ABSTRACT

Cerebral arteries play an important role in the regulation of cerebral blood flow through autoregulation to supply oxygen and nutrients for the brain and neurons. Although investigation of the activity of cerebral blood vessels has a long and distinguished history, there exists a deficit in the pharmacology of the isolated mouse cerebrovascular system.

In this thesis, we designed protocols to determine whether the mouse middle cerebral artery (MCA) possessed similar properties with respect to myogenic control and responsiveness to vasoconstrictors and vasodilators. We found that the intrinsic tone of the mouse MCA was evoked at low pressures with no significant additional constriction occurring at higher pressures (>50mmHg). Inhibition of nitric oxide (NO) and endothelin-1 (ET-1) altered the extent of pressure-induced myogenic tone. Unlike the general insensitivity of cerebral arteries to adrenergic receptor stimulation in most other species, the mouse MCA constricted to α-adrenergic receptor activation. Interestingly, 5-HT and histamine, which are potent vasomotor factors, did not elicit any effect on the mouse MCA. In summary, the mouse MCA has a pharmacological profile that is distinct from other species, including humans; however, similar to findings in other cerebral arteries, the mouse MCA shows intracellular sensitization to Ca$^{2+}$ following receptor activation.

Mouse is the most commonly used species for constructing a genetically modified model to explore the intricacies of cerebrovasculature system; therefore, our study provides pertinent input for the further characterization of the pathophysiology and dysfunction in cerebral vasculature.
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LIST OF ABBREVIATIONS AND UNITS

The following abbreviations, definitions and units have been used throughout this thesis.

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<th>Definition</th>
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<tbody>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BK</td>
<td>bradykinin</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>calcium ion</td>
</tr>
<tr>
<td>[Ca(^{2+})](_i)</td>
<td>intracellular Ca(^{2+}) concentration</td>
</tr>
<tr>
<td>[Ca(^{2+})](_e)</td>
<td>extracellular Ca(^{2+}) concentration</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CaM</td>
<td>Ca(^{2+})-calmodulin</td>
</tr>
<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>chloride ion</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanidine monophosphate</td>
</tr>
<tr>
<td>CO(_2)</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>EC(_{50})</td>
<td>effective dose eliciting 50% response</td>
</tr>
<tr>
<td>ECE</td>
<td>endothelin-converting enzymes</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelium-dependent relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii (Latin, 'and others')</td>
</tr>
<tr>
<td>ET(_A)</td>
<td>endothelin type A receptor (on vascular smooth muscle)</td>
</tr>
<tr>
<td>ET(_B)</td>
<td>endothelin type B receptor (on endothelium)</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>water</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est (Latin, 'that is')</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>K(^+)</td>
<td>potassium ion</td>
</tr>
<tr>
<td>K(_{Ca})</td>
<td>Ca(^{2+})-sensitive K(^+) channel</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L</td>
<td>litre(s)</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NG-nitro-L-arginine-methyl ester</td>
</tr>
<tr>
<td>m</td>
<td>meter(s)</td>
</tr>
<tr>
<td>M</td>
<td>mole(\text{-}1)</td>
</tr>
<tr>
<td>MCA</td>
<td>middle cerebral artery</td>
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</tbody>
</table>
MetHb ................. methemoglobin
MgCl₂ ................ magnesium chloride
Min ...................... minute(s)
MLC ...................... myosin light chain
MLCK .................... myosin light chain kinase
MLCP .................... myosin light chain phosphatase
n ........................ number of animals
NaCl ..................... sodium chloride
NE ........................ norepinephrine
NOS ...................... nitric oxide synthase
O₂ ........................ molecular oxygen
OxyHb .................... oxyhaemoglobin
p value ................... probability (of incorrectly rejecting the null hypothesis)
PE ........................ phenylephrine
PH ......................... logarithmic unit measuring acidity
PKC ...................... protein kinase C
RBC ...................... red blood cell
PCO₂ ..................... carbon dioxide tension
PGI₂ ..................... prostacyclin
PO₂ ...................... oxygen tension
PRP ...................... platelet-rich plasma
PSS ...................... physiological salt solution
ROC ...................... receptor-operated channel
RyR ...................... ryanodine
SAH ...................... subarachnoid hemorrhage
SD ........................ standard deviation of the mean
SEM ........................ standard error of the mean
SOD ...................... superoxide dismutases
SR ........................ sarcoplasmic reticulum
VSMC .................... vascular smooth muscle cell
VSP ...................... vasospasm

Mathematical prefixes
k ....................... kilo \(10^3\)
c ....................... centi \(10^{-2}\)
m ....................... milli \(10^{-3}\)
μ ....................... micro \(10^{-6}\)
n ....................... nano \(10^{-9}\)
ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Ismail Laher, for the experience I have gained working with him. His knowledge, guidance and profound insight have sharpened my critical thinking during the course of my research. I would also like to thank the members of my committee: Dr. Catherine C. Y. Pang and Dr. Xiaodong Wang for reading my thesis and offering advice.

I had the incredible good fortune to have known Farzad Moien-Afshari, a wonderful colleague and friend in the lab. He was always willing to set aside time to help out with things I was working on. It would have been difficult to accomplish what I did without his cheerfulness and willingness help.

I also want to acknowledge my previous colleagues Anie Min and Adrian Hui who taught me the techniques and were willing to help me anytime. I would like to thank Shaila Jamaluddin Merchant who encouraged me all the time and provided invaluable help for my thesis. The short but enjoyable time working with her in the lab is unforgettable.

Most importantly, I would like to send my deepest thanks to my family: my parents, my brother and my husband who always stood by me and without whom none of this would have been possible. Their unconditional love and support are the most valuable thing I have had in my life.

Finally, I would like to acknowledge the financial support from Natural Sciences and Engineering Research Council of Canada.
CHAPTER 1
CEREBRAL VASCULATURE AND AUTOREGULATION

1.1 INTRODUCTION
Although the brain is only about 2% of the total body weight in humans, it consumes 15-20% of the total cardiac output due to the high-energy demand of brain cells and neurons, and the inability of brain to store essential nutrients (oxygen and glucose). Nerve cells begin to die within 3-5 minutes thereby causing irreversible brain damage if the brain is completely deprived of blood. To provide a sufficient and uninterrupted blood supply, cerebral circulation possesses a characteristic feature to maintain relatively constant blood flow in the brain over a wide range of arterial blood pressures (Busija and Heistad 1984a). The independence of cerebral blood flow (CBF) from arterial blood pressure is called cerebral autoregulation, which is a relatively robust response and occurs even following the development of atherosclerosis (Heistad et al. 1980). Autoregulation is critical to satisfy the metabolic needs of the brain despite the fluctuations in arterial blood pressure. Autoregulation also protects downstream vessels from damage resulting from arterial hypertension (Faraci and Heistad 1990). It has been reported that a variety of modulators, including neurogenic, metabolic and myogenic factors, are involved in the autoregulatory response. Among these regulators, myogenic factors may be predominant during arterial hypertension, while other factors are likely more important during the decline of blood pressure. Autoregulation of CBF is easily abolished by trauma, hypoxia or other noxious stimuli in the brain.

1.2 NEUROGENIC REGULATION IN CEREBRAL VASCULATURE
Cerebral blood vessels are extensively innervated with inputs from sympathetic parasympathetic and sensory fibers (Busija and Heistad 1984a). It has also been reported that central pathways, existing exclusively within the brain itself, innervate cerebral resistance vessels; however, evidence has shown that cerebral autoregulation occurs despite removal of
various neural influences, suggesting that the nerve supply of the cerebral arteries is not necessary for this response (Rapela et al. 1967, Waltz et al. 1971). Although the relationship between neurogenic modulation and cerebral autoregulation is controversial, it is clear that nerve stimulation modifies the autoregulation curve, shifting it toward higher pressure levels. Other features such as the speed of vascular response, may also be modulated by the neurogenic factors (Salanga and Waltz 1973).

1.3 METABOLIC REGULATION IN CEREBRAL VASCULATURE

The brain has a high and rather stable global metabolic rate of oxygen consumption in sleep, resting wakefulness, and while performing motor and/or sensory work. Only in pain and in anxiety are increases seen in total cerebral oxygen uptake (by 20% to 30%) (Ingvar et al. 1976, Kety 1975). Cerebral blood flow, a main determinant of the oxygen and glucose supply, is also relatively high (approximately 50ml/100g/min) and stable with increases in pain and anxiety. Since oxygen tension (PO₂) variations around the normal level (approximately 100 mmHg) do not influence CBF, or even tend to increase during enhanced brain activity, it is unlikely that the suppressed local oxygen is the principal messenger to adjust flow to match metabolism. However variations in arterial CO₂ tension (PCO₂) exert a profound influence on CBF. Hypercapnia causes dilatation, while hypocapnia induces constriction. (Gotoh et al. 1961). On the other hand, marked hypoxia causes clear-cut cerebral vasodilatation (see detailed discussion below). The additional factors that couple flow to metabolism are H⁺, K⁺, Ca²⁺ and adenosine concentration.

1.4 MYOGENIC RESPONSE IN CEREBRAL AUTOREGULATION

1.4.1 INTRODUCTION

Myogenic response is defined as a phenomenon in which blood vessels respond to transmural pressure elevation with constriction, and to pressure reduction with dilation. This behavior is inherent to smooth muscle and is independent of neural, metabolic and hormonal influences.
(Johnson 1978). The myogenic response is a unique property of resistance vessels (with lumen diameter of 70-200μm) by which blood flow and blood pressure are regulated. Although the myogenic response is mainly observed in arterioles, it can be occasionally demonstrated in arteries, venules, veins and lymphatics.

1.4.2 PHYSIOLOGICAL SIGNIFICANCE
The most important functions of the vascular myogenic response are the establishment of basal vascular tone and autoregulation of blood flow and capillary hydrostatic pressure. Basal vascular tone establishes an underlying arteriolar constriction upon which other control mechanisms produce vasodilation or vasoconstriction. The local regulation of blood flow protects capillary beds from large increases in hydrostatic pressure during variation in systemic arterial pressure (Johnson 1978).

1.4.3 MECHANISMS UNDERLYING THE MYOGENIC RESPONSE
Uchida and colleagues have been credited with the initial suggestion that the myogenic response may reflect an improved excitation-contraction coupling resulting from membrane depolarization and increased Ca$^{2+}$ permeability (Uchida and Bohr 1969). Although the exact signal transduction mechanisms underlying myogenic tone remain to be elucidated, it is clear that the phenomenon resides within the vascular smooth muscle cell, and can be modulated by a variety of mechanisms, such as vascular smooth muscle depolarization, mechanosensitive channels, nonselective cation channels, K$^+$, Cl$^-$, and voltage gated Ca$^{2+}$ channels (Davis and Hill, 1999). Moreover, other possibilities have also been suggested, including the control of myosin light chain-phosphorylation, the smooth muscle binding proteins caldesmon and calponin, protein kinase C (PKC), G-proteins, the adenylate cyclase, arachidonic acid pathways, and the cytoskeleton and extracellular matrix (Davis and Hill, 1999).
1.5 ENDOTHELIUM

1.5.1 INTRODUCTION
Endothelium is located at the interface between the blood and the vessel wall. The endothelial cells are in close contact and form a layer that prevents blood cell interaction with the vessel wall. Moreover, the dynamic tissue performs many other active functions, such as the secretion of vasoactive substances (endothelin-1, nitric oxide) to regulate vascular smooth muscle tone, and hence CBF, the regulation of coagulation, leukocyte adhesion, and vascular smooth muscle cell proliferation and migration.

1.5.2 BLOOD-BRAIN BARRIER
In most of vessels, endothelial tissue has small spaces between each individual cell, thereby substances can move readily between the inside and the outside of the vessel. However, in the brain, the endothelial cells are joined by tight junctions making up the so-called blood-brain barrier (BBB), which allows some materials (glucose, iron, amino acids, peptides, small organic acids) to access the brain, but prevents others from passing. The BBB has several important functions: 1) prevents the brain from "foreign substances" in the blood that may injure the brain tissue; 2) protects the brain from hormones and neurotransmitters in the rest of the body; 3) maintains homeostasis in the brain.

1.5.3 ENDOTHELIUM AND MYOGENIC TONE
The hypothesis that vascular endothelium was prerequisite to myogenic responsiveness was suggested by Harder (Harder, 1987) and was confirmed by Katusic who showed that endothelium removal from canine basilar arterial rings prevented the active force development by stretch. However, subsequent studies demonstrated that the mechanical denudation of endothelium did not retard the generation of the myogenic response on various types of vessels.
Currently, it is widely accepted that the myogenic response is the consequence of the mechanical stimuli that directly act on the vascular smooth muscle to evoke vasoconstriction independent from the intact endothelium. On the other hand, the stretch or distension of vascular wall augments the production of endothelium-derived vasoconstrictors or decreased the release of an endothelium-derived relaxing factor which may modify the myogenic response (Meininger et al., 1992).

1.6 MEMBRANE POTENTIAL

1.6.1 INTRODUCTION
Electrophysiological events occurring at the plasma membrane regulate many cellular functions within excitable cells. With respect to vascular smooth muscle cells, one of the principal events primarily regulated by changes in resting membrane potential (E_m) is activation of contractile elements and cell shortening. The precise degree of E_m involved in activation of smooth muscle cells varies depending upon the vascular types as well as the species.

The resting E_m of cerebrovascular smooth muscle cell studied in an organ bath is between –60 to –70mV (Harder 1980; Lombard et al. 1986). This is maintained largely by the cell permeability to K^+ (Hirst, 1989). Three physiologically relevant types of channels which may play a role in the regulation of E_m have been identified; they are delayed rectifier K^+ channel (Okabe et al. 1987), Ca^{2+}-sensitive K^+ channel (K_{Ca}) (Benham et al. 1986; Wilde and Lee 1989), and ATP-sensitive K^+ channel (Standen et al. 1989). The K_{Ca} channel is activated as intracellular Ca^{2+} increases, but may be sensitive to extracellular Ca^{2+} as well. The ATP-sensitive K^+ channel is turned off as intracellular ATP increases beyond some critical value and is not only sensitive to ATP but also regulated by a variety of intracellular mediators including pH and PO_2 (Ashcroft 1988; Davies 1990).
1.6.2 PHYSIOLOGICAL SIGNIFICANCE

1.6.2.1 Membrane potential and vasomotor response

Membrane potential is an important determinant of intracellular \( \text{Ca}^{2+} \) concentration ([\( \text{Ca}^{2+} \)]_i), hence regulating the vasomotor response. The hyperpolarization mediated by the extrusion of \( \text{K}^+ \) reduces the open probability of \( \text{Ca}^{2+} \) channels, leading to vasorelaxation. On the other hand, when \( \text{K}^+ \) channels close there is resultant depolarization and vasoconstriction. The classical model of electromechanical coupling considers that membrane potential determines [\( \text{Ca}^{2+} \)]_i. In turn, [\( \text{Ca}^{2+} \)]_i also can regulate membrane potential by modulating the open probabilities of ion channels, suggesting that there is a network of positive and negative feedback loops regulating [\( \text{Ca}^{2+} \)]_i as well as membrane potential (Carl et al. 1996).

1.6.2.2 Membrane potential and transmural pressure

Increasing transmural pressure in an isolated cerebral arterial segment depolarizes muscle cells, induces spontaneous action potential generation, and reduces internal diameter (Harder 1984). Either pressure or the subsequent mechanical deformation of the vascular muscle wall activates arterial muscle by depolarizing the membrane and increasing \( \text{Ca}^{2+} \) permeability.

The “sensor” translating the mechanical stimulus into the observed biophysical process in arterial muscle is not yet well defined (Smeda and Daniel 1988; Osol and Halpern 1985; Halpern and Osol 1985). One of putative “pressure-sensors” is a “stretch-activated” ion channel either in the vascular muscle, endothelial cell, or both. The majority of mechanosensitive ion channels are nonspecific and exhibit conductance for multiple ion species with the greatest level of selectivity being either for cations or anions (Morris 1990). These mechanosensitive channels are considered as plausible mediators for the muscle cell depolarization and action potential generation observed in cerebral arteries upon elevations in transmural pressure.
1.6.2.3 Membrane potential and hypoxia
In the cerebrovascular system, hypoxia primarily increases the open probability and mean open time of $K_{Ca}$ channels which hyperpolarize vascular smooth muscle cell to mediate vasorelaxation, thereby increasing CBF. Hypoxia may also reduce tone by stimulating the activity of an ATP sensitive $K^+$ channel (Daut et al. 1990). When the level of intracellular ATP drops due to hypoxia, there is an augmentation of signal $K^+$ channel activity, which increases the outward current, hyperpolarizes and then relaxes arterial muscle.

1.6.2.4 Membrane potential and PCO$_2$/pH
Reduction of extracellular pH either by increases in PCO$_2$ or in H$^+$ concentration dilates cerebral arteries. This dilation is mediated by membrane hyperpolarization in isolated cerebral arteries (Harder 1982). Reduction of PCO$_2$ or elevation of pH depolarizes and contracts cerebral arteries. Both of these effects are apparently mediated at least in part by changes in the conductance of $K^+$. In patch-clamped isolated cerebral arterial muscle cells, reduction of pH from 7.4 to 7.20 greatly increases the peak outward current by 35% (Harder and Madden 1985).

1.7 VASOACTIVE MEDIATORS
1.7.1 NITRIC OXIDE
1.7.1.1 Synthesis, storage and release
In 1980, Furchgott and Zawadzki discovered that nitric oxide (NO) is the endothelium-dependent relaxing factor (EDRF)(Furchgott and Zawadzki, 1980). Endogenous nitric oxide (NO) is converted from L-arginine by nitric oxide synthase (NOS); in addition, NO may also be formed from endogenous nitrate ion. Being a gaseous mediator, NO is not stored in cells but produced in response to a number of agonists, such as acetylcholine, bradykinin and substance P. After being produced, NO rapidly diffuses from its synthesis site to surrounding tissues. The
family of NOS consists of three isoforms known as: neuronal NOS (nNOS), found in epithelial and neuronal cells; endothelial NOS (eNOS), formed in endothelial cells, and inducible (iNOS), produced in macrophages and smooth muscle cells.

1.7.1.2 NO in the cerebrovasculature

The relaxation of cerebral smooth muscle by NO released from endothelial cells is due to the stimulation of soluble guanylate cyclase in smooth muscle cell, which results in the formation of cyclic guanidine monophosphate (cGMP) (Rapoport and Murad 1983). The latter activates cGMP-dependent protein kinase, which leads to an upregulated extrusion of Ca$^{2+}$ and vasodilation (Lincoln, 1994). Moreover, cGMP-independent pathways such as activation of $K_{Ca}$ channels, inhibition of vasoconstrictor (20-hydroxyeicosatetraenoic acid) production, and direct inhibitory effects on Ca$^{2+}$ influx and Ca$^{2+}$ release are also responsible for the NO-induced relaxation (Alonso-Galicia et al. 1997; Blatter and Wier 1994; Bolotina et al. 1994; Kannan et al. 1997).

1.7.2 ACETYLCHOLINE

1.7.2.1 Synthesis, storage and release

Acetylcholine (ACh) is synthesized from acetyl-CoA and choline by the enzyme choline acetyltransferase. The availability of choline may serve as the rate-limiting step for the synthesis of ACh. After synthesis, ACh is transported into and stored in synaptic vesicles of the nerve ending. The release of ACh from vesicles requires the entry of Ca$^{2+}$ and the stimulation of an interaction between proteins associated with the vesicles and the membrane. The action of ACh is terminated by metabolism to acetate and choline by the enzyme acetylcholinesterase.

1.7.2.2 ACh in cerebrovasculature
ACh-induced vasorelaxation is due to the presence of muscarinic receptors located on endothelial cells. In cerebral arteries, the stimulation of these receptors, primarily M_3 subtype, increases the influx of Ca^{2+} that activates the synthesis of NO from L-arginine and elicits vasodilation (Moncada and Higgs 1995). In the absence of endothelium, ACh induces vasoconstriction by its direct action on smooth muscle cells mediated by the reduction of cyclic adenosine monophosphate (cAMP), the stimulation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) release, and the activation of G-protein.

Cholinergic vasodilation may be impaired by multiple mechanisms in the ischemic brain and may play a role in the pathogenesis of cerebrovasculature disease. Moreover, a deficit of cerebral ACh is a prominent finding in Alzheimer's disease (Geaney et al. 1990).

1.7.3 ENDOTHELIN

1.7.3.1 Synthesis, storage and release

Endothelins (ETs) are peptide vasoconstrictors produced from the cleavage of big ET-1 by endothelin-converting enzyme (ECE) which is primarily located in endothelial cells of blood vessels. Although ET-1 is mainly produced in endothelial cells, the synthesis also occurs in other cell types such as vascular smooth muscle cells. Under normal condition, small amount of ET-1 is produced and released from cellular vesicles to mediate vascular tone in vivo. In pathological conditions, such as hypoxia and vasospasm, ET-1 production is greatly upregulated (Weir, 1999).

Three different ET peptides (ET-1, ET-2, ET-3) with minor variations in amino acid sequence have been identified in humans. ETs act by at least three different receptor subtypes including ET_A, ET_B-1, and ET_B-2. The ET_A and ET_B-2 receptor are localized in vascular smooth muscle cells and mediate the vasoconstrictor effect (Arai, 1990; Sakurai, 1990). The ET_B-1 subtype
also occurs on vascular endothelial cells and induces the endothelium-dependent vasodilator effect.

1.7.3.2 ET-1 in the cerebrovasculature
ET-1 is a potent and long-lasting constrictor which functions from the adventitial but not the luminal side of cerebral arteries (Yanagisawa, 1988; Masaki, 1991). ET-1 acts by a Ca\(^{2+}\)-dependent mechanism partly mediated by the activation of PKC and phospholipase A\(_2\) (Murray, 1992). Also, ET causes Ca\(^{2+}\) influx via a voltage-operated Ca\(^{2+}\) channel (Luscher, 1992). Moreover, ET-1 activates smooth muscle proliferation and is involved in some forms of hypertension and a number of cerebrovascular disorders, including vasospasm.

1.7.4 5-HYDROXYTRYPTAMINE
1.7.4.1 Synthesis, storage and release
5-Hydroxytryptamine (5-HT) is synthesized through a 2-step process involving a tetrahydrobiopterin-dependent hydroxylation reaction catalyzed by tryptophan-5-monooxygenase and a decarboxylation catalyzed by aromatic L-amino acid decarboxylase. 5-HT is located in several tissues including circulating platelets, central neurons, and cerebral mast cells. After release from serotonergic neurons, most of the released 5-HT is recaptured by an active reuptake mechanism.

1.7.4.2 5-HT in the cerebrovasculature
The serotonergic innervation of cerebral blood vessels was first postulated in 1976 (Chan-Palay 1976; GOTOH et al. 1961). However, the existence of a serotonergic innervation of the cerebrovasculature remains to be elucidated as studies also showed consistent results suggesting that the serotonergic innervation was in fact the noradrenergic sympathetic innervation (Saito and Lee 1987).
As 5-HT does not cross the BBB, its influence on the cerebral circulation cannot be directly assessed. Depending on the sites of investigation and the methods employed, 5-HT elicits vascular relaxation and constriction. Most studies on the vascular effects of 5-HT showed that this amine was a powerful constrictor agent, while 5-HT was also reported to induce vasodilation in small pial arteries (Harper and MacKenzie 1977). The function of 5-HT is exerted upon its interaction with specific receptors identified as 5HT1.7. The majority of cerebral arteries express a 5-HT1-mediated contractile response, although mixed 5-HT1/5-HT2 characteristic is also observed. 5-HT is involved in the pathogenesis of cerebral vasospasm following subarachnoid hemorrhage and also in some cerebrovascular diseases such as stroke, trauma, and migraine (Pappius 1991).

1.7.5 HISTAMINE
1.7.5.1 Synthesis, storage and release
Histamine is formed from histidine by histidine decarboxylase and is mainly stored in lungs, skin and intestinal mucosa. Histamine is released from mast cells by a secretory process during inflammatory or allergic reactions.

1.7.5.2 Histamine in the cerebrovasculature
Intravascularly applied histamine may not affect CBF as long as blood pressure is kept within the autoregulatory range since circulating histamine does not readily cross the BBB (Oldendorf 1971). Extravascularly applied histamine induced pial arterial dilation in most species, while contractile action of histamine was also found in cerebral arteries (Miranda et al. 1992; Ottosson et al. 1988b; Toda 1990; Van Riper and Bevan 1991).
Histamine produces its action via specific receptors, which are H1, H2, and H3. Histamine dilates blood vessels by acting on H1 receptor in humans and a combined action on H1 and H2 receptors in some experimental animals. Histamine-induced vasodilation is partly endothelium-
dependent, whereas at high concentrations, it produces vasoconstriction in cerebral arteries which does not involve specific histamine receptors. Histamine can stimulate inositol phospholipid hydrolysis which results in synthesis of IP\textsubscript{3} and then release of Ca\textsuperscript{2+} from the intracellular store to activate the contractile apparatus (Berridge and Irvine 1984). Additionally, activation of an unselective cation channel which consequently upregulates the influx of Ca\textsuperscript{2+} may also be involved in the contractile action of histamine (Karashima and Kuriyama 1981).

Due to its potent enhancement of permeability and dilatory effects on cerebral vessels, an increased concentration of histamine may induce brain edema. Whether histamine acts as a mediator of vasospasm is still under debate (Table 1.1).

1.8 FACTORS INVOLVED IN THE REGULATION OF VASOMOTOR RESPONSE

1.8.1 CALCIUM

The importance of Ca\textsuperscript{2+} in smooth muscle constriction and arteriolar tone was directly established three decades ago (Uchida 1969; Nakayama, 1982). The advent of Ca\textsuperscript{2+} sensitive fluorescent dyes together with video-based imaging and photometer techniques allows researchers to investigate Ca\textsuperscript{2+} dynamics in resistance vessels.

Smooth muscle contraction or dilation is mainly controlled by the changes in intracellular [Ca\textsuperscript{2+}]. Intracellular Ca\textsuperscript{2+} increases during contraction as a consequence of Ca\textsuperscript{2+} influx from the extracellular space or its release from intracellular stores. Binding molecules for Ca\textsuperscript{2+} in the myoplasm and in the sarcoplasmic reticulum (SR) act as cell buffers which endow a relatively large capacity for Ca\textsuperscript{2+} storage with the low free concentrations in the cells (Horowitz et al. 1996).

The sarcolemma is a barrier across which a 10,000-fold concentration gradient exists between extracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{e}) and [Ca\textsuperscript{2+}]\textsubscript{i}. Ca\textsuperscript{2+} enters the sarcoplasm via receptor-operated channels (ROCs), voltage-dependent channels (L type), and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger.
Ca\textsuperscript{2+} is removed from the sarcoplasm by a plasma membrane ATPase as well as the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger.

The second Ca\textsuperscript{2+}-integrating system is in the SR which is an intracellular Ca\textsuperscript{2+} pool with more than 10 times the Ca\textsuperscript{2+} needed to trigger a single contraction. At least two types of Ca\textsuperscript{2+} channels are present in the SR. One is sensitive to IP\textsubscript{3}; the other type is sensitive to ryanodine (RyR) and caffeine. The efflux of Ca\textsuperscript{2+} from the Ca\textsuperscript{2+} store is linked to the binding of the second messenger IP\textsubscript{3} or RyR to their receptors on the SR. The reduction in stored Ca\textsuperscript{2+} stimulates the release of a Ca\textsuperscript{2+} influx factor, which facilitates the entry of Ca\textsuperscript{2+} through the plasmalemma via depletion-operated Ca\textsuperscript{2+} channels. On the other hand, the drop in lumenal free Ca\textsuperscript{2+} makes it easier for sarcolemmal Ca\textsuperscript{2+} ATPase to pump Ca\textsuperscript{2+} into the SR against a lesser concentration gradient.

1.8.2 MYOSIN LIGHT CHAIN KINASE AND MYOSIN LIGHT CHAIN PHOPHATASE

A major factor in the regulation of vasomotor action is the phosphorylation of myosin. The degree of myosin phosphorylation depends on the relative activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (Stull et al. 1998). MLCK (105 kDa) is a Ca\textsuperscript{2+}-dependent protein kinase and its primary regulator is Ca\textsuperscript{2+}/Ca\textsuperscript{2+}-calmodulin (CaM). When Ca\textsuperscript{2+} binds to calmodulin, there is a conformational change rendering it capable of activating MLCK, which subsequently phosphorylates serine 19 of the myosin light chain (MLC). The phosphorylation MLC greatly increases actin-activated myosin Mg-ATPase activity, which ultimately initiates smooth muscle contraction.

1.8.3 Rho KINASE

The Ca\textsuperscript{2+}-mediated pathway has long been regarded as the principle mechanism by which MLC phosphorylation is regulated; however, a second pathway regulating the phosphorylation state of MLC was found by analyzing muscle cells. Studies show that various physiological stimuli
can induce smooth muscle contraction also in the absence of an increase in the free cytosolic 
Ca\textsuperscript{2+} concentration (Bradley and Morgan 1987; Himpens et al. 1990; Rembold 1990). 
Subsequent studies revealed that this “Ca\textsuperscript{2+}-independent” regulation occurs through the 
inhibition of myosin phosphatase and involves the monomeric GTP-binding protein RhoA 
(Bradley and Morgan 1987; Hirata et al. 1992; Gong et al. 1996; Somlyo and Somlyo 1994). 
Activation of RhoA leads to the activation of Rho-kinase which in turn phosphorylates the 
regulatory myosin-binding subunit of myosin phosphatase and results in the inhibition of the 
increased actomyosin interaction can be achieved through Ca\textsuperscript{2+}-mediated MLCK activation and 
through Rho-dependent inhibition of RLC dephosphorylation, both leading to increased RLC 
phosphorylation.

1.9 THE TECHNIQUES FOR THE INVESTIGATION OF CEREBROVASCULAR 
FUNCTIONS

During 1930s, Forbes and Fog performed the first systematic attempts to analyze 
cerebrovascular smooth muscle reactivity using a cranial window technique, comprised of a 
craniotomy and the superfusion of artificial cerebrospinal fluid on the exposure area. This 
preparation could visualize pial arteriole diameter hence examine the alterations of cerebral 
circulation following changes in blood pressure and resulting from the administration of 
various agents (Purves, 1972). It was not until the beginning of 1970s that isolated vessel 
techniques were developed (Nielsen and Owman 1971). Nowadays, the techniques (isometric 
and isotonic preparation) enable more careful and precise quantitation in resistance arteries. 
Particularly, the isotonic preparation more closely mimics the physiological condition and 
serves as an useful approach to distinguish the effect of a desired objective from flow, 
metabolic, neural, and endothelial influences in the cerebrovasculature.
CHAPTER 2
PHARMOCOLOGY OF ISOLATED MOUSE MEDDLE CEREBRAL ARTERY

2.1 INTRODUCTION

Cerebral arteries supply oxygen and glucose to the brain; remove waste products and regulate the ionic environment of the cerebral circulation (Thorin et al. 1997a). They also play an important role in the regulation of cerebral blood flow through autoregulation. Although much is known about the cerebral circulation, many essential mechanisms of autoregulation and features of pathological changes in the cerebral circulation, such as subarachnoid hemorrhage and stroke, have not been elucidated (Table 1.1). Due to these shortcomings, various animal models have been developed to improve our understanding of the pathophysiology of the cerebral circulation with the goal of establishing strategies for the prevention and treatment of cerebral circulatory disorders. To better understand cerebral ischemia, mice lacking both alleles for neurotrophin 4 or deficient in a single allele for brain-derived neurotrophic factor were selected (Endres et al. 2003). Likewise, in order to identify mechanisms of oxygen-induced cerebral vasoconstriction, mouse models either overexpressing or lacking extracellular superoxide dismutase (SOD) were constructed (Demchenko et al. 2002). By overexpressing transforming growth factor-beta1, transgenic mice were also used to explore the etiology of cerebrovascular abnormalities prominent in Alzheimer's disease (Buckwalter et al. 2002). Thus there are a variety of murine models of cerebrovascular disease where a number of molecular and structural elements of cerebral disorders have been gleamed.

However, the pharmacological properties of mouse cerebral arteries have not been described, and there exists a deficit in our knowledge of the mouse cerebrovascular system. Thus, we designed protocols that would determine whether the cerebral blood vessels of the mouse possessed similar properties with respect to myogenic control and responsiveness to...
vasoconstrictors and vasodilators. We undertook this study by using two forms of vasoconstrictors -- increases in transmural pressure because pressure-induced regulation of artery diameters is a key component of cerebral autoregulation, and receptor agonists known to have vasoconstrictor efficacy in the cerebral circulation of other species.

2.2 HYPOTHESES AND OBJECTIVE

My hypothesis was that the mouse cerebral circulation should respond to changes in transmural pressure as well as vasoconstrictors, such as 5-HT and vasodilators, such as ACh, in a comparable manner as for cerebral vessels from other species. Likewise, we also examined the effects of several known endothelium-dependent vasodilators. In addition, I used the pressurized isolated mouse middle cerebral artery (MCA) to simultaneously measure changes of $E_m$ and arterial diameter or changes in intracellular Ca$^{2+}$ and arterial diameter in response to these vasoactive stimuli.

2.3 MATERIALS AND METHODS

2.3.1 TISSUE PREPARATION

The mice used in this study were housed in a temperature- and humidity-controlled environment with a 12-hour light/dark cycle and free access to food and water. Male CD-1 mice weighing between 38 -- 45g were injected with sodium pentobarbital (30mg/kg) and heparin sulfate (500U/kg) intraperitoneally. After loss of all reflexes, the mouse was decapitated and the brain was removed from the cranium and placed in ice-cold (4°C) physiological salt solution (PSS). The mouse MCA (intraluminal diameter, 80 - 90µm) was dissected and transferred to the chamber of a pressure myograph that contained two glass micropipettes (tip diameter 35 -- 45µm). The distal cannula was occluded and the proximal one was connected to a pressure servo system (Living Systems Instrumentation, Burlington, VT, USA). The vessel
was mounted on the proximal cannula with a single strand of braided 4-0 silk suture, and blood was removed by gently perfusing PSS into the vessel. The other end of the vessel was then mounted on the distal cannula.

Each vessel was bathed in PSS, which continually circulated at a flow rate of 20 -- 30 ml/min from a reservoir. A gas mixture containing 95%O₂ and 5% CO₂ was used to aerate the PSS. After gassing, the pH was maintained between 7.35 -- 7.45. A circulating water bath and a glass heat exchange coil were used to maintain the temperature between 36.5 -- 37°C. After the vessel was positioned between the tips of the two cannulae, the chamber was placed on the stage of an inverted microscope. A Video Dimension Analyzer measured inner diameter and data were recorded on a personal computer.

2.3.2 EXPERIMENTAL PROTOCOL

2.3.2.1 Myogenic tone and endothelial factors

After a MCA was mounted, intravascular pressure was gently increased to 80 mmHg at which point the bath temperature was raised to 37°C. The artery segment was equilibrated for 60 minutes at 80 mmHg during which time the vessel spontaneously developed pressure-induced myogenic tone. The pressure-diameter relationship was obtained by increasing the intraluminal pressure from 10mmHg to 110mmHg in 10mmHg increments, and 5 minutes allowed for the vessel to achieve a new steady-state diameter at each new pressure.

To determine the role of nitric oxide (NO) in modulating the intrinsic tone, NG-nitro-L-arginine-methyl ester (L-NAME) (10μM), a NO synthase (NOS) inhibitor, was added to the external reservoir for 30 minutes and the pressure-diameter curve was obtained in an identical way.
To further examine whether endothelin contributes the generation of pressure-induced tone, bosentan (1μM), an ET<sub>A</sub> and ET<sub>B</sub> receptor antagonist, was applied following the same procedure in a separate series of experiments and the pressure-diameter curve was recorded.

2.3.2.2 Vasoconstrictor effects
All responses to vasoconstrictors were studied at 20mm Hg. The constrictor effects of endothelin-1 (ET-1, 10pM - 0.1μM), phenylephrine (PE, 1nM - 0.1mM), U46619 (10pM - 10μM), 5-HT (1nM - 10μM), and histamine (1nM - 10μM) were studied by making cumulative additions to the external reservoir to construct cumulative concentration-response curves. In addition, the responses to various concentrations of KCl were examined.

2.3.2.3 Vasodilator effects
MCA was pretreated with PE (5μM) at 20mm Hg. After a sustained constriction was evoked, tissue was exposed to ACh (1nM - 50μM) added to the external reservoir, and the final maintained diameters were recorded. To further clarify whether NO was involved in the response to ACh, the vessel was exposed to L-NAME (1μM) for 15 minutes following washout of ACh. The identical protocol was used to study the vasodilator effects of bradykinin (BK, 0.1nM - 10μM), and substance P (1pM - 0.1μM). Moreover, in other experiments, 5-HT (1nM - 10μM), and histamine (1nM - 10μM) were also added in PE pre-constricted vessels to determine possible vasodilatory effects in mouse MCA.

To study whether NO is involved in the vasodilatation of 5-HT and histamine, L-NAME (1μM) was incubated for 15 minutes after the pre-constriction by PE (5μM) at 20mm Hg.

At the end of each experiment, PSS was substituted with calcium-free PSS to obtain the maximal passive diameter of each vessel.
2.3.2.4 Measurement of intracellular Ca$^{2+}$ concentration

Mouse MCAs were first cannulated in a pressure-myograph chamber as described above. The arteries were then incubated with the Ca$^{2+}$-sensitive fluorescent dye, fura-2 AM 5µM (20µl of anhydrous DMSO was used to dissolve 50µg fura-2 AM and was diluted with 10ml of PSS to yield a final concentration of 5µM), and pluronic acid (0.01%; wt/vol) for 30 minutes at room temperature in oxygenated PSS followed by washout with PSS for 30 minutes at 37°C (Coombes et al. 1999; Gollasch et al. 1998; Knot and Nelson 1998b; Lagaud et al. 2002a; Lohn et al. 2001b). Excitation was performed using a 75w xenon arc. The excitation wavelength was alternatively switched between 340 - 380 nm using a diffraction grating, and the emission was recorded at 510nm. The emitted fluorescence was converted to relative measures of [Ca$^{2+}$], using Felix quantitative ratio fluorescence software (Photon Technology International; Monmouth Junction, NJ). The measurements in each vessel were normalized to the maximum recorded in response to 60 mM KCl. Ratio was measured and compared at different bath concentrations of PE (10nM -10µM) and KCl (20,40,60,and 80mM). In these experiments, changes in intracellular Ca$^{2+}$ and changes in arterial diameter were recorded simultaneously.

2.3.2.5 Measurement of membrane potential

Mouse MCA was studied using a pressure myograph as described above. The chamber was then transferred to the stage of an inverted microscope (Ziess, Axiovert 25) where changes in $E_m$ and in internal diameter were recorded simultaneously. $E_m$ was measured in vascular smooth muscle cells (VSMCs) of arteries by inserting microelectrodes pulled from borosilicate glass with filament (OD: 1.2mm, ID: 0.69mm) using a Flaming/Brown micropipette puller (model p-97, Sutter Instrument Co.). Microelectrodes were backfilled with 3M KCl solution (tip resistance 100 - 150 MΩ) and mounted on an electrode holder (Axon Instrument, Inc.) connected to a micromanipulator (MP-285, SUTTER Instrument Comp.), which was used to
move the electrodes towards the arterial wall for insertion. A reference electrode (Ag-AgCl) was positioned in the solution inside the chamber. Microelectrodes pierced the vessels from the adventitial side and the electrical signal obtained was amplified using an AxoClamp 2B amplifier (Axon Instruments, Inc.). The amplified signal was continuously monitored and recorded by a personal computer using Axoscope 8.1 software (Axon Instruments, Inc.). $E_m$ was measured at different transmural pressures and also after 15 - 20 minutes incubation with PE or different concentrations of KCl. The criteria for accepting a record were i) a sharp negative deflection in potential upon entry; ii) stable recording for at least one minute after entry; and iii) a sharp positive deflection to 0mV upon exiting from the recorded cell.

2.3.2.6 Solutions and chemicals
The PSS consisted of the following (in mM): NaCl 119, KCl 4.7, KH$_2$PO$_4$ 1.18, NaHCO$_3$ 24, MgSO$_4$·7H$_2$O 1.17, CaCl$_2$ 1.6, glucose 5.5 and EDTA 0.026. Ca$^{2+}$-free solution was PSS containing no CaCl$_2$ and 2.0 mM EGTA. Equimolar concentrations of K$^+$ replace Na$^+$ for depolarizing solutions of KCl. U46619 was obtained from Cayman Chemical (Ann Arbor, MI). Fura2-AM and Pluronic® F127 were from Molecular Probes. Other drugs were purchased from Sigma (St. Louis, MO).

2.3.2.7 Expression of results and statistical analysis
Myogenic tone at each pressure was expressed as a percent constriction=$100\% \times [(D_{Ca^{2+}}\text{-free} - DPSS)/D_{Ca^{2+}}\text{-free}]$, where $D$ is the diameter in calcium free ($D_{Ca^{2+}}\text{-free}$) or Ca$^{2+}$ containing PSS (DPSS). Percent dilation was calculated using the equation $100\% \times [(D_d - D_b)/(D_{Ca^{2+}}\text{-free} - D_b)]$, where $D$ is the diameter upon stabilization after drug addition (d), baseline (b) or Ca$^{2+}$ free.

Constrictor responses of arteries loaded with fura2-AM were not different from those without the incubation with the dye.
All results were expressed as mean ±SE of n experiments. Data were analyzed with NCSS 2000 and PASS 2000 software using analysis of variance (ANOVA) and/or repeated-measures ANOVA with multiple comparisons performed by Bonferroni’s test when appropriate. LogEC50 (pD2) was calculated by Graphpad Prism, version 3.02. The results of statistical tests were considered statistically significant at \( p<0.05 \).

2.4 RESULTS

2.4.1 MYOGENIC TONE OF MOUSE MIDDLE CEREBRAL ARTERY

Myogenic tone of mouse MCA was initiated at 10mmHg where the arteries were constricted by 3.4±2.1% (n=15). As intravascular pressure was increased, myogenic constriction increased to 20.6±2.4% (n=15) at 90mmHg, which was not significantly different from the myogenic constriction that reached a plateau at 50mmHg (Fig. 2.1A). The addition of L-NAME (10μM) to arteries equilibrated in PSS potentiated the pressure-induced constriction at all pressures between 20 and 110 mmHg. At 90mmHg, L-NAME potentiated vascular tone by 12.4±0.4%(n=4). The myogenic response of vessels equilibrated with bosentan (1μM) was attenuated by 7.2±1.8%(n=4) at 90mmHg(Fig. 2.1B).

2.4.2 VASOCONSTRICTOR RESPONSES

Application of a depolarizing solution of raised \( K^+ \) evoked constriction (Fig. 2.2A). Constriction occurred with low concentrations of KCl, such that with 8mM the constriction was 3.6±2.1%(n=6) in the mouse MCA. With cumulative additions of KCl, the maximum constriction achieved was 63.3±3.3% at 66mM; increasing the concentration of KCl to 114mM did not increase tone further. The EC50 for KCl was 31.0 ± 4.1mM.

The contractile response to ET-1, a combined ETₐ and ETₜ receptor agonist, was also examined. There was a significant contractile effect of ET-1 on the mouse MCA. ET-1 (50nM)
generated the greatest constriction (47.8 ± 4.2%, n=6) amongst all agonists studied. At this concentration, the arteries responded weakly to adrenergic receptor stimulation, such that only 2.9±0.6% constriction was elicited by PE, an α1-adrenoceptor selective agonist. Although PE could constrict vessels to 45.8 ±4.5%(n=8), a higher concentration (50μM) was required. U46619, a stable analog of thromboxaneA2, produced a 13.9±2.6%(n=8) constriction at a concentration of 50nM. The maximum constriction generated by U46619 (5μM) was 34.8±3.5%(n=8). The pD<sub>2</sub> values of ET-1, PE and U46619 are 8.8±0.5, 6.0± 0.3, and 7.1±0.3, respectively.

5-HT (10nM - 10μM, n=6), a potent vasoconstrictor of cerebral arteries in most other species (e.g. rabbit, dog, monkey, human etc.) did not induce any contractile response in mouse MCA (Fig. 2.4A). The inhibition of NO production did not unveil constriction to 5-HT (10μM, n=6) (Fig. 2.4B).

2.4.3 VASODILATOR RESPONSES

ACh-induced dilation of mouse MCA was studied in arteries that possessed an intact endothelium and had been pre-contracted by PE (5μM) or U46619 (10nM) at 20mmHg. ACh (1nM -- 50μM) induced a maximal dilation of 58.7±11.8% at 10μM (n=6), as shown in Fig. 2.3A. Using another MCA segment, a stronger concentration-related vasodilation was exhibited by the addition of BK (0.1 nM - 10 μM), which reached a peak value of 82.4±10.4% (n=4) at 10μM. Another endothelium-dependent vasodilator substance P (50nM) produced maximum dilation 42.9 ±9.2%, which was lower than the maximal vasodilation induced by BK and ACh. Inhibition of NO synthase with L-NAME (1μM) attenuated the vasodilatory responses induced by BK (Fig. 2.3B), ACh and substance P (data not shown). The pD<sub>2</sub> values of BK, ACh and substance P are 8.6±0.4, 7.4±0.5, and 10.3±1.5, respectively. Their efficacy with a decreasing order is BK >ACh > substance P.
Histamine either dilates or constricts the cerebral arteries of most species; however, the cumulative application of histamine (1nM - 10µM, n=6) in the mouse MCA failed to produce any vasomotor actions (Fig. 2.4A). The inhibition of NO production did not unmask histamine (10µM, n=6) to exhibit vessel constriction (Fig. 2.4C).

2.4.4 \([\text{Ca}^{2+}]_i\) AND ARTERIAL DIAMETER

The fluorescence ratio at 340/380 was measured in fura-2 loaded MCA as an indicator of \([\text{Ca}^{2+}]_i\). We compared the effects of PE (10µM) and KCl (40mM) in the same artery segments. These bath concentrations were equally effective in causing vasoconstriction (42.3 ± 2.3% vs. 45.8 ± 4.5%, respectively, n=5, paired t-test, p = 0.54) (Fig. 2.5A). The normalized 340/380 fluorescence ratio was higher in the presence of KCl 40 mM compared to PE 10µM (0.99 ± 0.003, n=6 vs. 0.92 ± 0.02, n=6, p< 0.01) (Fig. 2.5B).

2.4.5. MEMBRANE POTENTIAL AND THE EFFECTS OF TRANSMURAL PRESSURES

As the transmural pressure was increased, smooth muscle cell \(E_m\) became more positive. The \(E_m\) was significantly more negative at a low pressure of 20mmHg (-52.6 ± 0.90mV) compared to higher pressures: 40mmHg (-42.3 ± 1.99mV), 60mm Hg (-35.3 ± 2.12mV) and 80mmHg (-37.3 ± 1.75mV). The differences in \(E_m\) of smooth muscle cells at 60 and 80mmHg were not statistically significant (Fig. 2.6 A & B).

2.4.6. KCl AND AGONIST-INDUCED DEPOLARIZATION OF SMOOTH MUSCLE CELLS

The resting \(E_m\) of the mouse MCA at a transmural pressure of 20mmHg was -52.6 ± 0.9mV (n = 12). The addition of KCl depolarized smooth muscle cell in a concentration dependent manner: KCl 20mM (-38.5 ± 3.3mV, n=12), 40mM (-29.5 ± 1.1mV, n=15), 60mM (-30.6 ± 0.9mV, n = 19), and 80mM (-29.0 ± 1.4mV, n = 6). A significant difference in \(E_m\) between
20mM KCl and higher concentrations of K$^+$ was observed (Fig. 2.7). Likewise, PE (10µM) depolarized the membrane significantly in mouse MCAs (-43.4 ± 2.7, n = 15) compared to control (-52.6 ± 0.9, n=25, p<0.01)(Fig. 2.6A&2.8). Whereas at the same level of constriction, the $E_m$ was significantly more negative in the presence of PE (10µM) compared to 40mM KCl (-43.4 ± 2.7mV vs. -29.5 ± 1.1mV, n =15, p<0.01) (Fig. 2.8).

2.5 DISCUSSION

2.5.1. INTRINSIC TONE

Intrinsic tone results from intravascular pressure activating myogenic constriction of small arteries, and may be more important physiologically than the efficacy or potency of any single contractile or relaxant agonist in the regulation of basal vascular tone. In the cerebral circulation, the ability to autoregulate blood flow over a broad range of perfusion pressures is almost completely dependent on the regulation of intrinsic tone (Rosenblum and Wormley 1995a). Our data demonstrated that the intrinsic tone of the mouse MCA was evoked at low pressures with no significant additional constriction occurring at higher pressures (>50mmHg). This implies that myogenic vasoconstriction in the mouse MCA only partially protects the capillary vessels, and that at higher pressures; other protective mechanisms may be activated. Nevertheless, the maximum constriction generated by the raised intraluminal pressure in mouse MCA is similar to that exhibited in isolated cerebral arteries from different species which range between 20-30% (Wallis et al. 1996; Ishiguro et al. 2002)(Table2.2). The application of bosentan impaired the development of myogenic tone of mouse MCA; while L-NAME potentiated vessel constriction. Our results are compatible with previous studies, suggesting that intrinsic tone includes the influences of both vasoconstrictor (e.g. ET-1) and vasodilator (e.g. NO) molecules released from the vascular endothelium (Smeda and Payne 2003).
2.5.2 AGONIST-INDUCED VASOCONSTRICTION

Extracellular K\(^+\) concentration, which is 3-5mM in central nervous system interstitial tissue, increases to 10-12mM when neurons are activated. In pathological conditions, such as traumatic brain injury and stroke, extracellular K\(^+\) can increase to 50 -- 80mM to depolarize the vascular smooth muscle and cause constriction (Sykova 1983). In rat cerebral arteries, 21mM K\(^+\) induces dilation, and maximal constriction is produced by 81mM K\(^+\) (Golding et al. 2000). In the mouse MCA, we found that 20mM KCl elicited a constriction and concentration-dependent constrictions were maximal with 66mM KCl.

ET-1 elicits potent and long-lasting constriction. The vasoconstrictor potency of ET-1 exceeds that of PE and U46619 in the mouse MCA. The sensitivity of mouse MCA to ET-1 is similar to that reported in isolated human and canine cerebral arteries (Kaito et al. 1995), but lower than in cat isolated MCA (Saito et al. 1989) (Table2.2).

The cerebral vasculature has an extensive adrenergic innervation arising predominantly from the superior cervical ganglia. Under normal conditions, these nerves may not have an important role since cerebrovascular tone is largely influenced by changes in intraluminal flow and intravascular pressure (Busija and Heistad 1984b; Bevan and Laher 1991; Bevan 1997). Cerebral arteries from a number of species, including humans, are relatively insensitive to adrenoceptor vasoconstrictor agonists and frequently require high (mM) concentrations of agonists (Laher and Bevan 1985b). Compared to \(\alpha\)-adrenergic vasoconstriction in cerebral arteries from human, monkey, canine, and rabbit (Laher and Bevan 1985c; Sasaki et al. 1985a; Thorin et al. 1997b), the PE-evoked contractile response in mouse MCA is more pronounced. This suggests that adrenoceptor sensitivity of cerebral arteries may be species-related and/or the vasomotor response is mediated by a class of adrenergic receptors distinct from the classical ones (Laher, 1986).
Cerebral arteries from most species contract in response to 5-HT. The vasoconstriction ranges from 40 to 100% of the maximum constriction elicited by 124mM KCl (Young et al. 1986). In isolated human and cat cerebral arteries, 5-HT induces endothelium-independent constriction (Conde et al. 1991), while 5-HT causes an unstable contractile response in rabbit MCA (Thorin et al. 1997c). In our study, 5-HT did not provoke changes in arterial tone in the mouse MCA, and the inhibition of NO did not unmask any constrictions to 5-HT. This brings into question the usefulness of the mouse as a model for migraine and stroke research, diseases in which 5-HT is thought to play important roles (Nilsson et al. 1999; Wester et al. 1992).

2.5.3 ANTAGONIST-INDUCED VASODILATION

ACh induces vasodilation of isolated rabbit, feline and guinea pig MCAs (Brayden and Wellman 1989; Brayden 1990; Dong et al. 2000). ACh also relaxes the mouse MCA, and the sensitivity of mouse MCA to ACh is higher than that in human cerebral arteries (Tsukahara et al. 1989). Inhibition of NOS with L-NAME suppressed the Ach-, BK- and substance P-induced dilations in mouse MCA, indicating the involvement of the endothelial-dependent relaxing factors. BK has greater efficacy than ACh in isolated mouse MCA, a finding that is consistent with experiments conducted in vivo (Rosenblum and Wormley 1995b).

Histamine dilates isolated human (Ottosson et al. 1988a) monkey (Ayajiki et al. 1992) rat (Benedito et al. 1991b) cerebral arteries. Also histamine elicited vasoconstriction in rabbit cerebral arteries (Laher and Bevan 1985a, Gokina and Bevan 2000). However, our study showed that the histamine was unable to evoke either dilation or constriction in the mouse MCA, either in the absence or presence of L-NAME (Fig. 2.4C). This finding also raises the question of choosing the isolated mouse MCA to investigate the etiology of brain edema and vasospasm in which histamine is one of putative mediators.
2.5.4 ARTERIAL WALL \( \text{Ca}^{2+} \) AND SMOOTH MUSCLE CELL MEMBRANE POTENTIAL

Depolarization induced by KCl causes vascular constriction by an increase in \( [\text{Ca}^{2+}]_i \) due to opening of voltage-gated \( \text{Ca}^{2+} \) channels (Nelson et al. 1990). Agonists such as PE induce arterial constriction through a combination of increased \( \text{Ca}^{2+} \) entry and augmented intracellular \( \text{Ca}^{2+} \) sensitization (Somlyo and Somlyo 1994). In our study both PE and KCl induced significant membrane depolarization; however, at the same level of constriction, the KCl-induced depolarization was significantly higher than PE. These findings combined with direct measurements of intracellular calcium indicating that a significant role for agonist-induced \( \text{Ca}^{2+} \) sensitization in cerebrovasculature.

Increases in transmural pressure also caused membrane depolarization of mouse MCA. This membrane depolarization may contribute to pressure-induced myogenic tone in mouse cerebral vessels. The mechanism of this effect could be attributable to depolarization-induced opening of L-type calcium channels in VSMC as demonstrated in rat cerebral artery. In rat MCA, both myogenic tone and membrane depolarization reach a plateau at a transmural pressure of 100mmHg (Knot and Nelson 1998a). However, our data indicate that both myogenic tone and membrane depolarization reach a plateau at 50-60mmHg.

2.6 CONCLUSION

In summary, this study focuses on the response of the mouse MCA to a number of pharmacological agents that are modulators in the cerebrovascular circulation under normal and pathophysiological states. We demonstrated that the mouse MCA i) constricts to \( \alpha \)-adrenergic receptor activation. This is unlike the general insensitivity of cerebral arteries to adrenergic receptor stimulation in most other species, including humans; ii) is insensitive to 5-HT, which is a potent constrictor in the cerebral circulation in other species; iii) is insensitive to histamine, which causes vasodilation in various species, and iv) increases in tone produced by PE is
accompanied by smaller changes in membrane potential and intracellular Ca\(^{2+}\) compared to tone produced by membrane depolarization with raised K\(^+\).
<table>
<thead>
<tr>
<th>Putative spasmogen</th>
<th>For</th>
<th>Against</th>
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<tbody>
<tr>
<td>Neurogenic factors</td>
<td>1) Innervation of adventitia of cerebral blood vessels</td>
<td>1) Loss of staining of nerves following exposure to blood does not correlate with VSP</td>
</tr>
<tr>
<td></td>
<td>2) Lesion of the A2 nucleus, an ascending pathway for NE release, prevents VSP</td>
<td>2) Sympathectomy and bilateral superior cervical ganglionectionomy does not reverse VSP</td>
</tr>
<tr>
<td>Adrenergic, Cholinergic or Peptidergic nerves</td>
<td>3) NE uptake altered by Hb</td>
<td>3) No obvious changes in sympathetic / parasympathetic perivascular neural networks found in rat VSP</td>
</tr>
<tr>
<td></td>
<td>4) Neurogenic vasodilation is suppressed by oxyHb</td>
<td>4) Cerebral arteries dilate to electrical stimulation in the presence of tetrodotoxin</td>
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<tr>
<td></td>
<td>5) Phentolamine inhibits VSP</td>
<td></td>
</tr>
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<td></td>
<td>6) Continuous electrical stimulation of the trigeminal ganglion causes vasodilation and increases CBF; a trigeminal lesion causes vasoconstriction.</td>
<td></td>
</tr>
<tr>
<td>Biogenic amines</td>
<td>1) Histamine and NE produce vasoconstriction</td>
<td>1) Cerebrovascular smooth muscle is relatively insensitive to α-adrenergic vasoconstrictors</td>
</tr>
<tr>
<td>Histamine, NE</td>
<td>2) NE metabolites in CSF detected in VSP</td>
<td>2) Multi-receptor has little effect on VSP</td>
</tr>
<tr>
<td></td>
<td>3) A NE periarterial nerve plexus is depleted of fluorescence by VSP</td>
<td>3) Contractility to NE and histamine of vasospatic vessels does not differ from control</td>
</tr>
<tr>
<td></td>
<td>4) NE uptake decreases to about 60% after SAH</td>
<td>4) Phenoxybenzamine does not reserve VSP</td>
</tr>
<tr>
<td></td>
<td>5) Selective lesions of the medullary catecholamine nuclei prevent VSP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6) Reduced tyrosine hydroxylase like immunoreactivity occurs in SAH</td>
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<tr>
<td>5-HT</td>
<td>1) Injection of 5-HT into the subarachnoid space causes VSP</td>
<td>1) Injection of blood and 5-HT into the subarachnoid space evokes only a transient constriction</td>
</tr>
<tr>
<td></td>
<td>2) 5-HT metabolism is activated in VSP</td>
<td>2) Chronic VSP is insensitive to the 5-HT antagonist (cyproheptadine)</td>
</tr>
<tr>
<td></td>
<td>3) Phenoxybenzamine prevents the 5HT constriction</td>
<td>3) No elevation of 5-HT in CSF after SAH</td>
</tr>
<tr>
<td></td>
<td>4) Pronounced network of 5-HT immunoreactive nerve fibers after SAH</td>
<td>4) Decline of 5-HT levels does not change the severity of VSP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5) Contractility of vasospatic vessels to 5-HT is</td>
</tr>
<tr>
<td>Eicosanoids</td>
<td>Prostaglandins</td>
<td>Thromboxanes</td>
</tr>
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<tr>
<td>6) Augmented 5-HT constriction in VSP is due to the suppressed release of NO</td>
<td>1) Prostaglandins (F₂α, E₂, A₁, B₁, B₂) cause constriction 2) Elevation of different prostaglandins occurs in SAH 3) PGI₂ is reduced in VSP 4) Sudoxicam and meclofenamate have a marked inhibitory effect on the development of VSP</td>
<td>1) Thromboxane A₂ is not increased in spastic vessels</td>
</tr>
</tbody>
</table>

**Prostaglandins**
- Prostaglandins (F₂α, E₂, A₁, B₁, B₂) cause constriction
- Elevation of different prostaglandins occurs in SAH
- PGI₂ is reduced in VSP
- Sudoxicam and meclofenamate have a marked inhibitory effect on the development of VSP

**Thromboxanes**
- Thromboxanes induce vasoconstriction
- Thromboxane synthetase inhibitors ameliorate VSP

**Leukotrienes**
- Leukotriene D₄ evokes vasoconstriction
- Intraventricular injection of intermediates of leukotriene induces VSP

**Endothelin**
- ET-1, big ET-1, ECE increased in SAH
- ETₐ and ETₐ receptor mRNA doubled in vasospastic cerebral arteries
- ET receptor binding increased after SAH
- Inhibitors of ET receptors and ECE retard VSP and inhibit the ischemic damage

**Blood and CSF**
- Incubation of blood-CSF mixture causes cerebral vasoconstriction
- Chronic VSP occurs after the application of RBCs and the degree of VSP is proportional to volume of RBC mass
- Hemolysate induces potent contraction
- PRP constriction due to 5-HT release
- Intracisternal injection of blood lacking RBCs does not produce VSP or narrowing

**Hb**
- OxyHb causes severe chronic VSP
<table>
<thead>
<tr>
<th><strong>Free Radicals</strong></th>
<th><strong>NO</strong></th>
<th><strong>OxyHb-induced contraction has similar pharmacology as xanthochromic CSF</strong></th>
</tr>
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<tbody>
<tr>
<td>Lipid peroxides are elevated in CSF post-SAH</td>
<td>1) Excessive production of NO causes cellular injury</td>
<td>2) OxyHb inhibits production of NO</td>
</tr>
<tr>
<td>SAH causes a marked elevation in CSF uric acid (xanthine oxidase product)</td>
<td>2) Nitrotyrosin and peroxynitrite contribute to chronic VSP</td>
<td>3) OxyHb stimulates release of endothelin and prostaglandin</td>
</tr>
<tr>
<td>Injection xanthine, xanthine oxidase, ferric chloride, metHb, iron and EDTA mixture into the cisterna magna produces vasoconstriction</td>
<td>3) Endothelial cells provide sufficient NO to damage their own cellular function</td>
<td>4) OxyHb produces other potential vasoconstrictors such as hemin, iron, and bilirubin</td>
</tr>
<tr>
<td>SAH generates lipid peroxides</td>
<td>4) Innervation by NOS-containing nerve fibers</td>
<td>5) OxyHb can auto-oxidize to release $O_2^-$ and produce $OH^-$</td>
</tr>
<tr>
<td>CSF Glutathione peroxidase increases in VSP</td>
<td>5) Hb vasoconstriction occurs via the reduction in NO delivery</td>
<td>6) OxyHb-induced contraction has similar pharmacology as xanthochromic CSF</td>
</tr>
<tr>
<td>Gene transfer of extracellular SOD attenuates</td>
<td>6) Inducible NOS is upregulated in VSP</td>
<td>7) Hb damages perivascular nerves</td>
</tr>
<tr>
<td>1) Lipid peroxides are elevated in CSF post-SAH</td>
<td>1) NO is a vasodilator</td>
<td>8) Hb is synergistic with $K^+$, ATP, 5-HT, fibrin degradation products, and hypoxia</td>
</tr>
<tr>
<td>SAH causes a marked elevation in CSF uric acid (xanthine oxidase product)</td>
<td>2) Nitrotyrosin and peroxynitrite contribute to chronic VSP</td>
<td>9) Hb increases intracellular Ca$^{2+}$</td>
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<td>3) Endothelial cells provide sufficient NO to damage their own cellular function</td>
<td>10) Hb present in spastic vessel walls</td>
</tr>
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<td>3) Endothelial cells provide sufficient NO to damage their own cellular function</td>
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**Superoxide, Hydroxyl radical** | **Superoxide, Hydroxyl radical** | **Free Radicals** |
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<tr>
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<td>5) CSF Glutathione peroxidase increases in VSP</td>
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<td>6) Gene transfer of extracellular SOD attenuates</td>
<td>6) Gene transfer of extracellular SOD attenuates</td>
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2) Most studies use impure Hb in vitro | 2) Most studies use impure Hb in vitro | 2) Most studies use impure Hb in vitro |
3) Hb contains endotoxin, stromal proteins, and phospholipids that cause vasoconstriction and inflammation | 3) Hb contains endotoxin, stromal proteins, and phospholipids that cause vasoconstriction and inflammation | 3) Hb contains endotoxin, stromal proteins, and phospholipids that cause vasoconstriction and inflammation |
4) Hb contractile potency is increased after combination with low-molecular-weight components of erythrocytes | 4) Hb contractile potency is increased after combination with low-molecular-weight components of erythrocytes | 4) Hb contractile potency is increased after combination with low-molecular-weight components of erythrocytes |
5) OxyHb inhibits production of NO | 5) OxyHb inhibits production of NO | 5) OxyHb inhibits production of NO |
6) OxyHb stimulates release of endothelin and prostaglandin | 6) OxyHb stimulates release of endothelin and prostaglandin | 6) OxyHb stimulates release of endothelin and prostaglandin |
7) OxyHb produces other potential vasoconstrictors such as hemin, iron, and bilirubin | 7) OxyHb produces other potential vasoconstrictors such as hemin, iron, and bilirubin | 7) OxyHb produces other potential vasoconstrictors such as hemin, iron, and bilirubin |
8) OxyHb can auto-oxidize to release $O_2^-$ and produce $OH^-$ | 8) OxyHb can auto-oxidize to release $O_2^-$ and produce $OH^-$ | 8) OxyHb can auto-oxidize to release $O_2^-$ and produce $OH^-$ |
9) OxyHb-induced contraction has similar pharmacology as xanthochromic CSF | 9) OxyHb-induced contraction has similar pharmacology as xanthochromic CSF | 9) OxyHb-induced contraction has similar pharmacology as xanthochromic CSF |
10) Hb damages perivascular nerves | 10) Hb damages perivascular nerves | 10) Hb damages perivascular nerves |
1) Excessive production of NO causes cellular injury | 1) Excessive production of NO causes cellular injury | 1) Excessive production of NO causes cellular injury |
2) Nitrotyrosin and peroxynitrite contribute to chronic VSP | 2) Nitrotyrosin and peroxynitrite contribute to chronic VSP | 2) Nitrotyrosin and peroxynitrite contribute to chronic VSP |
3) Endothelial cells provide sufficient NO to damage their own cellular function | 3) Endothelial cells provide sufficient NO to damage their own cellular function | 3) Endothelial cells provide sufficient NO to damage their own cellular function |
4) Innervation by NOS-containing nerve fibers | 4) Innervation by NOS-containing nerve fibers | 4) Innervation by NOS-containing nerve fibers |
5) Hb vasoconstriction occurs via the reduction in NO delivery | 5) Hb vasoconstriction occurs via the reduction in NO delivery | 5) Hb vasoconstriction occurs via the reduction in NO delivery |
6) Inducible NOS is upregulated in VSP | 6) Inducible NOS is upregulated in VSP | 6) Inducible NOS is upregulated in VSP |
1) NO is a vasodilator | 1) SOD and catalase fail to protect against oxyHb-induced VSP | 1) SOD and catalase fail to protect against oxyHb-induced VSP |
2) Lipid peroxidation may be a result of VSP rather than its cause | 2) Lipid peroxidation may be a result of VSP rather than its cause | 2) Lipid peroxidation may be a result of VSP rather than its cause |
3) Inhibitors of lipid peroxidation do not prevent VSP | 3) Inhibitors of lipid peroxidation do not prevent VSP | 3) Inhibitors of lipid peroxidation do not prevent VSP |
4) Allopurinal prevents the elevation in uric acid but does not inhibit VSP or vascular damage | 4) Allopurinal prevents the elevation in uric acid but does not inhibit VSP or vascular damage | 4) Allopurinal prevents the elevation in uric acid but does not inhibit VSP or vascular damage |
<table>
<thead>
<tr>
<th></th>
<th>VSP</th>
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<tbody>
<tr>
<td></td>
<td>7) Some free radical scavengers ameliorate VSP through affecting oxyHb constriction</td>
</tr>
<tr>
<td></td>
<td>8) Vitamin E prevents SAH cerebral hypoperfusion</td>
</tr>
</tbody>
</table>

**Abbreviation:**
- **ATP:** Adenosine triphosphate
- **CBF:** Cerebral blood flow
- **CSF:** Cerebral spinal fluid
- **ET:** Endothelin
- **ECE:** Endothelin converting enzyme
- **EDTA:** Ethylenediaminetetraacetic acid
- **Hb:** Hemoglobin
- **5-HT:** 5-hydroxytryptamine
- **metHb:** methemoglobin
- **NO:** Nitric oxide
- **NOS:** Nitric oxide synthase
- **NE:** Norepinephrine
- **OxyHb:** Oxyhaemoglobin
- **PGI₂:** Prostacyclin
- **PRP:** Platelet-rich plasma
- **RBC:** Red blood cell
- **SAH:** Subarachnoid hemorrhage
- **SOD:** Superoxide dismutases
- **VSP:** Vasospasm
Table 2.1 Comparison of vasoactive factors in different species

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Human</th>
<th>Monkey</th>
<th>Rat</th>
<th>Rabbit</th>
<th>Canine</th>
<th>Feline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tone</td>
<td>20.6±2.4%</td>
<td>23.4±4.2%</td>
<td></td>
<td>38.0±2.0%</td>
<td>19.7 ± 2.2%</td>
<td>13.2%</td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>(+) 8.8±0.07</td>
<td>(+) 8.9</td>
<td></td>
<td>(+) 8.5</td>
<td>(+) 8.2</td>
<td>(+) 8.08</td>
<td>(+) 9.81−10.86</td>
</tr>
<tr>
<td>PE</td>
<td>(+) 6.0±0.1</td>
<td>Insensitive</td>
<td></td>
<td>(Sasaki, et al, 1985)</td>
<td>Small, Unstable contraction</td>
<td>(+) 4.8</td>
<td>(Sasaki et al., 1985)</td>
</tr>
<tr>
<td></td>
<td>(Laher and Bevan, 1985)</td>
<td></td>
<td></td>
<td>(Sasaki et al, 1985)</td>
<td>(Thorin et al., 1997)</td>
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<tr>
<td>U-46619</td>
<td>(+) 7.1±0.1</td>
<td>7.5</td>
<td>(Wallis et al, 1996)</td>
<td>9.4</td>
<td>(Sasaki, et al, 1985)</td>
<td>(+) 8.9</td>
<td>(Sasaki, et al., 1985)</td>
</tr>
<tr>
<td>5-HT</td>
<td>(N/A)</td>
<td>(+) 7.8</td>
<td>(Conde et al., 1991)</td>
<td>(+) 6.8</td>
<td>(McCulloch, 1984)</td>
<td>Unstable contraction</td>
<td>(+) 8.5</td>
</tr>
<tr>
<td>ACh</td>
<td>(-) 7.4±0.2</td>
<td>(-) 5.7</td>
<td>(Tsukahara et al, 1989)</td>
<td>(-) 5.8</td>
<td>(Momoi et al, 2003)</td>
<td>(-) 6.3</td>
<td>(Gilbert et al., 2001)</td>
</tr>
<tr>
<td>BK</td>
<td>(-) 8.6±0.2</td>
<td>(-) 7.89</td>
<td>(-) Watters, 1971)</td>
<td>(-) Dacey et al., 1988)</td>
<td>(-) Whalley, 1983)</td>
<td>(+) Whalley et al., 1987)</td>
<td>(-) 6.6</td>
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<tr>
<td>Substance</td>
<td>1983)</td>
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<tr>
<td>P</td>
<td>10.3±0.7 (Wallis et al., 1996)</td>
<td>(N/A) (Dacey et al., 1988)</td>
<td>(-) (Stubbs et al., 1992)</td>
<td>(-) 8.9 (Edvinsson et al., 1981)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>(N/A) 7.3 (Ottosson et al., 1988)</td>
<td>(-) 5.3 (Ayajiki et al., 1992)</td>
<td>(+) (Gokina and Bevan, 2000)</td>
<td>(+) 5.3 (Kitamura et al., 1995)</td>
<td>(-) 5.3 (Edvinsson, 1975)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(+): constriction; (-): dilation; (N/A): no response; Values are $-\log ED_{50}$
Fig. 2.1. Myogenic tone of isolated mouse cerebral arteries. (A) As the intraluminal pressure was increased at 5 minutes intervals, mouse MCA's (n=15) contracted spontaneously and achieved a new steady-state diameter. The maximum active constriction was $20.6 \pm 2.4\%$ at 90mmHg, which was not significantly different from the myogenic constriction that reached a plateau at 50mmHg. (B) Representative trace of effect of L-NAME, a NOS inhibitor, and bosentan, an ET$_A$/ET$_B$ receptor antagonist, on the myogenous tone mouse MCA. The addition of L-NAME (10μM) potentiated pressure-induced contraction (n=4), while the myogenic response of vessels equilibrated with bosentan (1μM) was attenuated (n=4).
Fig. 2.2. Agonist-induced constriction of the isolated mouse MCA. (A) Vasoconstriction was induced at concentrations as low as 8mM and the EC$_{50}$ of KCl was 31.0±10.0mM. Maximum constriction (63.3±3.3%, n=6) occurred with 66 mM KCl. (B) Concentration response curves for the constrictor effect of three distinct receptor activators studied at 20mmHg. Cumulative concentrations of agonists were applied vessels allowed to reach stabilized constriction. Maximum constriction (47.8±4.2%) was evoked by ET-1 (50nM, n=6). PE was able to generate its greatest constriction (45.8±4.5%) at 50μM (n=8). U46619 elicited its highest constriction (34.8±3.5%) at 5μM (n=8). The pD$_2$ values of ET-1, PE and U46619, were 8.8±0.2M, 6.0±0.1M and 7.1±0.1M, respectively. The rank order of efficacy is ET-1 >PE >U46619 (repeated measures ANOVA, p<0.01).
Fig. 2.3. Endothelium-dependent vasodilation in mouse MCA. (A) Cumulative concentrations were applied and vessel diameters allowed to reach a maintained value. The greatest vasodilator response (82.4 ± 5.1%) occurred with 5μM BK (n=4), while maximal vasodilation with ACh was 58.7 ± 6.0% (n=6) and with substance P was 42.9 ± 5.3% (n=5). (B) A representative recording shows the inhibitory action of L-NAME (1μM) to the dilation evoked by BK (0.1nM -- 10μM) after pre-constriction with U46619 (10nM). In the presence of L-NAME, BK-induced vasodilation was greatly attenuated. Maximal vasodilation occurred with SNP (1μM), a directly acting vasodilator that is a NO donor.
Fig. 2.4. Lack of responses to 5-HT and histamine in the mouse MCA. (A) Trace showing that 5-HT (10µM) and histamine (10µM) induced neither constriction, nor dilation in PE (1µM) pre-constricted mouse MCA (n=6). (B) L-NAME (1µM) was applied for 15 minutes before addition of PE (1µM). 5-HT (10µM) was not able to constrict the vessel further, whereas vasopressin (0.5µM) generated additional tone (n=6). (C) After washout, L-NAME (1µM) was again incubated for 15 minutes and histamine (10µM) did not evoked additional tone either, whereas vasopressin (0.5µM) elicited additional vasoconstriction, and SNP (1µM) caused maximal dilation (n=6).
Fig. 2.5. Simultaneous changes in arterial diameter and changes in the 340/380 fluorescence ratio in mouse MCA loaded with fura-2. (A) After being incubated with the Ca$^{2+}$-sensitive fluorescent dye, fura-2 AM and pluronic acid, mouse MCAs was challenged with increasing concentrations of KCl (20mM -- 80mM, n=5) and PE (10nM -- 10μM, n=5). A representative trace shows that a more pronounced rise in the fluorescence ratio occurred with KCl-induced constriction compared to PE. (B) Although PE (10μM) and KCl (40mM) caused similar constriction (42.3 ± 2.3% vs. 45.8 ± 4.5%, n=5, paired t-test, $p = 0.54$), the normalized 340/380 fluorescence ratio was higher in the presence of KCl 40 mM compared to PE 10μM (0.99 ± 0.003 vs.0.92 ± 0.02, n=5, $p< 0.01$).
Fig. 2.6. Recordings of smooth muscle membrane potential in mouse MCA. (A) $E_m$ was measured in vascular smooth muscle cells (VSMCs) of the mouse MCA by inserting

![Graph showing membrane potential changes with transmural pressure.](image)

### B

<table>
<thead>
<tr>
<th>Transmural Pressure (mmHg)</th>
<th>Membrane Potential (mV)</th>
</tr>
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<tbody>
<tr>
<td>20</td>
<td>-58</td>
</tr>
<tr>
<td>40</td>
<td>-45</td>
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<tr>
<td>60</td>
<td>-36</td>
</tr>
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<td>80</td>
<td>-27</td>
</tr>
</tbody>
</table>

Fig. 2.6. Recordings of smooth muscle membrane potential in mouse MCA. (A) $E_m$ was measured in vascular smooth muscle cells (VSMCs) of the mouse MCA by inserting
microelectrodes. The measurement was done either at rest (PSS) or in the presence of PE (10μM) at a transmural pressure of 20mmHg. A sharp negative deflection signaled the entry of microelectrode into a VSMC, and a sharp positive deflection to 0mV indicated microelectrode exit from the recorded cell. (B) Depolarizing effect of stepwise-increases in transmural pressure on smooth muscle $E_m$ of mouse MCA. At low pressure (20mmHg), the $E_m$ was significantly more negative (-52.6 ± 1.0mV) compared to higher pressures: 40mmHg (-42.3 ± 2.0mV), 60mm Hg (-35.3 ± 2.1mV) and 80mmHg (-37.3 ± 1.8mV). The differences in $E_m$ of smooth muscle cells at 60 and 80mmHg were not statistically significant. The numbers in parentheses represent the sample size. (Repeated measures ANOVA with multiple comparisons performed by Bonferroni’s test *$p<0.01$ compared to other pressures, ** $p<0.01$ compared to other pressures).
Fig. 2.7. Depolarizing effect of increasing concentrations of KCl on smooth muscle $E_m$ of mouse MCA. The application of different concentrations of KCl shows concentration-dependent depolarization. Mouse MCA smooth muscle cells were significantly more depolarized at KCl 20mM (-38.5 ± 3.3mV, n=12), 40mM (-29.5 ± 1.1mV, n=15), 60mM (-30.6 ± 0.9mV, n = 19), and 80mM (-29.0 ± 1.4mV, n = 6) compared to control -52.6 ± 0.9mV(n=25), which was the resting $E_m$ of 5mM KCl (PSS) at 20mmHg (p<0.01). Moreover, the $E_m$ of 20mM KCl had a significant difference compared to that of higher concentrations of KCl. The numbers in parentheses represent the sample size. Repeated measures ANOVA with multiple comparisons performed by Bonferroni’s test, *$p$<0.01 compared to other concentrations. **$p$<0.01 compared to higher concentration.
Fig. 2.8. A comparison of the depolarizing effects of PE (10μM) and KCl (40mM) in mouse MCA. PE and KCl caused similar changes in arterial tone; whereas, the depolarization evoked by PE was significantly less than that of KCl (-43.4±2.7mV vs. -29.5±1.1mV). The numbers in parentheses represent the sample size. Repeated measures ANOVA with multiple comparisons performed by Bonferroni’s test. *p<0.01 compared to PE and KCl, **p<0.01 compared to PE.
References


Chan-Palay,V. 1976. Serotonin axons in the supra- and subependymal plexuses and in the leptomeninges; their roles in local alterations of cerebrospinal fluid and vasomotor activity. Brain Res. 102: 103-130.


