Cell. Mol. Life Sci. 64 (2007) 2863 – 2886
1420-682X/07/222863-24
DOI 10.1007/s00018-007-7254-0

Cellular and Molecular Life Sciences

Review

The endocrine role for chromogranin A: A prohormone for peptides with regulatory properties

K. B. Helle[a, *], A. Corti[b], M.-H. Metz-Boutigue[c] and B. Tota[d]

[a] Department of Biomedicine, University of Bergen, Jonas Lies vei 91, 5009 Bergen (Norway),
Fax: +47 55586340, e-mail: karen.helle@biomed.uib.no
[b] Department of Oncology and IIT Network Research Unit of Molecular Neurosciences, San Raffaele Institute, Milan (Italy)
[c] INSERM, U575, Strasbourg (France)
[d] Department of Cell Biology, University of Calabria, Cosenza (Italy)

Received 1 June 2007; received after revision 11 July 2007; accepted 12 July 2007
Online First 27 August 2007

Abstract. Chromogranin A (CgA) belongs to the granin family of uniquely acidic secretory proteins co-stored and co-secreted with other hormones and peptides in elements of the diffuse neuroendocrine system. The granins arise from different genes and are characterized by numerous sites for post-translational cleavage into shorter peptides with postulated regulatory properties. This review is directed towards endocrine aspects of CgA and its biologically active peptides. There is ample evidence from *in vitro* studies of distinct effects and targets for three CgA-derived peptides, vasostatin-I, pancreastatin and catestatin. Endocrine regulations are indicated from *in vivo* studies, consistent with the postulated prohormone function of CgA for peptides with regulatory properties. Most of the effects fit into patterns of direct or indirect, inhibitory modulations of major functions, implicating CgA peptides in regulation of calcium and glucose metabolism, cardiovascular functions, gastrointestinal motility and nociception, tissue repair, inflammatory responses and as host defense peptides in the first phase of microbial invasions.

Keywords. CgA peptides, vasostatins, chromofungin, pancreastatin, catestatin, cateslytin, parastatin, calcium and glucose metabolism.

Introduction

Chromogranin A (CgA) is a well-established member of the granin family of genetically distinct and uniquely acidic proteins that are ubiquitous in secretory cells of the nervous, endocrine and immune system [1]. Numerous pairs of basic amino acids indicate potential sites for cleavage by the prohormone convertases PC1/3 and PC2 that occur as co-stored components of neurosecretory granules [2]. Numerous cleavage products of the granins have been identified, some of them with biological activities [1]. Although an endocrine role for the released granins remains to be unambiguously established, some of their cleavage products have been postulated to participate as regulators of homeostatic processes, inflammatory reactions and the innate immunity.

CgA was the first granin to be characterized as a uniquely acidic protein co-stored and co-released with the catecholamine hormones from the bovine adrenal medulla [3, 4]. Virtually identical forms of CgA were

* Corresponding author.

associated with different tissues such as the adrenal medulla [5], the parathyroid gland [6] and the gastro-entero-pancreatic endocrine system [7, 8], however with some differences in primary sequence and post-translational modifications. For instance, the bovine protein ($bCgA_{1–431}$) is shorter than the human ($hCgA_{1–439}$) and rat proteins ($rCgA_{1–448}$) [9]. Notably, the N- and C-terminal domains, $CgA_{1–76}$ and $CgA_{316–431}$, being highly conserved sequences in mammals [5, 10, 11], suggested that CgA might serve as a prohormone for shorter fragments postulated to have regulatory properties [11, 12]. Our present knowledge of the phylogenetic distribution of CgA rests largely on the immunochemical cross-reactivity with polyclonal and monoclonal antibodies to defined sequences of the bovine CgA. Immunoreactive CgA-like proteins have been demonstrated in mammals, birds, amphibians, fish and invertebrate classes down to the protozoans [13–16]. This implies a widespread conservation of immunological epitopes in CgA despite marked species-specific differences in primary sequence, even among mammals [17]. In addition, within a given tissue the molar ratio of CgA to other granins may differ in species-specific patterns. For example, in the human and porcine adrenal medulla the molar CgA/CgB ratio is 1:1, while it is 10:1 in the bovine gland [5]. Hence, although the mammalian adrenal medulla may release CgA, this release may subserve different, species-specific functions.

The physicochemical properties and structural characteristics of CgA have directed investigators to extensive studies along two main lines: (A) Does this protein participate in the intracellular events leading to formation of the condensed cores for exocytosis from the hormone storage granules? (B) Does CgA, once released, exert specific functions *via* the circulation and/or the extravascular space, as the intact molecule and/or as a prohormone for peptides with distinct effects other than those of their co-stored and co-released neurotransmitters and hormones? With respect to the first line of investigations, there is accumulating support for the importance of CgA and other granins in sorting of proteins to the regulated secretory pathway [18]. The exocytotic release of adrenomedullary CgA with the co-stored catecholamine hormones [3, 19] is also a well-established concept [20]. This review focuses on the second line, the prohormone concept [12, 21–23], implying that intact CgA in the extracellular space is processed into smaller peptides, acting locally and/or at some distance from the site of release. Although a coherent picture has yet to be drawn of an endocrine role for the stress-induced release of CgA and the CgA-derived peptides from elements of the diffuse neuroendocrine system, experimental support for the prohormone concept is steadily on the rise.

The prohormone concept

The discovery that pancreastatin (PST), a CgA-derived peptide ($bCgA_{248–293}$) that was first purified from porcine pancreas, was able to inhibit the glucose-evoked insulin secretion from pancreatic beta-cells [7], initiated the concept of a prohormone function for CgA [12].

In endocrine and neuroendocrine cells, most peptide hormones are produced from larger, inactive precursors through limited proteolysis at pairs of basic amino acids residues during their transport through the exocytotic pathway [2]. This molecular mechanism corresponds to the intracellular processing of CgA, and chromaffin granules have been successfully used as a model for regulated secretory vesicles that contain enzymes for the biosynthesis of peptides hormones. Several proteases were identified in chromaffin granules including the neuroendocrine-specific carboxypeptidase E/H that removes basic residues from neuropeptides intermediates, the prohormones convertases and finally the Lys/Arg-aminopeptidases [2]. In the bovine chromaffin granules the proteolytic processing of CgA occurs at 13 sites, 5 of them at the N-terminal side, 2 in the middle and the rest at the C-terminal end. These sites are located at residues 3–4, 64–65, 76–77, 78–79, 115–116, 247–248, 291–292, 315–316, 331–332, 350–351, 353–354, 358–359 and 386–387 [24, 25]. Among the resulting fragments several biological activities have been demonstrated (Fig. 1).

There may also be species-, cell- and tissue-specific processing of CgA, as in the rat [26] and in the human gastrointestinal tract [27–29].

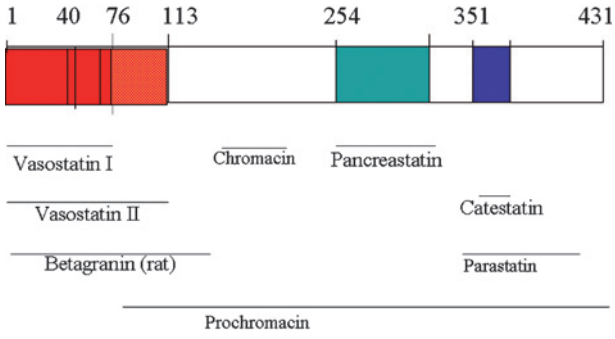

Figure 1. Schematic illustration of biologically active chromogranin A (CgA)-derived peptides originating from the stress-activated, diffuse neuroendocrine system [24, 25].

Table 1. Reported actions of CgA-derived peptides, indicating endocrine functions directed towards counteraction of tissue- and organ-specific activations involved in homeostatic regulations.

	Vasostatin I	PST	Catestatin	Parastatin
Calcium metabolism	inhib	inhib	nd	inhib
Carbohydrate metabolism	nd	inhib	nd	nd
Cardiovascular functions	inhib	nd	inhib	nd
Innate immunity	inhib	nd	inhib	nd
Pain	act/inhib	nd	nd	nd
Tissue repair	act	nd	nd	nd

Inhibition (inhib), activation (act), not demonstrated (nd).

The biologically active CgA-derived peptides

Since 1986 evidence for biological activity in CgA-derived peptides has accumulated. First out was PST [7], thereafter the rat betagranin [30], VS-I ($CgA_{1\text{-}76}$) and VS-II ($CgA_{1\text{-}113}$) [31, 32], parastatin [33] prochromacin and chromacin [34, 35] and finally, catestatin [36].

To date only one granin peptide, the CgA-derived catestatin, has been reported to act *via* a classical type of receptor, the nicotinic acetylcholine receptor [36]. Similarly sized membrane binding proteins in the 70–80-kDa range have been reported for VS-I [37, 38] and PST [39]. Further identification of these membrane proteins as classical receptors has so far remained elusive. Nevertheless, reports on modulatory effects of CgA peptides are numerous, affecting a wide range of tissues and functions, as summarized in Table 1.

Although an overall pattern for the regulatory potencies of these CgA peptides is yet to be recognized, their *in vitro* activities strongly suggest participation not only in homeostatic processes such as regulation of calcium and glucose metabolism [40] and cardiovascular functions [41–43], but also in inflammatory reactions [44, 45], pain relief [46], tissue repair [47, 48], gastrointestinal motility [46, 49–51] and in the first line of defense against invading microorganisms [52–55]. The possible involvement of CgA and some of its peptides in mechanisms of disease has been reviewed [56, 57].

The purpose of this review is to arrive at a comprehensive picture of the potentials and possible mechanisms of actions of the CgA-derived peptides, *in vitro* and *in vivo,* in the normal and diseased states. The potentials of vasostatins, PST and catestatin as modulators and/or regulators of tissue-specific functions and possible mechanism of actions in animal models and in humans, are also discussed.

Circulating prohormone and peptides

A range of radioimmunological assays (RIAs) has been employed to quantify the CgA levels in bodily fluids, making use of highly different antibodies, resulting in divergent results as a reflection of differences in interspecies cross-reactivity. Most of the antibodies raised to the human CgA detect the large, near mid-sequence domain ($CgA_{115\text{–}232}$) between VS-II and PST [35, 58], as illustrated in Fig. 1. A performance assessment of three commercial kits for circulating human CgA [59] indicates significantly different properties and specificities of the antibodies [47, 58–61], a factor that should be taken into consideration when comparing results from different clinical studies of plasma and serum CgA.

In normal human plasma CgA ranges from 0.5 nM [62], 2 nM [63, 64] and 5 nM [59, 65, 66], reflecting the specificities of the antibodies used for the RIAs, still lower than the value of 7 nM for CgA in bovine serum [67]. A similar range (1–6 nM) has been reported for CgA in mammals using antisera to selected, highly conserved CgA domains, such as VS-I, betagranin, catestatin and the near-terminal peptide GE-25 [10, 67, 68]. The plasma concentration of the free N-terminal domain (vasostatin-like immunoreactivity) was much higher in the rat (3 nM [10]) and the pig and horse (2 nM) [67] than in healthy humans (0.5 nM) [67]. A range of immunoreactive catestatin-containing forms occurs in human plasma [69], giving rise to mean plasma levels of 1.3 nM catestatin compared to 5 nM for catestatin-containing prochromacin ($CgA_{113\text{–}439}$) [65] when assayed with a range of region-specific antibodies to the human CgA [59].

In contrast, the concentration of amidated PST in rat plasma is low (0.4 nM) and still lower (5 pM) in healthy humans [70]. Three PST forms, containing the amidated and biologically active C terminus, have been identified in human plasma, namely $CgA_{259\text{–}294}$, $CgA_{223\text{–}294}$ and $CgA_{220\text{–}294}$; none of them is completely identical to the human PST ($CgA_{250\text{–}301}$). Higher plasma PST levels (19 pM) occur in obese individuals with type 2 diabetes [70]. This level was not reduced after a 2-month regimen of weight reduction (~7 kg). Higher than normal

Table 2. Circadian rhythms in plasma CgA in healthy individuals, essential hypertensives and adrenalectomized patients before and 120 min after insulin-induced hypoglycemia.

	(*n*)	Plasma CgA (ng/ml)		
		Morning	Afternoon	Night
Controls	54	44	57*	65***
+ hypoglycemia	54	80*	–	–
Essential hypertensives	83	43	56***	–
+ hypoglycemia	8	60*		
Adrenalectomized	6	34*	46**	–

CgA molecular mass 46 kDa; 46 ng/ml = 1 nM. Values are means.
*$p<0.05$, **$p<0.003$, ***$p<0.0001$ for difference from respective morning controls. Morning (0800), afternoon (1800), night (2300) (*n*) number of patients. Data from [75].

plasma PST (14 pM) also occurs in patients with primary hyperparathyroidism [71].

Sympathoadrenal and other neuroendocrine sources

CgA-like immunoreactivity is ubiquitous in normal human endocrine tissues, with a rank order (μg/g wet wt): adrenal medulla > pituitary > pancreas > stomach > small intestine > brain frontal cortex > parathyroid > thyroid, corresponding to 0.04–25 % of the immunoreactivity in the adrenal medulla [72]. It was suggested that the adrenal medulla was the main source for the 1.4-fold increase in plasma CgA in response to insulin-induced hypoglycemia in normal individuals. However, only intense stimulation of chromaffin cells or sympathetic nerves resulted in measurable changes in plasma CgA, consistent with human sympathetic nerves containing 97 times less CgA by weight than the human adrenal medulla [73]. Importantly, plasma CgA still persists in patients with unilateral or bilateral adrenalectomy [74, 75], also speaking against the adrenal medulla and sympathetic nerves as the sole source of plasma CgA (Table 2). Although a range of selective stimuli for hormone secretion failed to change plasma CgA, the non-selective suppression of these secretions by somatostatin reduced plasma CgA by 48 % [74], in accordance with a considerable contribution to plasma CgA from non-sympathoadrenal, endocrine sources expressing circadian rhythms also in the adrenalectomized patients [75] (Table 2).

The endocrine nature of the mammalian heart relates to atrial co-secretions of natriuretic peptides and CgA/CgB, as demonstrated in the rat [76]. Four N-terminal CgA-derived peptides ($CgA_{4–113}$, $CgA_{1–124}$, $CgA_{1–135}$ and $CgA_{1–199}$) have recently been characterized from rat heart homogenates, consistent with cleavage into peptides containing the homologous vasostatin-I motif within betagranin (rat $CgA_{1–127}$) [77] (Fig. 1). Moreover, myocardial CgA is expressed and colocalized with brain natriuretic peptide (BNP) in biopsies from patients with dilated cardiomyopathies and hypertrophic myopathies [78], indicating constitutive release of CgA in parallel with the constitutive release of BNP from the hypertrophied human ventricles.

Neuroendocrine tumors

A human pheochromocytoma was the first source of purified human CgA for the RIA [79] used to diagnose a wide range of neuroendocrine tumors [80, 81]. As illustrated in Table 3, the mean plasma levels of CgA in pheochromocytoma and other neuroendocrine tumors are consistently above normal [82] and may reach up to 1000-fold higher levels in individual cases of carcinoid tumor [80].

Table 3. Mean plasma CgA in hypertension, pheochromocytoma and neuroendocrine tumors.

	n	mean plasma CgA (ng/ml)[a]
Controls	130	49
Essential hypertension	48	51
Adrenocortical tumors	25	92
Pheochromocytoma	20	254
Other neuroendocrine tumors	28	469

[a] Two-sandwich RIA, anti-$hCgA_{145–245}$ for (*n*) number of controls and patients. Data from [82].

Inflammatory conditions

After the original work by O'Connor and Bernstein [58] showing that sera of patients with neuroendocrine tumors have increased levels of CgA, many investigators have detected increased serum levels of CgA also in patients with systemic diseases, including renal and hepatic failure, cardiac arrest and essential hypertension [56]. More recently, it has been reported that increased levels of CgA may also occur in the blood of patients with diseases associated with

inflammation such as heart failure and rheumatoid arthritis [63, 64]. In patients with chronic heart failure (CHF), circulating CgA is increased up to 10–20 nM (500–1000 ng/ml), depending on the severity of the disease and is an independent predictive factor for mortality [64]. While CgA does not correlate with hormones known to be activated in CHF, such as catecholamines, vasopressin, endothelins and components of the renin-angiotensin-aldosterone system [83], CgA correlates with soluble tumor necrosis factor (TNF) receptors (sTNFRs), which are sensitive markers of systemic inflammation [63]. The good correlation between CgA and sTNFRs and the lack of correlation with neuroendocrine variables [63] suggest that circulating CgA reflects systemic inflammation much better than neuroendocrine activation in CHF.

Rheumatoid arthritis (RA) is another inflammatory condition associated with increased levels of CgA [84]. Serum levels of CgA were particularly elevated in RA patients with severe extra-articular manifestations. Also, in these patients, serum levels of CgA correlate with circulating sTNFR-I and sTNFR-II. TNF-α activation is a primary mechanism in the pathogenesis of RA. Interestingly, treatment of patients with anti-TNF antibodies was associated with significant clinical responses and loss of correlation between CgA and sTNFRs [84].

The tissue origin of circulating CgA in CHF and RA is unknown. While in patients with neuroendocrine tumors the source of abnormally high levels of CgA is often the tumor itself, the tissue origin of CgA in CHF may be more complex. Recent work has shown that myocardial CgA is expressed and colocalized with BNP in ventricles of failing heart [78], suggesting constitutive release of co-release of CgA and BNP from the hypertrophied human ventricles as a result of increased wall stress [85]. The cellular source of CgA in RA is more obscure. The correlation between CgA and sTNFRs observed in heart failure, RA and cancer (A. Corti, unpublished observation) suggests that a regulatory link exists between CgA secretion and certain inflammatory conditions. Moreover, polymorphonuclear neutrophils (PMN) have emerged as a significant source of intact and processed forms of CgA, vasostatins [53] and catestatins [55]. When PMN accumulate at sites of inflammation and are stimulated by lipopolysaccharide (LPS), these cells may provide CgA peptides for paracrine modulations of the relevant tissue-specific targets.

In vivo responses to CgA and CgA peptides

CgA and VS-I

The first evidence for an endocrine effect of CgA and vasostatin peptides was obtained *in vivo* in a mouse model [44]. Intraperitoneal injection of TNF-α in non-toxic concentration was used to induce extravasation of trypan blue/albumin complexes through liver vessels in mice harboring subcutaneous tumors genetically engineered to produce human CgA, compared with mice harboring CgA-negative tumors and wild-type mice. Vascular leakage of the dye after administration of TNF-α was evident in controls and in mice with CgA-negative tumors but not in mice bearing the CgA-producing tumors. Their blood levels of CgA were in the range 7–20 nM. Noteworthy, these levels are within the range of plasma CgA in patients with neuroendocrine tumors (Table 3) [86] and CHF [63, 64]. Natural human CgA (0.7 μg), VS-1 (human recombinant STA-$CgA_{1\text{-}78}$) (3 μg) and synthetic peptide $CgA_{7\text{–}57}$ (3 μg) could also inhibit TNF-induced dye extravasation in the liver of normal mice [44]. Although these studies revealed a novel activity of CgA in protection of endothelial integrity when challenged by TNF-α, the high concentrations required (above 7 nM) indicate a relevance mainly for elevated plasma CgA, *e.g.*, during inflammatory provocations in CHF [63, 64], RA [84], in adrenomedullary hyperplasia [87] and in a range of neuroendocrine tumors [81] (see Table 3). The mechanism behind this protection was further elucidated using cultured endothelial cells [44, 45], as reviewed below (see *Endothelial integrity*).

PST

The first indication of an endocrine effect of PST derives from the pig [7, 88]. The peptide, later shown to be identical to the mid sequence of CgA (Fig. 1), was extracted from the pig gut and shown to inhibit the first phase of the glucose-induced insulin secretion from the porcine pancreas [12]. Although the physiological relevance of PST in humans has been questioned [89], there are two reports [70, 90] indicating an endocrine role for PST in man. However, these two studies represent different experimental designs and concentrations of infused PST, yielding highly diverging results. The first study [90] was based on the effects of intravenous infusion of 10 pmol/kg/min for 3 h of the human C-terminal PST (hP-16) on oral glucose tolerance on a background of 80 pM PST-like material in plasma. However, on the background of hP-16 infusion, the observed reductions of the peaks of plasma glucose and insulin were contradictory to the hypothesis of a PST-induced inhibition of the first phase of the glucose-induced insulin-release. A stim-

ulation of somatostatin secretion was suggested as a possible alternative for the observed PST reduction of plasma glucose and insulin. This hypothesis has yet to be tested experimentally.

The endocrine role of PST in humans has also been approached by a different experimental design [70]. Using a forearm perfusion technique, PST was given and blood samples collected *via* the cannulated brachial artery. The effects of a 20-min infusion of PST (local concentration 200 nM), insulin (70 μU/min) and, finally, insulin + PST were studied in healthy male controls. PST infusion at this concentration, *i.e.*, 40 000 times above the baseline plasma PST of 5 pM, reduced the arterio-venous (A-V) glucose difference and inhibited glucose uptake without affecting blood flow. While insulin markedly enhanced glucose uptake, A-V difference and forearm blood flow, the presence of PST did not change any of the insulin-mediated effects. PST also markedly reduced the uptake of free fatty acids (FFA) and the A-V difference in FFA, again without significant effect on the forearm blood flow, modifying neither the insulin action on uptake of FFA nor the increase in forearm blood flow induced by insulin. PST had, on the other hand, no effect on its own either on the A-V difference or the uptake of any of the 20 natural amino acids, or on the insulin-reduced spillover of alanine, aspartic acid and ornithine. Thus, these *in vivo* results at supranormal concentrations of PST in the human forearm resembled those previously obtained *in vitro* in rat adipocytes [91], and may indicate a dysglycemic role for PST on the subcutaneous adipocytes in the forearm. However, the postulated inhibitory role of PST on the first phase of glucose-stimulated insulin release from the human pancreas [7] still awaits experimental support.

Catestatin

To establish to what extent the inhibitory effects of catestatin on catecholamine release *in vitro* [36] could be demonstrated *in vivo*, tests were carried out in rats [92]. Intravenous injections of catestatin were given to yield an extracellular concentration of 6 μM, about 30 times above the IC_{50} of 200 nM [36]. The result was a hypotension in response to activation of sympathetic outflow by electrical stimulation. However, this vasodepression persisted in presence of α and β adrenoceptor blockade, speaking against an inhibition of catecholamine release. On the contrary, catestatin infusion increased plasma adrenaline by 11-fold, and also blunted the pressor response to exogenous neuropeptide Y agonists, while being without effect on plasma noradrenaline. Another CgA peptide, chromostatin ($CgA_{140–160}$), was without similar effects on these parameters. On the other hand, endogenous circulating histamine was increased 21-fold by the catestatin infusion and mimicked both the epinephrine elevation and the vasodepressor actions of catestatin. Moreover, a histamine H1 receptor antagonist blocked the vasodepressor response to catestatin and the elevation in plasma adrenaline. Accordingly, the endocrine effect of catestatinin the rat *in vivo* was that of an indirect vasodilatation, mediated by histamine release acting *via* a H_1 receptor and seemingly unrelated to the autocrine inhibition of catecholamine release by catestatin in the adrenal medulla *in vitro*. The assumption of catestatin-induced stimulation of histamine release *in vivo* was subsequently supported by the demonstration that catestatin is a potent stimulator of histamine release from rat mast cells *in vitro* [93] (see *Catestatin* section in *Structure-function relationships*).

Arterial infusion of catestatin at 1 μM in the forearm perfusion study [70] was compared with PST at 200 nM for *in vivo* effects on glucose uptake. Contrary to PST, catestatin was without effect on either glucose uptake or A-V difference, while slightly, yet significantly, enhancing the forearm blood flow. Thus, catestatin *in vivo* appeared to be without effects on carbohydrate metabolism in humans. Catestatin was not tested on other aspects of the PST-modified processes in this human model. Whether the observed enhancement of forearm blood flow by catestatin in man reflected an inhibition of catecholamine release from the sympathoadrenal system or a stimulation of histamine release from subcutaneous mast cells remains to be answered.

Possible consequences of deficiency in the catestatin domain have been analyzed in Chga null mice in two reports, revealing diverging results with respect to adrenomedullary morphology and endocrine functions, yet agreeing on elevated excretion of catecholamines [94, 95]. While other dense-core granule proteins were elevated, suggesting a compensatory mechanism for the lack of Chga gene expression [94], an elevated blood pressure in the Chga null mice could be rescued by exogenous catestatin or by humanization of the Chga null mice, implying a hypotensive effect of the CgA-derived catestatin [95]. However, it was not clarified whether the catestatin effect was secondary to a histamine release from mast cells, as in the rat [92]. Although exocytotic co-release of CgA and catecholamines from the adrenal medulla is well established [19], elevated plasma CgA is not a normal finding in essential hypertension (see Tables 2 and 3). Moreover, elevated plasma CgA appears to reflect systemic inflammation much better than neuroendocrine activation in heart failure [63]. Thus, in view of the complex patterns of vasostatin effects on the heart

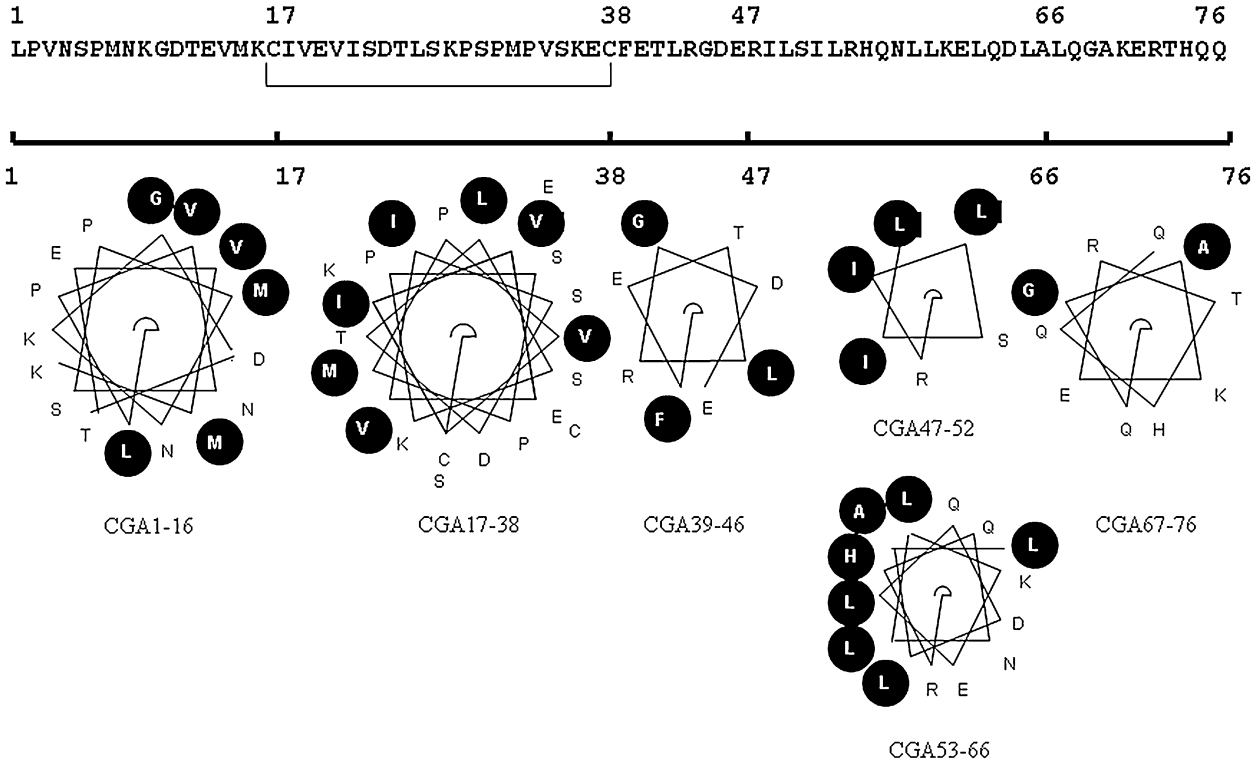

Figure 2. Helical wheel prediction of the structure of $CgA_{1–76}$, comprising the three amphipathic domains of VS-I, $CgA_{1–16}$, $CgA_{17–38}$ and $CgA_{53–66}$. [96, 97].

and vascular endothelium (see *Cardiovscular processes* below), the causal relationship between lack of CgA and hypertension may be more complex than so far suggested from the effects of exogenous catestatin.

Structure-function relationships

VS-I

Using bioinformatic programs for predictions of secondary structure according to the Garnier algorithm [96], about 74 % of VS-I is compatible with an α-helical orientation. The helical wheel predictions reveal an amphipathic character for three domains, $CgA_{1–16}$, $CgA_{17–38}$ and $CgA_{47–66}$ (Fig.2) [96, 97]. A hydrophobic domain comprising $L_1M_{15}M_7V_{14}V_3G_{10}$ (right to left) and a hydrophilic domain $E_{13}K_9K_{16}$ indicates an amphipathic property of the $CgA_{1–16}$ domain. In addition, the cysteine-bridged loop domain $CgA_{17–38}$ exhibits amphipathic characteristics indicated by a large hydrophobic region comprising $V_{34}V_{19}L_{26}I_{22}I_{18}M_{32}V_{21}$ (right to left) in opposition to a hydrophilic region spanning over $K_{28}D_{24}E_{20}$. Thus, the N-terminal domain of VS-I ($CgA_{1–40}$) comprises two amphiphathic regions, while the remarkable hydrophobic region at the C terminus ($CgA_{47–66}$) contains the third ($CgA_{53–66}$), in accordance with our previous report [54].

Highly different effects have been assigned to these three domains, suggesting structure-function relationships. A series of synthetic peptides, comprising one or several of the α-helical sequences have been examined to establish their contributions to the biological activities reported for the naturally derived VS-I in the various tissues and cells, *i.e.*, $CgA_{1–64}$, $CgA_{7–57}$, $CgA_{4–16}$, $CgA_{1–40}$ and $CgA_{47–66}$.

Rat $CgA_{1–64}$

The rat $CgA_{1–64}$ comprising all three amphipathic helices of VS-I, has been tested in the Langendorff preparation of the rat heart. Unlike the human recombinant VS-1, the rat peptide causes coronary vasodilatation [77], suggesting distinct region and species-specific vascular sensitivity towards vasostatins [41, 98, 99]. In other respects the rat $CgA_{1–64}$ peptide revealed inhibitory effects on all inotropic parameters in the rat heart [99].

Human recombinant $CgA_{7–57}$

The human recombinant $CgA_{7–57}$ revealed VS-1-like effects *in vivo,* inhibiting dye extravasation in TNF-α-treated wild-type mice by a mechanism implicating the 53–57 epitope [44]. In the eel [100]

and frog hearts [42] $CgA_{7–57}$ exerted negative inotropic effects similar to that of VS-1, and this was also the case in the rat colon in which $CgA_{7–57}$ effectively inhibited motility *via* stimulation of primary inhibitory neurons [51]. Thus, the sequence $CgA_{7–57}$ appears to be sufficient for the respective responses to VS-I in the human endothelium, the eel and frog hearts and the inhibitory neurons in the rat gastrointestinal tract.

$CgA_{4–16}$

$CgA_{4–16}$ has so far only been tested *in vitro*. The bovine and frog $CgA_{4–16}$ vary markedly in primary sequence, the frog peptide being more acidic than the bovine peptide [101]. Intriguingly, the bovine peptide was more effective than the frog peptide in eliciting negative inotropy in frog hearts [58]. The bovine peptide enhanced acid-induced gastrointestinal pain but inhibited motility in human [46, 49] and rat preparations [50], indicating a stimulatory role for the very N terminus of CgA in inflammatory pain.

$CgA_{1–40}$

$CgA_{1–40}$ combines the first and second amphipathic domains (Fig. 2). A new range of inhibitory activities have been reported for this peptide, *i.e.*, of parathyroid hormone (PTH) release from the bovine parathyroid cells at low plasma Ca^{2+} [37, 38, 102, 103], of vascular contractility in human and bovine blood vessels [31, 37, 41, 104] of myocardial inotropy in vertebrate hearts [42, 43, 99, 100, 105, 106], of cell volume regulation in rat submandibular salivary glands (Kanli and Helle, unpublished findings) and of fungal growth [107]. Hence, a wider and different range of targets responds to $CgA_{1–40}$ than to the first amphipathic domain $CgA_{4–16}$ alone. As common for small hydrophobic and amphipathic peptides, $CgA_{16–40}$ showed a secondary structure by circular dichroism spectra when shifting from buffer to a stabilizing, hydrophobic solvent [102]. Moreover, $CgA_{18–37}$ was shown to bind to the membrane of chromaffin granules at pH 5.5 [108] and to be responsible for the CgA binding to these membranes [109], consistent with a membrane affiliating property of the cysteine-bridged loop [102].

$CgA_{47–66}$

The C-terminal region of VS-I, unique in several aspects, is cationic, hydrophobic and contains the third amphipathic α-helix $CgA_{53–66}$ (Fig. 2). A number of quite different activities have been assigned to this domain, *e.g.*, activating adhesion of mouse fibroblasts and coronary arterial smooth muscle cells [48]. Other effects are inhibitory, *i.e.*, on endothelial barrier dysfunction induced by inflammatory agents for which the epitope $CgA_{53–57}$ in the amphipathic helix is essential [44], and on growth of microorganisms, notably of fungi [53, 54]. Moreover, a helical wheel prediction suggested an amphipathic α-helix for the calmodulin (CaM)-binding synthetic peptide $CgA_{40–65}$ [110]. In accordance with the potent antifungal activity of $CgA_{47–66}$, this peptide has been named chromofungin [54]. As reviewed above, this peptide also inhibits myocardial inotropy in the frog [43] and the intracellular rise in calcium in the pressure-autoregulating rat posterior cerebral artery [98]. Hence, a wide variety of tissues and cells respond to chromofungin. Analogous to the two other domains in VS-I, chromofungin modulates seemingly unrelated cellular processes. The putative mechanisms behind these effects are discussed separately in *Vasostatins* below.

PST

The predominant PST corresponds to the amidated sequences of bovine ($bCgA_{248–293}$) and human CgA ($hCgA_{240–288}$), respectively. The discovery and characteristics of PST have been reviewed elsewhere [111]. The biological activity of PST resides in the C terminus that is the best conserved among mammals [112]. The interspecies homology is relatively low, being 70% between mammals [113] and there are also tissue-specific differences in phosphorylation [114], being highest in the pancreas.

The primary sequence of the 49-amino acid peptide [7], GWPQAPAMDGAGKTGAEEAQ PPEGKGAREH SRQEEEEETAGAPQGLFRG, indicates a proline-rich, overall negatively charged molecule, with, however, hydrophobic N- and C-terminal regions. The secondary structures of the full-length peptide and the biologically active C-terminal domain hP-16 (EEEEETAGAPQGLFRG) have yet to be reported.

Catestatin

Catestatin ($CgA_{344–364}$) is a 21-residue long, cationic and hydrophobic peptide [36] located at the N-terminal end of parastatin [33] and derives from intragranular and extragranular processing of the prohormone [36, 115, 116]. Several distinct biological activities have been assigned to the catestatin domain, *i.e.*, inhibiting catecholamine secretion from bovine chromaffin cells [36], PTH secretion from porcine parathyroid cells [33, 117] and bacterial and fungal growth [55], while activating histamine release in a mastoparan-like manner from rat mast cells [93]. Unlike the helical structure of mastoparan [118], the primary sequence of human catestatin, SSMKLSFRAR AYGFRGPGPQL, forms a loosely coiled structure with an electropositive Arg-rich loop that stabilizes the hydrophobic form assumed to be essential for its catechol-

amine release-inhibitory action [115]. Intriguingly, the mast-cell stimulating effect of catestatin is analogous to a series of cationic and hydrophobic stimulators of histamine release from mast cells such as mastoparan and substance, P. A receptor-independent, mastoparan-like membrane interaction for catestatin [93, 118] is therefore implicated, distinct from the non-competitive inhibition of the nicotinic cholinergic receptor [36]. Moreover, the growth-inhibitory effect of catestatin on bacteria, fungi and yeasts also resides in the N terminus of catestatin, $CgA_{344–358}$, accordingly named cateslytin [55] (see *First defense against microbial invasions* below).

Putative mechanisms for endocrine effects

Amphipathic properties, receptor-independent activation and cell penetration

A number of cationic peptides have the ability to form α-helices when associating with the hydrophobic environment of membrane lipids [119, 120], rapidly killing bacteria and fungi by selective perturbations in the lipid bilayer of the negatively charged microbial membranes. Such hydrophobic interactions are assumed to occur by carpet-like modes in microorganism, while forming pores and channels in barrel-stave manners in animal cells [119, 120]. On the other hand, powerful antimicrobial cationic peptides of the hairpin β-stranded category such as the protegrins may form pores or channels when inserted into lipid bilayers as monomers, dimers and oligomers [121–123].

Intriguingly, two of the antimicrobial domains of CgA, VS-I [53, 54] and catestatin [55], acquire significantly different secondary structures in the hydrophobic environment of cytoplasmic membranes, *i.e.*, amphipathic α-helices and a loosely coiled structure [115], respectively. On the other hand, when isolated from mammalian extracts VS-I [32] and catestatin [124] display similar solubility property characteristics of membrane-affiliated peptides, both peptides being recovered in the 60–80 % methanol fraction from Sep-Pak C_{18} columns [124]. Concentration-dependent dimer formation is a property shared by VS-1 [61] and $CgA_{1–40}$ [67] with some of the pore-forming antimicrobial peptides [121–123], consistent with amphipathic helical structures predicted for these two vasostatins.

The first interactions between vasostatins and monolayers of phosphatidyl choline (egg lecithin) and $CgA_{47–66}$ [54] and $CgA_{1–40}$ [114] were demonstrated with peptides in the subnanomolar range at 20°C in buffered saline at pH 7.4, mimicking the conditions used in the antimicrobial assays [97]. Peptide-induced increases in surface pressure were obtained with both peptides, consistent with peptide penetration into the hydrophobic environment. Importantly, at 37°C, relevant for mammalian membranes, $CgA_{47–66}$ but not $CgA_{1–40}$, induced a conspicuous increase in surface area in monolayers of phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine (PS) at low nanomolar concentrations of peptides in the phosphate-buffered physiological saline surface [125]. Under these conditions VS-1 revealed a unique property. Unlike the cationic and amphipathic helical $CgA_{47–66}$, the entire VS-1 molecule with its three helical domains specifically increased the surface area of dipalmitoyl PS and disteatoryl PS in transit from the gaseous to the liquid phase, consistent with enhanced fluidity of the monolayers. Moreover, the specificity for saturated PS species strongly suggests electrostatic as well as hydrophobic interactions between the vasostatin peptides and membrane-relevant phospholipids at physiological conditions.

The specific affinity of VS-I for PS calls for further considerations. An enhanced exposure of PS on the surface of mammalian cells takes place as a result of a collapse of the phospholipid asymmetry in the plasma membrane [126] and occurs as a general feature of senescence and apoptosis, as evident in aging erythrocytes, platelets and tumor cells [127, 128]. PS is also externalized in activated, viable immune cells [129]. In contrast to the immune cells in which PS is located in lipid rafts, PS in apoptotic cells appear excluded from the structurally modified lipid rafts that are involved in the externalization of PS [130–132]. It is therefore not unlikely that VS-I may modulate the PS-dependent phagocytosis of apoptotic cells by activated immune cells. Moreover, the monolayer experiments indicate that intact VS-I may also interact with PS-enriched domains located outside the lipid rafts and caveolae where a range of receptors may cluster in cell and tissue-specific patterns [130–132].

Receptor-independent cell penetration into microbial and mammalian membranes has been observed for a series of cationic and amphipathic peptides frequently used as drug carriers [133–137], and antimicrobial peptides implicated in self defense and innate immunity [138]. VS-I and VS-1 as well as the negatively charged $CgA_{1–40}$ and the positively charged chromofungin domains exhibit potent antifungal activities associated with penetration into the fungi [53, 54, 107], as to be expected from their potential for adopting α-helices in hydrophobic environments. In contrast, catestatin adopts a loosely coiled structure in a hydrophobic environment [115], penetrates microbial membranes [55], inhibits catecholamine release from chromaffin cells [36] and stimulates histamine release from mast cells. Moreover, analogous to the cationic

and amphipathic wasp venom, mastoparan [93], catestatin activates mast cell release *via* heterotrimeric G proteins in a receptor-independent manner [118]. Thus, the membrane translocating potentials of VS-I and catestatin suggest that these two amphipathic and/or hydrophobic domains of CgA may interfere with subcellular signaling pathways in receptor-independent manners that appear largely inhibitory in microorganisms, while inhibiting or activating animal systems in cell- and tissue-specific patterns.

In vitro targets, regulated processes and putative mechanisms

Calcium metabolism: Regulation of PTH secretion

PTH is a primary homeostatic regulator of plasma Ca^{2+} levels, the hormone being co-stored and co-released with CgA [6]. While the release of PTH is stimulated by low plasma Ca^{2+} levels or IL-8 *via* the CXC2 type of G protein coupled receptor at normal, 1 mM Ca^{2+} [139], it is inhibited *via* two different pathways, (1) by high plasma Ca^{2+} activating the calcium sensor [140], and (2) by three of the CgA-derived peptides at low plasma Ca^{2+}, namely VS-I and $CgA_{1–40}$ [38, 102, 103], PST [141, 142] and parastatin [33, 117]. The inhibitory mechanism for high plasma Ca^{2+} in human parathyroid cells involves an increase in intracellular Ca^{2+} sufficient for enhanced efflux of K^+ *via* a tetraethylammonium (TEA)-sensitive, calcium-regulated K^+ channel [140]. The resulting hyperpolarization brings about a cessation of the PTH release. The second mechanism for inhibition of PTH is mediated by VS-I derived from the co-secreted CgA at low plasma Ca^{2+}. This autocrine inhibition is as effective as the physiologically high concentrations of Ca^{2+} [103]. A Gαi subunit in the signaling pathway has been implicated by the blocking effect of pertussis toxin (PTX). By analogy, the inhibitory effect of VS-I might be a hyperpolarization mediated *via* a Gαi subunit coupled to opening of another K^+ channel. Partial inhibition of PTH secretion at low plasma Ca^{2+} could also be obtained with PST in porcine [141] and bovine parathyroid cells [142], but this inhibition was regarded as physiologically irrelevant due to the low degree of CgA processing into PST in this tissue.

In the porcine parathyroid yet another highly conserved CgA domain, parastatin ($pCgA_{347–419}$), was shown to inhibit the co-secretion of PTH and CgA [33], but at a higher concentration range than with VS-I in the bovine parathyroid cells. The full inhibitory effect of parastatin on this PTH release resided in a synthetic peptide corresponding to the first 19 N-terminal residues [33, 117], close to catestatin ($CgA_{344–364}$) [36]. Hence, the autocrine inhibition of PTH release from parathyroid cells by the catestatin domain, as demonstrated in the porcine gland, suggests that three different CgA peptides, vasostatin I, PST and catestatin, may modulate PTH release at low plasma Ca^{2+}, presumably leading to hyperpolarization *via* different signaling pathways activated by their distinctly different primary sequences and secondary structures.

Carbohydrate metabolism: PST as a regulator in pancreas, hepatocytes and adipocytes

The islet cells of the endocrine pancreas represent together with the liver and adipose tissue the essential components in the homeostatic regulation of plasma glucose. Inhibition of the first phase of the glucose-stimulated secretion of insulin from the porcine pancreas by PST [7] was therefore sensational, implicating for the first time CgA in homeostasis. Glucose-stimulated insulin release has since been shown to be mediated *via* the rise in intracellular ATP causing depolarization *via* inhibition of an ATP-regulating K^+-channel [143, 144]. Whether PST inhibition involves a hyperpolarization, *e.g.*, by opening of this or other K^+ channels, emerges as an intriguing possibility for the inhibitory effect of this peptide on glucose-stimulated insulin secretion.

The regulatory effects of PST on liver and adipose tissues are to date best documented *in vitro*, as extensively reviewed elsewhere [111, 145]. In the rat adipocytes PST inhibits insulin-mediated glucose transport, glucose utilization and lipid synthesis, being associated with modulation of intracellular Ca^{2+} levels and some of the G protein subunits. A PTX-sensitive basal and insulin-stimulated protein synthesis by PST indicates involvement of a Gαi subunit. In hepatocytes as well as adipocytes the PST modulated glucose and lipid metabolism is assumed to occur *via* a glycoprotein receptor for PST physically coupled to the Gq/11 protein-phospholipase C (PLC)-β pathway. Analogously, a cross-talk with insulin signaling *via* protein kinase C (PKC) might explain the PST effects on protein synthesis [146]. Hence, pancreatic α and β cells as well as hepatocytes and adipocytes appear as likely targets for PST, activating a range of subcellular signaling pathways *via* distinct G protein subunits. In contrast, the inhibitory effect of PST on growth and proliferation in hepatoma cells is mediated *via* endothelial nitric oxide synthase (eNOS), neuronal (n)NOS, cGMP and PKC, implicating endothelial and neuronal contributions to the inhibitory mechanisms [147].

Cardiovascular processes

A range of inhibitory responses to VS-I in vascular and cardiac elements has been reported. The first experimental models were human blood vessel segments [31, 148], later extended to the pressure-activated bovine coronary and adrenal resistance arteries [41, 104]. Several models of the vertebrate heart have been introduced, *e.g.*, the frog [105, 106], the eel [100, 106] and the rat [99] myocardium. There is also accumulating support for inhibitory effects of VS-1 on endothelial cells when activated by proinflammatory agents [44, 45]. Common to these models are inhibitory responses to the highly conserved VS-I domain in activated preparations, whether by pharmacological, mechanical or electrical activation. Some targets and signaling pathways have been identified, such as involvement of K^+-channels, PTX-sensitive Gαi protein subunits and NO production in tissue-specific patterns. These findings are reviewed in the following subsections.

Vasodilatation. The vasoinhibitory effect of VS-I was initially discovered using isolated segments of the human internal thoracic artery and saphenous vein [31, 37, 148]. These segments responded to the naturally derived bovine VS-I + VS-II and synthetic VS-I by suppression of the potent vasoconstriction elicited by endothelin-1 (ET-1). The inhibitory effect was independent of the endothelium and extracellular Ca^{2+}, affecting the maximal, sustained tension response, but not the potency for ET-1. Importantly, the synthetic peptide $CgA_{1–40}$ mimicked the VS-I-induced suppressions of a depolarizing concentration of K^+ or a noradrenaline-evoked tension response, but was without inhibitory potency on the sustained response to ET-1. Thus, two different mechanisms leading to vasodilatation might be activated by the intact VS-I, one inherent in $CgA_{1–40}$ and the other in the C-terminal region, possibly the chromofungin domain. Inhibitory effects of VS-I and $CgA_{1–40}$ were also evident in the pressure-activated resistance arteries of the bovine adrenal and coronary circulation [41, 104]. The autoregulated basal tone in both arteries was dependent on extracellular Ca^{2+} and largely sensitive to 4-aminopyridine, indicating a tonic influx of Ca^{2+} and pressure-activated K_v channels. The VS-I evoked dilatations, being independent of other activators, persisted at moderately elevated extracellular $[K^+]$ (8–16 mM) in both arteries [41, 148]. Moreover, the dilator effect of $CgA_{1–40}$ in the coronary artery was abolished by several antagonists to K^+ channels, such as TEA, glibenclamide and Ba^{2+}. Thus, the $CgA_{1–40}$ evoked inhibition of the autoregulated tone in the bovine coronary artery appeared to involve hyperpolarization *via* concerted contributions from opened K_{Ca}, K_{ATP} and K_{IR} channels. At 8–16 mM $[K^+]$ the inhibitory effect of $CgA_{1–40}$ was diminished by PTX, suggesting in addition an involvement of a Gαi subunit. Accordingly, in the pressure-activated bovine resistance arteries, the naturally occurring VS-I appeared to have a direct dilator potential, closely similar to that of $CgA_{1–40}$ and thus acting *via* the N-terminal, loop-containing domain, involving several types of K^+ channels and a Gαi subunit as early steps in the signal pathway. Species differences may, however, be important, as indicated from a report on the pressure-activated, autoregulating rat posterior cerebral artery [98], in which vasodilator effects were not apparent, either with the bovine $CgA_{1–40}$ or the rat $CgA_{7–57}$ peptide.

Endothelial integrity. In vivo experimental evidence, reviewed above (see *Neuroendocrine tumors*), suggests that CgA plays a role as a regulator of vascular permeability, preventing TNF-α-induced vascular leakage of macromolecules [44]. Studies on the mechanism of action showed that the endothelial lining of vessels is the primary target of both TNF-α and CgA. For instance, *in vitro* experiments showed that 4–40 nM CgA could inhibit the TNF-α-induced flux of radiolabeled albumin through endothelial cell monolayers (HUVEC), suggesting that CgA can protect the integrity of the endothelial barrier from TNF-α pro-permeabilizing activity [44].

Structure-activity relationships studies, carried out with CgA and CgA fragments, showed that the bioactive site is located in VS-I [44]. Furthermore, *in vitro* studies with HUVECs showed that CgA and its N-terminal fragments can inhibit TNF-α-induced cytoskeletal reorganization and inter-cellular VE-cadherin down-modulation, a transmembrane protein important for cell-cell adhesion [44]. VS-1 could also inhibit TNF-α-induced gap formation in arterial endothelial cells of bovine pulmonary (BPAEC) and coronary (BCAEC) origin [45], suggesting that VS-I can affect endothelial barrier dysfunction in venous as well as arterial vascular beds.

Interestingly, VS-1 also partially inhibits thrombin-induced and VEGF-induced permeability of HUVECs [44]. One possibility is that CgA acts on signaling molecules or intracellular components of endothelial cells that are critical for TNF-α, VEGF and thrombin activity. Accordingly, in BPAEC, p38MAP kinase phosphorylation induced by TNF-α, thrombin or PTX was markedly attenuated in the presence of VS-1, the inhibitory effect corresponding to that of the p38MAP kinase inhibitor SB203580 [45]. Moreover, the inhibitory activity of VS-1 may be associated with the p38MAP kinase signaling cascade *via* a PTX sensitive, presumably a Gαi-coupled,

mechanism. Although it has been shown that endothelial cells can internalize 1 nM ^{125}I-labeled CgA [149], the receptors or the molecular targets responsible for the biological effects of CgA and VS-I on endothelial cells are still unknown.

Negative myocardial inotropy. The heart acts as an integrator between the adrenal medulla and sympathetic nerve terminals being a preferential target for adrenergic stimuli, especially under stress [150]. Several lines of evidence indicate that CgA-derived vasostatins exert negative inotropy under basal and isoprenaline-stimulated conditions [42, 43, 100, 105, 106]. Thereby, the vasostatins may serve as inhibitory, cardio-regulatory principles of relevance for cardiac homeostasis both under normal and physio-pathological conditions.

Locally derived vasostatins might exert autocrine/paracrine regulation of cardiac function, analogous to the marked negative inotropic effects of VS-1 and synthetic vasostatin peptides in vertebrate hearts [99, 106]. In the rat heart, CGA is stored in non-adrenergic myoendocrine atrial cells containing atrial natriuretic peptide [76] and in Purkinje fibers of the rat atrium and ventricle containing the calcium channel α1E subunit [151]. CgA-derived fragments may also originate from sympathetic nerve termini in the heart [152]. Four vasostatin-containing CgA peptides have recently been extracted from rat heart, $CgA_{4–113}$, $CgA_{1–124}$, $CgA_{1–135}$ and $CgA_{1–199}$, in addition to intact CgA and larger sized fragments containing the C terminus [77], indicating a less extensive CgA processing in the heart than in the adrenal medulla. Most amphibian hearts lack a coronary system and are suitable models for specific myocardial effects of cardio-active agents. VS-1, VS-2 (human recombinant STA-CgA_{1-115}) and $CgA_{7–57}$ exerted negative inotropism under basal conditions, while counteracting the positive inotropism elicited by the β-adrenergic agonist isoproterenol (ISO) in the isolated and perfused avascular frog (*Rana esculenta*) heart working at physiological loads [105]. In addition, the specific inhibition of cardiac contractility and the counteraction of the β-adrenergic inotropism were most potently elicited by VS-1, $CgA_{7–57}$ and CgA_{1-40SS}, implicating the disulfide-bridged region as a requirement for the marked negative inotropism whether mechanically activated or stimulated by ISO [43].

Interestingly, the least effective inhibitor, the frog $CgA_{4–16}$ is more acidic than the bovine homologue, while there is a complete homology between the frog, porcine, bovine and human $CgA_{14–29}$ and $CgA_{40–62}$ [101]. Hence, the modulation of myocardial contractility in the frog heart by structurally different regions of frog and bovine VS-I is consistent with a functional conservation of this domain.

The isolated Langendorff -perfused rat heart represents a well-characterized experimental model for coronary contribution to cardiac performance. While the ISO-dependent positive chronotropism was unaffected by VS-1 and reduced by VS-2, both peptides counteracted the cardio-stimulatory effects of ISO without modifying either the β-adrenergic-dependent coronary dilation or the ouabain-induced positive inotropism. A detailed analysis has revealed a non-competitive type of antagonism of VS-1 on the myocardial β-adrenergic response [99].

Interestingly, significant negative inotropic effects of the synthetic rat $CGA_{1–64}$ have been demonstrated in the Langendorff heart preparation, associated, however, with a positive chronotropism and a significant reduction of the coronary pressure (Angelone et al., unpublished findings). This finding confirms the vasodilation reported for the bovine $CgA_{1–40}$ on segments of bovine coronary resistance arteries [41] as well as of this peptide and the synthetic bovine VS-I on segments of intrathoracic artery and saphenous vein exposed to VS-1 [31, 37, 148], while contrasting the absence of coronary activity by VS-1 in the rat heart [99]. Distinct species-specific vascular sensitivities towards vasostatin peptides may account for the observed differences in coronary responses despite their similar effects on all inotropic parameters. These data, together with the results obtained with VS-1 and $CgA_{7–57}$ in the avascular eel and frog hearts, strongly support the view that the inotropic activity of the N-terminal domain of CgA is highly conserved and that neither the N-terminal amino group nor the C-terminal carboxylic group of VS-I are critical for this activity.

Tumor growth, cell adhesion and tissue remodeling

Studies on the tumorigenic properties of mouse lymphoma and mouse mammary adenocarcinoma cell lines transfected with human CgA cDNA showed that CgA secretion was associated with decreased tumor growth rate, increased tumor necrosis and multi-nodular growth pattern [153]. This suggests that CgA expression by tumor cells can affect tumor development and tissue architecture. Interestingly, CgA expression did not affect the proliferation index of these tumor cells *in vitro* [153]. Thus, the effect on tumor growth is apparently indirect and host-mediated. One possibility is that CgA may affect the complex interplay between neoplastic cells and one or more components of the tumor stroma, *e.g.*, vascular cells, immunocytes, fibroblasts or the extracellular matrix. For instance, given the notion that CgA can affect endothelial permeability and vascular leakage,

as reviewed above, one possibility is that CgA inhibited tumor growth by decreasing the transport of nutrients and growth factors from the vascular compartment into the tumor interstitium.

Stromal fibroblasts could represent another important target in tumors. These cells are important for production of extracellular matrix and tissue remodeling in tumor growth. *In vitro* cell adhesion assays have shown that CgA, isolated from human pheochromocytomas, can inhibit the adhesion of human and mouse fibroblasts to plates coated with collagen I, collagen IV, fibronectin or fetal calf serum [47, 48, 154, 155]. It is therefore possible that CgA affects the tumor architecture by modulating the physiology of stromal fibroblasts within tumors.

A growing body of evidence suggests that CgA is proteolytically processed in different manners in different tumors [59]. Interestingly, proteolytic cleavage of CgA with plasmin [116], an enzyme involved in extracellular proteolysis and tissue invasion in cancer [156], decreases its anti-adhesive activity and induces pro-adhesive effects in fibroblast adhesion assays [154]. Maximum pro-adhesive effect occurs when the K-K $_{77–78}$ dibasic site is cleaved leading to production of VS-1 [154]. Accordingly, plates coated with 30–300 nM VS-1 promote fibroblast adhesion and spreading [48, 157]. It would therefore appear that CgA might work as a negative modulator of fibroblast adhesion as well as a precursor of positive modulators, depending on proteolytic processing. The hypothesis that CgA affects remodeling of other organs, such as endocrine glands, blood vessels or heart in CHF patients [158–161] deserves to be investigated.

Structure-function studies showed that the region 47–64 is critical for the pro-adhesive activity on fibroblasts [48]. This region is 100 % conserved in human, equine, bovine, porcine, and mouse CgA [21]. Circular dichroism analysis of $CgA_{47–68}$ [48] and ^{1}H NMR studies of $CgA_{47–66}$ [54] suggest that the region 47–51 forms a short hydrophobic helix, followed by an amphipathic helix (residues 53–66) (Fig. 2). Interestingly, this region is adjacent to an RGD sequence (residues 43–45), an integrin binding motif often present in extracellular matrix proteins involved in cell adhesion. However, a recombinant $CgA_{7–439}$ mutant in which RGD was replaced with RGE induced pro-adhesive effects after tryptic digestion as the wild-type molecule [48]. Although an accessory role of integrins cannot be totally excluded, this observation suggests that this site, not conserved in mouse and rat, is unlikely to play a major role in the anti/pro-adhesive activity of CgA and its fragments.

Innate immunity

Similarities have been highlighted between pathogen recognition, signaling pathway and effector mechanisms of innate immunity in *Drosophila* and mammals [162]. Now, more than 700 cationic peptides isolated from numerous species with largely various structures have been characterized in several tissues, cells and biological fluids, and it has become clear that innate immunity is an evolutionary ancient defense mechanism. They are usually expressed as pro-peptides that undergo subsequent proteolytic processing to release these antimicrobial molecules. Some peptides are constitutively expressed, while others are strongly inducible by microbial signature molecules, inflammation or tissue injury such as Crohn's disease [163].

First defense against microbial invasions. The physical characteristics supporting antimicrobial activity include net positive charge, hydrophobicity, secondary structure and flexibility allowing the transition from peptide conformation in solution to membrane interaction [164]. Antimicrobial peptides are classified as inhibitors for microbial cell metabolism [165], induction of apoptosis *via* mitochondrial membrane disruption, as lytic peptides disrupting the membrane structure [166] and as inhibitors for proteins, DNA and RNA synthesis [167]. Different models of membrane lysis by host defense peptides have been proposed (barrel-stave, carpet, detergent, toroidal pore and aggregate models) and correspond to different types of events (formation of a transient channel, membrane micellization and membrane depolarization) [168].

During the last decade a range of new natural antimicrobial peptides has been derived from the processing of chromogranins and from proenkephalin-A and ubiquitin, which are secreted with catecholamines upon stimulation of chromaffin cells from the adrenal medulla [169–171]. These new antimicrobial peptides are integrated in the concept that the adrenal medulla is an important factor for immunity. Thus, adrenaline and neuropeptide Y, released from the adrenal medulla, regulate immunity systemically. In addition to adrenaline, the adrenal medulla contains and release large amounts of IL-6 and TNF in response to inflammatory stimuli such as LPS, IL-1α and IL-1β. The discovery of the presence of TLRs on cells of the adrenal cortex raise the interesting possibility that the adrenal glands might have a direct role in the response to pathogens, activation of innate immune response and clearing of infectious agents [172].

The natural bovine VS-I displays both antibacterial and antifungal activities and is active against Gram-positive bacteria, filamentous fungi and yeasts cells at

the micromolar range [53]. Furthermore, several CGA-derived fragments and VS-I are secreted by neutrophils stimulated by the leukocidin of Panton-Valentin [53]. To characterize the structural parameters of VS-I that are important for the antimicrobial activities, the peptide was digested by the protease Glu-C of *Staphylococcus aureus* and the resulting fragments were tested for their antimicrobial activities. The most active short peptide named chromofungin corresponds to the sequence $CgA_{47–66}$, revealing an amphipathic helical conformation [54]. The antifungal activity of chromofungin was analyzed with rhodaminated peptide by confocal microscopy showing that this peptide destabilizes the plasma membrane and penetrates by pore formation into fungi and yeast cells. Following destabilization of microbial cell walls and membranes, the internalized chromofungin was assumed to interfere with intracellular targets such as CaM-dependent systems [54]. CaM binds a variety of peptides, hormones, toxins and enzymes in a calcium-dependent manner and the intact CgA and also the shorter peptide $CgA_{40–65}$ bind CaM in presence of calcium, being responsible for CaM binding to the chromaffin granule membrane [110]. By comparison of the sequence of chromofungin ($CgA_{47–66}$) with the CaM-binding domains of various target enzymes, a similarity (60 %) between chromofungin and a model peptide could be demonstrated [54]. This alignment suggested that chromofungin may represent a membrane-translocating CgA domain that is able to interact with CaM in a variety of cells, thus affecting the activity of a range of CaM-dependent enzymes. One of these, the phosphatase activity of calcineurin (CaN), plays a crucial role in hyphal growth. The fact that chromofungin possess the ability to completely inhibit CaN phosphatase activity at a concentration of 250 μM [54] suggests that the destabilization of fungal wall and plasma membrane together with the possible intracellular inhibition of CaM-dependent enzymes may represent the mechanism by which VS-I and chromofungin exert antifungal activity.

Moreover, synergistic effects of chromofungin with other antifungal peptides are also likely. For instance, at local infectious sites, the V8 protease of, *S. aureus* may generate antifungal peptides by proteolysis of the respective precursors, CgA for chromofungin and ubiquitin for ubifungin. Interestingly, when these two antimicrobial peptides were combined, their individual activities were maximized [173].

Circulating catestatin and its fragments derive not only as secretory products of chromaffin cells but also from neutrophils in the form of the intact CgA, $CgA_{79–439}$ and others catestatin-containing peptides when stimulated by the leukocidin of Panton-Valentine [55]. The shortest of the catestatin peptides corresponded to the fragment $CgA_{340–394}$, serving as a substrate for thrombin to cleave at positions 353–354 and 358–359.

Taking into account the highly cationic nature of catestatin we hypothesized that this CgA peptide and its N-terminal active domain ($CGA_{344–358}$) might also display antimicrobial activities [55]. Indeed, the active core of catestatin ($CGA_{344–358}$), named cateslytin (RSMRLSFRARGYGFR) revealed growth inhibitory potencies on the Gram-positive bacteria *Micrococcus luteus* and *Bacillus megaterium* (MIC of 0.8 μM and 2 μM, respectively), the Gram-negative bacteria, *E. coli* D22 (MIC 8 μM) and a variety of filamentous fungi, e.g., *Neurospora crassa* (MIC 1.2 μM). In addition, cateslytin was also active against several forms of yeasts (*Candida*) [55]. Importantly, at concentrations below 100 μM no hemolytic activity could be observed.

The molecular mechanism involved in the antifungal activity of cateslytin has been elucidated using confocal microscopy, analyzing the interaction of synthetic rhodamine-labeled peptide with the membranes of *Aspergillus fumigatus* [55]. Cateslytin is able to rapidly and efficiently penetrate the fungal cell wall as illustrated by internalization and compartmentalization of 1 μM rhodaminated cateslytin within 2 min. With a 5 μM concentration for 1 h, rhodaminated cateslytin was localized in vacuoles. The effects of cateslytin on growth of hyphal tips in developing, *N. crassa* was followed using time-lapse video microscopy. In absence of peptide, *N. crassa* grows normally by the extension of the fungi and the formation of new filaments. As soon as the peptides had penetrated into the developing fungus, accumulated rhodaminated cateslytin blocked the growth and development of hyphens [55], consistent with efficient peptide penetration into the fungal cytoplasm.

There is now increasing evidence in favor of antimicrobial potencies of cationic peptides providing protection *in vivo* against a wide variety of infections. In many cases the anti-infective properties of the cationic host defense peptides relate to their activities as regulators of immune responses, suggesting a potential for these peptides in management of infections [164]. While some are potent antimicrobials in their own right, the antimicrobial activity of others may be prevented by physiological variables such as host proteases, divalent cations and polyvalent anions, *e.g.*, glycosaminoglycans. Hence, the cationic host defense peptides are important effector molecules of the innate immune system. They are also able to enhance phagocytosis, stimulate prostaglandin release, neutralize the septic effects of LPS, promote recruitment and accumulation of various immune cells at inflam-

matory sites, promote angiogenesis and induce wound repair. Finally, host-defense peptides of mammalian origin have also been demonstrated to have an active role in activation of the adaptive immune response by being chemotactic for human monocytes and T cells [174–176], and by exhibiting adjuvant and polarizing effects in dendritic cell development [177].

Microglial activation and neuronal death. The resident macrophages in the nervous system, the microglial cells, support neuronal survival and differentiation by releasing and stimulating production of neurotrophins in astrocytes [178]. Microglia plays a major role in the immune response by their secretion and response to cytokines and by their properties as mononuclear phagocytes associated with neurological disorders [179]. However, while an acute activation of microglial activity is beneficial to the host, prolonged activation may damage particularly sensitive neighboring neurons, implicating microglia in the inflammatory processes in chronic neurodegenerative disorders [180]. In a series of experiments the effects of bovine CgA and recombinant human CgA and VS-1 [181] were shown to activate cultured rat microglia in a manner analogous to but not identical to LPS. Both agents triggered secretion of heat-stable, diffusible neurotoxins and accumulation of NO and TNF-α, suggesting that CgA might trigger microglial responses involved in neurological degeneration. For instance, senile plaques of Alzheimer's brain are characterized by activated microglia and immunoreactivity for CgA [182] and in amyotrophic lateral sclerotic mice CgA expression is induced in activated astrocytes, probably acting as a chaperone promoting secretion of superoxide dismutase [183] Moreover, the neuronal signaling pathway may involve the death receptor Fas, p38MAPK and mitochondrial cytochrome c [184].

Finally, it has been shown that CgA, up-regulated in many neurological disorders, may be involved in neuronal apoptosis *via* its activation of microglia and subsequent induction of inflammatory processes [185–187].

Gastrointestinal inflammation, pain and motility. Inflammatory mediators participate in controlling the activity of CgA-storing enterochromaffin cells. When injected intraperitoneally or applied locally acetic acid may produce inflammatory, somatovisceral pain *in vivo* [46] and reduce motility in human [49] and rat [50] colonic segments. The number of acid induced abdominal constrictions (writhes) increased in a dose-dependent manner in response to i.p injected CgA_{4-16}, although the peptide was without intrinsic effect [46]. The synthetic peptide CgA_{1-16} appears to display pronociceptive activity *via* afferent spinal neurons, suggesting involvement of L-type calcium channels and direct or indirect activation of inflammatory cells [46]. Acetic acid alone abolished the spontaneous contractile activity and decreased the excitatory component of the tonic response to transmural nerve stimulation in the isolated human colonic segments [49]. In this preparation CgA_{4-16} reduced the inhibitory effects of acetic acid in a manner that was insensitive to tetrodotoxin (TTX) or L-NAME/apamin, yet blocked in presence of BAYK8644 and CgA_{4-16} [49]. L-type of Ca^{2+} channels appeared to be targeted by acetic acid and counteracted by CgA_{4-16} both on the smooth muscle and the afferent nerve terminals. In the rat distal colon with intact mucosa *in vitro* [50], acetic acid induced a transient hyperactivity followed by a decrease in tone, the first phase being sensitive to TTX and capsaicin, while the second phase was sensitive to BAYK8644 but insensitive to L-NAME/apamin. Neither CgA_{4-16} nor CgA_{47-66} separately affected the smooth muscle motility, although pre-treatment with CgA_{4-16} increased the duration of the excitatory component and reduced tone inhibition by acetic acid while pre-treatment with CgA_{47-66} had a contrasting effect and only on the excitatory phase. In absence of mucosa, acetic acid only decreased tone, but in a manner that was sensitive to BAYK8644 and CgA_{4-16}, consistent with a counteracting effect of CgA_{4-16} on the acetic acid-induced inhibition of primary, presumably nitrergic afferents *via* L-type calcium channels.

An alternative approach, using intraluminal pressure as the stimulus, was applied to rat proximal colon *in vitro* [51]. VS-1 and CgA_{7-57} in the low nanomolar range produced a concentration-dependent, progressive decrease in the mean amplitude of the spontaneous contractions in the circular layer of smooth muscle without affecting the resting tone. The peptide effects were abolished by four antibodies directed to different sites in the VS-I sequence. Pretreatment with either L-NAME, ODQ, apamin or TTX reduced the inhibitory response to VS-1, suggesting a partial stimulation of inhibitory, nitrergic afferents terminating on the circular layer of the colonic smooth muscle. Hence, these four studies agree on suppressive effects of the intact VS-1 and CgA_{7-57} [51] as well as of CgA_{4-16} [46, 47, 50] on elements of the gastrointestinal tract *via* activation of primary inhibitory, presumably nitrergic afferents in addition to a direct inhibition of smooth muscle contractility.

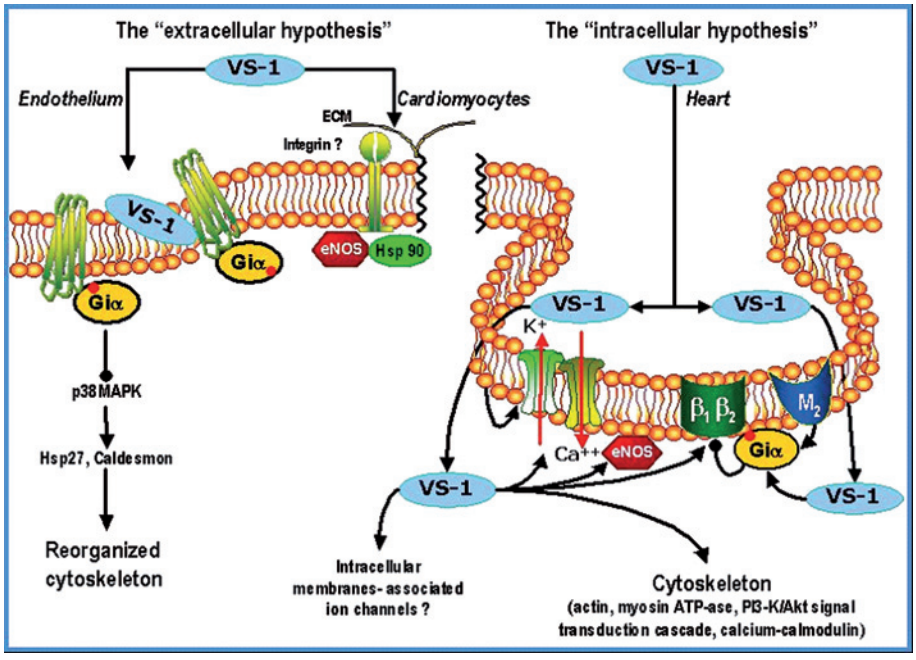

Figure 3. A cartoon of our current hypothesis for VS-I responses in the cardiovascular system *via* receptor-independent membrane perturbations (the extracellular hypothesis) and translocations (the intracellular hypothesis), affecting subcellular signaling outside and inside lipid rafts and caveolae [188].

Putative membrane targets and signaling mechanisms

Vasostatins

Activation of microglia and the subsequent induction of inflammatory processes appear to involve the VS-I domain of CgA, activating iNOS and releasing TNFα and neurotoxic factors that subsequent lead to neuronal death *via* the death receptor Fas, p38MAPK and mitochondrial cytochrome c [178–186]. In the gastrointestinal tract afferent nitrergic neurons respond to VS-I, $CgA_{7–57}$ and $CgA_{4–16}$, presumably *via* activation of Ca^{2+}-channels and nNOS as part of the inhibitory response of these peptides on motility [47, 49, 50, 51]. The cartoon (Fig. 3) is based on the ability of VS-I to interact *via* its amphipathic helical properties in a receptor-independent manner to enhance membrane fluidity and modulate receptor coupling (the "extracellular hypothesis") and when translocated to modulate ion channels, receptors and enzymes in endothelial, endocardial and myocardial cells (the "intracellular hypothesis").

In vascular endothelial cells the inhibitory effects of VS-I appear to be modulatory, protecting the coupling of the Gαi subunit to a tonic inhibition of p38MAPK by inflammatory agents such as TNF-α, thrombin and PTX [189]. In vascular smooth muscle cells, hyperpolarization by opening of a range of inwardly rectifying K^+ (Kir) channels [190] may contribute to the vasodilator response to VS-I and $CgA_{1–40}$, as indicated by the inhibitory effects of PTX, Ba^{2+}, 4-aminopyridine, TEA or glibenclamide on the vasostatin-evoked vasodilatations in the pressurized bovine coronary arteries [41].

Receptor clustering to lipid rafts and caveolae are characteristics of the vascular endothelium and the structural elements of the heart. Here the Ca^{2+} and K^+ channels, eNOS, β1 and β2 receptors and the muscarinic M2 receptor are located [130–133], all known to be involved in various inhibitory transduction cascades triggered by chemical or physical stimuli [191]. Other functionally important membrane components, such as the PTX-sensitive Gαi/o proteins, have also been located within caveolae. The downstream transduction mechanisms so far implicated in vasostatin-mediated cardiac inhibition, involves one or more of the components clustered in lipid rafts and/or caveolae, discussed briefly below.

Calcium and potassium channels. An involvement of calcium influx has been implicated from the inhibitory effects of lantanium and diltiazem on the negative inotropic effects of vasostatins in the eel and frog hearts [42, 100]. The eel and frog cardiomyocytes, unlike the mammalian counterparts, lack the transverse tubular system and depend exclusively on extracellular Ca^{2+} for tension development [192, 193]. Hyperpolarization via opening of several Kir channels may also contribute to the negative inotropy in the frog heart, suggested by the blocking effects of Ba^{2+}, 4-aminopyridine, TEA or glibenclamide on the inhibitory effects of vasostatins [42]. In contrast, in the working eel heart, the VS-1-mediated negative ino-

tropism is abrogated only by TEA and glibenclamide [100].

Endocardial- and endothelium-mediated mechanisms: NO-cGMP signaling. The eel and frog ventricles are highly trabeculated and lined by an extensive endocardial endothelium (EE) as a barrier between the cardiac lumen and the subjacent myocardium. Both hearts are under a tonic EE-dependent, negative inotropic influence *via* a NO-cGMP transduction pathway that can be also activated by mechanical and chemical endoluminal stimuli [194, 195]. In the frog the vasostatin-induced negative inotropism involves neither the EE nor the G protein or NO-cGMP systems [42]. In contrast, in the eel the same inotropic effects are mediated by PTX-sensitive G proteins and require an EE-NO-cGMP signal transduction mechanism. In the rat, on the other hand, the VS-1-induced negative inotropism is dependent on NO-cGMP-protein kinase G (PKG) and Gαi/o protein. These data suggest that, whatever the subcellular signaling route, VS-1 exerts a negative inotropic effect on vertebrate hearts. Enhancing NOS activity, either through a direct control of eNOS, or through modulation of Gαi/o proteins, is one alternative, another effector being PKG, controlling intracellular calcium homeostasis and utilization. PKG may also exert a feedback regulation of Gαi/o proteins, thereby generating a circuit of interactions converging to depress contractility. PST, the CgA-derived peptide known as a counter-regulatory agent of insulin action, has also been reported to affect cardiac function through interaction with GTP binding proteins (G proteins) [196].

On the whole, these comparative results emphasize the importance of species-specific differences in EE properties, affecting peptide binding, internalization and trans-endocardial transport. Scavenger receptors have been reported in the EE of teleost hearts [197], which through an interaction with a number of peptides, including VS, could trigger an EE-mediated negative inotropy. Recently, a novel calcium-independent mechanism of eNOS activation has been demonstrated to involve caveolae-mediated endocytosis induced by the albumin-binding protein gp60 and activation of downstream Src, Akt and PI3K pathways [198]. A similar mechanism, by an interaction with caveolar domains [100] and internalization in endothelial cells [44], might explain the VS-1-dependent NOS activation in the EE of eel and rat hearts. Intriguingly, VS-I was ineffective on the basal contractility on rat papillary muscle, while reducing the effect of ISO stimulation by 27 % [199]. Moreover, removal of EE and inhibition of NO synthesis and PI3K activity abolished the anti-adrenergic effect of VS-1, indicating that the anti-adrenergic effect in the rat heart is mainly due to a PI3K-dependent-NO release by endothelial cells rather than to a direct action on the cardiomyocytes. Moreover, two different pathways appear to mediate the protective activity of VS-1 against ischemic insults in the rat heart, one *via* A1 receptors and the other by NO release, both converging on PKC [200].

Cytoskeletal involvement. In eel and frog hearts the VS-1-mediated negative inotropy is abolished by a number of inhibitors of cytoskeleton reorganization, cytochalasin-D, Wortmannin, butanedione 2-monoxime (BDM) and *N*-(6-aminohexil)-5-chloro-1-naphthalenesulfonamide (W7) [106], and for cytochalasin-D and W7 confirmed in the isolated Langendorff rat heart (Angelone *et al.*, Cardiac cytoskeleton is modulated by recombinant vasostatin-I, submitted). In 3-D cultured adult rat cardiomyocytes VS-1 modulated interactions between the cells and the extracellular matrix, leading to a different cellular localization of HSP90 associated with eNOS [201]. Therefore, changes in cytoskeletal dynamics may play a crucial role in the negative inotropic response to vasostatins.

The "anti-β-adrenergic", non-competitive antagonism. The functional non-competitive antagonism between VS-1 and the ISO-elicited increase in cardiac performance in the rat heart [99] differs significantly from the non-competitive inhibition by catestatin ($CGA_{344–364}$) on the nicotine cholinergic receptor-induced release of catecholamines from chromaffin cells [36]. In line with the hypothesis that vasostatins may target localized signal-transduction domains at the cell membrane in a receptor-independent manner, at least two possible mechanisms may underlie the inhibition of the ISO-mediated positive inotropy. A modulation of the β-adrenergic receptor coupling to adenylate cyclase *via* an inhibitory G protein, might be triggered by vasostatins independent from the ligand binding site or other modulations of the downstream intracellular, although in distinct species-specific manners. Alternatively, the "anti-adrenergic" effect of VS-1 may be due to a PI3K-dependent release of NO from EE [199, 200] rather than to a modulation of the receptor coupling in the cardiomyocytes.

In fungi there is evidence for a growth inhibitory action of $CgA_{47–66}$ (chromofungin) *via* its structural similarity to calmodulin binding sequences and inhibition of CaM-stimulated enzymes such as CaN (phosphatase B) [54].

PST

The responses to PST in adipocytes, hepatocytes and pancreatic cells are also complex. In hepatocytes, PST activates PTX-sensitive and insensitive Gα subunits, subsequently activating NOS, IP3 and PKC pathways to inhibit protein and glycogen synthesis [111, 145]. In adipocytes, PST inhibits insulin receptor signaling *via* the PTX-insensitive Gαq/11 subunit, PKC activation and prevention of GLUT4 translocation and glucose uptake [91]. In pancreas, PST may inhibit glucose-induced release of insulin by opening of the K^+ATP channel [143, 144].

Catestatin

Four distinct mechanisms of action are at present indicated for this cationic and hydrophobic, loosely coiled peptide. The first indicates a non-competitive inhibition of the nicotinic acetylcholine receptor, blocking the calcium-dependent catecholamine release from the adrenomedullary chromaffin cells and subsequently also the acetylcholine-induced desensitization of the receptor [36]. The second mechanism closely resembles the receptor-independent histamine release from mast cells by mastoparan and other cationic peptides *via* a PTX-sensitive, Gαi subunit [93]. The third mechanism relates to the membrane translocating ability of catestatin, effectively inhibiting a wide range of fungi and Gram-negative and Gram-positive bacteria [55]. Finally, the inhibiting effect of catestatin containing moiety of parastatin on PTH release from the porcine parathyroid gland cells [33] suggests that the catestatin domain, by its receptor-independent, membrane translocating potencies may modify hormone release also from other endocrine tissue than the few so far examined.

Concluding remarks

As presently reviewed, endocrine regulations by three of the CgA-derived peptides are indicated from *in vivo* and *in vitro* studies. Consistent with its prohormone function, CgA is released to the circulation from the diffuse neuroendocrine system in response to a variety of external and internal stimuli and is processed into at least three peptides with regulatory properties, VS-I, PST and catestatin. Each of these products may modulate a range of processes of significance for maintenance of bodily functions. Hence, CgA emerges as a pluripotent prohormone for modulation of homeostatic processes, notably in response to excessive stimulations such as inflammations, growth of neuroendocrine tumors and the first phase of microbial invasion.

A clue to the mechanisms behind the multitude of cellular processes to be modulated by at least one of the CgA derived regulatory peptides, *e.g.*, VS-I, appears to be its membrane translocating potential. By its amphipathic, α-helical structure in hydrophobic environments this peptide may interact with phospholipids such as PS, eliciting receptor-independent perturbations of normal signaling pathways in species and tissue-specific patterns.

Acknowledgements. Financial support from The Tordis and Fritz Riebers Legacy (to, K.B.H.) and from Associazione Italian per la Ricerca sul Cancro (to, A.C.) is most gratefully acknowledged. Thanks are also due to, G. Serck-Hanssen and, A. Blois, Department of Biomedicine, University of Bergen, for valuable comments, to, D. Colin (Institut de bactériologie, Strasbourg, France) for help in collecting secretions from human neutrophils, G. Nullans and, B. Guérold (Inserm U575, Strasbourg France) for peptide synthesis, and Claire Gasnier for sequencing (Inserm U575, Strasbourg France).

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