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PO Box 117
221 00 Lund
+46 46-222 00 00

Vascular effects of neuropeptides and UDP- β -S and alterations after stroke

ANDRÉ ERDLING

DEPARTMENT OF CLINICAL SCIENCES, LUND | FACULTY OF MEDICINE | LUND UNIVERSITY





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Vascular effects of neuropeptides and UDP- β -S and alterations after stroke

André Erdling



LUND
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DOCTORAL DISSERTATION

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Abstract The aim of this thesis is to investigate the changes in vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) and P2Y ₆ -mediated signaling following experimental subarachnoid hemorrhage (SAH) and transient middle cerebral artery occlusion (tMCAO). VIP and CGRP are among the most powerful vasodilators known to man while P2Y ₆ mediates strong vasoconstriction in cerebral blood vessels. By describing the potential changes in these receptor/agonist systems, we hope to better describe the balance between vasoconstriction and vasodilation following stroke. Stroke is a common and severe disorder of the central nervous system, where disturbances in blood supply induce ischemic injury. Ischemia and the following cascade of inflammation cause not only neuronal death but also induce changes in blood vessels and surrounding tissue. We have previously demonstrated an increase in the expression of several vasoconstrictive receptor systems following both ischemic and hemorrhagic stroke. These changes are hypothesized to induce a more vasoconstrictive state, which reduce blood flow and may enhance ischemic damage in the near-ischemic zone surrounding the ischemic core. Most previous studies have focused on traditional vasoconstrictors such as endothelin, angiotensin and serotonin, while less focus has been put on more locally acting vasoconstrictors such as purines. Even less effort has been applied to the investigation of vasodilatory receptor systems and their plasticity following ischemia. The first two papers making up the basis for this thesis investigate the physiological function of CGRP, VIP and the closely related pituitary adenylate cyclase-activating polypeptide (PACAP) in rat middle cerebral arteries (MCAs). Wire myography and pressurized arteriography evaluate changes in wall tension and vessel diameter along with measurements of intracellular calcium. Immunohistochemistry is used to determine receptor localization. Based on these experiments we conclude that CGRP and VIP receptors are located on the smooth muscle cells and not on the endothelial cells of the rat MCA. VIP and CGRP both induce a sustained vasorelaxation which correlates with a decrease in intracellular calcium. It was also demonstrated that VPAC ₁ and VPAC ₂ receptors are predominant over PAC ₁ receptors. The last two papers of this thesis investigate changes in receptor expression and agonist effect in rat MCAs following either experimental SAH or transient MCA occlusion. Wire myography was used to determine changes in wall tension and western blot and/or flow cytometry was used to evaluate alterations in receptor expression. We demonstrate an enhanced vasodilatory response to VIP following tMCAO but not after SAH. CRGP-mediated vasodilation is completely unaffected by SAH or tMCAO. An enhanced expression of the P2Y ₆ receptor following both types of stroke was demonstrated, however contraction was only enhanced after tMCAO.			
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*The brain is the most important organ
you have, according to the brain.*

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Original articles

This doctoral thesis is based on the following articles, which are referred to in the text by their Roman numerals (I-IV).

- I. **Erdling A**, Sheykhzade M, Maddahi A, Bari F, Edvinsson L. VIP/PACAP receptors in cerebral arteries of rat: characterization, localization and relation to intracellular calcium. *Neuropeptides* 2013; 47(2):85-92.
- II. **Erdling A**, Sheykhzade M, Edvinsson L. Differential inhibitory response to telcagepant on α CGRP induced vasorelaxation and intracellular Ca^{2+} levels in the perfused and non-perfused isolated rat middle cerebral artery. *J Headache Pain* 2017;18(1):61.
- III. **Erdling A**, Johansson SE, Maddahi A, Arkelius K, Ansar S, Edvinsson L. Alterations in vasomotor responses to VIP and CGRP following focal and global cerebral ischemia in rat middle cerebral artery. *Submitted*
- IV. **Erdling A**, Johansson SE, Radziwon-Balicka A, Ansar S, Edvinsson L. Changes in P2Y₆ receptor mediated vasoreactivity following focal and global ischemia. *Submitted*

Abbreviations

5-HT _{1B}	5-hydroxytryptamine receptor 1B
AC	adenylate cyclase
ADP/ATP	adenosine diphosphate/adenosine triphosphate
AM	adrenomedullin
AT ₁	angiotensin II type 1 receptor
CaM	calmodulin
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor like receptor
ERK _{1/2}	extracellular signal-regulated kinase 1/2
ET _B	endothelin receptor type B
IP ₃	inositol triphosphate
MCA	middle cerebral artery
MEK _{1/2}	mitogen-activated protein kinase kinase 1/2
PAC ₁	pituitary adenylyl cyclase-activating polypeptide type 1 receptor
PACAP	pituitary adenylyl cyclase-activating polypeptide
PLC	phospholipase C
RAMP	receptor activity modifying protein
SAH	subarachnoid hemorrhage
tMCAO	transient middle cerebral artery occlusion
UDP/UTP	uridine diphosphate/uridine triphosphate
UDP-β-S	uridine- 5'- O- (2- thiodiphosphate)
VIP	vasoactive intestinal peptide
VPAC _{1/2}	vasoactive intestinal peptide receptor 1/2
VSMC	vascular smooth muscle cell

Introduction

Stroke is one of the leading causes of death and disability in the developed world and accounts for 6.6 million deaths per year. Persisting neurological deficit such as physical disability, speech impairment and cognitive disability are other serious side effects which make stroke a top five cause of reduction in disease free living years.

Cerebral ischemia affects not only the neurones, but also trigger an inflammatory response which is responsible for blood brain barrier dysfunction and dysregulation of vascular function which in turn endanger neurones in the near ischemic zone known as the penumbra. These cells are salvageable if blood flow is restored, a feature that distinguishes them from cells inside the ischemic core.

Treatment options for ischemic stroke are rather limited and mainly consist of thrombectomy or thrombolysis if the cause is thromboembolic. The amount of stroke patients that are eligible for this therapy depends on several factors. First, there is a narrow time window where therapy is effective. Furthermore, hemorrhagic transformation of the infarct area pose a risk of worsened injury following thrombolysis.

Subarachnoid hemorrhage, which constitutes 5-7% of all stroke cases, lack effective treatment, especially against vasospasm and delayed ischemia following the primary insult.

Combined efforts to develop neuroprotective agents that could either extend the time window for thrombectomy/thrombolysis, salvage neurones in the penumbra and/or prevent cerebrovascular dysfunction, vasospasm and delayed ischemia in humans have yet been unsuccessful.

This thesis is aimed at describing the potential changes in receptor systems favouring either vasodilation or vasoconstriction following stroke and thus identify potential drug targets for re-establishing vascular function. Previous attempts to do this have focused heavily on traditional vasoconstrictors such as angiotensin, endothelin, serotonin, and their respective receptor systems. The experiments in this thesis are focused on the neuropeptides VIP and CGRP which are strong vasodilators and the locally acting purine receptor P2Y₆ which mediates a strong vasoconstrictive response in cerebral vessels. The first part of the thesis describes the vasodilatory actions of CGRP and VIP with respect to receptor localization and changes in intracellular calcium while the latter part describes changes in receptor

expression and sensitivity to either VIP, CGRP or the P2Y₆ agonist UDP-β-S following ischemia in two rat stroke models.

Pathophysiology of stroke

Stroke is a common disease, causing millions of deaths worldwide each year¹. It is also one of the leading causes of disability-adjusted life year (DALY)^{1,2} which is measure of death and disability in a population. Stroke is a pathological state caused by the sudden cessation of blood flow to a part of the brain, ultimately causing neuronal death and loss of function. The cause for the interrupted blood flow can be either ischemic due to thromboembolism or due to hemorrhage (Figure 1). Impaired blood flow causes hypoxia which triggers the pathological pathways of the ischemic cascade and ultimately causes irreversible neuronal damage. This is followed by disruption of cell membranes and leakage of intracellular contents into the extracellular space, triggering inflammation, leukocyte infiltration and loss of vascular and blood-brain barrier function³⁻⁵.

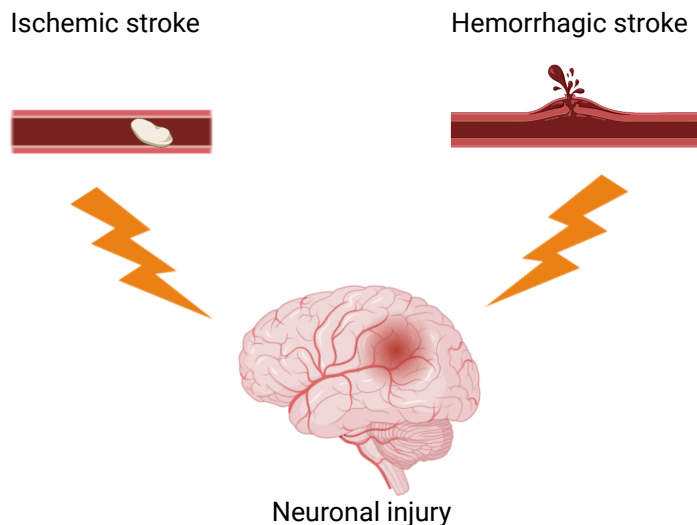


Figure 1. Illustration of different types of stroke.

Ischemic stroke is caused by a thrombus or embolus occluding a blood vessel while hemorrhagic stroke is caused by the sudden rupture of a cerebral blood vessel. Created using BioRender.com.

Ischemic stroke

The most common form of stroke is caused by the sudden occlusion of a blood vessel, either by a thrombus or an embolus, resulting in immediate loss of oxygen and glucose supply to the neuronal tissue downstream of the lesion. Hypoxia triggers the changes described above in the ischemic core and cells within this area are injured and die within a few minutes⁶. A larger volume of brain tissue wreathing the ischemic core, the penumbra, can be restored to normal function if cerebral blood flow is reinstated.

Therapies aimed at restoring blood flow, such as thrombectomy or thrombolysis have revolutionized the treatment of ischemic lesions but have to be attempted as soon as possible to have any effect⁷. Although reperfusion of ischemic tissue is critical for restoring function, it can paradoxically cause secondary damage, known as ischemia/reperfusion injury.

Hemorrhagic stroke/SAH

Subarachnoid hemorrhage (SAH) constitutes a subgroup of hemorrhagic stroke and makes up 5-7%⁸ of all stroke cases. It is the result of a ruptured cerebral artery with bleeding occurring under the arachnoid membrane of the brain. The rupture can be either traumatic or, more commonly, the result of an aneurysm. The risk for aneurysm formation increases with certain genetic risk factors (e.g., connective tissue disorders) and smoking, hypertension, drug abuse and other lifestyle factors. Proinflammatory activation at areas subjected to high shear stress is one of the features of aneurysm formation and seem to be closely related to atherosclerotic processes including chronic inflammation and accumulation of lipids in the vessel wall along with activation of the complement system.

The period immediately after aneurysm rupture is characterized by a steep increase in intracranial pressure (ICP) which typically lasts for a few minutes. The ICP then falls to a level slightly higher than before aneurysm rupture. The rapid increase in ICP can reach levels exceeding the blood pressure and thus cause a short period of virtually zero cerebral perfusion after which perfusion pressure returns to near normal levels⁸. Cerebral blood flow (CBF), however, remains impaired due to impaired autoregulation and acute vasoconstriction causing further ischemic damage. This initial phase of early brain injury (EBI) which usually resolves within 72 hours may be followed by a phase of delayed cerebral ischemia which reaches its peak at day 6-8 after the initial insult. Inflammation is induced by ischemia as for ischemic stroke, but also by blood entering the subarachnoid space.

Initial treatment of SAH is aimed at controlling the bleeding aneurysm⁹, either by open surgical techniques or by endovascular coiling. The calcium channel blocker nimodipine is recommended as a first-line treatment for preventing delayed ischemia/vasospasm¹⁰. Nimodipine has only minor effect on angiographic vasospasm and its neuroprotective effects may rather be due to protection against

Ca²⁺ toxicity at a cellular level¹¹. Manifest vasospasm is usually treated with induced hypertension and hypervolemia (HHT, hypertensive hypervolemic therapy), which can decrease neurological deficit in up to 80% of all cases^{12,13}.

The cerebral circulation and cerebral blood flow regulation

The brain, cerebellum and brainstem receive blood from both the carotid arteries and the vertebral circulation via the basilar artery. The internal carotid arteries join the basilar artery via communicating arteries and form the circle of Willis which delivers blood to the major cerebral arteries, including the right and left middle cerebral arteries.

The cerebral blood vessels are innervated by sympathetic, parasympathetic and sensory neurons, each of which contains multiple neuronal messenger molecules¹⁴. The role of these perivascular nerves in the regulation of the cerebral circulation has been extensively discussed, and they are now considered modulators and regulators of cerebrovascular tone and blood flow¹⁵. The importance and role of the individual neuronal messenger in the maintenance of CBF and vascular tone remains unclear, especially in pathological states such as during and after ischemia. Both thromboembolic stroke and SAH are associated with upregulation or increased expression of vasoconstrictive receptors, causing increased vascular tone which can contribute to delayed ischemia and cerebrovascular dysfunction, including vasospasm, after ischemia¹⁶⁻¹⁸. There is also an innate response in the vascular wall that responds to increases in transmural pressure by increasing vascular tone. This myogenic response, commonly referred to as myogenic tone, is a primary feature of resistance arteries and arterioles and offers a resting level of vascular smooth muscle activity, ensuring that the vessel can both dilate and contract when needed.

The CBF is tightly regulated and remains constant in healthy subjects despite variations (50 – 150 mmHg) in cerebral perfusion pressure¹⁹, allowing matching of metabolic supply and demand. This autoregulation is thought to be mediated by a combination of myogenic, neurogenic, metabolic, and endothelial factors but the exact mechanisms remain elusive. Metabolic control is due to the vasodilatory properties of CO₂ and hydrogen ions produced locally and is primarily considered a feature of the microvasculature. Endothelial control of CBF is mediated by locally acting substances such as nitric oxide (NO), endothelin and thromboxane A₂.

Autoregulation of CBF is frequently disturbed in pathological conditions. Hypertension can, for instance, cause a rightward shift of the cerebral perfusion pressure span in which autoregulation is effective, and thus require a higher blood pressure than in healthy subject to maintain adequate CBF. Impaired autoregulation

is also a feature of intracranial vascular pathology, including ischemic and hemorrhagic stroke, where damage to arterioles and capillaries, changes in receptor density and endothelial dysfunction is thought to diminish the adaptive response to changing perfusion pressures^{5,20}.

Physiology of vascular contraction

Vascular tone is primarily determined by the level of cytosolic free calcium, $[Ca^{2+}]_i$, within the vascular smooth muscle cells (VSMCs) of the arterial wall. A rise in cytosolic calcium induces a conformational change in the protein calmodulin (CaM) allowing it to form a complex with myosin light chain kinase (MLCK) which is then activated. The Ca^{2+} /CaM/MLCK complex is then able to phosphorylate myosin light chains, promoting the formation of cross-bridges between myosin heads and actin, ultimately causing contraction (Figure 2). Ca^{2+} is thus essential for smooth muscle contraction and the regulation of intracellular concentrations of Ca^{2+} affects excitation-contraction coupling. The actions of MLCK are opposed by myosin light chain phosphatase (MLCP) which catalyses the dephosphorylation of the myosin light chains and reduce cross-bridge formation. Inhibition of MLCP sensitizes the contractile apparatus to Ca^{2+} and thus increases contractile force at a given Ca^{2+} level. MLCP is mainly inhibited by signaling through the RhoA/Rho-kinase (ROCK) pathway. The actions of both MLCK and MLCP are under influence of several other intracellular messengers which convey stimuli from surface receptors.

Ca^{2+} enters the cell from the extracellular space or is released from intracellular Ca^{2+} stores in the sarcoplasmic reticulum (SR). Extracellular Ca^{2+} influx is mainly mediated by the opening of voltage dependent L-type Ca^{2+} channels (LTCC), but there are other channels that also regulate intracellular Ca^{2+} , for instance transient receptor potential type C and V channels (TRPC and TRPV). Ca^{2+} release from the SR is mediated through activation of inositol triphosphate (IP_3) receptors or ryanodine receptors (RyR).

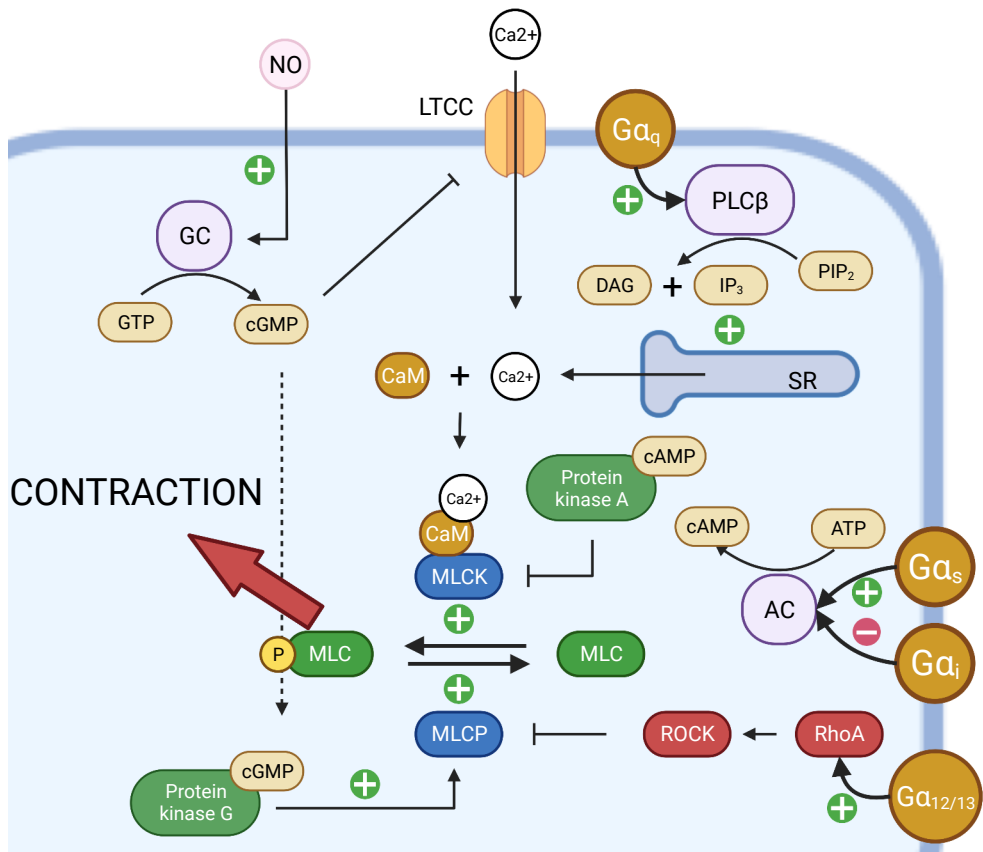


Figure 2. Overview of pathways regulating smooth muscle contraction.

Adenylyl cyclase (AC), adenosine triphosphate (ATP), calmodulin (CaM), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate, diacylglycerol (DAG), G-protein alpha subunit q,s,i and 12/13 (G_{α_q} , G_{α_s} , G_{α_i} and $G_{\alpha_{12/13}}$ respectively), guanylate cyclase (GC), guanosine triphosphate (GTP), inositol triphosphate (IP3), L-type calcium channel (LTCC), myosin light chain (MLC), myosin light chain kinase (MLCK), myosin light chain phosphatase (MLCP), nitric oxide (NO), phosphatidylinositol 4,5-bisphosphate (PIP2), phospholipase C (PLC), rho GTPase, type A (RhoA), rho-associated protein kinase (ROCK), saroplasmic reticulum (SR). Created using BioRender.com.

Smooth muscle relaxation can be induced by several mechanisms including sequestration or removal of intracellular Ca^{2+} , hyperpolarization, removal of stimuli favouring contraction, increased MLCP activity and inhibition of plasma membrane Ca^{2+} channels. During relaxation, receptor- and voltage-operated Ca^{2+} channels in the plasma membrane close resulting in diminished Ca^{2+} entry into the cell. Na^+/Ca^{2+} exchangers are also located on the plasma membrane and aid in lowering intracellular Ca^{2+} . The sarcoplasmic reticulum and the plasma membrane contain Ca^{2+} -ATPases (e.g., SERCA, sarco/endoplasmic reticulum Ca^{2+} -ATPase) that remove Ca^{2+} from the cytosol. Activation of protein kinase A (PKA) by rise in cAMP also lowers myosin light chain kinase activity by phosphorylation.

VIP and PACAP

Vasoactive intestinal peptide (VIP) is a 28-amino acid peptide that belongs to a family of structurally related hormones including glucagon, secretin, and the closely related pituitary adenylate cyclase-activating polypeptide (PACAP)^{21,22}. VIP and PACAP are widely distributed within the nervous system²³ and can be found in perivascular nerves surrounding the cerebral vasculature²⁴. Their biological effects are mediated by three distinct receptors, VPAC₁ and VPAC₂ which have equal affinity for VIP and PACAP, and PAC₁ which binds VIP with much lower affinity than PACAP which is a full agonist at this receptor²⁵⁻²⁸. VPAC_{1/2}- and PAC₁ receptor activation induces vasodilation by activation of PKA due to an increase in cAMP catalysed by G_{α_s} induced activation of adenylate cyclase (AC). PKA then activates K_{ATP} and BK-channels, causing smooth muscle cell hyperpolarisation and relaxation. VPAC₁/VPAC₂/PAC₁ receptor activation in non-vascular tissues has also been linked to activation of other intracellular second messenger systems including phospholipase C (PLC) and phospholipase D (PLD)²⁷ (Figure 3).

VIP and PACAP in stroke

Several studies have shown that VIP and PACAP may play a role as neuroprotective agents in various settings of cerebral ischemia and injury²⁹⁻³¹. The administration of these peptides in close proximity to the cerebral insult seem to reduce inflammation and increase both angiogenesis and neurogenesis which seem to enhance recovery³²⁻³⁴. A more direct neuroprotective effect may be present since both VIP and PACAP have an important physiological role in the regulation of vascular tone.

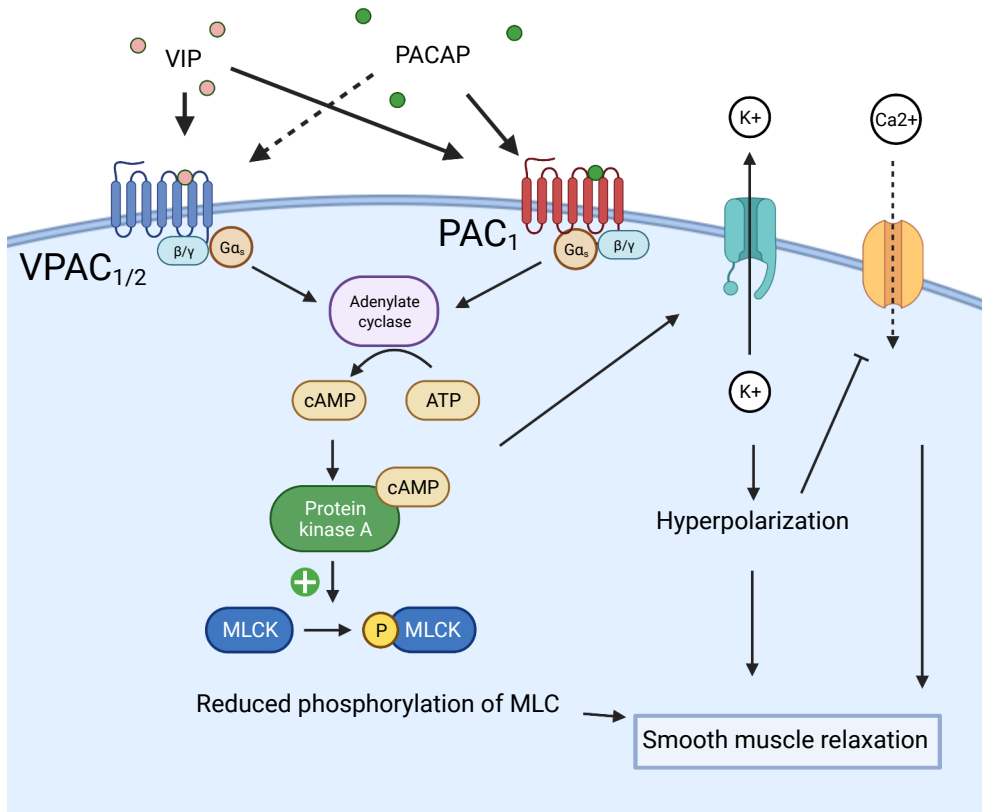


Figure 3. Intracellular signal pathways following VPAC_{1/2} and PAC₁ receptor activation in smooth muscle cells. Adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP), G-protein alpha subunit s (G_{αs}), myosin light chain (MLC), myosin light chain kinase (MLCK), myosin light chain phosphatase (MLCP), pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP). Created using BioRender.com.

CGRP

Calcitonin gene-related peptide (CGRP), a 37-amino acid neuropeptide, is one of the most potent endogenous vasodilators identified to date. CGRP belongs to the calcitonin family of peptides, which also includes calcitonin, amylin and adrenomedullin. CGRP has, since its discovery in 1982³⁵, been found throughout the central and peripheral nervous systems, especially in perivascular nerves. The peptide exists in two isoforms, α - and β CGRP of which the former is more abundant in the central and peripheral nervous systems while β CGRP is thought to play a larger role in the enteric nervous system^{36,37}. CGRP is commonly co-localized with substance P, in sensory and perivascular nerves³⁸.

The vasodilatory effects of CGRP, which are especially profound in the microvasculature and cerebral arteries³⁹⁻⁴¹, are exerted through the CGRP receptor which is a complex consisting of the G-protein coupled calcitonin receptor-like receptor (CLR), a single transmembrane receptor activity modifying protein (RAMP₁) and a receptor component protein (RCP), which is thought to facilitate the interaction between the transmembrane receptor complex and the intracellular G-proteins⁴², mainly G α s which activates adenylate cyclase. Increased levels of cAMP activate protein kinase A (PKA) resulting in opening of K_{ATP} and K_{Ca2+} channels and subsequent hyperpolarization of smooth muscle cells. In some blood vessels, for instance in the rat aorta³⁹, CGRP-induced vasodilation is caused by release of nitric oxide from the endothelium rather than a direct effect on the smooth muscle cells. The vasorelaxant effect of CGRP on feline cerebral arteries is clearly endothelium independent which is also true for human cerebral, meningeal and temporal arteries⁴³⁻⁴⁵.

CLR may also form receptor complexes with other RAMPs. CLR/RAMP₂, for instance, has a high affinity for adrenomedullin (AM) and constitutes the AM₁ receptor⁴⁶ while CLR/RAMP₃ binds both CGRP and AM and form the AM₂ receptor⁴⁷.

CGRP in stroke

The role of CGRP in ischemia is poorly defined, but the peptide is considered a safeguard against excessive vasoconstriction⁴⁸. Recent development of CGRP-inhibitors has risen some concern that the use of such drugs could increase the risk for cerebral ischemia by inhibiting CGRP signaling under physiological conditions. This is somewhat supported by a sporadic case report⁴⁹ and a recent study where CGRP receptor antagonists worsened outcome following tMCAO in mice⁵⁰. A recent abstract from a retrospective study on migraineurs however reports no increase in stroke incidence among those treated with CGRP inhibitors⁵¹. The involvement of CGRP in SAH and especially in vasospasm is a bit better defined. CGRP levels in cerebrospinal fluid (CSF) and jugular vein blood⁵² are increased in

the acute phase of SAH and low levels of CGRP in CSF seems to correlate with increased risk of vasospasm⁵³ further supporting the role of CGRP as a safeguard against excessive vasoconstriction⁵⁴.

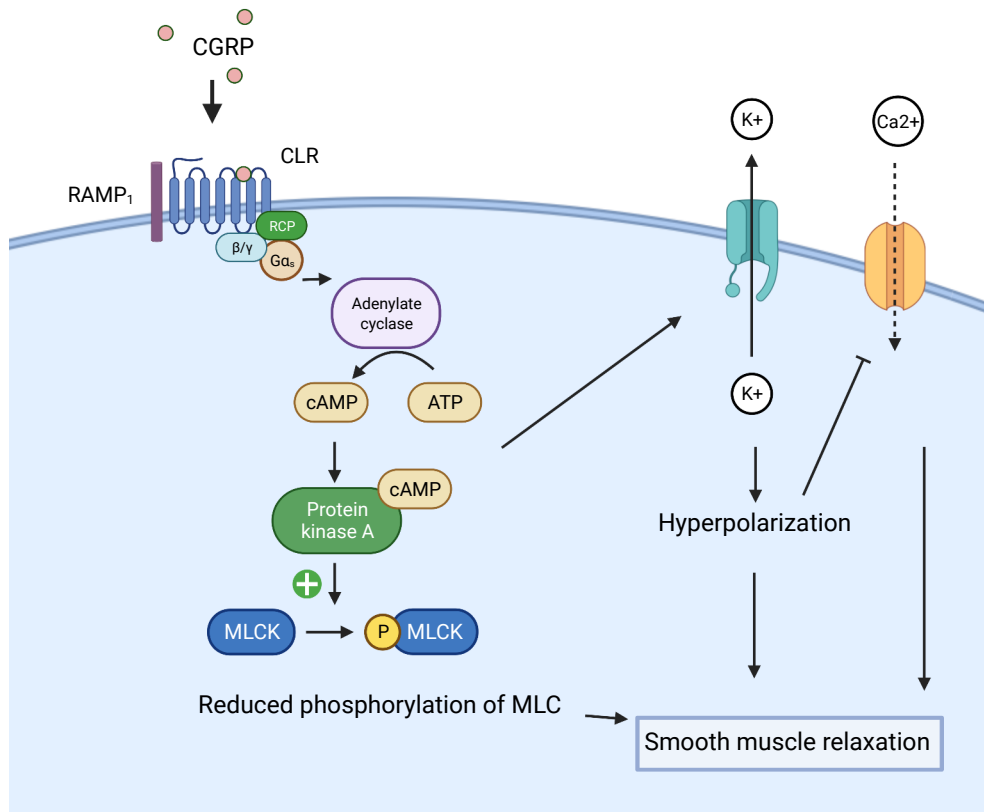


Figure 4. Intracellular signal pathways associated with CGRP receptor activation.

Adenosine triphosphate (ATP), calcitonin gene-related peptide (CGRP), calcitonin receptor-like receptor (CLR), cyclic adenosine monophosphate (cAMP), G-protein alpha subunit s ($G\alpha_s$), myosin light chain (MLC), myosin light chain kinase (MLCK), myosin light chain phosphatase (MLCP), receptor activity modifying protein 1 (RAMP₁), receptor component protein (RCP). Created using BioRender.com.

Purinergic signaling

Nucleotides (e.g., ATP, ADP, UTP and UDP) and nucleosides (e.g., adenosine) are involved in a wide variety of biological processes including regulation of local blood flow and can be released in the extracellular space in response to stimuli such as shear stress or hypoxia. Nucleotides and nucleosides may also be released as co-transmitters from sympathetic and parasympathetic nerves or from circulating platelets or red blood cells⁵⁵. Nucleotides are hydrolysed by a family of ectonucleotidases, named nucleoside triphosphate diphosphohydrolases (NTPDases)⁵⁶ and the conversion of triphosphate nucleotides into diphosphate nucleotides adds to the complexity of purinergic signaling.

Nucleotide and nucleoside receptors are ubiquitous and most mammalian cells express one or more of the seven ionotropic P2X-receptors (P2X₁₋₇), eight G-protein coupled P2Y-receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄) or four G-protein coupled adenosine A₁, A_{2A}, A_{2B} or A₃ receptors⁵⁷⁻⁵⁹.

Vascular smooth muscle cells and vascular endothelial cells are no exception and contain a mix of these receptors which help regulate vascular tone. The exact receptor composition varies with size and localization of the blood vessel. Endothelial purinoreceptors induce vasodilation in response to nucleotides released locally in response hypoxia and shear stress, and the vasodilation is mediated by increased synthesis of NO, EDHF and prostaglandins⁶⁰. Perivascular nerves act directly on smooth muscle purinoreceptors by releasing nucleotides that induce smooth muscle contraction. This results in a purinergic “dual control” of vascular tone.

Activation of purinergic receptors has also been linked to vascular inflammation and long-term trophic events such as cell proliferation and differentiation associated with cardiovascular disease.

Each purinoreceptor has a different ligand profile, P2Y₆ is for instance almost exclusively activated by UDP while P2Y₂ has similar affinity for both ATP and UTP. The P2Y₆ receptor, which is expressed on human and rat cerebral vascular smooth muscle^{61,62}, is coupled to G α_q and induces signaling through the inositol triphosphate pathway which activates phospholipase C and culminates in the release of Ca²⁺ from intracellular storages^{63,64}. Coupling to G $\alpha_{12/13}$ has also been implied, causing activation of the Rho/ROCK pathway, culminating in inhibition of MLCP activity, thus increasing calcium sensitivity^{65,66}.

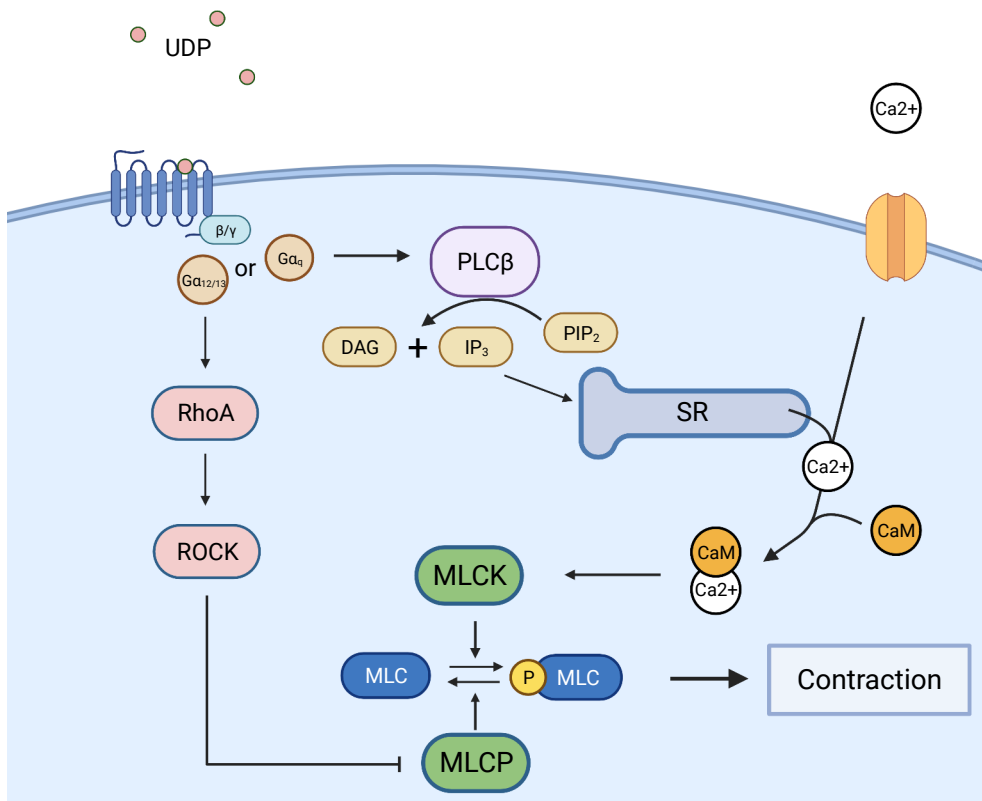


Figure 5. Intracellular pathways associated with P2Y₆ receptor activation.

Calmodulin (CaM), diacylglycerol (DAG), G-protein alpha subunit q, and 12/13 (Gα_q and Gα_{12/13}), inositol triphosphate (IP₃), myosin light chain (MLC), myosin light chain kinase (MLCK), myosin light chain phosphatase (MLCP), phosphatidylinositol 4,5-bisphosphate (PIP₂), phospholipase C (PLC), rho GTPase, type A (RhoA), rho-associated protein kinase (ROCK), sarcoplasmic reticulum (SR), uridine diphosphate (UDP). Created using BioRender.com.

Furthermore, P2Y₆ receptors have been reported to act as mechanoreceptors and may thus play an integral part in the myogenic response to increasing transmural pressures⁶⁵.

Aims

The general goal of this thesis is to expand the knowledge about vascular receptor plasticity following stroke. The following aims were specifically addressed:

1. To assess and evaluate the distribution and function of VIP receptors within the middle cerebral artery in rats with special focus on changes in intracellular calcium.
2. To assess and evaluate the distribution and function of CGRP receptors within the middle cerebral artery in rats with special focus on changes in intracellular calcium.
3. To evaluate potential changes in receptor density, localization, and function of CGRP and VIP receptors following experimental SAH and tMCAO.
4. To evaluate plasticity in receptor density, localization, and function of the P2Y₆ receptor following experimental SAH and tMCAO.

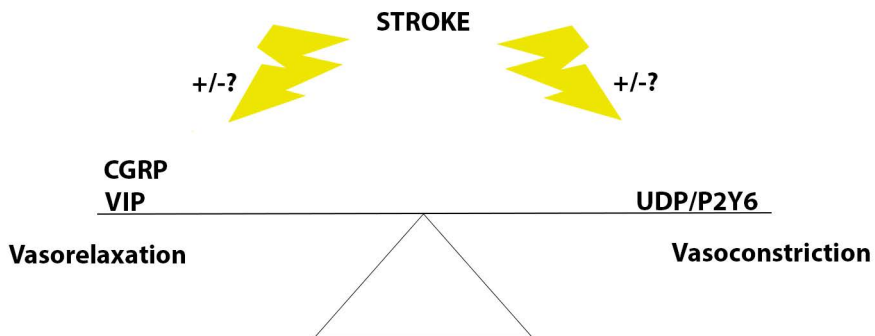


Figure 6. Illustration of main hypothesis.

Our main hypothesis is that both SAH and tMCAO induces changes in vasodilating (VIP and CGRP) receptor systems as well as in vasoconstricting receptor systems (purinergic/P2Y₆) and that these changes either reinforce or counteract each other.

Methods

Ethics

All experiments and procedures were performed in full compliance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Experimental procedures performed in Sweden were approved by the Lund-Malmö Institutional Ethics Committee under the Swedish National Department of Agriculture (M153-15 and M188-12) and experiments performed in Denmark were approved by the Danish Animal Experimentation Inspectorate (licence 2011/561-2025). Paper III and IV were also in full compliance with the more recent ARRIVE 2.0 guidelines.

Table 1. Overview of in vivo and in vitro techniques in the present thesis

	Animal surgery	Wire myography	Pressurized arteriography	Immuno-histochemistry	Intracellular calcium measurements	Flow cytometry	Western blot
Paper I		X	X	X	X		X
Paper II		X	X	X	X		
Paper III	X	X		X			X
Paper IV	X	X		X		X	

Animal surgery procedures (Paper III-IV)

Transient middle cerebral artery occlusion

Unilateral transient middle cerebral occlusion (tMCAO) was induced in rats as described in detail previously^{67,68}. In short, male Wistar rats were anesthetized by spontaneous inhalation of isoflurane in an oxygen/nitrous oxide mixture. An incision in the midline of the neck exposed the right common carotid artery along with the carotid bifurcation and the internal and external carotid arteries. A silicon rubber-coated monofilament was then advanced from the external carotid artery, through the internal carotid artery and onwards until the tip occluded the proximal part of the right MCA (Figure 7). Proper occlusion was confirmed by a prompt drop in flow as measured by a transcranial laser-doppler probe. Anesthesia was discontinued after subcutaneous administration of a local anaesthetic in all open wounds. Two hours after the occlusion, the rats were re-anesthetized and the

occluding filament was removed allowing prompt reperfusion which was confirmed by the return of flow/laser-doppler signal.

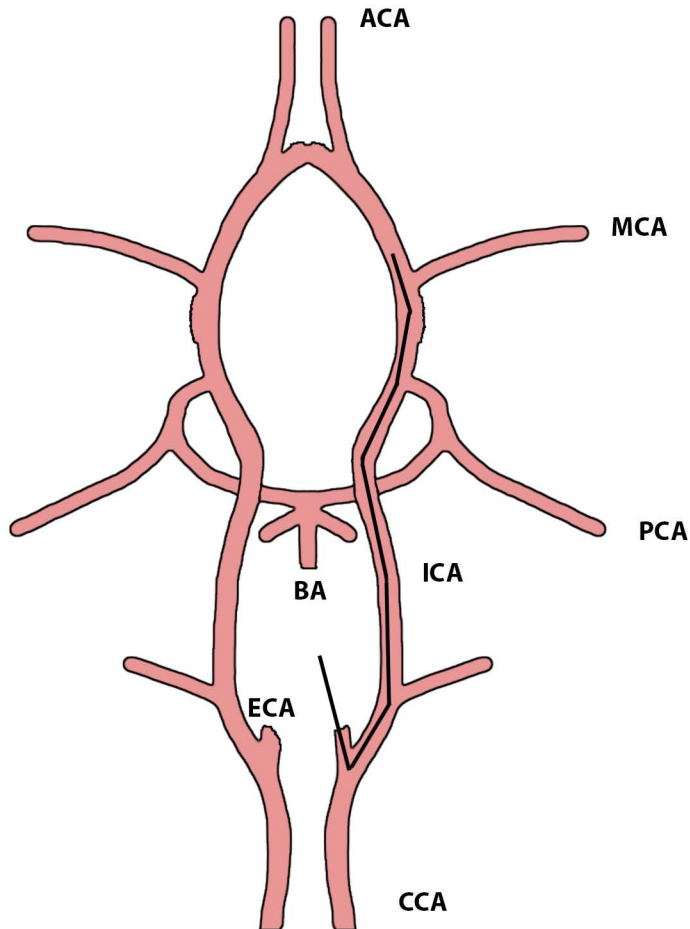


Figure 7. Major vessels of the rat cerebral circulation and position of microfilament during MCA occlusion. Anterior cerebral artery (ACA), middle cerebral artery (MCA), posterior cerebral artery (PCA), common carotid artery (CCA), internal carotid artery (ICA), external carotid artery (ECA) and basilar artery (BA).

SAH surgery and sham surgery

Experimental SAH was induced in male Sprague-Dawley rats as described previously⁶⁹. In short, rats were anesthetized with either isoflurane, halothane, or a mixture of fentanyl, fluanisone and midazolam and mechanically ventilated. A tail artery catheter was used to monitor arterial blood pressure and a laser-doppler probe

attached to the dura mater through a drill hole was used to monitor cerebral blood flow. A catheter placed in the cisterna magna allowed monitoring of intracranial pressure. Blood (250-300 μ L) was injected anterior to the optic chiasm through a cannula, increasing ICP to the mean arterial pressure level. This step was omitted for the sham animals.

Harvest of Cerebral arteries

All operated rats were anesthetized and sacrificed by decapitation 48 hours after the surgery. The MCAs were carefully isolated from the brains and used for myograph experiments or snap frozen at -80°C for further examination by immunohistochemistry, western blot or flow cytometry.

Wire myography (Paper I-IV)

The contractile responses of rat middle cerebral arteries upon stimulation with VIP, PACAP, CGRP or UDP- β -S were evaluated in vitro by mounting freshly harvested cylindrical artery segments in a wire myograph system⁷⁰ (Figure 8). The blood vessels were, in short, mounted on two parallel steel wires running through the lumen of each vessel segment. The steel wires were then connected to a force transducer and an adjustable micrometer, allowing for precise force measurement and careful adjustment of the distance between the steel wires. The whole setup was submerged in a physiological salt solution which was continuously aerated with an oxygen/carbon dioxide mixture (95/5%). Each vessel segment was normalized to attain 90% of the diameter corresponding to a “blood pressure” of 100 mmHg. Viability and contractility of the smooth muscle layer was evaluated by exposure to a potassium rich (60 mM K^+) solution causing smooth muscle cell depolarisation and contraction. This potassium evoked response served as measure of the contractility for each segment. The endothelial function of each vessel segment was tested by precontracting the vessel segment with 5-hydroxytryptamine (5-HT) and then adding the endothelium dependent vasodilator carbachol. Vessel segments used for studies on vasodilating receptor systems were then precontracted using the thromboxane A_2 analogue U46619 (9,11-dideoxy-9a,11a-methanoepoxy $\text{PGF}_{2\alpha}$)(Sigma–Aldrich, Germany).

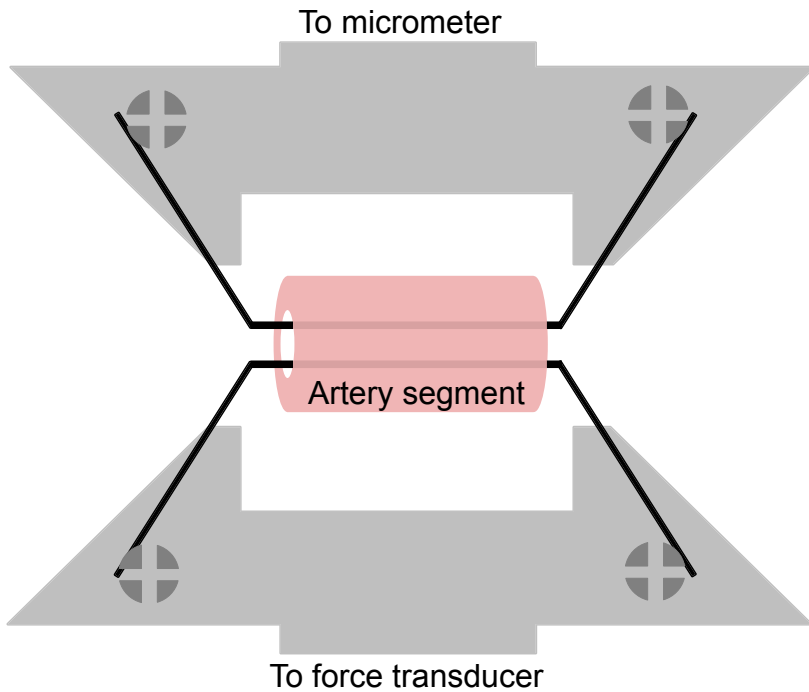


Figure 8. Wire myograph setup.

Pressurized arteriography (Paper I-II)

Pressurized arteriography was used in addition to wire myography in paper I and II to examine the vascular actions of VIP, PACAP and CGRP. Advantages of pressurized myography include the ability to separate the lumen of a vessel from its abluminal side. The main disadvantage is that it is more complicated and time consuming to perform.

In short, 4-6 mm long artery segments were mounted between two glass cannulae and tied in place with a thin nylon thread. The setup was then mounted in an

arteriography system (Living Systems, Burlington, USA) and immersed in a physiological buffer solution (kept at +37°C and continuously aerated by 95% O₂ and 5% CO₂ thus keeping pH at 7.4). The inflow and outflow cannulae were then connected to fluid reservoirs placed at the appropriate height to achieve a transmural pressure of 85 mmHg and a luminal flow of approximately 100 microliters per minute.

The vessel segments were visualized by a microscope (at 600-fold magnification) connected to a digital camera (Axis, Lund, Sweden), which recorded vessel diameter every second and evaluated it in the computer software Mary™ (Nihil KB, Lund, Sweden).

Following mounting, the perfused vessels were allowed to equilibrate and attain a stable tone. Endothelial viability was tested by adding ATP (10⁻⁵ M) to the luminal perfusate.

At the end of each experiment, the vessels were treated with calcium free buffer solution to elicit maximal dilatation which then was used as a reference point.

Immunohistochemistry (Paper I-IV)

Immunohistochemistry is used to visualize localization and expression of proteins in MCA sections. The method is based upon two sets of antibodies; one that detects the protein of interest and one that binds to the first antibody and conjugates with a dye which then can be visualized when exposed to light of an appropriate wavelength.

The papers in this thesis used MCAs which were fixed in 4% paraformaldehyde in phosphate buffer solution (PBS) for 1 h, followed by rinsing in Soerensen's phosphate buffer with increasing concentrations (10 – 25%) of sucrose. The fixed specimens were then placed into Tissue TEK (Gibo, Invitrogen A/S, Taastrup, Denmark), frozen on dry ice and sectioned into 10 µm-thick slices, which were rehydrated in PBS containing 0.25% Triton X-100 for 15 minutes before incubation overnight at +4° C with their primary antibodies. On the following day, the sections were subjected to secondary antibodies for 1 hour at room temperature, washed with PBS and mounted with Vectashield mounting medium (Vector laboratories, Inc. Burlingame, CA, USA) containing 4,6-diamino-2-phenylindole (DAPI) that stains cell nuclei.

Immunoreactivity was visualized and photographed using a light- and epifluorescence microscope (Nikon 80i; Tokyo, Japan) at the appropriate wavelength. The same procedure was used for the negative controls except that the primary antibodies were omitted resulting in no staining in the tissue except for auto-fluorescence in the internal elastic lamina.

Intracellular calcium measurements (Paper I-II)

Changes in intracellular calcium concentrations can be measured using the calcium sensitive fluorescent dye FURA-2. The FURA-2 molecule displays a shift in maximum excitation wavelength from ~380 nm when completely free from Ca^{2+} to ~340 nm when fully saturated with Ca^{2+} . The emission wavelength remains the same at ~510 nm. The concentration of Ca^{2+} in any given moment can be calculated using the ratio of emission when excited at 340 nm over the emission when excited at 380 nm⁷¹. Ratios can then be calibrated to emission signals in the presence of known free Ca^{2+} concentrations (for instance 0 and 5 mM).

The calcium measurements in paper I and II were conducted in the dark on middle cerebral artery segments mounted in a wire myograph placed on the stage of an inverted microscope (Leica DMIRBE, Germany). The arteries were loaded with the FURA-2 (in its cell membrane penetrating acetoxymethyle ester form, FURA-2AM) for 45 minutes at +37° and the loading procedure was performed twice before the vessels were washed and equilibrated for 15 minutes in buffer solution. This also allowed the FURA-2AM to be converted to active FURA-2 by intracellular esterases.

Excitation of the Ca^{2+} /FURA-2 complex was achieved by illuminating the vessel segments with a xenon arc lamp at 340 and 380 nm, respectively. The emitted light was passed through filters (500 - 530 nm) and detected by a photomultiplier (PTI: Photon Technology International, Germany). During the experiments, in which the vessels were precontracted with U44619 (10^{-7} M) and then subjected to incremental concentrations of CGRP or VIP, fluorescence signals and force signals were continuously captured and processed by a computer (FeliX32 program, Photon Technology International, Monmouth Junction, NJ, USA).

The actual calcium concentrations were calculated according to the following equation:

$$[Ca^{2+}]_i = K_d \times \beta \times [(R - R_{min}) / (R_{max} - R)]$$

where K_d is the dissociation constant of the FURA-2/ Ca^{2+} complex (224 nM at +37°C)⁷¹, R is the measured background-corrected ratio between emission at 340 nm excitation and emission at 380 nm excitation, R_{max} and R_{min} are background-corrected ratios under Ca^{2+} -saturating and Ca^{2+} -free conditions, respectively. The β is the ratio of emission signals when excited at 380 nm during determination of R_{min} and R_{max} , respectively.

Flow cytometry (Paper IV)

Flow cytometry can be used to determine expression of proteins in a specified population of cells. In short, tissue is placed in a lysis buffer which dissolves tissue into single cells. Antibodies targeted at a marker specific for the cell type of interest (in this case SM22 α for smooth muscle cells) is then added along with markers for viable cells and the sought protein. A secondary set of antibodies conjugated to a fluorescent dye is then added and the cell suspension is then analysed by a fluorescent-activated cell sorting (FACS) machine sorting the cells into different populations depending on whether they express fluorescent markers for viability, markers identifying them as smooth muscle cells and/or markers for expression of the sought protein. This method is quantitative and very powerful for determining expression of proteins on a defined cell type.

Western blot (Paper I and III)

Western blots are used to detect and identify specific proteins in a sample. The method consists of three steps; separation of proteins by size, transfer to a membrane and marking the sought protein with a primary and secondary antibody allowing it to be visualized. The first step after obtaining the tissue of interest is to break it down and lysate the cells. The lysed sample is then mixed with sodium dodecyl sulphate (SDS) which denatures the proteins and add negative charge. The sample is then allowed to migrate through a gel in an electric field (gel electrophoresis) which separate the proteins by size. The separated proteins are then transferred to a membrane, usually composed of nitrocellulose. The membrane is then incubated with antibodies specific to the protein of interest. A secondary antibody conjugated to either a radioactive or fluorescent dye and aimed at the primary antibody is then

added allowing detection and quantification of the relative levels of protein on the membrane.

Illustrations and statistics

All graphs and pictures were created using either GraphPad Prism (version 6, 8 and 9)(GraphPad Software, San Diego, CA, USA), Microsoft PowerPoint (Microsoft Corporation, Redmond, WA, USA), Adobe Illustrator, Adobe Photoshop (Adobe, San Jose, CA, USA) or the web based tool BioRender (www.biorender.com). Statistical analyses and curve fitting were unexceptionally performed using GraphPad Prism (version 6, 8 and 9). Details regarding statistical tests used in papers I-IV are described in the material and methods section in each paper. Statistical significance was accepted for all $p < 0.05$. All n refers to the number of unique animals used in each experiment.

Results and discussion

VIP mediated vasorelaxation (Paper I and III)

VIP and PACAP are closely related neuropeptides which are thought to help regulate cerebral blood flow and modulate inflammation. Their receptors are hence thought to be potential targets for drugs aimed at preventing neurodegeneration and protection against ischemia⁷². In paper I, we demonstrate the vasorelaxant effects of VIP and PACAP on rat MCAs and correlate the dilatory effect of VIP to diminishing levels of intracellular calcium (Figure 9A). We also demonstrate the inhibitory effect of PG99-465 and PACAP6-38 on VIP induced vasorelaxation (Figure 9B and C). Based on the order of potency of the agonists and the lack of response when agonists were given luminally (Table 2), we also suggest the presence of VPAC₁ and/or VPAC₂ receptors rather than PAC₁ receptors on the vascular smooth muscle cells.

Table 2. Pressurized vs wire myograph experiments

Agonist	Max dilatory response, %
VIP wire	32.71 ± 0.89
VIP ab	24.86 ± 7.25
VIP lum	-4.08 ± 3.71
PACAP-27 ab	25.08 ± 3.89
PACAP-27 lum	5.70 ± 3.20
PACAP-38 ab	10.29 ± 3.75
PACAP-38 lum	-1.85 ± 1.62

Maximum dilatory responses to VIP, PACAP-27 and PACAP-38 when administered abluminally (ab), luminally (lum) or in the wire myograph system (wire).

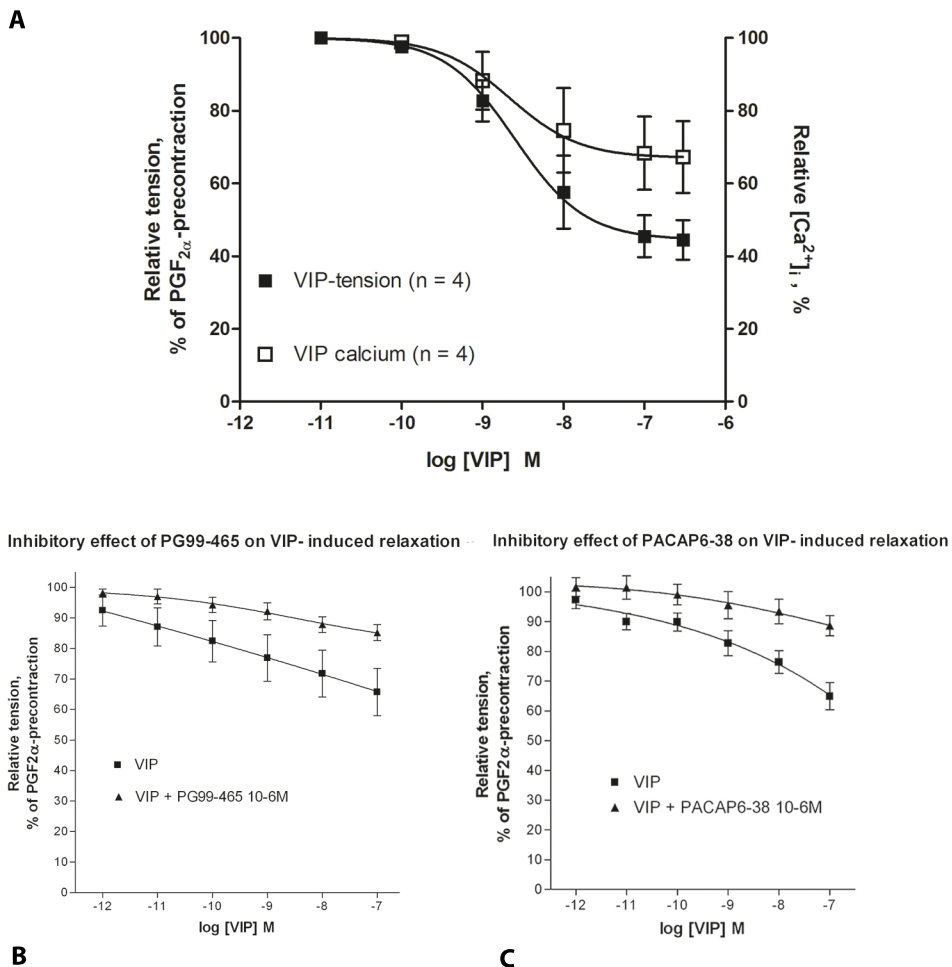


Figure 9. Concentration-response curves demonstrating (A) the vasorelaxant effect of VIP and its association with intracellular calcium levels, (B) the inhibitory effect of PG99-465 on VIP-induced vasorelaxation, and (C) the inhibitory effect of PACAP 6-38 on VIP-induced vasorelaxation on rat MCAs.

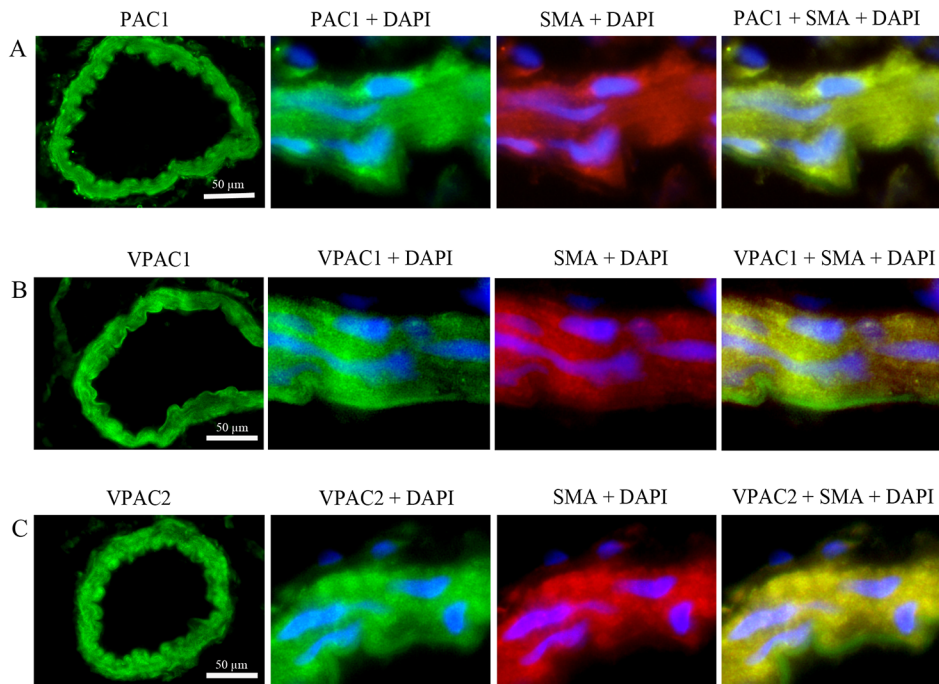


Figure 10. Triple immunostaining demonstrating the co-localization of smooth muscle actin (SMA) and PAC₁, VPAC₁ and VPAC₂, respectively. DAPI (blue) is a nucleus marker in sections of rat MCAs.

Changes in receptor systems favouring vasoconstriction following ischemia have been described previously^{16,17,68,73–79}, but plasticity in receptor systems favouring vasodilation are less defined. In paper III we investigate changes in vasomotor responses to VIP following either experimental SAH or transient MCA occlusion. Our results contain the first description of increased vasodilatory responses to VIP following tMCAO, as indicated by an increase in E_{max} . We demonstrate a right shift of the second part of the biphasic VIP concentration-response curve for both SAH and tMCAO which may indicate decreased signaling through PAC₁ receptors, either by decreased receptor expression or by decreased ligand-receptor interaction. It may also be noted that there was a significant change in pEC_{50} in phase 2 for the contralateral to the tMCAO. This may reflect a more widespread change in vessel phenotype, possibly caused by metabolic disturbances^{80,81} in a state known as diaschisis⁸². This phenomenon highlights the need for true sham animals rather than the use of the contralateral blood vessel in tMCAO models of stroke.

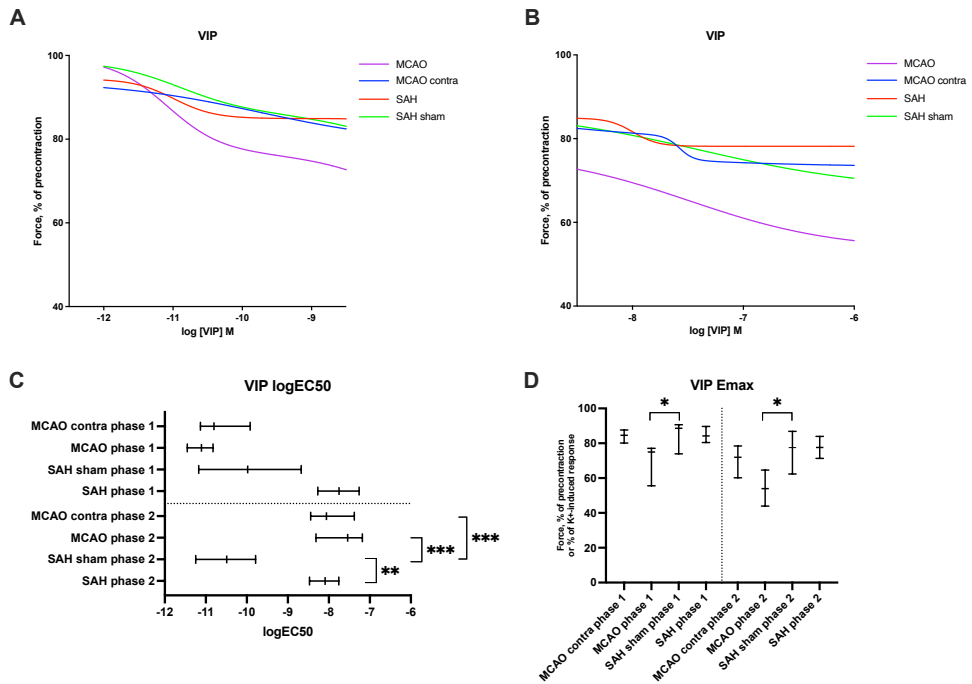


Figure 11. Wire myograph experiment showing the bi-phasic vasodilatory effects of VIP on the middle cerebral artery, separated into phase 1 (A) and phase 2 (B). Only the fitted curves are presented. pEC₅₀ and E_{max} for VIP following tMCAO (n = 7 (ipsilateral) or 8 (contralateral)), SAH (n = 10) or sham SAH surgery (n = 6) are presented in (C) and (D), respectively. Data is presented as median and IQR. $p < 0.05$ (*) / $p < 0.01$ (**) / $p < 0.001$ (***).

We were not able to detect any significant changes in expression of the receptor proteins VPAC₁, VPAC₂ or PAC₁ following either tMCAO or SAH. tMCAO was however associated with increased levels of RAMP₁ and RAMP₃ which may associate with the receptor proteins and alter their ligand affinity or the intracellular signaling⁸³. VPAC₁-RAMP₂ interaction is associated with increased PLC activity through G α_q -mediated signaling⁸⁴. An increased expression of RAMP₁ and RAMP₃ may reduce this association and thus cause a state of increased AC activity and less production of IP₃ which could potentially enhance vasodilation.

It may also be noted that western blot is less sensitive when compared to methods such as flow cytometry, especially when using whole tissue samples containing not only smooth muscle cells but also endothelial cells and glial cells.

The mitogen activated protein kinase kinase 1/2 / extracellular signal regulated kinase 1/2 (MEK/ERK_{1/2}) signaling pathway is heavily implied as a main mediator of the detrimental changes occurring immediately after both global and focal ischemia and is thought to regulate expression of several vasoconstrictive receptor proteins^{16,79}, inflammatory mediators^{85,86} and cellular responses to oxidative stress.

To complicate things further, MEK/ERK_{1/2} also seem to regulate some of the processes associated with recovery, such as neurogenesis and angiogenesis^{87,88}. In an experimental stroke study it has been demonstrated that treatment with a MEK1/2 inhibitor in the early phase of stroke does not prevent the recovery processes in the later phase of stroke⁸⁹.

It seems likely that changes in VIP-induced vasoreactivity rely on activation of a non-MEK/ERK_{1/2} pathway since previous work have determined that this pathway is activated in both SAH and tMCAO models of stroke¹⁶ and the present changes in vasoreactivity and RAMP protein levels seem to occur only in the tMCAO animals.

VIP plays an important role in neuronal and cardiac remodeling after ischemia and its protective actions may be exerted through anti-apoptotic and anti-inflammatory⁹⁰⁻⁹² mechanisms as well as through neurotrophin⁹³⁻⁹⁵ release. VIP has also been shown to reduce SMC proliferation after carotid ligation in mice⁹⁶.

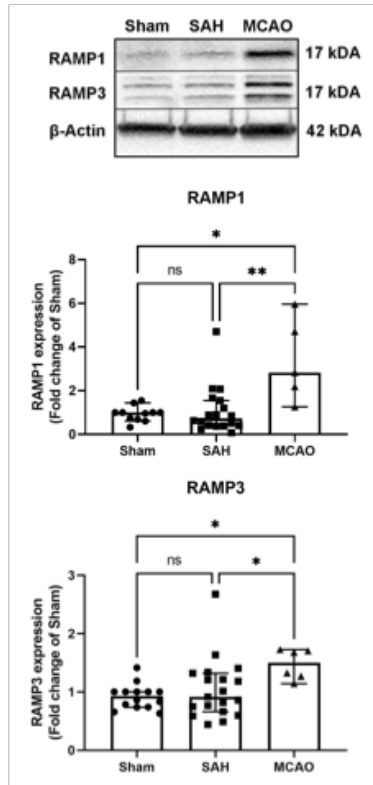


Figure 12. RAMP₁ and RAMP₃ protein expression demonstrated by representative western blots and quantification graphs. The protein expression was normalized to β-actin (loading control). An increase in expression of RAMP₁ and RAMP₃ following tMCAO but not SAH was observed. Data is presented as median and IQR. $p < 0.05$ (*) / $p < 0.01$ (**).

CGRP mediated vasorelaxation (Paper II and III)

CGRP is a neuropeptide with very strong vasorelaxant properties. It also has a key role in migraine and other cluster headaches⁹⁷ and has been implied in the prevention of vasospasm following SAH⁵³. Paper II describes the dilatory actions of CGRP on the cerebral vasculature with special focus on changes in intracellular calcium. From the results obtained, we conclude that the CGRP receptor is expressed only on the smooth muscle cells (Figure 13) and that the induced dilatation (Figure 14 and 15B) correlates with a dose dependent decrease in intracellular calcium levels (Figure 15A). We also describe how the CGRP receptor antagonist telcagepant thwarts both

the dilatory effects of CGRP and the corresponding changes in intracellular calcium levels (Figure 14 and 15).

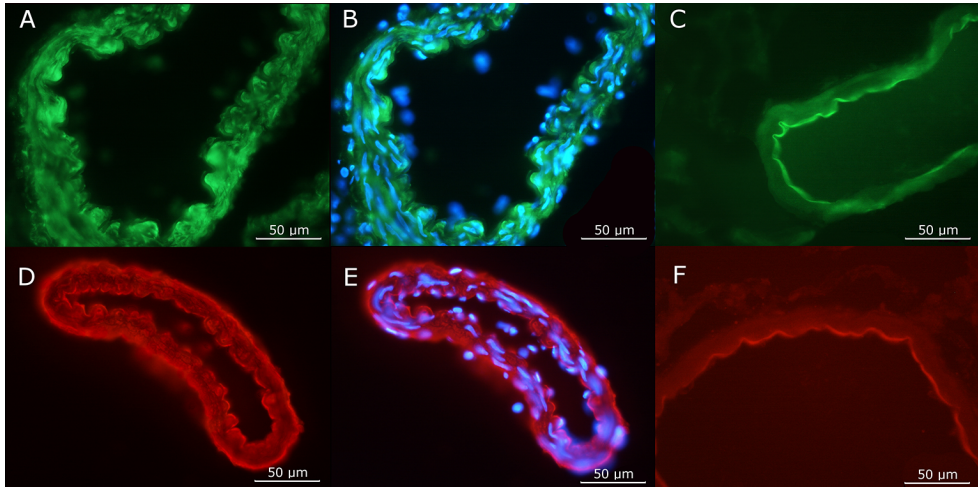


Figure 13. Cross-sections of rat MCAs demonstrating expression of CLR (A and B) and RAMP₁ (D and E). Negative controls (C and F). A nucleus stain (DAPI) has been added to (B) and (E).

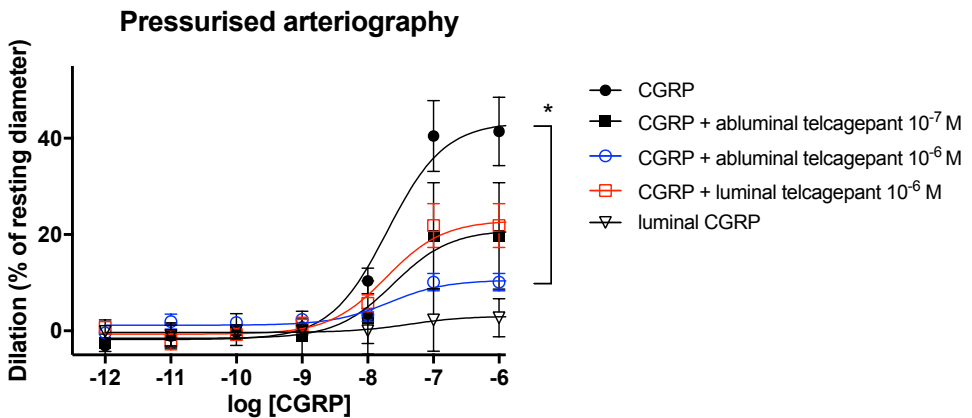


Figure 14. Pressurised arteriography experiment demonstrating the vasorelaxant effect of abluminal, but not luminal CGRP and the dose- and localization dependent inhibitory effect of telcagepant. Values are presented as means \pm SEM, n = 4-12. * = p < 0.05.

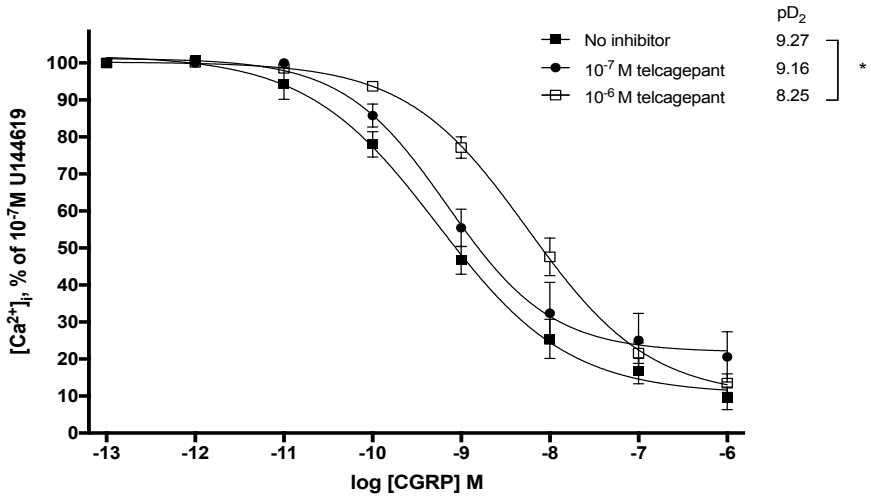
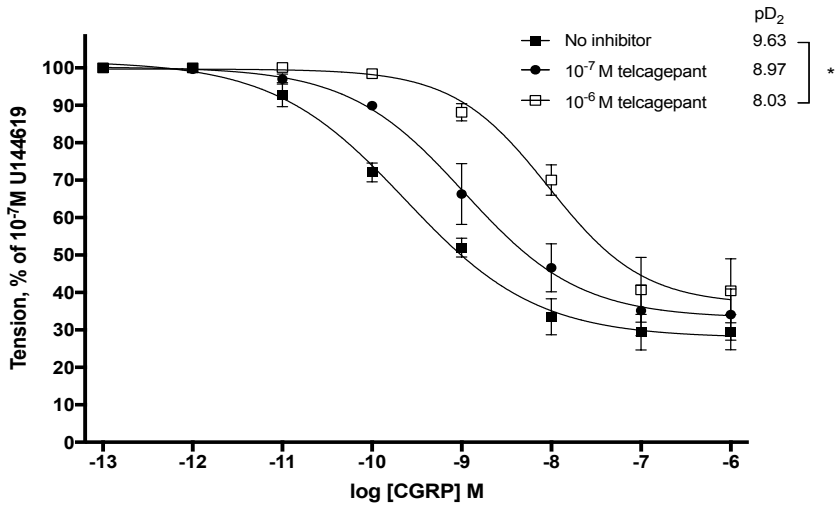
A**B**

Figure 15. CGRP concentration-response curves demonstrating (A) intracellular Ca^{2+} -levels and (B) tension with or without telcagepant, $n = 3 - 6$, data presented as means \pm SEM.

The vasodilatory responses to CGRP were unaffected by both tMCAO and SAH (Figure 16) which further supports the important role of CGRP as a protective agent in states of excessive vasoconstriction. Treatment with CGRP antagonists seems to increase neurological damage following cerebral ischemia, at least in mice⁵⁰, while the loss of CGRP signaling seems safe under non-ischemic conditions⁵¹. We were not able to detect any changes in CLR expression after tMCAO but report an increase in RAMP₁ and RAMP₃ levels (Figure 12). All receptor protein levels remained unchanged after SAH.

The lack of changes in receptor expression and sensitivity to CGRP after SAH supports the idea that CGRP depletion rather than receptor downregulation may be a contributing factor in the pathogenesis of vasospasm. A recent study investigating CGRP responses in rat basilar arteries and MCAs following SAH indeed concluded that vascular responses to CGRP are maintained and that capsaicin induced release of CGRP is impaired after SAH⁷⁴.

The apparent difference in responses (E_{max}) to CGRP in the wire myograph (Figure 15B) and pressurised arteriography system (Figure 14) is partly due to the inadequacy in comparing changes in wall tension (wire myography) and vessel diameter (pressurized arteriography) since those are not readily comparable. The need for a precontracting agent is also a possible confounder which makes comparison between the two difficult.

Pressurized arteriography thus seems the more physiological, yet less established method for study of vasodilating substances. It is also more time consuming and may be most useful when dealing with receptor systems expressed on both the abluminal and luminal side of the blood vessel.

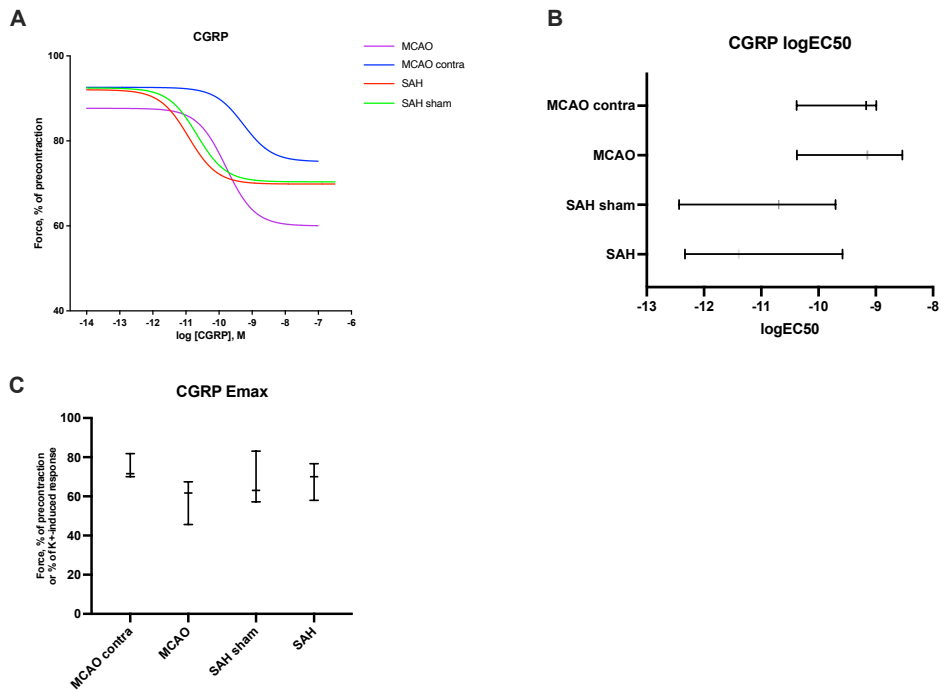


Figure 16. Wire myograph experiment showing the vasodilatory effects of CGRP (A) on the middle cerebral artery. Only the fitted curves are presented. pEC50 and Emax for CGRP following tMCAO (n = 10), SAH (n = 14) or sham SAH surgery (n = 9) are presented in (B) and (C), respectively. Data is presented as median and IQR.

UDP- β -S mediated vasoconstriction (Paper IV)

Purinergic receptors are important for regulation of myogenic tone⁹⁸. The P2Y₆ receptor has previously been found on the smooth muscle cells of both rat and human cerebral arteries and mediates a strong vasoconstrictive response upon stimulation by its agonist UDP⁶¹. In paper IV we report increased expression of P2Y₆ receptors on vascular smooth muscle cells following both SAH and tMCAO (Figure 17). Vasoconstrictive responses to the stable P2Y₆ receptor agonist UDP- β -S were, however, only enhanced after tMCAO (Figure 18). We were not able to detect any expression of P2Y₆ receptors on the endothelium (Figure 19). The enhanced responses to P2Y₆-mediated signaling thus seem to add yet another pro-constrictive receptor system to those previously described^{16,17,68,73–79}, and these changes seem to favour a vasoconstrictive vascular phenotype following focal or global cerebral ischemia.

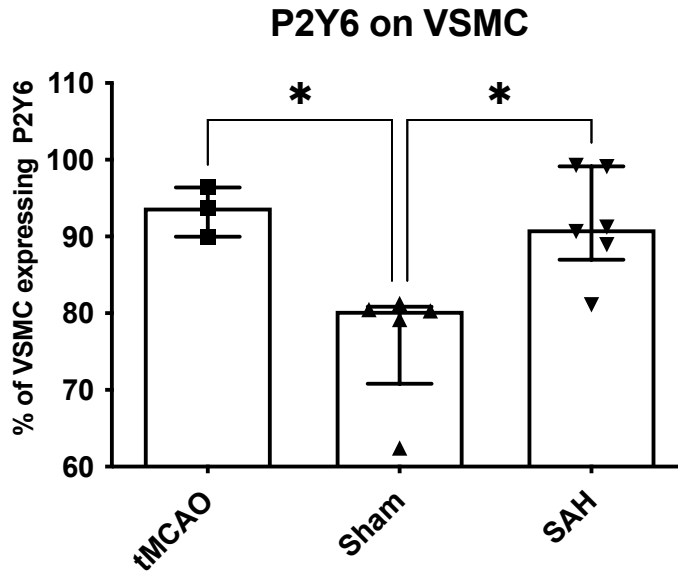


Figure 17. Scatter/box plot demonstrating the expression of P2Y₆ receptors on vascular smooth muscle cells determined by flow cytometry. Data are presented as median and IQR, n = 3-6, * = p < 0.05.

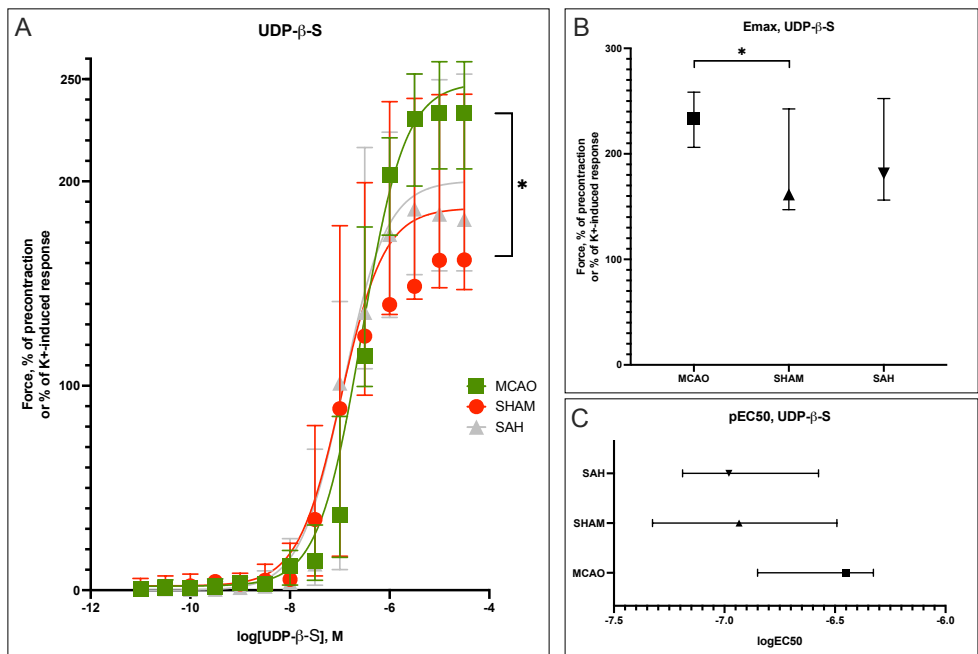


Figure 18. Wire myograph experiments demonstrating the vasoconstrictive actions by cumulative application of UDP-β-S on the middle cerebral artery following tMCAO (n=10), SAH (n=11) or sham surgery (n=9) (A). Emax and pEC50 are presented in (B) and (C) respectively. Data are presented as median and IQR, * = p < 0.05

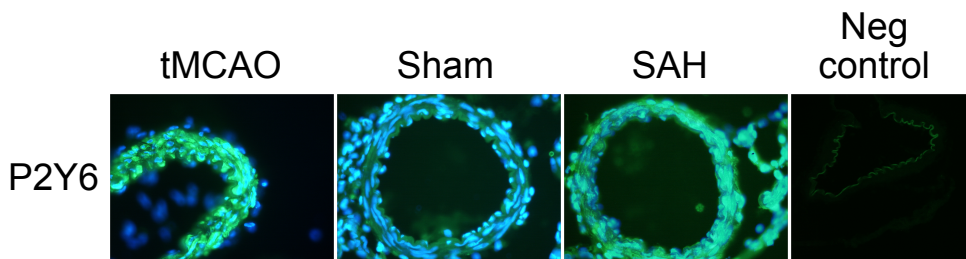


Figure 19. Cross-sections of rat MCAs demonstrating the presence of P2Y₆ receptors on VSMCs but not on the endothelial cells after tMCAO, SAH and sham surgery respectively.

The discrepancy between tMCAO induced upregulation of the receptor protein and the concomitant increase in vasoconstrictive responses versus the SAH induced expression of the receptor protein while maintaining normal responses to UDP-β-S warrants further investigation. It may reflect a slower progression of vascular changes following SAH when compared to tMCAO but could also be caused by

P2Y₆ receptor expression being regulated by another pathway than MEK/ERK_{1/2} which regulates ET_B, 5-HT_{1B} and AT₁ receptor expression. An additional possibility is that the receptors in the SAH model remain internalized to a greater extent and thus are unable to interact with UDP-β-S.

Strengths and limitations

The papers in this thesis contribute to increased knowledge regarding the plasticity of CGRP and VIP receptors in two different settings of cerebral ischemia. Utilizing two different stroke models is a major strength of this thesis and highlights both common and differing features of global and focal ischemia. The thesis also contains the first description of enhanced P2Y₆-mediated signaling following cerebral ischemia.

A weakness of this thesis, especially concerning papers III and IV is the lack of conformity in anesthetic method. No less than three different anesthetic regimen have been used. There was, however, no inter-group difference in vascular responses between animals based on anesthetic regimen.

It is important to remember that results from animal studies cannot be readily translated to other species. The past decade illuminates this very well as hundreds of therapeutic agents targeting at cerebral ischemia have shown promising results in animals just to be proven to be useless in human subjects. Animal studies still have a role in describing mechanisms underlying both physiological and pathological processes.

Comparing different strains of laboratory rats can be problematic since responses to ischemia may vary^{99,100}. Changes in vascular receptors, however, seem consistent as up-regulation of ET_B, 5-HT_{1B} and AT₁ receptors following ischemia is present in both SD and Wistar rats^{17,68,75,77}. The development and extension of a penumbra also seems independent of rat strain¹⁰⁰ indicating that the main difference in rat strains may lie in neuronal sensitivity to ischemia rather than differences in vascular responses¹⁰⁰.

Main conclusions

The following conclusions can be made from the papers constituting the basis for this thesis:

- VIP receptors are mainly expressed on the smooth muscle cells of the rat middle cerebral artery with VPAC₁/VPAC₂ being much more abundant than PAC₁.
- VIP receptor activation causes a dose dependent vasorelaxation which closely correlates to intracellular calcium levels.
- tMCAO induces changes not only in the occluded MCA, but also in the contralateral vessel rendering this vessel unsuitable as a control vessel.
- tMCAO but not SAH enhances the vasodilatory responses to VIP, either due to increased expression of one or both VPAC receptors, a shift from one VPAC to the other, or by interaction with RAMP₁ or RAMP₃.
- The CGRP receptor complex is expressed on the smooth muscle cells and not on the endothelial cells of the rat middle cerebral artery.
- CGRP causes dose dependent vasorelaxation which is closely related to decreasing levels of intracellular calcium. Telcagepant antagonizes both vasorelaxation and changes in intracellular calcium.
- CGRP-mediated vasoreactivity is unaffected by both tMCAO and SAH, lending support to the notion that CGRP depletion rather than receptor downregulation may contribute to vasospasm following SAH.
- tMCAO increases expression of P2Y₆ receptors on cerebrovascular smooth muscle cells, increasing the vasoconstrictive response to the UDP analogue UDP- β -S. A similar change in receptor expression was observed after SAH, but there was no enhanced vasoconstrictive response.

Concluding remarks

SAH and ischemic focal stroke seem to result in a broad, almost general, upregulation of vasoconstrictive receptors within the cerebral vasculature. From our present results we can conclude that this upregulation of receptors seems to include at least one *vasodilatory* receptor system, at least after tMCAO. In addition, we have identified yet another receptor system which may add to the vasoconstrictive responses after ischemia. The net effect of these changes may contribute to the delayed ischemic injury which endanger the penumbra and can cause delayed cerebral ischemia.

The increase in both P2Y₆ receptor expression and P2Y₆-mediated contraction is well in line with previous findings for other vasoconstrictive receptor systems. There was an increase in receptor expression in the SAH model as well, but there was no significant change in either E_{\max} or pEC_{50} which differs from previous findings related to other vasoconstrictive receptor systems where ischemia, whether global or focal, induce the same changes in phenotype in the cerebral blood vessels. Up-regulation of the P2Y₆ receptor may nevertheless contribute to impaired tissue perfusion and may hence be a target for therapy aiming to improve perfusion.

The evidence of changes, not only favouring vasoconstriction, but also promoting vasodilation may add further targets for therapy against vascular dysregulation following ischemia and shed additional light on the delicate balance regulating vascular homeostasis under both physiological and pathological conditions. VIP and its receptors are of certain interest given their neuroprotective effects combined with the increased vasodilatory response following ischemia. Enhanced VIP signaling may thus *kill two birds with one stone* and achieve neuroprotection along with increased tissue perfusion. Further studies on the subject are warranted, especially to delineate which signaling pathway is responsible for the vascular changes.

The vast number of mediators regulating vascular tone adds to the complexity of CBF regulation and it is unlikely that a single receptor system is responsible for the pathological changes in tissue perfusion seen after ischemia. The findings in the present thesis provides additional therapeutic possibilities in demonstrating up-regulation in an additional vasoconstrictive receptor system as well as demonstrating enhanced signaling through a vasodilatory receptor system.

Future perspectives

This thesis has contributed to the current knowledge of vascular receptor plasticity in stroke, but it has also generated new questions and perspectives that warrants further investigation.

First, I would like to investigate whether P2Y₆ receptor up-regulation is associated with activation of the MEK/ERK_{1/2} pathway described for ET_B, 5-HT_{1B} and AT₁ receptors. The timeframe for changes in P2Y₆ receptor plasticity also warrants further investigation, especially since SAH caused increased expression of the receptors but failed to change the agonist induced response at the 48-h point in our experiments. *If* there is a progressive but delayed increase in UDP induced vasoconstriction following SAH it would be interesting to evaluate whether this coincides with the development of vasospasm and whether vasospasm can be prevented by an UDP antagonist.

It would also be interesting to investigate the mechanisms behind the enhanced vasodilation caused by VIP after tMCAO. An assay measuring AC and PLC activity could be used to investigate whether increased RAMP₁ and RAMP₃ correlate with reduced VPAC₁-RAMP₂ interaction. Finding the signaling pathway responsible for the changes in VIP vasoreactivity is also an obvious goal. A partial goal along that road would be to exclude the possibility that enhanced VIP signaling is the result of the same MEK/ERK_{1/2} pathway that induce the vasoconstrictive state mentioned above.

I would also like to use the more sensitive and specific flow cytometry method to better define the changes in receptor protein expression following tMCAO and SAH. One of the benefits of flow cytometry is the ability to discriminate between cell types and it would thus be easy to distinguish between changes in protein expression in SMCs and endothelial cells.

Finally, I would like to investigate the changes in VIP and P2Y₆ vasoreactivity and receptor expression in human tissue.

Svensk sammanfattning

Miljontals människor världen över drabbas årligen av stroke i någon form. Bara i Sverige drabbas ca 30 000 personer årligen. Det primära insjuknandet beror vanligen på att en propp bildats i något av de blodkärl som förser hjärnan med blod (hjärninfarkt), men hos ett mindre antal uppstår i stället blödningar mellan hjärnhinnorna då något av hjärnans kärl spricker (subaraknoidalblödning). Den sistnämnda gruppen har sämst prognos med en 30-dagarsdödlighet på upp till 45%. Flertalet patienter som drabbas av en propp drabbas också av någon form av funktionsnedsättning till följd av den hjärnskada som uppstår. Det verkar också som om hjärnans blodkärl genomgår en förändring som gör att de fungerar sämre än vanligt och har svårare att reglera och dirigera hjärnans blodflöde. Patienter vars blodkärl har spruckit kan dessutom råka ut för kramp i det drabbade blodkärlet vilket orsakar ytterligare hjärnskador.

Från att inte kunna behandlas över huvud taget har man på senare år kunnat erbjuda behandling som löser upp eller mekaniskt avlägsnar en eventuell propp och detta har förbättrat prognosen för denna undergrupp av patienter markant. Tyvärr måste sådan behandling ges inom ett visst tidsintervall för att vara effektiv. Mycket energi har därför lagts på att försöka finna ett läkemedel som kan förhindra eller åtminstone mildra effekten av den syrebrist som hjärnan drabbas av. För att utveckla sådana läkemedel är det viktigt att kartlägga de förändringar som sker såväl i hjärnans nervceller, dess stödjevävnad och i de blodkärl som förser hjärnan med blod.

Tidigare studier har visat att en rad receptorer för kärksammandragande substanser ökar kraftigt efter både proppar och blödningar i hjärnan. Målet med avhandlingen som helhet är att närmare undersöka funktionen hos två blodkärlsvidgande och ett kärksammandragande receptorsystem samt undersöka om funktionen i dessa receptorsystem påverkas av antingen hjärninfarkt eller subaraknoidalblödning. För att undersöka detta har vi i delarbete I och II beskrivit hur de kärlvidgande receptorsystemen i fråga fungerar i friska blodkärl från råttor. Delarbete III och IV undersöker sedan närmare på vad som händer med receptorer och deras funktion i råttor som drabbats av propp eller subaraknoidalblödning.

Sammanfattningsvis har vi beskrivit lokaliseringen för och kopplingen mellan receptorer för signalsubstanserna VIP, PACAP och CGRP och förändringar i intracellulärt calcium, samt beskrivit förändringar i receptoruttryck och -funktion

för VIP-, CGRP- och P2Y₆-receptorn i hjärnans kärl efter antingen experimentellt skapad hjärninfarkt eller subaraknoidalblödning.

Vi drar slutsatsen att hjärninfarkt ökar blodkärlens känslighet för signalsubstansen VIP samt ökar känsligheten för P2Y₆-medierad kärlsammandragning. Subaraknoidalblödning ökade visserligen mängden P2Y₆-receptorer på kärlen, men ökade inte dess kärlsammandragande effekt. Hjärninfarkt visade sig också öka halterna av några receptormodifierande proteiner som möjligen kan bidra till den ökade känsligheten för VIP.

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