altered by nifedipine, NE concentration– response curve (CRC) was slightly displaced to the right, n=6 (Fig. 24B).

We next used Gd^{3+} (10 μM) to test the effect of SOC and stretch – activated channels. In the MA, Gd3+ did not exhibit any effect on NE CRC, (n=3) (Fig. 25A). However, in the MV, Gd^{3+} displaced the NE CRC to the right without changing the E_{max}, n=5 (Fig. 25B).

Decreased sensitivity to IP_3 receptor blockade in MV. IP_3 is produced by the activation of PLC by Gq and liberation of IP_3 and diacyl glycerol. IP_3 acts on the IP_3 receptors on smooth endoplasmic reticulum in SMC, leading to the activation of Ca^{2+} release from the stores (Minneman et al. 1988). To determine the relative contribution of this pathway in adrenergic responses of MA and MV, we used the IP_3 receptor antagonist 2-APB (20 and 50 μM). At 20 μM, only a slight inhibition of NE responses in MA was detected (Fig. 26A). However, at 50 μM 2-APB, a right-ward shift of the NE CRC was observed, with significant

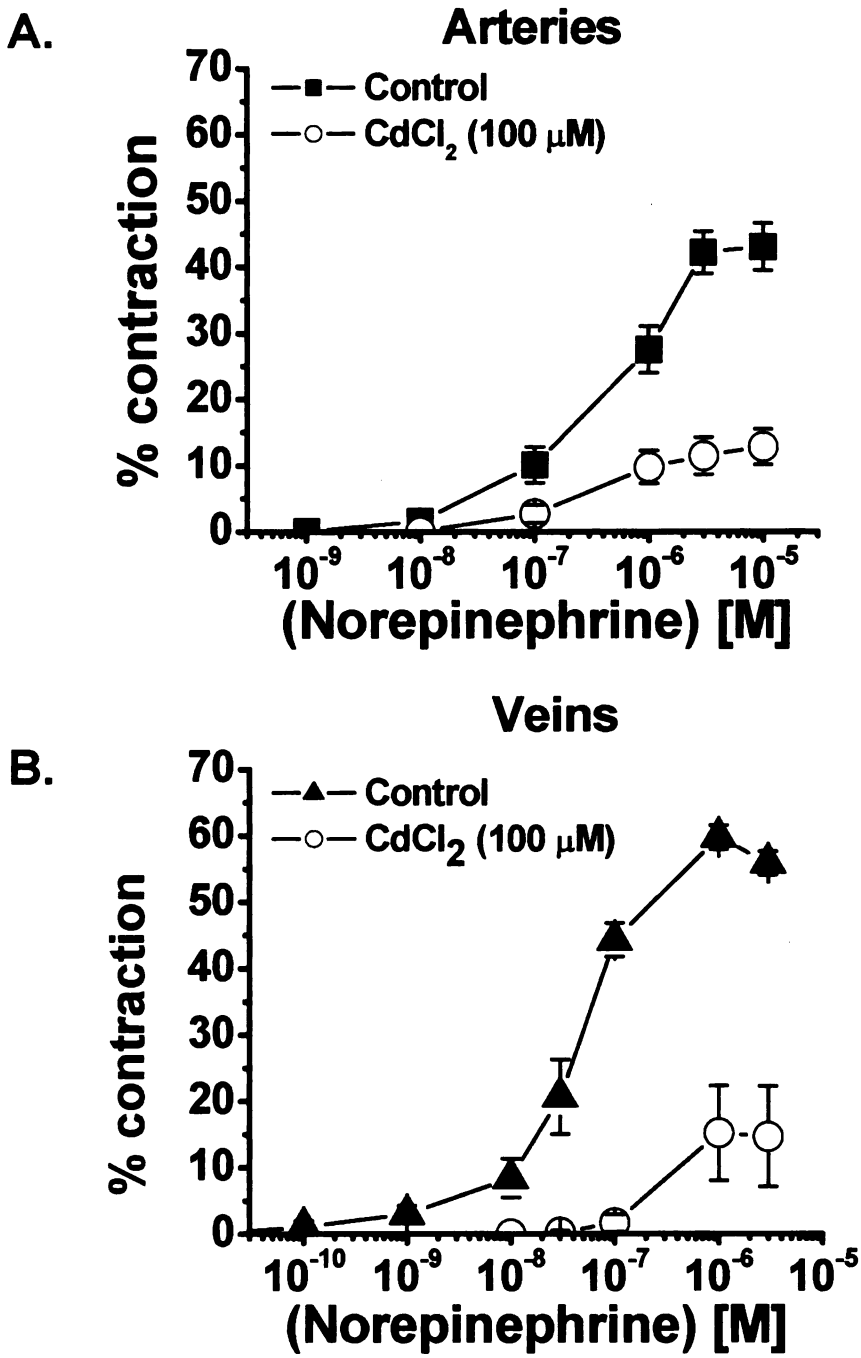


Fig. 23. NE responses in MA and MV are dependent on Ca^{2+} influx from extracellular space. Cadmium chloride (100μM), the non – selective Ca^{2+} channel blocker significantly attenuated NE responses in MA (A). In MV, cadmium produced even larger inhibitory effect; NE responses were abolished at low agonist concentrations and vessels exhibited attenuated constriction at higher NE concentrations (B).

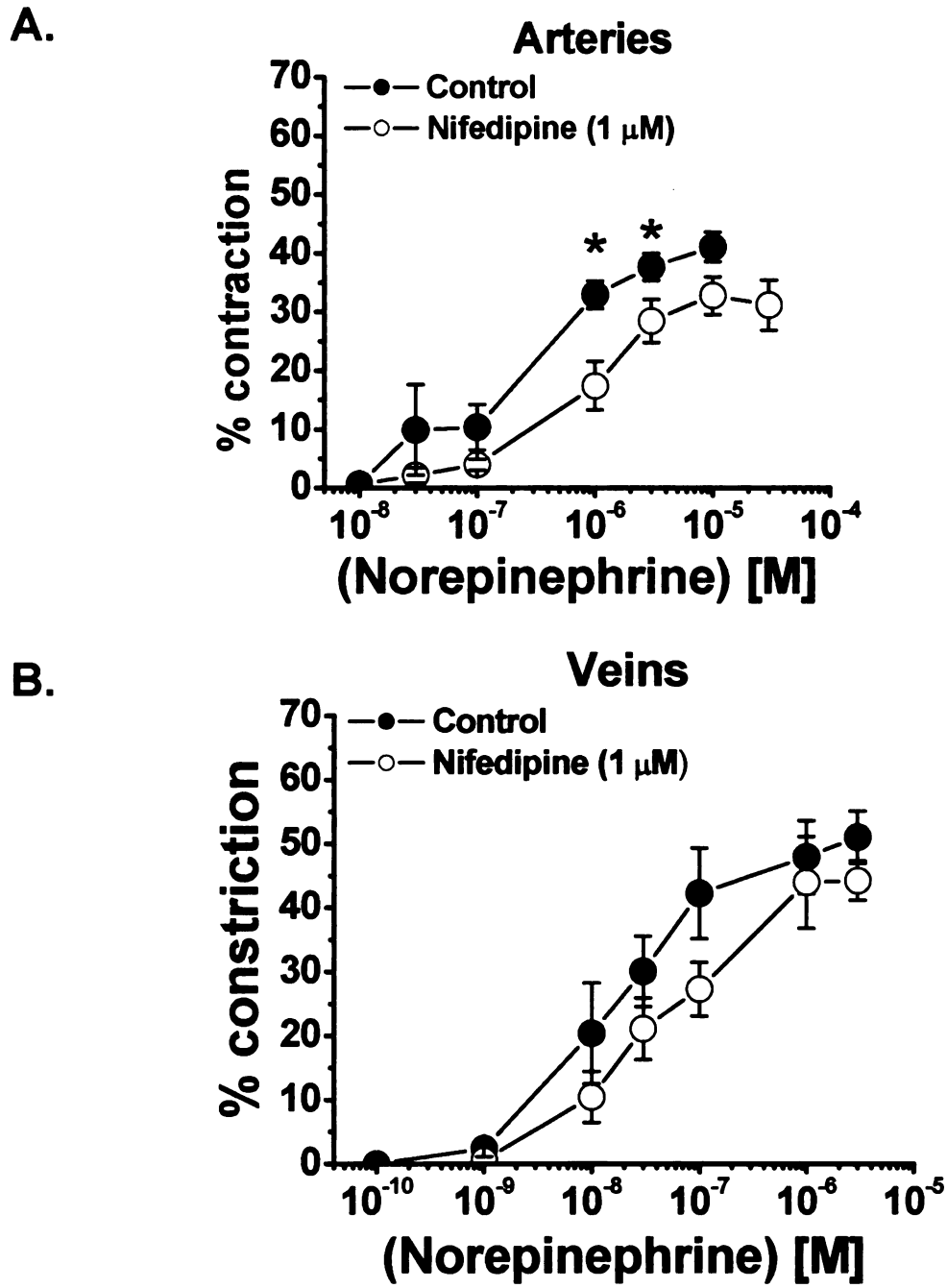


Fig. 24. L-type VOCC play a more important functional role in MA than MV in adrenergic responses. Nifedipine (1μM) shifted the CRC to NE to the right and significantly attenuated constriction to NE at larger NE concentration (A). Nifedipine also produced a slight right-ward shift of NE CRC in MV, although the inhibitory effect on venous constriction did not reach statistical significance (B).

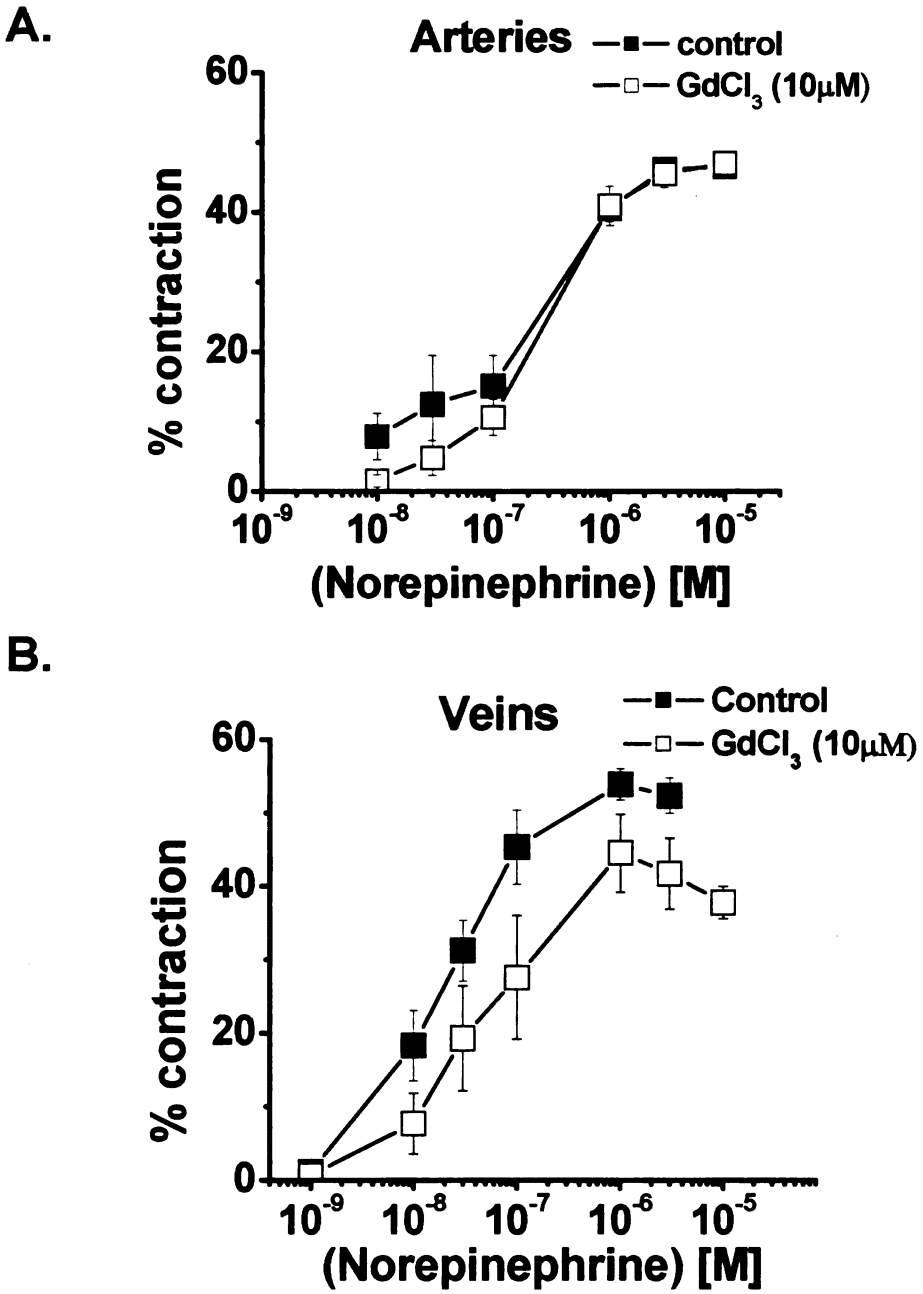


Fig. 25. SOC contribute to venous, but not arterial constriction to NE. Gadolinium chloride (Gd^{3+}) (10μM), blocker of Ca^{2+} - induced Ca^{2+} release activated channels (CRAC) and SOC had no effect on NE responses in MA (A). CRC to NE was shifted to the right in MV in the presence of gadolinium (B). Although the inhibition was not statistically significant, attenuation of NE responses suggest some functional involvement of these channels in functional responses to NE in MV.

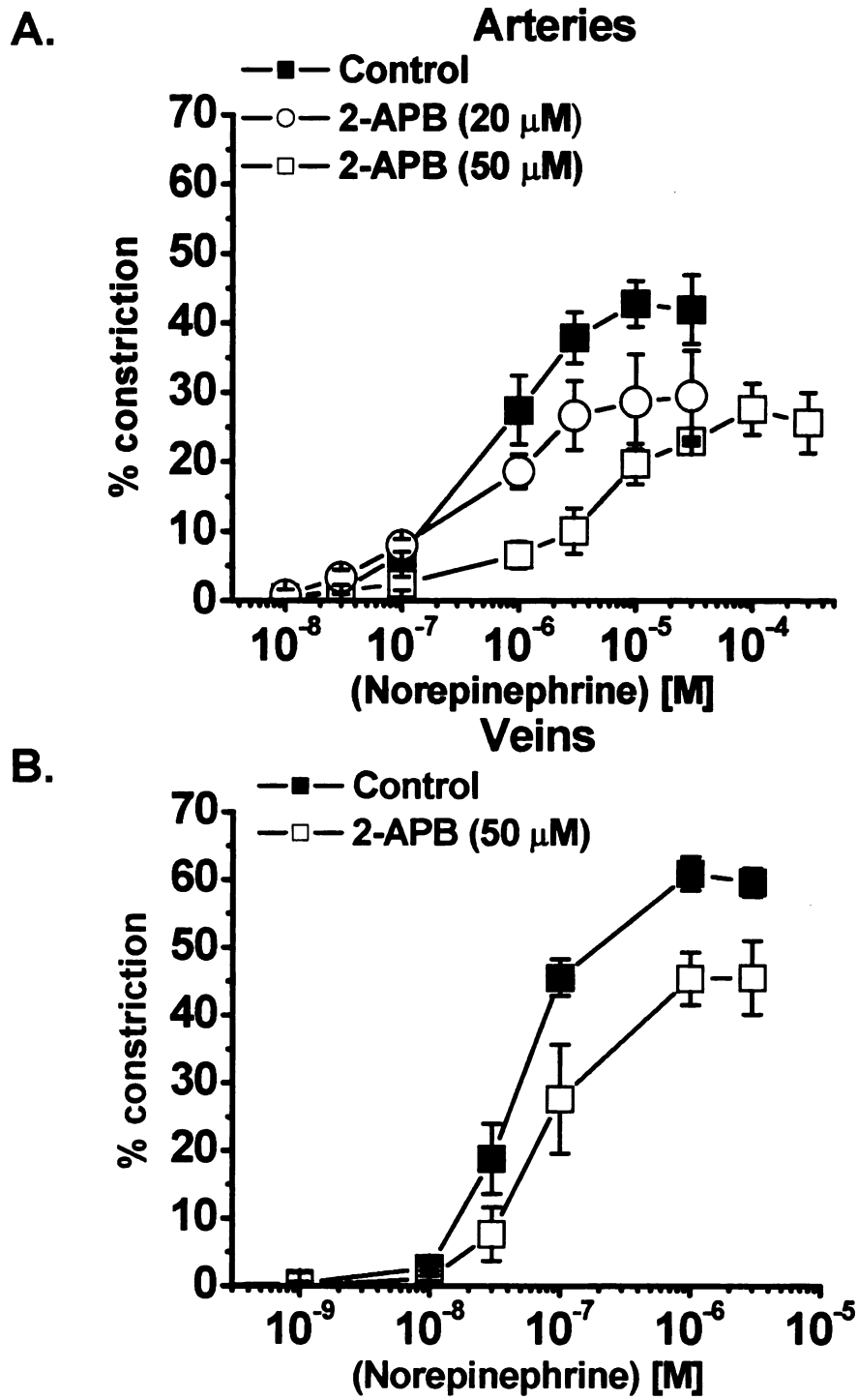


Fig. 26. Effect of IP_3 receptor antagonist 2-APB on NE – mediated constriction of MA (A) and MV (B). 20μM 2-APB only slightly attenuated responses to NE in MA. However, 50μM 2-APB significantly attenuated constriction to NE in MA. 2-APB was much less potent inhibitor of functional responses in the MV.

inhibition of maximal constriction, n=4. A different effect of 2-APB was observed in the MV. 2-APB only started to produce some inhibitory effect in the MV at 50μM that was similar to the effect of 20μM in the MA (Fig. 26B). Higher concentration of 2-APB was not employed due to potential non-specific effect of this drug.

α_2AR and activation of Ca^{2+} influx. In order to determine whether activation of α_2AR by NE couples to Ca^{2+} influx in MV, we utilized thapsigargin (1μM) to deplete intracellular Ca^{2+} stores. NE or PE was applied before and after thapsigargin treatment and the % reduction from control response was analyzed for both agonists. Thapsigargin produced a large constriction (57 ± 2 %) of MV that deteriorated very slowly while thapsigargin was still present (~50 min). While responses to PE were attenuated more than responses to NE (79 ± 5 for PE *vs* 57 ± 13 % reduction for NE, n=6), there was not a statistically significant difference between the two groups (Fig. 27).

Even though the difference between the magnitude of NE and PE responses after thapsigargin treatment did not reach statistical significance, there was a trend suggesting that NE induced constrictions are more dependent on extracellular Ca^{2+} than PE induced constrictions of MV. Data presented above indicate that NE induced constrictions of MV use both L-type VOCC and Gd^{3+}-sensitive Ca^{2+} channels. To test whether activation of α_2AR by NE leads to activation of these Ca^{2+} channels in MV, we tested the combined effect of Gd^{3+} (10 μM) and nifedipine (1 μM) in the presence or absence of α_2AR antagonist

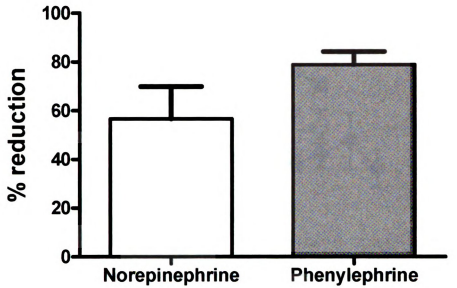


Fig. 27. NE – mediated constriction is more dependent on Ca^{2+} influx than PE – induced constriction of MV. Responses to NE (1μM) and PE (1μM) were recorded before and after thapsigargin treatment (1μM) (vessels were exposed to thapsigargin for 50min – time required for MV to relax and stabilize the diameter). Depletion of thapsigargin – sensitive Ca^{2+} stores significantly reduced responses to both NE and PE, although the % reduction of PE responses was larger.

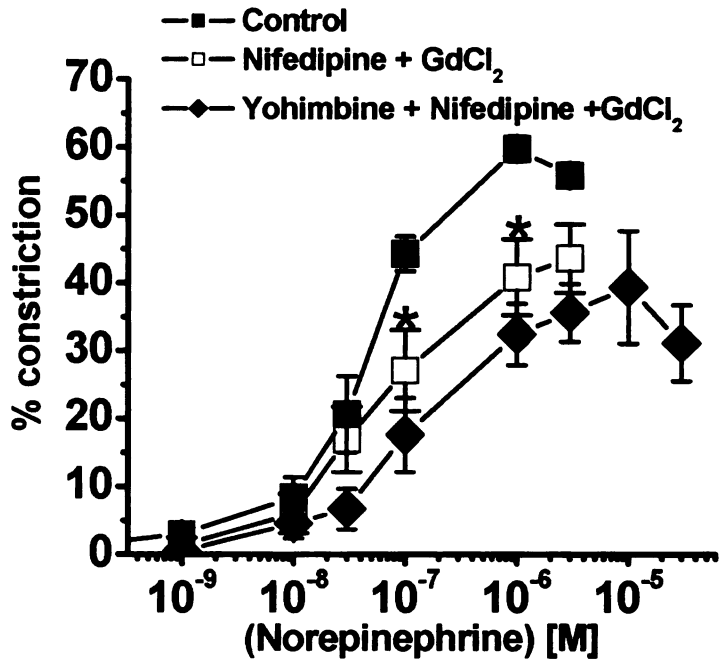


Fig. 28. α_2AR and activation Ca^{2+} channels in MV. Combined inhibition of L-type VOCC and SOC by nifedipine and Gd^{3+} produced a significant inhibition of NE responses in MV. When the α_2AR antagonist yohimbine was applied together with nifedipine and Gd^{3+}, NE CRC was shifted slightly, but not significantly to the right compared to nifedipine and Gd^{3+} alone.

yohimbine (0.3 μM). Fig. 28 represents the combined effect of nifedipine and Gd^{3+} on NE responses in MV, n=6. NE responses were significantly inhibited in the presence of Gd^{3+} and nifedipine at 0.1 and 1μM NE. Yohimbine did not further shift the NE CRC in the presence of Gd^{3+} and nifedipine.

Involvement of Rho kinase in adrenergic responses of MA and MV. We hypothesized that increased involvement of Rho kinase in MV during agonist – induced constriction might also play a role in the differences in sensitivity to NE; increased activation of Rho kinase would increase Ca^{2+} sensitivity of contractile apparatus and therefore constriction. The Rho kinase inhibitor Y27632 (10 μM) was continuously applied to blood vessels and NE CRC were constructed for MA and MV. In MA, Y27632 produced a significant attenuation of NE responses at all concentrations (Fig. 29A). The maximal response to NE was inhibited by 49.1±10.3 % from 34.4±4 to 17.0±1.8 (n = 3). Similarly, Rho kinase plays important functional role in venous responses to NE. In the presence of Y27632, maximal response to NE was reduced by 24.5%±7.0 from 47.1±2.3 to 35.1±3, (Fig. 29B, n = 5).

Calcium sensitivity of NE - mediated responses in MA and MV. To test for the differences in Ca^{2+} sensitivity between MA and MV during adrenergic responses, we constructed cumulative CRC to Ca^{2+} in the presence of constant concentration of NE, 0.03 μM for MV and 0.3 μM for MA (concentration representing EC_{50} for NE in the two vessels). Initially, we equilibrated the blood vessels in 0 Ca^{2+} solution/1mM EGTA for 15 min. This

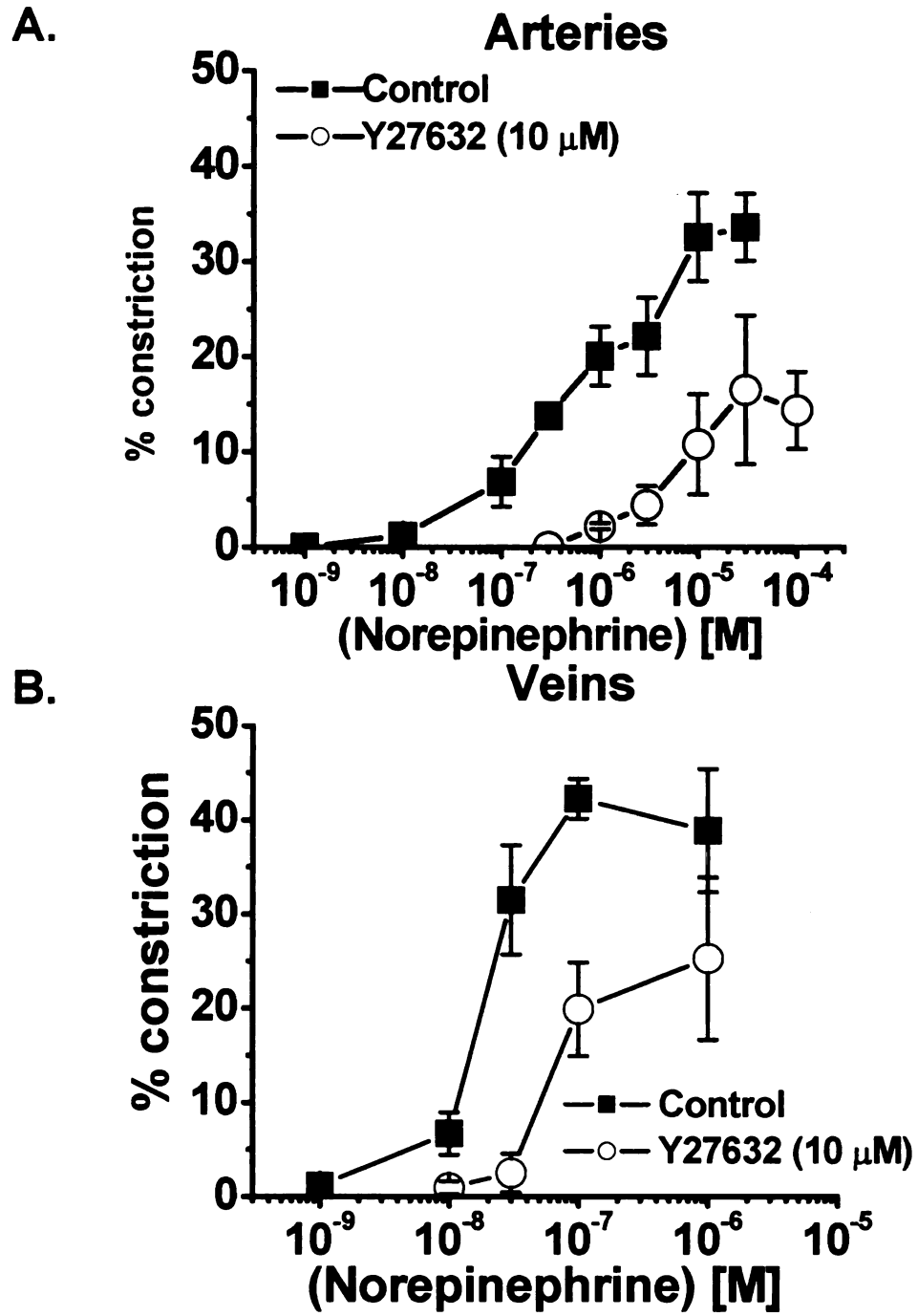


Fig. 29. Rho kinase pathway plays an important functional role in adrenergic responses of both MA and MV. Rho kinase inhibitor Y27632 (10μM) was continuously superfused over blood vessels and NE responses of blood vessels were measured. Y27632 produced a significant inhibition of NE – mediated constriction of both MA and MV, indicating that enhanced sensitivity of MV to adrenergic stimulation is not due to more prominent role or Rho pathway.

initial treatment depleted intracellular Ca^{2+} in both blood vessels, because NE did not evoke a constriction at the end of this incubation. Both vessels started to constrict at Ca^{2+} concentration of 0.01 μM (Fig. 30). We observed larger response in MV compared to MA at 1 μM $CaCl_2$ ($p<0.05$). However, differences between the responses at higher $CaCl_2$ concentrations were not statistically significant. Both MA and MV reached 100% of the control response at 100 μM $CaCl_2$ (25 times lower than the Ca^{2+} concentration in standard Krebs' solution). With further increases of $CaCl_2$, constriction in both MA and MV became larger than the control suggesting increased Ca^{2+} sensitivity after the initial Ca^{2+} depletion.

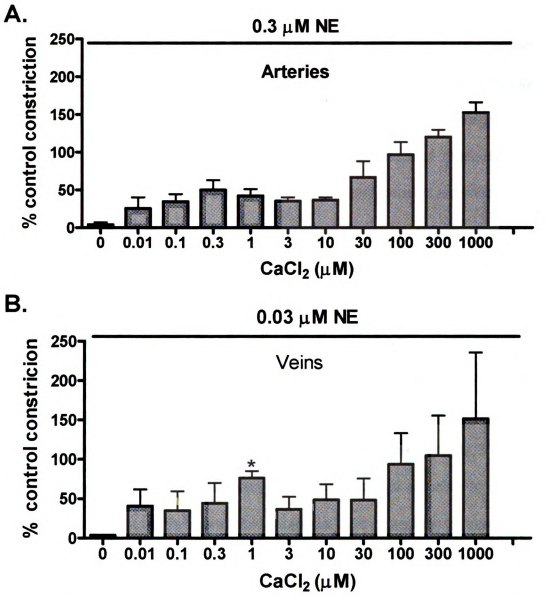


Fig. 30. MA and MV exhibit similar Ca^{2+} sensitivity during NE – mediated responses. MA and MV were incubated in buffer lacking Ca^{2+} in the presence of EGTA (1mM). After 15 min wash that producing Ca^{2+} depletion in both vessels, cumulative CRC to $CaCl_2$ were constructed in the presence of constant concentrations of NE; 0.3μM for MA and 0.03μM for MV, concentrations representing approximately the EC50 for NE in these vessels. Both MA and MV exhibited bi-phasic response to $CaCl_2$ and except at 1μM $CaCl_2$, the differences between MA and MV were not statistically significant. Data are presented as % of control response to the given NE concentration in Ca2+ containing buffer (2.5mM).

Discussion

Previous studies established that MV are more sensitive to adrenergic stimulation than MA. The aim of the present study was to clarify the differences in Ca^{2+} utilization between MA and MV during adrenergic stimulation that may contribute to differential functional responses of these two vessels. Because activation of α_2ARs enhances constrictions caused activation of α_1AR in MV, we also tested the hypothesis whether α_2ARs in MV couple to Ca^{2+} channels and Ca^{2+} influx. This additional Ca^{2+} influx activated by α_2AR could potentially underlie the mechanism of synergistic interaction between α_1 – and α_2AR, as has been previously described for other preparations including tail artery, cat nictating membrane and cauda epididymis of the guinea pig (Xiao and Rand, 1989; Haynes and Hill, 1996).

Pressure enhances the reactivity of MA but not MV. To study Ca^{2+} signaling in MA and MV, we utilized pressurized system due to the effect of intraluminal pressure on membrane potential and reactivity of vascular SMCs. Because MV are physiologically low pressure system, the difference in the adrenergic reactivity between pressurized and non - pressurized MV was not as large as the difference noted for MA. While the EC_{50} for NE was shifted ~2.5 times to the right in MV, the maximal response of MV has been significantly increased. Therefore, the rightward shift does not represent a decrease in the sensitivity of MV. As expected, we observed larger difference in the CRC for NE between pressurized and non-pressurized MA. This result is plausible because the difference in the pressure between pressurized and non-pressurized MA was

~ 60mm Hg as opposed to ~8mm Hg for MV. Stretch-activated channels in the SMCs become more active when the intravascular pressure increases and acts as mechanical force on the SMCs. This leads to increased cation influx and lowering of the membrane potential that regulates the activity of multiple channels. The decreased membrane potential enhances the blood vessels reactivity.

To determine the potential role of $G_{i/o}$ proteins in the α_1 – α_2AR interaction, we used PTX treatment to inactive $G_{i/o}$ and measured its effect on NE responses in MV. Our findings indicate that other G proteins than $G_{i/o}$ likely mediate this interaction, since no effect of PTX on NE responses has been detected while α_2AR antagonists shifted the NE CRC curve to the right. This result is somehow surprising as multiple studies indicate that pressor responses in the vasculature mediated by α_2AR involve activation of $G_{i/o}$ (Nichols et al., 1989; Aburto et al., 1995) and α_2AR – mediated potentiation of α_1AR signaling in cauda epidymis involves PTX – sensitive proteins (Haynes and Hill, 1996). While incomplete inactivation of $G_{i/o}$ proteins would prevent us from detecting an effect of this inhibitor if $G_{i/o}$ proteins were involved, it is unlikely the treatment was ineffective because it has been performed at high concentration of PTX for 2 hours. Same protocol has been shown to be effective in quinea-pig myenteric neurons (Bian and Galligan, 2007). In addition, studies in our laboratory on rat MA employing the same protocol (Stacie Demel, unpublished) have shown that PTX is effective in inhibiting functional coupling of prejunctional α_2AR in DOCA-salt rats. However, in SHAM rats this treatment did not result into complete

inactivation of $G_{i/o}$, perhaps due to larger $G_{i/o}$, reserve in the SHAM animals. These studies indicate that this protocol might not be universally effective and perhaps this is related to the amount of the $G_{i/o}$, proteins in the given preparation. In the study performed by Haynes and Hill, tissue was incubated for 24h in PTX - containing Dulbecco's modified eagle medium, although the concentration of PTX was lower (200 ng/ml). This prolonged incubation would not have been possible to perform with MV due to their fragility. To probe the involvement of $G_{i/o}$, studies show that bolus injection of PTX *in vivo* 24 – 48 hours prior to the actual experiment is an effective way to inactivate the proteins and perhaps this would be a viable option for our future studies.

Role of Ca^{2+} influx in adrenergic responses of MA and MV. We further investigated functional involvement of Ca^{2+} channels in NE induced constriction of MA and MV. While MV seem to rely more on intracellular Ca^{2+} stores than MA (chapter 4), I predicted that α_2AR in MV couples to a Ca^{2+} channel and thereby enhances the total Ca^{2+} elevation caused by NE. I also predicted that I would detect differences in the Ca^{2+} channels involved in adrenergic responses between MA and MV. The non-selective Ca^{2+} channel blocker $CdCl_2$ inhibited NE responses in MA and MV. However, the nature of the inhibition differed between the two vessels; while $CdCl_2$ produced concentration-dependent inhibition of NE responses in MA, MV were more sensitive to $CdCl_2$. MV did not respond to NE in the presence of $CdCl_2$ at low agonist concentrations and even though we observed some constriction at higher NE concentrations, the response was almost completely abolished at 10μM NE. These data suggest

that in the MA, $CdCl_2$ blocks a Ca^{2+} entry mechanism that directly participates in the αAR-mediated responses. However, an important mechanism of $CdCl_2$ action in MV might be depletion of Ca^{2+} stores because there did not seem to be a dose – dependent inhibition of NE responses in MV as in MA. Because the time required to perform CRC in the vessels was ~1h and 45 min, it is possible that capacitative Ca^{2+} entry was compromised leading to depletion of intracellular stores and therefore inhibition of constriction. Cadmium has also been shown to inhibit receptor – operated channels – the transient receptor potential channel 6 (TRPC6) that are activated by αAR stimulation and these channels might play important functional role in vascular constriction of both MA and MV (Inoue et al. 2001).

However, $CdCl_2$ not only blocks Ca^{2+} channels, it also blocks Ca^{2+} transporters. $CdCl_2$ modulates the activity of Na^+-Ca^{2+}-exchanger (NCX) where it competes with Ca^{2+} for import into the cell, thereby inhibiting Ca^{2+} influx (Trosper and Philipson, 1983; Le et al. 2005). NCX works as a bidirectional exchanger; while at resting membrane potentials its function is to maintain low Ca^{2+} levels by pumping out Ca^{2+} it can also promote Ca^{2+} influx during constriction. This can be mediated by coupling of NSCC TRPC6 to NCX, where localized increases of Na^+ reverse Ca^{2+} transport producing Ca^{2+} influx. Studies in Chapter 5 indicate, that NCX can mediate Ca^{2+} influx during αAR-mediated constriction in MA. Therefore, it is possible that $CdCl_2$ inhibited the NCX - mediated Ca^{2+} influx, inhibiting constrictions in MA. In contrast, the role of NCX in NE-induced constrictions of MV is not as prominent, although we can not exclude the possibility that reverse

mode of NCX also contributes to Ca^{2+} influx in MV. Based on our current results, MV are more sensitive to $CdCl_2$ which because it has an additional site of action in MV that is not present in MA. .

We tested the effect of nifedipine to asses the role of VOCC in NE-induced constrictions of MA and MV. Nifedipine produced a greater inhibition of NE induced constrictions in MA compared to MV. The role of L-type Ca^{2+} channels in NE constriction in arteries has been established previously (Mukundan and Kanagy, 2001). In addition, these channels might play a greater functional role in MA in response to sympathetic stimulation, because P2X receptors are known to activate Ca^{2+} influx through these channels in MA in response to ATP released from sympathetic nerves (Lagaud et al. 1996). In contrast, only αAR are activated by sympathetic nerve stimulation in MV and P2X receptors do not contribute to neurogenic constriction of MV (Luo et al. 2003; Park et al. 2007).

Next I used $GdCl_3$ to determine if NSCC contributes to NE constriction. $GdCl_3$ blocks the stretch-activated channels that are NSCC. $GdCl_3$ had no effect on NE responses of MA. However, in MV $GdCl_3$ shifted the NE CRC to the right suggesting some functional role of these NSCC in NE-mediated constriction of MV. My studies indicate that $GdCl_3$ blocks SOC entry in MV (Chapter 5) and also other studies show, that $GdCl_3$ is an effective blocker of SOC in other systems (Trebak et al. 2002). On the other hand, $GdCl_3$ might be less effective as a blocker of receptor–operated Ca^{2+} channels (ROC), whereas 2-APB is more potent inhibitor of this Ca^{2+} entry pathway (Trebak et al. 2002).

2-APB may exhibit dual inhibitory action in MV where it acts an IP_3 receptor antagonist on the ER and therefore inhibits the Ca^{2+} release stimulated by $G_{q/11}$ proteins (Minneman, 1988). In addition, the IP_3 receptor is required for conformational coupling of SOC and activation of SOC entry. In this model, IP_3 receptors sense depletion of intracellular Ca^{2+} stores and transmit this message to SOC via direct protein – protein interaction (Kiselyov et al. 1998). Therefore, 2-APB could prevent this interaction and activation of SOC. However, other studies indicate that 2-APB can directly bind to and inhibit SOC (Dobrydneva and Blackmore, 2001, Broad et al. 2001). In my studies, 2-APB did not abolish, but significantly inhibited NE – stimulated responses in MA. $GdCl_3$ had no effect on NE induced constrictions of MA indicating no functional role of SOC in these responses. Therefore, it is likely that the IP_3 receptors are the only target for the action of 2-APB in MA. An important question remains, however, about the role or ROC since these channels are also inhibited by 2-APB and Cd^{2+}. A slightly different result was obtained in rat MA, where application of 2-APB inhibits PE-induced constriction by 95% (Lamont and Wier, 2004). While this inhibition was greater than what we have observed (about 55% inhibition), it is possible that species-dependent differences, as well as some differences between the signaling of PE and NE could account for the differences in the potency of 2-APB between our result and this study. 2-APB was much less effective in MV. This result is unexpected as MV are dependent on intracellular Ca^{2+} stores for NE-induced constriction. Even though thapsigargin treatment did not completely abolish responses to NE and PE in MV, constriction was inhibited by more than

50%, suggesting that significant portion of the response is dependent on thapsigargin – sensitive Ca^{2+} stores and the established mechanism of Ca^{2+} release from the stores mediated by Gq–coupled receptors involves IP_3 – dependent mechanism (Minneman, 1988). Therefore, one explanation of this result would that IP_3 receptors in MV are less sensitive to 2-APB, or perhaps there is a larger IP_3 receptor reserve on SER in the venous myocytes. Considering that MV posses larger Ca^{2+} reserve in the stores than MA and it takes longer for Ca^{2+} depletion in the absence of extracellular Ca^{2+}, it is possible that the receptors regulating this Ca^{2+} release might exhibit differential sensitivity to simulation in MA than MV. Another interpretation of this result would be that there is a small contribution of IP_3–mediated Ca^{2+} release required for NE induced constriction of MV. Intracellular Ca^{2+} release could also be produced by Ca^{2+}-induced Ca^{2+} release (CIRC) by the action of Ca^{2+} on ryanodine receptors. In this model, stimulation of αARs would lead to initial Ca^{2+} rise due to the activation of Ca^{2+} channels (the yet unidentified Ca^{2+} channels or transporters – NCX - blocked by Cd^{2+}), followed by CIRC. Another alternative explanation for the low sensitivity to IP_3 blockade in MV would be modulation of Ca^{2+} sensitivity of the contractile apparatus (discussed below) as the main mechanism regulating the vascular tone in MV. However, this mechanism is quite unlikely to be the major pathway because of the large sensitivity of NE induced constrictions of MV to Cd^{2+} and thapsigargin.

Another factor to consider when interpreting the data from MV is the specificity of 2-APB action. Even though its effect was not as large as in the MA,

it still inhibited NE responses. Since SOC may directly participate in NE-induced constriction of MV, perhaps the action of 2-APB might not be exclusively ascribed to inhibition of IP_3 receptors. The concentration used in these studies was at the high end and therefore, we can not completely exclude the possibility that the inhibition of NE was produced by the action of 2-APB on SOC. Similarly to the result in MA, 2-APB could inhibit ROC component in MV. ROC that are composed of TRPC6 are known to be activated by αAR via a DAG related mechanism. Considering that $CdCl_2$ also inhibits TRPC6 channels, the role of these channels needs to be further explored. Utilization of the compound SK&F96356, the more specific blocker for ROC should help to asses this possibility. Unfortunately, current pharmacological tools do not allow for direct identification of channels and their subunits that form SOC and ROC and techniques such as direct electrophysiological measurements would only be able to give a more specific answer about the composition and functional properties. These channels might be composed of multiple TRPC homologues and different composition yields different functional properties and sensitivities to channel blockers. The specific properties of these channels in MV could perhaps be exploited in the future for more selective targeting of venous SMCs with the goal of increasing vascular capacitance.

Role of α_2AR in Ca^{2+} influx. To determine whether NE induced constrictions are more dependent on Ca^{2+} influx than PE responses in MV due to functional coupling of α_2AR to Ca^{2+} channels, I used thapsigargin to deplete intracellular Ca^{2+} stores. I then compared the relative reduction of NE and PE

constrictions. My data indicate that NE responses in MV might be slightly more dependent on Ca^{2+} influx, perhaps due to contribution of α_2AR in NE but not PE constriction. Because our studies suggested that both L-type VOCC and SOC might contribute to NE-mediated responses, we tested the combined effect of the two channel inhibitors on NE-induced constrictions in the absence and presence of yohimbine. Combined nifedipine and $GdCl_3$ treatment produced significant inhibition of NE-induced constrictions. When yohimbine was applied together with the channel blockers there was no additional inhibition of the NE constriction. These data suggest that the indirect contribution of α_2AR in MV may be based on activation of Ca^{2+} entry. This type of mechanism would be consistent with results from previous studies that suggest activation of L-type of Ca^{2+} channels as the underlying mechanism enhancing contractility of α_1AR by the action of α_2AR (Haynes and Hill, 1996, Xiao and Rand, 1989).

α_2AR are regulated to larger degree by myogenic tone than α_1AR (Faber and Meininger, 1990). It is possible that activation of Ca^{2+} channels by α_2AR would be subject to myogenic regulation, because activity of L-type Ca^{2+} channels is dependent upon intravascular pressure and membrane potential. In situations where intravascular pressure would be increased, increased activity of L-type Ca^{2+} channels would be present. Release of NE by SNS would than perhaps produce greater constriction due to increased functional coupling of α_2AR to Ca^{2+} channels.

Ca^{2+} sensitivity of MA and MV during adrenergic stimulation. Differences in Ca^{2+} sensitivity between MA and MV could also account for

differences in functional responses between the two blood vessels. Rho kinase is the most important regulator of Ca^{2+} sensitivity and differential activation of this pathway could also play a role in differential regulation of arterial and venous tone. Indeed, a previous study suggested that inactivation of this pathway produces greater inhibition of agonist induced constriction in veins than in arteries (Robertson et al. 2007). However, these studies were not performed on mesenteric vasculature. In addition, α_2AR – mediated constriction in palmar lateral vein as well as in some other blood vessels involves activation of Rho pathway (Roberts, 2004), providing a testable hypothesis for α_2AR action in MV. Rho kinase might be more important in NE-induced constrictions of MA compared to MV given the larger inhibition of functional response in MA. These data suggest that more pronounced activation of Rho kinase pathway in MV likely does not account for higher adrenergic responsiveness of MV. In these studies, we did not test the possibility whether Rho kinase pathway is activated by α_2AR. However, the mechanism of inhibition of NE responses by Y27632 was different than that produced by yohimbine. Rho kinase inhibition suppressed maximal responses to NE, while yohimbine did not attenuate the maximal constriction to NE. However, additional inhibitors of the Rho kinase pathway should be tested because Y27632 is know to suppress some non-Rho kinase mediated effects.

In addition, we obtained $CaCl_2$ CRCs in the presence of a constant concentration of NE to determine possible differences Ca^{2+} sensitivity during NE–mediated constriction of MA and MV. Initially, the blood vessels were washed in

buffer in the absence of Ca^{2+}, resulting into depletion of Ca^{2+} stores. After this wash, MA and MV were no longer able to respond to NE due to Ca^{2+} depletion. There were no substantial differences in $CaCl_2$ sensitivity between MA and MV. We observed a bi-phasic response in both MA and MV when NE was present; there was an initial peak constriction at 0.3μM $CaCl_2$ in MA and at 1 μM $CaCl_2$ in MV. After this initial peak, responses tapered off and starting at 10μM $CaCl_2$, responses were increasing in dose – dependent manner in both MA and MV. It is interesting that at the larger Ca^{2+} concentration that was still smaller than concentration in regular Krebs' buffer, responses of both MA and MV were significantly larger than control responses before the start of experiments. Perhaps, this was due to increased Ca^{2+} sensitivity of contractile proteins after the initial Ca^{2+} depletion.

Because the Ca^{2+} concentration in the buffer does not reflect the Ca^{2+} concentration in the cytosol, a direct relationship between Ca^{2+} sensitivity and contractility in MA *vs* MV can not be established from this experiment. However, initial Ca^{2+} depletion followed by small increases in Ca^{2+} likely affects Ca^{2+} storage in the SER and the magnitude of Ca^{2+} influx during NE stimulation. Our experiments with Cd^{2+} provide direct evidence that both MA and MV are very dependent on Ca^{2+} influx during adrenergic stimulation. It is likely, that Ca^{2+} influx and Ca^{2+} storage will be inhibited to similar degree in MA and MV by low Ca^{2+} concentration in the buffer. Therefore, the fact that we did not observe any major differences between MA and MV in NE responses at different Ca^{2+} concentrations suggests that there are no major differences in Ca^{2+} sensitivity of

Ca^{2+} - dependent excitation – contraction coupling between the two vessels. Rather, differences in Ca^{2+} utilization appear to play a more important role in differences in responsiveness to contractile agents.

Conclusions.

Overall, these data suggest that adrenergic responses of MA and MV activate Ca^{2+} influx pathways but there are important differences in Ca^{2+} influx mechanisms and utilization. In the MA, NE activates NCX and perhaps ROC, but IP_3 – mediated mechanism of Ca^{2+} release from the endoplasmic reticulum also plays an important role in functional responses. In the MV, Ca^{2+} influx is crucial and may be mediated by ROC and NCX due to high sensitivity of NE responses to Cd^{2+}. In contrast, MV may be more dependent on Ca^{2+} - induced Ca^{2+} release as opposed to IP_3 – mediated Ca^{2+} release due to low sensitivity to IP_3 receptor blockade. In addition, α_2AR in MV may couple to Ca^{2+} influx, leading to greater functional responses of MV when this receptor is activated.These differences in Ca^{2+} utilization might provide basis for differential kinetics of constriction between the two blood vessels, with veins exhibiting faster rise of constriction to adrenergic stimulation than arteries. In addition, differential Ca^{2+} utilization is more likely to explain the differences in adrenergic sensitivity between MA and MV as opposed to differential Ca^{2+} sensitivity of the two blood vessels.

CHAPTER 5

Differential Calcium Handling between in Murine Mesenteric Arteries and Veins

ABSTRACT

Murine mesenteric veins (MV) are more sensitive to adrenergic stimulation than mesenteric arteries (MA). The mechanism underlying this artery vein difference was studied using MV pressurized to 8 mm Hg and MA to 60 mmHg *in vitro*. The norepinephrine EC_{50} value for MV constriction was 0.05 ± 0.01 μM in MV and 0.5 ±0.1 μM in MA. We investigated differences in Ca^{2+} handling that might contribute to differences in MA vs MV adrenergic reactivity. While both MA and MV constricted to NE and depolarizing agent KCl, MV exhibited a slower relaxation than MA to both NE and depolarizing agent KCl. In normal Ca^{2+} solution, thapsigargin (1 μM) caused a large phasic followed an a smaller tonic constriction of MV. In Ca^{2+}-free media, the tonic but not the phasic constriction was abolished. The tonic constriction was inhibited by La^{3+} and Gd^{3+} but not Cd^{2+}. Thapsigargin caused a small, transient constriction of MA with no tonic response. This constriction was abolished in Ca^{2+} free buffer. After Ca^{2+} depletion, replenishment of Ca^{2+} caused a concentration – dependent constriction of MV but not MA.

Oubain (0.5 mM) enhanced thapsigargin-induced constriction of MA and MV. NE responses were inhibited by 61% in MV and by 94% ($P<0.05$) in MA by thapsigargin. However, NE responses after in the presence of thapsigargin and ouabain were abolished in MV but reduced in MA only by 38%, indicating that facilitation of Ca^{2+} influx by ouabain after intracellular Ca^{2+} depletion is sufficient for NE to contract MA, but not MV. Caloxin 1b1 (200 μM) a plasma membrane Ca^{2+}-ATP-ase 4 (PMCA4) blocker did not afftect thapsigargin

responses in MA. Caloxin 1b1 potentiated and prolonged NE responses in MV. Caloxin 1b1 did not affect NE responses in MA.

These data indicate that MV have larger Ca^{2+} stores than MA and activation of SOC differentially affects the tone of MA and MV. Ca^{2+} removal after constriction is more efficient in MA where NCX facilitates Ca^{2+} efflux. PMCA4 in addition to NCX contribute to Ca^{2+} removal in MV. These differences may contribute to increased reactivity of MV to NE.

INTRODUCTION

The mechanisms that regulate venous tone are incompletely understood. While there are many studies focusing on the regulation of venous tone, most of them use larger diameter conductance veins like the vena cava or saphenous vein (Lee et al., 2001; Aburto et al., 1993). Small veins, particularly the ones from splanchnic bed are the most important capacitance bed as they store approximately 25% of total blood volume and are densely innervated by sympathetic nerves. Increases in sympathetic nerve activity can rapidly shift blood from the highly compliant splanchnic veins to the less compliant arteries (Pang, 2001).

While the sympathetic nervous system (SNS) regulates tone in mesenteric arteries (MA) and veins (MV), there are several functional differences in the responses to SNS stimulation between the two vessels. SNS-induced constriction of MV is mediated α_1AR and α_2ARs, while $P2X_1$ receptors for ATP and α_1AR mediate constrictions in MA (Luo et al., 2003; Park et al. 2007; Perez-Rivera et al., 2007).

Even though differential contribution of αARs subtypes in MA and MV may contribute to the increased sensitivity of MV to adrenergic stimulation, our studies indicate that differential receptor expression alone does not fully explain the enhanced sensitivity of MV relative to MA. Previous studies indicate, that both MA and MV equally express all three α_1AR subtypes (Perez-Rivera, unpublished). Furthermore, immunocytochemistry studies on freshly dispersed vascular SMCs revealed that MA and MV express multiple α_2AR subtypes at

comparable levels. Previous work in a heterologous expression system failed to reveal an interaction between a_1AR and a_2AR as was observed in the MV (Perez-Rivera et al., 2007), indicating that factors other than receptor expression contribute to increased reactivity of MV compared to MA. MV are more sensitive than MA to most other vasoconstrictors including ATP and endothelin-1 (ET-1), suggesting a more general enhanced venous reactivity (Galligan et al., 2001; Xu et al., 2007).

In this study, we attempted to identify differences in Ca^{2+} signaling between MA and MV that may contribute to increased adrenergic sensitivity of MV. Differences in Ca^{2+} influx and extrusion regulate vascular constriction. There are two main Ca^{2+} efflux pathways in the vascular (SMCs): the high capacity Na^+-Ca^{2+}-exchanger 1 (NCX1) and low capacity plasma membrane (PM) ATP-driven Ca^{2+} pump (PMCA) (Schuh et al., 2003). Four isoforms of PMCA have been identified and two isoforms (PMCA 1 and 4) are localized to vascular SMCs (Schuh et al., 2003; Pande et al., 2006). In aortic SMCs, the NCX1 exchanger accounts for ~90% of Ca^{2+} efflux released from intracellular stores (Lynch et al., 2008). NCX is regulated by the Na^+-K^+ –ATP-ase (NKA) via its influence on the transmembrane Na^+ gradient. The relative contribution of these two pumps in Ca^{2+} export in MA and MV is not known.

NCX1 is a bi-directional transporter and while its action is crucial in Ca^{2+} extrusion from the SMCs after constriction in addition to maintaining low Ca^{2+} at resting membrane potentials, it can also facilitate Ca^{2+} influx during receptor mediated constriction. αAR are known to activate transient receptor

potential channels 6 (TRPC 6) that carry the non-selective cation current (NSCC) (Inoue et al., 2001). Opening of TRPC6 causes local accumulation of Na^+ that can reverse the NCX and facilitate Ca^{2+} influx during constriction (Poburko et al., 2007). This suggests that NCX performs a dual function is SMCs, facilitating both Ca^{2+} import and export. This dual role of NCX has been confirmed in mice overexpressing NCX; the ventricular myocytes from these mice exhibit an increased clearance of Ca^{2+} from cells in addition to prolonged time required to reach peak $[Ca^{2+}]_i$ (Yao et al., 1998).

To maintain intracellular Ca^{2+} concentration within a narrow range, NCX and PMCA indirectly regulate activity of one another. If activity of NCX1 is compromised by gene deletion of NKA alpha-2 isoform, PMCA activity is increased (Lynch et al., 2008). Changes in the activities of these pumps affect vascular tone. For example, receptor-mediated contraction is enhanced whereas vasodilatation mediated by forskolin or SNP is inhibited in animals lacking NKA α-2 isoform (Shelly et al., 2004). Similarly, differences in the level of expression of PMCA lead to altered dynamics of Ca^{2+} handling and efflux that affect responses to receptor stimulation (Liu et al., 1996).

Any residual Ca^{2+} spillover in SMCs is taken up into smooth endoplasmic reticulum (SER) by smooth endoplasmic reticulum Ca^{2+} ATP-ase (SERCA). SER store content is indirectly influenced by PMCA and NCX that regulate cytosolic Ca^{2+} (Golovina et al., 2003). Selective inhibition of NKA α-2 leads to local increases of Na^+ in the cytoplasm and in turn local increases in

Ca^{2+} by NCX. This results into increased Ca^{2+} uptake by SERCA and increased store loading.

When SERCA is blocked, Ca^{2+} from the stores is unloaded leading to Ca^{2+} depletion. This results into stimulation of store – operated channels (SOC) that normally replenish the stores after Ca^{2+} is released (Leung et al., 2008). However, if Ca^{2+} entering the cell is not taken up by SERCA due to its inhibition, other pathways must remove this residual Ca^{2+}. Even thought intracellular Ca^{2+} is elevated in most types of blood vessels after SERCA blockade, it does not always result into vascular constriction (Leung et al., 2008). Whether SOC directly serve any functional purpose or whether their only purpose is to replenish the stores is still a subject of controversy.

Since there is a large degree of interdependence and adaptability of the various components of Ca^{2+} signaling to maintain Ca^{2+} homeostasis, to handle this function more efficiently, expression of the NKA, NCX and SOC is compartmentalized to small microdomains of the PM that overlie SER. However, PMCA is more uniformly distributed in the PM (Juhaszova et al., 1996; Ambudkar, 2006).

It is interesting that receptor-mediated constriction in mouse aorta is potentiated in mice lacking the NKA α-2 isoform. In contrast, KCl-mediated constriction is not affected in these KO mice (Shelly et al., 2004). The authors suggested that increased Ca^{2+} load in these mice leads to increased release of Ca^{2+} for any given inositol triphosphate (IP_3) produced by receptor stimulation, resulting into enhanced contraction. However, since KCl responses are mediated

predominantly by Ca^{2+} influx through voltage – operated calcium channels (VOCC) with little utilization of Ca^{2+} stores, no difference in the responses between WT and KO animals were detected.

These studies indicate how alterations in the Ca^{2+} handling processes may affect vascular contractility. Differences in the Ca^{2+} handling between MA and MV might also be contributing to enhanced functional responses of MV. Therefore, we investigated the relative intracellular Ca^{2+} loads in MA and MV by inhibiting SERCA. Our studies suggest that activation of SOC differentially affects MA and MV. By utilizing inhibitors of the two Ca^{2+} efflux pathways PMCA and NCX, differences in their relative contribution to relaxation and Ca^{2+} homeostasis in MA and MV were assessed. These studies revealed that there are profound differences in the way MA and MV handle Ca^{2+} and in addition to differences in the functional receptors, this may be another important contributor to the differential regulation of venous and arterial tone.

MATERIALS AND METHODS

Animals. C57/Bl6 male mice (25-30g) were purchased from Charles River Breeding Laboratories (Portage, MI). In the animal care facility, mice were maintained according to the standards approved by the Michigan State University All-University Committee on Animal use and Care. Mice were individually housed in clear plastic cages with access to standard chow (Harlan/Teklad 8640 Rodent Diet) and tap water.

In Vitro Preparation of Mesenteric Vessels. Mice were anesthetized, and the small intestine with its associated mesenteric vessels was removed and placed in oxygenated (95% oxygen, 5% carbon dioxide) Krebs' physiological saline solution of the following composition (millimolar): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, and 11 glucose. In some experiments Ca^{2+} was omitted and the Ca2+ chelator EGTA (100 μM) was added to quench residual Ca^{2+}. A piece of the intestine with associated vessels was removed and pinned flat in a silicone elastomer-lined (Sylgard; Dow Corning, Midland, MI) petri dish. A section of mesentery containing vessel close to the mesenteric border (2^{nd} or 3^{rd} order vessel) was cleaned using fine scissors and forceps. MA and MV were isolated for study by carefully clearing away the surrounding fat tissue. Small piece of a vessel (1.5-2mm) was transferred to a recording chamber containing Krebs-filled micropipettes. After the vessel was fastened to the glass pipettes using ophthalmic sutures, the recording bath containing the preparation was mounted on the stage of an inverted microscope (Olympus CK-2; Tokyo,

Japan) and superfused with warm (37°C) oxygenated Krebs' solution at a flow rate of 7 ml/min. The vessels were than pressurized to reach intravascular pressure of 60 mm Hg in MA and 7mm Hg in MV by adjusting the height of Krebs'-filled syringe connected to the micropipette on which the vessel was mounted. All preparations were allowed a 20-min equilibration period during which the vessels relaxed to a stable resting diameter.

Video Monitoring of Vessel Diameter. The output of a black- and-white video camera (Hitachi model KP-111; Yokohama, Japan) attached to the microscope was fed to a framegrabber card (Picolo, Euresys Inc., TX, USA) mounted in a personal computer. The video images were analyzed real-time using Diamtrak Edge-tracking software (version 3.5, Diamtrak, Adelaide, Australia), which tracks the distance between the outer edges of blood vessel in the observation field. Changes in vessel diameter of 1 μm could be resolved.

Drug application. Drugs were applied in known concentrations by addition to the superfusing Krebs' solution or from a flow tube (0.3 mm tip diameter) positioned directly over the MA or MV. Solution flowed through the tube at 0.5 ml/min. All drugs were purchased from Sigma-Aldrich (St. Louis, MO). Caloxin 1b1 was synthesized at the MSU Research Technology Support Facility based on previously published sequence (Pande et al., 2006).

Data Analysis. Constrictions of blood vessels are expressed as percentage constriction from the resting diameter. Half-maximal effective agonist concentration (EC_{50}) and maximum response (E_{max}) were calculated from a least-

squares fit of individual agonist concentration-response curves using a logistic function from Origin 7.0 (Origin-Lab Corp., Northampton, MA): $Y = \{[E_{min} - E_{max}]/[1+(x/EC50)^n]+E_{max}$, where E_{min} is the minimum response and was constrained to zero and *n* is the slope factor. All data are expressed as mean ± S.E.M. Statistical differences between groups were assessed by Student's two-tailed unpaired *t* test. **Determination of relaxation rate**: Blood vessel diameter was recorded during constriction to NE and KCl. For NE, the decay phase was fitted by the non-linear regression function using Clampfit and the time constanst for individual relaxation segments were recorded. For KCl, linear regression was used due to oscillations in vessel diameter during relaxation. The slope representing vascular relaxation was used to estimate the rate of constriction decline. Because MV desensitized after they reached peak constriction to KCl, the desensitization phase was not considered in the calculations.

Isolation of Vascular Smooth Muscle Cells (SMCs). MA and MV veins from male C57B6 mice (25-30 g) were harvested and cells were isolated according to previously described protocol {Jackson, 1997 #15}. Briefly, the blood vessels were cleaned of surrounding fat and connective tissue in a cold dissociation buffer of the following composition (millimolar): (140 NaCl, 5 KCl, 1 $MgCl_2$, 10mM HEPES and 10mM glucose, 10mM Bovine serum albumin, pH adjusted to 7.4). Tissues were than incubated with papain in the presence of dithioerythrol (1 mg/ml) for 35 minutes at 37°C and this incubation was followed by digestion in a collagenase (1.9U/ml) /elastase (0.15mg/ml) solution with soybean trypsin inhibitor (1mg/ml). Digested vessels were than washed twice to

remove residual enzymes using solution of the following composition (millimolar) (135NaCl, 4KCl, 1$MgCl_2$, 2$CaCl_2$, 10HEPES, 10 glucose, pH adjusted to 7.4) before they were dispersed using 1ml eppendorf pipette.

Immunocytochemical analysis. Cells were allowed to attach to poly-D-lysine – coated coverslips for 30min-1hr after dispersion. SMCs were fixed using Zamboni fixative for 30 minutes. After fixation, cells were rinsed in phosphate buffered saline (0.1 M PBS, pH 7.4) and incubated with blocking serum (4% serum in PBS; pH 7.4) to diminish nonspecific binding. After overnight incubation with primary antibody (Table 1) at room temperature in PBS containing 0.1% triton, cells were incubated for 1 h with secondary antibodies at room temperature. Coverslips were then washed in PBS and staining was visualized using a Nikon TE2000-U inverted microscope. Photographs were taken using a SPOT Insight Color Mosaic camera (Mager Scientific, Inc.) with MetaImaging Series software. Controls with no primary antibodies were used to ensure that binding is specific. Rabbit antiserum for NCX was purchased from Swant, Bellinzona, Swithzerland.

Calcium imaging. Dispersed SMCs were kept in a tube on ice before they were used. Cells were used for up to 4hrs after isolation. 40 min prior to calcium recording, cells were incubated in HEPES solution with 1μM Fluo-4 on ice. 10min prior to recording, 50μl of cell suspension was applied on top of a coverslip mounted in a chamber on the stage of confocal microscope to give sufficient time for cells to attach (Leica TCS SL confocal microscope, Leica

Microsystems, Bannockburn, IL). Cells were continuously perfused at room temperature with HEPES buffer of the following composition (millimolar): 147 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 Glucose pH = 7.4) and after 3 min equilibration, drug treatments were begun Fluo-4 was excited using an argon laser and a wavelength of 488 nm. Emissions were collected in the range of wavelengths between 450 and 500 nm. Images (512 x 512) were obtained every 3 seconds for 3 minutes. Each experiment consisted of 5 pre-drug (baseline) images followed by thapsigargin (1μM) application.

RESULTS

NE induced constriction of MA and MV. Concentration response curves for NE in MV were left-shifted compared to MA; the EC_{50} for NE in MV was 0.05±0.01 μM compared to 0.5 ±0.1 μM in MA, ($P<0.01$, n=6). In addition, the maximal constriction was significantly higher in MV than MA (59 ± 2% vs. 44 ± 4%, n=6, $P<0.01$, Fig. 31A).

MV exhibit slower relaxation than MA. NE induced constrictions in MV were longer-lasting than MA (Fig. 31B). The time necessary for the vessels to relax was significantly different between MA and MV; 227.5±26s and 678±108s for MA and MV, respectively ($P<0.01$). When non-linear regression was used to fit the decay phase of constriction in MA, it was best described by two time constants, whereas in MV it was best described by 3 time constants. The averaged time constants were much larger in MV than in MA (table 3). To confirm that the decay of constriction was due to actual relaxation, we continuously applied NE and recorded the diameter changes. Neither MA nor MV desensitized to 1 μM NE, indicating that our results represent actual relaxation of blood vessels (Fig. 31C).

To determine whether the longer relaxation time of MV is specific for NE responses or if it represents more general mechanism of relaxation, the relaxation rates of MA and MV to the depolarizing agent KCl (60 mM) were compared. Maximal responses to KCl amounted to 50 ± 2% in MA and 53 ± 5% in MV. However, during the application of KCl, MV desensitized after reaching

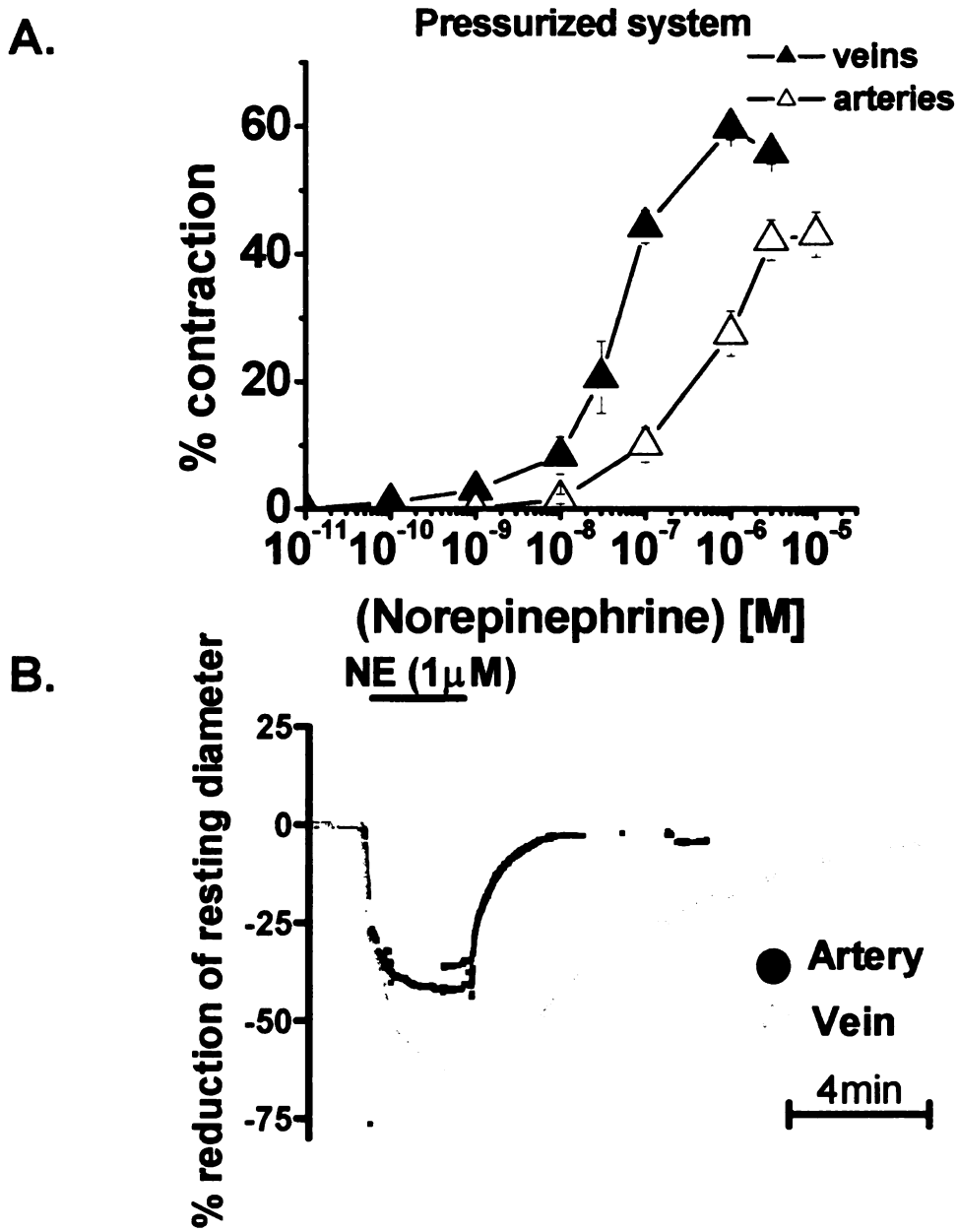
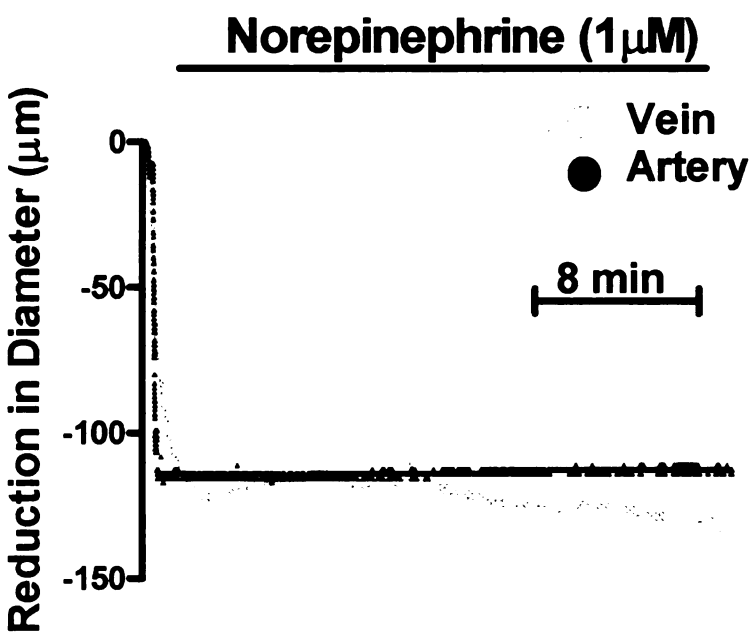


Fig. 31A. MV are more sensitive to NE than MA. EC50 to NE is significantly shifted to the left in MV and maximal constriction is larger in MV. MA were pressurized to 70mm Hg, while MV were exposed to pressures of 8mm Hg. (A) Responses to adrenergic stimulation are longer-lasting in MV than in MA (B). 30ml of 1μM NE solution was applied to pressurized MA and MV to develop a stable constriction. Changes in the vessel diameter including the wash-out period were recorded. The decay phase of the constriction was significantly slower in MV than in MA, $p<0.005$ (B).

C.



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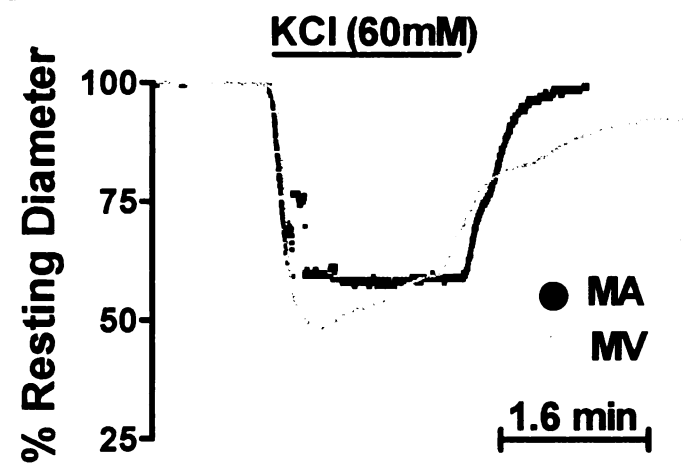


(Fig. 31 continued) The decay of constriction to NE was entirely due to relaxation and not due to desensitization, because neither MA nor MV desensitized when NE was continuously applied (C). Similarly, when 30ml of 60mM KCl was applied, the relaxation rate was significantly slower in the MV than in MA (D). However, MV but not MA exhibited a small degree of desensitization during the application of KCl that resulted into bi-phasic decay phase. Since the first phase represented a combination of both desensitization and relaxation, the second phase representing only vascular relaxation was taken into account when relaxation rates were calculated.

Table 3. Decay phase of NE constriction is longer and composed of more components in MV compared to MA. Decay of constriction was fitted by standard exponential equation and time constants of each component were recorded.

Decay constants of NE response			
	time constant 1 (s)	**time constant 2 (s)**	**time constant 3 (s)**
Arteries	32.65±17.89	38.49±17.44	
Veins	297.8±70.44	135.6±45.5	65.79±44.6

their maximal response to KCl, resulting in a biphasic decline of constriction (Fig. 31D). To estimate the rate of relaxation in MV, only the second phase of MV relaxation curve was used. MA did not desensitize to KCl. The decline of constriction was more complex for KCl than NE in that many vessels exhibited oscillations of their diameter during relaxation, making it difficult to fit by non-linear regression. Therefore, to get a rough estimate for comparative purposses, I used linear regression to calculate the rate of constriction decline. The slope of relaxation was significantly larger in the MA than MV; 2 ±0.1 in MA (n=6) versus 0.3 ± 0.08 in MV (n=4), $p<0.005$.

Responses of MA and MV to SERCA blockade. Application of thapsigargin (1 μM) alone caused a 57 ± 2% constriction of MV, Fig. 32B, n=12, table 3. The vessel maintained a tonic constriction and relaxed very slowly to its baseline after washout of thapsigargin (45-55 min). NE (1μM) responses in MV were inhibited by 61% after thapsigargin treatment. In Ca^{2+} free medium, thapsigargin caused a transient constriction of MV (Fig. 32C) but the tonic constriction was abolished. In addition, I tested 0.1- and 10μM concentrations of thapsigargin. These concentrations produced similar effect to 1μM thapsigargin (not shown).

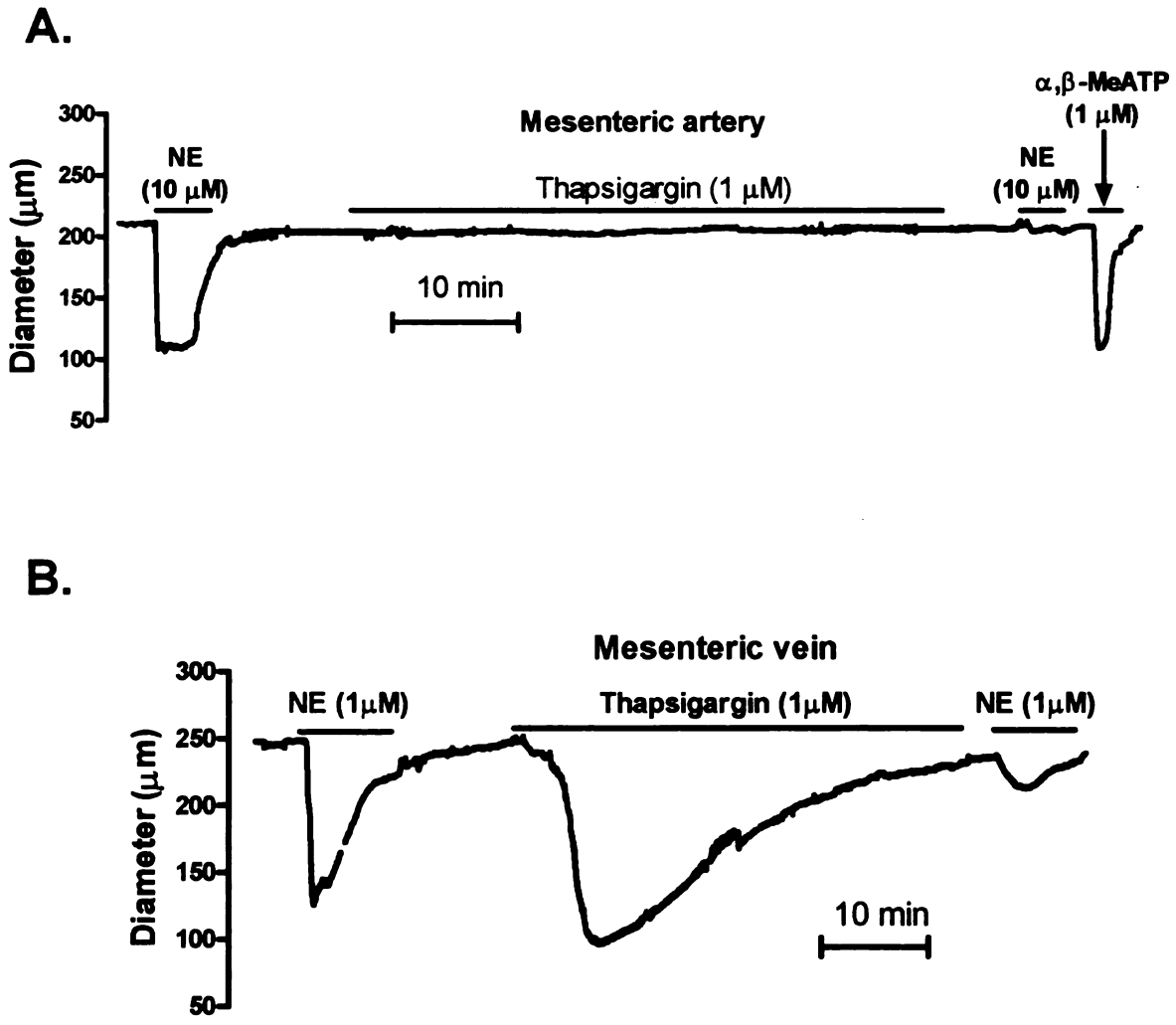
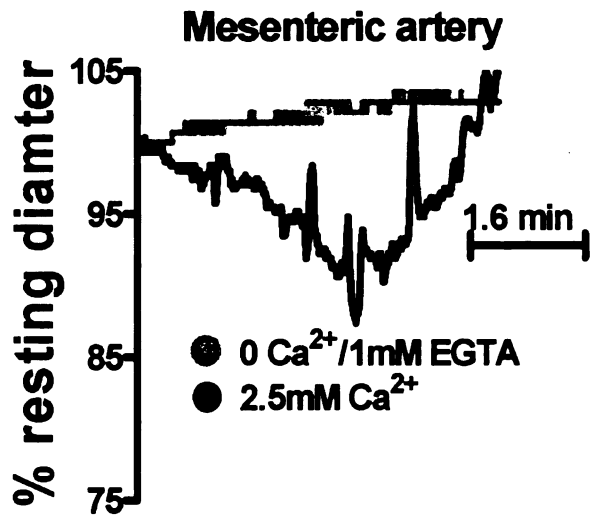


Fig. 32. MV, but not MA develop a strong constriction in response to SERCA blockade thapsigargin (1μM). Thapsigargin was applied using microapplicator and responses of blood vessels to NE (1μM) were recorder before and after thapsigargin exposure. Responses of MV to thapsigargin were always larger than to NE and constriction to thapsigargin decayed very slowly in MV (~45-55 min). Response to NE was reduced by over 60% in MV after thapsigargin treatment (B) (Table 1), but was almost abolished in MA (A). In contrast to MV, MA did not contract in response to thapsigargin.

C.



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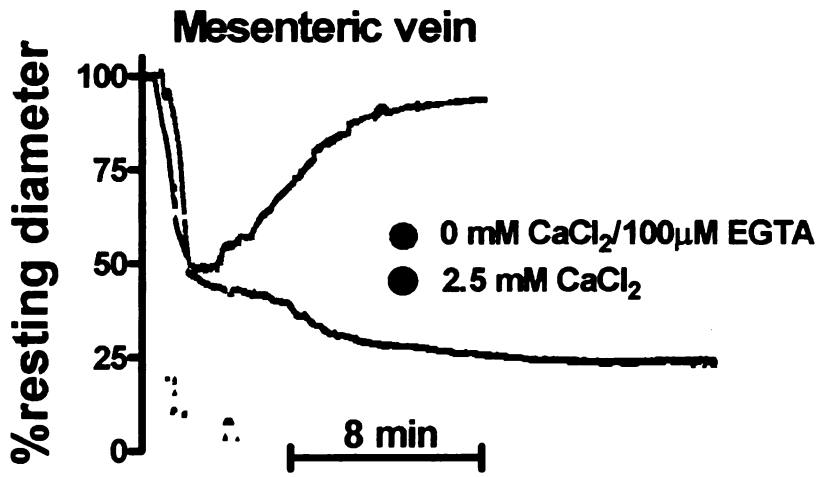


(Fig. 32 continued) Superfusion of thapsigargin produced similar results than microapplication, but response of MA produced a transient and oscillatory constriction that was still much smaller than in MV, black trace, (C). This transient constriction of MA was abolished in the absence of extracellular Ca^{2+} (grey trace) .Constriction to thapsigargin in MV consisted of two components; the first component likely represented Ca^{2+} unloading from SER, because this transient constriction was not abolished in Ca^{2+} free media. However, in the absence of extracellular Ca^{2+}, sustained phase of constriction to MV was abolished, implicating the dependence of this phase on Ca^{2+} influx (D).

Thapsigargin did not constrict MA (Fig. 32A) but prolonged thapsigargin application reduced NE (10 μM) constrictions by 94% (Fig. 32A, Table 4), which was significantly more than in MV ($P<0.05$). However constrictions of MA caused by the $P2X_1$ receptor agonist, α,β-methylene ATP (1 μM) were unaffected by thapsigargin. α,β-methylene ATP–induced constrictions of MA depend on extracellular Ca^{2+} entry (Gitterman and Evans, 2002). When thapsigargin was applied by superfusion instead of microapplication, small oscillatory constriction developed in MA. This response was abolished in the absence of Ca^{2+} in the extracellular buffer (Fig. 32C). 10μM thapsigargin produced same effect as 1μM thapsigargin (not shown).

To determine the direct effect of thapsigargin on $[Ca^{2+}]_i$ in individual SMCs, we performed calcium imaging on freshly dispersed SMCs from MA and MV. Thapsigargin caused a transient increase in $[Ca^{2+}]_i$ in MA SMCs followed by small oscillations and a slightly elevated baseline (Fig. 33A). However, venous SMCs responded to thapsigargin with a more sustained increase in $[Ca^{2+}]_i$ that slowly declined back to baseline (Fig. 33B), paralleling the data from contractility experiments. The Ca^{2+} elevation and decline in MV SMCs closely paralleled the constriction and its decline in MV.

In certain blood vessels, activation of SOC by $[Ca^{2+}]_i$ depletion is coupled to constriction while in others increased SOC activity does not produce constriction. We used Ca^{2+}-free medium to deplete intracellular $Ca^{2+}{}_i$ in order to activate SOC. After a 10 min incubation in this buffer, we performed cumulative concentration-response curve to $CaCl_2$. In MA, addition of $CaCl_2$ produced little

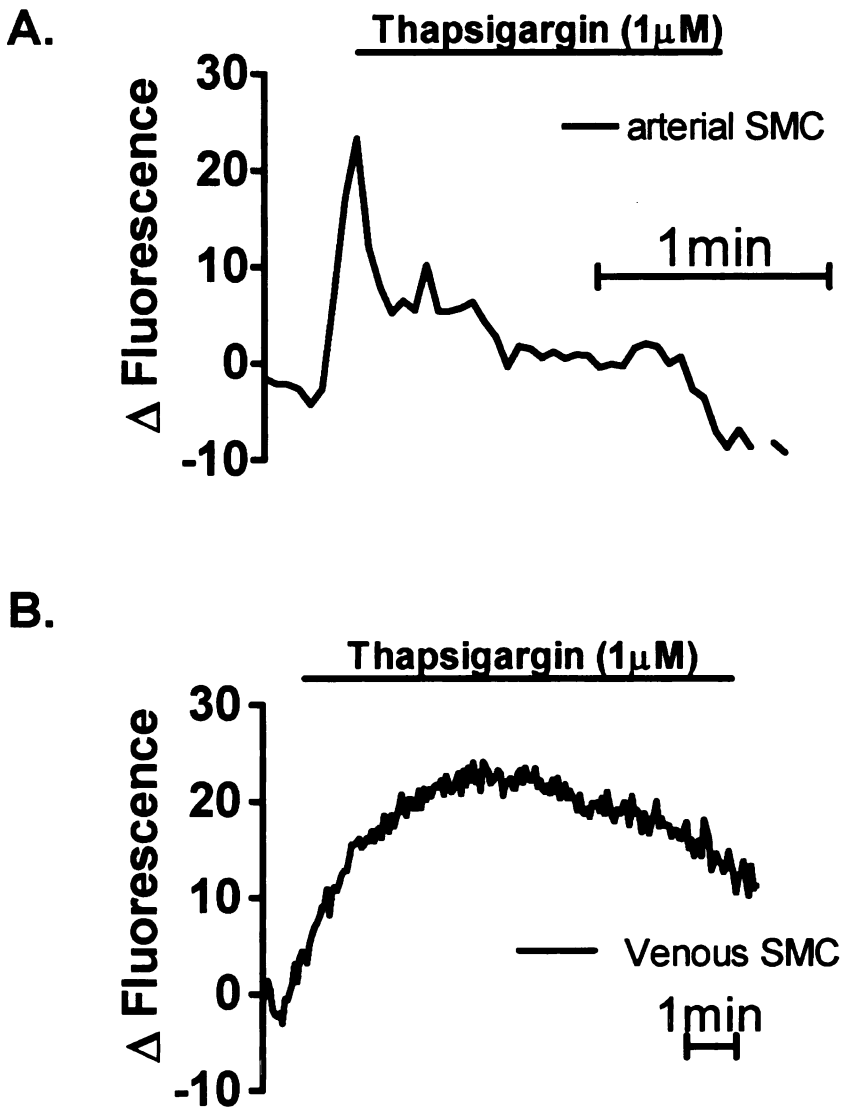


Fig. 33. SERCA blockade causes elevation of $[Ca^{2+}]_i$ in dispersed SMCs from MA and MV. Representative traces of Ca^{2+} elevation during the exposure to 1μM thapsigargin in arterial (A) and venous (B) SMC go along with the data from contractility experiments, where thapsigargin induced a sustained constriction in MV but not in MA.

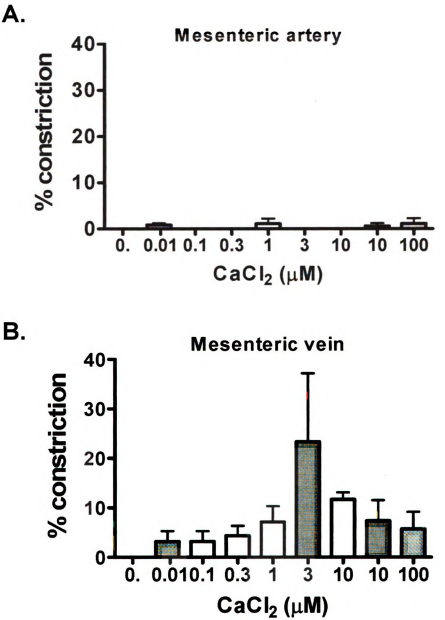
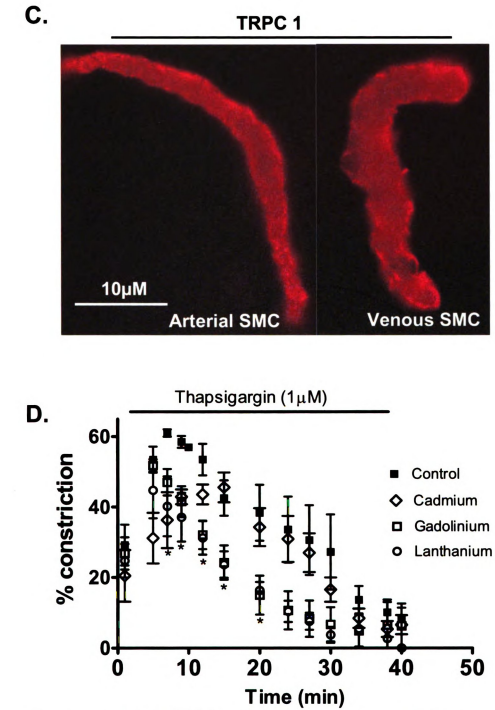


Fig. 34. Store-operated channels are coupled to constriction in MV, but not in MA. Blood vessels were equilibrated in 0 extracellular Ca^{2+} solution/1mM EGTA for 15min. After this period, the vessels were no longer able to respond to NE. Incremental increases of $CaCl_2$ concentration up to 3μM produced a concentration-dependent constriction in MV (B). In contrast, MA did not respond with a constriction after 0 Ca^{2+} buffer was replaced with increasing concentrations of $CaCl_2$ (A).



(Fig. 34 continued) TRPC1, the main component of SOC is expressed in the membrane of both arterial and venous SMCs (C). Application of Cd^{2+}, Gd^{3+} or La^{3+} did not inhibit the initial constriction produced by thapsigargin in MV. However, the tonic phase of constriction was significantly inhibited by La^{3+} and Gd^{3+}, but not by Cd^{2+} (D).

or no constriction (Fig. 34A) while MV response with a robust concentration–dependent constriction (Fig. 34B).

TRPC1 may mediate capacitative Ca^{2+} entry during SOC stimulation by SERCA blockade or after Ca^{2+} depletion (Brueggemann et al., 2006). Indeed, TRPC1 seems to be the major component of SOC in many cell types including vascular SMCs (Ambudkar et al., 2007). We performed immunocytochemistry on freshly dispersed vascular SMCs from MA and MV to determine the distribution of this channel. We detected a strong expression of TRPC1 in the membrane of both MA and MV (Fig. 34C), indicating that MA also express this channel.

We further investigated the nature of the SOC in MV by employing pharmacological tools. First, we tested the effect of Cd^{2+}, a non-selective Ca^{2+} channel blocker, on thapsigargin responses in MV. In the presence of Cd^{2+} (100 μM) MV still responded to thapsigargin and the tonic phase of response has not been abolished or inhibited. We also used $GdCl_3$ (Gd^{3+}) (10μM) blocker of stretch – activated channels and SOC. In the presence of this cation, MV still responded to thapsigargin with large constriction. However, the tonic phase of thapsigargin response has been significantly attenuated (n=6), (Fig. 34D).

MV are less efficient than MA in clearing intracellular calcium. Slow relaxation rates after constriction in MV and the data obtained following SERCA blockade led to hypothesis that MV do not clear intracellular Ca^{2+} as efficiently as MA. To test this hypothesis, we performed experiments in the

Table 4. Effect of SERCA blockade on the tone and NE-mediated responses in MA and MV. MV responded to thapsigargin exposure with strong constriction. NE responses after thapsigargin incubation were reduced in MV by 61%, but almost abolished in MA (reduced by 94%). In the presence of indirect inhibition of NCX-mediated Ca^{2+} export by ouabain, MA responded to thapsigargin with 10% constriction. Ouabain potentiated NE responses of MA after thapsigargin exposure, with constriction inhibited by only 38% after thapsigargin exposure.

	MA (% constriction)	MV (% constriction)
Thapsigargin	0.27±0.27	57.31±1.88
NE control response	43.46±1.9	50.18±1.3
NE response after Thapsigargin	2.55±1.23	19.65±7.1
Thapsigargin+ouabain	10.25±0.7	66.8±1.9
NE response after Thap + Oua	27.08±1.9	7.6±2.6
Ouabain	4.7±1.6	3.9±1.7

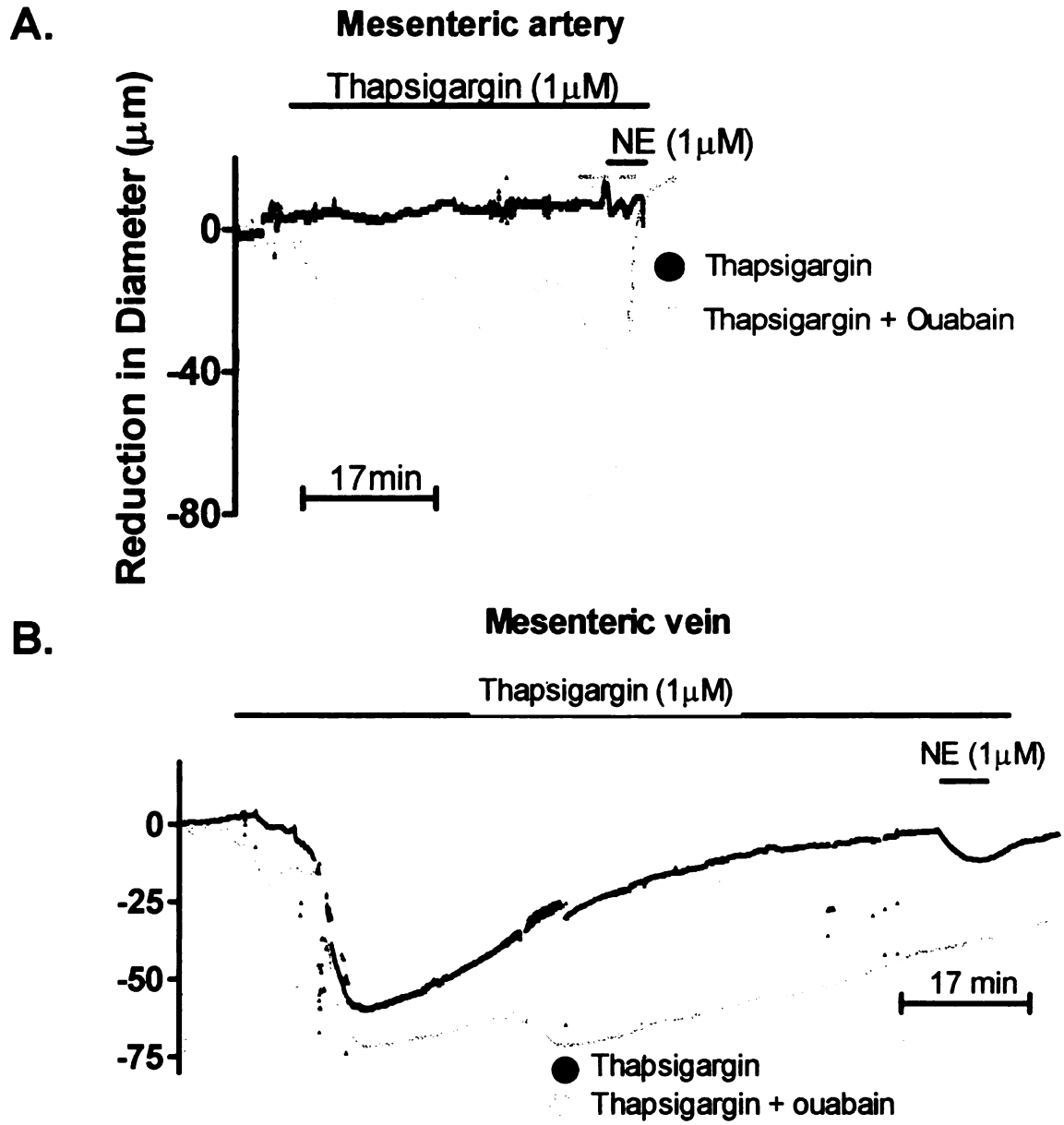
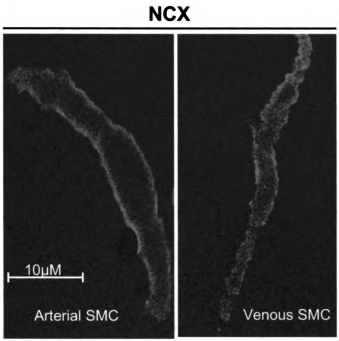


Fig. 35. Ouabain potentiates constriction of MA and MV to thapsigargin. In the presence of indirect inhibition of NCX1-mediated Ca^{2+} efflux by ouabain (0.5mM), MA constricted 10.25±0.7% when exposed to thapsigargin. In addition, in the presence of ouabain, strong oscillatory constriction developed in response to NE after thapsigargin exposure (27% *versus* 2.5% in the absence of ouabain) (A). Ouabain also increased the maximal constriction to thapsigargin in MV. In addition, the decay phase of constriction was increased in the presence of ouabain in MV (B).

C.



(Fig. 35 continued) Immunocytochemical staining of freshly dispersed arterial and venous SMCs indicated the expression of NCX in the membrane of both cells (C).

presence of ouabain, blocker of the NKA. NKA inhibition causes intracellular Na^+ accumulation inhibiting the Ca^{2+} extrusion function of NCX. We next tested the hypothesis that more efficient Ca^{2+} removal by NCX contributes to the low responsiveness of MA to thapsigargin. MA and MV were pre-treated with ouabain (0.5 mM). Ouabain caused a small constriction of MA (Table 4) which stabilized after 10 min. Thapsigargin (1 μM) applied to MA in the presence of ouabain caused a sustained, 8-12% constriction (Fig. 35A, Table 4).Even though the responses of MV to thapsigargin were strong and sustained, ouabain further potentiated these responses, $P<0.05$ (Fig. 35B, Table 4). As described above thapsigargin blocks NE-induced constraction of MA. However, when MA were exposed to ouabain and thapsigargin, NE caused large amplitude phasic constrictions (Fig. 35A, Table 4). Ouabain did not potentiate the peak NE-induced constriction of MV after thapsigargin treatment, but it did delay recovery after NE and thapsigargin washout (Fig. 35B, Table 4). We performed immunocytochemical analysis of NCX in freshly dispersed SMCs from MA and MV. We detected this protein the membrane of both SMCs (Fig. 35C).

Inhibition of PMCA4 pump in MA and MV. PMCA4 is the predominant isoform expressed in vascular SMCs and caloxin1b1 inhibits this enzyme (Pande et al., 2006). Caloxin1b1 (200 μM) did not constrict MA and thapsigargin did not constrict caloxin1b1 pretereated MA. We next used caloxin1b1 to test for the involvement of PMCA4 in clearing Ca^{2+} after NE (1 μM) constriction. Caloxin 1b1 (200 μM for 10 min), did not alter the rate of decay of the NE constriction after NE washout (n=3, Fig. 36A). In contrast, caloxin 1b1

constricted MV (n=4, Fig. 36B) and potentiated NE-induced constractions in MV. Also, caloxin1b1 treatment prolonged the recovery of NE-induced constriction after NE washout (Fig. 36B).

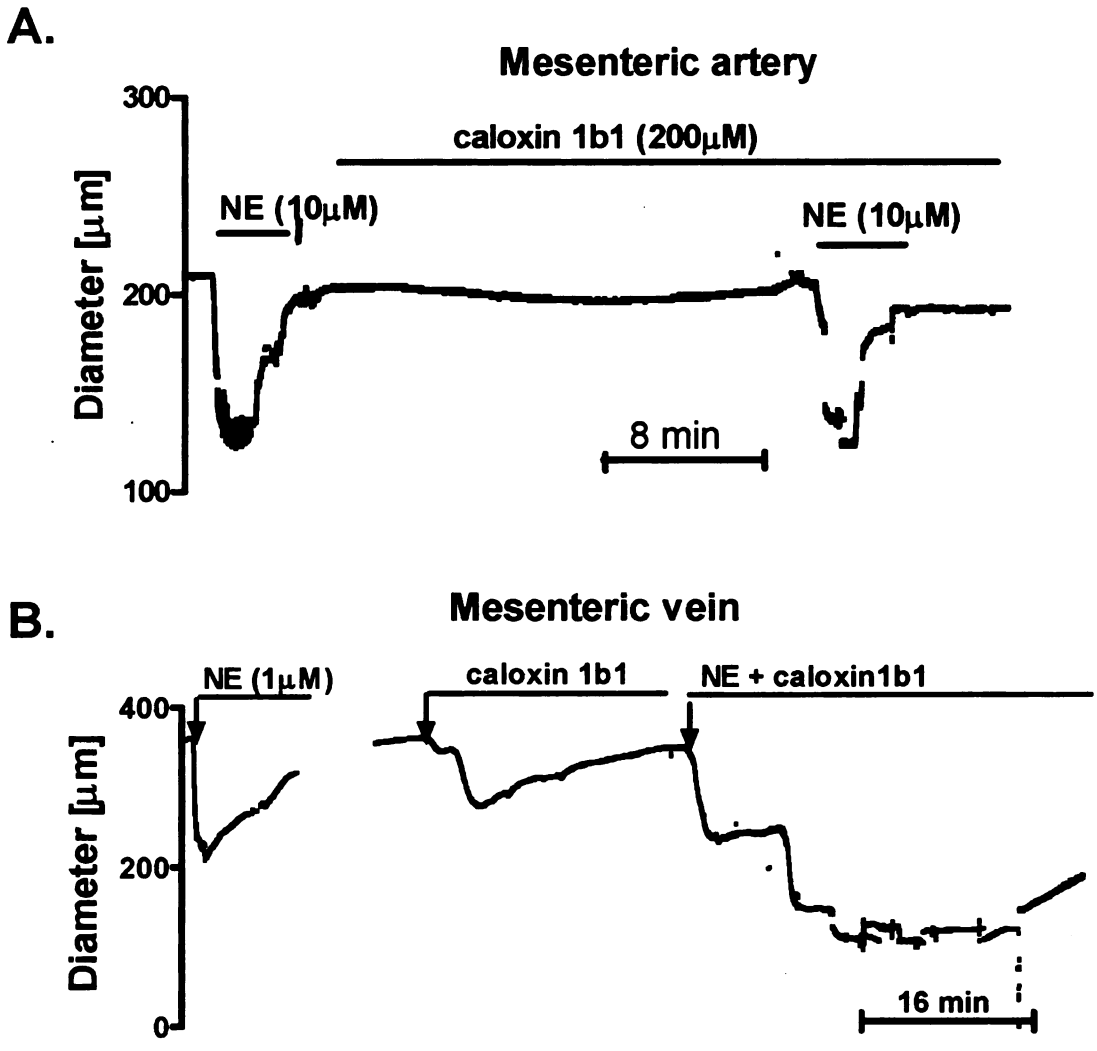


Fig. 36. Inhibition of PMCA 4 affects Ca^{2+} homeostasis and potentiates NE responses in MV. NE (1 in MV or 10μM in MA) was applied before and at the end of caloxin 1b1 exposure that took 20min. MA did not respond to caloxin 1b1 (A). In addition, response to NE has not been affected by this PMCA pump inhibitor. In contrast, application of caloxin 1b1 (200μM) by itself produced constriction in MV. In the presence of caloxin 1b1, response to NE was enhanced and the relaxation phase was also significantly prolonged in MV (B).

DISCUSSION

MV are more sensitive than MA to the constrictor effects of NE. Previous studies indicated differences in receptors mediating functional responses between MA and MV where α_2ARs indirectly contribute to NE constriction in MV but not in MA (Perez-Rivera et al., 2007). However, in addition to receptor differences there are additional mechanisms underlying this pronounced sensitivity of MV to many vasoconstrictor agents. In these studies, we studied the differences in Ca^{2+} clearance to asses its contribution to differential regulation of arterial and venous tone.

MV exhibited a 10-fold higher sensitivity to adrenergic stimulation than MA. and the rate of relaxation from NE - induced constrictions is slower in MV than MA. Because MV also relaxed more slowly from constriction mediated by KCl, the slow time course is probably due to differences in Ca^{2+} clearance rather than slow dissociation of NE from α_1AR or α_2AR in MV. In contrast to MA, MV desensitized to application of 60 mM KCl and MV exhibited a bi-phasic relaxation after KCl constriction. The fact that responses in MV are longer lasting than in MA can be due to less efficient Ca^{2+} removal from cytosol after vascular constriction. By employing specific drugs that interfere with Ca^{2+} handling we investigated this possibility.

Ca^{2+} stores in MA and MV. Thapsigargin constricted MV but not MA. The thapsigargin constriction in MV had two components; an early transient constriction due to Ca^{2+} release from intracellular stores that persisted in the

absence of extracellular Ca^{2+} and a sustained constriction that was abolished in the absence of extracellular Ca^{2+}, suggesting involvement of Ca^{2+} influx. It has been proposed that Ca^{2+} influx during SERCA blockade is a good marker for SOC in vascular SMCs (Leung, 2008). The sustained constriction caused by thapsigargin was insensitive to Cd^{2+}, so it is possible that Ca^{2+}-release activated Ca^{2+} channels (CRAC) or other SOC mediate the Ca^{2+} influx in MV during SERCA blockade. Indeed, previous studies showed that Gd^{3+}, but not Cd^{2+}, blocked SOC during SERCA blockade (Ma et al., 2000). While the early transient constriction caused by thapsigargin was not altered in the presence of Gd^{3+} and La^{3+}, two cations that can block SOC, the tonic constriction of MV was inhibited by these ions. Other studies showed that SERCA blockade in vascular SMCs causes a large, transient increase in intracellular Ca^{2+} that corresponds to ER Ca^{2+} unloading. This transient response is followed by plateau that represents the balance between Ca^{2+} entry through SOC and Ca^{2+} efflux mediated by NCX, PMCA and mitochondrial buffering (Golovina et al., 2003). SOC are opened by store depletion (Golovina and Blaustein, 2000).

Calcium imaging results indicated, that arterial SMCs respond to thapsigargin with a transient Ca^{2+} increase that returned to baseline faster than in MV. These data indicate that MV posses a larger Ca^{2+} reserve in the SER. Also the fact that constriction of MV to NE was not completely abolished after thapsigargin incubation as it was in the MA (MV maintained ~40% constriction) supports the idea of larger intracellular Ca^{2+} store in MV than MA.

Potential implications of larger Ca^{2+} reserve in enhanced functional responses of MV. It is interesting to see that MV and MA do not desensitize to continuous application of NE. However, MV, but not MA desensitize to application of KCl. A profound difference is that during KCl-mediated constriction, Ca^{2+} utilized in vessel constriction comes mostly from extracellular stores through VOCC. In contrast, activation of receptors such as αARs is coupled to both intracellular Ca^{2+} stores and Ca^{2+} influx pathways, with Ca^{2+} stores likely contributing more to constriction in MV. In the light of our data it is likely that MV poses larger intracellular Ca^{2+} reserve as MA. This might be a consequence of the inefficient removal of cytosolic Ca^{2+} in MV to maintain homeostasis. Therefore more Ca^{2+} is taken up by SERCA, increasing the SERs Ca^{2+} content. In fact, studies suggest that if Ca^{2+} efflux pathways are inhibited by knocking – out NKA alpha-2 isoform or by employing agents that inhibit NKA alpha-2, SERCA activity and SER Ca^{2+} store loading are increased (Golovina et al., 2003; Edwards and Pallone, 2007). The increased Ca^{2+} content in SER of MV would cause increased Ca^{2+} release at any given level of IP_3 generated compared to MA, leading to a larger constriction. Indeed, it has been previously proposed that enhanced Ca^{2+} content in stores results into larger Ca^{2+} mobilization and functional responses (Shelly et al., 2004).

Activation of SOC in MA and MV. Besides the profound difference in the magnitude of constriction between MA and MV, an additional difference between the responses of MA and MV to thapsigargin was the sustained constriction in MV. The lack of a tonic constriction in MA could be explained by

two separate mechanisms: 1) MA have more efficient Ca^{2+} buffering mechanisms and/or 2) SOC activation in MA might not induce constriction as in MV. As NE-induced constriction in MV are longer – lasting than in MA, the first mechanism may be more likely.

Differences in Ca^{2+} buffering between MA and MV. To test the hypothesis that Ca^{2+} efflux pathways are more efficient in MA, we used oubain to block NKA causing indirect inhibition of NCX1 by altering the Na^{+} gradient. Indeed, in the presence of ouabain, thapsigargin caused a sustained constriction of MA, although this response was smaller than in MV. This result suggests that Ca^{2+} efflux by the NCX contributes to quick removal of Ca^{2+} in MA. The fact that mice lacking the NKA α-2 isoform exhibit diminished Ca^{2+} clearance by the NCX1 exchanger (Lynch et al., 2008) further supports our conclusion. Similarly, transgenic mice overexpressing NCX1 have increased clearance of Ca^{2+} from the cytosol (Yao et al., 1998). We also detected a potentiated response to thapsigargin in the presence of ouabain in MV, suggesting that even though NCX might not be as efficient in MV as in the MA, it contributes to Ca^{2+} removal. This is further supported by immunocytochemical analysis, where NCX protein was detected in membrane of both MA and MV. However, this type of analysis is not quantitative and therefore differences in expression of NCX can not be excluded as the contributor to differential Ca^{2+} clearance by NCX between MA and MV.

Differential role of SOC between MA and MV? While previous results support increased Ca^{2+} extrusion in MA compared to MV, differences in dynamics of Ca^{2+} removal can not fully account for differences in responses to

SERCA blockade and SOC activation. Even though blockade of SERCA in most vascular beds increases Ca^{2+} concentration, it is not always accompanied by vascular constriction (Leung, 2008). Similar to our results, thapsigargin was found to elevate Ca^{2+} concentration in rat MA, although it did not produce constriction (Snetkov et al., 2003). It is possible that SOC might be coupled to constriction only in some blood vessels and MV but not MA are the ones that exhibit this property. We detected TRPC1 - the main component of SOC in the membrane of both MA and MV suggesting similar expression of channels mediating SOC. SOC are activated by intracellular Ca^{2+} store depletion and it is interesting that reintroduction of Ca^{2+} into the solution after Ca^{2+} depletion by Ca^{2+} free buffer caused concentration–dependent constriction of MV but not MA. However, after reaching a threshold concentration of Ca^{2+} (3 μM), constriction of MV started to diminish. This was likely due to store replenishment and diminished Ca^{2+} entry through SOC. These data further support a differential role of SOC and Ca^{2+} handling between MA and MV. In fact, our previous studies suggested that SOC may play some functional role in NE – mediated responses of MV, but not MA.

Role of NCX in Ca^{2+} influx in NE responses of MA and MV. Thapsigargin- treatment abolished NE responses in MA. However when thapsigargin and ouabain were both present, NE induced constrictions of MA persisted. This result suggests that Ca^{2+} influx is sufficient to mediate NE-induced constriction of MA even though Ca^{2+} stores are depleted. However, MV did not respond to NE in the presence of ouabain, indicating that perhaps MV are

more dependent on intracellular Ca^{2+} than Ca^{2+} influx during αAR-mediated constriction.

The constriction of MA to NE in the presence of ouabain after thapsigargin treatment was likely mediated by Ca^{2+} influx through NCX. This constriction was abolished in the absence of extracellular Ca^{2+}. Indeed, NCX is recognized as a mediator of Ca^{2+} influx during agonist-stimulated constriction (Poburko et al., 2007). NCX requires functional coupling with TRPC6, non-selective cation channels activated by α_1ARs (Inoue et al., 2001). Perhaps in the absence of ouabain, NCX1 activity is not sufficient to support NE-mediated constriction of MA when Ca^{2+} stores are depleted. However, in the presence of ouabain, high intracellular Na^+ concentration resulting from NKA inhibition and activation of TRPC6 channels by α_1ARs potentiates NCX activity, driving Ca^{2+} influx. Ca^{2+} entry induces strong, oscillatory constriction as we have observed in MA. Studies from mice overexpressing NCX1 further support the physiological relevance of the bi-directional Ca^{2+} transport mediated by NCX1; the ventricular myocytes from these mice exhibit an increased rise of intracellular Ca^{2+} during constriction in addition to faster Ca^{2+} removal during relaxation (Yao et al., 1998). Since we did not observe similar effect in the MV, it is likely that the NCX plays a less prominent role in αAR-mediated constriction of the MV. Instead, constriction of MV is more dependent on intracellular Ca^{2+} stores, likely mediated by Ca^{2+} - induced Ca^{2+} release.

Role of PMCA4 in Ca^{2+} extrusion of MA and MV. We used an inhibitor of the PMCA4 to asses the role of this Ca^{2+} efflux pathway in MA and

MV. In contrast to the effect of ouabain in MA, thapsigargin did not constrict MA in the presence of caloxin 1b1, a blocker of PMCA4. In addition, caloxin 1b1 did not have any effect on NE-mediated constriction of MA. These results suggest that PMCA4 does not play a major role in Ca^{2+} efflux in MA. While PMCA may play dominant role in Ca^{2+} extrusion in certain SMCs like the uterine mycytes (Shmigol et al., 1999), NCX accounts for the majority of Ca^{2+} removal in arterial SMCs (Lynch et al., 2008).

Caloxin 1b1 by itself caused constriction and potentiated NE responses in MV. In addition, it prolonged the recovery period after NE washout in MV, implicating PMCA4 in Ca^{2+} buffering in MV. Inhibition of PMCA4 has also been found to potentiate phenylephrine-mediated constriction in rat aorta (Pande et al., 2006), suggesting that this transporter is active during agonist stimulation. However, PMCA is active even during resting conditions in MV, maintaining low Ca^{2+} levels. Overall, agonist induced constrictions of MV are very sensitive to treatments that disrupt Ca^{2+} buffering systems MA are much more efficient in Ca^{2+} buffering that may prevent constriction caused by Ca^{2+} accumulation.

Summary and conclusions. Altogether, our data indicate that there are differences in Ca^{2+} handling between MA and MV during vascular constriction and relaxation. Responses of MA to vasoconstrictors wash out more quickly than in MV, because mechanisms responsible for clearing intracellular Ca^{2+} are more efficient than in MV. NCX is not as efficient in MV in Ca^{2+} removal and this is likely related to the function of other transporters in venous SMCs that regulate Na^{+} gradient and NCX activity. Function of PMCA4 in addition to NCX

compensates for the slower Ca^{2+} extrusion process in MV. There is a basal Ca^{2+} influx in MV, perhaps through SOC and PMCA4 significantly contributes to maintenance of Ca^{2+} homeostasis during resting state. These differences might have significant implications in the hemodynamics; quick regulation and adjustments of arterial resistance are necessary to prevent hypoxia. If the time required for resistance arteries to relax was not tightly regulated, detrimental consequences could occur. On the other hand, the gradual filling of veins due to slower relaxation of venous SMCs seems to be crucial in preventing abrupt filling of the highly compliant veins. Fast filling of veins with large volume of blood would likely lead to frequent and highly uncomfortable syncope.

In addition to Ca^{2+} removal, NCX also plays an important role in functional responses in MA, facilitating Ca^{2+} influx during constriction. NCX might also play a role in Ca^{2+} influx during constriction of MV, but activation of this influx is not sufficient for functional responses; MV rely more on intracellular Ca^{2+} and posses larger Ca^{2+} store reserve and exhibit some dependence on SOC during functional responses of MV. In contrast, activation of SOC in MA mainly serves to replenish Ca^{2+} stores and activation of SOC is not coupled to vascular constriction. Overall these differences in Ca^{2+} handling mechanism in addition to differences in functional receptors likely contribute to larger reactivity of MV to vasoconstrictor agents.

Chapter 6.

General Discussion

General Discussion.

Overall relevance of studies

Cardiovascular disease (CVD) causes 17 million deaths per year globally and is one of the most common causes of deaths in the developed world (WHO, 2006). Hypertension is highly prevalent risk factor for CVD in the industrialized world. Increased longevity together with the prevalence of contributing factors such as obesity, unhealthy diet and physical inactivity, hypertension is becoming increasingly common health problem (Yusuf et al., 2001). The proportion of global disease burden caused by hypertension is very significant (Fig. 37, WHO, 2003) and it is estimated that 7.1million premature deaths worldwide can be attributed to hypertension each year (WHO, 2006). Hypertension along with atherosclerosis and thrombosis are the major contributors to vascular dysfunction. Because vascular dysfunction compromises organ function, it may cause the development of cerebrovascular disease, ischemic heart disease and cardiac and renal failure. It many cases, these end-point diseases might be preventable with antihypertensive therapy (MacMahon et

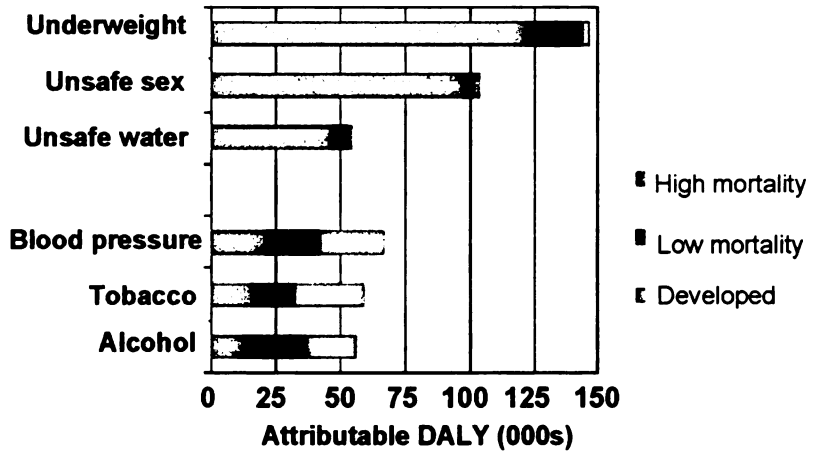


Fig. 37. global distribution of disease burden attributable to six major risk factors. WHO, 2003 (DALYs- disability – adjusted life year).

al., 1990). Studies from randomized clinical trials suggested that reduction of blood pressure can attenuate the risk of stroke and coronary heart disease by 42 and 14%, respectively (Collins et al., 1990). Despite significant improvement in the management of hypertension in the developed world, the cause of hypertension remains unknown in 90-95% of cases. The remaining 5-10% cases may be attributed to secondary hypertension due to kidney abnormalities, certain cancers or congenital heart defects.

Sympathetic nervous system (SNS) and its regulation is a major focus in hypertension research, because most hypertensive patients have elevated SNS activity (Anderson et al., 1989, Schlaich et al., 2004), supporting a neurogenic origin of hypertension. Elevated sympathetic tone can result from increased firing rate of sympathetic nerves (Matsukawa et al., 1991), decreased function of the NE transporter that leads to larger NE spillover (Schlaich et al., 2004) or diminished baroreflex function so that arterial pressure increases are not efficiently buffered (MAP) (Matsukawa et al., 1991). In addition, neurohumoral factors such as angiotensin II, sodium and mineralocorticoids can affect SNS activity (Perondi et al., 1992). A causal relationship between SNS overdrive and hypertension is also demonstrated by the effectiveness of sympatholytic agents like α- and β-adrenergic antagonists in lowering BP (Goodman and Gillman, 2006).

Blood pressure is a function of two variables that are controlled by autonomic nervous system: total peripheral resistance (TPR) and cardiac output (CO) (Medical Physiology, Boron). While TPR is a function of arterial tone and

resistance, CO is dependent on several variables: end-diastolic volume, myocardial contractility and heart rate. Myocardial contractility and heart rate are determined by both sympathetic (SNS) and parasympathetic division of autonomic nervous system. End-diastolic volume represents the volume reached by the ventricular chamber before contraction and is determined by venous pressure. In turn, venous pressure is related to blood volume and tone of the venous smooth muscle cells (SMC). The significant contribution of venous tone to blood pressure regulation points to the importance of studies that investigate their neural control mechanisms and signal transduction involved in contraction.

Reduction of venous capacitance occurs in hypertension (Edmunds, et al., 1989, Ricksten et al., 1981; Ferrario et al., 1970; London et al., 1985) accompanied by increased CO. Similar results have been obtained in experimental models of hypertension, including 2-kidney, 1-clip hypertensive rats (Edmunds et al., 1989) and spontaneously hypertensive rats (SHR) (Ricksten et al., 1981). While CO is increased only transiently during hypertension development and returns back to normal, the redistribution of blood from capacitance veins to arteries will have profound effect on the hemodynamics of the circulation. When increased tissue perfusion exceeds metabolic demands, no metabolic gain for the tissue will be provided. However, damage to blood vessels and organs may ensue. Therefore, autoregulatory mechanisms cause the blood flow to tissues and organs to return to normal when high MAP tries to increase the flow. These autoregulatory mechanisms convert any tendency of high CO hypertension into high resistance hypertension (Guyton et al., 1989).

Studies suggest that the autoregulatory mechanisms are not mediated by autonomic nervous system and mechanism that account for the increase in TPR can include metabolic or myogenic responses to the increased perfusion (Lombard et al., 1989; Folkow et al., 1982). The increased TPR is the mechanism responsible for the of high blood pressure in the more advanced stages of hypertension (Ferrario et al., 1970; Smith et al., 1979; Cowley et al., 1992).

Venous capacitance is determined by two main factors: 1) structure and 2) contractile activity of venous vascular smooth muscle cells (venomotor tone) (Safar et al., 1985). It has been noted, that venous elasticity is reduced in hypertension {Walsh, 1969 #302}. Even though structural characteristics of veins play an important role in determining the capacity of veins for a given blood volume, the regulation of venous tone might be more important with respect to the etiology of hypertension. The measure of venomotor tone - mean circulatory filling pressure (MCFP) - is an index of venous capacity for any given blood volume (Yamamoto et al., 1983). A change in the MCFP reflects primarily a change in the upstream distending pressure (veins and venules). Several studies using various models of hypertension (2 kidney-1clip, SHR) have provided evidence that MCFP is increased in the initial as well as more advanced stages of this disease (Edmunds et al., 1989; Martin et al., 1998).

In addition to the role of veins in the etiology of hypertension, the incidence of other venous diseases is very high. For example, orthostatic intolerance that affects ~ 500,000 Americans results from inappropriate response

of the autonomic nervous system to the postural changes and may involve inadequate regulation of venous tone. Other venous diseases that affect significant proportion of population involve deep veins thrombosis and varicose veins.

My studies investigated the mechanisms that are involved in the regulation of venous tone, with the focus on comparative studies of αAR signaling in MA and MV. While many previous studies indicated that veins are more sensitive to sympathetic stimulation than arteries, the mechanism that underlies enhanced venous reactivity has not been clarified. Because enhanced sympathetic drive occurs in hypertension, veins would be affected more than arteries.

While a goal in hypertension research is to detect its causes and signaling mechanisms that are changed in the disease process, it is equally important to characterize the basic principles that are involved in the regulation of blood pressure under non-hypertensive conditions. Thorough understanding of the basic regulatory mechanisms involved in the regulation of arterial and venous tone will lead to better awareness of the mechanisms that may be deregulated in disease state. Whether increased vascular tone can be causative of hypertension or just a consequence of changes that occur during its' etiology, control of vascular tone is central to the regulation of blood pressure.

α_1 - α_2AR interaction in MV. α_2ARs contribute to the regulation of venous tone, while in the resistance vasculature only α_1AR mediates constriction caused by NE released from sympathetic nerves. I hypothesized that differential

receptor expression between MA and MV contributes to differences in adrenergic reactivity between MA and MV. I found that α_2AR in MV indirectly contributes to NE– mediated responses. While direct activation of α_2AR by α_2AR agonist UK14304 did not cause constriction in MV, stimulation of α_2AR enhanced the responses mediated by α_1AR in MV. These initial findings suggested that the synergistic effect of α_2AR in MV might be an important contributor to venous adrenergic sensitivity. I hypothesized that MA do not express α_2AR and therefore lack this synergistic mechanism in response to adrenergic stimulation. Since the functional role of α_{1D}AR in MV has been established previously in our laboratory and our current studies indicated, that α_{2C}AR is the major α_2AR subtype contributing to the interaction in MV we focused specifically, although not exclusively on interaction between these two receptors. In order to investigate the molecular mechanism of this interaction, we utilized heterologous expression system of HEK 293 cell in functional and expression studies because most of our studies would have not been feasible in vascular smooth muscle cells (SMCs). MV are very fragile and enzymatic isolation of venous SMCs yields small number of cells that only survive for few hours after tissue dispersion. Another factor is tissue culture of MV SMCs. If SMCs are maintained in a media containing serum, they quickly dedifferentiate and loose their SMC phenotype.

Lack of α_1 - α_2AR interaction HEK-293 cells. There are multiple advantages in employing HEK293 cell system in studies of protein interactions and signaling. As opposed to primary culture of SMCs, they grow fast and are easy to transfect. Control over the expression of proteins of interest provides a

great advantage over native system that has not been characterized in such a great detail. However, overexpression of receptors in HEK293 cells is often criticized for producing non-specific effects that would not occur at physiological levels of expression. In my studies, I was able to demonstrate that interaction only occurs between specific receptors, suggesting that certain functional and structural specificity was maintained when the receptors were overexpressed.

In the light of the emerging concept of receptor heterodimerization as ubiquitous process for efficient receptor trafficking, expression and functionality, we predicted that the synergistic α_1 – α_2AR interaction involves heterodimer formation. Most αAR subtypes have been shown to dimerize as well as heterodimerize. Heterodimerization of α_{1D}AR with α_{1B}- and β_2AR has been well documented in promoting receptor trafficking to the plasma membrane and thereby enhancing functional coupling of this receptor. Although there are many reports in the literature documenting synergistic interaction between α_1 – and α_2AR (Haynes and Hill, 1996, Xiao and Rand, 1989, Reynen et al., 2000), no study investigated receptor dimerization as a potential mechanism underlying this phenomenon.

We generated a stable cell line expressing hemaglutinin–tagged α_{1D}AR. Because co-localization of two proteins is a good indication/prerequisite for their physical association, we screened for receptor co-localization between α_{1D} – and the three α_2AR subtypes. As our control, we utilized β_2AR that is known to heterodimerize with α_{1D}AR. While co-expression of β_2AR caused redistribution of α_{1D}AR from intracellular compartment to the plasma membrane with strong co-

localization of the two receptors, immunocytochemical analysis did not suggest similar effect of the three α_2ARs on α_{1D}AR. Because some studies suggest that dimer formation might be enhanced by agonist stimulation, we performed NE treatment of co-expressing cells and studied redistribution of receptors after their activation. NE treatment induced a profound internalization of α_{2A}– and α_{2B}AR, but these two receptors did not co-internalize with α_{1D}AR. By employing ICC, we were not able to detect internalization of α_{1D}– and α_{2C}AR, likely due to their predominant intracellular expression. In contrast, β_2AR internalization was accompanied by co-internalization of α_{1D}AR in small vesicles. Several receptors that form heterodimers co-internalize as a heterodimer and therefore these studies provided further evidence against the existence of heterodimer between α_{1D}- and α_2AR subtypes.

Lastly, we employed a more quantitative approach to study the effect of α_2AR on membrane expression of α_{1D}AR. Luminometer surface expression assay selectively quantified the amount of HA-tagged receptor on the cell surface, providing an objective evaluation of receptor distribution. While β_2AR significantly enhanced membrane expression of α_{1D}AR, we did not observe any effect of α_2ARs on α_{1D}AR membrane expression. While these studies seemed to suggest, that receptor dimerization might not be the underlying mechanism for the functional interaction we observed in MV, we can not completely exclude this possibility. Perhaps, the dimerization was below the detection limits of our methods. Direct experiments involving receptor co-immunoprecipitation would be required to exclude the possibility of receptor heterodimerization. In the light of

my data, however, it appeared to be more reasonable to investigate the receptor interaction at the level of calcium signaling instead of performing biochemical tests based on no evidence of physical interaction. I further performed functional studies in HEK293 cells to determine, whether the functional interaction detected in MV could be simulated in this system. This would allow us to further employ this system in the investigations of the mechanism. However, even these studies failed to detect the α_1-α_2AR functional synergism, while β_2AR enhanced the calcium signal mediated by α_{1D}AR.

Even though studies in the HEK-293 cells led to a series of negative data, the information provided by these studies is very valuable in the context of our studies. These experiments helped us to exclude several possibilities how the functional interaction might occur. Based on these data, it is most likely that the two receptors are not sufficient for the manifestation of the functional synergism. Originally we hypothesized that α_1- and α_2AR form a single functional entity that exhibits enhanced coupling to intracellular messengers and functional responses. However, if this was the case we should be able to detect the interaction in any cellular context, including HEK-293 cells. Ca^{2+} mobilization is a good measure of adrenergically – mediated responses in both vascular SMCs and in HEK293 cells. While both types of cells posses intracellular calcium stores that can be activated in IP_3 – dependent manner, there are differences in adrenergically – mediated Ca^{2+} utilization between the two cell types. While αARs utilize intracellular Ca^{2+} through IP_3 – dependent mechanism in vascular SMCs, they also activate Ca^{2+} influx through multiple pathways that might not be

present in HEK293 cells. Even more importantly, α_2AR seems to be more dependent on Ca^{2+} influx than α_1AR during vascular constriction and it was shown to potentiate the signaling of α_1AR (Xiao and Rand, 1989, Haynes and Hill, 1996). Indeed, our studies seem to suggest that α_2AR in MV also couples to Ca^{2+} influx.

While HEK293 cells endogenously express Ca^{2+} channels including receptor – operated and store operated channels (ROC and SOC), these channels might differ from those activated by αAR in MV. SOC and ROC are most likely composed of multiple TRPC channels (Bugaj et al., 2005). Expression of TRPC1, TRPC3, TRPC4, TRPC6 has been detected in HEK293 cells (Shi et al., 2004, Garcia and Schilling, 1997). Combination of different TRPC that determines the functional properties of SOC/ROC might be characteristic for each cell type and tissue (Garcia and Schilling, 1997). This conclusion is further reinforced by our experimental data that indicate that even though both MA and MV express functional SOC (SERCA inhibition is an established mechanism of SOC activation SERCA blockade increases intracellular Ca^{2+} in both vessels), their properties and coupling to contraction varies between MA and MV. Therefore, even though HEK293 cells express multiple calcium channels, these channels might still poses different functional characteristics than the ones expressed in MV. In addition, the accessory proteins involved in regulation of these channels might differ in the two cellular contexts. Furthermore, HEK293 cells do not seem to express endogenous L-type VOCC that are present in excitable cells including vascular SMCs (Berjukow et

al., 1996) indicating that there are differences in expression of some important Ca^{2+} regulatory proteins. Considering that our data indicate, that the L-type VOCC might be a downstream target of α_2AR in MV, presence of this channel might be critical for the functional interaction.

How does expression of αAR relate to functional responses of MA and MV? While we predicted that only MV but not MA express α_2AR, our studies did not confirm this hypothesis. We detected all three α_2AR subtypes in the SMCs of MA with pretty strong membrane localization. Since no functional role for these receptors has been detected *in vitro*, these receptors might be functionally uncoupled in MA as has been suggested by previous studies (Daniel et al., 1991). On the other hand, caution must be taken when extrapolating data from *in vitro* studies to *in vivo* studies. Other neurotransmitters and hormones are present under *in vivo* conditions that may alter the responses of particular receptor to its agonists. For example, signal transduction pathways activated by angiotensin II synergistically interact with α_2AR signaling, enhancing vasoconstriction to angiotensin II (Gao et al., 2003, Jackson et al., 2005). Since angiotensin II is present in the blood, it could also potentially modulate responses of α_2AR in the mesenteric vasculature under *in vivo* conditions.

In addition, coupling of α_2AR but not α_1AR to vascular constriction is very sensitive to metabolic factors including pH. Carbon dioxide (CO_2) – mediated acidosis was found to selectively inhibit α_2AR–mediated constriction of skeletal muscle arteries and veins, while no effect on α_1AR-mediated constriction was observed (McGillivray-Anderson and Faber, 1990). While our experiments were

performed in oxygenated Krebs' buffer, we can not completely exclude the possibility that alterations in the extracellular environment during tissue removal and preparation could have affected pH of the Krebs' buffer – even small changes of temperature itself lead to changes in pH. While these external factors would be hard to control, they could also have a potential effect on our functional studies of α_2AR. It would be important to find out, whether changes in α_2AR signaling under *in vitro* conditions introduced by slight fluctuations of pH are reversible or not. It would be also interesting to know, whether some variability in the experimental conditions during tissue extraction and preparation could have contributed to the variability in the responses to α_2 agonist UK 14304 in MV. While we did not observe constriction to UK 14,304 in many MV or the constriction was negligible (< 5%), in certain MV we detected constriction as high as 18%.

Differences in the expression of αAR between MA and MV. Overall, data from immunocytochemistry experiments do not support increased α_1- and α_2AR expression in MV as a basis for enhanced sensitivity of adrenergic responses in MV. While previous studies suggested, that MV possess a larger receptor reserve compared to MA due to their increased resistance to alkylating agent phenoxybenzamine (Perez-Rivera et al., 2004), these results could also be explained by more efficient stimulus – response coupling in MV compared to MA. In addition, α_1AR subtypes were expressed at larger levels in the membrane of MA SMCs compared to MV SMCs, further suggesting that activation of smaller

amount of receptors increased constriction of MV. The α_1AR is the dominant functional adrenergic receptor in MA and MV.

Certainly, there are some limitations that have to be considered when interpreting immunocytochemical data. While we tested the specificity of antibodies for the αAR in transfected HEK-293 cells, the environment in primary SMC is different. The amount of receptors in heterologous systems greatly exceeds the amount expressed in native systems. Therefore, positive staining in native systems requires higher sensitivity and specificity of primary antibodies than in the heterologous systems. Therefore, even though our data suggest that the antibodies we used are sensitive and selective in transfected HEK-293 cells, they might not work as efficiently in native SMCs.

Differential effect of enzymes on the membrane of arterial and venous SMCs and its effect on antibody staining. Even though the membrane of venous SMCs was not as smooth as arterial SMCs after vascular digestion, we could still detect equally strong membrane staining of certain proteins in the membrane of venous SMCs as in arterial SMCs. For example, pan-cadherin staining was equally bright in arterial and venous SMCs, even though the membrane was not as smooth. In addition, TRPC1 staining was stronger in the membrane of venous SMCs than arterial SMCs and NCX was detected at similar level in both MA and MV SMCs. These data suggest that less intense membrane staining of certain receptors in MV is likely not caused by damaged membrane caused by the exposure to the enzymes used to prepare isolated SMCs.

α_{2C}AR KO mice. Because our initial pharmacological studies indicated that α_{2C}AR is the main functional α_2AR in MV, we performed functional studies in the MV of α_{2C}AR KO mice. Based on our hypothesis we predicted that responses in these MV would be right – shifted. In contrast, there was no difference in the EC_{50} and E_{max} between the WT and KO MV. As discussed in Chapter 2, the lack of effect in these mice could be attributed to physiological compensation, that is frequently observed in genetically – manipulated animals. There are also examples how a deficiency in one adrenergic receptor subtype results into upregulation of other adrenergic receptor. Studies utilizing femoral arteries from showed that chloroethylclonidine, an α_{1B}AR antagonist, was more effective in inhibiting NE-mediated contractile responses in α_{1D}AR KO mice than in WT arteries. These authors concluded that there was an increased functional role for the α_{1B}AR in the α_{1D}AR KO compared to WT mice (Zacharia, Hillier et al. 2005). In the KO MV, yohimbine still caused the rightward shift in the CRC similar to WT MV, suggesting that α_2AR other than α_{2C}AR mediates this inhibition. Data from studies using subtype selective antagonists suggested, that α_{2A}ARs take over the function of α_{2C}AR in the KO mice.

Physiological implications of α_2AR in MV. While the relative contribution of α_1- and α_2AR in sympathetically – mediated contraction varies between different vascular beds, studies have consistently found that NE responses in systemic arteries are predominantly mediated by α_1AR, whereas α_2AR mediate adrenergic responses in small arterioles and in veins. Even though α_2AR is not the dominant functional receptor in MV, it enhances the function of

α_1AR and its contribution to adrenergic responses and regulation of venous tone might be important from physiological perspective. Moment – to – moment adjustments in vascular capacitance are required to maintain homeostasis and this is dependent on the integration of adrenergic and local metabolic and to smaller degree myogenic determinants. It is possible that α_2AR plays a regulatory role in the MV, enhancing the function of α_1AR when increased venous tone is required to meet the demands of the circulation. Conversely, α_2AR function may be down-regulated under certain circumstances and in this case, it would not enhance α_1AR functionality, resulting into smaller effect of SNS drive on venous SMCs tone. α_2ARs are more sensitive to metabolic and myogenic regulation than α_1AR (McGillivray-Anderson and Faber, 1990, Faber and Meininger, 1990) and these factors could directly modulate venous responsiveness to SNS by affecting functional coupling of α_2AR. In addition, α_2AR exhibits differential sensitivity to SNS stimulation than α_1AR and therefore its activity might be dependent on the level of sympathetic drive. Studies suggest that low frequency nerve stimulation preferentially constricts vessels where α_2AR mediates responses to NE (Ohyanagi et al., 1991). It is also interesting that in our studies, α_2AR was able to potentiate α_1AR function only at low concentration of PE, with no effect on maximal responses when concentration of PE was high. Perhaps, α_2AR enhances α_1AR function only when stimulus from the sympathetic nerves is very low. Since studies have confirmed, that SNS activity is elevated in hypertension (Schlaich et al., 2004), perhaps targeting α_2AR under these conditions would be a useful and selective venous target in hypertension

treatment. However, more studies about the receptor subtypes in MV need to be performed to develop a selective α_2AR antagonist that does not act in the brainstem.

Our studies addressed the functional responses of αARs to circulating catecholamines. Additional studies are needed to address the contribution of α_2AR in venous responses to direct SNS stimulation to evaluate the physiological significance of this receptor in mediating the actions of SNS in MV.

DIFFERENCES IN Ca^{2+} UTILIZATION BETWEEN MA AND MV: ROLE OF α_2AR.

We further investigated Ca^{2+} signaling involved in NE responses to identify the differences in Ca^{2+} utilization between MA and MV that could be potentially attributed to α_2AR signaling in MV, enhancing sensitivity of MV. Differential dependence on Ca^{2+} from the stores and Ca^{2+} from extracellular compartment can also affect the functional responses.

Role of α_2AR and Ca^{2+} influx in MV. Responses to PE were inhibited to larger degree than NE responses after depletion of intracellular Ca^{2+} by thapsigargin suggesting that contractile responses to NE that also involve activation of α_2AR in MV, utilize more extracellular Ca^{2+} than PE responses. Because nifedipine and Gd^{3+} exhibited some inhibitory effect on NE responses in MV, we tested the hypothesis whether the α_2AR in the MV couples to Ca^{2+} influx through these channels. Our studies suggested that in the presence of the two inhibitors, α_2AR antagonist yohimbine no longer significantly shifted the EC_{50} for

NE to the right in MV. This mechanism would go along the lines of previous studies, where the potentiating effect of α_2AR is based on activation of Ca^{2+} channels (Haynes and Hill, 1996, Xiao and Rand, 1989).

As mentioned above, α_2AR are regulated to larger degree by myogenic tone than α_1AR (Faber and Meininger, 1990). It is possible that activation of Ca^{2+} channels by α_2AR would be also a subject to myogenic regulation, because activity of L-type Ca^{2+} channels is highly dependent upon intravascular pressure and membrane potential. Increased pressure would lead to increased activity of L-type Ca^{2+} channels. In addition, if α_2ARs were stimulated, its coupling to L-type Ca^{2+} channels would lead to enhanced functional responses under given conditions.

Store-operated channels (SOC) in MA and MV. MV reacted very sensitively to inhibition of smooth endoplasmic reticulum Ca^{2+} - ATPase (SERCA), whereas only small and transient response was observed in MA. Our studies indicated that two mechanisms are responsible for the differential responses to SERCA blockade. I) MV posses larger Ca^{2+} reserve, while Ca^{2+} gets depleted very quickly in MA and II) SERCA blockade activates Ca^{2+} influx through SOC and this influx is coupled to constriction in MV but not MA. The increased Ca^{2+} reserve in MV can potentially influence the magnitude of functional responses in MV. Studies suggest that when Ca^{2+} load in the stores is enhanced there is an increased release of Ca^{2+} at any given level of IP_3 produced. While we do not have appropriate data to attribute the enhanced

sensitivity of MV purely to this mechanism, it certainly contributes to functional differences between MA and MV. The larger stores could also explain the fact, why MV desensitize to KCl but no desensitization to NE is observed. While NE responses in MV are dependent on Ca^{2+} stores (see below), KCl mediated constriction depends on Ca^{2+} influx through VOCC. NE generates a large Ca^{2+} signal in venous SMC likely due to continuous refilling of the stores through SOC, providing for continuous constriction. In contrast, Ca^{2+} influx during KCl exposure is not sufficient to induce constant constriction of MV. However, in MA constriction to KCl does not desensitize, suggesting that Ca^{2+} influx through VOCC provides more powerful signal in MA than in MV. It is interesting that MV were less sensitive to IP_3 receptor inhibition by 2-APB than MA. Three mechanisms might potentially explain it: I) the IP_3 receptor is less sensitive in MV or perhaps there is a greater IP_3 receptor reserve on SER in MV. In addition, as our studies suggest MV posses larger Ca^{2+} stores than MA and this could lead to sufficient release of Ca^{2+} at a given IP_3 produced even though the majority of IP_3 receptors are blocked or II) MV do not utilize this pathway in adrenergic responses. However, our studies with thapsigargin suggested that after depletion of intracellular Ca^{2+} stores, only a fraction of control NE response is maintained. This would be inconsistent with the idea that MV do not utilize intracellular Ca^{2+} stores. No other receptor that would be coupled to GPCR has been characterized on SER. However, Ca^{2+} could be also released from SER by Ca^{2+} - induced Ca^{2+} - release (CICR) by its action on ryanodine receptor. In this model, αAR in MV could activate Ca^{2+} channels/transporters (large sensitivity of NE

responses to Cd^{2+}) and Ca^{2+} influx would activate CICR, providing for sustained constriction. Lastly (III), involvement of messengers modulating the Ca^{2+} sensitivity of the contractile apparatus in MV may be the dominant mechanism regulating the contractility of MV. We only performed few experiments to address this issue (see below) and therefore more data are needed to make a decisive conclusion about this potential mechanism.

SOC are coupled to constriction in MV. The tonic but not the initial phasic constriction to thapsigargin in MV was inhibited by SOC blockers Gd^{3+} and La^{3+} and in media lacking Ca^{2+}. These results suggested that activation of SOC in MV induces vascular constriction. While also MA express TRP channels that contribute to SOC and SERCA blockade also increases intracellular Ca^{2+} in MA likely through SOC activation, activation of these channels did not induce constriction of MA even though $[Ca^{2+}]_i$ was elevated. While SOC Ca^{2+} entry has been consistently shown to induce increases in $[Ca^{2+}]_i$ in the vasculature, activation of these channels by SERCA blockade did not consistently induce constriction in systemic arteries and arterioles. Examples of vessels where increased $[Ca^{2+}]_i$ caused by SERCA blockade does not lead to vascular constriction include rat MA, renal artery and coronary artery (Naganobu and Ito, 1994, Snetkov et al., 2003). It is particularly interesting, that in rabbit cerebral arteriolar SMCs, SERCA blockade and KCl lead to similar increases in $[Ca^{2+}]_i$ even though KCl and not SERCA blockade produce constriction {Flemming, 2002 #2}.

The profound differences in the responses to SOC activation between MV and MA and the above mentioned vessels may be based on physiological and anatomical differences between the vessels. Perhaps in the MA, SOC are functionally compartmentalized and serve to refill Ca^{2+} in endoplasmic reticulum, without close localization and coupling to contractile proteins. In addition, MA posses efficient mechanisms other than SERCA, that remove the accumulating Ca^{2+} from the cytosol. In contrast, SOC may directly activate contractile proteins in MV. In fact, we detected some functional role of SOC in NE responses in MV but not MA, suggesting that activation of SOC also has functional implications in sympathetic responses. However, more studies are required to explain the heterogeneity in responses to SOC activation between MA and MV. Additionally, it would be very interesting to find out the physiological implications of this heterogeneous regulation of arterial and venous tone by SOC activation.

Bi-directional role of NCX in MA. Our studies indicate that NCX works as bi-directional Ca^{2+} transporter in MA. It is more efficient in Ca^{2+} removal from the cytosol in MA than in MV and also seems to play an important role in Ca^{2+} influx during NE–mediated constriction of MA. Even when thapsigargin–sensitive Ca^{2+} stores were depleted, NE was still able to induce constriction of MA when Ca^{2+} influx was potentiated by ouabain. This effect of ouabain was not observed in MV, suggesting that intracellular Ca^{2+} stores are absolutely necessary for adrenergic responses in MV.

Responses to NE were greatly inhibited by Cd^{2+} in both MA and MV, although the kinetics of inhibition by Cd^{2+} differed between the two vessels. While

Cd^{2+} exhibited a dose–dependent inhibition of NE responses in MA, Cd^{2+} abolished constriction to NE at NE concentrations that produced maximal constriction in MV, suggesting the involvement of Ca^{2+}-dependent mechanisms inhibited by Cd^{2+} in MV. Cd^{2+} competes with Ca^{2+} for the same regulatory binding sites on NCX, but with several orders of magnitude larger affinities (Le et al., 2005). Inhibitory effects of Cd^{2+} on NE responses in MA and MV could be at least partially attributed to inhibition of NCX. This is further supported by the fact nifedipine does not fully account for the effect of Cd^{2+} (reduction of NE responses was much smaller), and also SOC likely do not participate in NE constriction of MA (no effect of Gd^{3+} in MA).

Immunocytochemical analysis confirmed that NCX is expressed in the membrane of MA. We also detected NCX in the membrane of venous SMCs. These data further indicate that NCX plays an important functional role in MA and MV (see below). NCX–mediated Ca^{2+} entry in MA might be an important characteristic of functional responses in MA. Increased activity of reverse mode NCX could enhance Ca^{2+} influx and increase TPR.

It is interesting that a recent study has proposed a role of NCX1 (the dominant isoform expressed in the vasculature) in the etiology of salt-sensitive hypertension. The selective inhibitor of NCX that preferentially blocks Ca^{2+} entry mode (SEA0400) reduced blood pressure in salt-sensitive hypertension in rat. In addition, mice heterozygous for NCX1 mutation resisted the development of salt-sensitive hypertension while blood pressure in transgenic mice overexpressing NCX1 was more salt-sensitive (Iwamoto et al., 2004). The data suggested that

the NCX1 in the vasculature causes increased Ca^{2+} influx resulting in increased vascular tone. This finding might also have clinical relevance, because the plasma level of endogenous ouabain and other cardiotonic steroids is elevated in large proportion (~50%) of hypertensive individuals (Manunta et al., 1999). Ouabains are produced and stored by the cortex of adrenal gland (Laredo et al., 1995). Endogenous ouabain has been shown to be identical to the plant – derived ouabain and other structurally related endogenous ouabains have also been identified. Among those are the cardenolides that include digoxin and ouabain. The second group includes bufadienolides with 19-norbufalin, telocinobufagin and marinobufagenin (Schoner and Scheiner-Bobis, 2007). Increased NE overflow due to enhanced SNS activity together with enhanced endogenous ouabain levels could greatly potentiate signaling of αAR in hypertension.

Further studies are needed to determine the relative contribution of NCX1 – mediated Ca^{2+} influx in MA and MV. Utilization of SEA0400 would help to directly asses this. While our studies suggest that NCX plays an important Ca^{2+} influx role in MA, it might also facilitate Ca^{2+} influx in MV. The studies by Iwamoto described above (NCX1 overexpression, salt–sensitive hypertension) did not address the contribution of TPR and CO in hypertension development. Since salt–sensitive hypertension models also exhibit increased venous tone and decreased capacitance, it would be interesting to find out the role of NCX in venous SMCs in the pre-hypertensive and hypertensive states.

Less efficient Ca^{2+} removal in MV than in MA. Our studies indicated that responses of MV decay more slowly than in MA. While inhibition of the plasma membrane Ca^{2+}-ATPase 4 (PMCA4) did not affect constriction to NE nor did it attenuate the relaxation in MA, it greatly potentiated constriction to NE in MV and also prolonged the constriction decay phase. It is likely that NCX is the dominant Ca^{2+} - clearance pathway in MA, whereas PMCA is likely involved in Ca^{2+} homeostasis (inhibition of this transporter causes venous constriction) as well as Ca^{2+} clearance after constriction in MV because of the less efficient Ca^{2+} efflux by NCX in MV. NCX has a large capacity for Ca^{2+} export, whereas PMCA is the low capacity Ca^{2+} export system. Therefore, the relative contributions of these two efflux pathways in MA and MV has the potential to explain, why contractile responses of MV that reflect intracellular $[Ca^{2+}]_i$ decay more slowly in MV. Even though the NCX more efficiently removes Ca^{2+} from the cytosol in MA, this is not due to higher NCX expression in MA compared to MV. Perhaps, differences in other transporters that influence the Na^+ gradient across the plasma membrane might differentially regulate the activity of NCX in MA and MV.

More efficient Ca^{2+} export mechanisms in MA compared to MV would have great implications in human physiology. While the diameter of resistance arteries must be regulated in a fast manner, such that if too long time was required to relax arteries, it could potentially cause tissue ischemia, a slower rate of Ca^{2+} removal and relaxation in MV could provide for gradual filling of veins with blood instead of very large amount of blood being shifted to veins

instantaneously. Since veins are much more compliant than arteries, fast shifts of large volumes of blood into the veins could cause syncope.

Ca^{2+} sensitivity of MA and MV. Our data did not indicate that the contractile proteins of MV are more sensitive to Ca^{2+} than in MA. The CRC to Ca^{2+} in the presence of constant NE concentration was almost identical in both vessels. While the extracellular Ca^{2+} concentration does not reflect intracellular Ca^{2+} levels, it is likely that after Ca^{2+} depletion, Ca^{2+} release and Ca^{2+} influx at low Ca^{2+} concentrations are inhibited to similar degree in both vessels. Therefore, these studies provided only indirect evidence that there are no major differences in Ca^{2+} sensitivity. Ca^{2+} imaging studies could provide more conclusive evidence against/for the existence of such mechanism.

Rho kinase. Our data indicate that Rho kinase plays an important functional role in adrenergic responses of both MA and MV, perhaps even greater role in MA due to larger inhibition of adrenergic responses by the inhibitor in MA. Therefore, it is unlikely that the enhanced sensitivity of MV could be attributed to an increased role of this pathway that leads to Ca^{2+} sensitization in venous SMCs. Further studies should be performed in MA and MV from hypertensive animals to asses the potential upregulation of Rho pathway in the veins of hypertensive animals and its contribution to hypertension.

Role of altered Ca^{2+} sensitivity in hypertension. Ca^{2+} sensitization and enhanced vascular contractility occur in hypertension. A recent study has proposed an important role for $G_{12/13}$ signaling and its downstream activation of

Rho kinase in salt-sensitive hypertension. While selective abrogation of $G_{12/13}$ in the smooth muscle cells did not alter basal blood pressure, it prevented the development of DOCA-salt hypertension in these transgenic mice. On the other hand, selective abrogation of Gq_{11} not only prevented hypertension development, but also reduced basal blood pressure (Wirth et al., 2008). While these data indicate a greater role for Gq in regulation of blood pressure under basal conditions, selective reduction of blood pressure under hypertensive conditions by $G_{12/13}$ abrogation provides a promising target for treatment of hypertension. Indeed, studies suggest that independent of the cause of hypertension, activation of the Rho kinase pathway seems to be the downstream signaling target in many animal models and inhibition of this pathway can attenuate blood pressure (Uehata et al., 1997, Seasholtz and Brown, 2004, Seko et al., 2003).

Overall Conclusions and Implications.

Studies described in this dissertation aimed to clarify the mechanism behind the enhanced adrenergic sensitivity of MV. Multiple hypotheses were tested and contributed to a better overall understanding of the differences in adrenergic regulation of MA and MV.

a. α_2AR indirectly contributes to adrenergic responses in MV but not MA and enhances the functional coupling of α_{1D}AR. While direct stimulation of α_2AR does not induce constriction in MV, blocking this receptor shifts the concentration – response curve to NE to the right. The α_2AR subtype mediating this interaction in WT mice is predominantly α_{2C}AR subtype. In the

α_{2C}AR KO mice, another α_2AR subtype compensates for the loss of α_{2C}AR function.

b. The mechanism underlying the α_1–α_2AR interaction likely does not involve direct interaction between the two receptors through receptor heterodimerization. No co-localization, co-trafficking or effect on the membrane expression of α_{1D}AR has been detected in HEK-293 cells. In addition, stimulation of the two receptors when co-expressed did not lead to the functional interaction, suggesting that this synergism is specific for certain cellular phenotypes like the MV and occurs at the level of intracellular messengers. While MA also express α_2AR subtypes, this interaction was not detected in these arteries.

-differential receptor expression does not account for the interaction and differences in adrenergic sensitivity of MA and MV

-SMCs from MV express smaller amount of the α_1AR subtypes in the membrane than MA, suggesting more efficient stimulus – response coupling of αARs instead of increased receptor reserve as the underlying mechanism for enhanced sensitivity of MV

c. α_2AR promotes Ca^{2+} influx through L-type VOCC and SOC and perhaps some other yet unidentified Ca^{2+} channel and this action facilitates increased Ca^{2+} accumulation and contraction. This mechanism may underlie the functional interaction between α_1 – and α_2ARs in MV

d. MV posses larger Ca^{2+} reserve in the SER than MA. This can lead to increased Ca^{2+} release from the endoplasmic reticulum at any given IP_3 produced by the stimulation of αAR. MV are more dependent on intracellular

Ca^{2+} than MA. When the stores are depleted, potentiation of Ca^{2+} influx by NCX facilitates responses to NE in MA, but not in MV. Intracellular Ca^{2+} stores are necessary for NE response in MV. IP_3 – independent CICR may also prove to be important Ca^{2+} release mechanism in MV.

e. SOC are coupled to contractile responses in MV but not in MA. SERCA inhibition activates SOC and this results into profound constriction of MV, while the vascular tone in MA is unaffected. This may be attributed to differential spatial organization of Ca^{2+} handling proteins between MA and MV, where SOC can directly activate proteins of the contractile apparatus in the MV. While SOC are present and their activation enhances intracellular Ca^{2+} in MA, they may be more compartmentalized in the MA. In addition, SOC appear to play some functional role in adrenergic responses of MV, but not MA.

f. MA posses more efficient alternative pathways to remove Ca^{2+} that enters the cell through SOC compared to MV when SERCA in inhibited. NCX contributes to Ca^{2+} removal in both MA and MV, although in MA, this process is more efficient. These differences in Ca^{2+} handling contribute to the differential responsiveness to SERCA blockade between MA and MV. However, the more efficient Ca^{2+} removal by NCX in MA can not be attributed to increased expression of NCX protein, because large expression of NCX was detected in the plasma membrane of both arterial and venous myocytes.

-Responses to contractile agonist are longer lasting in MV than MA, further indicating less efficient Ca^{2+} efflux pathways in MV than MA. Not only NCX, but also PMCA contributes to Ca^{2+} removal in MV. PMCA also contributes

to the maintenance in Ca^{2+} homeostasis during resting conditions in MV. Less efficient Ca^{2+} removal could also contribute to increased Ca^{2+} accumulation during agonist stimulation and enhanced functional responsiveness.

Altogether, these studies suggest that while there may not be major differences in the expression of αAR between MA and MV, αAR in MV are more efficiently coupled to contractile responses in vascular capacitance vessels. Previous studies did not suggest that there are any major differences in the contractile proteins between MA and MV (Yamboliev et al., 2002), indicating that differences in signal transduction and Ca^{2+} handling are indeed the likely candidates explaining the differences in vascular reactivity.

All five points described above could contribute to larger sensitivity of MV than MA. When SNS activity is slightly elevated in pre-hypertensive subjects, it may selectively target capacitance vasculature. Considering that α_2AR enhances α_1AR signaling at low stimulation thresholds, α_2AR antagonists may selectively block an increase in venous tone when baseline SNS activity is slightly elevated.

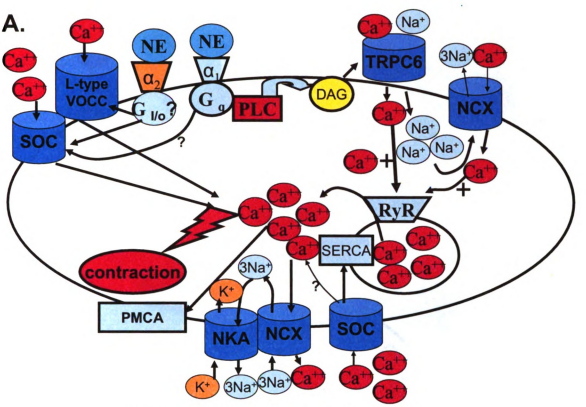
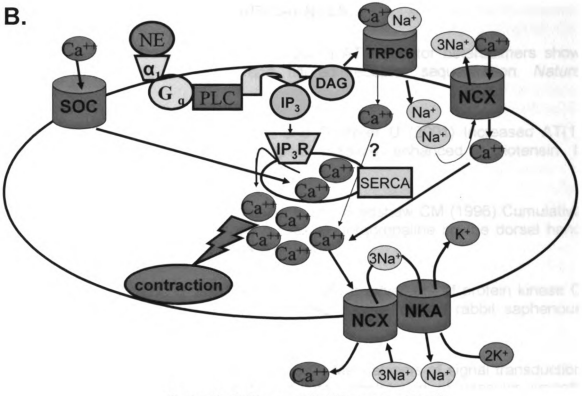


Fig. 38A. Model of the proposed adrenergic receptor signaling and calcium coupling in venous smooth mucle cell. Activation of α_1AR by NE leads to production of IP_3 and DAG. DAG activates receptor – operated channels (ROC, likely TRPC6) that results into Na^+ and Ca^{2+} influx. Ca^{2+} may either act on ryanodine receptors to activate Ca^{2+}-induced Ca^{2+} release (CICR). The accumulation of Na^+ in small microdomains could reverse the activity of NCX, driving additional Ca^{2+} influx and CICR, producing constriction. α_2AR is also activated by NE and couples to Ca^{2+} influx through L-type voltage – operated Ca^{2+} channels (VOCC) and/or store operated channels (SOC). SOC can also directly participate in vascular constriction. During relaxation, Ca^{2+} is taken up by plasma membrane Ca^{2+} - ATPase (PMCA) and reverse mode NCX. The activity of NCX and therefore Ca^{2+} export is regulated by other transporters that regulate Na^+ gradient across plasma membrane, like the Na^+/K^+-ATPase.



(Fig 38 continued). Model of the proposed adrenergic receptor signaling and calcium coupling in arterial smooth mucle cell (B). α_1AR in arteries activate phospholipase C and production of IP_3 and DAG. IP_3 activates IP_3 receptors on smooth endoplasmic reticulum, leading to Ca^{2+} release from the stores. DAG activates ROC, perhaps TRPC6, producing Ca^{2+} and Na^+ influx in localized regions near the plasma membrane. Whether this Ca^{2+} is sufficient to produce constriction remains to be evaluated. However, increased concentration of Na^+ causes NCX to operate in its reverse mode, producing Ca^{2+} influx that directly participates in arterial contraction. SOC refill the Ca^{2+} stores when Ca^{2+} is depleted. However, they do not directly participate in arterial constriction. During relaxation, Ca^{2+} extrusion is very efficient and NCX contributes to this process.

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