Mechanisms of cellular synchronization in the vascular wall

Mechanisms of vasomotion

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1. PREFACE

This thesis is based on the work carried in the Vascular Smooth Muscle group at the Institute of Physiology and Biophysics, Aarhus University. This thesis is focused on a mechanistic understanding of cellular synchronization in the small resistance arteries. I have started this work because of my general interest in vasomotion, a phenomenon of synchronized activity in the vascular wall which has been known for more than 150 years. In spite of the long history and suggestions that vasomotion is important for pathological states the studies of vasomotion have been mostly descriptive. Development of new experimental techniques such as small artery myography, intracellular Ca^{2+} imaging and electrophysiological approaches brought new possibilities to the studies of cellular mechanisms of vascular synchronization.

I have used these advanced methods to characterize vasomotion in detail and have suggested and tested a model for generation of vasomotion in the rat mesenteric artery. The suggested model is one of several models of vasomotion but it has strong experimental support and is supplemented by the mathematical modeling published by our group. Two key elements for the synchronized oscillation in the mesenteric small arteries a cGMP-dependent Ca^{2+}-activated Cl^- current and the electrical intercellular communication were further explored in my research. I have characterized the cGMP-dependent Ca^{2+}-activated Cl^- current suggested by our model for vasomotion and demonstrated this current in different vascular beds. Using a novel siRNA approach I have then shown the association between this current and bestrophin-3 protein expression *in vivo* and *in vitro*. Based on these results I suggested the molecular identity of this current and its significance for smooth muscle cell synchronization by a membrane potential-dependent mechanism. The studies of intercellular communication in the vascular wall are lacking specific and effective tools to manipulate these intercellular contacts. I have performed comprehensive studies to analyze the action of the most commonly used gap junction blockers and demonstrated that vasomotion can be used as a "readout" for intercellular communication. Using this approach I demonstrated that inhibition of the ouabain-sensitive Na^+/K^+-ATPase uncouples smooth muscle cells in the vascular wall and suggested the mechanism responsible for this electrical uncoupling. In my studies on the role of the ouabain-sensitive Na^+/K^+-ATPase for vascular function I suggested the presence of Na^+/K^+-ATPase-based signalosome which also includes the Na^+/Ca^{2+}-exchanger, gap junctions and the ATP-dependent K^+ channels. These studies provide a useful tool for manipulations intercellular communication in the small arteries.

The thesis includes the following previous publications:

I. Peng H, Matchkov V, Ivarsen A, Aalkjaer C, Nilsson H. Hypothesis for the initiation of vasomotion. Circ Res. 2001; 88(8): 810-815.

II. Rahman A, Matchkov V, Nilsson H, Aalkjaer C. Effects of cGMP on coordination of vascular smooth muscle cells of rat mesenteric small arteries. J Vasc Res. 2005; 42(4):301-311.

III. Matchkov VV, Aalkjaer C, Nilsson H. A cyclic GMP-dependent calcium-activated chloride current in smooth-muscle cells from rat mesenteric resistance arteries. J Gen Physiol. 2004; 123(2): 121-134.

IV. Matchkov VV, Aalkjaer C, Nilsson H. Distribution of cGMP-dependent and cGMP-independent Ca^{2+}-activated Cl^- conductances in smooth muscle cells from different vascular beds and colon. Pflugers Arch. 2005; 451(2): 371-379.

V. Matchkov VV, Larsen P, Bouzinova EV, Rojek A, Boedtkjer DM, Golubinskaya V, Pedersen FS, Aalkjaer C, Nilsson H. Bestrophin-3 (vitelliform macular dystrophy 2-like 3 protein) is essential for the cGMP-dependent calcium-activated chloride conductance in vascular smooth muscle cells. Circ Res. 2008; 103(8): 864-872.

VI. Matchkov VV, Rahman A, Peng H, Nilsson H, Aalkjaer C. Junctional and nonjunctional effects of heptanol and glycyrrhetinic acid derivates in rat mesenteric small arteries. Br J Pharmacol. 2004; 142(6): 961-972.

VII. Matchkov VV, Rahman A, Bakker LM, Griffith TM, Nilsson H, Aalkjaer C. Analysis of effects of connexin-mimetic peptides in rat mesenteric small arteries. Am J Physiol. 2006; 291(1): H357-H367.

VIII. Matchkov VV, Gustafsson H, Rahman A, Briggs Boedtkjer DM, Gorintin S, Hansen AK, Bouzinova EV, Praetorius HA, Aalkjaer C, Nilsson H. Interaction between Na^+/K^+-pump and Na^+/Ca^{2+}-exchanger modulates intercellular communication. Circ Res. 2007; 100(7): 1026-1035.

IX. Glavind-Kristensen M, Matchkov V, Hansen VB, Forman A, Nilsson H, Aalkjaer C. K_{ATP}-channel-induced vasodilation is modulated by the Na,K-pump activity in rabbit coronary small arteries. Br J Pharmacol. 2004; 143(7): 872-880.

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3. LIST OF ABBREVIATIONS

AA	arachidonic acid
ADP	adenosine diphosphate
ANO1	anoctamin-1; see also TMEM16A
ATP	adenosine triphosphate
AVP	arginin-vasopressine
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BK	big-conductance Ca^{2+}-activated K^+ channels
$[Ca^{2+}]_i$	intracellular calcium concentration
CaCC	Ca^{2+}-activated Cl^- channels

CAMKII	calmodulin kinase II
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CICR	Ca^{2+}-induced Ca^{2+} release
Cx	connexin
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
EDHF	endothelium-derived hyperpolarizing "factor"
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
GAP	connexin-mimetic peptides
GJ	gap junctions
IAA-94	R(+)-[(6,7-Dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy]acetic acid
$I_{Cl(Ca)}$	Ca^{2+}-activated Cl^- current
$I_{Cl(Ca,cGMP)}$	cGMP-dependent Ca^{2+}-activated Cl^- current
IK	intermediate-conductance K^+ channels
IP_3	inositol 1,4,5-trisphosphate
IP_3-R	IP_3-sensitive channels
K_{ATP}	ATP-dependent K^+ channels
K_{Ca}	Ca^{2+}-activated K^+ channels
MAPK	mitogen-activated protein kinase
MLC	myosin light chain
NCX	Na^+/Ca^{2+} exchanger
NO	nitric oxide
NPY	neuropeptide Y
PKG	protein kinase G
PKC	protein kinase C
PLC	phospholipase C
PLA_2	phospholipase A_2
RYA-R	ryanodine-sensitive channel or receptor
siRNA	small interfering RNA
SK	small-conductance K^+ channels
SMCs	smooth muscle cells
SR	sarcoplasmic reticulum
TMEM16A	transmembrane protein 16A, see also ANO1
TRP channel	transient receptor potential channel
VDCCs	voltage-dependent Ca^{2+} channels
VMD	vitelliform macular dystrophy

4. INTRODUCTION

A blood circulation system in complex, multicellular organisms should satisfy the metabolic demands of all cells in the body. This demand varies widely with location of the tissues and with time, and is affected by changes in environmental and internal parameters over a considerable range. Therefore, it is important to have a very precise regulation of blood flow that is achieved by the combined effects of multiple interacting mechanisms, including sensitivity to pressure, flow rate, metabolite levels, and neural signals. Flow regulation requires the sensing of metabolic and hemodynamic conditions, and the main effectors of this regulation are the arterioles and small arteries, which are located proximally to the tissue that they supply. Arterial pressure falls markedly while passing these vessels [1], which demonstrates that they are responsible for a significant part of total vascular resistance in the circulation [2-4]. These small arteries are therefore known as resistance arteries. Abnormal changes in peripheral vascular resistance were shown to be associated with a number of pathological conditions including hypertension and diabetes, which underlines the importance of understanding their function.

Arterial resistance is under constant control of numerous regulatory systems, such as neurogenic and hormonal influences as well as a broad range of local and intrinsic factors. These regulatory mechanisms are not functioning independently but rather are deeply integrated into each other, modulating the final vascular responses. Nevertheless, the final effect of all these regulations is the change in the vessel diameter, i.e. vascular resistance, which depends on the contractile status of smooth muscle cells in the vascular wall. Whether smooth muscle cells are relaxed or constricted depends on the level of myosin light chain (MLC) phosphorylation by MLC kinase activated by the Ca^{2+}-calmodulin complex [5]. Thus, the contractile status of smooth muscle depends on the intracellular calcium ($[Ca^{2+}]_i$) level as well as on the sensitivity to $[Ca^{2+}]_i$ of proteins involved in the dynamic process of MLC phosphorylation-dephosphorylation. Many agonists and local stimuli, e.g. noradrenaline and transmural pressure, act in both directions: by increasing $[Ca^{2+}]_i$ via membrane influx and release from intracellular Ca^{2+} stores, and by the sensitizing the contractile apparatus to prevailing Ca^{2+} level [6-9].

$[Ca^{2+}]_i$ and membrane potential in smooth muscle cells are in a reciprocal relation [10], i.e. membrane depolarization opens the voltage-dependent L-type Ca^{2+} channels which are the major pathway for Ca^{2+} influx [11], while increase in $[Ca^{2+}]_i$ stimulates a Ca^{2+}-dependent Cl^- conductance on the smooth muscle cell membrane [12-15]. In contrast to some other tissues, e.g. skeletal muscles [16], Cl^- in smooth muscle cells is not distributed passively across the plasma membrane, but accumulates actively inside the cell [17;18]. This makes the equilibrium potential for Cl^- less negative than resting membrane potential in smooth muscle cells. Therefore, Ca^{2+}-activated increase in Cl^- conductance will lead to Cl^- efflux across the plasma membrane and depolarize smooth muscle cells (SMCs) [19-24]. Although the degree to which the resulting depolarization contributes to contraction of smooth muscles is not known [25], the depolarizing Ca^{2+}-activated Cl^- conductance counterbalances to a certain extent a Ca^{2+}-activated K^+ current [12;26-31] which tend to hyperpolarize and relax smooth muscle cells.

Although various external signals changing SMCs contractility are obviously important for both long-term and short-term regulation of arterial diameter, an internal ability of SMCs to alter the vascular wall tone in response to physical factors at least as important [2;32;33]. In reality, the combination of myogenic and non-myogenic factors creates the final vascular tone, which can be both stable and varying over time. Rhythmic changes in the vascular tone, known as vasomotion, were observed in different vessels but are clearly more prevalent in small arteries and arterioles [34]. Vasomotion is one of the most mysterious and fascinating vascular responses although only very limited information regarding the generating mechanism was available until recently[35]. The importance of such knowledge is obvious since the changes in the rhythmic activities in the vascular wall have been associated with several pathologies [34]. Thus, it has been shown that vasomotion is more prevalent or pronounced in hypertension. Studies on both animal models and humans indicate a tight coupling between the high blood pressure and the ability of vessels to oscillate [36-39]. Vasomotion is reduced in different forms of diabetes [40]. It is noteworthy that certain oral antidiabetics (e.g. metformin) markedly stimulate vasomotion in diabetes [41]. Altogether this suggests that vasomotion is of pathophysiological, and tentatively of prognostic, interest.

Recent studies significantly improved our understanding of vasomotion [34;35;42-44]. Several models for initiation of vasomotion were suggested and received experimental support (I and [45-55]). We have suggested a model for vasomotion in the mesenteric small arteries which is based on synchronization of intracellular Ca2+ oscillations by a membrane potential related phenomenon (I). The key elements for this synchronization are the depolarizing

Ca^{2+}-activated Cl^- current which projects changes in $[Ca^{2+}]_i$ into membrane potential oscillations and gap junctions which enables spreading of the depolarization between the smooth muscle cells. The following detailed studies of these key players provide a better understanding of their role in the generation of vasomotion and their molecular identities (V, VII and VIII). This brings us to a new molecular level in our understanding of the phenomenon of vasomotion.

5. THE MODEL FOR THE GENERATION OF VASOMOTION IN RAT MESENTERIC SMALL ARTERY (PAPERS I AND II)

An outstanding motor phenomenon in the vasculature: 150 years of research

Rhythmic contractions, which are known for many organs from the heart to the gastrointestinal and urinary tracts, are also described in blood vessels where they are termed 'vasomotion'. Vasomotion is sometimes used as a broad term which describes any vasomotor response, i.e. a change in the vascular diameter [56], but it is also used exclusively to describe spontaneous, rhythmical changes in the vascular diameter or tone. Although both applications of the term are still in use, the majority of vascular physiologists prefer to confine the term vasomotion to the rhythmic oscillatory behavior of the vascular wall [34;44;57-60].

This "outstanding motor phenomenon observed in peripheral vascular structures" [61] was first described in 1852 *in vivo* in study of bat wing circulation [62]. This observation of rhythmic contraction and dilatation was ascribed to a natural state of veins while the ability of arteries to oscillate on its own was seriously doubted. The evidence accumulated during the following 100 years proved, however, that vasomotion is a phenomenon common for both arteries and veins [63;64], and this led to the classical study on vasomotion by Nicoll and Webb in 1955 [61]. This study postulated that SMCs function during vasomotion as independent effectors modulated by changes in their immediate environment [61]. Nicoll and Webb made a large effort to study these regulatory factors which they subdivided into the nerve impulses, the specific or general chemical substances, and physical phenomena, such as temperature and pressure. They concluded that all these factors have only modulatory function and regulate the frequency and characteristics of vasomotion which has an intrinsic nature [61]. This conclusion is still valid and there is no doubt that vasomotion is an intrinsic function of the vascular wall [34;42;60;65].

During the last years vasomotion has been observed by many researchers in many, if not all, vascular networks under certain conditions. Being essentially characterized *in vivo* [61;62], vasomotion research remained to be quite descriptive due to technical limitations over a long period of time. A significant advancement was made by the development of modern techniques for both *in vivo* and *in vitro* studies, such as myography of small (few hundred micrometer diameter) vessels [44;57;66-69], electrophysiological approaches for membrane potential measurement and patch clamping of single ionic currents (I, VI, VII, VIII and [70-74]), intracellular ion imaging and confocal microscopy (VII, VIII and [43;53;54;75-80]), laser-Doppler flowmetry [81], immunohistochemistry and molecular biological methods (VII and [74;82-85]). In spite of great progress the cellular mechanism for vasomotion remained a matter for debate. The fact that this discussion has 150 years' history indicates the many problems which researchers have had and still have in the experimental studies of vasomotion. Vasomotion is often unpredictable, making it difficult to standardize results and to draw generalized conclusions. This has led to intense scientific debates between research groups whether some treatment really stops or induces vasomotion, or just brings the vessel to a state where oscillations in tone are not possible [86;87]. The appearance of vasomotion depends on the type of blood vessel, the nature of stimulation and is also very sensitive to the experimental procedure, i.e. form of anesthesia, solutions, preparations and physical conditions [35;65].

Is it possible at all to generalize the appearance of vasomotion?

Being regulated by multiple factors which in variable combinations can give different results, vasomotion is difficult to evaluate by analogy to many other biological responses where an intensity of stimulus can be correlated to the strength of the response. The fact that the same artery under certain conditions can develop different types of oscillations, makes the situation even more complicated. As described previously, the inhibition of one oscillator in the vascular wall will not necessary lead to elimination of vasomotion. On the contrary, this can unmask another oscillator, which was suppressed by the 'dominating' oscillator and this will initiate vasomotion with other characteristics than before [87;88]. Thus, several oscillators in the vascular wall are interacting with each other in a complicated manner. The final outcome of these interactions might depend on experimental conditions. Caution should be therefore taken when different reports on vasomotion are compared and a number of different parameters should be taken into account.

Interestingly, non-invasive *in vivo* measurements detect several different types of oscillations simultaneously in the same vascular bed [89]. Although it was previously suggested that these oscillations have different origin, e.g. cardiac, respiratory, myogenic, neurogenic and endothelial types [90], they may also represent different types of intrinsic myogenic or myoendothelial oscillations which can be seen *in vitro* depending on experimental conditions (I, II and [87;88]). *In vivo* oscillations termed myogenic have the same frequency as vasomotion normally observed in vitro on the arterial segment (I and [90]) but other types of oscillation can also be induced.

It is obvious that the studies of vasomotion *in vivo* have great physiological significance but are limited in the possibilities to provide mechanistic insight. *In vitro* experiments can give the mechanistic insight although the meaning of 'physiological conditions' is significantly reduced *in vitro*. Isolated arterial segments provide the possibility to study vasomotion without mechanical, hormonal and neurogenic influence from the rest of body.

Oscillators in the vascular wall.

The mechanism of vasomotion may vary between different species and within the same species between different vascular beds. Several models for the generation of vasomotion have been suggested and are receiving strong experimental support (I and [34;35;42;43;46-48;50-52;55;91]). It is necessary to accept that the complexity of the vascular wall makes it impossible to exactly reproduce vasomotion by theoretical modeling. On the other hand, the modeling of the process helps to highlight the major components which are important for vasomotion and also it helps to suggest and predict possible interventions [46;47].

Virtually all existing models for the generation of vasomotion are based on the presence of oscillators [35;92]. It is generally accepted that the release of Ca^{2+} from intracellular stores and the following synchronization through coupling of oscillations in SMCs are the basis of vasomotion. With respect to the mechanism, the putative oscillators can be subdivided into cytosolic and membrane oscilla-

tors [34;42]. As it can be appreciated from the name, the cytosolic oscillator originates from the cytoplasm. The current view is that low concentrations of an agonist can induce transients of $[Ca^{2+}]_i$ increases which are not necessarily associated with membrane potential changes (I and [78;93]) but strictly depend on the SR function [35;42;60;65]. Depending on the vessel studied and the type of stimulation, Ca^{2+} is released either via inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release and/or via ryanodine-sensitive channels.

This localized initial rise in $[Ca^{2+}]_i$ appears in specific regions of the cell and propagates along the cytoplasm in a wave-like manner [48]. The Ca^{2+} waves do not represent simple diffusion of Ca^{2+} but require regeneration by Ca^{2+}-induced Ca^{2+} release (CICR). The Ca^{2+} waves appear spontaneously under resting conditions [48;48;79;94] and when low concentrations of contractile agonists are applied (I and [43;95-97]). Stimulation of adrenoreceptors causes initially transient Ca^{2+} waves, with a typical frequency of 0.01-0.2 Hz (I, II and VII). These Ca^{2+} waves are uncoordinated between neighboring cells and show a considerable heterogeneity between different SMCs in the arterial wall (I, II and [48]). When $[Ca^{2+}]_i$ is integrated over an entire cell with time, these Ca^{2+} waves appear as rhythmical oscillation in $[Ca^{2+}]_i$ but due to their asynchrony have little effect on the global $[Ca^{2+}]_i$ changes across the entire arterial wall or on tension (I).

The CICR allows $[Ca^{2+}]_i$ to propagate over substantial distance without decrement in strength [98;99]. Both IP_3- and ryanodine-sensitive channels are theoretically suitable for the CICR and these have received experimental proof (I and [78;97;99]). There is a general suggestion that the IP_3 channels stimulated by IP_3 produced by agonist stimulation are essential for the initial $[Ca^{2+}]_i$ rise which then can propagate by means of IP_3- or ryanodine channels, or by interaction of both types [34;42;60;65]. Thus, in rabbit inferior vena cava [97], in cultured aortic SMCs [75] and in rat portal vein [100] the blockade of IP_3-channels stops Ca^{2+} waves. Similarly, acute inhibition of ryanodine channels blocks the Ca^{2+} waves in rat mesenteric artery (I), in cultured aortic SMCs [75], in rat tail artery [99] and in rabbit inferior vena cava [94]. Interestingly, chronic downregulation of the ryanodine channels in rat tail artery did not affect Ca^{2+} waves while acute application of ryanodine stopped it [101] suggesting that one source of Ca^{2+} release can be sufficient for propagation of Ca^{2+} waves and can compensate for the lack of another.

The transience of the Ca^{2+} waves is based on the following inhibition of the Ca^{2+} release. This is ascribed to a number of mechanisms, such as inhibition of IP_3 channels with the high $[Ca^{2+}]_i$ [102;103] and/or by low luminal SR Ca^{2+} [104], an adaptive inactivation of ryanodine channels [105] and a time-dependent inactivation of both IP_3 and ryanodine channels [106]. The temporal characteristics of the inhibition determine the frequency of oscillations. This is supported by the observation that the frequency of Ca^{2+} oscillations has normally a limit and does not increase continuously with increasing agonist concentration [48;94].

An increase in agonist stimulation increases the number of SMCs responding with the Ca^{2+} waves and leads to SMCs synchronization [48;78;94;99;107;108]. Synchronization of SMCs within the vascular wall gives rise to global oscillations in $[Ca^{2+}]_i$ and vasomotion (I and [43;96;108]). The global Ca^{2+} oscillations represent a uniform rise in $[Ca^{2+}]_i$ throughout the cell. Significant changes in membrane potential are essential to induce such global synchronized Ca^{2+} influx through the voltage-dependent Ca^{2+} channels (VDCCs). Consistent with this, vasomotion was shown to be associated with oscillations in membrane potential in all vessels where it has been measured (I, VI and [7;71;72;86;93;109-111]) with the exception of irideal arterioles [68;74].

To be synchronized SMCs need to be coupled to allow a coordinating signal to quickly spread between the cells. There is no doubt that intercellular gap junctions are the key elements for such synchronization. It has been documented experimentally that interruption of gap junctions desynchronizes Ca^{2+} transients and membrane potential oscillations and stops vasomotion, but is without effect on the Ca^{2+} waves (VI, VII, VIII and [112;113]). This suggests an essential role of gap junctions in synchronization and entrainment of the Ca^{2+} oscillations [45;47;48]. The nature of this signal which spreads through the gap junctions is, however, debatable. The synchronization can be mediated by transfer of small signaling molecules between SMCs. Depending on the model for synchronization, current (I and [46;47]) or $[Ca^{2+}]_i$ [45] have been suggested as major candidates. The movement of $[Ca^{2+}]_i$ between SMCs seems to be small since the Ca^{2+} waves in one cell were not shown to initiate the Ca^{2+} waves in other, neighboring cells (I and [48;94;96]). This can be due to limited number of gap junctions between SMCs in the vascular wall [114;115] or due to a low (a few hundred nanomolar) concentration gradient (i.e. driving force) of $[Ca^{2+}]_i$ between two SMCs in comparison to the gradients between cytosol and extracellular Ca^{2+} or Ca^{2+}stored in the SR. A high buffering capacity of the cytosol will also prevent spreading of the Ca^{2+} signal between the cells. Thus, Ca^{2+} flux between two cells is unlikely to significantly affect the global $[Ca^{2+}]_i$. The electrical current is therefore the more likely candidate to substantially affect the membrane potential and induce massive Ca^{2+} influx through the VDCCs (I and [46;47]).

Based on the current knowledge one of three generalized mechanism for generation of vasomotion can be suggested by combining the parameters discussed above. In all suggested models the Ca^{2+} release from the SR is essential for vasomotion. In one rare case, seen only for irideal arterioles [68;74], no voltage-dependent membrane channels are involved. The critical dependence of such voltage-independent vasomotion on phospholipase C and A2 pathways suggested their function as oscillators (Fig. 1A). The feedback loop will result here in oscillations due to biphasic regulation of the IP_3 channels by $[Ca^{2+}]_i$ [68]. These oscillations are independent of membrane voltage but can induce oscillations in membrane potential on a secondary basis. This suggests that SMCs are not synchronized by means of voltage but coupled by movement of second messengers [45]. Whether the kinetics of second messenger movements is consistent with the speed sufficient for information transfer between the SMCs necessary for vasomotion is unclear.

Alternatively, in most of other blood vessels vasomotion is voltage-dependent because the influx of Ca^{2+} through the VDCCs is essential for the synchronization of individual oscillators. This synchronization can arise from an interplay between membrane conductances (membrane oscillators) or between cytosolic and membrane oscillator. The first might be due to temporary shifted activation by $[Ca^{2+}]_i$ of the Ca^{2+} activated Cl^- channels and the Ca^{2+}-dependent K^+ channels. This is possible because of different voltage-, Ca^{2+}- and time-dependence of Cl^- and K^+ channels (Fig. 1B) [26-31;116]. This suggestion is based on the observation that in hamster cheek pouch arteries inhibition of K^+ membrane conductance abolishes vasomotion [117]. It is important to note that the involvement of K^+ and Cl^- is not mandatory and several other membrane transporters have been suggested to act as membrane oscillators, e.g. the Na^+/K^+-ATPase [66] and TRP channels [118].

Finally, oscillations can appear due to activation of a depolarizing current which is stimulated by oscillating $[Ca^{2+}]_i$. This depolarizing

current will lead to the VDCCs opening, Ca^{2+} influx and synchronization of the global Ca^{2+} oscillations by membrane potential (Fig. 1C). Although significant discrepancies between different groups were reported, this model provides suitable explanation for a large part of reports on vasomotion in rat mesenteric arteries (II and [43;54;66;69;76;78;86;87;96;110;112;119;120]). I dedicated my research to improve the understanding of this oscillation type in the rat mesenteric small arteries.

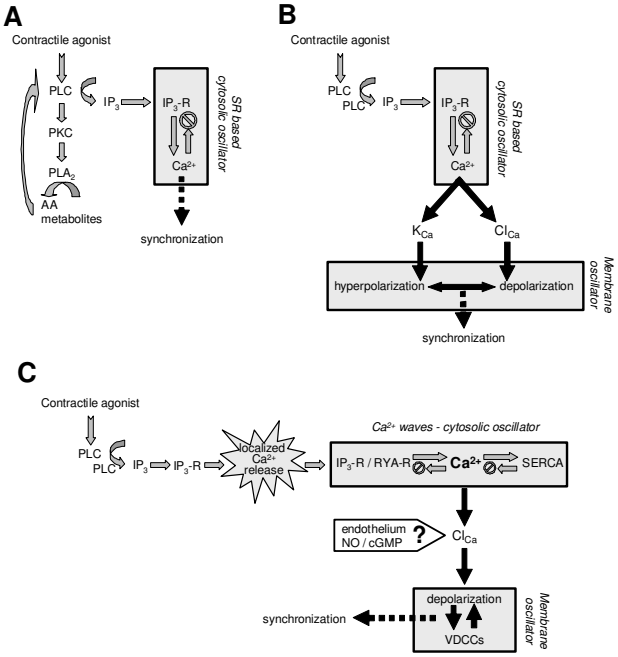

Figure 1

Sequences of events suggested for three different general models for the initiation of vasomotion. **Panel A** illustrates the voltage-independent model where the interaction between phospholipase C (PLC), phospholipase A2 (PLA2) and protein kinase C (PKC) amplifies the IP_3 signal. The elevated IP_3 level can induce oscillation in $[Ca^{2+}]_i$ due to biphasic regulation of the IP_3 channels by $[Ca^{2+}]_i$. SMCs can be synchronized by the movement of second messengers between the cells. AA is arachidonic acid. **Panel B** shows the pathway suggested for voltage-dependent oscillations. CICR can affect two membrane conductances (for example, Ca^{2+}-activated K^+ channels (K_{Ca}) and Ca^{2+}-activated Cl^- channels (CaCC)) which have opposite effect on the membrane voltage. Neighboring SMCs will be then synchronized by membrane potential changes. **Panel C** shows an interaction between cytosolic and membrane oscillators. IP_3 induces the local Ca^{2+} release which gives rise to Ca^{2+} waves through CICR. Transiently elevated $[Ca^{2+}]_i$ stimulates a depolarizing membrane current, possibly the Ca^{2+}-activated Cl^- current. The following depolarization opens VDCCs, induces a global Ca^{2+} influx which in turn affects the membrane potential and enhances the possibility of oscillations. This model has experimental support where vasomotion was shown to be endothelium-dependent (I and II). This can be due to steep cGMP-dependence of the Ca^{2+}-activated Cl^- current (III, IV and [121]). This endothelium-dependence is however still matter of debate [44].

Hypothesis for the initiation of vasomotion in rat mesenteric small arteries (Paper I)

Agonist-induced responses of rat mesenteric small artery in vitro

Rat mesenteric small arteries are popular for studies the structure and function of resistance arteries, due to their easy accessibility and a large number of long branches of different diameters [2;115;122;123]. However, these arteries have unique properties in comparison to other small arteries. They have virtually no intrinsic myogenic tone which is often observed in other resistance arteries [9;124-129]. Mesenteric small arteries contribute, nevertheless, significantly to the total peripheral resistance [130-132] where the sympathetic nervous control of smooth muscle contraction is of major importance [80;122;133]. Mesenteric small arteries are heavily innervated and sensitive to sympathetic neurotransmitters [ATP, noradrenaline (NA) and neuropeptide Y (NPY) [134-137]] as well as to a number of other contractile agonists, such as vasopressin [138;139], endothelin [140], thromboxane [141] and some vasoactive peptides [142]. This agonist-induced receptor-coupled stimulation of vascular contractility involves elevating $[Ca^{2+}]_i$ as well as a sensitization of myofilaments to $[Ca^{2+}]_i$ [143]. $[Ca^{2+}]_i$, elevated either by transmembrane Ca^{2+} influx, or by release from the SR, can then either directly activate the contractile filaments or indirectly alter cell excitability by affecting ion channel activity in the plasma membrane [65;144;145]. In the mesenteric small arteries noradrenaline is the most often used contractile agonist (I, II, VI and [7;43;54;57;66;70;86;87;96;119;120;140;146-148]).

Development of myograph technique revolutionized the experimental use of small arteries *in vitro* [149]. Before this technique was developed, *in vitro* research was limited to strips and rings of large, conduit arteries [122]. Development of small vessel myographs allowed arteries with diameter of few hundreds micrometers and below to be studied [2]. In myographs changes in the wall tension or diameter are measured under isometric or isobaric conditions, respectively, to evaluate the vascular response to the stimulation. Although many researchers suggest that the isobaric conditions more closely resemble situation *in vivo*, in practice the difference between these two methods is not so dramatic: the arteries show similar passive pressure-diameter characteristics, although under isobaric conditions they are more sensitive to agonist stimulation [150]. Nevertheless, the isobaric conditions (i.e. pressure myograph) are preferable for studies of vascular wall autoregulation. Moreover, under these conditions researchers receive the possibility to monitor changes in different parts of arterial segment independently. Thus, pressure myograph was a suitable technique for our study of partial synchronization in the vascular wall (II).

Wire myography has an advantage over isobaric myography in experiments where the accurate and reproducible determination of basal tension is essential [2]. This method allows normalization of the arteries in each experiment by determination of the passive length-tension relationship and then setting the internal diameter to a value that gives maximal force development. Thus, the normalization sets all vessels in the same standard conditions which are utilized for almost all studies employing isometric myography of resistance arteries [2]. In our studies (I, II, VI-IX) the vessel diameter was set to 90 % of the value vessel would have had *in vivo* under transmural pressure of 100 mmHg [149]. These standardized isometric conditions are ideal for interventional studies of vasomotion.

The observation that submaximal stimulation by different contractile agonists can induce rhythmic oscillations in tone suggests a primary role of SMC activation for initiation of vasomotion rather than a specific effect from a certain receptor. Vasomotion in rat mesenteric small arteries has been seen with electrical field stimulation of sympathetic nerves, where they are suggested to be due to noradrenaline release [80], in response to administration of exogenous noradrenaline (I, II, VI and [7;43;54;57;66;70;86;87;96;119;120;140;146-148]), vasopressin [151] and NPY [137] (Fig. 2). Although stimulation with the thromboxane analog U46619 or endothelin-1 is reported to fail to induce vasomotion in rat mesenteric small arteries [140], the presence of endothelin-induced vasomotion in other vascular beds, e.g. cat arterioles [152], and our unpublished observation (Fig. 2C) in the rat mesenteric small arteries could suggest an importance of different experimental

conditions. The inconsistence could also be due to the steepness of the concentration-response curves for U46619 and endothelin-1 which makes it difficult to achieve a reasonable submaximal level of tone.

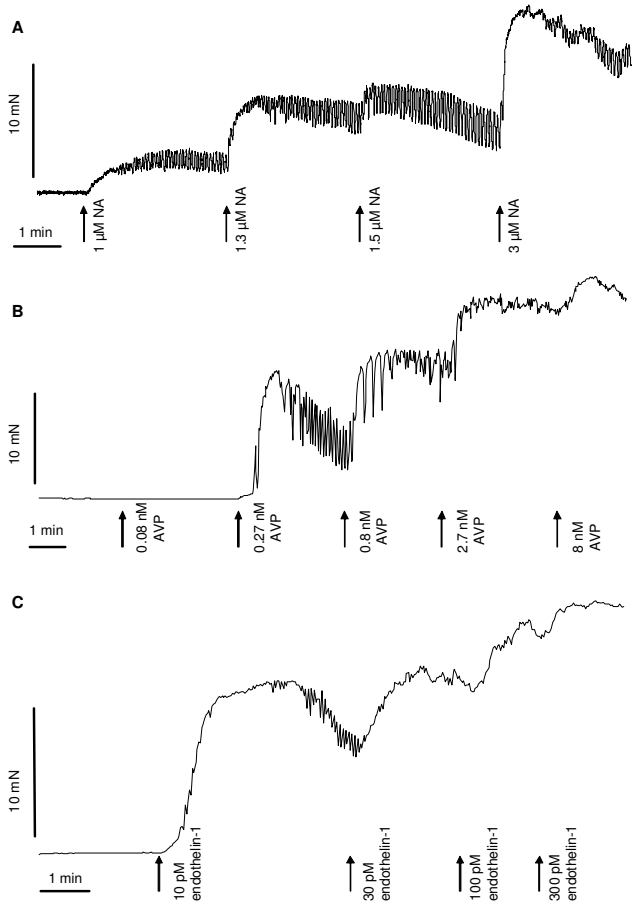

Figure 2
In spite of the different kinetics of contraction, vasomotion induced by different contractile agonists has a similar pattern. **Panel A** shows a cumulative stimulation with increasing concentrations of noradrenaline (NA). **Panel B** shows vasomotion in response to arginin vasopressin (AVP). **Panel C** shows response to endothelin-1. Arteries were studied *in vitro* under isometric conditions.

Vasomotion is normally seen over nearly the entire spectrum of vascular tone, though their characteristics may change with the tone. This is especially true for the amplitude of oscillations while the frequency does not change much at different levels of tone. Since maximal amplitude is achieved at about 50% of maximal tone, this is a standard level of contraction where vasomotion is normally being studied (I, II and [87;110]).

$[Ca^{2+}]_i$ imaging in the vascular wall in vitro
Development of new techniques, first of all live fluorescence microscopy, improved our understanding of the sequence of events leading to vasomotion (I) (Fig. 1C). The possibility of loading the arterial wall with fluorescent dyes was greatly improved with development of acetoxymethyl ester (AM) dye forms. Prior introduction of the fluorescent dyes into the cells was a harmful procedure including temporary membrane disruption with detergents or voltage pulses [153]. The membrane permeable AM-form becomes an impermeable, hydrophilic form inside the cell after the AM group is cleaved away by endogenous esterases. Available fluorescent dyes have various properties making them useful for different applications. Thus, we have used the Ca^{2+} ratiometric (dual excitation) dye Fura-2 (I, VI and VII) which is a practical tool for the continuous real-time monitoring of global $[Ca^{2+}]_i$ events [154]. The ratiometric properties allow a conversion of the fluorescence ratio signal into $[Ca^{2+}]_i$ (VI) although this calibration does not necessary contribute further important information and often calibration is not done (VII). The fluorescence ratio depends on several parameters, e.g. temperature, pH and ionic strength, which modify the dissociation constant for Ca^{2+} binding to Fura-2. This uncertainty is a disadvantage to the calibration method and it is necessary to assume that the dissociation constant is unchanged during the study.
To record $[Ca^{2+}]_i$ changes with Fura-2 we used a conventional epifluorescent microscopic technique which does not allow monitoring of $[Ca^{2+}]_i$ dynamic at the cellular and subcellular levels. This techniqual limitation can be overcome with the laser confocal microscopy approach. Combining a high numerical aperture objective and ability to move the focal point this approach makes it possible to record live images of the individual SMCs in the vascular wall mounted in the specially designed wire myograph over time (II, VII, VIII and [48;146]). The narrow focal plane complicates recording of $[Ca^{2+}]_i$ during even slight movement, i.e. the region of interest can move out of focus when the artery constricts. The movements can be inhibited chemically, e.g. wortmannin inhibits the myosin light kinase and therefore contraction, or by sustained hyperpolarization, e.g. pinacidil opens the ATP-dependent K^+ channels. These methods of inducing stabilization however limited the ability to study vasomotion, i.e. the oscillation in tension. Therefore, most of the confocal data in our studies were obtained without these drugs because under the isometric conditions the movements are negligible (I, II, VI-VIII).
Due to techniqual limitations (lack of suitable excitation wavelengths) we were not able to use Fura-2 dye in our confocal studies. The non-ratiometric Ca^{2+} dye Calcium Green-1 was used instead (I, II, VI-VIII). Calcium Green-1 increases in intensity upon binding to Ca^{2+} without a shift in the wavelength where emission is seen. This increases the probability of interference from movement artifact. In the experiments where this risk was especially high, e.g. measurement of subcellular Ca^{2+} dynamic in very small region of interest, we combined two Ca^{2+} dye indicators to perform semi-ratiometric $[Ca^{2+}]_i$ measurements (VIII). Thus, elevated $[Ca^{2+}]_i$ results in increased fluorescence intensity of Calcium Green-1 and decreased fluorescence intensity of Fura Red. The combination of these two calcium indicators allows ratiometric analysis of $[Ca^{2+}]_i$ changes relatively independent from movements.

$[Ca^{2+}]_i$ transients in smooth muscle cells induced by an intracellular oscillator
Our paper by Peng et al. (I) clearly illustrates that Ca^{2+} waves within the individual SMCs precede synchronized oscillations in $[Ca^{2+}]_i$ and vasomotion (Fig. 3). Similar asynchronous $[Ca^{2+}]_i$ waves preceding the rise in tension were previously seen in rat tail artery [99] and rabbit vena cava [94] SMCs. We have also detected $[Ca^{2+}]_i$ waves in some SMCs of un-stimulated arteries [48]. Increasing noradrenaline concentration recruits SMCs into an oscillatory mode. The frequency of these asynchronous $[Ca^{2+}]_i$ waves varied between SMCs (I and II) but was constant over time [48]. This means that even during repeated stimulation the characteristic fre-

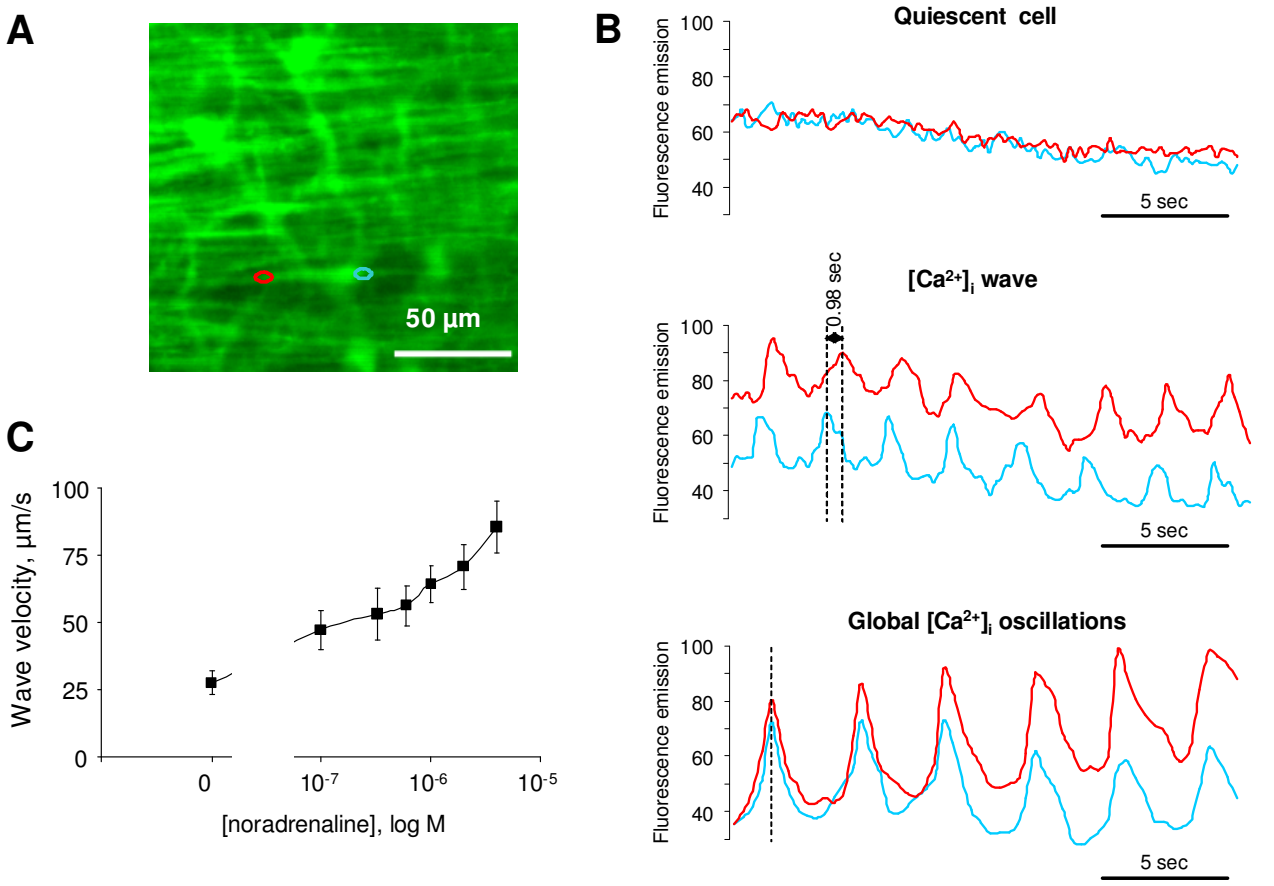

Figure 3
$[Ca^{2+}]_i$ events in SMCs in rat mesenteric small artery studied using confocal microscopy. **Panel A** shows $[Ca^{2+}]_i$ image of arterial wall loaded with Calcium Green 1/AM. **Panel B** illustrates different stages of $[Ca^{2+}]_i$ in one SMC from panel A. $[Ca^{2+}]_i$ was measured in regions of interest (ROI) placed in two distant (about 40 µm) points within the cell as indicated by corresponding colors. Upper panel shows the quiescent state of cell before noradrenaline administration. Middle panel illustrate $[Ca^{2+}]_i$ waves stimulated with noradrenaline before synchronization occurred. The 0.98 sec delay in the peak fluorescence gives wave velocity of 40.8 µm/s. Lower panel shows the global $[Ca^{2+}]_i$ oscillations observed when the synchronization occurred. **Panel C** shows increase in the wave velocity with increasing noradrenaline concentration. An average of 4 independent experiments, at least 5 cell in each.

quency of individual cells remained fairly constant. This observation indicated the phenotypic heterogeneity of SMCs in the vascular wall with respect to the Ca^{2+} dynamics [48;79]. The source for this heterogeneity is unclear [46;48]. Ca^{2+} waves also differ in direction and dynamics (I and [48;79]). The waves in individual SMCs move in different directions, they can be initiated in the cell end or somewhere near the center and spread to non-excited parts of the cell, but they do not spread between the cells. The Ca^{2+} waves spread with different velocities between 12 and 175 µm/s with a median of 36 µm/s (I) (Fig. 3C) and their frequency increases with the noradrenaline concentration. This frequency is usually slower although overlap with the frequencies of synchronized oscillations consistent with the suggested model (I).

It is generally accepted that Ca^{2+} wave generation needs a functional SR [35]. We have shown that this is also the case for mesenteric small arteries. Interruption of the SR function stopped the Ca^{2+} waves (I and [88]). In line with this observation, the increase in noradrenaline concentration and, thus, IP_3 production increases the velocity of the Ca^{2+} waves (Fig. 3C). In contrast, we found that another source for $[Ca^{2+}]_i$ rise, an extracellular Ca^{2+} influx is not necessary for the appearance of Ca^{2+} waves (I). Inhibition of Ca^{2+} influx with VDCCs inhibitors or Ca^{2+}-free bath solution preserves Ca^{2+} waves for some period of time. Ca^{2+} waves disappeared eventually after 10 to 60 minutes of Ca^{2+} influx inhibition (I). This was probably due to loss of some Ca^{2+} from the cell by pumping across the membrane. Similar conclusions were made previously by other groups in studies on rat tail artery [99] and rabbit venous [94] SMCs. Interestingly, the Ca^{2+} waves were also preserved in SMCs hyperpolarized by opening the ATP-dependent K^+ channels with pinacidil (I) suggesting its independence not only from the Ca^{2+} influx but also from the membrane potential. Thus, based on the experimental facts, consistent with other reports [78;94;99], we can conclude that the Ca^{2+} waves are initiated by an intracellular oscillator and propagated by an intracellular mechanism (I). The Ca^{2+} waves are seen in the absence of synchronization between SMCs, i.e. when vascular tone is static. These asynchronous waves shift to global $[Ca^{2+}]_i$ oscillations when SMCs synchronize and vasomotion appears (I).

Transition from waves to global $[Ca^{2+}]_i$ oscillations

In agonist-stimulated rat mesenteric small arteries spontaneous synchronization occurs after a variable period of time (normally within 30 seconds – 5 minutes), if the endothelium layer is intact (I) or if sufficient concentration of a membrane-permeable analog of cGMP (II) is present in the bath. During synchronization the Ca^{2+} waves change to global Ca^{2+} oscillations, which are seen in all SMCs in the arterial wall (I and [48]). These synchronized oscillations in $[Ca^{2+}]_i$ lead to the rhythmic contractions. Analysis of $[Ca^{2+}]_i$ dynamics showed that during these global $[Ca^{2+}]_i$ oscillations (I and [48]) $[Ca^{2+}]_i$ rose simultaneously (within the limits of the temporal resolution) throughout the whole cell (Fig. 3).

In spite of significant difference in the dynamic characteristics, we concluded that the Ca^{2+} waves function as pacemakers for the global Ca^{2+} oscillations and, thus, vasomotion (I and [66;76]). This is similar to a number of other reports which demonstrate that when either release or uptake of Ca^{2+} into the SR is inhibited vasomotion, the synchronized Ca^{2+} oscillation and the Ca^{2+} waves disappear [68;74;88;93;155;156]. In contrast to the Ca^{2+} waves, the global Ca^{2+} oscillations are dependent on Ca^{2+} influx; the blockade of VDCCs, immersion into the Ca^{2+}-free bath and hyperpolarization with the ATP-dependent K^+ channel opener all stopped vasomotion in the rat mesenteric arteries and, even more interesting, transformed the global Ca^{2+} oscillation back to the asynchronous Ca^{2+} waves (I). Thus, the synchronized activity in the mesenteric artery wall is voltage-dependent (I and II). Vasomotion and synchronized Ca^{2+} oscillations are accompanied with rhythmic changes in membrane potential with the same frequency that were observed in rat mesenteric arteries (II, VI, VII and [7;54;70;86]) and many other blood vessels [71;72;74;93;109]. Moreover, the oscillations in membrane potential are shown to precede the oscillations in $[Ca^{2+}]_i$ and the wall tension (I and [35]).

Although each cycle of contraction in vasomotion starts from depolarization [35] following by a rise in $[Ca^{2+}]_i$ which leads to smooth muscle contraction, the means by which transition from Ca^{2+} waves to global oscillations in Ca^{2+} and membrane voltage occurs remains to be explained. Based on the previously reported measurements (II, VI, VII and [7;54;70-72;74;86;93;109]) there is a little doubt that SMCs synchronize by membrane potential dependent mechanism. An electrical signal is probably the only signal fast enough to synchronize SMCs over a long arterial segment. It is important to mention that the mesenteric small artery wall is equipped with low-resistance channels of gap junctions [115;157-160]. The gap junctions between of SMCs undoubtedly mediate the electrical coupling between cells which has been documented experimentally by different techniques. In the studies using putative blockers of gap junctions vasomotion was shown to be inhibited after uncoupling SMCs (VII and [96;112;113]) and the global Ca^{2+} oscillations were replaced by unsynchronized Ca^{2+} waves (VII). The observation of an irregular arterial vasomotion in cremaster muscle arterioles in connexin 40 knockout mice is also consistent with the importance of intercellular communication in synchronization [59]. It is obvious in this context that the regulation of gap junctional conductance could be an important regulator of vasomotion. Little is known, however, about such regulation, although some potential regulatory elements have been suggested, e.g. cGMP, interaction with other membrane transporters such as Na^+/Ca^{2+}-exchanger, K^+ channels and Na^+/K^+-ATPase (II, VIII and [66;70]).

The key element for our model for vasomotion in the rat mesenteric small arteries is the suggestion that the intracellular and membrane oscillators interact with each other to produce phase locking of the individual cells (I). This is possible because the intracellular oscillations in Ca^{2+} induce rhythmic changes in membrane potential. We suggested (I) that Ca^{2+} activates a depolarizing current on the cell membrane. The consequent depolarization increases Ca^{2+} influx through the VDCCs and the likelihood for CICR, which amplifies the depolarization that spreads through the gap junctions to the neighboring SMCs. This depolarizing current enhances Ca^{2+} influx in the neighboring cell which increases the probability of Ca^{2+} release and, thus, entrains the Ca^{2+} release and forms the basis for vasomotion. The relaxing part of the vasomotion cycle might be caused by hyperpolarizing current generated by the Ca^{2+}-sensitive K^+ channels which have slow activation kinetics, strong voltage-dependence and low sensitivity to $[Ca^{2+}]_i$ [30;145;161-167] and therefore activate substantially only when the membrane depolarizes and $[Ca^{2+}]_i$ is significantly elevated. It is important in this respect that the K^+ channel blockers have only modulating effect on vasomotion (I and [66]). This could be because several different types of channels are involved in the repolarization. Another reason for the repolarization could be a slow-down of Ca^{2+} release due to emptying of the SR in the combination with an active removal of Ca^{2+} from the cytoplasm [97]. A decrease of Ca^{2+} release from the SR could be due to a refractoriness of the Ca^{2+}-release channels [98].

The transition of Ca^{2+} waves to the synchronized oscillations in the rat mesenteric small arteries is only possible if the endothelial layer is intact or if a sufficient concentration of cGMP is present (I, II and [87]). The fact that a fixed concentration of intracellular cGMP could, at least partially, compensate for the absence of endothelium suggests that oscillations originate in SMCs and that the endothelium is not directly involved in the generation of oscillations under normal conditions (II and [70;88;108;168;169]). A similar importance of endothelium was previously observed in other vessels [89;170-175], while in some vessels, e.g. rabbit mesenteric and ear arteries, aorta and hamster cheek pouch [155;175-178], vasomotion was shown to be potentiated by endothelium removal or NO production blockade. The reason for such inconsistencies is unknown and can be ascribed to significant differences in experimental protocols or to the variability in the mechanism of vasomotion in different vasculatures. It is quite natural to suggest that several endothelium-derived factors have influences on vasomotion and the role of each of the factors depends on the vasculature and the experimental conditions. Our studies together with other groups reports show a promoting role of the NO/cGMP pathway for vasomotion in the rat mesenteric small arteries (II and [70;88;96;108;168;169]). The importance of cGMP for the entrainment of the intracellular Ca^{2+} oscillations via a membrane potential changes led us to suggest the presence of cGMP-dependent Ca^{2+}-activated depolarizing membrane conductance (I). This suggestion was supported by our membrane potential measurements demonstrating that caffeine-induced Ca^{2+} release from the SR could depolarize SMCs only if the endothelium was present or if the loss of endothelium was compensated by a membrane permeable-analog of cGMP (I). We later identified this conductance as the cGMP-dependent Ca^{2+}-activated Cl^- conductance (III and [146;179]).

The model for generation of vasomotion

Our comprehensive study of vasomotion in the rat mesenteric small arteries together with other studies in this field lead us to suggest a detailed model for the initiation of vasomotion in this vessel (I). Our model (Fig. 4) suggests that agonist stimulation of SMCs induces intermittent release of Ca^{2+} from the SR which activates a Ca^{2+}-dependent depolarizing Cl^- channel in the mem-

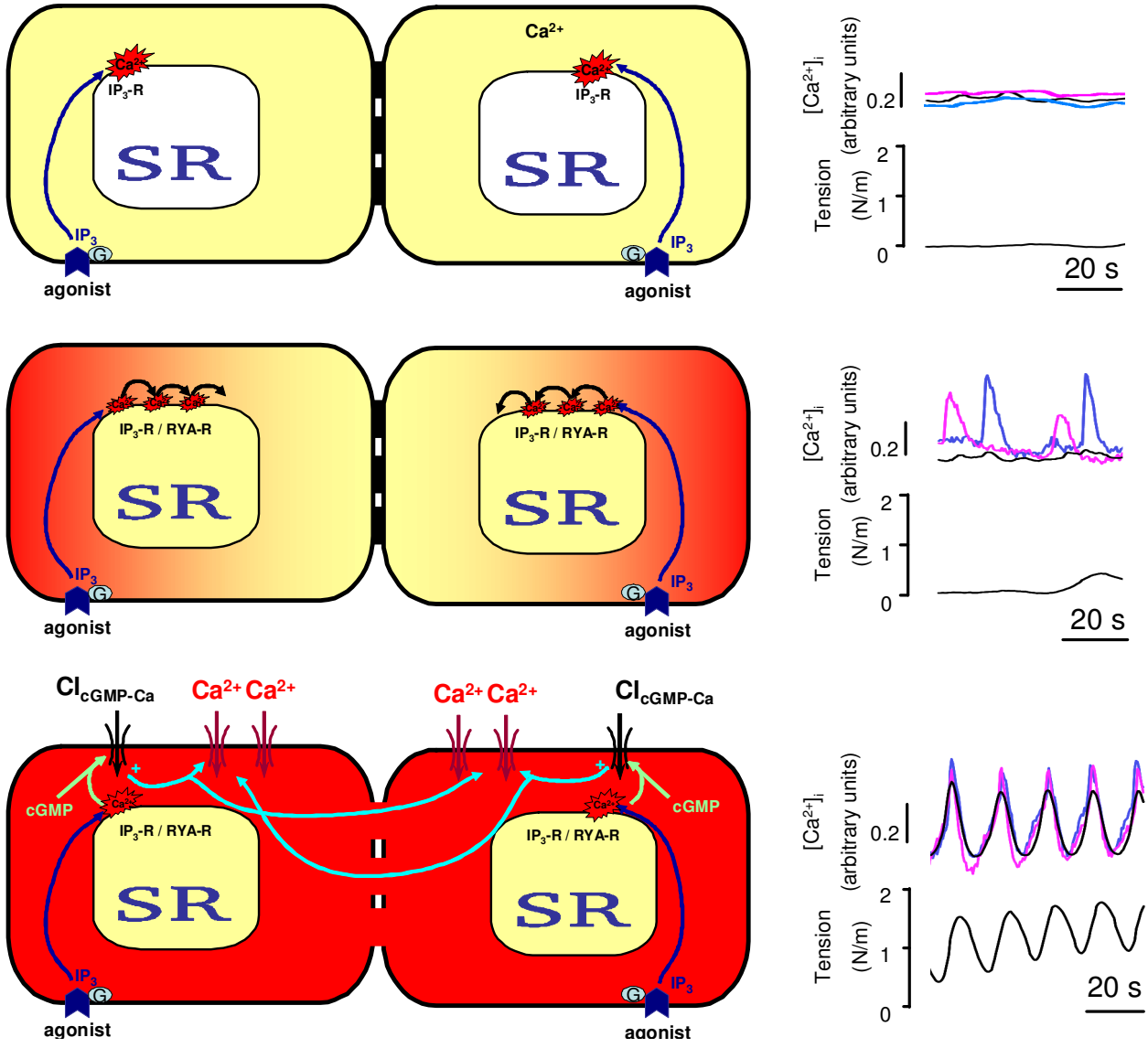

Figure 4
Model for the generation of vasomotion we suggest based on our experimental data (I, II). Left panel represents the schematic sequence of events during the initiation of vasomotion, the right panel shows changes in $[Ca^{2+}]_i$ in two individual SMCs (shown in two colors), the global $[Ca^{2+}]_i$ in the vascular wall (black line) and changes in the wall tension. Initial stimulation with contractile agonist increases IP_3 production and stimulates localized Ca^{2+} released from the SR (upper panel). This is not accompanied with any changes in the global $[Ca^{2+}]_i$ and in force. This local Ca^{2+} release can initiate the Ca^{2+} waves amplified by the CICR (middle panel) causing unsynchronized Ca^{2+} transients but not contractions. In the presence of a substantial amount of cGMP a rise in $[Ca^{2+}]_i$ can stimulate the cGMP-dependent Ca^{2+}-activated Cl^- channel (bottom panel) and depolarize the membrane. Membrane depolarization opens the VDCCs, induces global Ca^{2+} oscillation and synchronizes SMCs via gap junctions. This leads to vasomotion.

brane only when cGMP is present. While this may occur randomly in different SMCs, the individual cells become synchronized by entrainment of Ca^{2+} release from SR in individual cells. When this occurs in a sufficient nu
mber of cells, a depolarization is evoked which spreads through the gap junctions (I and [46;47]).

A complex action of cGMP in the generation of vasomotion (Paper II)

In accordance with our model for the generation of vasomotion the presence of a sufficient amount of intracellular cGMP is necessary for transition of the Ca^{2+} waves to synchronized oscillation in the vascular wall (I). This role of the endothelium/cGMP pathway in the synchronization of SMCs is somewhat controversial: cGMP/endothelium has been shown to have both potentiating (I and [70;170;171]) and suppressing [67;180] action, depending on the vasculature type and the experimental protocols. In an attempt to understand this controversy we tested whether cGMP has a more complex role in the initiation of vasomotion (II) than those suggested by our initial model (I) where some of the parameters were simplified [46-48]. Our detailed study of vasomotion "beating" and the concentration-dependent effect of 8Br-cGMP on the oscillation parameters, e.g. the frequency and amplitude of oscillations, led us to two major conclusions (II). The first finding is that a state of partial coupling between SMCs is possible and that intermediate concentrations of cGMP can provide it. The second is that the stimulation of Ca^{2+}-activated Cl^- channel is only one of several different targets of cGMP in its complex modulation of vasomotion (II). The partial coupling of SMCs in the vascular wall was seen as a 'beating' oscillation in endothelium-denuded arter-

ies under isometric conditions after addition of intermediate concentrations of 8Br-cGMP (30-100 μM) (II). The phenomenon can be explained by the presence of two or more regions oscillating with different frequencies. This was confirmed in isobaric experiment with pressurized arteries where different segments of a denuded artery oscillated with different frequencies at intermediate cGMP concentrations (II). Importantly, this phenomenon is rarely seen in the endothelium-intact rat mesenteric small arteries [181]. Moreover, the frequencies between different pieces of the endothelium-intact vessels are the same even when these to the pieces are not coupled to each other [110].

The partial synchronization was accompanied by a dissociation of oscillations in membrane potential and tension (II). When a glass electrode was implanted in one SMC the membrane potential oscillations often differed from the 'summarized' force oscillations. Similar observation was also made for the $[Ca^{2+}]_i$ dynamics (II). At the state of intermediate cGMP concentration islands of cells having synchronous oscillations of $[Ca^{2+}]_i$ were observed (II and [48]). Even in the presence of a high (300 μM) concentration of 8Br-cGMP some SMCs were quiescent during synchronous oscillations in $[Ca^{2+}]_i$ in their neighbors [48]. Other SMCs had an extra transient which were not synchronized and was in the form of a Ca^{2+} wave (II). The observed extra $[Ca^{2+}]_i$ waves were not seen in arteries with intact endothelium suggesting that endothelium provides more than NO/cGMP. The endothelium can also provide additional means of synchronization in the vascular wall, which is also suggested by the observation that vasomotion is not always stopped by blockade of NO production (II and [57]). This might be explained by an essential role of an endothelium-derived hyperpolarizing factor because its inhibition by charybdotoxin and apamin blocked vasomotion [57]. This was, however, not the case in another study [66] and in our own experiments (unpublished observation).

The cGMP molecule has multiple effects on vascular function. Thus, cGMP-dependent phosphorylation of connexins 37 and 43 [182] reduces intercellular coupling but cGMP enhance the intercellular communication via connexin 40 [183-185]. It is therefore possible that cGMP affects vasomotion via an effect on intracellular coupling [115]. It has been shown previously that the coupling resistance between SMCs is much higher than that between endothelial cells [186]. Thus, the removal of endothelium also abolishes a low-resistance pathway for current synchronization and therefore potentially makes it more difficult to entrain synchronized islands of SMCs located distantly.

In our cGMP concentration-effect study we also observed that cGMP had a direct effect on $[Ca^{2+}]_i$ dynamic by reducing the number of oscillating cells and the frequency of Ca^{2+} release (II). The exact reason for this suppressing action of cGMP was not studied, but it has been reported previously that cGMP inhibited SR-dependent Ca^{2+} transients via stimulation of Ca^{2+} extrusion mechanisms, i.e. the Ca^{2+}-ATPase [187] and the Na^+/Ca^{2+}-exchanger [188], as well as through direct inhibition of the IP_3 pathway [189;190]. Increased Ca^{2+} extrusion could explain the reduction in the frequency of oscillation. Thus, similar to our findings, stimulation of NO/cGMP production by acetylcholine and sodium nitroprusside in tail artery reduces $[Ca^{2+}]_i$ dynamics even in hyperpolarized arteries [191]. We suggested that Ca^{2+} waves, being the pacemaker for vasomotion, have a strong influence on the frequency of the synchronized oscillations. It is therefore possible to expect that cGMP, by affecting the frequency of the Ca^{2+} waves, will also reduce the frequency of vasomotion. We confirmed this experimentally by showing that the frequency of vasomotion decreased gradually with an increasing 8Br-cGMP concentration (II).

Electrophysiological approaches in the studies of vasomotion

Registration of electrical events over the cell membrane is one of the essentials for understanding vasomotion. The measurements of membrane potential in isolated arterial segment supported the suggested theory about transition of $[Ca^{2+}]_i$ waves into the synchronized global Ca^{2+} oscillations by means of a membrane potential dependent mechanism (I and II). Moreover, simultaneous measurements of membrane potential, $[Ca^{2+}]_i$ and isometric force showed the initial role of membrane potential changes in the sequence of events leading to the rhythmic contractions [35]. The great advantage of this method is an ability to record membrane potential without isolating the cells [7]. Thus, a sharp glass electrode can be impaled in smooth muscle cell located in its natural environment in the vascular wall. It is therefore possible to combine membrane potential recording with isometric force and $[Ca^{2+}]_i$ measurements, although the problems with movement artifacts are also relevant for this method. Although the myosin light chain kinase inhibitor wortmannin can be used for stabilization, normally recordings under isometric conditions are made without wortmannin. We controlled the location of the electrode tip by continuous recording of electrode resistance (input resistance) by current pulse injection (VI and VII). The recorded input resistance is an indicator of both the membrane resistance of the impaled cell and the resistance of intercellular contacts within the vascular wall. We have, therefore, used input resistance for qualitative evaluation of intercellular coupling within the intact vascular wall (VI and VII).

The suggested presence of a cGMP-dependent Ca^{2+}-activated depolarizing current based on the membrane potential measurements (I) received support in our voltage clamp studies where we characterized the current (I, III-V). Under voltage clamp conditions the current injected to clamp the potential reflects the ionic current across the cell membrane [192;193]. A major disadvantage of voltage clamp is the difficulty of applying it to vascular smooth muscle cells in situ. In comparison with the conventional sharp electrode used for membrane potential measurements, the voltage clamp electrode has low tip resistance and "patch" the cell membrane forming a very high-resistance seal with the membrane surface [194]. The formation of a gigaohm seal is essential for successful patch clamp experiments and this means that only "clear" membrane surfaces can normally be used [195]. Vascular wall cells are not "clean" and additional procedures, e.g. enzyme "shaving" of vessels surface are necessary [195;196]. Even if it will be possible to patch the vessel without enzymatic digestion (as it is the case for some arterioles) this will not give much advantage over the conventional membrane potential measurement due to the "space phenomenon", i.e. inability to control the voltage over a piece of tissue containing more than a few cells [192;195;197]. This problem can however be solved by chemical uncoupling the cells in the vascular wall [196;198]. In summary, some modification of the conventional technique provides the possibility to patch SMCs in the vascular wall but the necessary manipulations will significantly modify the cells.

The cells for conventional patch clamp can be isolated by enzymatic digestion of the vessel. The method of digestion depends on the type of artery and the type of conductance which is studied. There is no one ideal isolation technique which is suitable for all studies. Different combinations of collagenase, elastase and a broad-spectrum protease, papain, are normally used to digest different vessels [195]. Papain is know to damage some types of Ca^{2+} conductances but is good for studies of K^+ currents [199]. Although Ca^{2+} conductances are resistant to the collagenase/elastase diges-

tion, the isolated cells show hyperreactivity and are constrict to any stimulus which makes difficult to patch them [200].
After the gigaseal is obtained several patch clamp configurations can be formed [195]. Rupture of the membrane under the tip forms a low resistant pathway between the intracellular environment and the pipette solution. The configuration I have mostly used in my studies is the "whole-cell" technique because it enables me to record the current from the entire cell membrane. Under the whole-cell configuration the contents of the cell and the pipette exchanges within a few minutes which allows control of the intracellular environment [201]. This property is helpful for effective isolation of single ionic conductances in macroscopic mode. I have dialyzed the cells with a cesium rich solution to establish more complete block of K^+ conductances which otherwise could contaminate the recordings (III and IV). Similarly the intracellular messengers and other big signaling molecules can be added via the pipette in defined concentrations, e.g. I applied intracellularly membrane-impermeable cGMP (III-V), ATP (IX), protein kinase G (III) and controlled intracellular Ca^{2+} by Ca^{2+}-chelators (III, V, VIII). Importantly, diffusion also occurs from the cell to the pipette, and significant loss of important cellular component can be observed [201-203]. This is the explanation for rundown of several membrane currents, e.g. Ca^{2+}-activated Cl^- current [204]. Washing out of diffusible second messengers explains also the disruption of receptor-channel coupling seen in the whole-cell experiments [12]. This problem with the loss of intracellular constituents is partially overcome using the perforated patch clamp where the membrane patches only permeabilized for monovalent ions by antifungal drugs, nystatin and amphotericin (I and [205]). Permeabilized patch does not allow addition of bigger molecules via the pipette solution resulting in pure control of $[Ca^{2+}]_i$, ATP/ADP ratio, etc. Noteworthy, the conventional patch clamp configuration does not seem to provide a complete control over larger organic molecules (e.g. cAMP and ATP) which have low washout rates partially because of their size [206;207] and partially because of the microdomain structures [208;209]. Moreover, ion concentration was also suggested to be poorly controlled in some subcellular compartments. Thus, the possibility to change local $[Ca^{2+}]_i$ is strongly dependent on the type of Ca^{2+} chelator and the distance to the signaling target [210].
Other modifications of patch clamp allow direct measurements of the conductance of a single channel and provide detailed information on channel biophysical properties which can not be obtained from the whole-cell macroscopic recordings. This can be done in "cell-attached" mode when other cellular constitutes can directly modulate channel behavior [121]. Only poor control of voltage over the patch membrane and no control of the intracellular environment are limiting the use of this method. A single-channel can also be studied in a cell-independent manner when the membrane patch is completely isolated from the cell in "inside-out" and in "outside-out" modes [195;211]. These methods permit identification of distinct channel subtypes when the macroscopic currents are not easy to distinguish [195;211]. Under these conditions there is a tight control of the environment on both sides of the membrane which is a clear advantage but a drawback at the same time since single channels in excised patches behave differently because of lack of many intracellular constituents which play regulatory and modulatory roles. Therefore, caution should be taken when relating the patch clamp data to functions of the intact vessels.
Patch clamp is used not only for studies of membrane currents but also for evaluation of intercellular communication. Evaluation of electrical coupling can be seen under conditions when visual dye coupling fails to detect intercellular communication [72;212]. Electrical coupling between two cells can be determined by dual patch clamp [213;214]. This technique is more challenging than conventional single electrode recording. Alternatively, monitoring the membrane capacitance of coupled cells by single electrode patch clamp can give insight to the cell coupling (VII, VIII and [215;216]). Cell membranes store a charge as a capacitor. Therefore, when cells are electrically coupled the total electrically coupled membrane surface is increased and membrane capacitance increases likewise (VIII). Uncoupling of coupled cells decreases capacitance. The great advantage of this method is that it enables intercellular coupling to be estimated under conditions where only one of the coupled cells is patched [215;216]. Importantly, this way to estimate electrical coupling can not be used easily for quantitative measurements because of non-linear relation between the measured total capacitance and intercellular resistance [217]. Nevertheless, intercellular resistance changes can be estimated by a complicated recalculation of the measured parameters [217-219].

6. A Cl^- CHANNEL IN VASOMOTION? (PAPERS III, IV, V)

The characterization of a unique cGMP-dependent Ca^{2+}-activated Cl^- current in SMCs from rat mesenteric small arteries (Paper III)

A cGMP-dependent Ca^{2+}- activated depolarizing conductance was predicted by the model for the initiation of vasomotion in rat mesenteric small arteries (I).We recorded this cGMP-depended current initially in permeabilized whole-cell mode (I). This type of recording makes it difficult to manipulate the intracellular environment and to characterize the conductance in detail. We chose, therefore, the conventional whole-cell patch-clamp method which allowed us to manipulate both intracellular and extracellular environments. Using this method we characterized the cGMP-dependent current in detail (III). Piper and Large published a description of the same conductance at the single channel level [121;220].

$[Ca^{2+}]_i$-dependence

Similar to the permeabilized patch-clamp experiments, caffeine-induced Ca^{2+} release induced under the conventional ruptured patch-clamp conditions a few hundred pA inward current only when micromolar cGMP was added into the pipette solution (I and III). This cGMP-dependent, Ca^{2+}-activated inward current can be also stimulated by increasing $[Ca^{2+}]_i$ by other means, e.g. stimulation of extracellular Ca^{2+} influx (III) or dialysis of the cell with high-Ca^{2+} solution (IV and V). Importantly, this current can also be stimulated by noradrenaline via the G-protein-coupled adrenoreceptor in the cell-attached mode [121]. Chelating Ca^{2+} with a low concentration of BAPTA or EGTA abolished the cGMP-dependent current induced by caffeine (III) suggesting that Ca^{2+} can be released at a distance (>100 nm) from the channel [210] and that there is no need for a very close interaction between the SR and the channel.
The cGMP-dependent inward current was steeply Ca^{2+} dependent, as was shown with the single channel recordings [121]. The current was detectable already at 50 nM $[Ca^{2+}]_i$ and reached its maximum at 100 nM with half-maximal activation at 74 nM. This $[Ca^{2+}]_i$-dependence was shown to be mediated via calmodulin which increased the open probability of the channel in a calmodulin kinase II (CaMKII) independent pathway [220]. The surprisingly high sensitivity for $[Ca^{2+}]_i$ suggests that either under the physiological conditions Ca^{2+} sensitivity is under control of second mes-

sengers, as it is known for other Ca^{2+}-activated ion channels, or the channel is regulated by local spatially restricted Ca^{2+} changes rather than global $[Ca^{2+}]_i$.
We observed sometimes during caffeine stimulations a superimposed outward current due to an activation of big-conductance Ca^{2+}-activated K^+ channels (BK) (III), since it was abolished by specific blockers, e.g. charybdotoxin or iberiotoxin [221]. It also has been shown previously that Ca^{2+}-activated outward (K^+) and inward (Cl^-) conductances can superimposed on each other [27-29]. The appearance and prevalence of the BK current depended on the membrane voltage and became significant at voltages positive to -60 mV, when it was distant from the K^+ equilibrium potential (E_K=–78.5 mV). To avoid complications in the analyses of the Cl^- conductance, we had K^+ channel blockers, charybdotoxin and barium chloride, in the bath during almost all our experiments. Another reason to have these K^+ blockers in the bath was to compensate for the non-specific effects of niflumic acid on the membrane conductance (IV and [222]) (see below).

An absolute requirement for cGMP
There is no doubt that caffeine induces the inward current by elevation of $[Ca^{2+}]_i$ only in the presence of intracellular cGMP (III). This requirement for cGMP was absolute under whole-cell configuration but a small inward current was seen under permeabilized conditions in the absence of 8Br-cGMP (I). This can be due to remaining endogenous cGMP. It is, therefore, important that the single channel recordings clearly indicate an absolute requirement for cGMP [121]. The sensitivity to cGMP (in micromolar range) was not significantly different under whole-cell (III) and single channel configurations [121]. Although the detected sensitivity for cGMP is similar to that described for cyclic-nucleotide-gated channels [223;224] this channel is not one of them, because we have shown that the channel is activated via phosphorylation by a cGMP-dependent protein kinase (PKG) which is not the case for the cyclic-nucleotide-gated channels (III and [121]). Key evidence for this was that a cGMP-independent constitutively active catalytic fragment of PKG Iα [225] activated the Ca^{2+}-activated inward current in the absence of cGMP (III). The catalytic subunit of PKG I was produced by trypsinization [225] but it is not possible to specify to which extent it depends on the two PKG I variants, i.e. PKG Iα and PKG Iβ [226]. The role of the variants is, however, important since the dominating smooth muscle variant, PKG Iα [227], is highly sensitive to cGMP with half-maximum activation at 0.1 µM [226]. This is much higher than the sensitivity detected in our studies and that of Piper & Large (III and [121]). Thus, since PKG Iβ is also expressed in SMCs and is at least 10-times less sensitive to cGMP, PKG Iβ is the most likely candidate for the activation of the cGMP-dependent Ca^{2+}-activated inward current. The two PKG variants are involved in distinct signaling pathways [228;229]. Thus, it is possible that cGMP pathways divide into the well-known NO-mediated relaxation via cGMP with involvement of PKG Iα, and another pathway via PKG Iβ which is associated with rhythmic membrane depolarization via the cGMP-dependent Ca^{2+}-activated depolarizing current (I). This is consistent with previous suggestions that both Ca^{2+} and cGMP signaling are spatially restricted in SMCs [65;228-232].

A novel cGMP-dependent Ca^{2+}-activated Cl^- current
Since the equilibrium potential for K^+ was negative to the holding potential in our experiments, K^+ cannot be a charge carrier for the inward current, but Na^+, Ca^{2+} and Cl^- ions are candidates (E_{Na}=+68 mV; E_{Ca}=+88 mV; E_{Cl}=0 mV) (III). However, neither Ca^{2+}-free extracellular solution nor Ca^{2+} channel blockers affected the cGMP-dependent current. Na^+ was also excluded from the candidates based on substitution experiments (III). In contrast, the close correlation between the equilibrium potential for Cl^- (E_{Cl}) and the current reversal potential (E_{REV}) indicates that the cGMP-dependent Ca^{2+}-activated inward current is carried by Cl^- (III and [121]).

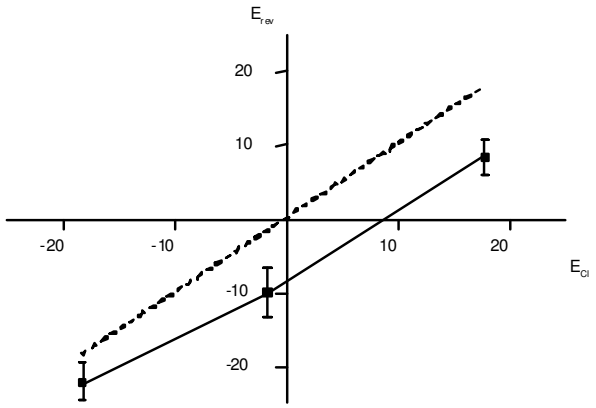

Figure 5
E_{REV} is plotted against calculated E_{Cl}. The dashed line is the identity line, expected for ideal Cl^- channel. Bath solution contained the following cations (in mM) 6 K^+, 145 Na^+, 0.1 Ca^{2+}. Pipette solution contained (in mM) 132 K^+, 10 Na^+, 0.01 Ca^{2+}. Cl^- concentration was varied as described previously (III). Vertical bars indicate SEM. Note the difference of the experimental results from the identity line. This difference disappeared when symmetrical CsCl solution was used instead (III).

To estimate the anion selectivity in the whole-cell configuration we used CsCl solutions to suppress the Ca^{2+}-activated K^+ channels and buffering $[Ca^{2+}]_i$ at 900 nM. Under these conditions, E_{REV} corresponds fairly well to E_{Cl} [24;233;234] indicating a pronounced, although not perfect, selectivity for anions (III). It is not apparent, however, whether some deviations from the identity line reflect cations passing through the channel, or if they result from an insufficient control of intracellular ion concentrations. In general, a Cl^- channel displays a 5-50 time selectivity for anions over cations. This is the case for, e.g. cystic fibrosis transmembrane conductance regulator (CFTR) [235], $GABA_A$ and glycine receptors [236] and the voltage-gated Cl^- channels [237-239]. However, a more appropriate name might be a Ca^{2+}-activated anion channel.
When we used complex solutions, containing K^+, Na^+, Ca^{2+} and Cl^- ions, the correlation between E_{Cl} and E_{REV} was no longer ideal (Fig. 5), indicating less than perfect anion selectivity. The downward shift from the identity line seen under these conditions indicates a significant influence of K^+ conductance which has a negative E_K relative to E_{Cl}. A relatively poor selectivity for anions over for cations was previously described for the Ca^{2+}-activated Cl^- channels (CaCCs) [240]. More information about the ion conducting properties comes from the studies of relative halide permeability. This was normally evaluated under bi-ionic substitutions, i.e. when Cl^- on one side is substituted for another anion, and this results in the E_{REV} change [240;241].
The relative halide permeability of most CaCCs increases with the ionic radius and normally follows the sequence $I^->Br^->Cl^->F^-$. The Cl^- channels are also permeable to polyatomic non-halide anions, such as thiocyanate (SCN^-), acetate and aspartate. SCN^- is usually more permeable than Cl^-, while acetate and aspartate are known to be less permeable due to their size [240;242]. To enter the channel

pore, anions have to be partly dehydrated and the energy necessary for dehydration must be supplied by a binding site within the pore. Wright and Diamond [243] suggested that a binding site with a low electrical field strength (where the interaction of ions with the channel is weaker than interaction with water) provides this energy and, thus, defines the typical permeability sequence for the anion channel. In the simple terms this would indicate that I^-, which is the large ion, binds better within the pore than F^-, which is small, and, therefore, is more permeable. Importantly, the relative permeability can not be used as a measure of an ion flux and describes only an ability of an ion to enter the channel pore. The flux of ions is determined mainly by the strength of interaction within the pore. High-affinity binding slows down ion flux (the ion "sticks" in the pore) and reduces channel conductance. Conductance is evaluated from the current-voltage relationship [240] but is rarely studied in details. When it has been studied, I^- was either shown to be more conductive than Cl^- [235;244] or no difference was detected [245;246]. This indicates that I^- binds in the channel pore with either similar or weaker strength than Cl^-.
Keeping this in mind, we identified both the relative conductances and permeabilities for the cGMP-dependent Ca^{2+}-activated Cl^- current ($I_{Cl(Ca,cGMP)}$) in SMCs (III). The sequences were similar to those of the 'classical' Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) but not identical, e.g. we found $Br^->I^-$. This is possible when permeabilities for I^-, Br^- and Cl^- are close [243] which was the case in our study (III). This might also be the reason for the inconsistency with results achieved in the single channel recordings, where the relative permeability sequence was $Cl^->I^-$, but again the I^-/Cl^- ratio was close to 1 [121]. When the channel permeability was placed against the hydration energy of the ions (Fig. 6) the slope was shallow compared to that reported for other CaCCs [15;240;241;247]. This indicates that in contrast to other CaCCs [240;241;247], the hydration energies is not a major determinant of the ion permeability. Which other parameters are of significance for the cGMP-dependent CaCC permeability and conductance remains unclear, but we failed to find a correlation with ion dimensions [248]. Also the binding affinity could not explain the observations since the recalculated relative affinities for the binding site in the channel were equal for all halides (III). Obviously, ionic radius is playing a role when ion dimensions become very big, as is the case with aspartate. Acetate, in contrast, is still permeable in accordance with its hydration energy. These results suggest that the channel pore is bigger than 4.7 Å (acetate size) but smaller than 5.8 Å (aspartate size) [248].

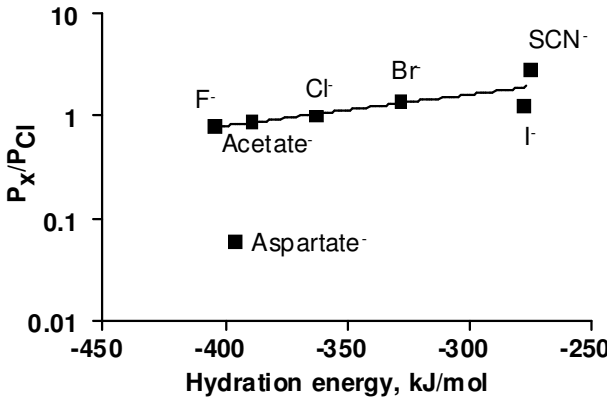

Figure 6
The permeability of anions (P_X) measured relative to Cl^- permeability (P_{Cl}) (III) correlated reasonably, but with a shallow slope, to the hydration energy of the anions.

Another interesting observation we made was that SCN^- has high permeability but low conductance suggesting a high affinity to the channel binding site (III and [240]). Similar properties were previously shown for the native $I_{Cl(Ca)}$ in the Xenopus oocytes [240] and, surprisingly, for GABA receptors where SCN^- induced pore blockade [236]. The latter observation might suggest a possible structural similarity between the cGMP-dependent CaCC and the $GABA_A$ receptors (V).

The voltage and time independence
In addition to $[Ca^{2+}]_i$, the open probability of many CaCCs is determined by membrane voltage. This was not, however, the case in our voltage-clamp experiments where the $I_{Cl(Ca,cGMP)}$ changed linearly with the membrane voltages (III and IV). Similar results were obtained from single channel recordings [121]. It is possible that the high $[Ca^{2+}]_i$ we used in the studies (III and IV) can be the reason, since voltage-dependence is known to be $[Ca^{2+}]_i$-dependent; current rectification is prominent at lower $[Ca^{2+}]_i$ but is almost absent at micromolar $[Ca^{2+}]_i$ [15;241;247]. We tested the possibility of Ca^{2+} dependence of the voltage-dependency and found that this was not the case: the $I_{Cl(Ca,cGMP)}$ was voltage-independent through the entire $[Ca^{2+}]_i$ range (V). The observation that the cGMP-independent 'classical' $I_{Cl(Ca)}$ had the $[Ca^{2+}]_i$ sensitive outward rectification under these experimental conditions suggests that our finding is not an artifact of the experimental protocol but a unique property of the channel. Some previously described $I_{Cl(Ca)}$ also have a weak or even absent the voltage-dependence at submaximal $[Ca^{2+}]_i$ [249-251]. The reason for these discrepancies is unknown.
The $I_{Cl(Ca,cGMP)}$ has no obvious time dependence (III and IV): as soon as $[Ca^{2+}]_i$ and cGMP are sufficient for activation, the channel is activated and the $I_{Cl(Ca,cGMP)}$ depends only on the driving force for Cl^-. Therefore, voltage steps in SMC dialyzed with cGMP and high $[Ca^{2+}]_i$ produce an immediate Cl^- current which is not different between the early and the instantaneous phase (III and IV). The absence of a tail current after repolarization also demonstrates that the gating mechanism is time- and voltage-independent.

Sensitivity to blockers
None of several putative blockers, known to block other Cl^- conductances, were found to be specifically effective for the $I_{Cl(Ca,cGMP)}$. Thus, DIDS and IAA-94 were without effect on the $I_{Cl(Ca,cGMP)}$ (III) when applied in concentrations known to inhibit other Cl^- conductances [15;241;247]. The most common blocker of the $I_{Cl(Ca)}$ [20;252-254], niflumic acid, also had no effect on the $I_{Cl(Ca,cGMP)}$ (III, IV, V and [121]). This is consistent with previous reports on insensitivity of some of Ca^{2+}-activated Cl^- currents [255] and even a potentiating action of niflumic acid on the Cl^- conductance [116;256-258]. This dual effect of niflumic acid on the $I_{Cl(Ca)}$ is of interest because recent data suggest an overlapping pharmacology of the Ca^{2+}-activated Cl^- and the Ca^{2+}-activated K^+ (BK) currents [116;222]. It has been shown recently that niflumic acid can potentiate both the BK current and, under certain conditions, the sustained Ca^{2+}-activated Cl^- current (III, IV and [116;256-258]), while the BK channel modulators can also affect the Ca^{2+}-activated Cl^- current [258]. Therefore, some similarities in the molecular structure have been suggested [116], although the effect of a physical interaction between the two proteins is also possible. Importantly, these non-specific effects appeared only when the blockers were applied

separately. When charybdotoxin is applied first, niflumic acid produces effective inhibition of the $I_{Cl(Ca)}$. Thus, the combination of blockers is needed to achieve inhibition of the $I_{Cl(Ca)}$. In our experiments (III-V), the bath solutions contained charybdotoxin to avoid the potentiating effects of niflumic acid on the BK and Ca^{2+}-activated Cl^- currents. The inhibition of the classic $I_{Cl(Ca)}$ supports action of niflumic acid on this conduction (III-V).
Since several Cl^- conductances were previously reported to be sensitive to multivalent cations, we tested the effect of divalent cations on the $I_{Cl(Ca,cGMP)}$ and found that micromolar Zn^{2+} effectively inhibited the current while Co^{2+} was without effect (III and [121]). Zinc was previously used to inhibit voltage-gated Cl^- channels but at higher concentrations [259;260]. Interestingly, the sensitivity to micromolar Zn^{2+} is one of the fingerprints for $GABA_A$ sensitive Cl^- conductance [261;262]. Zinc inhibits $GABA_A$ channels by binding to the selectivity rings in the channel pore [263;264] again suggesting a possible similarity in the pore structure between putative cGMP-dependent CaCC and the $GABA_A$ channel.

Two distinct Ca^{2+}-activated Cl^- currents co-exist in SMCs (Papers III and IV)

The current conductance
The characterization of the $I_{Cl(Ca,cGMP)}$ clearly indicated that the current we described (III) was unique in comparisons to other known, so called "classical", $I_{Cl(Ca)}$ [15;241;247]. The unique position of the $I_{Cl(Ca,cGMP)}$ in the group of the $I_{Cl(Ca)}$ was strongly supported by the finding that both types of current are present in the same SMCs (III and V). Also single channel recording revealed the presence of both currents [121;265]. The classical Cl^- conductance was much smaller (~3 pS) than the $I_{Cl(Ca,cGMP)}$ conductance which was ~20 pS [121]. This interesting feature is in accordance with previous observations indicating that at least two distinct types of anion conductances can be detected in the cell membrane. The low-conductive CaCCs is the most reported type. This conductance is detected in the various tissues including SMCs [265-268]. The reports on the large Ca^{2+}-activated Cl^- conductance are sparser.
In the beginning of the 1990's Benos and co-authors identified in bovine trachea a membrane protein constructed of 38- and 64-kDa polypeptides which incorporated in a lipid bilayers gave 25-30 pS $I_{Cl(Ca)}$ without any voltage-dependence [269]. Interestingly, this reconstituted channel did not need any other intracellular messengers but Ca^{2+} for its activation and was completely inhibited by 100 µM DIDS. In contrast, other authors reported a large $I_{Cl(Ca)}$, which depended on protein kinase G activation, and explained the Cl- current rundown by washout of intracellular messengers [270-272]. No voltage-dependence was shown for these $I_{Cl(Ca)}$. It should be noted, that CaCCs with conductance >100 pS was previously reported too, although most of these conductances belong to intracellular Cl^- channels [273;274].

The difference in pharmacology
The cGMP-dependence was used to distinguish between the Ca^{2+}-activated Cl^- currents when they were activated in the same SMCs (III-V and [121;220]). This differentiation was possible also based on distinct pharmacological properties. Thus, the $I_{Cl(Ca,cGMP)}$ had low sensitivity to niflumic acid in concentrations which inhibited the classical $I_{Cl(Ca)}$ (III). In contrast, the classical $I_{Cl(Ca)}$ was insensitive to micromolar Zn^{2+}, the blocker of the $I_{Cl(Ca,cGMP)}$ (III-V and [121;220]). This allowed us to design a protocol where the two currents were measured and evaluated during the same voltage clamp of SMC (III). The protocol was then carefully tested for unwanted effects of the blockers, e.g. niflumic acid, cGMP and Zn^{2+} (IV and V).
The $[Ca^{2+}]_i$ sensitivity of the $I_{Cl(Ca,cGMP)}$ [121] is surprisingly high in comparison to previously published values for other $I_{Cl(Ca)}$ [15;241;247]. Most of the $I_{Cl(Ca)}$ reported in the SMCs have half-maximal activation at 0.2-0.6 µM of $[Ca^{2+}]_i$ and achieve maximal activation at 0.6-1 µM of $[Ca^{2+}]_i$ [23;246;265;275]. This broad range can probably be explained by a variability of mechanisms (a direct activation or by phosphorylation via Ca^{2+}/CaM-dependent protein kinase II) for Ca^{2+}-activation within the heterogeneous group of the CaCCs [247;276;277], although this has not been studied systematically.
In our experiments we dialyzed SMCs with a solution containing ~0.9 µM free $[Ca^{2+}]_i$, which was enough for activation of both Ca^{2+}-activated Cl^- conductances (III and IV). When sensitivities of the two types of the Cl^- current were directly compared, both Cl^- currents were maximally activated at 0.3-0.9 µM $[Ca^{2+}]_i$, but the $I_{Cl(Ca,cGMP)}$ showed a higher $[Ca^{2+}]_i$ sensitivity (V). No current reduction was seen with supra-maximal $[Ca^{2+}]_i$, as it was shown previously for single channel recording [220].

Distribution of the currents in SMCs of different origin
The two different Ca^{2+}-activated Cl^- conductances might be caused by the same protein where phosphorylation by PKG changes the channel properties. PKG can thus act as a switch between different channel modes. This is, however, not the case, since the cGMP-dependent and the classical Ca^{2+}-activated Cl^- currents have different expression profiles in SMCs. This is also supported by differences between these two Ca^{2+}-activated Cl^- conductances in pharmacology, single-channel conductance and ion selectivity.
The $I_{Cl(Ca,cGMP)}$ was first characterized in SMCs freshly isolated from the rat mesenteric small arteries (III). It was then detected in SMCs isolated from other vascular beds and in visceral SMCs (IV). In most vessels, e.g. aorta, tail artery, femoral artery and vein, middle cerebral artery, renal artery, portal vein, superior mesenteric artery and colon, the $I_{Cl(Ca,cGMP)}$ was accompanied with the classical $I_{Cl(Ca)}$ in variable proportions (IV).
Importantly, due to different voltage-dependences the relative size of two currents was strongly dependent on the membrane voltage at which the currents were measured (IV). The classical $I_{Cl(Ca)}$ showed a strong outward rectification. Thus, for the comparison of the two Cl^- currents, it is important to define at which membrane voltage the currents are compared. It seems more relevant to compare the currents under conditions close to physiological membrane potentials. We, therefore, compared the Ca^{2+}-activated Cl^- currents at membrane voltages close to the resting membrane potential of SMCs (IV and V). Under these conditions SMCs from cerebral arteries and femoral vein had dominating $I_{Cl(Ca,cGMP)}$, while in the myocytes from tail and mesenteric arteries and portal vein these two currents are not different. Importantly, the pulmonary arteries had virtually no $I_{Cl(Ca,cGMP)}$ (III). It remains unclear whether this has physiological significance, but we have used this observation to search for the gene responsible for $I_{Cl(Ca,cGMP)}$ (V).

The Cl^- conductance in the SMCs
If Cl^- is distributed passively across the plasma membrane intracellular Cl^- concentration should be ~10-15 mM. This is consistent with the data in skeletal muscles [16], but not the smooth muscles. Measured with intracellular electrode [14;278-281], ^{36}chloride efflux [282;283] and a fluorescent dye [284] in different SMCs, the $[Cl^-]_i$ is surprisingly high within the range between 30 and 50 mM. Thus, the estimated E_{Cl} will be between -40 and -25 mV which is above

resting membrane potential in the majority of smooth muscles [285;286]. This suggests active transport of Cl^- into the SMCs. There are at least two transport mechanisms which contribute to intracellular Cl^- accumulation: Cl^-/HCO_3^- exchange [14;287] and the Na^+-K^+-Cl^--co-transport [288;289]. In addition to these well-known types of Cl^- transports, a third form of transport, the so called 'pump III', is suggested from the work of Chipperfield and co-authors [290-292]. Neither the molecular identity nor the driving force for the 'pump III' is known.

Opening of Cl^- channels will depolarize SMCs and, therefore, induce smooth muscle contraction. Indeed, a role of Cl^- efflux for agonist-induced contraction has been shown in several types of smooth muscle and the role of CaCCs was emphasized [19-24]. If Cl^- is important for contraction, the contraction should depend on the Cl^- gradient across the plasma membrane. Several studies have shown that replacement of extracellular Cl^- with non-permeate anions can amplify agonist-induced depolarization and contraction [25;125;293], but this is not always the case. Substitution of extracellular Cl^- was shown to have only mild effects on both resting and stimulated membrane potentials [294] and no significant effect on contraction in rat mesenteric arteries [87]. A growing number of reports suggest a variable significance of Cl^- for SMC contraction depending on the type of vasculature and stimulation [293].

Ca^{2+}-activated Cl^- conductance: an important function of unknown origin

The CaCCs, first described more than 25 years ago [295-297], are found at the functional level in most SMCs where they have been characterized electrophysiologically and pharmacologically [15;247;276]. Despite their obvious importance and the great interest in this group of channels their molecular identity is still under debate [247;277;298].

Why does the question of molecular identity remain open?

Several problems have made it difficult to pinpoint the molecular counterparts of endogenous $I_{Cl(Ca)}$.

- Heterologous expression has often been used to show that a protein is a bona fide CaCC. This technique has several drawbacks and the major of them is the absence of an expression system that has no endogenous Cl^- conductance. This endogenous Cl^- conductance often seems to be upregulated by over-expression of a candidate protein, resulting in false positives. Thus, the most widely used expression system, the Xenopus oocytes, expresses a huge amount of an endogenous Ca^{2+}-activated Cl^- conductance and is therefore difficult to use for characterizing a candidate.
- Compounds blocking or activating the conductance of interest are needed for differentiation of the $I_{Cl(Ca)}$ from other membrane currents and, especially, other Cl^- currents. However, available drugs are lacking specificity [116;299]. There are currently attempts to overcome this problem by using molecular biological tools, such as antisense DNA, knockout models, siRNA knock-downs, and specific antibodies.
- Most of the currently known membrane channels are multimers composed of several different subunits. Some of the subunits form the channel pore, while others are involved in the regulation, but all of them may be essential to produce the current. This means that the heterologous expression may be unable to reproduce the endogenously observed current. This is a well-known problem even for the Cl^- channels that have already been cloned, such as CFTR, $GABA_A$ receptors and ClC channels.
- Finally, a broad spectrum of CaCC properties are found [15;241;247]. This variability suggests that the CaCCs are a heterogeneous group of different protein families. Three to four different classes of CaCCs have been suggested [277]. Some CaCCs are directly activated by physiological concentrations of $[Ca^{2+}]_i$, while others are modulated by CaMKII [239]. The Cl^- channels with large, over 100 pS channel pore conductance form possibly a special class of the CaCCs [270;273;274;300].The cGMP-dependent CaCC can be considered another independent class, although it shares many similarities with the group of channels directly regulated by $[Ca^{2+}]_i$ (III, V and [121;277]).

The problems described above complicate the search for the molecular identity of the CaCCs and indicate that only a complex strategy can be successful. It suggests also that caution should be taken with respect to conclusions made based upon only one research strategy, since it can be misleading as it has been recently shown in an example with CLCA candidate gene [301].

The potential candidates for the role of the Ca^{2+}-activated Cl^- channel

CLCA

In spite of these problems, some molecular candidates for CaCCs have been suggested over the past years. The CLCA protein was the first notable candidate to mediate an $I_{Cl(Ca)}$ [255;302]. The first member of the CLCA family was cloned from a bovine tracheal cDNA library by immunoscreening with an antibody against a Ca^{2+}-activated anion channel [255]. The following evidence for its function as a CaCC was collected [255;302-305]:

- Heterologous expression of various isoforms of CLCA generated membrane currents which activated by high $[Ca^{2+}]_i$.
- The heterologously expressed current was sensitive to the classic Cl^- channels blockers such as niflumic acid and DIDS.
- The heterologously expressed CLCA protein carried Cl^- ions, but this was shown under Na^+ and K^+ free conditions.

This evidence is, however, not enough to accept CLCA as a valid candidate for the CaCC structure, especially because several inconsistencies were also identified.

- The channel structure remains unclear. Although mutagenesis analyses identified potential protein domains which might be responsible for forming a pore, the deletion of these domains did not affect the current [306].
- There was a number of concerns regarding membrane localization of CLCA, and CLCA also has been suggested to function as an adhesion molecule or a secreted protein [307-309].
- There are significant phenotypical differences between characteristics of the classical $I_{Cl(Ca)}$ and the current induced by heterologous expression of CLCA [310].
- The CLCA-associated current has been shown to be activated by $[Ca^{2+}]_i$ in much higher concentrations than are known to activate the endogenous $I_{Cl(Ca)}$ [310]. Greenwood and colleagues have solved this problem by co-expressing the CLCA protein with the β-subunit of the Ca^{2+}-activated K^+ channel [311]. This co-expression 'normalized' the Ca^{2+} sensitivity and voltage-dependence suggesting the lack of a regulatory subunit in the CLCA expression system.
- Importantly, the correlation of $I_{Cl(Ca)}$ and the CLCA expression in different tissue is not convincing. For example, mouse Ehrlich tumor ascites cells have the $I_{Cl(Ca)}$ but do not express CLCA [310].

However, in spite of these concerns, it is too early to exclude the CLCA protein family from the list of potential candidates for CaCCs.

ClC channels

ClC channels are the family of voltage-gated Cl^- channels [239;312]. One member of this family, ClC-3, was shown previously to be regulated by $[Ca^{2+}]_i$ via CaMKII activation [313;314]. The expression of ClC-3 produces an outward-rectifying, slightly time-dependent $I_{Cl(Ca)}$. It has been shown, however, that although the CaMKII-activated Cl^- conductance is gone in ClC-3 knockout mice, the $I_{Cl(Ca)}$ is still present [315]. This suggests that ClC-3 could be responsible for the CaMKII-activated Cl^- current, but another pore-forming protein conducts the directly-Ca^{2+}-activated Cl^- current.

TWEENTY

The heterologous expression of mammalian homologues for Drosophila flightless gene TWEENTY has been shown previously to induce the $I_{Cl(Ca)}$ [316]. The TWEENTY-associated Cl^- channels have an unusually high conductance of >100 pS and, therefore, belong to the distinct group of maxi-Cl^- channels. The maxi-Cl^- currents, which were found endogenously in neurons [270], kidneys [274] and skeletal muscles [273;300] have biophysical properties similar to the TWEENTY-associated Cl^- conductance. There is, however, no correlation between the TWEENTY-homologous gene expression and the appearance of smaller Ca^{2+}-activated Cl^- conductances.

TMEM16A

TMEM16A gene belongs to a family assembled from bioinformatics analyses [317;318] and has a number of different names, e.g. TAOS2, DOG-1, OVRAOV2 and NGEP, which are given because of its association with different forms of cancer [319]. After identification as a putative CaCC [320-322], the protein produced by TMEM16A gene was termed 'anoctamin-1' (ANO1) because of the anion selectivity of this eight transmembrane domain structure [320-322]. The characterization of ANO1 was received as a breakthrough in the search for the CaCCs identity [298;319] because this protein most closely reproduces the native properties of endogenous $I_{Cl(Ca)}$. Several strong arguments support it.

- ANO1 was predicted to have eight transmembrane domains and was shown to be localized in the plasma membrane [320;322].
- ANO1 has been shown directly to produce Cl^- current in response to G-protein-coupled receptor stimulation (via endothelin, angiotensin II, muscarinic, histamine and purinergic receptors) [320-322].
- The biophysical properties and halide conductance ($I^- > Br^- > Cl^- > F^-$) of the ANO1-overexpressed current [319-322] are similar to the endogenous $I_{Cl(Ca)}$ [15;241;247;321]. The ANO1-associated Cl^- current shows time-dependent activation and outward rectification which reduces with increase in $[Ca^{2+}]_i$. The detected single-channel conductance of ANO1 Cl^- current (~8 pS) [320] corresponds to the well-known small conductances of the classical $I_{Cl(Ca)}$ [241;247].
- The overexpressed ANO1-associated Cl^- current has been shown to be sensitive to low concentrations (10 µM) of classical Cl^- channel blockers, such as DIDS and niflumic acid [320]. These concentrations are, however, somewhat different from the sensitivities of native $I_{Cl(Ca)}$. Thus, in many tissues the sensitivity to DIDS and niflumic acid was shown to be significantly higher or lower [15;116;241;247;276]. Interestingly, measuring Cl^- conductance as I^- influx, Caputo and co-authors reported sensitivity of ANO1 to niflumic acid much closer to endogenous $I_{Cl(Ca)}$ sensitivity [321]. Finally, Schroeder and co-authors reported the concentrations inhibiting half of the ANO1-associated Cl^- current to be ~30 µM for niflumic acid and ~24 µM for DIDS [322], which again is consistent with previous reports [15;116;241].
- The Cl^- current associated with ANO1 is sensitive to $[Ca^{2+}]_i$, although $[Ca^{2+}]_i$ inducing half-maximal activation (2.6 µM) is higher [320] than that described for many native $I_{Cl(Ca)}$ [15;241;247]. Schroeder and colleagues also evoked the ANO1-associated $I_{Cl(Ca)}$ in oocytes permeabilized with a Ca^{2+}-ionophore by increasing extracellular Ca^{2+} to 5 mM but detailed characterization of the Ca^{2+} sensitivity was not done [322]. In contrast, other groups have showed a significant activation of the ANO1-associated $I_{Cl(Ca)}$ already at 0.2-0.6 µM $[Ca^{2+}]_i$, although the complete concentration-response study has not been performed [319;321;322]. Nevertheless, native $I_{Cl(Ca)}$ with half-maximal activation by $[Ca^{2+}]_i$ >1 µM were reported previously as well as current activated half maximally at ~0.3-0.6 µM $[Ca^{2+}]_i$ [15;241;247]. In addition, similar to the native $I_{Cl(Ca)}$, the Ca^{2+}-sensitivity of the ANO1 current was voltage-dependent and the current was inhibited at extreme (>10 µM) $[Ca^{2+}]_i$ [320].
- Mutagenesis analyses identified the potential protein region which forms the channel pore. Mutations in the putative ion selectivity filter or agents modifying sulfhydryl groups affected ion conductance [320;321].
- $I_{Cl(Ca)}$ was significantly reduced after downregulation of ANO1 expression with siRNA [320;321].
- ANO1 expression was shown in the tissues, such as salivary and submandibulary glands, pancreas, lungs, airway epithelia, kidney, retina and neurons, where the $I_{Cl(Ca)}$ is important [320-322]. Expression of ANO1 was shown to be associated with changes in the organ function. However, the downregulation of ANO1 with siRNA suppressed only part of secretory function (<50%). These points out that ANO1 might not be the only protein mediating a Cl^- conductance in the same tissue.

Thus, although ANO1 is the best candidate for the classical $I_{Cl(Ca)}$ some inconsistencies are present. Currently, there are only few publications which characterize ANO1 as a Cl^- channel in detail [320-322]. Some differences between these studies and between described ANO1-associated Cl^- current and native $I_{Cl(Ca)}$ could be due to differences in clones used in these studies (xenopus vs. human cDNA) and differences in the expression systems. There are, however, some critical points, which cannot be explained by differences in the experimental setup.

- Among 10 mammalian members of the ANO gene family, ANO7 was suggested to have a transcript of soluble protein without transmembrane domains [323]. This raises the question whether all family members belong to transmembrane channel proteins or they serve entirely different functions.
- ANO proteins do not have apparent Ca^{2+}-binding sites and it is unclear how this channel can be activated by Ca^{2+} [247].

Bestrophins

After ANO1 has been suggested to be the classical CaCCs [320-322], the previous favorite, bestrophin [324-326], did not lose its interest. The first member of the bestrophin family, Best-1, was identified more than 10 years ago as a gene responsible for vitelliform macular dystrophy (VMD, Best's disease) [327] but its cellular function remained unclear for some time. Four members of the bestrophin family were identified in the mammalian genome and many homologous in genomes of invertebrates and even prokaryotes [328-330]. Previously two parallel nomenclatures had been developed: one based on the VMD gene abbreviation –VMD2, VMD2-like1, VMD2-like3 and VMD2-like2; another is Best-1, Best-2, Best-3 and Best-4, respectively. It is confusing that the VMD2-like3 gene corresponds to Best-3, while VMD2-like2 is Best-4. This is because previously VMD2-like2 (Best-4) was suggested to be a pseudo-gene in mouse [331], although this seems rather unique for

the mouse genome and is not the case for other species (V). Recently, a 'Best' nomenclature was suggested for uniform use by the HUGO and the Mouse Genome Database [330;332].

Bestrophins were previously suggested to be secondary active transport proteins [333] or proteins associated with the Ca^{2+} channels [334-336]. However, based on the protein homology in the ion selectivity filters to ligand-gated anion channels such as the GABA and glycine receptors, bestrophin was cloned as a putative Cl^- channel [324]. Heterologous expression of bestrophins produced the $I_{Cl(Ca)}$ and several lines of evidence suggested that bestrophins are bona fide Cl^- channels [325;326;337-339].

- Bestrophins expressed in different cell types produce a similar $I_{Cl(Ca)}$ [324;325]. If overexpression induced upregulation of endogenous current, one should not expect the same kind of current in different cell types. Different bestrophin isoforms produce Cl^- currents with different characteristics, even when expressed in the same type of cells [324;337].
- Co-immunoprecipitation revealed that bestrophins form multimeric complexes composed of 4-5 subunits, as one would expect for an ion channel [325]. Bestrophin proteins have been predicted to have several transmembrane domains in plasma membrane and this has been confirmed by biotinylation experiments [334;337;340-343].
- Point mutations and sulfhydryl group modifications within a putative pore domain modified the bestrophin-associated $I_{Cl(Ca)}$ providing strong argument for the channel structure [326;344].
- Bestrophins are quite sensitive to $[Ca^{2+}]_i$, with half-maximal activation about 200 nM. Thus, they can be activated by slight changes in physiological $[Ca^{2+}]_i$ [324;337;345]. Bestrophin proteins contain domains which can be responsible for $[Ca^{2+}]_i$ sensitivity, possibly both via direct Ca^{2+} action and/or via Ca^{2+}-dependent phosphorylation [345-348]. The predicted Ca^{2+}/CaMKII sites are, however, of low stringency [347].
- Bestrophins have been shown to respond with Cl^- currents to G-protein-coupled receptor stimulation [328;349-351].
- Knockdown of bestrophins with siRNA abolishes the endogenous $I_{Cl(Ca)}$ in cell cultures and in the intact tissue (V and [352;353]).
- Mutations in Best-1, which affect the $I_{Cl(Ca)}$, have been shown to be associated with several ophthalmological conditions, first of all with vitelliform macular dystrophy [332].

In light of the variability of the $I_{Cl(Ca)}$, it is likely that ANO and bestrophins are two different families of proteins forming the pore of a CaCCs [319;332]. The exact separation of the $I_{Cl(Ca)}$ between these two protein families remains to be elucidated. Currently, bestrophins have as many arguments as ANO proteins in favor of being the CaCCs, but possibly due to longer research story bestrophins have accumulated a number of concerns regarding their function.

- The heterologously expressed bestrophins have different current characteristics compared to the native $I_{Cl(Ca)}$ [324;325;337;338;343;344;347;354;355]. This difference could be explained by the importance of a multimeric structure with different isoforms or by lack of relevant subunits in the heterologous systems.
- The observation that a large fraction of heterologously expressed bestrophin appeared intracellularly contradicts its function as a membrane channel [324;328;350]. However, the antibodies raised against bestrophins are very poorly characterized. In addition, the experiments with biotinylation contradicts this finding [334;337;340-343].
- There is no detailed study of single channel conductances of the bestrophin-associated Cl^- current. Drosophila ortologs of bestrophin-1 have 2 pS conductance [355]. The only know conductance of mammalian bestrophin, mouse bestrophin-2, is surprisingly small (~0.3 pS), suggesting that other regulatory subunits were lacking in the host cell of the overexpression model [348].
- siRNA-induced knockdown eliminates the $I_{Cl(Ca)}$, although a significant portion of the protein (~50 %) is still present (V and [350]). Whether this represents the specific protein turn-over in respect to the transient siRNA-induced mRNA degradation remains unknown. It is also unknown why all the current disappears while there is still 50% of protein.
- Overexpression of bestrophins affects endogenous Ca^{2+} channels [334;336] suggesting a regulatory function of bestrophins in line with CFTR which function both as a channel and as a regulatory protein [356].
- Finally, Best1 knockout mice do not have macular dystrophy [335]. It has been suggested that VMD is caused by gain-of-function mutations in Best1, although the species difference might also be relevant.

Bestrophin-3 properties are close to the $I_{Cl(Ca,cGMP)}$ characteristics (Papers IV, V)

Detailed comparison of biophysical and pharmacological properties of the $I_{Cl(Ca,cGMP)}$ (III-V and [121;220]) with the known characteristics of the bestrophin-associated Cl^- currents [324;337;338;342-344;347;348;350;352-355] reveals many similarities [15;241;247] but also some differences can be seen. The comparison, however, is partially complicated by lack of a comprehensive study of each bestrophin isoform [332] and because some of the characteristics are different not only between isoforms but also between different splice variants and between species. Thus, Best-3 is widely variable, at least 5-6 different splice variants have been identified and their distribution varies between tissues [357;358]. In addition, it is important to remember, that most of the characteristics are obtained by heterologous expression. Thus, presence or absence of regulatory proteins in the host cells can be critical for the observed functions.

The comparisons I have made do not point to one of the isoforms as the best candidate for the cGMP-dependent CaCC. We found, however, interestingly that Best-3 is the most variable and broadly expressed family member, which is potentially under strong intracellular regulation [331;357;358]. Several regulatory sites were found on the Best-3 C-terminal [343;345;354]. We found previously sites for phosphorylation by protein kinase G, predominantly in the C-terminal (V).

Using sets of specific primers, we analyzed SMCs of different origin for the expression of bestrophins in the rat (V). The expression of all four bestrophins was seen at the mRNA level. This is a surprising result since Best-4 was previously suggested to be a pseudogene in rodents based on the presence of premature stop codons in the mouse genomic cDNA [357]. However, the PCR product (~200 bases) was verified by sequencing, although the entire open reading frame has not been obtained (unpublished). Best-3 showed the strongest expression of the four bestrophins in the mesenteric small arteries between other family members (V). A Best-3 band was detected in the rat mesenteric small arteries, aorta and A7r5 cells but only insignificant expression was seen in the pulmonary arteries.

As it can be seen from Table 1, the tissue distribution of the bestrophins is still controversial due to the problems of alternative

splicing [357;358] and lack of well-characterized antibodies. Even in well-studied tissues such as the eye and some epithelia there is poor correspondence between results obtained using different techniques and in different laboratories [332]. We characterized a commercially available antibody to Best-3 by expressing immunizing peptide fused to eGFP expressing plasmid (V). Recently, the specificity of this Best-3 antibody was supported by use of antibody against another epitope on the Best-3 protein (gift from prof. Kunzelmann, unpublished). Western blot identified the expression profile seen in RT-PCR of SMCs of different origin. No Best-3 protein was detected in the pulmonary arteries (V) (Fig. 7). This profile of rBest-3 expression is similar to distribution of $I_{Cl(Ca,cGMP)}$ (III and IV) suggesting that rBest-3 might be associated with this current.

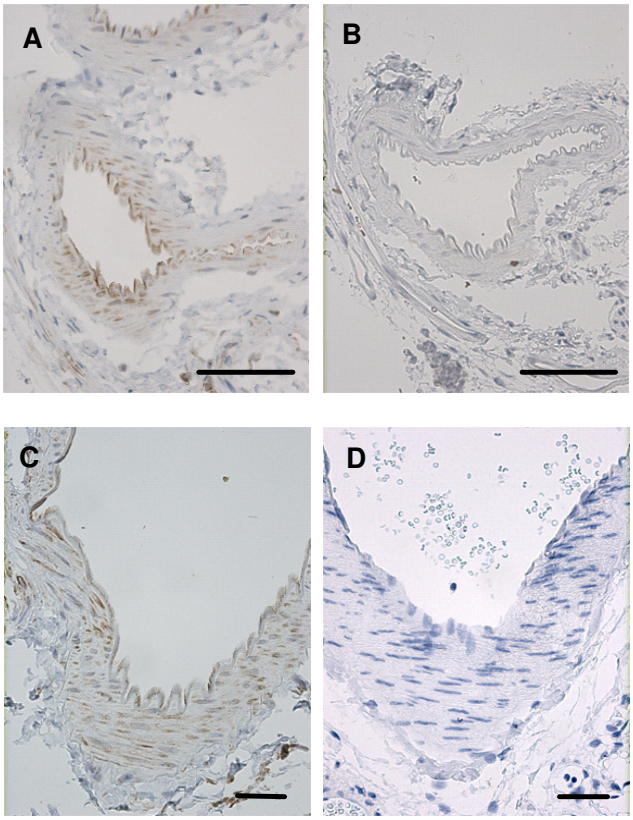

Figure 7
Bestrophin-3 expression was identified immunohistochemically in several arteries. **Panel A** shows staining of rat mesenteric small artery with bestrophin-3 antibody, **panel B** the same artery stained with bestrophin-3 antibody preincubated with immunizing peptide. **Panel C** demonstrated expression of bestrophin-3 in the kidney artery and **panel D** shows absence of bestrophin-3 expression in the pulmonary artery. Bars represent 50 µm.

Bestrophin-3 is associated with the $I_{Cl(Ca,cGMP)}$ in SMCs (Papers V)

To address the question of whether the $I_{Cl(Ca,cGMP)}$ is associated with Best-3, we used small interference RNA (siRNA). Since siRNA can have several off-target and non-specific effects [359;360], we performed several controls to ensure the specificity of siRNA action (V). Only the specific siRNAs had effect on the mRNA and protein expression in cultured aortic SMCs A7r5 and in the rat mesenteric small arteries (V). The degree of downregulation was not, however, consistent between mRNA and protein: 80% reduction mRNA was accompanied with only 55% of protein reduction. We do not have an explanation for this, although it could be due to different time-scales for mRNA and protein degradations. Interestingly, a similar tendency was seen when Best-1 was downregulated in colonic epithelial cells [350].

In accordance with our previous findings (III and IV), SMCs expressed two Ca^{2+}-activated Cl^- currents: the classical $I_{Cl(Ca)}$ and the $I_{Cl(Ca,cGMP)}$. Downregulation of Best-3 specifically suppressed the $I_{Cl(Ca,cGMP)}$ without affecting the classical $I_{Cl(Ca)}$ (V). These experiments strongly suggest a direct association between Best-3 expression and the $I_{Cl(Ca,cGMP)}$. Interestingly, cGMP-dependence was previously shown only for human Best-1 [352;361]. In contrast, the mouse Best-3 variant expressed in the heart was not shown to be cGMP-dependent [362]. Whether this is due to tissue-specific differences or due to species variability remains to be determined. Importantly, the mouse Best-3 variant from heart expressed heterologously produced $I_{Cl(Ca)}$ of significant amplitude under whole-cell configuration [358;362]. This is contrasted by data from Hartzell and colleagues who showed that expression of the full-length clone of mouse Best-3 does not produce any significant Cl^- current consequent to autoinhibition by the C-terminal [343;354]. This autoinhibitory cytoplasmic C-region contains several sites for phosphorylation (including sites for PKG-dependent phosphorylation) and mouse Best-3 with deleted C-terminal produced a large Cl^- current.

Several other groups have recently used siRNA technique to prove association between the bestrophin proteins and the $I_{Cl(Ca)}$. This was, however, only done in cell cultures [350;351;353;355;361;363]. We are the first who showed that Best-3 expression can be downregulated *in vivo* and this is accompanied with loss of a specific Cl^- current - the $I_{Cl(Ca,cGMP)}$ (V). Whether the cGMP-dependency is specific for the splice variant expressed in the rat SMCs or is a general characteristic of Best-3 remains to be studied.

Table 1. **Comparison of the characteristics of different bestrophin isoforms and the $I_{Cl(Ca,cGMP)}$. mBest - mouse ortolog; hBest - human ortolog; dmBest - *Drosophila melanogaster* ortolog; ceBest - *Caenorhabditis elegans* ortolog gene.**

	$I_{Cl(cGMP-Ca)}$	**Bestrophin-1 (VMD2)**	**Bestrophin-2 (VMD2-like1)**	**Bestrophin-3 (VMD2-like3)**	**Bestrophin-4 (VMD2-like2)**
Cl^- conductance	Yes (**III** and **V**)	Yes [325;326;336;339;347;351-353;363;364]	Yes [324;325;337;339;344;351;364]	Yes [326;354;358;362;364]	Yes [326;347;364]
Halide permeability	$Br^- > I^- > Cl^-$ (**III**) or $Cl^- > I^-$ [121]	$I^- > Br^- > Cl^-$ [325;341;342;349]	$I^- > Br^- > Cl^-$ [324;337;338;348]	$I^- > Cl^-$ [362] or $I^- > Br^- > Cl^-$ [354]	not tested
cGMP-dependence	activation; EC_{50}≈3-6 μM (**III**, **V** and [121])	activation[352;361]; possibly explain current run-down in whole cell[325]	not tested	no effect in the mouse heart [362]	probably, no effect [347]
Ca^{2+}-sensitivity	EC_{50}≈75 nM [121]	activation [325;339;341;349;352;355;363] EC_{30}≈140nM [341;345]; inhibition >1μM [351]	activation EC_{50}≈200-400 nM [324;337;339;348;349;351]; inhibition at >1μM [351],	activation [349;354;358] EC_{50}≈200 nM [362]	EC_{50}≈200 nM [347]
Action of $[Ca^{2+}]_i$	directly, without Ca^{2+}/CaMKII [220]	binding to the CaM-like motif [345], might be phosphorylation [355]	probably, direct action [347;348]	probably, direct action [347]	probably, direct action [347;348]
Conductance block with SCN^-	Yes (**III**)	No [341;342;355]	Yes [337;338;344]	No (for mBest-3) [354;362]	not tested
Voltage-dependence	No (**III**-**V** and [121])	No (mBest-1 [351]); Small (hBest-1 [325;326;336;339;341;342;350;353;355;364] and dmBest-1 [325;363]); Inward rectification (ceBest-1 [325;326])	No (xBest-2 [324], mBest-2 [337;338;344;348;351;358;364], hBest-2 [325;339]); small (hBest-2 [364] and dmBest-2 [355])	No (mBest-3 [343;354;358;362] and hBest-3 [364]); Inward rectification (hBest-3 [326] and mBest-3 [354])	Small (hBest-4 [326;364]); Inward rectification (hBest-4 [354])
Sensitivity to niflumic acid	No effect of 100 μM; 100% inhibition by 1 mM (**III** and [121])	~50% inhibition by 100 μM [350;353]	EC_{50}≈10 μM [348]	~65% inhibition by 100 μM (mouse) [348]	not tested
Sensitivity to DIDS	No effect of 200 μM; 80% inhibition by 1 mM (**III**)	60-80% inhibition by 100 μM [325;350-353;361]	EC_{50}≈3 - 100 μM, voltage-dependent block [337;338;351]	~70% inhibition by 100 μM (mouse) [358;362]	not tested
Sensitivity to Zn^{2+}	EC_{50}≈2-6 μM (**III**-**V** and [121])	No [361]	not tested	not tested	not tested
Cell localization	membrane current (**III**-**V** [121;220])	Cell membrane[325;341;342], many intracellular [326;328;339;349]	cell membrane (partially) [324;328;337;348;349]	cell membrane (partially) [326;343;362]	cell membrane (partially) [326;349]
Tissue expression	vascular and colonic SMCs (**IV**)	RPE; lung; submucosal glands; endothelium; SMCs; neurons; airways, colonic and kidney epithelia; heart; testis [331;348;350-352;362;365]	RPE; retina; lung; gut; liver; spleen; airways and olfactory epithelia [324;331;348;350;351;362]	(≥5 splice variants) heart; testis; exocrine glands; kidney; lung; liver; pancreas; spleen; lymph node; brain; intestine; skeletal muscles [331;348;350;358;362]	colon [329;331;363]

Bestrophin-3 is important for vasomotion.

Although we have shown that Best-3 is expressed in rat mesenteric small arteries and is associated with the $I_{Cl(Ca,cGMP)}$ (V), the important question remains to be answered: what is the function of this Best-3-associated Cl^- current? Since our essential interest for the $I_{Cl(Ca,cGMP)}$ arises from the model of vasomotion (I), we hypothesized that this channel might be important for synchronization of SMCs by membrane potential changes [46;47]. This suggests a Cl^- dependence of vasomotion. Indeed, this was the case [87]. Substitution of Cl^- with impermeable anions abolished vasomotion in the mesenteric small arteries, consequent with a critical role of membrane potential for the global synchronized oscillations in the vascular wall. Thiocyanate was previously shown to inhibit the $I_{Cl(Ca,cGMP)}$ (III) and we demonstrated later that SCN^- abolished vasomotion by stopping oscillations in membrane potential and desynchronizing $[Ca^{2+}]_i$ oscillation [87]. Surprisingly, we have also seen inhibition of the classical $I_{Cl(Ca)}$ in the inward direction by SCN^-, which is in contrast to previous reports showing stimulation of the $I_{Cl(Ca)}$ with full substitution of Cl^- by SCN^- [366-368]. This inconsistence prevents us from distinguishing the importance of the two Ca^{2+}-activated Cl^- conductances for vasomotion. The use of the Cl^- conductance blockers, DIDS and Zn^{2+}, in the myograph experiments did not make the picture more clear [87]. We expected that these blockers would inhibit one or both types of

the Ca^{2+}-activated Cl^- currents, but instead vasomotion changed properties and became endothelium-independent. This endothelium-independent vasomotion was still inhibited by SCN^- suggesting an importance of a Cl^- conductance [87]. Other Cl^- conductance blockers can not be used because of their unspecific inhibitory effect on the vessel contractility. Thus, pharmacological tests and anion substitutions demonstrated the importance of Ca^{2+}-activated Cl^- conductance for the generation of vasomotion [87].
The lack of specific tools makes it, however, impossible to differentiate between the role of the classic $I_{Cl(Ca)}$ and the $I_{Cl(Ca,cGMP)}$. One of the ways to differentiate between the two Ca^{2+}-activated Cl^- conductances will be a specific removal of the channel from cell membrane in situ [369]. We reported previously the successful downregulation of Best-3 in the mesenteric small arteries *in vivo* which was accompanied with significant inhibition of the $I_{Cl(Ca,cGMP)}$ (V). When arteries, downregulated for Best-3, were tested in an isometric myograph (unpublished), no change in contractility was seen. However, the arteries with downregulated Best-3 had significantly suppressed amplitude of vasomotion without changes in oscillation frequency. Vasomotion suppressed by Best-3 downregulation was endothelium-dependent and addition of 8Br-cGMP to the endothelium-denuded arteries reproduced vasomotion, but still with a significantly lower amplitude. These unpublished results are consistent with our initial model for the generation of vasomotion [46;47].

7. INTERCELLULAR COMMUNICATION SYNCHRONIZES SMOOTH MUSCLE CELLS IN THE VASCULAR WALL (PAPERS VI AND VII)

Gap junctions

Gap junctional communication, in contrast to the chemical synapse, involves direct exchange of information between adjacent cells [370]. Cell-to-cell communication through gap junctions (GJ) exists in most animal cells and is essential for many important biological processes where synchronization of cells is important including rapid transmission of electric signals to coordinate contraction of cardiac and smooth muscle and the intercellular propagation of Ca^{2+} waves within a tissue.
GJs consist of two hemichannels, aggregates of six transmembrane connexin proteins, that dock to each other forming a channel between the adjacent cells [157;158;371;372]. Recombinant gap junction channels revealed that there is a tight seal between the two hemichannels [373;374]. The wall of each hemichannel creates an approximately 2 nm-diameter aqueous pore that allows diffusion of molecules of about 1 kDa between the cytoplasm of adjacent cells [375;376]. The commonly used nomenclature distinguishes connexins (Cx's) by their molecular mass deduced from their respective cDNAs (www.genenames.org/genefamily/gj.php).
Hemichannels are homomeric, when composed from a single Cx type, or heteromeric, when they contain different Cx's. Furthermore, homotypic and heterotypic GJ channels were shown to be formed depending on the types of hemichannels in two interacting cells [157;158;377]. Given that at least 24 mammalian Cx's have been characterized to date, a number of structurally and, thus, potentially physiologically distinct GJ channels may be formed to provide a diversity of intercellular communication. The diversity of Cx combinations confers distinct regulatory and biophysical properties to GJ channels [377]. Heteromeric hemichannels and heterotypic GJ channels have been shown in expression systems and in some cell cultures [378-381] but whether they are present *in vivo* remains unknown. Some electron microscope studies suggested the presence of heteromeric hemichannels and heterotypic GJ between endothelial cells [382-384] but, unfortunately, these studies do not differentiate between real mixtures of Cx types in one truly heteromeric GJ channel and different homotypic GJ channels mixed in one GJ plaque. The potential heterogeneity in the GJ channels could have functional importance, for example to provide the base for asymmetrical transfer of dyes between endothelial and smooth muscle cells [385].
For technical reasons, most biophysical properties of intercellular communication are studied on homotypic GJ channels demonstrating symmetric properties. These properties are, however, quite different depending on the type of Cx's [377]. In general, all homotypic GJ channels display voltage sensitivity. But Cx37 is more sensitive to the transjunctional voltage than Cx43 [379;386]. Cx40 is shown to have intermediate voltage sensitivity [387]. For these "vascular" Cx's the transjunctional voltage providing half-maximal conductance is between 30 and 80 mV suggesting that the voltage sensitivity of GJ channels is not a major determinant of the dynamic changes in intercellular coupling under physiological conditions. The exception for the vascular Cx's is Cx45 which has half maximal conductance already at 20 mV transjunctional potential [387]. When a transjunctional voltage is applied, the initial current decays with time to a steady-state level, indicating a significant time-dependence of the conductance [388]. Importantly, although the voltages modifying the GJ conductance are outside the usual physiological range, this can be modified by intracellular second messengers and ligands [389]. Interestingly, Cx's have a broad spectrum of unitary conductances, which suggests different functions. Thus, for the 'vascular' Cx's the sequence Cx37>Cx40>Cx43>Cx45 (from ~400 pS to ~25 pS) is suggested [377]. This makes Cx37 and Cx40 the more attractive candidates to mediate the conductive vascular responses [390]. Whether this suggests an involvement of the charge selectivity filter remains to be studied. Nevertheless, it has been shown that Cx43 forms rather non-selective ion pores while Cx40 and Cx45 have some anion selectivity [391;392] and, furthermore, the selectivity of Cx37 depends nonlinearly on ion concentrations [393]. Although the biophysical properties of GJ formed by different Cx's are quite variable and could suggest different functional properties they, probably, represent the real endogenous properties only to a certain extent.
Of special physiological interest is the permeability of GJ channels to such important ions as H^+ and Ca^{2+}. Both of these ions were shown in high concentration to close GJ channels but could permeate at low, physiological concentrations [394;395]. There is evidence that Ca^{2+} and H^+ can affect the GJ gating synergistically but other studies suggest independent action of these ions [396]. The following order of pH sensitivity for Cx's was suggested (with pKa~7) Cx45>Cx37>Cx43>Cx40 [397] which is an intrinsic property of the GJ hemichannels [398]. Some data suggest that low intracellular pH affects gating via an increase in $[Ca^{2+}]_i$ which in turn modulates the GJ gating by activation of calmodulin [399;400]. Although the question regarding Ca^{2+} and H^+ sensitivity is still open, it is clear that the sensitivity is dependent on the Cx composition of the GJ channel. Thus, Ca^{2+} blocks Cx43-formed GJ channels in a concentration-dependent manner in the range from 150 to 600 nM [401]. The inhibition was later shown to be dependent on calmodulin but not on CaMK II, with a half-maximal inhibition at 310 nM Ca^{2+} which makes Ca^{2+} a potent regulator at physiological conditions [402].
In addition to mediating electrical continuity, GJ channels are also known to be permeable to a variety of cytoplasmic molecules [389]. Being aqueous intercellular channels, GJ provide partial cytoplasmic continuity between cells. GJ channels were shown to be

freely permeable to second messenger molecules. Thus, hemichannels formed of Cx43 and Cx43 homotypic GJ channels have been shown to be permeable to ATP and ADP, cAMP, adenosine, IP_3 and Ca^{2+} [403-405]. Interestingly, even siRNA molecules were shown to be transferred through the GJ [406]. The general view that large channels, such as Cx's, act as molecular sieves and discriminate between molecules by their size, contradicts with the simple comparison of Cx's pore diameters and permeabilities of uncharged molecules [389]. Thus, the permeability cannot be accounted by the permeating molecule's size and some affinity of the relevant molecules to the GJ channel pore is likely [407]. It is important that the same second messengers, which permeate through GJ channels, have been shown to modulate intercellular communication between coupled cells [408]. Thus, it remains unclear whether this permeability has significance for organ function. Several factors, such as a short lifetime of second messengers and their small concentration gradient, suggest that they would not diffuse rapidly between the cells [389]. Some publications suggest that oscillatory changes in signal molecule concentration (primarily Ca^{2+}) observed in coupled cells occur due to spread of the oscillatory signal through GJ channels (I and [85;409;410]) and although diffusion of signaling molecules can be involved in the synchronization of some slow processes, spread through GJ's of an electrical signal seems to be the best candidate (see chapter about vasomotion), at least in vascular tissue (I).

Intercellular communication in the vascular wall

Communication between cells is important for coordination of cellular behavior in the vessel wall. Cx's have a major influence on vascular function and are key factors in integration and regulation of intercellular communication. Signaling via GJ channels spreads along the same type of cells in the vascular wall (homocellular coupling of either SMCs or endothelial cells) or from one cell layer to another (heterocellular coupling between SMCs and endothelial cells). There is evidence for both types of signaling [159;182;411]. While longitudinal, homocellular signaling is important for vascular conductive responses playing a role in blood flow redistribution [182], heterocellular myoendothelial gap junctions are suggested to be responsible for an endothelium-derived hyperpolarizing "factor" (EDHF) [412;413]. Also regular oscillations of vascular tone or diameter – vasomotion (I, VI and [85]) – require intercellular communication, and GJ channels are of key importance for these.

At least three type of Cx's (Cx37, 40 and 43) have been identified in the vasculature (VII and [159;182;414;415]). Although Cx45 has been suggested to be expressed in the vasculature [414;416-419] this is quite controversial [420], and it should be noted here that some Cx45 antibodies have been shown to cross-react with Cx43 protein [421]. Although only a limited number of Cx subtypes are expressed in the vasculature, their distribution varies through the circulation and between different cell types, as well as between species [158;159;182;414;422]. The mapping of Cx in the vascular wall is difficult and is mostly studied using specific antibodies. It should also be noted that immunohistochemistry is not sufficient and immunoelectron microscopy is the only definitive method available to demonstrate Cx's at morphologically defined gap junctions [383;384]. Identification of expressed Cx can be affected by plaque size [382;384]; the larger the number of colocalized GJ channels the more visible they will be on staining with an antibody. Indeed, big and numerous GJ plaques between vascular endothelial cells are often described [83;414] but the GJ between SMCs appear as single channels or in smaller plaques [422-424]. Therefore, GJ channels between SMCs are rarely identified on a morphological basis and information about Cx's in SMCs is relatively limited compared to the extensive and varied Cx expression in endothelial cells of different vessels [182;414;420;425].

A pattern of Cx distribution can be suggested, at least for larger vessels [384;414;426]. Thus, Cx40 is mostly found in the endothelium, while its appearance in SMCs is rarely reported. Cx37 is found to be more uniformly distributed (VII and [426-428]). The distribution and prevalence of Cx43 has been most extensively studied. Cx43 is expressed in both endothelial and SMCs and is the most abundant vascular Cx [418]. The expression of Cx43 falls in endothelium and disappears in the media with reduction of arterial size along different vascular beds [429] including rat mesenteric (VII and [422]) and renal circulations [430;431]. Similar observations made with mouse Cx40 [432] suggests that this is a general phenomenon for Cx distribution with the vascular size [422;426].

The suggestion that endothelial cells are more extensively linked via GJ channels than the accompanying SMCs [433;434] is supported by functional data showing that the vascular cells do not communicate electrically to a similar extent. It has been suggested that the endothelial layer may provide a more efficient pathway for a longitudinal electrical signal because of the anatomical shape and the orientation of a single cell [435]. The intensity of functional intercellular coupling is difficult to evaluate using dye transfer technique. Nevertheless, dye movement was seen between both SMCs and endothelial cells in most studies [424;436], whereas in some reports it was limited to endothelial or myoendothelial GJ channels [71;385;385;437]. Coupling between SMCs has been directly confirmed by measurements of electrical conducted responses along endothelium denuded arteries [427;438], although the importance of SMC homocellular GJ channels for the conducted response is questioned and probably depends on the type of stimulus [439-441].

Searching for specific inhibitors of intracellular communications (Papers VI and VII)

Drugs specifically affecting GJ conductance are essential for demonstration of a functional role of intercellular communication. Although a number of different compounds have been suggested to have a specific action on GJ channels, the effects of most of them are questioned. GJ channels are regulated at many levels including conductance, trafficking, synthesis and downregulation. On this basis many drugs and compounds have been suggested to modulate the GJ activity [372;408;442-445]. Thus, drugs affecting the intracellular ion concentration, e.g. Na^+, Ca^{2+}, H^+ and Mg^{2+}, have been shown to modulate GJ conductance [445;446]. Inhibition of the Na^+/Ca^{2+} exchanger or Na^+, K^+-pump has been suggested to act via ionic changes on GJ conductance (VIII and [447;448]). Since endogenous amines, e.g. histamine, adrenaline and noradrenaline were also shown to modulate GJ conductivity via regulatory pathways, drugs acting on the cAMP- and cGMP-activated protein kinases, protein kinases C, mitogen-activated protein kinase, tyrosine kinase and others can potentially modulate gating of GJ channels [449-451]. Although these types of drugs are broadly used in the research of modulation of intercellular communication, it is obvious that they have no specificity and, therefore, are not always useful tools in studies of GJ's.

Several other groups of chemicals are suggested to have direct action of the GJ channels, possibly without involvement of complicated regulatory pathways. A group of lypophylic drugs includes the inhalation narcotics halothane and isoflurane, as well as long-chain alcohols such as heptanol and ochtanol, and a num-

ber of organic acids and their derivates: glycyrrhetinic, myristoleic, decaenoic and palmitoleic acids [408;446]. The action of these lypophylic drugs is explained by their incorporation into lipid bilayers leading to impairment of GJ conductance. Thus, heptanol has been reported to reduce coupling by reducing the open probability of GJ channels through conformational changes induced by the interaction between connexin and plasma membrane [452;453]. The inhibitory action of heptanol (KD ~160 μM) was shown to be reversible [454]. Heptanol is commonly used to block intercellular communication in the vasculature [455-457], in spite of a considerable number of reports questioning the specificity of its action at physiologically relevant concentrations [113;186;458-460]. For this reason, we studied in detail the action of heptanol in the mesenteric small arteries (VI). We found that a concentration of heptanol (150 μM), which has insignificant effect on intercellular communication, increases K^+ membrane conductance, induces hyperpolarization, directly inhibits the Ca^{2+} current, reduces $[Ca^{2+}]_i$ and lowers tension development. Only at concentrations above 200 μM heptanol significantly affected intercellular communication between SMCs but also depolarized SMCs (VI). Consistent with the previous reports we found both heptanol-induced depolarization [461] and hyperpolarization [127], and suggested that nonjunctional effects of heptanol depended on both heptanol concentration and the vasculature type.

Also derivatives of glycyrrhetinic acid, 18α- and 18β-glycyrrhetinic acid, which are commonly used to block GJ's have been suggested to have nonjunctional effects [462;463;463-466]. 18α-glycyrrhetinic acid has been used as a gap junction inhibitor [467;468] in concentrations of ~50 μM [469] and 18β-glycyrrhetinic acid has been suggested to be 10 times more potent [470]. We have also found that 18β-glycyrrhetinic acid at micromolar concentrations inhibited intercellular communication (VI), similar to previous reports [113;372;471]. 18β-glycyrrhetinic acid has, however, several nonjunctional effects; it depolarizes the membrane, reduces $[Ca^{2+}]_i$ and relaxes the arteries (VI). This is consistent with observations made by other researchers [463;466;472]. Effects of heptanol and 18β-glycyrrhetinic acid on membrane conductance could be explained by a lypophylic action of these drugs independent of their effect on intercellular communication since it was also seen under single cell conditions (VI). In contrast to previous reports [127;465;473], we did not find any significant nonjunctional effects of 18α-glycyrrhetinic acid on SMCs (VI). We suggested, however, the 18α-glycyrrhetinic acid acts specifically on the myoendothelial GJ, similar to previous studies [83;467;474].

Another approach to block GJ channels is the use of peptides that mimic the extracellular loops of Cx's [375]. The synthetic connexin-mimetic peptides (GAP) are peptide fragments corresponding to short sequences of one of the extracellular loops of Cx's [475]. It was found that sequences of 10 to 14 amino acids are the most efficient to specifically inhibit intercellular communication while longer peptides show less efficiency and specificity [476]. Despite GAP peptides having been intensively used in the functional studies of GJ communication, the mechanism underlying their action remains unresolved. It has been shown that GAP peptides do not affect expression, synthesis and de novo formation of GJ channels [477-479]. This suggests that GAP peptides should act directly on the GJ channel. Although prolonged exposure with GAP peptides was shown to disturb docking of GJ channel formed from Cx40 and Cx43 in lymphocytes [480;481], this is a slow process which is unlikely responsible for a quick (within minutes) functional effects of GAP peptides [479]. One of the fast responses, which have been suggested to be involved in the GAP peptide action, is an assembly/disassembly of connexons into GJ channels. GAP peptides could disturb the docking of two hemichannels either sterically or electrostatically. This could either prevent new docking of the GJ channels (Cx's have a rapid turnover [482]) or dissociate already coupled Cx's, leading to GJ channel breakdown. Alternatively, GAP peptides may directly interact with GJ channels and alter channel gating. It is important to notice here that different types of Cx's were suggested to dock each other to form heteromeric channels [158;378]. How GAP peptides preserve specificity in this situation remains unknown.

A number of functional reports support either one or another possible mechanism of GAP peptide action. Thus, GAP peptides inhibit transfer of fluorescent dye between cells [469;479], electrical communication (VII and [483]) and propagation of Ca2+ waves across groups of cells (VII and [484]), which is consistent with the specific blockade of intercellular communication. If GAP peptides uncouple already formed GJ channels, their effect will be seen immediately. This seemed dubious, however, since time was always needed to achieve the uncoupling effect [485]. Nevertheless, we reported previously that GAP peptides uncouple electrically coupled cultured SMCs within minutes and explained the longer time of action in the vascular wall (15 to 40 minutes) by the time necessary for diffusion of the big peptide molecules into the arterial media (VII). Another interesting observation in this study is that uncoupling by GAP peptides is accompanied with an increase in $[Ca^{2+}]_i$ and depolarization of SMCs. This cannot be just an unspecific effect, as was the case for 18β-glycyrrhetinic acid (VI), because GAP peptides do not affect membrane conductance of single SMCs (VII). It has been shown that under physiological conditions uncoupled GJ connexons are closed [486]. The docking of the extracellular loops of the Cx protein to extracellular loops of another Cx protein opens connexons and leads to GJ channel forming. It is, therefore, possible that GAP peptides mimic its action and by uncoupling of GJ channels create transiently open connexons on the cell membrane. These 'leaky' connexons close with time, possibly by some environmental stimuli, for example elevation of Ca^{2+} [486-488]. This hypothesis is not fully consistent with the observation that GAP peptides block ionic conductance through the single, "non-paired" connexons [485;489]. Although these experiments were made under special experimental conditions, which provoke opening of connexons, they suggest that GAP peptides could have some other actions on the connexons, possibly through a steric block of the channel, which is not specific to a certain type of connexins [485].

Unfortunately, the question regarding the specificity of GAP peptides is still a very complicated. Negative results are unfortunately not often published, although there are exceptions [85]. Several others refer to potential nonjunctional effects of GAP peptides [84;85;85;425]. It is, however, difficult to define functionally such nonjunctional effects, since some of them, e.g. depolarization or changes in $[Ca^{2+}]_i$, might be consequences of junctional actions of GAP peptides (VII). Finally, caution should be taken in the analyses and explanations of data received with GAP peptides, since their action depends not only on Cx expression, type of tissue etc., but also on experimental conditions, origin of the peptides, the way the peptides were cleaned and the buffers in which they were stored.

Functional consequences of SMCs uncoupling are consistent with the model for generation of vasomotion

In spite of the debate regarding the specificity of GAP peptides, they still remain the currently most specific compounds available for studies of GJ communication in the vascular wall. The GAP

peptides have been used to inhibit an EDHF response [84;85;412;425;490], a conducted response along the artery, and synchronization of SMCs in the vascular wall (VII and [85;113]).
We have used a triple combination of GAP peptides because three different types of Cx's were found expressed in the mesenteric small arteries (VII). Such 'combined' treatment was recently shown to be effective in hepatic arteries from rat [490]. Consistent with the previous findings [113] we have shown that GAP peptides stop vasomotion. A study of $[Ca^{2+}]_i$ dynamics indicated that this was not due to an effect on intracellular Ca^{2+} handling (VII). Consistent with the model for vasomotion (I), inhibition of intercellular communication abolished synchronization of SMCs, although asynchronous Ca^{2+} waves were still present in the SMCs. Also endothelium-dependent hyperpolarization was diminished in the presence of the GAP peptides suggesting inhibition of myoendothelial gap junctions (VII).

8. REGULATION OF INTERCELLULAR COMMUNICATION BY THE NA^+-PUMP (PAPERS VIII AND IX)

Modulatory protein interactions with connexins

Recent studies have shown that Cx's can have either direct or indirect interactions with other plasma membrane ion channels or membrane transport proteins with important functional consequences. Although the precise molecular nature of these interactions has yet to be defined, their consequences may be critical for normal tissue homeostasis. Thus, Cx43 and Cx45 have been suggested to be parts of a multiprotein complex containing CFTR [491] where Cx's are regulated by TNF-α via the tyrosine kinase c-Src [492]. Cx's have also been shown to interact with aquaporins: Cx45 and Cx56 are consistently collocalized with aquaporin-0 in lens epithelial cells [493], and Cx43 interacts functionally with aquaporin-4 in the mouse brain [494].
In the vasculature Cx's comprising GJ channels are recognized in close spatial association with small and intermediate conductance Ca^{2+}-activated K^+ channels (SK and IK) [84;495;496]. This interaction was suggested to be of specific functional importance for endothelium-dependent hyperpolarization [425]. Endothelial cells in the mesenteric small arteries are coupled by Cx37, Cx40 and Cx43. All three Cx type homocellular endothelial GJ channels were found spatially close to SK, while for myoendothelial GJ Cx37 was found associated with IK [84;495;496]. Interestingly, the SR and its IP3 channels were also found closely associated with vascular GJ channels [425]. Finally, the Na^+, K^+-ATPase was recently also shown to be colocalized with GJ channels [495]. Importantly, intercellular communication was reported to be reduced by blockers of the Na^+, K^+-ATPase (VIII and [54;66;497-500]).

Na^+, K^+-ATPase - membrane transporter and regulator of cell activity

The Na^+, K^+-ATPase, also called the Na^+-pump, can be dubbed "an enzyme of life" because of its essential role for cell life and death. The Na^+-pump is a ubiquitous membrane transport protein responsible for establishing and maintaining high K^+ and low Na^+ in the cytoplasm which is required for normal resting membrane potential. The ionic homeostasis maintained by the Na^+, K^+-ATPase is critical for numerous cellular functions and processes, such as cell growth, differentiation, movement, secretion and volume regulation. The list of the cellular tasks which are possible because of the Na^+-pump is constantly growing. Thus, recently a central role of the Na^+-pump for the different forms of cell death was recognized [501]. There is also a growing list of evidence for functions of the Na^+-pump as a signal transducer and activator of gene transcription [502;503]. Whether the pumping and signal transduction activities of the Na^+, K^+-ATPase are independent functions of the same protein molecules or they are integrative is a matter of debate.

Regulatory microdomains containing Na^+-pump in vascular SMCs.

Ouabain is a well-known specific inhibitor for Na^+-pump [504;505]. The dominant Na^+-pump in rodent SMCs is the α1 isoform, which is relatively ouabain-resistant [506]; micromolar concentrations of ouabain block only the ouabain-sensitive α2- and α3-isoforms [507], which are expressed at low levels in rat vasculature [508-510]. It is generally accepted that all living cells express at least α1 and one more isoform of the Na^+-pump [511-513]. Thus, skeletal, cardiac and smooth muscle cells co-express α1 and α2 isoforms, while neuronal tissues expresses α1 and α3 isoforms of the Na^+-pump. Similarly, although it has been found that renal epithelia expresses mostly the α1 isoform, the expression of the α2 isoform has recently been shown [514]. Specific functions of the different α isoforms are suggested by the findings that α2/α3 isoforms are localized in plasma membrane microdomains, i.e. spatially restricted areas in the plasma membrane [502;511;515].
These α2/α3 isoform/containing membrane microdomains were previously shown to be associated to the "junctional" sarcoplasmic/endoplasmic reticulum (SR/ER) and include the Na^+/Ca^{2+} exchanger (NCX) as the one of key players in the microdomain function [504]. The α1 isoform of the Na^+ pump, in contrast, is more widely and homogeneously distributed in the plasma membrane, but is apparently excluded from the microdomains [516]. The SR-associated microdomain was previously shown to constitute a spatially-restricted environment [65;78;517-519] under the plasma membrane that acts as a functional unit [520]. The restriction of Na^+ and Ca^{2+} diffusion enables the appearance of concentration gradients between these restricted spaces and the bulk cytosol [65;521]. Interestingly, the α2/α3 isoforms of the Na^+-pump have much lower affinities for Na^+ than the α1 isoform [522]. This suggests that different α isoforms will rise $[Na^+]_i$ more in restricted spaces controlled by α2/α3 isoforms of Na^+-pump than the global $[Na^+]_i$ which is under α1 isoform control. Thus, these α2/α3 Na^+-pump associated microdomains are well organized to control the local Na^+ electrochemical gradient which can influence Ca^{2+} homeostasis via the co-localized NCX isoform 1 [504;511]. This links cellular Ca^{2+} to Na^+ concentration; a spatially restricted rise in Na^+ will lead to a localized elevation of Ca^{2+}. Such interactions in ion metabolism do not only control local Ca^{2+} but also affect global $[Ca^{2+}]_i$ via modulation of the SR/ER load. This microdomain-regulated Ca^{2+} has many downstream signals, e.g. gene expression, cytoskeleton mobilization, membrane conductances, contraction and secretion.
The interaction described above explains some well-known cellular effects of ouabain, such as arrhythmias and decrease in conduction velocity in the heart [447;448;523;524] and SMC tone elevation [78;521;525;526]. Another type of ouabain-induced signaling independent of changes in intracellular ion concentrations was characterized recently in various cells, including SMCs and kidney epithelia [502;527-532]. In this pathway the Na^+, K^+-ATPase acts as a receptor for ouabain or endogenous ouabain-like compounds. Binding of ouabain induces activation of multiple signal transduction pathways, including the activation of Src kinase and tyrosine phosphorylation of the epidermal growth factor receptors and other

proteins, followed by the activation of Ras, the Ras/Raf/MEK/MAPK cascade, and increased production of reactive oxygen species [502;529-533].

The Na^+, K^+-ATPase is shown to be organized together with interacting proteins in a microdomain called signalosome [534-537]. These signalosomes have been shown to be restricted to caveolae [538]. Exposure to nanomolar ouabain increased binding of both Na^+, K^+-ATPase and Src kinase to the caveolae coating protein, caveolin-1 [530]. Moreover, the ouabain-bound Na^+, K^+-ATPase is able to recruit and assemble both Src and caveolin-1 into the signalosome. Thus, the Na^+, K^+-ATPase is also an important signal transducer that not only interacts and regulates protein kinases, but also functions as a scaffold, capable of bringing the receptor and effectors together to form functional signalosomes [533]. Significantly, the activation of these regulatory responses by ouabain occurs at concentrations which exerts no inhibition of Na^+, K^+-ATPase pumping activity [534;539-541]. It has been shown that the Na^+, K^+-ATPase dependent Src kinase activity is maintained in cells expressing a pumping-null rat α1 mutant isoform [542]. The data indicate that there is a pool of α1 non-pumping Na^+, K^+-ATPases interacting with the Src signaling cascade.

It is important to point out that involvement of the Na^+, K^+-ATPase in the signaling cascade does not exclude a role for its ion pumping function in ouabain-induced effects. Moreover, since intracellular Na^+ regulates the conformation of the Na^+, K^+-ATPase (e.g., the E1 state), it is possible that changes in intracellular Na^+ concentration could also regulate the formation of the Na^+, K^+-ATPase/Src complex, and thus cellular Src activity [533]. This integrative hypothesis remains to be proved experimentally.

Ouabain-sensitive isoform of the Na^+-pump is involved in regulation of gap junction channels in the mesenteric small arteries

Vasomotion and synchronized $[Ca^{2+}]_i$ oscillations can be used as a noninvasive way for evaluation of intercellular communication. It has been shown previously that blockade of GJ leads to desynchronization of $[Ca^{2+}]_i$ transients and inhibition of vasomotion (VI, VII and [113;479]). Ouabain in micromolar concentration is also known to abolish vasomotion [110] even in rodent tissues. This means that the observed effect of ouabain must be mediated by one of the two ouabain-sensitive isoforms of the Na^+-pump, α2 and/or α3 [506]. We have detected expression of the α2 isoform of the Na+-pump in mesenteric small arteries (VIII), which is consistent with previous findings [508;520;525;526;543;544]. In our detailed study we found that the effect of ouabain on vasomotion is due to electrical uncoupling of SMCs from each other; single SMCs continued to oscillate in unsynchronized fashion (VIII). Low concentrations of ouabain have previously been shown to interrupt intercellular communication between cells in a number of tissues including smooth muscles [500;545] and cardiac myocytes where they produce arrhythmias [524], but the mechanism of this action is unknown.

Our study demonstrated that the uncoupling effect of ouabain is mediated through an interaction between the ouabain-sensitive Na^+-pump and the Na^+/Ca^{2+}-exchanger (VIII). This conclusion is based on a patch-clamp study of electrically-coupled cultured aortic smooth muscle cells (A7r5). We further demonstrated that the Na^+-pump modulates cell coupling via changes in the $[Ca^{2+}]_i$ (VIII). This is consistent with the high sensitivity to Ca^{2+} of Cx43 abundantly expressed in A7r5 cells [479]. Since micromolar concentrations of ouabain were not shown to affect global ion concentrations [507] we suggested the involvement of local Ca^{2+} signaling in microdomains. This is consistent with previous suggestions regarding the role of the ouabain-sensitive Na^+-pump in the regulation of local $[Ca^{2+}]_i$ [516;521;544] because of a close structural association with the Na^+/Ca^{2+}-exchanger [508;525].

In accordance with the model we suggested for the interaction between the Na^+-pump, the Na^+/Ca^{2+}-exchanger and gap junctions, an inhibition of the Na^+-pump should lead to SMC uncoupling (VIII). This was not, however, the case when we attempted to stop the pumping activity by omission of extracellular K^+. We therefore explored the possibility that a leak of K+ from the cytoplasm through K_{ATP} channels can provide K^+ for the ouabain-sensitive Na^+-pump, functionally linking these two transporters (VIII). Such an association requires that the proteins are physically located near to each other. This is strongly supported by our study on coronary arteries where we demonstrated the opposite interaction, i.e. that K_{ATP} channels are regulated by the ouabain-sensitive Na^+,K^+-pump either via modulation of the local K^+ or the local intracellular ATP concentration (IX). Such a modulation can only be possible in spatially restricted subsarcolemmal areas. A similar interaction has been suggested previously to be present in the renal proximal tubule [546], in pancreatic β-cells [547], in skeletal muscle [548] and in the heart [549].

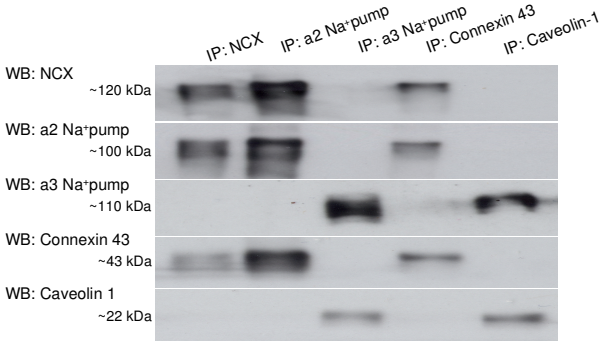

Figure 8

Preliminary co-immunoprecipitation experiments suggested a physical interaction between the Na^+/Ca^{2+} exchanger, α2 isoform of the Na^+-pump and connexin 43 but not with the α3 isoform of Na^+-pump and caveolin-1. The immunoprecipitates with each of these five proteins were tested on Western blot.

Lipid rafts and caveolae are present at high densities in endothelium and smooth muscles [550-553]. Caveolae/rafts harbor a subset of membrane proteins and signal transduction molecules, which allows locally restricted, high-fidelity signaling [550]. Interestingly, the ouabain-sensitive isoforms of the Na^+-pump [530], the NCX-1 [554], K_{ATP} channels [555] and gap junctions [556] have all been shown to be localized in the caveolae/rafts, although their interactions within these microdomains have never been studied. We have recently studied this interaction directly by using immunoprecipitation assay (Fig. 8). Interestingly, our preliminary results suggest that the α2 isoform of Na^+-pump, Na^+/Ca^{2+} exchanger, Cx43 interact physically with each other but not with caveolin-1. This suggests that this putative microdomain is located outside the caveolae, although it is possible that it may move to caveolae in response to stimulation, e.g. increase in $[Ca^{2+}]_i$ [530]. The dynamics of this interaction remain to be studied.

Caveolae have also been implicated as sites of assembly and regulation of signalosomes organized by α1 Na^+, K^+-ATPase. However, recently both α1 and α2 isoforms have been localized in caveolae in SMCs from pulmonary arteries, although only the α1 isoform of the Na^+, K^+-ATPase was seen in the caveolin-free

membrane fraction [557]. This functional study showed that the α2 isoform of the Na^+, K^+-ATPase plays a critical role in modulating the local $[Ca^{2+}]_i$ via an interaction with the Na^+/Ca^{2+} exchanger as well as by some other ways, e.g. Na^+ channels, L-type Ca^{2+} channels and Na^+/H^+ exchanger [557].

9. PERSPECTIVES

The studies included in this thesis lead in general to a deeper understanding of mechanisms involved in smooth muscle cell synchronization in the arterial wall (II-IX). They illustrate the complexity of coordinated activities in the vascular wall and a dramatic need for specific tools which allow manipulation of single membrane transporters involved in the synchronization. Comprehensive studies are, therefore, often required when one or another membrane transporter function should be highlighted. For example, no truly specific blocker for Ca^{2+}-activated Cl^- conductance is available currently. This means that pharmacological approaches attempting to demonstrate chloride channel function need to be combined with additional tests, e.g. patch-clamp or molecular biological studies (V and [87]). A similar problem is seen during manipulations of intercellular communication where caution should be taken in interpreting the results of pharmacological experiments (VI-IX). RNA interference is a technique that has great potential for solving these problems [360]. Our previous study demonstrates the efficiency of siRNA to downregulate the mRNA, protein and function of the target of interest (V). The current use of siRNA in vascular physiology is, however, greatly limited by difficulties in transfection of smooth muscle cells in situ [558]. We were the first to suggest the technique for *in vivo* transfection of arteries with siRNA (V). Our recent experiments show, however, that caution should be taken in using this method because of different untargeted responses and serious optimization is necessary for each new gene target. Nevertheless, this technique seems to have great potential both for basic research and clinical applications. There is no doubt that it should be further optimized to be more effective and less traumatic for the tissues. This can then be used for studying specific membrane transporters in the vasculature, e.g. the role of different isoforms of the Na^+, K^+-ATPase, the type of connexin forming gap junctions involved in smooth muscle cell synchronization and the proteins associated with Ca^{2+}-activated Cl^- conductances. The great advantage of this method in comparison to other approaches, such as pharmacological tools and genetically modified animals, is that it is quite specific, and that it can be used for acute and reversible down-regulation which limits compensatory changes in other proteins. In the future, it might also be used for correction of disease-causing vascular abnormalities [559].

The discovery that a specific form of the Na^+, K^+-pump is involved in the regulation of intercellular communication is of interest because of the potential role of endogenous ouabain-like compounds for blood pressure regulation [560;561] although this is still under debate [562;563]. Although our study indicated that the Na^+, K^+-pump is regulating intercellular communication via local $[Ca^{2+}]_i$ homeostasis, the detailed mechanism of this action remains unclear (VIII). It remains to be unraveled whether this local $[Ca^{2+}]_i$ acts directly on gap junctions or via other second messengers pathways [502;511;516;521;532;564]. It will also be important to clarify the time scale for ouabain action since the functional effects are quick and transient while ouabain binding has a much slower, long-lasting profile (VIII and [565]). Several processes initiated by ouabain could be involved, e.g. an acute inhibition of gap junction conductance following changes in the membrane transporters' expression and localization (VIII and [500]). Direct visualization of the membrane proteins will be necessary to solve this question. This could be helpful for understanding how ouabain-like substances are involved in elevating blood pressure [566-568].

One has to accept that the identification of the protein responsible for the Ca^{2+}-activated Cl^- conductance is still lacking whilst the number of potential candidates continues to grow [277;298]. Future studies involving mutagenesis in the putative pore-structures, detection of membrane localizations and endogenous characterization remain to be done [276;330;332]. It also remains to be shown how the potential protein candidates interact with each other and with other membrane proteins – for example, whether bestrophin and ANO1 proteins are both involved in the same type of Ca^{2+}-activated Cl^- current or represent two distinct channel families.

The accumulated knowledge suggests that transporters do not function independently but rather interact in a spatially and temporally restricted manner, organizing complicated cascades and functional pathways. These interactions may be either direct, via protein-protein interactions, or indirect, via interactions through signaling cascades or via modification of local ion concentrations. Most information presently available is based on functional data, which suggest that activity of one transporter locally can modulate the activity of another, but do not provide detailed insight into the mechanism of this interaction. It can therefore be suggested that future research will be focused on understanding these interactions. Such interaction of membrane transporters in functional and structural microdomains is a concept with substantial potential for enhancing our understanding of cell biology and is relevant probably for all cells in the body and hence for a variety of disciplines from the molecular biologic level to the clinic.

10. SUMMARY

Although the function of rhythmic contractions in the vascular wall – vasomotion – is still under debate, it has been suggested to play a significant role for tissue oxygen homeostasis and under pathological conditions where tissue perfusion is affected [35]. Vasomotion has further been suggested to be important for blood pressure control and has been shown to be reduced in diabetes. Vasomotion is initiated by the coordinated activation of smooth muscle cells (SMCs) in the vascular wall leading to rhythmic contractions. We have suggested the model for generation of this rhythmic activity (I) and have shown that vasomotion initiates via interaction between intracellular calcium released from the sarcoplasmic reticulum and changes in membrane potential. Rhythmic changes in intracellular calcium induce, under certain conditions (in the presence of sufficient concentration of cGMP (II)), changes in membrane potential that lock the electrically-connected SMCs into phase. Synchronized depolarization induces synchronous calcium influx and thus produces rhythmic contraction of blood vessels (I, II, VI-VIII and [46-48;87]).

I have demonstrated and characterized a new chloride channel in vascular SMCs (I), which has properties necessary to coordinate SMCs in the vascular wall (III). Chloride channels have been investigated for many years but remained somewhat in the shadow of cation channels. We know now the molecular structures of some chloride channels, i.e. GABA receptors, "cystic fibrosis transmembrane conductance regulator" (CFTR) and the ClC chloride channel family. There is one particular group of chloride channels, the calcium activated chloride channels (CaCCs), whose molecular structure is debated still. There are currently no pharmacological tools that activate or inhibit CaCCs with any significant selectivity.

The existence of CaCCs in almost all cells in the body has been known for many years based on electrophysiological and other functional studies. CaCCs have been suggested to be important for regulation of membrane potential and cellular volume, as well as for body homeostasis. CaCCs are well characterized in vascular tissues but only at the functional level [247]. The lack of their molecular structure makes it difficult to study the clinical significance of these channels.

Based on patch clamp measurements of ion currents, I have previously characterized in SMCs a chloride current with unique properties (III). This chloride current activates by cGMP, has very high sensitivity to calcium and can be inhibited by low concentrations of zinc ions, while the traditional inhibitors of CaCCs affect this current only at very high concentrations. This cGMP-dependent, calcium-activated chloride current has a linear voltage-dependence, which differs from previously characterized CaCCs, and it has characteristic anion permeability (III). This current has been detected in SMCs isolated from a number of different vascular beds but, importantly, it has not been detected in pulmonary arteries (IV). Moreover, this current has been shown in SMCs isolated intestine indicating its broad distribution. Based on unique characteristics I have suggested that the cGMP-dependent calcium-activated chloride current can synchronize SMCs in the vascular wall and that bestrophin protein could be the molecular substrate for this current.

Bestrophin has been characterized first as a gene in which mutations cause vitelliform macular dystrophy (VMD) or Best diseases [247]. Based on heterologous expression it has been suggested that bestrophin is a chloride channel [330]. This question is nevertheless controversial since caution should be taken in heterologous expression of calcium-activated chloride channel candidates [301]. The presence of chloride channels in virtually all living cells is an essential problem as well as the dependence of ion channel properties on the complex interaction of many cellular proteins.

I was the first who coupled the endogenous chloride current to one of four known bestrophin isoforms. PCR and Western blot studies on different blood vessels demonstrated the presence of bestrophin-3 protein with the exception of pulmonary arteries (V) (where the cGMP-dependent current is also absent (III and IV)). There was a strong indication that bestrophin-3 expression could be essential for the cGMP-dependent calcium-activated chloride current. To couple bestrophin-3 expression and this current I have used small interfering RNA (siRNA) technique to downregulate the expression of the candidate (bestrophin-3) and have studied the effect of this specific downregulation on chloride currents. I showed that bestrophin-3 expression is associated with the cGMP-dependent calcium-activated chloride current (V). This study does not tell us whether bestrophin-3 forms the channel or it is an essential subunit but the previous mutagenic experiments [247] suggested the first possibility.

Electrical communication between SMCs is essential for successful synchronization (I) and depends on channels between the cells called gap junctions. The majority of cardiovascular diseases (e.g. hypertension and atherosclerosis) are associated with defects in intercellular communications or in gap junction regulation. The molecular mechanisms responsible for these defects are unknown because of lack of specific experimental tools. Our comprehensive study on the often used gap junction inhibitors heptanol and 18β-glycyrrhetinic acid demonstrated unspecific effects of these drugs at the concentrations where they have no or little gap junctions effects (VI). Other drugs, e.g. 18α-glycyrrhetinic acid and connexin-mimetic peptides are better to inhibit gap junctions but also have demonstrated unspecific effects (VI and VII).

Previous studies suggested that channels and transporters in the cell membrane do not function independently but interact as functional units in the spatially restricted areas of the cell. I have demonstrated a close functional interaction between gap junctions and Na^+,K^+-ATPase, Na^+/Ca^{2+}-exchanger and ATP-dependent K^+ channels in the spatially restricted manner (VIII). I have shown that inhibition of the ouabain-sensitive Na^+, K^+-ATPase inhibits calcium efflux by the Na^+/Ca^{2+}-exchanger and this lead to the local elevation of intracellular calcium and inhibition of intercellular communications. This explains the inhibitory action of ouabain on vasomotion (VIII). I have also found that the ATP-dependent K^+ channel is an important player in this functional unit and this interaction is reciprocal, since K^+ channel supplies Na^+, K^+-ATPase with K+ ions (VIII) while the ATP-dependent K^+ channel current also regulates the Na^+, K^+-ATPase (IX).

This dissertation is based on nine scientific publications where I have suggested the model for generation of vasomotion and characterized the essential elements of this model.

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