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"The emerging role of Vasostatins and Hippocampal Cholinergic Neurostimulating Peptide: new putative antiadrenergic factors"

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Title:

"The emerging role of Vasostatins and Hippocampal Cholinergic Neurostimulating Peptide: new putative antiadrenergic factors"

TUTOR ***co-TUTOR***

Dr. M-H Metz-Boutique *Pr. Bruno Tota*

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Resumé de la thèse

Découverte du rôle de la Vasostatine-1 et de l'Hippocampal Cholinergic Neurostimulating Peptide en tant que nouveaux facteurs anti-adrénergiques

AVANT-PROPOS

Cette thèse est le fruit d'une collaboration entre l'équipe Neuropeptides Bioactifs (Responsable Dr. Marie-Hélène Metz-Boutigue) de l'unité Inserm U575 "Physiopathologie du Système Nerveux" dirigée par le Dr. Dominique Aunis et le laboratoire de Physiologie Cardiovasculaire de Calabre dirigé par le Pr. Bruno Tota dans le cadre de la cotutelle entre l'Université de Calabre et l'Université Louis Pasteur de Strasbourg. Cette collaboration a bénéficié d'un financement Egide (programme Galilée France-Italie).

INTRODUCTION

Lors de conditions normales et pathologiques, le cœur représente une cible majeure des systèmes sympathique (SNS) et parasympathique, ainsi que des catécholamines (CAs) circulantes. Un taux élevé de CAs est décrit capable d'induire des dommages nécrotiques au niveau du cœur (Raab, 1963; Tan et al., 2003; Goldspink et al., 2004). Ainsi, la réponse initiale du muscle cardiaque à un stress excessif et massif se traduit par une hypertrophie cardiaque (*i.e.,* un ajustement compensatoire prévenant une détérioration progressive de la fonction cardiaque) (Grossman et al., 1975; Chein, 1991). Cependant, depuis une dizaine d'années, il est apparu que le stress affecte

profondément le système cardiaque, conduisant lele cœur hypertrophié à une défaillance. En parallèle, afin de contrôler les volumes sanguins sortant du cœur et la pression sanguine systémique, il existe une activation du SNS et du système Rénine-Angiotensine (RAS) (Esler et al., 1997). Des évidences récentes ont également indiqué que, sans contrôle, ces cascades excitatrices ont des répercussions plus importantes que le stress cardiaque initial sur le processus de détérioration. Chez l'homme, lors d'une défaillance cardiaque, une intensification chronique de l'activation du SNS et des voies de signalisation adrénergique (*via* les CAs), représentent des pronostics négatifs et peuvent accélérer les processus pathologiques (Cohn et al., 1984).

Les recherches réalisées dans le domaine cardiovasculaire ont des applications cliniques évidentes. Elle permettent, par exemple la rationalisation des thérapies utilisant des substances affectant le système adrénergique, (traitement par les bêta-bloquants). L'objet de cette thèse concerne la découverte de deux nouveaux peptides cardiotrophiques qui pourraient représenter de nouvelles molécules naturelles cardioprotectrices lors d'états de stress. Ces molécules, la Vasostatine 1 (VS1) et l'Hippocampal Cholinergic Neurostimulating Peptide (HCNP), sont stockées avec les CAs dans les granules de sécrétion des cellules chromaffines de la médullo-surrénale et libérés dans la circulation lors des états de stress sous l'effet d'une stimulation nerveuse du nerf splanchnique ou par activation endocrine de l'axe hypothalamo-hypophyso-surrénalien.

Caractéristiques biologiques des vasostatines

Parmi les composants solubles de la matrice des granules de sécrétion des cellules chromaffines de la médullo-surrénale, la chromogranine-A (CGA) représente la protéine majoritairement représentée. Elle est stockée avec d'autres protéines incluant la proenképhaline, ainsi qu'avec les CAs dans les granules chromaffines, mais également dans le système neuroendocrine diffus qui est présent dans de nombreux organes incluant le cœur (Steiner et al., 1990). La CGA est une prohormone qui subit une protéolyse (intragranulaire et extra-cellulaire), produisant de nombreux peptides bioactifs. Cette maturation qui est tissu spécifique est principalement réalisée par les Prohormones Convertases (Metz-Boutigue et al., 1993). Les peptides dérivés de la CGA les mieux caractérisés d'un point de vue biologique correspondent à des fragments qui dérivent de la partie N-terminale de la protéine et qui incluent les vasostatines I (CGA_{1-76}; VS1) et II (CGA_{1-113} ; VS2). Les vasostatines (VSs) sont connues pour leur capacité à réduire le tonus vasculaire, pour antagoniser différents agents vasoconstricteurs incluant la noradrénaline (NE) (Helle et al., 2001).

Caractéristiques biologiques du HCNP

La PEBP (PhosphatidylEthanolamine-Binding Protein), protéine de 21kDa, a été initialement caractérisée dans le cerveau et la glande surrénale. Elle est distribuée de manière ubiquitaire dans le règne animal (Goumon et al., 2004) et est le précurseur du HCNP. Il s'agit d'une protéine initialement caractérisée dans le cytoplasme, mais plus tard localisée dans dans les membranes plasmiques et du réticulum. Par ailleurs, bien qu'elle ne possède pas de peptide signal elle a été identifiée dans des fluides biologiques

et en 2004 elle a été identifié dans les granules de sécrétion des cellules chromaffines de la médullo-surrénale et dans leurs sécrétions (Goumon et al., 2004). Cette protéine, également appelée Raf-1 Kinase Inhibitor Protein (RKIP) du fait de son interaction avec Raf-1, est impliquée dans plusieurs voies de signalisation incluant la modulation des protéines G hétérotrimériques (Vallée et al., 2003).

Le HCNP, qui correspond aux 11 premiers résidus de la PEBP, est un peptide généré dans l'hippocampe et qui est décrit pour augmenter la synthèse d'acétylcholine (Ojika et al., 2000). Par immunohistochimie il a été identifié dans les terminaisons nerveuses du cerveau de rat et de l'intestin. Il a également été identifié dans le liquide céphalo-rachidien de patients atteints d'Alzheimer, dans les sécrétions de plaquettes et dans le sérum (Goumon et al., 2004). Ces résultats, en plus des données indiquant la présence de la PEBP dans de nombreux organes, suggèrent que la PEBP et le HCNP pourraient représenter de nouveaux médiateurs endocrines capables de moduler les réponses physiologiques du cœur. Afin de tester cette hypothèse au niveau cardiaque, nous avons utilisé le modèle du cœur de grenouille isolé et perfusé. Ces travaux ont indiqué que, dans ce modèle, le HCNP exerce un effet inotropique négatif sur la performance cardiaque. Par ailleurs, le HCNP est également capable de contrecarrer l'action contractile positive (inotropisme positif) des agonistes bêta-adrenergiques (*i.e.,* isoprotérénol) (Goumon et al., 2004).

L'objet de cette thèse est de tester les propriétés cardiomodulatrices des vasostatines et du peptide HCNP. Nous avons utilisé le modèle de cœur de rat isolé et perfusé, également appelé modèle de Langendorff. Ce modèle permet d'analyser simultanément les effets d'agents cardioactifs, à la fois sur les performances myocardiaques (*i.e,*

chronotropisme et inotropisme) et sur la motilité coronarienne. Dans ce modèle, la perfusion réalisée dans des conditions isovolumiques permet une évaluation correcte de l'état lusitropique du cœur.

RESULTATS

Les travaux de recherche présentés dans cette thèse reposent sur 3 approches expérimentales :

1- les études physio-pharmacologiques (réalisées au sein du Laboratoire de Physiologie Cardiovasculaire de Calabre dirigé par le Pr. B. Tota)
2- les études cellulaires (réalisées dans l'équipe neuropeptides bioactifs de M.H. Metz-Boutigue)
3- l'analyse protéomique (réalisées dans l'équipe neuropeptides bioactifs de M.H. Metz-Boutigue).

Etude des vasostatines et de leurs peptides dérivés

Les études physio-pharmacologiques

Mes travaux ont permis de mettre en évidence un effet inotropique négatif de la VS1 recombinante humaine CGA_{1-78} sur le modèle de cœur de rat Langendorff, et ceci à des concentrations de l'ordre du nanomolaire (Cerra et al., 2005). Ainsi, la VS1 (11 - 165 nM) induit un effet inotropique négatif concentration-dépendent, effet qui est révélé par une réduction significative de la pression ventriculaire gauche (LVP) et associé à

une faible augmentation du rythme cardiaque (HR). A toutes les concentrations testées, la VS1 n'affecte pas la pression coronarienne (CP). Au contraire, des doses croissantes de VS2 humaine recombinante CGA_{1-115} (11-165 nM) ne possèdent pas d'effet sur la LVP et la RPP (Cerra et al., 2005). Nos travaux réalisés sur le cœur de mammifères ont confirmé les résultats obtenus sur le cœur de grenouille et d'anguille, indiquant un effet inotropique négatif de la VS1 (Corti et al., 2004; Imbrogno et al., 2004). Ces études ont également permis de décrire pour la première fois un effet lusitropique négatif pour la VS1 (Pieroni et al., 2005 soumis). De façon remarquable, dans la même préparation de cœur de rat, ce peptide ne contre pas seulement les effets inotropiques positifs des drogues bêta-adrénergiques, comme décrit pour l'anguille et la grenouille (Corti et al., 2004; Imbrogno et al., 2004), mais également les effets positifs exercés par une stimulation bêta-adrénergique sur la relaxation ventriculaire.

A l'heure actuelle, aucun récepteur pour les VSs n'a été décrit (Helle et al., 2001) et nous avons exploré si les actions des VSs sur la physiologie cardiaque impliquaient des mécanismes médiés par des récepteurs ou des interactions avec des protéines membranaires et intracellulaires. Sur le modèle du cœur de rat, le mécanisme inotropique négatif induit par la VS1 est aboli en présence d'agonistes des récepteurs adrénergiques (*i.e.,* phentolamine, propanolol), tandis qu'il n'est pas affecté par des antagonistes cholinergiques (*i.e*., atropine, pirenzepine, AFDX116) (Angelone et al., 2005). Ainsi, nous avons émis l'hypothèse que, l'inotropisme induit par la VS1 pourrait résulter des effets secondaires inhérents aux interactions électrostatiques des séquences poly-glutamiques de la VS1 avec des protéines régulatrices membranaires ou intracellulaires (*e.g*., sites allostériques des récepteurs adrénergiques). Nos travaux

suggèrent que les VSs (*i.e.,* les régions lipophilies) pourraient interagir avec des domaines lipidiques de la membrane plasmique (cavéoles, scavenger receptors, etc…), et ainsi activer une voie de transduction du signal. Un tel mécanisme a été postulé pour les activités anti-bactériennes et antifongiques de la VS1 et de ses fragments dérivés (*i.e*., la chromofungine ; CGA_{47-66}) (Lugardon et al 2001; Maget-Dana et al., 2002). Etant donné que la chromofungine est capable d'inhiber la calcineurine, qui est une phosphatase activée par la calmoduline (Lugardon et al., 2001), les interactions VS-calmoduline pourraient affecter l'activité des canaux ioniques (*e.g*., Ca^{++} and K^{+}) (Lugardon et al., 2001). Ainsi, les changements induits par la VS aux niveaux calcique et potassique pourraient être impliqués lors de l'effet inotropique négatif de ce peptide comme cela a été suggéré par l'étude sur l'anguille (Imbrogno et al., 2004) et la grenouille (Corti et al., 2004). Dans le modèle de cœur de rat Langendorff (Angelone et al., 2004), l'effet inotropique négatif induit par la VS1 est aboli par le blocage des récepteurs bêta-adrenergiques (*i.e*., protéines Gi/o et voie NO-cGMP-PKG), et pas cholinergiques. Sur la base de ces données, un projet de recherche a consisté à étudier le rôle de la VS1 dans la limitation de la nécrose induite par une ischémie et une reperfusion (I/R). Le préconditionnement, résultant d'une ischémie, est un phénomène pour lequel une brève ischémie (2-20 min) peut engendrer des mécanismes induisant à la protection contre les dommages cellulaires du myocarde lors d'une ischémie longue. Nos résultats ont indiqué que la VS1 limite les dommages causés par une I/R, et que cette activité protectrice est liée à la voie du NO. Si ces travaux se confirment, la cardioprotection médiée par la VS1 pourrait représenter un tremplin pour les études futures qui exploreraient le potentiel thérapeutique de cette molécule.

La culture cellulaire

Ces expériences ont été réalisées au cours de mon séjour à Strasbourg (automne 2004) en collaboration avec Dr Yannick Goumon (CR1 Inserm) et Elise Glattard (doctorante). Dans un premier temps, une approche utilisant la culture cellulaire et une technique d'immunocytochimie sur la lignée de cardiomyocytes de rat H9C2, a indiqué que la VS1 induisait une polymérisation du cytosquelette d'actine (10 nM-10 μM), suggérant un rôle important de la VS1 dans la modulation du réseau d'actine (Goumon et al., 2005 manuscrit en cours de rédaction). Au contraire, au cune modification du réseua d'actine est détectable après un traitement des cardiomyocytes par un peptide de la chromogranine B ($CGB_{614-626}$) ou l'isoprotérénol.

L'analyse protéomique

Une analyse protéomique a été réalisée sur des extraits protéiques de cœur de rat afin d'examiner la présence de CGA (et de ses fragments N-terminaux naturellement générés) pouvant exercer de façon endogène un effet modulateur de la physiologie cardiaque. Cette approche a nécessité l'utilisation de plusieurs techniques utilisées couramment dans l'équipe Neuropeptides Bioactifs (HPLC, gels mono et bidimensionnels, spectrométrie de masse TOF-TOF). La technique de spectrométrie de masse a nécessité l'utilisation des installations du laboratoire de Spectrométrie de Masse (Dir. Dr. Alain Van Dorsselaer; Cronembourg).

Les résultats obtenus ont indiqué pour la première fois la présence de fragments de CGA contenant la séquence de la VS1 et possédant différentes modifications posttraductionnelles (phosphorylations et O-glycosylations) (Glattard et al., 2005, soumis à Cardiovascular Research). Les fragments identifiés correspondent aux séquences : 4-113, 1-124, 1-135 et 1-199. La VS1 telle qu'elle est définie par rapport aux séquences de CGA humaine et bovine (CGA_{1-76}) ne peut pas exister chez le rat à cause de l'absence du site dibasique en position 77-78 et son remplacement par une séquence de polyglutamine. La localisation des modifications post-traductionnelles a été comparée avec celle qui avait été proposé par l'équipe Neuropeptides Bioactifs dans le cas des CGA bovine et humaine.

Etude du HCNP

Une autre partie de mon travail a porté sur les activités physio-pharmacologiques de l'Hippocampal Cholinergic Neurostimulatory Peptide (HCNP) sur le cœur de rat. Ces expériences, réalisées dans le laboratoire de Physiologie de l'Université de Calabre, en utilisant un peptide synthétisé dans l'Unité Inserm 575, ont indiqué un rôle inhibiteur de ce peptide sur l'activité contractile du cœur de rat (Angelone et al., 2005).

Nous avons étudié les effets du HCNP sur le cœur de rat (modèle de Langendorff). Cette étude a permis de montrer que ce peptide induisait un effet inotropique négatif de manière concentration dépendante et qui est observé par la diminution significative de la LVP et de la RPP, tandis que ce peptide ne modifie ni la HR, ni la CP. Par ailleurs, le HCNP s'est avéré contrecarrer le chronotropisme résultant

d'une exposition à des drogues bêta-adrénergiques, ainsi que l'inotropisme et la dilatation coronarienne (Angelone et al., 2005). L'analyse de l'interaction entre le HCNP et le système adrénergique a révélé que ce peptide représentait un antagoniste fonctionnel. Par ailleurs, il s'est avéré que l'effet inotropique négatif induit par le HCNP est augmenté en présence d'un stimuli cholinergique (*i.e.,* carbachol), agissant de façon synergique, ce qui démontre l'implication de ce peptide dans la transduction du signal cholinergique au niveau cardiaque. De plus, l'utilisation d'inhibiteurs muscariniques sélectifs a indiqué que le HCNP agissait comme un antagoniste compétitif de l'ACh au niveau du sous-récepteur M_2, sans aucune interaction avec les sous-types de récepteurs M_1-M_3. Ainsi, l'inotropisme médié par le HCNP est dépendante des protéines G. En résumé, ces données suggèrent que dans le modèle de cœur de rat, le HCNP pourrait agir comme un nouveau modulateur cholinergique *via* une interaction avec un système de transduction du signal, passant par une interaction directe avec un système de transduction activé par des proteines G couplées à des récepteurs muscariniques des cardiomyocytes.

CONCLUSIONS

Une étape importante de nos recherches s'est fixé comme objectifs d'examiner si l'action cardiotropique de VS1 implique des récepteurs membranaires ou des interactions avec d'autres protéines intracellulaires. Sur le cœur de rat l'inotropisme négatif de VS1 est aboli par la présence d'antagonistes adrénergiques tandis qu'il est insensible à l'inhibition cholinergique. Dans ces conditions, l'inotropisme induit par VS1 pourrait résulter d'interactions secondaires avec des protéines régulatrices localisées sur des sites allosétriques des récepteurs adrénergique plutôt que d'une interaction directe avec le récepteur lui-même. Ainsi le domaine lipophilique situé dans la séquence de la chromofungine (CGA_{47-66}) peut interagir avec des domaines lipidiques situés sur la membrane plasmique.

En conclusion, à la fois la VS1 et le HCNP, deux molécules impliquées dans le stress, représentent des modulateurs négatifs du cœur et sont capables de contrecarrer les signaux adrénergiques *in vitro*. Si l'on tient compte de ces effets, la VS et le HCNP pourraient agir conjointement pour réguler de manière fine les processus homéostatiques au niveau cardiaque et pourraient agir en tant qu'agents protecteurs vis-à-vis d'une hyperstimulation. Les activités anti-adrénergiques de la VS et du HCNP pourraient avoir une signification lors de réponse au stress, quand des organes tels que le cœur sont des cibles préférentielles des CAs. Etant donné que les séquences de la VS1 et le HCNP sont disponibles pour le design d'analogue, des études physio-pharmacologiques sont envisageables et pourraient avoir un impact biomédical à long terme.

Background

The heart is a major target of both sympathetic (SNSystem) and parasympathetic nervous activities and circulating catecholamines (CAs) under normal and physio-pathological conditions. Elevated CAs levels have long been known to induce necrotic damage in the heart (Raab, 1963; Tan et al., 2003; Goldspink et al., 2004). It is also well known that the initial response of the heart to prolonged and excessive stress is to enlarge morphologically to a state defined as cardiac hypertrophy, i.e. a compensatory adjustment preventing progressive deterioration of cardiac function (Grossman et al., 1975; Chien et al., 1991). However, often, as we have learned in the last decades, the stress will overwhelm the system, leading the hypertrophied heart to failing processes. Concomitantly, in an attempt to control cardiac output and systemic blood pressure, there is sustained heightened activation of the SNS and the Renin-Angiotensin System (RAS) (Esler et al., 1997). Recent evidence indicates that, if left uncontrolled, these excitatory cascades may be more important in the deterioration process than the actual stress placed on the heart. Importantly, in human heart failure, chronic heightened activation of the SNS and associated enhancement of adrenergic signalling pathways *via* the CAs, has adverse prognostic significance and may accelerate the pathological processes (Cohn et al., 1984).

Clearly, the outcome of the studies in this area of cardiovascular research has been of great clinical relevance, providing, for example, the rationale for anti-adrenergic drug therapy, including the beta-blockers, still amongst the world's most used drugs. The present work summarized studies with novel cardiotropic peptides that may represent new natural principles for heart protection under stress conditions. The two molecules, namely, Vasostatins (Vss) and Hippocampal

Cholinergic Neurostimulating Peptide (HCNP), are stored in the chromaffin granules from the adrenal medulla. VSs are known since several years while Hippocampal Cholinergic Neurostimulating Peptide (HCNP) is a novel peptide characterized by the research group at INSERM (Strasbourg) laboratories (Goumon et al., 2004).

Vasostatins. Chromogranin-A (CGA), the major member of the Chromogranin family, and other water-solubles proteins such proenkephalin-A, are co-stored with the CAs in the secretory granules of the chromaffin cells of the adrenal medulla and the diffuse neuroendocrine system present in many organs including the vasculature and the heart (Steiner et al., 1990). Upon adequate stimuli, particularly adrenergic stimulation, CGA and other secretory proteins are co-released exocytotically with CAs either in the circulation or at tissue level, and are predominantly processed by pro-hormone convertases in a tissue specific manner (Metz-Boutigue et al., 1993), thereby giving rise to several peptides. The best chemically and functionally characterized peptides have been the major N-terminal CGA-derived fragments, which include the vasostatins (VSs) corresponding to the bovine N-terminal sequences CGA 1-76 (vasostatin I; VS1) and CGA 1-113 (vasostatin II; VS2) and derive from cleavage at the first and second pair of basic amino acid residues. They have been so named for their ability to maintain blood flow by reducing the vascular tone, counteracting several vasoconstrictor agents, including noradrenaline (NE) (Helle, 2001). To characterize the cardiac actions of the peptides we have used the Langendorff preparation. This preparation allows the simultaneous analysis of the effects of cardioactive agents both on the myocardial performance (i.e, chronotropism and inotropism) and on the coronary motility. Lastly,

the perfusion at isovolumic conditions also allows a correct evaluation of the lusitropic state of the heart.

Physio-pharmacological studies. This part of the research has been carried out in the Laboratories of Cardiovascular Physiology of University of Calabria. These studies have demonstrated, on the Langendorff rat heart, a negative inotropic effect of human recombinant STA-CGA1-78 (here and throughout the text named VS1) (nanomolar range) (Cerra et al., 2005). VS1 (11 ÷165 nM) caused a concentration-dependent negative inotropic effect, revealed by a significant reduction of Left Ventricular Pressure (LVP), associated with a non significant small increase of Heart Rate (HR). The peptide significantly reduced Rate Pressure Product (RPP). At all concentrations, VS1 did not affect Coronary Pressure (CP). In contrast, increasing doses of human recombinant STA-CGA1-115 (named VS2) (11÷165 nM) did not change LVP and RPP (Cerra et al., 2005). Thus, we confirm on the mammalian heart the vasostatin-induced negative inotropic effects observed by our lab on eel and frog (Corti et al., 2004; Imbrogno et al., 2004). We also describe for the first time a negative lusitropic effect produced by VS1 (Pieroni et al., 2005 submitted). Remarkably, in the same rat heart preparation, the peptide counteracted not only the positive inotropic actions of beta-adrenergic drugs, in agreement with the findings reported in eel and frog (Corti et al., 2004; Imbrogno et al., 2004), but also the positive effects exerted by beta-adrenergic stimulation on ventricular relaxation.

Since at the moment nothing is known about the presence of specific receptors for VSs (Helle et al., 2001), we have also explored if their cardiotropic action involves a receptor-mediated mechanism or interaction with inter-and/or intracellular proteins. On

the rat heart, the VS1-dependent negative inotropism is abolished in the presence of alfa and beta adrenoreceptor antagonists (i.e., phentolamine, propanolol), while it is unaffected by cholinergic blockade (i.e., atropine, pirenzepine, AFDX116) (Angelone et al., 2005). Thus, we have hypothesized that rather than being directly related with a specific adrenergic receptor antagonism, the VS1-induced inotropism could be due to secondary interactions by electrostatic linkage between VS1 and inter- or intracellular regulatory proteins affecting, for example, allosteric sites of the adrenergic receptors. Indeed, the present evidence suggests that VSs through their lipophilic region may interact with some plasma membrane lipid domains (caveolae, scavenger receptors, etc…), thereby activating inter- and/or intracellular signal-transduction pathways. Such mechanism has been postulated for the antifungal and antibacterial activities displayed by VS1 and its derived fragments, particularly chromofungin, i.e. CGA47-66 (Lugardon et al 2001; Maget-Dana et al., 2002). Since chromofungin is also able to inhibit calcineurin, a phosphatase-activated calmodulin (Lugardon et al., 2001), VSs-calmodulin interactions may affect ion (e.g. Ca^{++} and K^{+}) channel activities (Lugardon et al., 2001). Conceivably, VS-induced calcium and potassium changes could be involved in the VS-mediated negative inotropism, as suggested by our work on eel (Imbrogno et al., 2004) and frog (Corti et al., 2004) hearts. In Langendorff rat heart preparations (Angelone et al., 2004) VS1-dependent negative inotropic effect is abolished by the blockade of β-adrenergic receptors, Gi/o protein and NO-cGMP-PKG pathway, but not by the cholinergic ones. On the basis of the above findings, we have also started to investigate a possible role of VS1 in limiting the extent of the necrosis induced by ischaemia and reperfusion (I/R). Ischemic preconditioning is a phenomenon

in which brief periods (2-20 min) of ischemia can evoke mechanisms leading to protection against myocardial cellular damage in the event of a subsequent prolonged period of ischemia. The results show that VS1 limits the damage caused by I/R and that the protective activity is mediated by NO signalling. The application of cardioprotective strategies, being clinically feasible, could represent a valuable tool in the hands of clinicians better than preconditioning. If confirmed, VS1-mediated cardioprotection, could provide the starting point for further studies designed to explore its potential therapeutical importance.

Cell culture and proteomic analysis. The studies on cell culture and the proteomic investigations were performed in the Inserm U575 "Physiopatologie du Système Nerveux" in the context of the Doctorate on co-tutelage in "Animal Biology" and "Aspects Moléculaires et Cellulaires de la Biologie" between the University of Calabria (Italy) and the Universitè Louis Pasteur of Strasbourg (France). Using cell culture and immunochemistry of cell line H9C2, we have found that VS1 induced remarkably actin polymerization at all concentration tested (10 nM÷10 μM), suggesting an important role of VS1 in modulating actin microfilaments network (Goumon et al., 2005 manuscript in preparation).

Futhermore, in collaboration with Inserm U575 we have used proteomic techniques including HPLC reverse phase chromatography, Western blot and tandem mass spectrometry analysis (TOF-TOF) in order to characterize endogenous N-terminal CGA-derived peptides in rat heart extract. The results have indicated for the first time the presence of new VS-containing peptides with different posttranslational

modifications such as phosphorilations and O-glycosylations (Glattard et al., 2005, submitted).

Another part of the research activities in collaboration with the Inserm laboratories (Strasbourg) has regarded the analysis of the physio-pharmacological effects of HCNP on the rat heart. The functional experiments, performed in the laboratories of Physiology at University of Calabria using HCNP synthesised at the Inserm U575, have revealed an inhibitory role of the peptide on the cardiac contractile performance of the rat (Angelone et al., 2005).

HCNP. Another novel aspect of the crosstalk between the nervous system and the heart is related to the PhosphatidylEthanolamine-Binding Protein (PEBP). In particular, it has been demonstrated that PEBP, a 21-kDa protein, ubiquitously distributed in the animal kingdom (Goumon et al., 2004), is precursor of a maturation product, the N-terminal Hippocampal Cholinergic Neurostimulatory Peptide. Importantly, PEBP, alternatively named Raf-1 Kinase Inhibitor Protein (RKIP) since it interacts with Raf 1, is involved in several signalling systems, including heterometric G proteins modulation (Vallée et al., 2003). The natural N-terminal peptide of mammalian PEBP is the undecapeptide "Hippocampal cholinergic neurostimulatory peptide" (HCNP), so named since it was purified from the rat hippocampus where it enhanced acetylcholine synthesis (Ojika et al., 2000). More recently, we have shown that both PEBP and HCNP are co-stored with CAs in the chromaffin secretory vesicles of the adrenal medulla, being then co-released in the circulation together with CAs upon adrenergic stimulation (Goumon et al., 2004). These results, together with the wide organ-tissue distribution of PBEP and HCNP, suggest that these substances may represent novel endocrine-humoral

modulators. In an attempt to test this hypothesis at the heart level, we have used as bioassay the isolated working frog heart, showing for the first time that HCNP exerts indeed inhibitory modulation on the contractile myocardial performance (i.e. exerts negative inotropism). At same time, it also counteracts the positive contractile action (positive inotropism) of classical beta-adrenergic agonists (isoproterenol; ISO) (Goumon et al., 2004).

More recently, we have studied the effects of HCNP on the rat heart (Langendorff isolated preparation). It has been demonstrated that HCNP caused a concentration-dependent negative inotropic effect, revealed by a significant decrease of LVP and the RPP, while it did not modify HR and CP. In addition, HCNP is able to counteract the beta-adrenergic-mediated positive chronotropism, inotropism and coronary dilation (Angelone et al., 2005). The analysis of the interactions between HCNP and the adrenergic system has revealed that the peptide exerts a functional but not receptorial competitive antagonism. The findings that HCNP-dependent negative inotropism is enhanced in presence of cholinergic stimuli (*i.e.* carbachol), acting in a synergic fashion, demonstrate the undecapeptide involvement in the cardiac cholinergic signal-transduction pathway. Moreover, using selective muscarinic subtype inhibitors, we provide evidence that HCNP acts as competitive antagonist of ACh at the M_2 subtype receptors level, without any significant interaction with the M_1-M_3 receptor subtypes. Remarkably, the HCNP-mediated inotropism is G protein-dependent. Taken together, these data suggest that in the rat heart HCNP may act as a new cholinergic modulator through direct interaction with the signal-transduction system activated by G-protein-coupled muscarinic receptor localized at the myocardiocyte sarcolemmal level.

Finally, both VSs and HCNP, two molecules important for the stress reaction, appear to act as negative heart modulators and are able to counteract the adrenergic signals. Taking into account these relevant and entirely unexpected effects, I postulate that VSs and HCNP may act together to finely tune at organ, tissue and cell level several homeostatic processes. Notably, they may act as potential counter principles for the protection of the organism against overstimulation at several levels. The "anti-adrenergic" actions of both VSs and HCNP may be of relevance in the stress response, when some organs like the heart are preferential targets of the CAs. Data on this aspect may represent a prerequisite for postulating, in the heart, these peptides as new modulators, exerting a homeostatic counteraction towards the adrenergic stimulation. Since the primary peptide sequences of VSs and HCNP are already available for analogue designs, physio-pharmacological studies in this direction may have interesting biomedical/medicinal impact.

Background in French

Mise en évidence du rôle de la Vasostatine-1 et de l'Hippocampal Cholinergic Neurostimulating Peptide en tant que nouveaux facteurs anti-adrénergiques

Le cœur représente une cible majeure des systèmes sympathique (SNS) et parasympathique, ainsi que des catécholamines (CAs) circulantes, à la fois lors de conditions normales et pathologiques. Un taux élevé de CAs est décrite pour induire des dommages nécrotiques au niveau du cœur (Raab, 1963; Tan et al., 2003; Goldspink et al., 2004). Ainsi, la réponse initiale du muscle cardiaque à un stress excessif et massif se traduit par une hypertrophie cardiaque (*i.e.,* un ajustement compensatoire prévenant une détérioration progressive de la fonction cardiaque) (Grossman et al., 1975; Chien et al., 1991). Cependant, depuis une dizaine d'année, il est apparu que le stress affecte profondément le système cardiaque, conduisant le cœur hypertrophié à une défaillance. De façon concomitante, afin de contrôler les volumes sanguins sortant du cœur et la pression sanguine systémique, il existe une activation du SNS et du système Renine-Angiotensine (RAS) (Esler et al., 1997). Des évidences récentes ont également indiquées que, sans contrôle, ces cascades excitatrices ont des répercussions plus importantes que le stress cardiaque initial sur le processus de détérioration. Chez l'homme, lors d'une défaillance cardiaque, une intensification chronique de l'activation du SNS et des voies de signalisation adrénergique (*via* les CAs), représentent des pronostiques négatifs et peuvent accélérer les processus pathologiques (Cohn et al., 1984).

Les recherches réalisées dans le domaine cardiovasculaire ont des applications cliniques évidentes. Elle permettent, par exemple la rationalisation des thérapies utilisant des substances affectant le système adrénergique, telle que l'utilisation des bêta-bloquants. Ce document résume les études réalisées à l'aide de deux nouveaux peptides cardiotrophiques qui pourraient représenter de nouvelles molécules naturelles cardioprotectrices lors d'états de stress. Ces molécules, la Vasostatine 1 (VS1) et l'Hippocampal Cholinergic Neurostimulating Peptide (HCNP), sont stockées avec les CAs dans les granules de sécrétion des cellules chromaffines de la médullo-surrénale.

Les vasostatines. Parmi les composants solubles de la matrice des granules de sécrétion des cellules chromaffines de la médullo-surrénale, la chromogranin-A (CGA) représente la protéine majeure. Elle est stockée avec d'autres protéines incluant la proenképhaline, ainsi qu'avec les CAs dans les granules chromaffines, mais également dans le système neuroendocrine diffus qui est présent dans de nombreux organes incluant le cœur (Steiner et al., 1990). Lors d'un stress ou d'une stimulation, la CGA et ses peptides dérivés sont secrétés avec les catécholamines dans la circulation sanguine ou au niveau tissulaire.

La CGA est une prohormone qui est soumise à une protéolyse intragranulaire, qui permet la génération de nombreux peptides bioactifs. Cette maturation qui est tissu spécifique est principalement réalisée par les Prohormones Convertases (Metz-Boutigue et al., 1993). Les peptides dérivés de la CGA les mieux caractérisés d'un point de vue biologique correspondent à des fragments qui dérivent de la partie N-terminale de la protéine et qui incluent la vasostatine I (CGA 1-76 ; VS1) et II (CGA 1-113 ; VS2). Les

VSs sont connues pour leur capacité à réduire le tonus vasculaire, pour antagoniser différents agents vasoconstricteurs incluant la noradrénaline (NE) (Helle et al., 2001). Afin de caractériser les propriétés cardiomodulatrices de ces peptides, nous avons utilisé le modèle de cœur de rat isolé et perfusé, également appelé modèle de Langendorff. Ce modèle permet d'analyser simultanément les effets d'agents cardioactifs, à la fois sur les performances myocardiaques (*i.e,* chronotropisme et inotropisme) et sur la motilité coronarienne. Dans ce modèle, la perfusion réalisée dans des conditions isovolumiques permet une évaluation correcte de l'état lusitropique du cœur.

Etudes Physio-pharmacologiques. Cette partie de mes recherches a été réalisée au sein du Laboratoire de Physiologie Cardiovasculaire de Calabre dirigé par le Pr. B. Tota. Mes travaux ont permis de mettre en évidence un effet inotropique négatif de la VS1 recombinante humaine CGA1-78 sur le modèle de cœur de rat Langendorff, et ceci à des concentrations de l'ordre du nanomolaire (Cerra et al., 2005). Ainsi, la VS1 (11 -165 nM) induit un effet inotropique négatif concentration-dépendent, effet qui est révélé par une réduction significative de la pression ventriculaire gauche (LVP) et associé à une faible augmentation du rythme cardiaque (HR). A toutes les concentrations testées, la VS1 n'affecte pas la pression coronarienne (CP). Au contraire, des doses croissantes de VS2 humaine recombinante CGA1-115 (11-165 nM) ne possèdent pas d'effet sur la LVP et la RPP (Cerra et al., 2005). Nos travaux réalisés sur le cœur de mammifères ont confirmé les résultats obtenus sur le cœur de grenouille et d'anguille, indiquant un effet inotropique négatif de la VS1 (Corti et al., 2004; Imbrogno et al., 2004). Ces études ont également permis de décrire pour la première fois un effet lusitropique négatif pour la VS1 (Pieroni et al., 2005 soumis). De façon

remarquable, dans la même préparation de cœur de rat, ce peptide ne contre pas seulement les effets inotropiques positifs des drogues bêta-adrénergiques, comme décrit pour l'anguille et la grenouille (Corti et al., 2004; Imbrogno et al., 2004), mais également les effets positifs exercés par une stimulation bêta-adrénergique sur la relaxation ventriculaire.

A l'heure actuelle, aucun récepteur pour les VSs n'a été décrit (Helle et al., 2001) et nous avons exploré si les actions des VSs sur la physiologie cardiaque impliquaient des mécanismes médiés par des récepteurs ou des interactions avec des protéines membranaires et intracellulaires. Sur le modèle du cœur de rat, le mécanisme inotropique négatif induit par la VS1 est aboli en présence d'agonistes des récepteurs adrénergiques (*i.e.,* phentolamine, propanolol), tandis qu'il n'est pas affecté par des antagonistes cholinergiques (*i.e*., atropine, pirenzepine, AFDX116)(Angelone et al., 2005). Ainsi, nous avons émis l'hypothèse que, l'inotropisme induit par la VS1 pourrait résulter des effets secondaires inhérents aux interactions électrostatiques des séquences poly-glutamiques de la VS1 avec des protéines régulatrices membranaires ou intracellulaires (*e.g*., sites allostériques des récepteurs adrénergiques). Nos travaux suggèrent que les VSs (*i.e.,* les régions lipophilies) pourraient interagir avec des domaines lipidiques de la membrane plasmique (cavéoles, scavenger receptors, etc…), et ainsi activer une voie de transduction du signal. Un tel mécanisme a été postulé pour les activités anti-bactériennes et antifongiques de la VS1 et de ses fragments dérivés (*i.e*., la chromofungine ; CGA47-66) (Lugardon et al 2001; Maget-Dana et al., 2002). Etant donné que la chromofungine est capable d'inhiber la calcineurine, qui est une phosphatase activée par la calmoduline (Lugardon et al., 2001), les interactions VS-

calmoduline pourraient affecter l'activité des canaux ioniques (*e.g.*, Ca^{++} and K^{+}) (Lugardon et al., 2001). Ainsi, les changements induits par la VS aux niveaux calcique et potassique pourraient être impliqués lors de l'effet inotropique négatif de ce peptide comme cela a été suggéré par l'étude sur l'anguille (Imbrogno et al., 2004) et la grenouille (Corti et al., 2004). Dans le modèle de cœur de rat Langendorff (Angelone et al., 2004), l'effet inotropique négatif induit par la VS1 est aboli par le blocage des récepteurs bêta-adrenergiques (*i.e.*, protéines Gi/o et voie NO-cGMP-PKG), et pas cholinergiques. Sur la base de ces données, un projet de recherche a consisté à étudier le rôle de la VS1 dans la limitation de la nécrose induite par une ischémie et une reperfusion (I/R). Le préconditionnement, résultant d'une ischémie, est un phénomène pour lequel une brève ischémie (2-20 min) peut engendrer des mécanismes induisant à la protection contre les dommages cellulaires du myocarde lors d'une ischémie longue. Nos résultats ont indiqué que la VS1 limite les dommages causés par une I/R, et que cette activité protectrice est liée à la voie du NO. Si ces travaux se confirment, la cardioprotection médiée par la VS1 pourrait représenter un tremplin pour les études futures qui exploreraient le potentiel thérapeutique de cette molécule.

Culture cellulaire et analyses protéomiques. Les études utilisant les cultures cellulaires et les analyses protéomiques ont été réalisées dans l'unité Inserm 575 "Physiopathologie du Système Nerveux" dirigée par le Dr. D. Aunis dans le cadre de la cotutelle entre l'université de Calabre et l'Université Louis Pasteur de Strasbourg (Pr. B. Tota et M.H. Metz-Boutigue).

Dans un premier temps, une approche utilisant la culture cellulaire et une technique d'immunocytochimie sur la lignée de cardiomyocytes de rat H9c2, a indiqué

que la VS1 induisait une polymérisation du cytosquelette d'actine (10 nM-10 μM), suggérant un rôle important de la VS1 dans la modulation du réseau d'actine (Goumon et al., 2005 résultats non publiés).

D'autre part, une étude utilisant les techniques de protéomie (HPLC, gels mono et bidimensionnels, spectrométrie de masse Time of Flight- Time of Flight) a été utilisée afin d'étudier les fragments de CGA N-terminaux présents dans des extraits de cœur de rat. Ces résultats ont indiqué pour la première fois la présence de fragments contenant la séquence de la VS1 et possédant différentes modifications posttraductionnelles (phosphorylations et O-glycosylations) (Glattard et al., 2005, soumis).

Une autre partie de la collaboration établie entre l'équipe du Dr. M.H. Metz-Boutigue a permis d'étudier les activités physio-pharmacologiques de l'Hippocampal Cholinergic Neurostimulatory Peptide (HCNP) sur le cœur de rat. Ces expériences, réalisées dans le laboratoire de Physiologie de l'Université de Calabre, en utilisant un peptide synthétisé dans l'Unité Inserm 575, ont indiqué un rôle inhibiteur de ce peptide sur l'activité contractile du cœur de rat (Angelone et al., 2005).

HCNP. La PEBP (PhosphatidylEthanolamine-Binding Protein), protéine de 21kDa, est distribuée de manière ubiquitaire dans le règne animal (Goumon et al., 2004) et est le précurseur du HCNP. Cette protéine, également appelée Raf-1 Kinase Inhibitor Protein (RKIP) du fait de son interaction avec Raf-1, est impliquée dans plusieurs voies de signalisation incluant la modulation des protéines G hétérotrimériques (Vallée et al., 2003). Le HCNP, qui correspond aux 11 premiers résidus de la PEBP, est un peptide généré dans l'hippocampe et qui est décrit pour augmenter la synthèse d'acétylcholine

(Ojika et al., 2000). Plus récemment, nous avons montré qu'à la fois la PEBP et le HCNP sont stockés avec les catécholamines dans les granules de sécrétion des cellules chromaffines de la médullo-surrénale bovine. Ceux-ci sont cosécrétés avec les catécholamines par les cellules chromaffines lors d'une stimulation (Goumon et al., 2004). Ces résultats, en plus des données indiquant la présence de la PEBP dans de nombreux organes, suggèrent que ces deux molécules pourraient représenter de nouveaux médiateurs endocrines. Afin de tester cette hypothèse au niveau cardiaque, nous avons utilisé le modèle du cœur de grenouille isolé et perfusé. Ces travaux ont indiqué que, dans ce modèle, le HCNP exerce un effet inotropique négatif sur la performance cardiaque. Par ailleurs, le HCNP est également capable de contrecarrer l'action contractile positive (inotropisme positif) des agonistes bêta-adrenergiques (*i.e.,* isoprotérénol) (Goumon et al., 2004).

Plus récemment, nous avons étudié les effets du HCNP sur le cœur de rat (modèle de Langendorff). Cette étude a permis de montrer que ce peptide induisait un effet inotropique négatif de manière concentration dépendante et qui est observé par la diminution significative de la LVP et de la RPP, tandis que ce peptide ne modifie ni la HR, ni la CP. Par ailleurs, le HCNP s'est avéré contrecarrer le chronotropisme résultant d'une exposition à des drogues bêta-adrénergiques, ainsi que l'inotropisme et la dilatation coronarienne (Angelone et al., 2005, soumis). L'analyse de l'interaction entre le HCNP et le système adrénergique a révélé que ce peptide représentait un antagoniste fonctionnel. Par ailleurs, il s'est avéré que l'effet inotropique négatif induit par le HCNP est augmenté en présence d'un stimuli cholinergique (*i.e.,* carbachol), agissant de façon synergique, ce qui démontre l'implication de ce peptide dans la transduction du signal

cholinergique au niveau cardiaque. De plus, l'utilisation d'inhibiteurs muscariniques sélectifs a indiqué que le HCNP agissait comme un antagoniste compétitif de l'ACh au niveau du sous-récepteur M_2, sans aucune interaction avec les sous-types de récepteurs M_1-M_3. Ainsi, l'inotropisme médié par le HCNP est dépendante des protéines G. En résumé, ces données suggèrent que dans le modèle de cœur de rat, le HCNP pourrait agir comme un nouveau modulateur cholinergique *via* une interaction avec un système de transduction du signal, passant par une interaction directe avec un système de transduction activé par des proteines G couplées à des récepteurs muscariniques des cardiomyocytes.

En conclusion, à la fois la VS1 et le HCNP, deux molécules impliquées dans le stress, représentent des modulateurs négatifs du cœur et sont capables de contrecarrer les signaux adrénergiques *in vitro*. Si l'on tient compte de ces effets, la VS et le HCNP pourraient agir conjointement pour réguler de manière fine les processus homéostatiques au niveau cardiaque et pourraient agir en tant qu'agents protecteurs vis-à-vis d'une hyperstimulation. Les activités anti-adrénergiques de la VS et du HCNP pourraient avoir une signification lors de réponse au stress, quand des organes tels que le cœur sont des cibles préférentielles des CAs. Etant donné que les séquences de la VS1 et le HCNP sont disponibles pour le design d'analogue, des études physio-pharmacologiques sont envisageables et pourraient avoir un impact biomédical à long terme.

Introduction

1. The Chromaffin cells.

Chromaffin cells of the adrenal medulla store catecholamines, nucleotides and proteins, the chromaffin vesicles (Trifaro, 1977). Upon stimulation, the soluble contents of the vesicles are released into the cell exterior by exocytosis (Sudhof and Jahn, 1991). Chromaffin vesicles are true organelles, which, in addition to their soluble contents (material for export), contain enzymes involved in catecholamine synthesis (i.e. dopamine β-hydroxylase), in maintaining a proton gradient across their membranes (i.e. ATPase), a catecholamine carrier, and a set of proteins (e.g. syntaxin and SNAP25) and cytosolic factors (NFS) to form the SNARE complex (Sudhof and Jahn, 1991). Chromaffin vesicles are present in chromaffin cells in at least two compartments: the release-ready vesicle pool and the reserve pool (Vitale et al., 1995). The traffic of vesicles between these compartments is subject to a fine regulation. Experimental evidence has suggested that a cortical F-actin filament network plays an important role in this regulation (Vitale et al., 1995).

Chromogranins are a group of acidic soluble proteins present in the core of catecholamine-storage vesicles. These proteins are widely distributed thoughout the neuroendocrine system. Three major proteins are well characterized: chromogranin A (CGA), chromogranin B (CGB) and secretogranin II (SGII). CGA is a prohormone precursor ans its proteolytic cleavage gives rise to numerous peptides that modify cellular functions (Helle et al., 1993). In the chromaffin cells CGA is also localized with other neuropeptides such as proenkephalin-A, neuropeptide Y, and phosphatidylethanolamine-binding protein (PEBP) (Goumon et al., 2004). PEBP, a

21-kDa protein is ubiquitously distributed in the animal kingdom (Goumon et al., 2004) and is precursor of a N-terminal peptide, the Hippocampal cholinergic neurostimulatory peptide (HCNP). Interestingly, both CGA and CGA-derived peptides, as well as PEPB and HCNP are co-stored with CAs in the chromaffin cells of the adrenal medulla, the diffuse neuroendocrine system and within several organs, including the heart. Upon adrenergic stimulation, all these proteins and peptides are co-released in the circulation together with CAs. This suggests that these peptides may represent novel endocrine agents.

The integrity of the catecholaminergic pathways is essential for a variety of normal and stress-induced homeostatic processes in humans. Alterations in these pathways, resulting in changes in circulating catecholamines or in other molecules co-stored and co-released with catecholamines, may lead to profound changes in autonomic, cardiovascular, neuroendocrine, metabolic and immune function (Cryer, 1980). The abnormalities in these systems have important implications for a variety of human diseases. Therefore molecules associated with catecholaminergic function may provide novel diagnostic and therapeutic strategies for human disease (Cryer, 1980).

2. The chromaffin cells as source of cardioregulatory peptides.

The secretory granules of the chromaffin cells, i.e. the neural crest-derived cells constituting the adrenal medulla and the diffuse neuroendocrine system, contain catecholamines (CAs) and a complex mixture of proteins and peptides (Goumon et al., 1998). The latter include several neuropeptides (e.g. neuropeptide Y and vasointestinal peptide) and prohormones. Upon adequate stimuli, these peptides are co-released with CAs either in the circulation from the adrenal medulla, or at organ level from the local endocrine, neuroendocrine and neuronal cells, thereby participating in a number of important homeostatic processes. In the last decade the main emphasis has been placed on the biochemical and functional characterization of the major constituents of the high molecular mass water-soluble proteins of the chromaffin granules, the chromogranin family and proenkephalin-A.

The chromogranin family consists of chromogranin A (CGA) and B (CGB) (Aunis and Metz-Boutigue, 2000). CGA, ubiquitously distributed in many mammalian tissues, including the heart (Steiner et al., 1990), is released exocytotically and rapidly processed by pro-hormone convertases and other proteases in a tissue-specific manner (Metz-Boutigue et al., 1993), giving rise to several peptides.

CGA is the precursor of numerous derived-peptides displaying several biological effects (Iacangelo and Eiden, 1995; Helle, 2004). Its sequence is

characterized by an high abundance of acidic residues and multiple consecutive basic residues, as well as monobasic residues representing cleavage sites for endopeptidases and carboxypeptidases (Metz-Boutigue et al., 1993). The kexin/subtilisin-like prohormone convertases PC1 and PC2 were shown to generate various CGA-derived fragments (Doblinger et al., 2003; Eskeland et al., 1996).

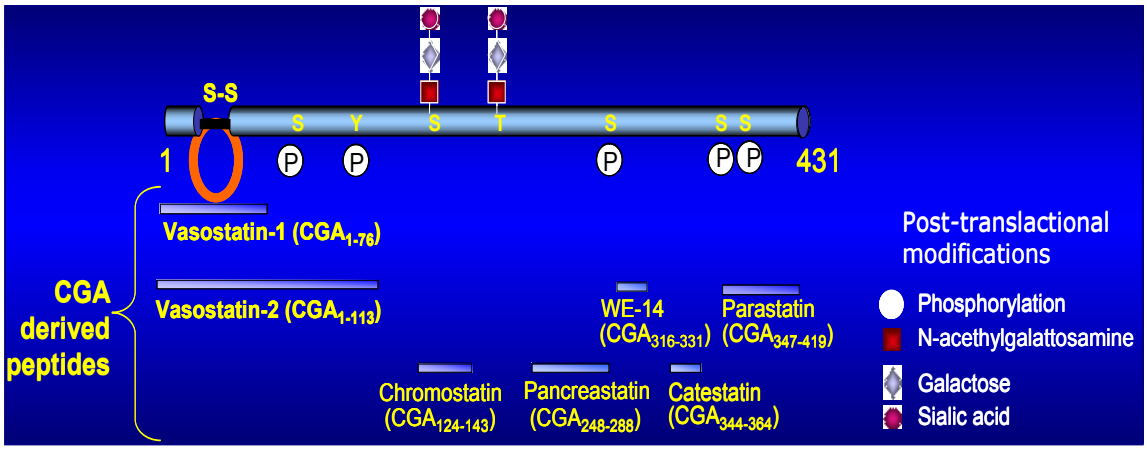

Fig.1 Chromogranin-A (human and bovine) sequence as precursor of small peptides (modified from Metz-Boutigue et al., 1993 and Taupenot et al., 2003).

CGA undergoes posttranslational modifications that were investigated in different species (human and bovine), leading to the characterization of phosphorylations and O-glycosylations modifications (Bauer et al., 1999; Gadroy et al., 1998; Strub et al., 1997; Zhang et al., 1997). As shown on bovine adrenal chromaffin cell model, it has been shown that CGA possess two O-glycosylations on residue Ser_{186} and Thr_{231} (Strub et al., 1997). Other studies have described the presence of two O-glycosylations on Ser_{167} and Thr_{231} (Bauer et al., 1999). Human CGA shares different posttranslational modifications and as shown in urine from cancer patients, CGA possesses di-, tri- and tetrasaccharids (Gadroy et al., 1998).

Since, CGA and its derived fragments are released into the blood (Nobels et al., 1998) and CGA levels are increased in cardiac pathology (Ceconi et a ,2002), the biological roles of these peptides were investigated. CGA-derived peptides, i.e. catestatin, pancreastatin, parastatin, vasostatins I and II, have been proposed as novel counter-regulatory hormones involved in adrenomedullary CAs release, plasma glucose and plasma calcium balance, innate immunity and vascular motility (Helle et al., 2001; Helle, 2004). Specifically, pancreastatin and catestatin have been suggested as components of a "zero steady-state error" counter-regulatory homeostatic mechanism (Koeslag et al., 1999). According to Koeslag and co-workers (1997), homeostasis is dependent on pairs of counter-regulatory hormones acting as a unit. There is evidence that the counter-regulatory pairs of hormones operate as 'integral' controllers. This means that during any steady-state disturbance, the controller always brings the controlled variable back to 'set point' (Koeslag et al. 1997; Saunders et al. 1998).

Vasostatin-1 (VS1) and vasostatin-2 (VS2) correspond respectively to the polypeptides CGA 1-76 and CGA 1-113 derived from cleavage at the first and second pair of basic amino acid residues of the N-terminal domain of CGA. The name Vasostatins (VSs) derives from their ability to maintain blood flow by reducing vascular contraction evoked in arterial and venous preparations by both high potassium and agonists such noradrenalin and endothelin (Aardal and Helle, 1992; Angeletti et al., 1994). Accordingly, they have been suggested as regulatory peptides protecting the vascular system against intense excitatory stimuli, such as those acting under stress responses (Helle and Angeletti, 1998). The finding that abnormal levels

of circulating CGA in patients with chronic heart failure represent an independent prognostic indicator of mortality and depend on the severity of the disease stresses the postulated role of VSs as stabilizers in cardio-circulatory homeostasis (Ceconi et al., 2002). Very recently, in absence of any information regarding their actions on the heart, we have studied the *in vitro* effects of exogenous VS1 and VS2 on the cardiac performance of eel, frog, and rat hearts as paradigms of differently arranged vertebrate hearts. Our work has indeed shown that the heart can also be an important target for VSs, since both peptides exert negative contractile effects (i.e. negative inotropism) under basal conditions, while counteracting the positive inotropism elicited by the beta-adrenergic agonist isoproterenol (ISO) (Corti et al., 2002; Imbrogno et al., 2004; Tota et al., 2003; Cerra et al., 2005). Moreover, structure-function analysis has revealed that the beta-adrenergic-mediated inotropism is potently antagonized by the intact STA-CGA 1-78, by the shorter fragment CGA7-57 and by the N-terminus CGA1-40 with intact disulfide bridge (Tota et al., 2003).

Another novel aspect of the crosstalk between the nervous system and the heart is related to the **PhosphatidylEthanolamine-Binding Protein (PEBP).** In particular, it has been demonstrated that PEBP, a 21-kDa protein, ubiquitously distributed in the animal kingdom (Goumon et al., 2004), is precursor of a maturation product, the **Hippocampal Cholinergic Neurostimulatory Peptide (HCNP).** Identified in wide range of organisms such as bacteria, yeast, plants, nematodes, drosophila and mammals (human, rat, mouse, and bovine) (Shoentgen and Jollès, 1995), it has been detected in many tissues including rat liver, kidney, testis, brain and adrenal gland, human platelets (Frayne et al., 1999; Ojika et al., 2000). PEBP has

been alternatively named Raf-1kinase inhibitor protein (RKIP), since it interacts with Raf 1(Yeung et al., 1999). Members of this protein family are involved in several signalling systems as documented by the mammalian PEBP, which has been shown to modulate the action of heterometric G proteins and to inhibit several serine proteases as well as the MAP kinase and the NF-kB pathways (Vallée et al., 2003). Particular attention has been focused on the N-terminal part of mammalian PEBP, the undecapeptide "Hippocampal cholinergic neurostimulatory peptide" (HCNP) (Fig. 2). HCNP is so named since it was purified from the rat hippocampus and enhanced acetylcholine (ACh) synthesis of medial septal nuclei in an explant culture system (Ojika and Appel, 1984; Ojika et al., 2000) (Fig. 2).

In cholinergic neurons, this action of HCNP results from a dose-dependent and time-dependent increase of both activity and Vmax of the enzyme choline acetyltransferase (ChoATase). In addition, there is evidence that HCNP induces the key enzyme required for ACh synthesis and influences the development of cholinergic phenotypes (Ojika et al., 1994).

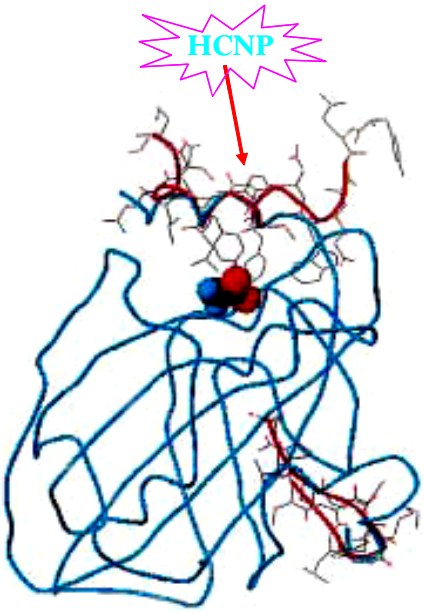

Fig. 2 Structural features of N-Pept and C-Pept of PEBP, blue tube; N-Pept (HCNP) and C-Pept red tube; the putative protein active site is materialised by the space filled representation of the acetate ligand (modify from Vallèe et al., 2003).

Studies on the relationships between structural and physicochemical features of HCNP and its precursor protein highlight an important dual function of this undecapeptide (Vallée et al., 2003). First, being the N-terminal region of PEBP, HCNP is able to interact with negatively charged membranes and, at the same time, by its hydrogen-bonding ability, it participates in the conformation and stability of the active site of PEBP. Second, after its specific cleavage, HCNP, free in solution, appears well structured at least in its central region (D3LSKW7), the conformation in this region being similar to that observed in crystallized PEBP (Vallée et al., 2003). Therefore, free HCNP is well suited to act as signal in target cells and tissues, thereby contributing to some of the biological functions of PEBP (Vallée et al., 2003).

Apart from their presence in the nervous tissue, both PEBP (e.g., adult rat: Frayne et al., 1999) and HCNP (e.g., young rat: Katada et al., 1996) have been immunolocalized in other organs and tissues, including the wall of blood vessels, the adrenal gland, the intestine and the kidney. More recently, using the medulla of bovine adrenal gland as a well characterized secretion model, Goumon et al. (2004) have demonstrated that PEBP and HCNP are also present in chromaffin cells (intragranular matrix) from which they are secreted with catecholamines into the circulation. In addition, they have been detected also in platelet secretion and in serum (Goumon et al., 2004), in the cerebrospinal fluid of Alzheimer patients (Tsugu et al., 1998) and in murine adipocytes (Kratchmarova et al., 2002). This wide organ and tissue distribution of HCNP and PEBP, together with their co-localization in the secretory granules of the chromaffin cells with other neuropeptides and

chromogranins (Kieffer et al., 2003), strongly suggest that HCNP and its precursor may represent novel endocrine agents. Using as bioassay an isolated and perfused frog heart preparation, our group has shown for the first time that HCNP exerts under basal conditions negative inotropism from 10^{-11} to 10^{-7} M and counteracts the adrenergic positive inotropism of ISO (Goumon et al., 2004). Importantly, these results have been confirmed also in the Langendorff isolated and perfused rat heart, a classical paradigm of mammalian experimental cardiology (Goumon et al., 2004; Angelone et al., 2005). These results suggest that HCNP might have a potential role as a negative cardiac modulator.

Materials and Methods

1. Isolated and perfused rat Langendorff heart

1.1 Animals

Male Wistar rats (Charles River Laboratories and Morini, Bologna, Italy S.p.A.) weighting 250 ÷ 350 g were housed three per cage in a ventilated cage rack system under standard conditions. Animals had food and water access *ad libitum*. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

1.2 Isolated heart preparation

Rats were anaesthetized with ethyl carbamate (2 g/Kg rat, i.p.). The hearts were rapidly excised and transferred in ice-cold buffered Krebs-Henseleit solution (KHs). The aorta was immediately cannulated with a glass cannula and connected with the Langendorff apparatus to start perfusion at a constant flow-rate of 12 ml/min. To avoid fluid accumulation the apex of the left ventricle (LV) was pierced. A water-filled latex balloon, connected to a BLPR gauge (WRI, Inc. USA), was inserted through the mitral valve into the LV to allow isovolumic contractions and to continuously record mechanical parameters. The balloon was progressively filled with water up to 80 μl to obtain an initial left ventricular end diastolic pressure of 5-8 mmHg (Javadov et al., 2000). Coronary pressure was recorded using another pressure transducer located just above the aorta. The perfusion solution consisted of a modified non re-circulating KHs containing 113 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4,

11 mM glucose, 1.1 mM mannitol, 5 mM Na-pyruvate. pH was adjusted to 7.4 with NaOH (1 M) and the solution was equilibrated at 37°C by 95 % O_2 – 5% CO_2. All drug-containing solutions were freshly prepared before the experiments. Isoproterenol hydrochloride (ISO), ouabain, phentolamine, propanolol, SR59230A, L-NMMA, L-NIO, Hb, ODQ, KT5823, W7, carbachol, atropine, pirenzepine and PTx (Pertussis Toxin) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Recombinant STA-hCGA-1-78 and STA-hCGA-1-115, named VS1 and VS2 respectively, were obtained by expression in *E.coli* cells (Corti et al. 1997). As described (Ratti et al. 2000), the products were purified by reverse-phase HPLC using a SOURCE 15 RPC column (Pharmacia-Upjohn), followed by gel filtration chromatography on a Sephacryl S-200 HR column (Pharmacia-Upjohn). The products were homogeneous and their purity was >95% by SDS-PAGE analysis (Fig.1). Endotoxin content of VS1 and VS2 was 0.015 and 0.075 units/µg, respectively, as measured by the quantitative chromogenic Limulus Amebocyte Lysate (LAL) test (BioWhittaker). AFDX116 was a generous gift from Boehringer Ingelheim (Biberach, Germany). Rat HCNP (rHCNP) was synthesized in "Inserm U575 Physiopathology of Nervous System", Strasbourg-France, according to the method previously reported (Lugardon et al., 2001). Haemodynamic parameters were assessed using a PowerLab data acquisition system and analysed using a Chart software (both purchased by ADInstruments, Basile, Italy).

2. Experimental protocols

2.1 Basal conditions

Cardiac performance was evaluated by analysing heart rate (HR, in beats/min), left ventricular pressure (LVP, in mmHg), which is an index of contractile activity, and the rate pressure product (RPP: HR x LVP, in 10^4 mmHg.beats/min), used as an index of cardiac work (Georget et al., 2003), +(LVdp/dt)MAX, i.e. the maximal rate of left ventricular pressure contraction, Ttp, i.e. the time to peak tension of the isometric twitch, which indicates changes in relaxation, -(LVdp/dt)MAX, i.e. the maximal rate of left ventricular pressure decline, HTR (Half Time Relaxation), and +(LVdp/dt)MAX/-(LVdp/dt)MAX, i.e. T/-t which indicates the ratio between maximal rates of contraction and relaxation (Vittone et al., 1994). The mean coronary pressure (CP, in mmHg) was calculated by averaging the coronary pressure during several cardiac cycles.

2.2 Drugs application

2.2.1 VS1 and VS2 stimulated preparations

Preliminary experiments (data not shown) obtained by the repetitive exposure of each heart to one concentration of human recombinant VS1, VS2 or rHCNP revealed absence of desensitisation. Thus, concentration-response curves were obtained by perfusing the cardiac preparation with KHs enriched with increasing concentrations of VS1or VS2 (11 ÷ 165 nM) or rHCNP (from 10^{-14} to 10^{-6} M) for 10 min.

2.2.2 Isoproterenol stimulated preparations

Cardiac preparations, stabilised for 20 min with KHs, were perfused with 5 nM isoproterenol (ISO) for 10 min and then washed-out with KHs. After returning to control conditions, each heart was perfused with KHs containing a single concentration of VS1or VS2 (11÷165 nM) plus 5 nM ISO for other 10 min.

To further describe the antagonistic action of VS1 toward the ISO-dependent stimulation, dose-response curves were generated by perfusing heart preparations with KHs enriched with increasing concentrations of ISO (10^{-10} ÷ 10^{-6} M) alone. These curves were then compared to those obtained by exposing other cardiac preparations to the same perfusion medium containing increasing concentrations of ISO (10^{-10} ÷ 10^{-6} M) plus a single concentration of VS1 (11, or 33, or 65 nM).

In order to evaluate whether rHCNP "antagonizes" the ISO-stimulation, dose-response curves of ISO (from 10^{-10} to 10^{-6} M) alone were compared to those obtained by exposing other cardiac preparations to the same perfusion medium containing increasing concentrations of ISO (from 10^{-10} to 10^{-6} M) plus a single concentration of rHCNP (10^{-11} M).

2.2.3 Ouabain stimulated preparations

Cardiac preparations, stabilised for 20 min with KHs, were perfused with 5 nM Ouabain (100 nM) for 10 min and then washed-out with KHs. After returning to control conditions, each heart was perfused with KHs containing a single concentration of VS1(33 nM) plus 100 nM ouabain for other 10 min.

2.2.4 Mechanism of action of VS1

Cardiac preparations, stabilised for 20 min with KHs, were perfused with 33 nM VS1 for 10 min and then washed-out with KHs. After returning to control conditions, each heart was perfused with KHs containing 33 nM of VS1 plus propanolol or phentolamine or SR 5923A or Pertussis toxin (PTx, inhibitor of Gi/0 protein) or L-NMMA (aspecific inhibitor of NOS) or L-NIO (specific inhibitor of eNOS) or Hb (scavenger of NO) or ODQ (inhibitor of sGC) or KT5823 (inhibitor of PKG) for other 10 min.

2.2.5 Cholinergic stimulated preparations

To obtain preliminary information on the cholinergic action of rHCNP toward the carbachol-dependent stimulation, dose-response curves were produced by perfusing the cardiac preparations with KHs enriched with increasing concentrations of rHCNP (from 10^{-12} to 10^{-7} M) alone. These curves were then compared with those obtained by exposing other cardiac preparations to the same perfusion medium containing increasing concentrations of rHCNP (from 10^{-12} to 10^{-7} M) plus a single concentration of carbachol (10 nM) or a single concentration of atropine (1 μM) or a single concentration of AFDX116 (100 nM) or a single concentration of pirenzepine (1 nM). To distinguish whether HCNP-dependent effects were not secondary to synthesizing activity of HCNP-stimulated ChAT, dose-response curves were produced by perfusing the cardiac preparations with KHs enriched with increasing concentrations of rHCNP (from 10^{-12} to 10^{-7} M) alone. These curves were then compared with those obtained by exposing other cardiac preparations to the same perfusion medium containing increasing concentrations of rHCNP (from 10^{-12} to 10^{-7}

M) plus a single concentration of bromoacetylcholine (BrACh, a specific inhibitor of ChAT) (10 nM) in presence of rHCNP.

2.2.6 Gi/0 Protein involvement

To verify the involvement of Gi/0 protein in the mechanism of action of rHCNP, dose-response curves were produced by perfusing the cardiac preparations with KHs enriched with increasing concentrations of rHCNP (from 10^{-12} to 10^{-7} M) alone. These curves were then compared with those obtained by exposing other cardiac preparations to the same perfusion medium containing increasing concentrations of rHCNP (from 10^{-12} to 10^{-7} M) plus a single concentration of Pertussis Toxin (PTx, inhibitor of Gi/0 protein) (0,01 nM) in presence of rHCNP.

2.3 Myocardial Protective Effects

After anaesthesia with uretane Wistar rats were decapitated. The hearts were excised, connected to a perfusion apparatus, perfused at constant flow with oxygenated Krebs-Henseleit buffer, paced at 280 b.p.m and kept at 37°C. Left ventricular pressure (LVP) and aortic pressure (AP) were measured. In the control AP was about 90 mmHg for a coronary flow of 20 ml/min. Developed LVP was taken as an index of contractility. Infarct size was determined with nitro-blue tetrazolium technique. The release of Lactic-dehydrogenase (LDH) was determined on samples of effluent fluid taken at regular intervals during reperfusion. The hearts were divided in four groups. Group I (n=5) was used as control. Before ischaemia, ischaemic preconditioning was performed in Group II (n=10) and VS1 at 80 nM concentration for 19 min was given to Group III (n=12). Finally a 10μM solution of

the NO-synthase inhibitor L-N-nitroarginine (L-NNA) was infused into the hearts of Group IV (n=4), 5 min before, during and 5 min after the end of the infusion of VS1.

2.4 Statistics

Data are expressed as the mean ± SEM. Since each heart represents its own control, the statistical significance of differences within-group was assessed using the paired Student's t-test ($p< 0.05$). Comparison between groups was made by using a one-way analysis of variance (ANOVA) followed by Duncan and Bonferroni's Multiple Comparison Test. Differences were considered to be statistically significant for $p<0.05$. To obtain a concentration–response curve for the ISO-induced increase of RPP, this parameter was expressed as a percentage: ISO maximum effect=100% and baseline=0%. The sigmoid concentration–response curves of ISO alone and of ISO plus VS1 were fitted using GraphPad Prism 4.02, and this provided, for each concentration–response curve, the –log of the concentration (M) producing 50% effect (EC_{50}) of ISO alone and of ISO plus VS1. The concentration-response curves of the reduction of LVP induced by rHCNP alone and by rHCNP plus carbachol or atropine or AFDX116 or pirenzepine were fitted using GraphPad Prism 4.02. This provided, for each curve, the –log of the concentration (in M) which induced the 50% effect (EC_{50}) of rHCNP alone and rHCNP plus carbachol, atropine, AFDX116 and pirenzepine. The same fitting procedure was used to calculate the EC_{50} of ISO alone and of ISO plus rHCNP on LVP and RPP.

3. Cell Culture and Immunochemistry

The cell line H9C2 (rat DB1X heart myoblast; ATCC) were grown in DMEM medium (Sigma) supplemented with 10% fetal calf serum (Sigma) and antibiotics (penicillin and streptomycin at 100 units/ml of medium). For cytochemistry experiments, glass slides were sterilized and coated with sterilized poly-L-lysine (0.1 mg/ml) during 10 min and then dried for at least 2 hours. After 2 days in culture in 24 wells plate, culture glass slides were treated during 2h with control medium, VS or isoproterenol or CGB. Cells were prefixed with 2% PFA/DMEM (37°C) for 2 min and then 10 min with 4 % PFA in phosphate buffer saline (PBS, 4°C). After three washes of 5 min (1ml/well) with PBS, cells were incubated with 250µl/well of phalloidin-TRIC in PBS (50µg/ml, Sigma). After three more washes of 5 min, cells were incubated 10 min with 250µl/well Hoechst 33258 (1/2000 in water; Sigma). Finally, cells were washed 3 times with PBS followed with one wash with water. Slides were mounted up side down in one drop of Mowiol 4-88.

4. Mass Spectra Analysis

Determination of mass was carried out on a Brucker BIFLEX^{TM} matrix-assisted laser desorption time-of-flight mass spectrometer (TOF-TOF) equipped with the SCOUT^{TM}. High Resolution Optics with X-Y multisample probe, a gridless reflector and the HIMAS^{TM} linear detector. This instrument has a maximum accelerating potential of 30 kV and may operate either in the linear or reflector mode. Ionization was accomplished with a 337-nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal from the detector was digitized at a sampling rate of 250 MHz in linear mode and 500 MHz in reflector mode using a 1 GHz digital oscilloscope (Lecroy model). The instrument control and data processing were accomplished with software supplied by Bruker using a Sun Sparc workstation. These studies were realized using as the matrix α-cyano-4-hydroxycinnamic acid (Sigma) prepared as a satured solution in acetone. Aliquots (0.5 µl) of the sample-matrix solution were deposited onto probe tips and air dried. After fast spreading and fast evaporation of the solvent, a thin layer of matrix crystals was obtained (Vorm and Roepstorff, 1994; Goumon, 1998). 0.7 µl of acidic water was add followed by 0.5 µl a micromolar analyte solution was applied to the matrix. Finally 0.2 µl of matrix prepared in 50% acetonitrile/water was add and allowed to dry under moderate vacuum.

Results

1. Isolated heart preparations

1.1 Basal conditions

After 20 minutes of equilibration, LVP was 89 ± 3 (mmHg), HR was 280 ± 7 beats/min, RPP was 2.5 ± 0.1 10^4 mmHg beats/min, CP was 63 ± 3 mmHg. To assess the endurance and the stability of the preparation, the performance variables were measured every 10 min showing that the heart is stable up to 180 min.

1.2 VS1 and VS2 stimulated preparations

Concentration-response curves of VS1 and VS2 were generated by exposing the cardiac preparations to increasing concentrations of each peptide, since exposure to single repeated doses of VS1 or VS2 revealed absence of desensitization (data not shown). The effects of both peptides reached their maximum at 5 min after drug application, remained stable until 15 min and then gradually decreased with time. Thus, cardiac parameters were measured at 10 min.

1.3 Inotropic effect

VS1 (11 ÷165 nM) caused a concentration-dependent negative inotropic effect, revealed by a significant reduction of LVP, associated with a non significant small increase of HR. The peptide from 33 to 165 nM significantly reduced RPP. At all concentrations, VS1 did not affect CP (Fig. 1).

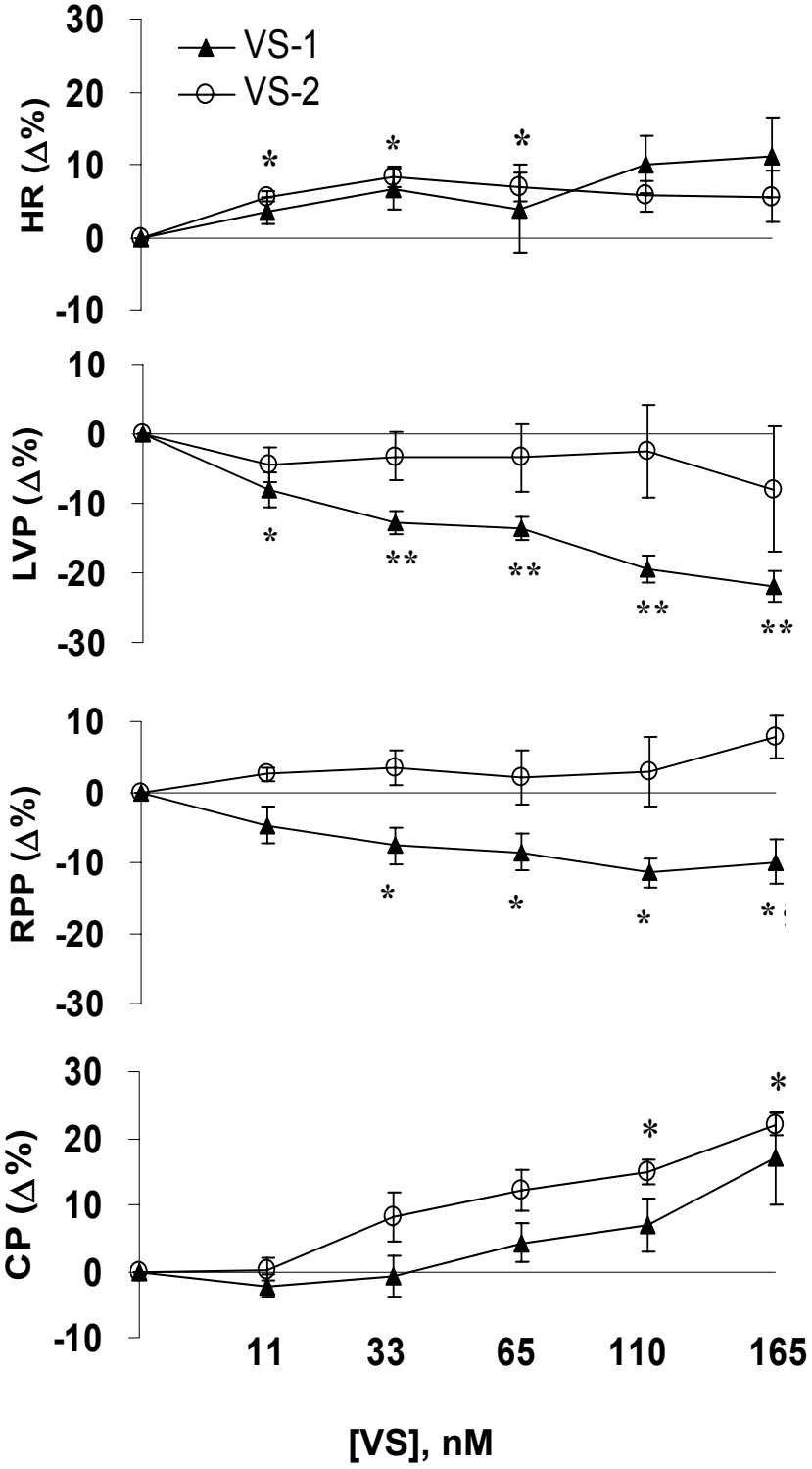

Fig. 1 Concentration-dependent responses of increasing concentrations (11 ÷ 165 nM) of VS1 or VS2 on HR (heart rate), LVP (left ventricular pressure), RPP (rate pressure product) and CP (coronary pressure) on the rat isolated and Langendorff perfused heart. Percentage changes were evaluated as means ± SEM of 8 experiments for each group. Significance of difference from control values (t-test);*=p<0.05, **=p<0.01. Comparison between groups (ANOVA, Duncan's test); §=p<0,05.

The peptide-dependent negative inotropism was also demonstrated by the reduction of both + (LVdP/dt) MAX, an index of contractility, and Ttp (Fig. 3). In contrast, increasing doses of VS2 (11÷165 nM) did not change LVP and RPP but significantly increased HR at 11, 33 and 65 nM. Of note, VS2 caused an increase of CP, significant at 110 and 165 nM (Fig. 1). The VS1 negative inotropic effect is not secondary to modifications in oxygen metabolism since the oxygen consumption does not change in the presence of the peptide (Fig. 2).

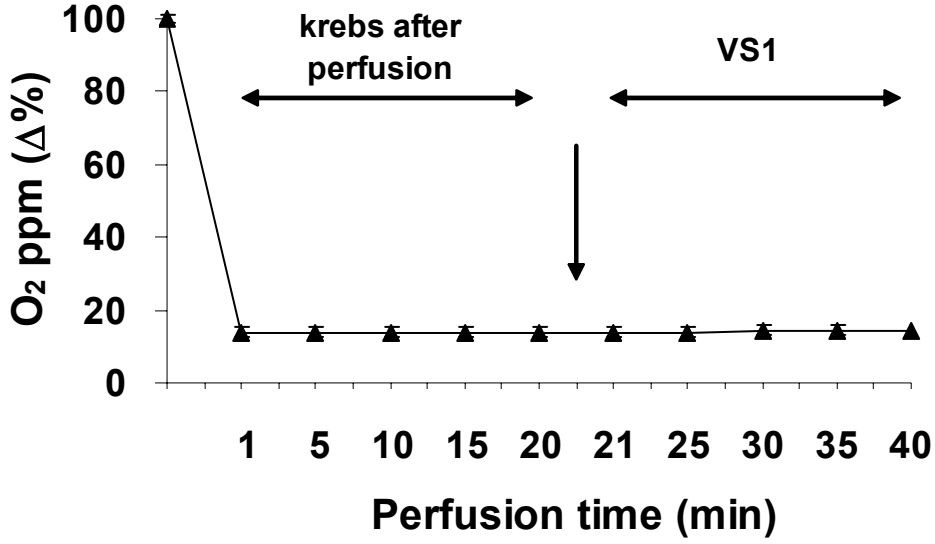

Fig. 2 Valuation of O_2 uptake (ppm) by VS1 treatment at 33 or 80 nM on the rat isolated and Langendorff perfused heart. Percentage changes were evaluated as means ± SEM of 7 experiments for each group.

1.4 Lusitropic effect

A detailed analysis of the VS1 myocardial effects revealed also a negative lusitropic action, represented by a significant reduction of –(LVdP/dt) Max and an increase of T/-t, HTR being unchanged (Fig. 3).

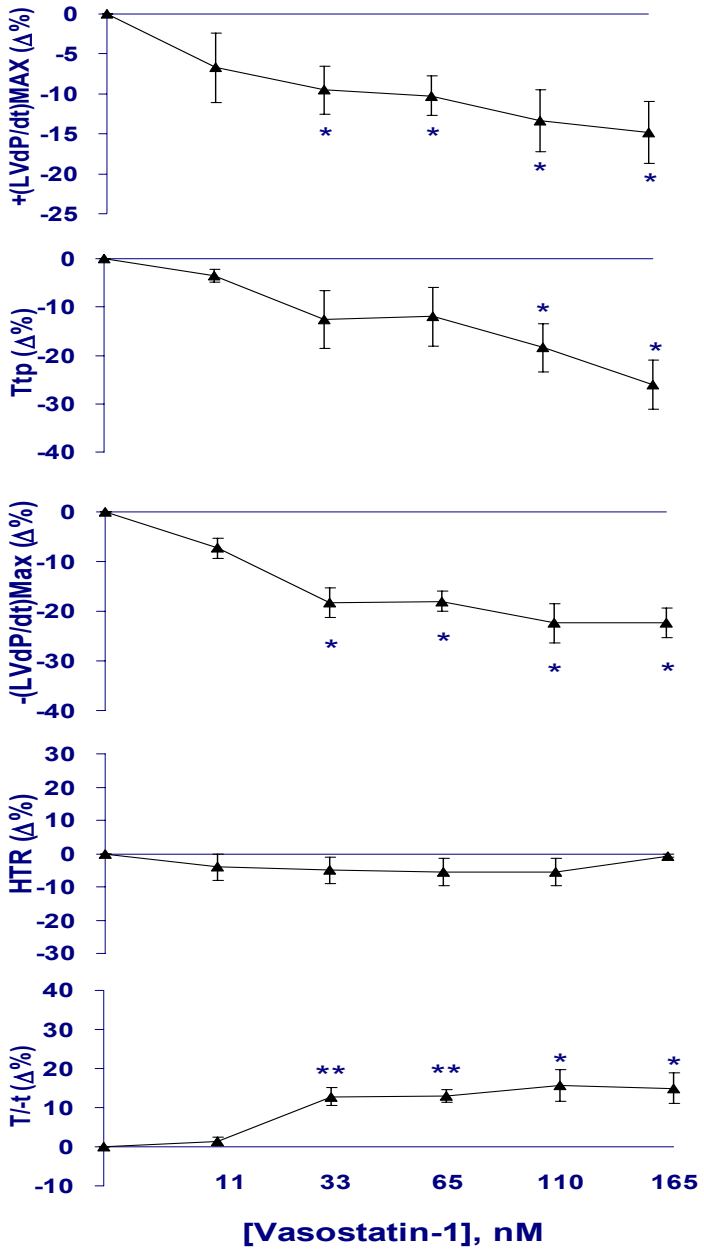

Fig. 3. Concentration-dependent responses of increasing concentrations (11÷165 nM) of Vasostatin-1 on +(LVdp/dt)MAX, Ttp, i.e. index of contractility, -(LVdp/dt)MAX, HTR (Half Time Relaxation) and T/-t or +(LVdp/dt)MAX/-(LVdp/dt)MAX, i.e. index of relaxation, on the rat isolated and Langendorff perfused heart. Percentage changes were evaluated as means ± SEM of 7 experiments. Significance of difference from control values (t-test);*=$p<0.05$, **=$p<0.01$.

1.5 Isoproterenol-stimulated preparations

The ß-adrenergic agonist ISO (5 nM) induced positive chronotropic and inotropic responses, associated with a significant vasodilation, indicated by increases of HR, LVP, RPP and the decrease of CP (Fig. 4a,4b). As already reported (Abbott and Lodola, 1992; Ali et al., 2004; Schiffman et al., 2002), the effects were significant until 5 min after application of the drug, then they gradually decreased with time. Preliminary experiments performed by consecutive exposures of ISO (5 nM) on each heart showed absence of desensitisation (data not shown).

To verify vasostatin ability to counteract the ISO-mediated positive chronotropic and inotropic effects as well as coronary dilation, hearts were perfused with KHs containing 5 nM ISO plus a single concentration of VS1 or VS2 (11÷165 nM). HR, LVP, CP and RPP were measured at 5 min from drug application.

Except for a small decrease of HR at 110 nM, the ISO-dependent positive chronotropism was unaffected by VS1. In contrast, VS2 significantly reduced the ISO-induced increase of HR and, at all concentrations, abolished the ISO-mediated positive inotropism. Moreover, VS1 blocked the ISO-mediated positive inotropism at 165 nM, and significantly inverted it at 11, 33, 65 and 110 nM. The stimulatory effect of 5 nM ISO on RPP was abolished by both VS1 and VS2 at all concentrations tested. VS1 and VS2 did not affect the ISO-dependent coronary dilation (Fig. 4a,4b). To verify whether vasostatin antagonism is selective against ISO stimulation, the effects of VS1 were evaluated against another positive inotropic agent, ouabain. Hearts were initially perfused with KHs containing 100 nM ouabain. They were then washed with KHs and, after their return to control conditions, were perfused with

100 nM ouabain plus VS1 at 33 nM. Ouabain-induced increase of RPP was not affected by administration of VS1 (ouabain: 9.38%, ouabain plus VS1: 10.2%; $p>0.5$)

To obtain further information on the antagonistic behaviour of VS1 against the ISO-dependent stimulation, heart preparations were perfused with KHs containing increasing concentrations of ISO ($10^{-10} \div 10^{-6}$ M) alone or with the addition of a single concentration of VS1 (11, or 33, or 65 nM). ISO significantly increased RPP from $5x10^{-9}$ to 10^{-6} M and significantly elicited a slight coronary dilation at $5x10^{-8}$ and $5x10^{-7}$ M. VS1 at 11 nM significantly reduced the ISO ($5x10^{-8}$ and 10^{-7}M)-mediated increase of RPP. The ß-adrenergic (at all ISO concentrations)-dependent increase of cardiac work was also significantly reduced by VS1 at 33 and 65 nM. In contrast, VS1 (at 11, or 33, and 65 nM) did not affect the ISO-dependent decrease of CP (Fig. 5). The analysis of the percentage of variations of RPP in terms of EC_{50} values of ISO alone and in presence of VS1 (11, or 33, or 65 nM) (Fig. 6) suggests a non-competitive type of antagonism of VS1.

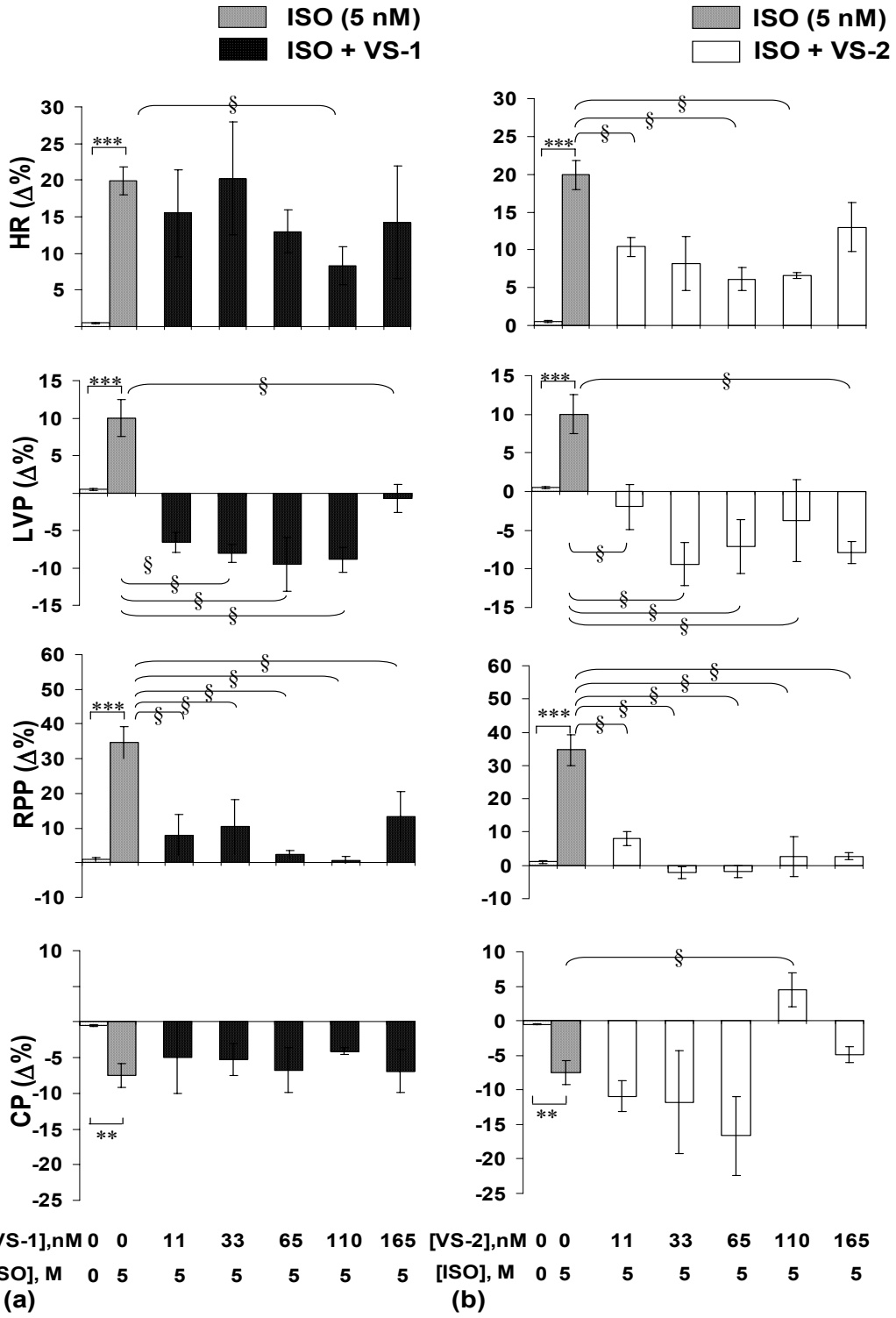

Fig. 4 a,b. Effects of human recombinant VS1 (a) and VS2 (b) on the positive response evoked by Isoproterenol (ISO 5nM, n=40) on HR (heart rate), LVP (left ventricular pressure), RPP (rate pressure product) and CP (coronary pressure) on the rat isolated and Langendorff perfused heart. Percentage changes were evaluated as means ± SEM of 4 experiments for each peptide concentration. Significance of difference from control values (t-test); **, ***=p<0.01. Comparison between groups (ANOVA, Duncan's test); §=p<0,05.

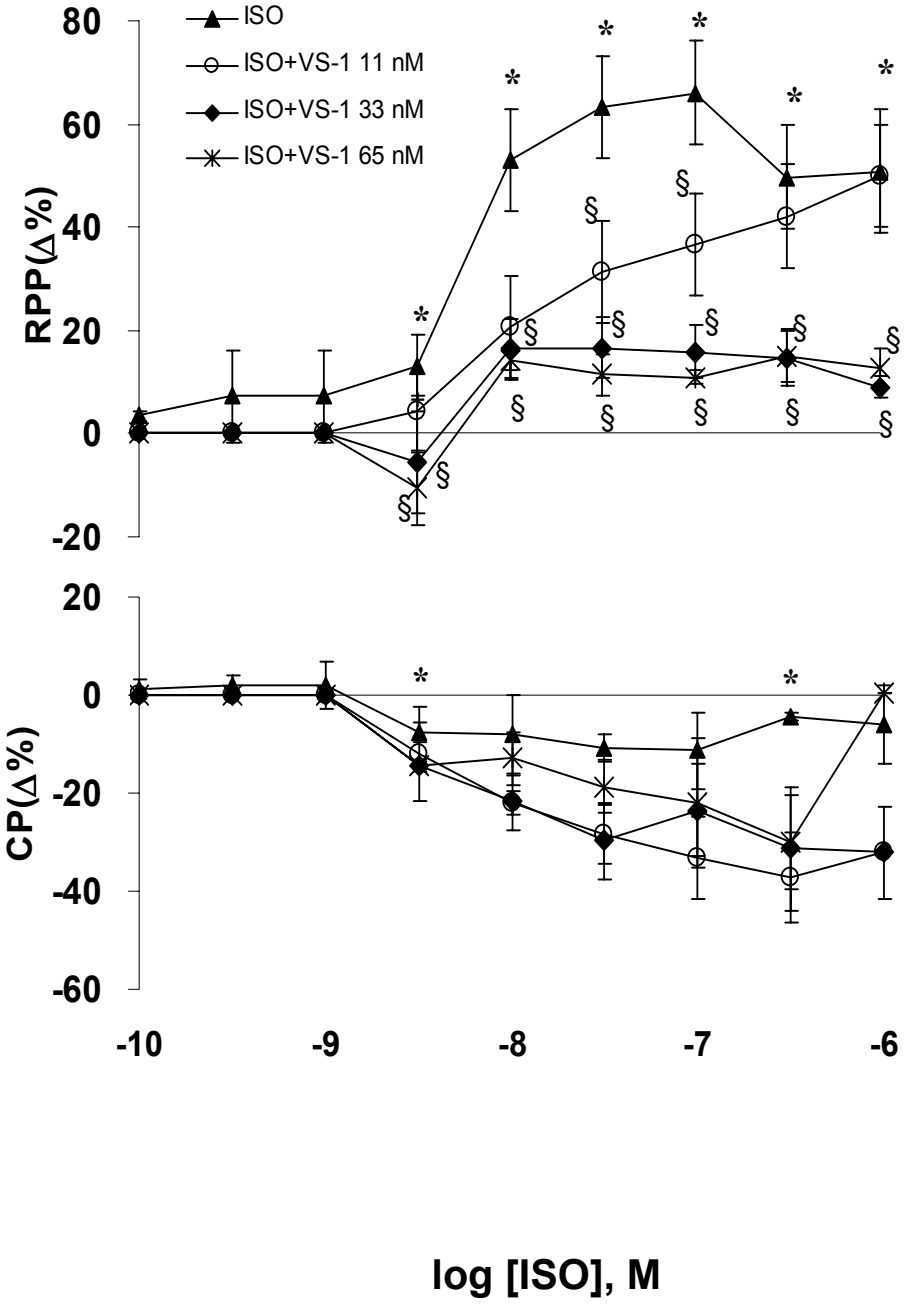

Fig. 5 Concentration-dependent responses of ISO alone (10^{-10}÷10^{-6} M) alone and of ISO plus of human recombinant VS1 (11, or 33, or 65 nM) on RPP (rate pressure product) and CP (coronary pressure) on the rat isolated and Langendorff perfused heart. Percentage changes were evaluated as means ± SEM of 7 experiments for each group. Significance of difference from control values (t-test); *=p<0.05. Comparison between groups (ANOVA, Duncan's test); §=p<0,05.

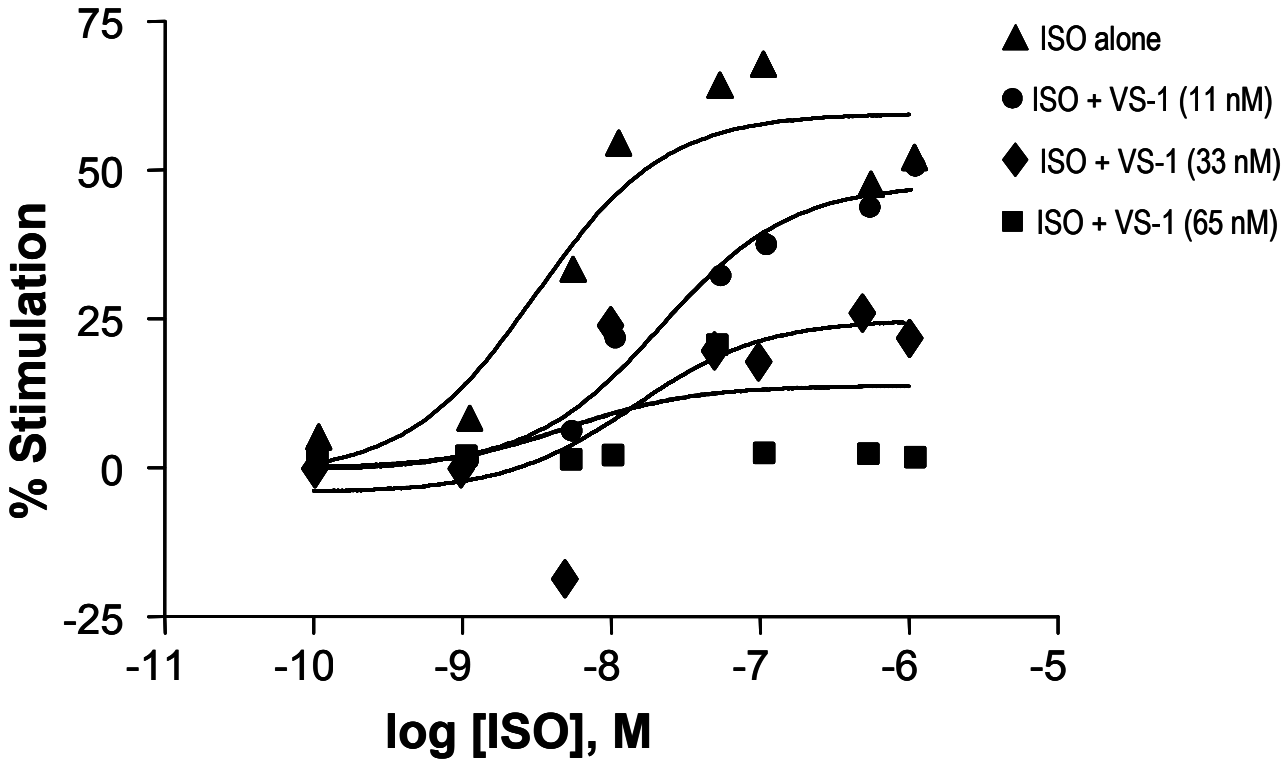

Fig. 6 The sigmoid concentration-response curves of ISO-mediated stimulation on RPP (rate pressure product) of ISO ($10^{-10} \div 10^{-6}$ M) alone and of ISO plus a single concentration of human recombinant VS1 at 11, 33 and 65 nM on the rat isolated and Langendorff perfused heart. Contraction is expressed as a percentage [baseline=0%, peak constriction by ISO and ISO plus STA-CGA1-78 =100%].
The EC_{50} values (in logM) of ISO alone was -8.5 ± 0.3 (r^2= 0.88), of ISO plus VS1 (11 nM, or 33 nM, or 65 nM) were -7.7 ± 0.15 (r^2= 0.97), -8.3 ± 0.5 (r^2= 0.67), -7.8 ± 0.78 (r^2= 0.55), respectively.

1.6 Mechanism of action of VS1

1.6.1 Membrane receptors

VS1-dependent cardiotropism was further analized to verify the involvement of membrane receptors. VS1-mediated negative inotropism was abolished by the inhibition of either α- or $\beta_{1,2}$-, or β_3-adrenergic receptor, but it was not modified by the muscarinic receptor antagonist Atropine (Fig. 7).

1.6.2 $G_{i/0}$ Protein

To verify the involvement of Gi/0 protein, the cardiac preparations were perfused with KHs containing Pertussis toxin (PTx) in the presence of VS1. Inhibition of Gi/0 proteins by PTx abolished VS1-mediated negative inotropism (Fig. 8).

1.6.3 NO-cGMP-PKG System

The involvement of the NO-cGMP signalling in the VS1-dependent cardiotropism, was examined by perfusing the cardiac preparations with haemoglobin (Hb) (NO scavenger), or L-NMMA or L-NIO (NOS inhibitors), or ODQ (guanylate cyclase blocker). VS1-mediated negative inotropism was abolished by both the NOS inhibition by L-NMMA and L-NIO, and by the removal of NO by Hb (Fig. 9).

Pretreatment with a specific inhibitor of soluble guanilate cyclase (ODQ), as well as inhibitor of PKG (KT5823), abolished VS1 mediated inotropism, indicating that the involvement of the cGMP-PKG component (Fig. 9).

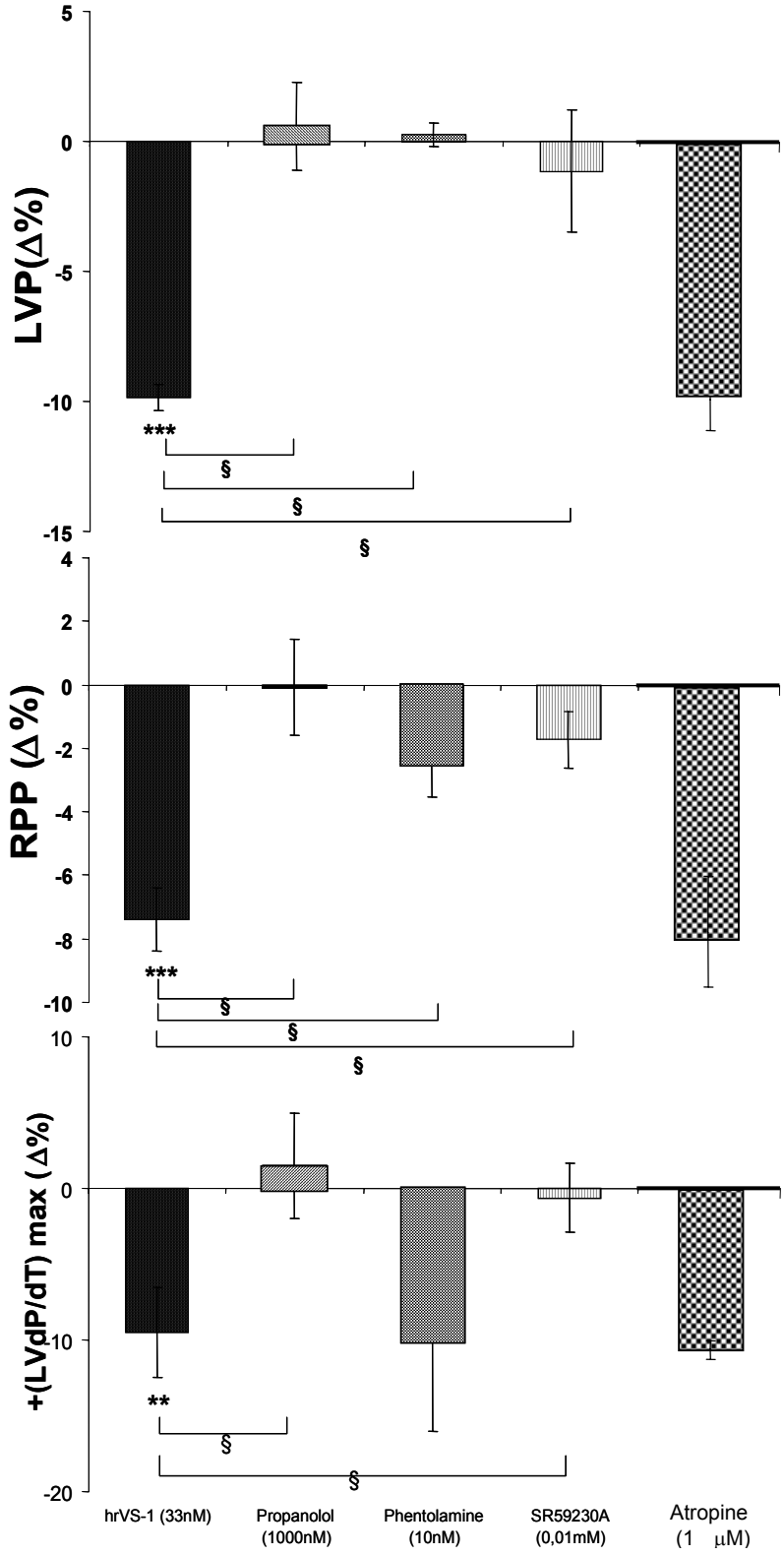

Fig. 7. Effects of human recombinant VS1 (33nM) alone and VS1 in presence of inhibitor of α-(Phentolamine) and $\beta_{1,2,3}$ adrenergic antagonists (Propanolol and SR59203, respectively) and of muscarinic receptor antagonist (Atropine) on LVP (left ventricular pressure), RPP (rate pressure product) and +(LVdP/dt)max on the rat isolated and Langendorff perfused heart. Percentage changes were evaluated as means ± SEM of 4 experiments for each peptide concentration. Significance of difference from control values (t-test); **, ***=p<0.01. Comparison between groups (ANOVA, Duncan's test); §=p<0,05.

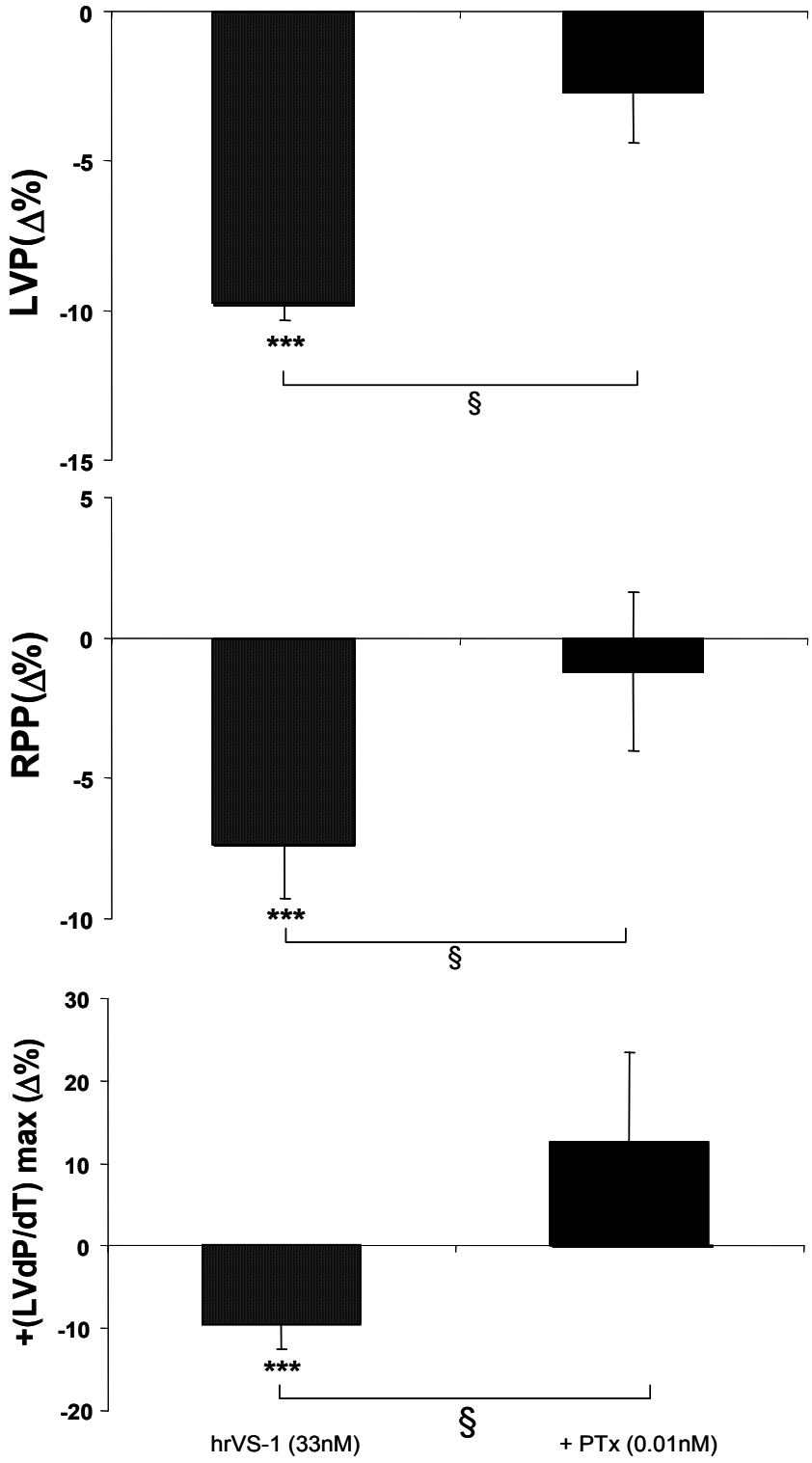

Fig. 8. Effects of human recombinant VS1 (33nM) alone and VS1 in presence of Pertussis Toxin (0.01 nM) on LVP (left ventricular pressure), RPP (rate pressure product) and +(LVdP/dt)max on the rat isolated and Langendorff perfused heart. Percentage changes were evaluated as means ± SEM of 4 experiments for each peptide concentration. Significance of difference from control values (t-test); **, ***=p<0.01. Comparison between groups (ANOVA, Duncan's test); §=p<0,05.

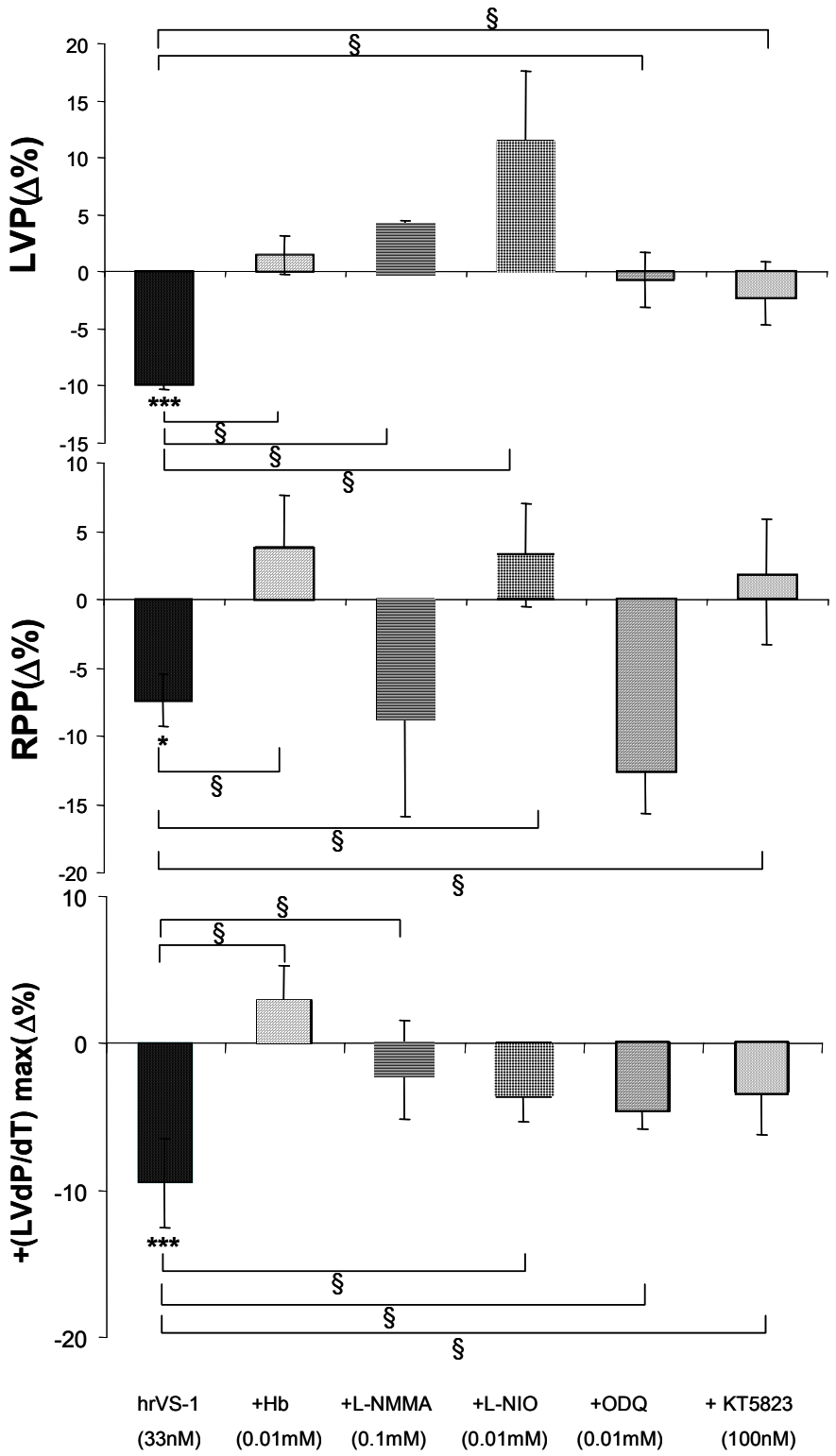

Fig. 9. Effects of human recombinant VS1 (33nM) alone and VS1 in presence of haemoglobin (Hb) (NO scavenger), or L-NMMA or L-NIO (NOS inhibitors), or ODQ (guanylate cyclase blocker) and inhibitor of PKG (KT5823) on LVP (left ventricular pressure), RPP (rate pressure product) and +(LVdP/dt)max on the rat isolated and Langendorff perfused heart. Percentage changes were evaluated as means ± SEM of 4 experiments for each peptide concentration. Significance of difference from control values (t-test); **, ***=p<0.01. Comparison between groups (ANOVA, Duncan's test); §=p<0,05.

1.7 Myocardial Protective Effects

Evaluation of the VS1 role in cardioprotection provided the following results: In Group I, necrosis concerned 66±16% of left ventricle and was reduced to 34±18% ($p<0.002$) in preconditioned Group II. In VS1 pretreated Group III, infarction was limited to 32±10% of the ventricle ($p<0.001$ vs. Group I and n.s. vs. Group II). In Group IV the protection by VS1 was completely suppressed by L-NNA. During reperfusion LVP recovery was proportional to the extent of protection. The release of LDH, measured to confirm the extent of the damage by I/R, was 1813±670 U/L in Group I, 711±714 in Group II, 780±226 in Group III ($p<0.01$) and 1129±322 in Group IV (n.s.). The results indicate that VS1 limits myocardial damage induced by ischemia and reperfusion to an extent similar to that of IP. Moreover, this protective effect appears to be mediated by the release of NO (fig. 10).

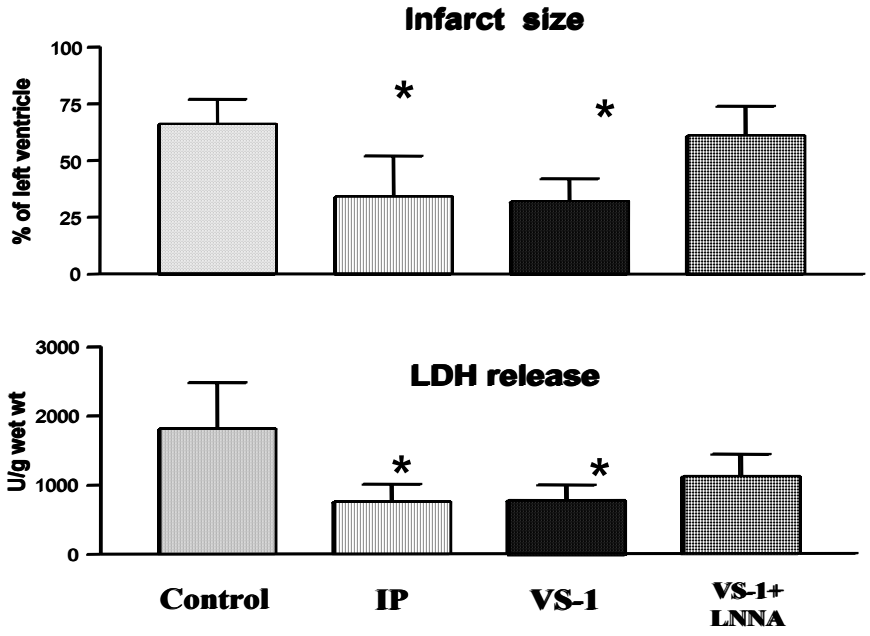

Fig. 10. Effects of human recombinant VS1 (80 nM) alone and in presence of LNNA (inhibitor of NOS) (10 μM) on cardioprotection by evaluation of infarct size and LDH release on the rat isolated and Langendorff perfused heart. Percentage changes were evaluated as means ± SEM of 7 experiments. Significance of difference from control values (t-test); **=$p<0.01$.

2. Cell Culture and Immunochemistry

These preliminary experiments have been realized in collaboration with Dr. Y. Goumon and E. Glattard in the Inserm U575 in Strasbourg, France. Using fluorescently tagged phalloidin, we showed that VS1 remarkably induced actin polymerization at all tested concentration (10 nM÷1 μM) (fig.11). In order to verify that the action of CGA-derived peptide is not secondary to osmotic effects, we tested an inert peptide CGB614-626 modify at different places and ISO . The effect induced by VS1 was not detectable after treatment with both CGB (fig.12 A) and Isoproterenol (fig.12 B).

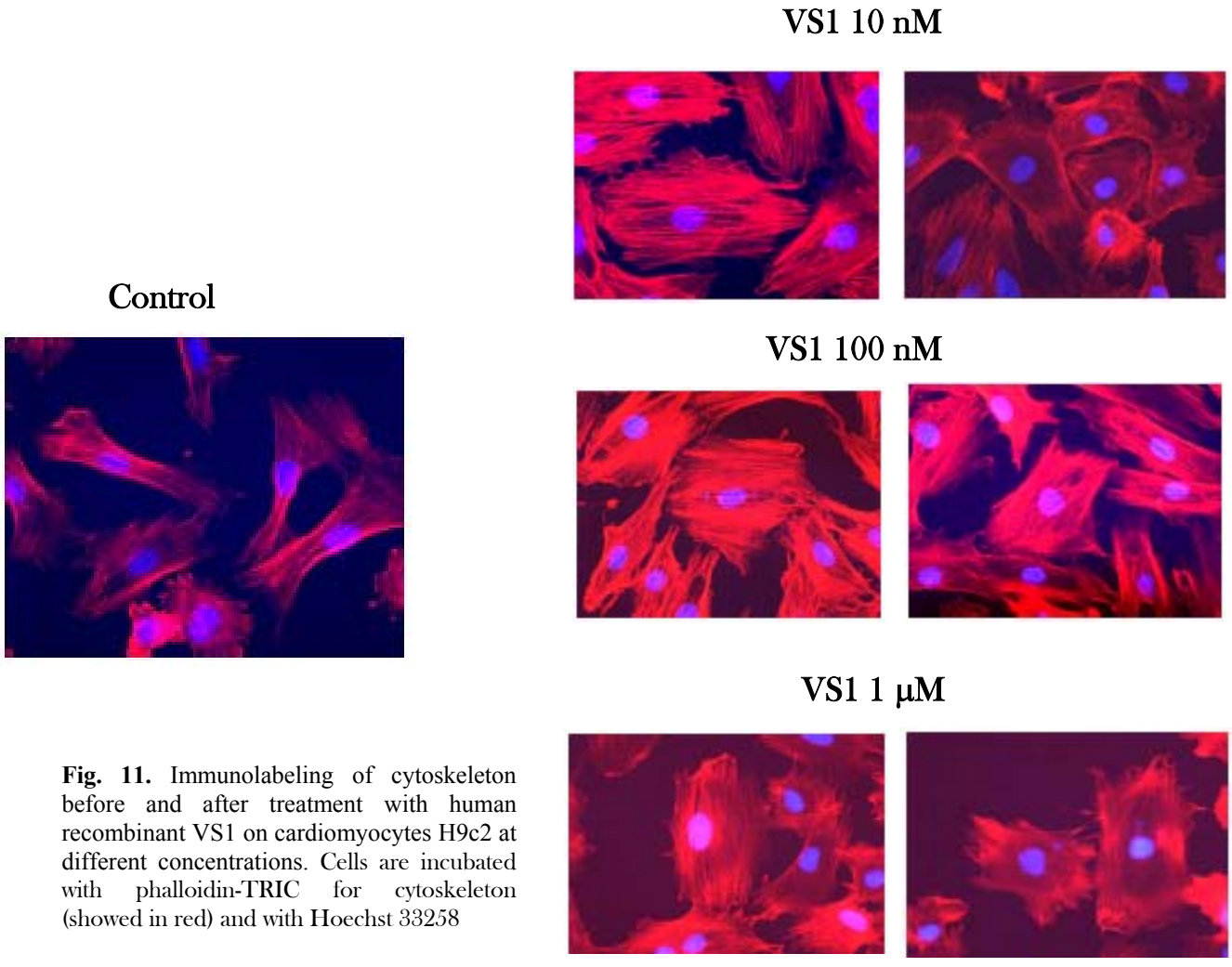

Fig. 11. Immunolabeling of cytoskeleton before and after treatment with human recombinant VS1 on cardiomyocytes H9c2 at different concentrations. Cells are incubated with phalloidin-TRIC for cytoskeleton (showed in red) and with Hoechst 33258

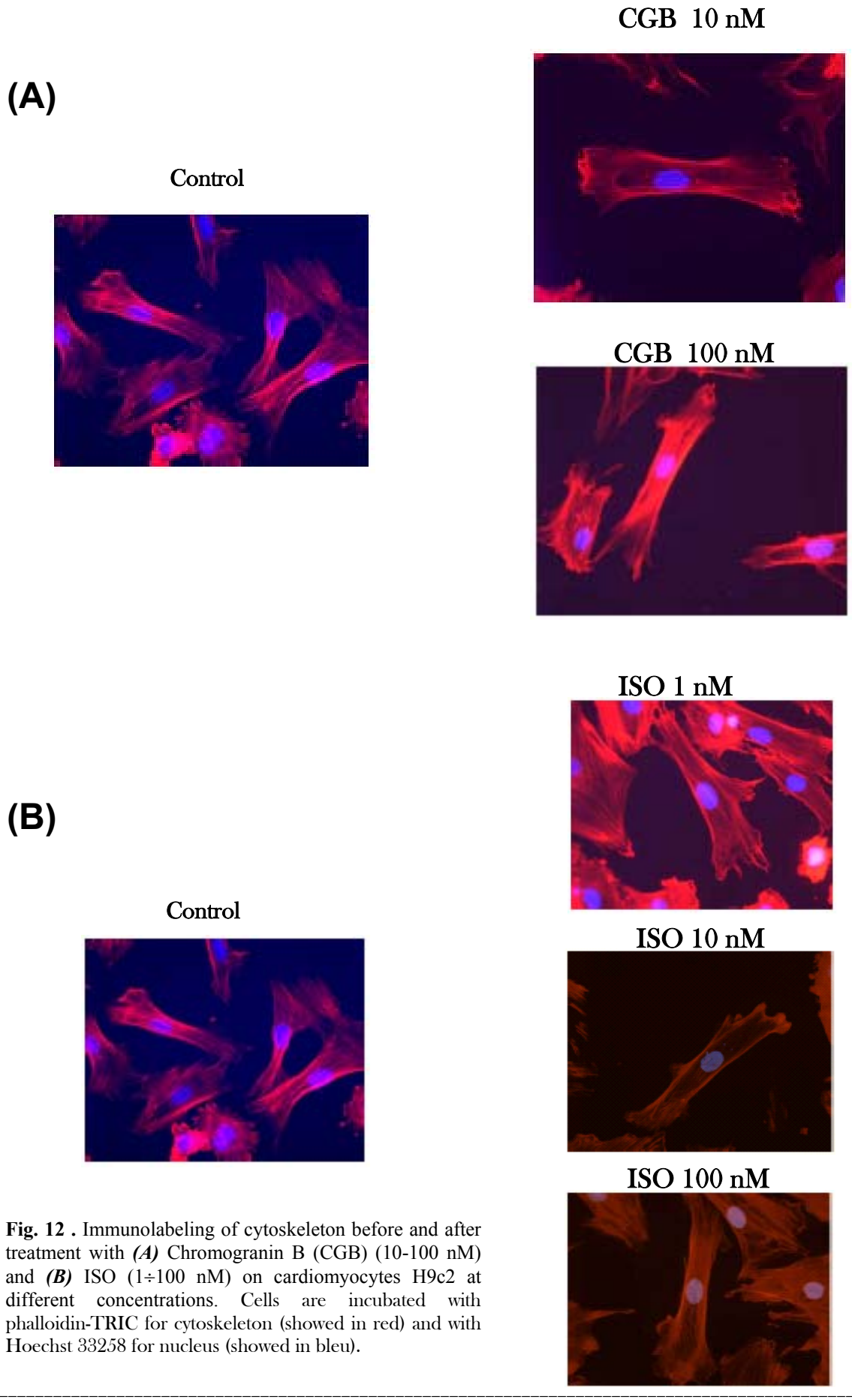

Fig. 12 . Immunolabeling of cytoskeleton before and after treatment with ***(A)*** Chromogranin B (CGB) (10-100 nM) and ***(B)*** ISO (1÷100 nM) on cardiomyocytes H9c2 at different concentrations. Cells are incubated with phalloidin-TRIC for cytoskeleton (showed in red) and with Hoechst 33258 for nucleus (showed in bleu).

3. Western Blot Analysis of CGA N-terminal Peptides Present in Rat Heart Extracts

This proteomic study has been performed in Strasbourg with the collaboration of Dr. Y. Goumon and E. Glattard. Rat heart extract (20 µg) was loaded on a 17% SDS-PAGE gel (Fig. 13). The gel was electrotransferred onto a PVDF membrane and submitted to a Western Blot using a rabbit anti-CGA_{4-16} antibody in order to examine the presence of CGA N-terminal fragments. Soluble proteins isolated from bovine intragranular chromaffin matrix were used as a control (Fig. 13, lane 1). This approach allowed us to determine the presence of three groups of bands in a range of 18-75 kDa (Fig. 13, lane 2 and labels): (*i*) one group including strongly labeled high molecular bands, (*ii*) a second group including other major bands and a last one (*iii*) containing the lightly labeled bands. Thus (*i*) includes *a* and *b* labels at a range of 80-50 kDa corresponding to the apparent molecular weight of the intact rat CGA (with and without posttranslational modifications) and several large N-terminal derived fragments. Such a migration is due to the presence of glutamic acid clusters which modifies the electrophoretic migration on SDS-PAGE, leading to the migration of the whole GGA (CGA_{1-448}, 48kDa) at an apparent molecular mass of 75 kDa (Fig. 13, *a* label) (Metz-Boutigue et al., 1997). The group (*ii*) includes *c*, *d* and *g* labels at 45, 37 and 27 kDa (Fig. 13) respectively and corresponds to shorter N-terminal CGA-derived fragments. The third group (*iii*) is composed of bands with a lighter labeling (Fig. 12; *e, f, h, i, j* and *k* labels) corresponding to 32, 30, 24, 23, 18 and 17 kDa apparent molecular masses, respectively. Interestingly, the 23 and 24 kDa bands

(Fig. 13, respectively *h* and *i* labels) might correspond to vasostatin II (VSII; rat $CGA_{1\text{-}128}$) that was previously described to migrate on polyacrylamide gel at an apparent molecular weight of about 21-23 kDa (McVicar et al., 2001). Thus, the rat 23 kDa band, was attributed previously to the $CGA_{1\text{-}128}$ (McVicar et al., 2001), but could also be attributed to $CGA_{1\text{-}124/128/130}$.

3.1 Purification and immunodetection of N-terminal CGA fragments

Rat heart extract (1 mg) was purified using the reverse phase HPLC technique. Each UV peak was collected and dotted (2/10) onto a nitrocellulose sheet before immunodetection with a rabbit polyclonal antibody anti-$CGA_{4\text{-}16}$ (Lugardon et al., 2001). Immunolabels were observed for the fractions 1, 6 and 10-14 (Fig. 14A).

In order to evaluate the apparent molecular mass of the CGA N-terminal fragments present in these fractions, an aliquot of each fraction (1 to 14; 2/5) was loaded on 17% SDS-PAGE gel, electrotransferred onto PVDF sheet, and immunodetected using anti-$CGA_{4\text{-}16}$. High molecular mass CGA immunoreactive fragments (75-70 kDa corresponding to entire CGA with and without posttranslational modifications and C-terminal truncated protein), were located in the more hydrophobic fractions as described for bovine CGA (Metz-Boutigue et al., 1993). Low molecular fragments (Fig.14; 22-25 kDa) were recovered in fractions 1 and 14, suggesting the presence of $CGA_{1\text{-}124/128/130}$.

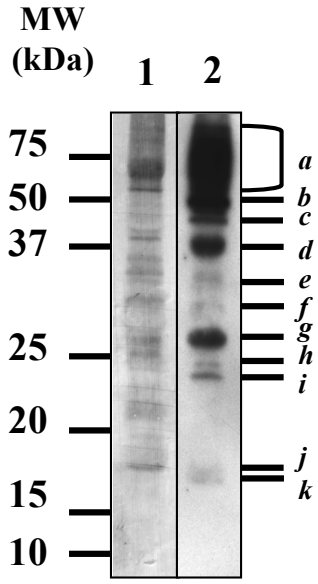

Fig. 13. Western blot analysis of CGA fragments present in rat heart extract. Samples were separated on 17% SDS-PAGE gel and electrotransferred onto a PVDF sheet. Immunodetection was carried out with a polyclonal anti-bovine $CGA_{4\text{-}16}$ antibody. Lane 1, control corresponding to intragranular proteins from bovine chromaffin cells (10μg). Lane 2, rat heart extract (20 μg).

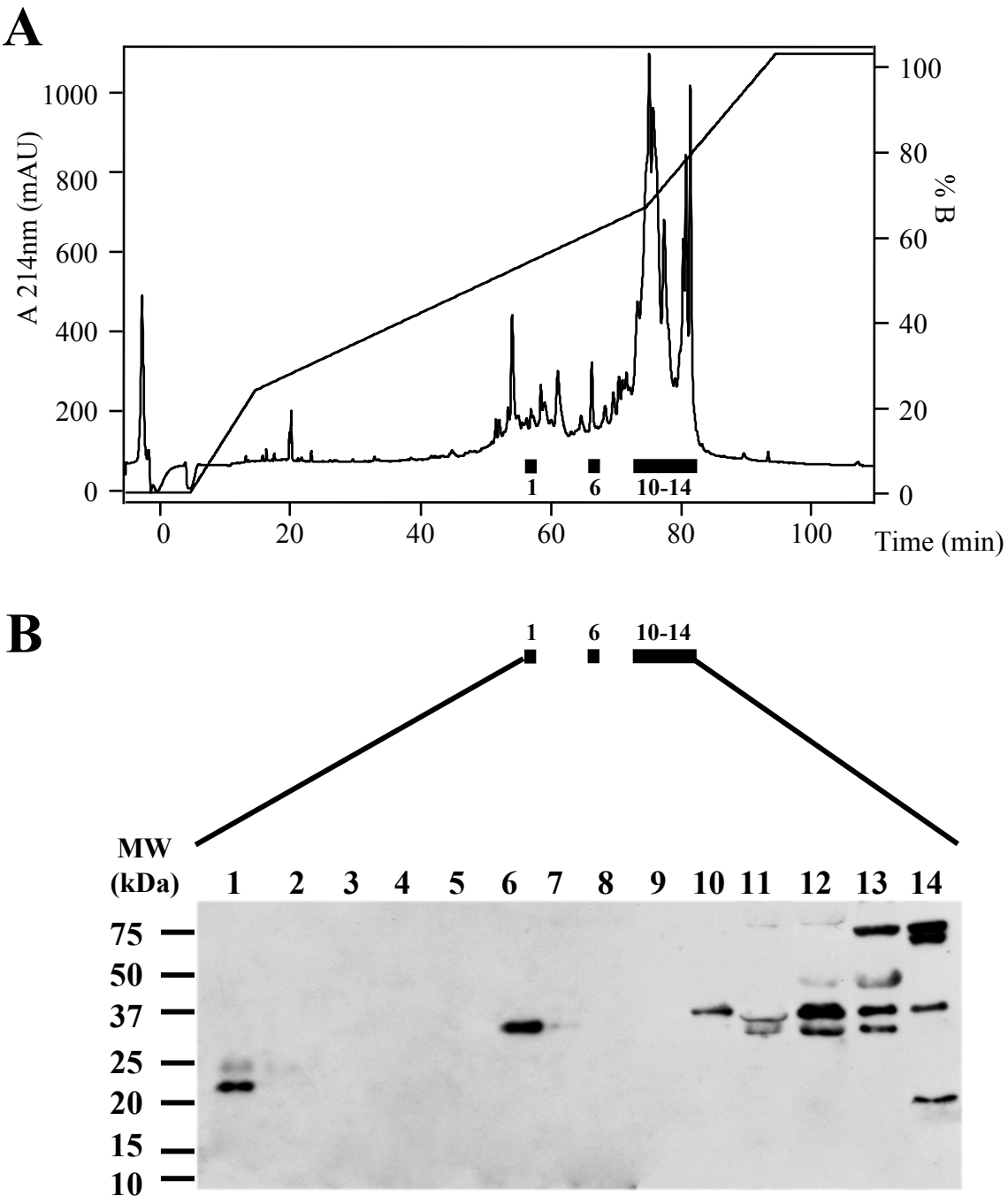

Fig. 14. HPLC purification of CGA fragments from rat heart extract. ***A***, 1 mg of rat heart extract was separated on a Macherey-Nagel reverse-phase Nucleosil 300–5C-18 column (4 x 250 mm). Aliquots of each resulting HPLC fractions (1 to 14) were dotted onto a nitrocellulose sheet and immunodetected with an anti-bovine $CGA_{4\text{-}16}$. Immunoreactive fractions are underlined on the chromatogram. ***B***, Eluted fractions 1 to 14 were separated by 17% SDS-PAGE gel and electrotransferred onto a PVDF sheet prior to immunodetection. Immunodetection was carried out with a polyclonal anti-bovine $CGA_{4\text{-}16}$.

3.2 Comparison CGA sequences from several species

Comparison with bovine and human CGA showed that the rat sequence contains three highly conserved amino acid sequences (Fig. 15, underlined)., The N-terminal rat $CGA_{1–76}$, including the VSI domain, displays 85% of identity and 93% of homology with the bovine CGA sequence, as well as 86% and 94% with the human sequence (ClustalW software). CGA_{94-106} possesses 84% of identity and 92% of homology with bovine and human CGA sequences. The rat C-terminal $CGA_{334-448}$ also shows 88% of identity and 98% of homology with the bovine CGA, and 91% of identity and 96% of homology with the human CGA. According, these results indicated a good conservation of the long N-terminal fragment CGA_{1-106}, suggesting also a conservation of the posttranslational modifications. Since bovine and human CGA possess posttranslational modifications, it was also investigated whether the N-terminal region of the rat CGA is modified. Thus, according to NetPhos 2.0 software, we examined the presence of putative phosphorylations on the serines located at residues 35, 50, 96, 133, 168, 182 and 185. Among these, the rat Ser_{96} corresponds to a previously described phosphorylated residue (Ser_{81}) in the bovine CGA sequence (Fig 16A) (Bauer et al., 1999; Zhang et al., 1997). In addition, the NetOGlyc 3.1 software allowed us to predict potential O-glycosylation on the residues Ser_{119}, Thr_{126}, Thr_{173}, Thr_{177}, Thr_{181}, Thr_{193} and Thr_{194}. Consensus N-glycosylation sites N-X-T/S (Kornfeld and Kornfeld, 1985) were also found (Asn_{107} and Asn_{175}; NetNGlyc 1.0).

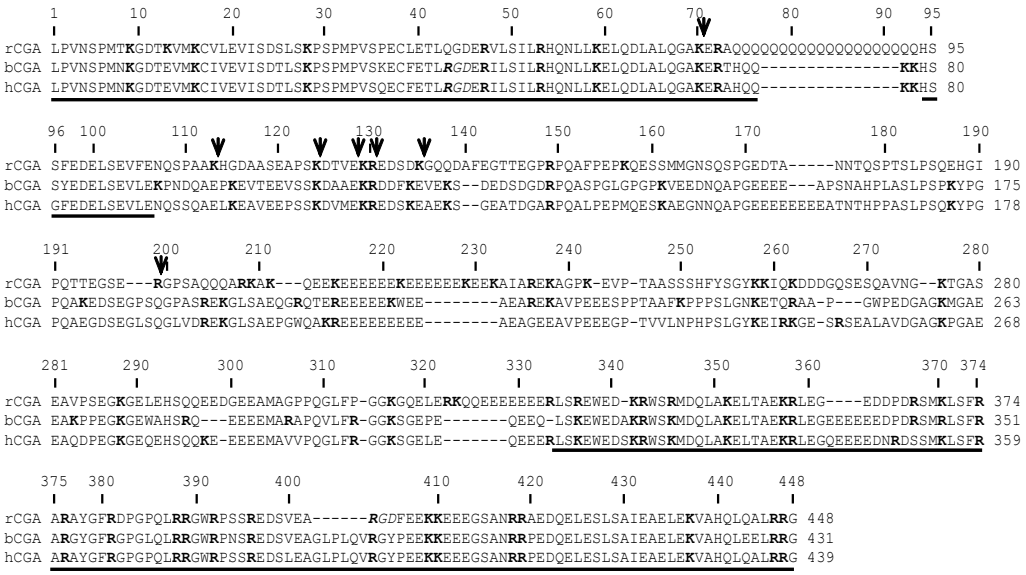

Fig. 15. CGA primary structure analysis. Sequence comparison of the CGA sequence from rat (r), bovine (b) and human (h) CGA. The underlined sequences correspond to highly conserved regions.

3.3 Characterization of N-terminal CGA fragments present in rat heart extract

Direct mass spectrometry analysis of the immunoreactive fractions was performed using TOF-TOF technique. We experimentally determine the masses of the N-terminal CGA-derived fragments and compared them with the theoretical mass in order to determine the possible posttranslational modifications (Table I). According to the NetPhos 2.0 software predictions and the previously described posttranslational modifications for other species, we expected both phosphorylations and glycosylations (Fig. 16A). Our results indicate the presence of phosphorylations and O-glycosylations, as well as oxidations. Phosphorylations were detected on

fragments CGA_{4-113}, CGA_{1-124}, CGA_{1-135} and CGA_{1-199}, whereas O-glycosylations (trisaccharide, NAcGal-Gal-NeuAc) were only found to be present on CGA_{1-135} (Table I). Oxidations were found on methionine residues 7, 15, 32 162 and 163 (Fig. 15 and 16A).

This approach provided the characterization of several fragments starting from the residues 1 or 4 of the CGA and ending at the amino acids 113, 124, 135 and 199 (Table I and Fig. 16B). Each C-terminal residue corresponds to potential cleavage sites (after a basic residue; Table I and Fig. 16).

HPLC Fraction	Molecular masses (Da)		Location	Cleavage site	Modifications
	Experimental	Calculated			
1	12,581.4	12,505.4	CGA4-113	V/N & K/H	P
12	14,009.9	13,864.9	CGA1-124	K/D	P + 3 Na^+
13	14,045.3				P + 2 O + 3 Na^+
12	15,278	15,167.5	CGA1-135	K/G	P + 2 O
12	15,866.6				O-Gly + 2 Na^+
13	15,868.9				
14	15,877.7				O-Gly + 2 O + 1 Na^+
10	22,092.1	21,948.5	CGA1-199	R/G	P + 3 O + 1 Na^+
11	22,097.6				

Table I: Mass spectrometry analysis of HPLC fractions. CGA-derived peptides were determined by comparing the experimental masses obtained by TOF-TOF MS analysis with the calculated values. O, oxidation. Na^+, sodium adducts. O-Gly, O-glycosylation. P, phosphorylation.

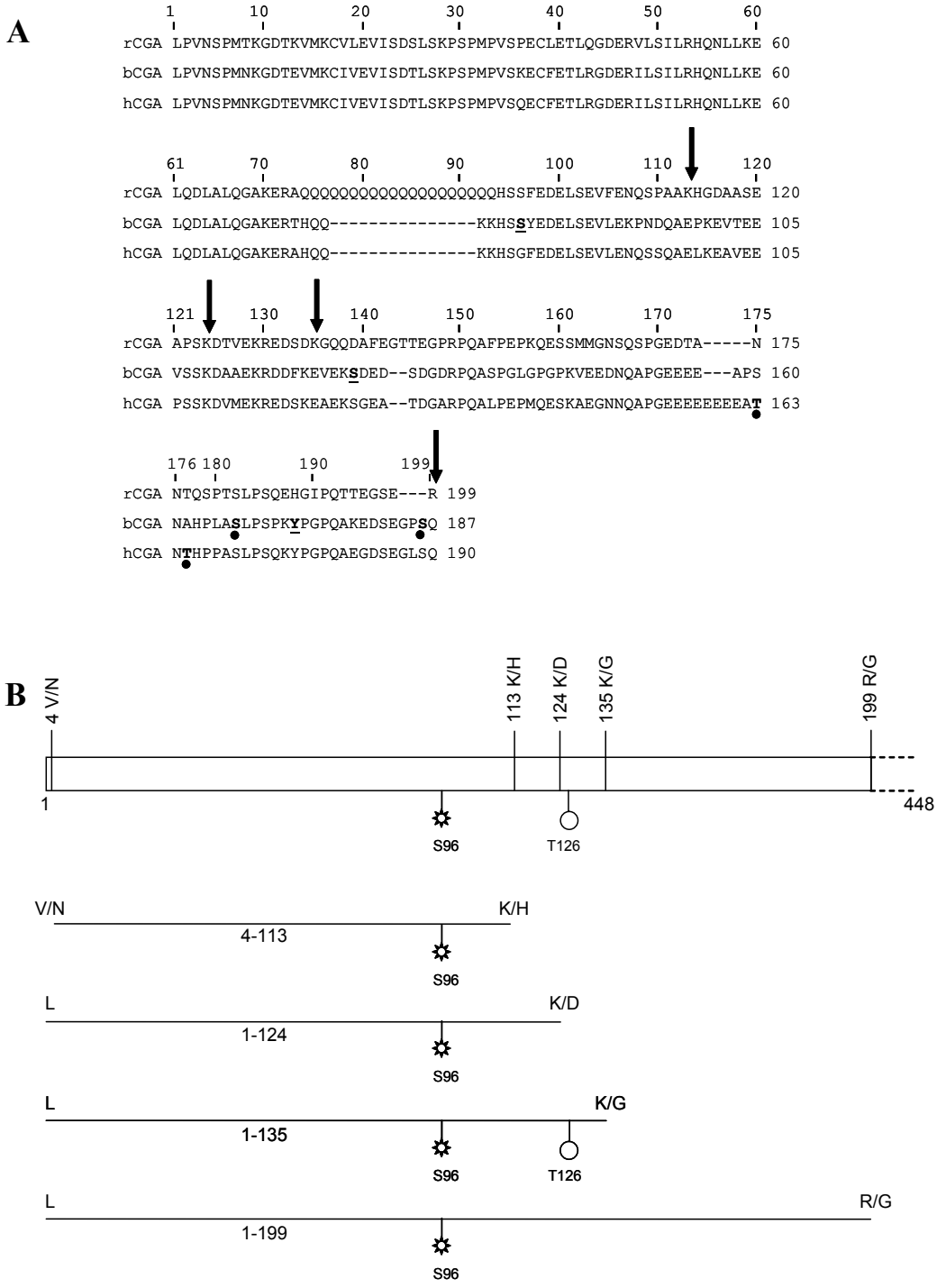

Fig. 16. Characterization of N-term CGA fragments present in rat heart extract. ***A*****,** Sequence alignment of rat (r), bovine (b) and human (h) N-ter CGA sequence. Phosphorylations were in bold underlined letters and O-glycosylation were labelled by black round shape. Localization of phosphorylations and glycosylations sites for bovine and human CGA were taken from the literature. Cleavage sites are located by arrows. ***B***, Mass spectrometry deduced N-terminal rat CGA fragments. ***C***, Putative location of the posttranslational modifications deduced from our data. , phosphorylation. O , O-glycosylation.

4. HCNP stimulated preparations

In absence of desensitization, concentration-response curves of rHCNP were generated by exposing the cardiac preparations to increasing concentrations of the peptide. Since the effects remain stable until 15 min then gradually decreased with time, cardiac parameters were measured at 10 min. rHCNP (from $5x10^{-13}$ to 10^{-6} M) caused a concentration-dependent negative inotropic effect, revealed by a significant decrease of LVP and RPP, without modification of HR. Notably, rHCNP did not affect CP (Fig. 17).

To establish whether HCNP-dependent effects were not secondary to synthesizing activity of HCNP-stimulated ChAT, rHCNP effects were evaluated in presence of a single concentration (10 nM) of bromoacetylcholine (BrACh), a specific inhibitor of ChAT. Blockade of ChAT by BrACh was without significant effect on the rHCNP-induced cardiac effects (Fig. 18).

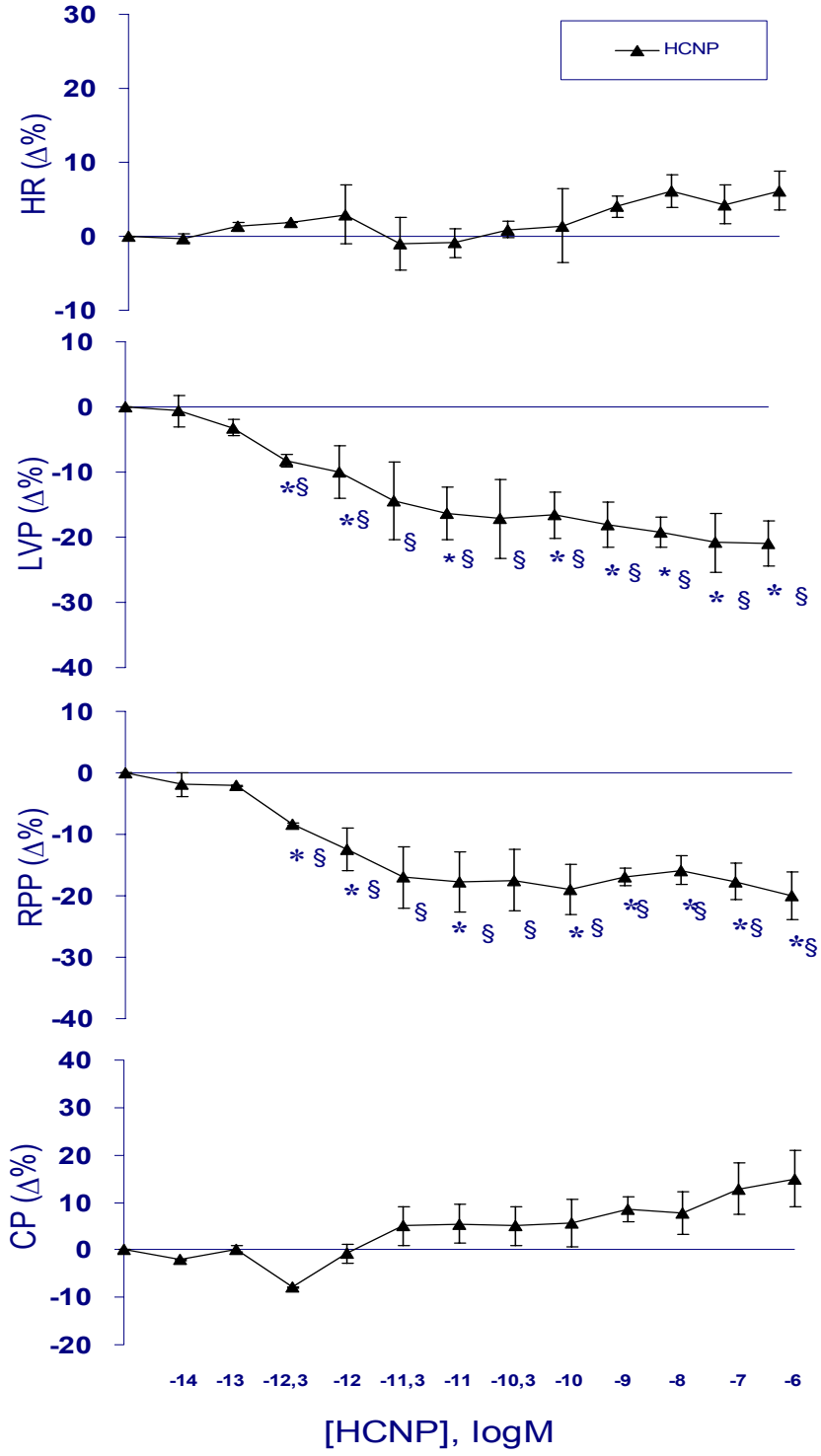

Figure 17. Concentration-dependent responses of rHCNP (from 10^{-14} to 10^{-6} M) on HR (heart rate), LVP (left ventricular pressure), RPP (rate pressure product) and CP (coronary pressure) on the isolated and perfused *Langendorff* rat heart preparation. Percentage changes were evaluated as means ± SEM of 13 experiments. Significance of difference from control values (t-test);*=$p<0.05$. Comparison between groups (ANOVA, Duncan's test); §=$p<0.05$.

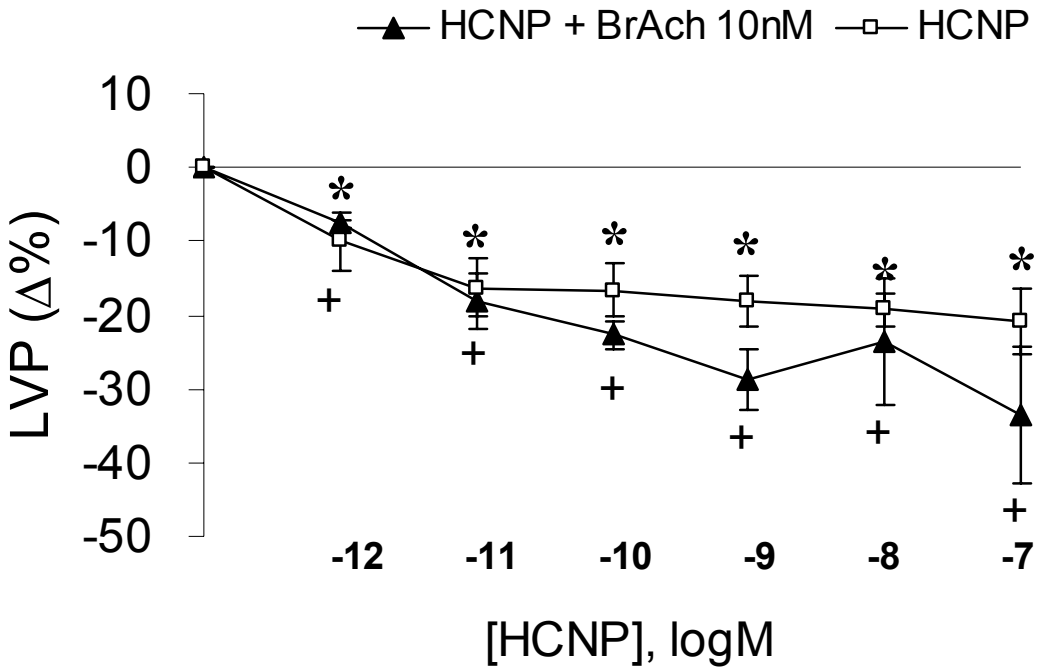

Figure 18. Concentration-dependent responses of rHCNP alone (10^{-12} ÷ 10^{-7} M) and of rHCNP (from 10^{-12} to 10^{-7} M) plus BrAch (10 nM) on HR, LVP, RPP and CP on the isolated and perfused *Langendorff* rat heart preparation. Percentage changes were evaluated as means ± SEM of 7 experiments for each group. Significance of difference from control values (t-test); $^{*,+}$=$p<0.05$. Comparison between groups (ANOVA, Duncan's test); §=$p<0.05$.

4.1 Cholinergic stimulated preparations

To investigate the interaction between rHCNP and intracardiac cholinergic signalling, hearts were perfused with KHs containing Carbachol (10 nM), a non selective cholinergic agonist which is resistant to the action of cholinesterases, plus increasing concentrations of rHCNP (from 10^{-12} to 10^{-7} M). Carbachol, at this concentration, did not modify the reduction of LVP and RPP induced by rHCNP from 10^{-12} to 10^{-11} M. However, it significantly increases the negative inotropism induced by rHCNP at higher concentrations (10^{-10} to 10^{-7} M). In the presence of carbachol, rHCNP did not affect HR, except for a small significant decrease observed at 10^{-10} and 10^{-9} M. At all concentrations, rHCNP plus carbachol induced a non significant increase of CP (Fig. 19). Atropine (1 μM), a competitive non-selective antagonist of muscarinic receptors, abolished the reduction of LVP induced by rHCNP at 10^{-12}, 10^{-8} and 10^{-7} M, while it reduces this effects at 10^{-11}, 10^{-10} and 10^{-9}M. Atropine also abolished or reduced the inhibitory effect of rHCNP on RPP, depending on the lower or higher peptide concentrations, respectively. Muscarinic receptor blockade did not affect HR or CP (Fig. 20). The computerized fitting of the curves indicated the percentages of variations of LVP obtained in the presence of rHCNP alone and rHCNP plus either carbachol (10 nM) or atropine (1 μM) (Fig.21). The sigmoid concentration-response curves and EC_{50} values (Fig. 21) showed a non-competitive antagonism of atropine, as well as a synergic action of carbachol on the rHCNP-mediated negative inotropism.

To explore the involvement of different muscarinic receptor subtypes, rHCNP effects were evaluated in presence of a single concentration (100 nM) of either

AFDX116 or pirenzepine, competitive selective antagonists of M_2- and M_1-M_3 subtype receptors, respectively. The treatment with AFDX116 abolished the reduction of LVP induced by rHCNP at 10^{-12}, 10^{-11} and 10^{-10} M, while significantly reducing it at 10^{-9}, 10^{-8} and 10^{-7} M. AFDX116 abolished the inhibitory effect of rHCNP on RPP at low peptide concentrations, with no effects at higher peptide concentrations. The treatment with the M_2-receptors antagonist induced both a reduction of HR at high concentrations and a vasoconstriction from 10^{-10} to 10^{-7} M (Fig. 22). In contrast, blockade of M_1-M_3 receptors by pirenzepine was without significant effect on the rHCNP-induced cardiac effects (Fig. 23)

The computerized fitting of the curves provides the percentage of variations of LVP obtained in the presence of rHCNP alone and of rHCNP plus AFDX116 (100 nM). The resulting sigmoid concentration-response curves and the EC_{50} values (Fig. 24) indicate competitive antagonism of AFDX116 on the rHCNP-mediated negative inotropism.

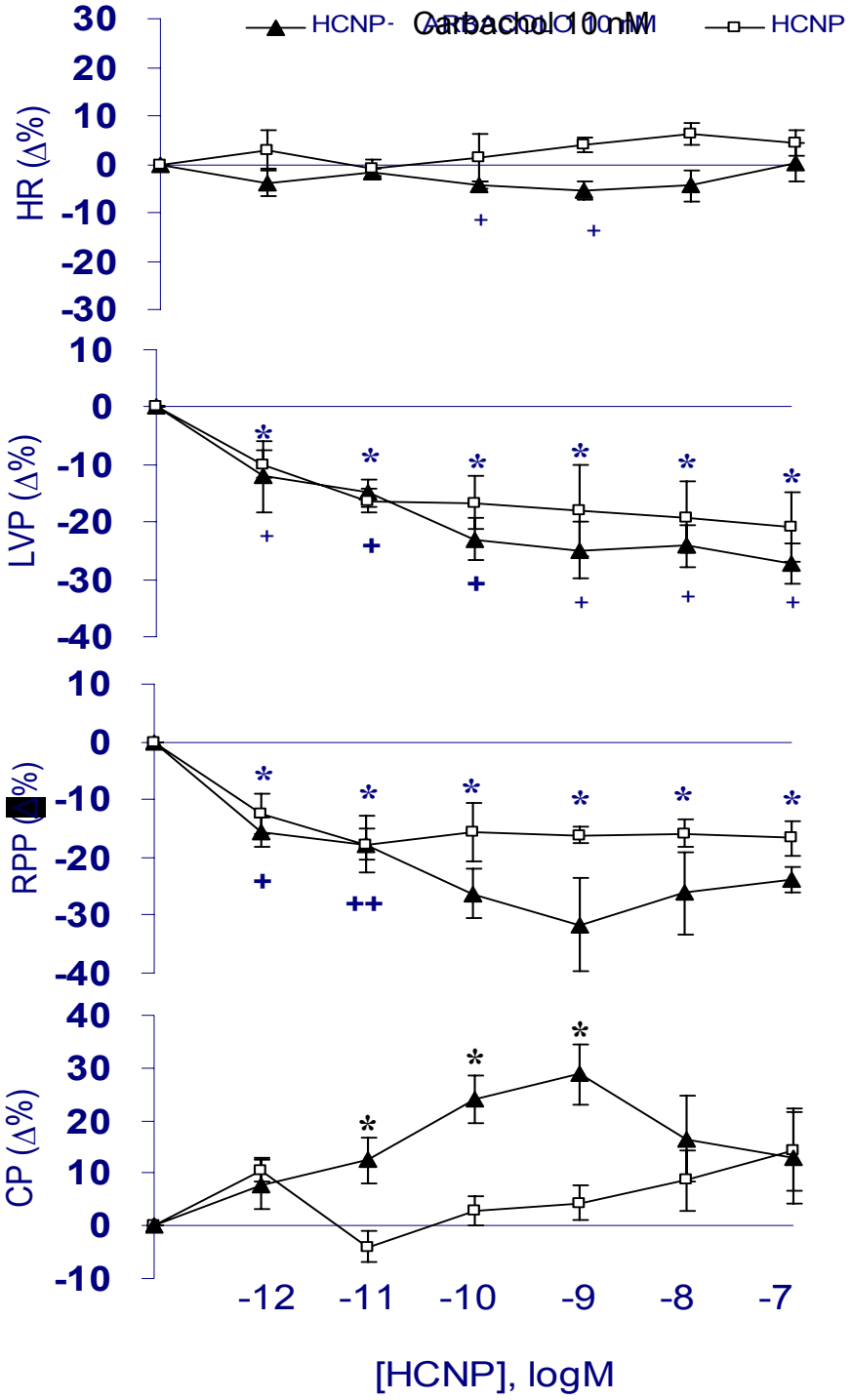

Figure 19 Concentration-dependent responses of rHCNP alone ($10^{-12} \div 10^{-7}$ M) and of rHCNP (from 10^{-12} to 10^{-7} M) plus Carbachol (10 nM) on HR, LVP, RPP and CP on the isolated and perfused *Langendorff* rat heart preparation. Percentage changes were evaluated as means ± SEM of 7 experiments for each group. Significance of difference from control values (t-test); $^{*,+}$=p<0.05. Comparison between groups (ANOVA, Duncan's test); §=p<0.05.

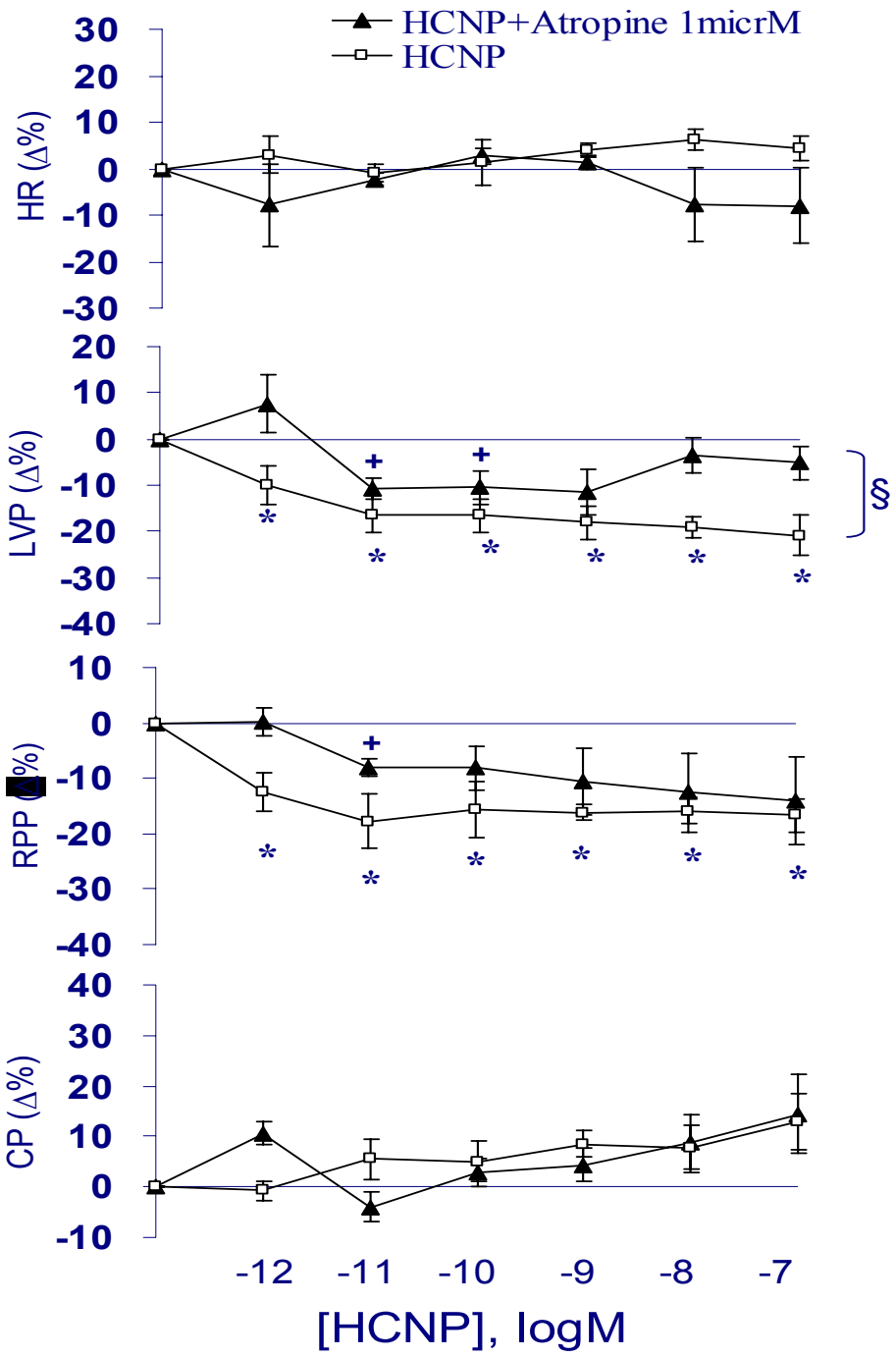

Figure 20 Concentration-dependent responses of rHCNP alone (from 10^{-12} to 10^{-7} M) and of rHCNP (from 10^{-12} to 10^{-7} M) plus atropine (1 μM) on HR, LVP, RPP and CP on the isolated and perfused *Langendorff* rat heart preparation. Percentage changes were evaluated as means ± SEM of 7 experiments for each group. Significance of difference from control values (t-test); $^{*,+}$=p<0.05. Comparison between groups (ANOVA, Duncan's test); §=p<0.05.

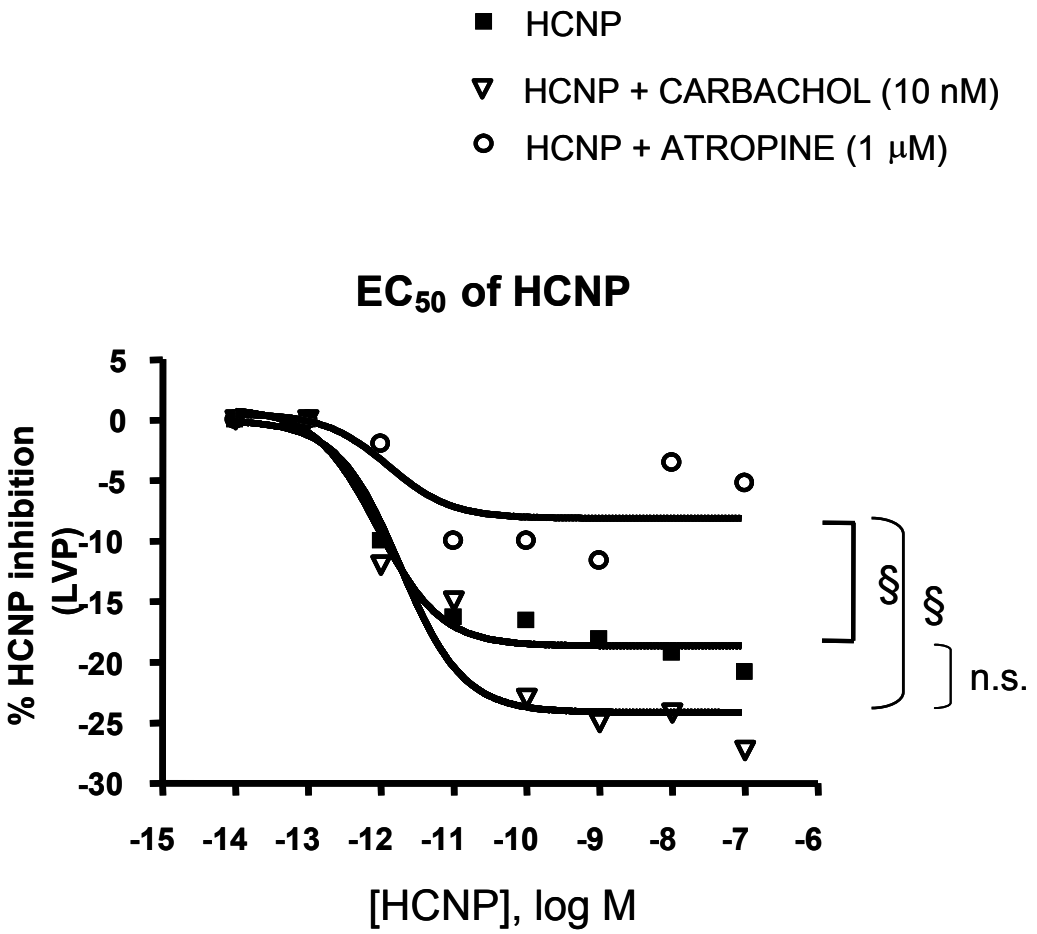

Figure 21. The sigmoid concentration-response curves of rHCNP-mediated inhibition on LVP of rHCNP alone (from 10^{-12} to 10^{-7} M) and of rHCNP (from 10^{-12} to 10^{-7} M) plus a single concentration of carbachol (10 nM) or atropine (1 μM) on the isolated and perfused *Langendorff* rat heart preparation. Inhibition of contractility is expressed as a percentage of LVP [baseline=0%, peak inhibition by rHCNP and rHCNP plus Carbachol or Atropine=-100%].
The EC_{50} values (in logM) of rHCNP alone was -12.05 ± 0.15 (r^2= 0.97), of rHCNP plus carbachol (10 nM) was -11.74 ± 0.28 (r^2= 0.91), of rHCNP plus atropine (1 μM) was -11.90 ± 0.77 (r^2= 0.64). Comparison between groups (ANOVA, Duncan's and Bonferroni's Multiple Comparison Test); §=$p<0.05$.

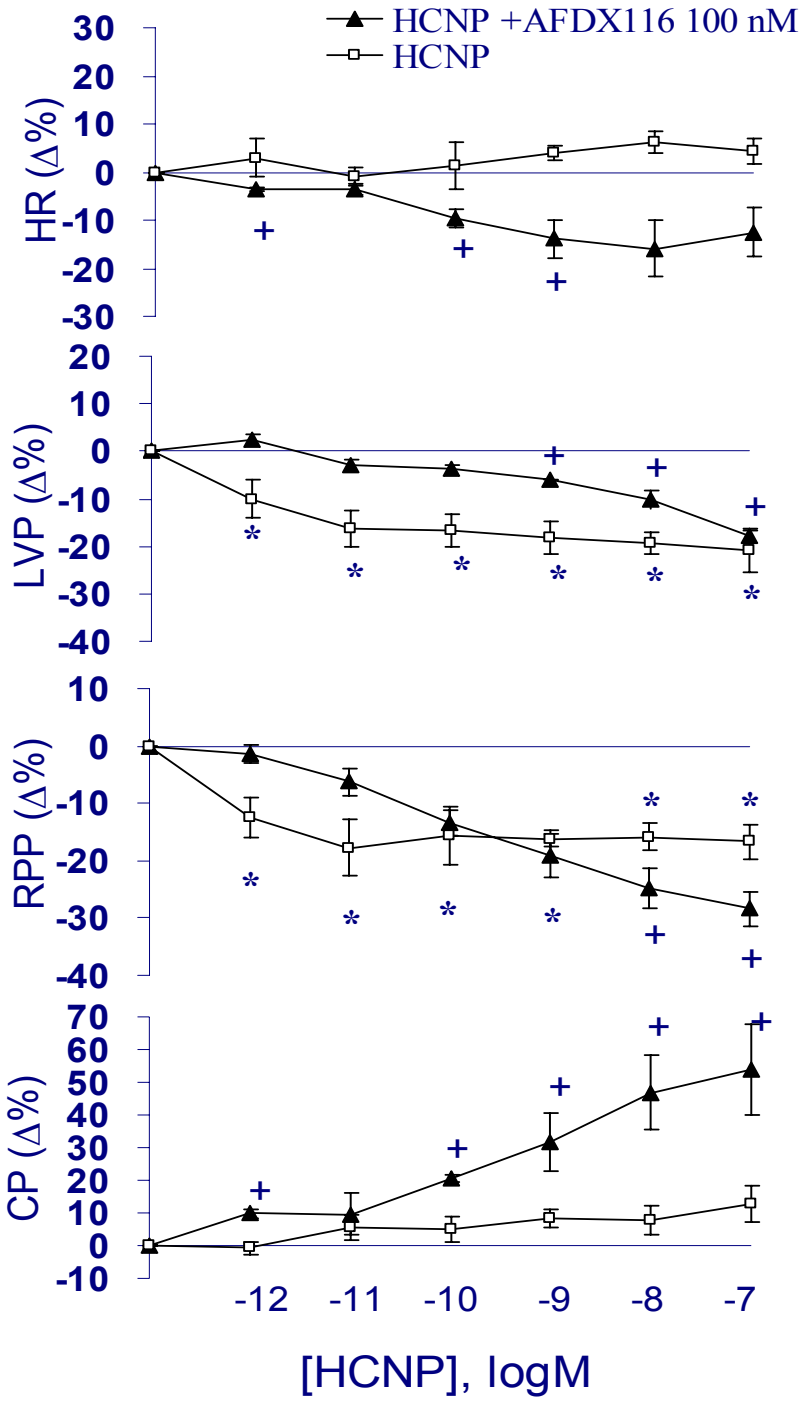

Figure 22 Concentration-dependent responses of rHCNP alone (from 10^{-12} to 10^{-7} M) and of rHCNP (from 10^{-12} to 10^{-7} M) plus AFDX116 (100 nM) on HR, LVP, RPP and CP on the isolated and perfused *Langendorff* rat heart preparation. Percentage changes were evaluated as means ± SEM of 5 experiments for each group. Significance of difference from control values (t-test); $^{*,+}$=p<0.05. Comparison between groups (ANOVA, Duncan's test); §=p<0.05.

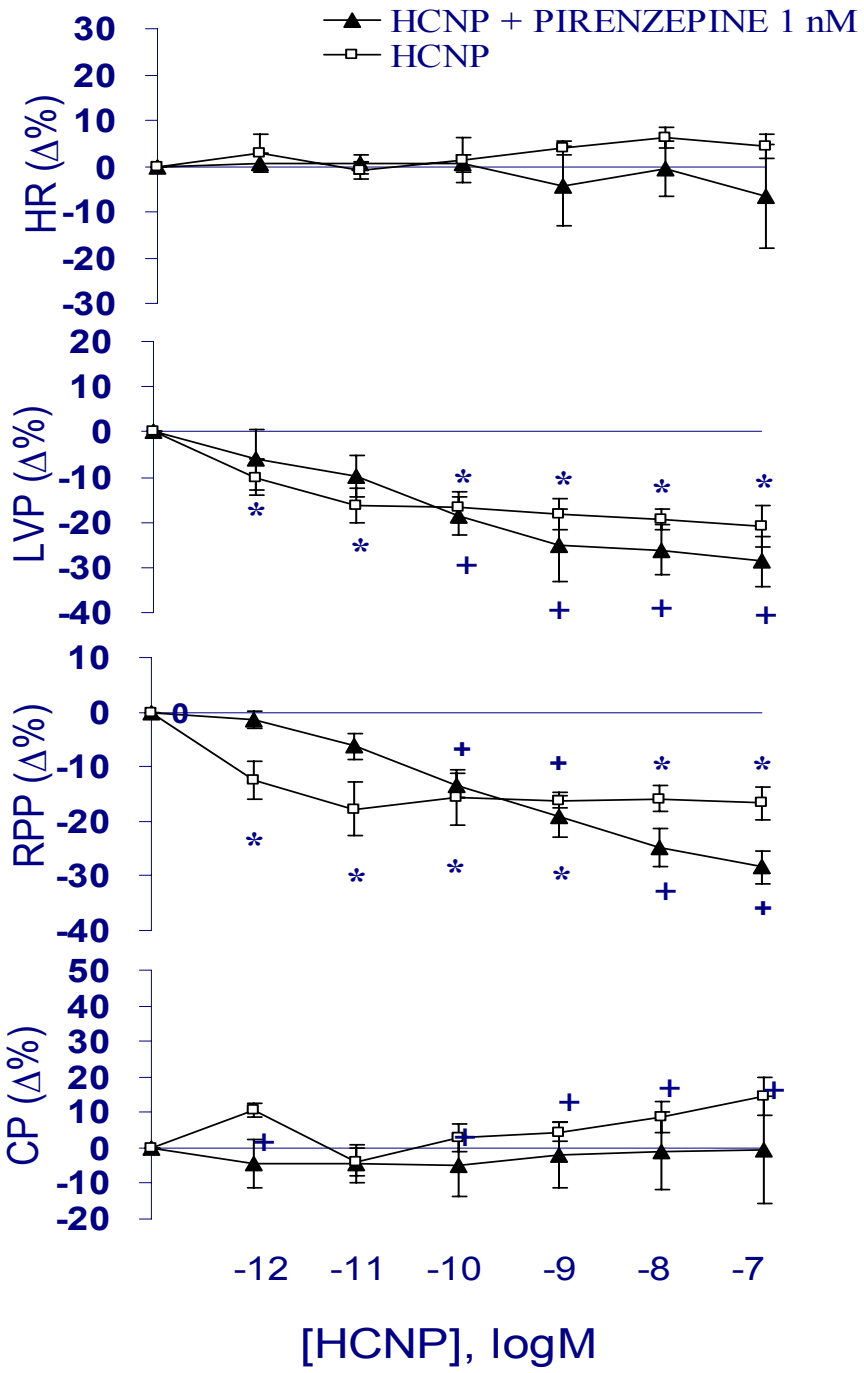

Figure 23 Concentration-dependent responses of rHCNP alone (10^{-12} to 10^{-7} M) and of rHCNP (from 10^{-12} to 10^{-7} M) plus pirenzepine (1 nM) on HR, LVP, RPP and CP on the isolated and perfused *Langendorff* rat heart preparation. Percentage changes were evaluated as means ± SEM of 5 experiments for each group. Significance of difference from control values (t-test); $^{*,+}$=$p<0.05$. Comparison between groups (ANOVA, Duncan's test); §=$p<0.05$.

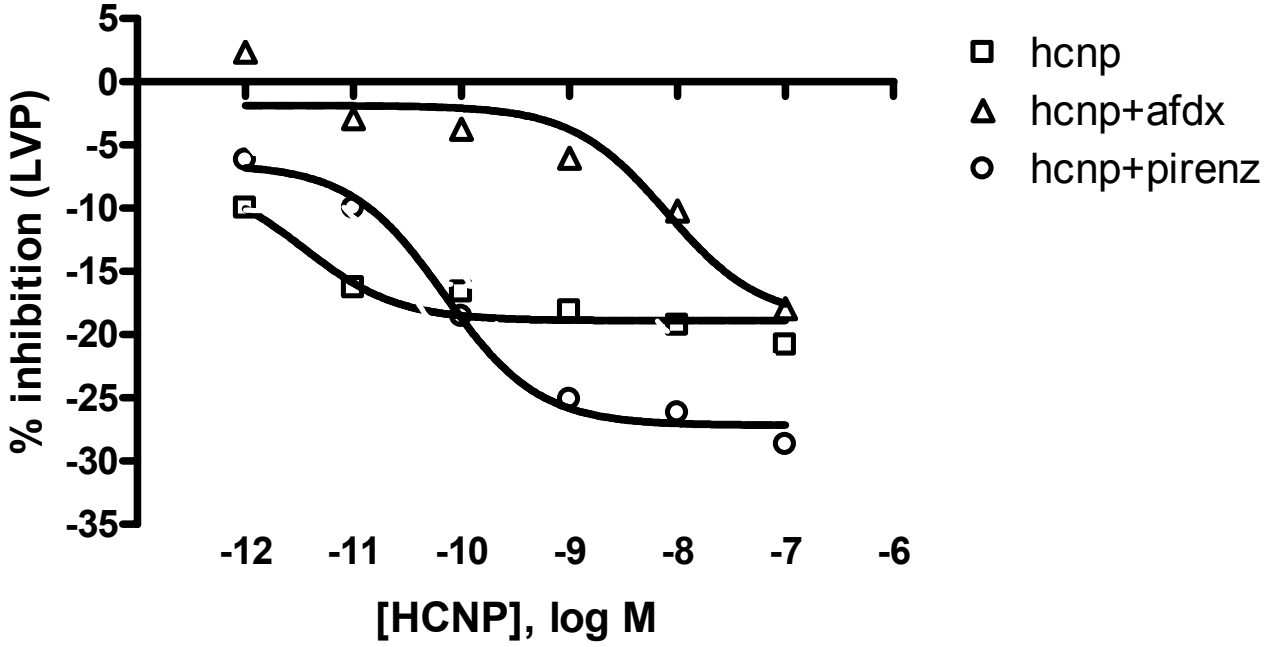

Figure 24. The sigmoid concentration-response curves of rHCNP-mediated inhibition on LVP of rHCNP alone (from 10^{-12} to 10^{-7} M) and of rHCNP (from 10^{-12} to 10^{-7} M) plus a single concentration of AFDX116 (100 nM) or a single concentration of pirenzepine (1 nM) on the isolated and perfused *Langendorff* rat heart preparation. Inhibition of contractility is expressed as a percentage of LVP [baseline=0%, peak inhibition by rHCNP and rHCNP plus AFDX116 or rHCNP plus pirenzepine= -100%].
The EC_{50} values (in logM) of rHCNP alone was -12.05 ± 0.15 (r^2= 0.97), of rHCNP plus AFDX116 (100 nM) was -8.17 ± 0.38 (r^2= 0.88), of rHCNP plus pirenzepine (1 nM) was10.42 ± 0.23 (r^2= 0.96). Comparison between groups (ANOVA, Duncan's Multiple Comparison Test); §=$p<0.05$.

4.2 Gi/o Protein involvement

To verify the involvement of Gi/o protein system, cardiac preparations were perfused with KHs containing PTx (0,01nM) in presence of rHCNP. PTx abolished the rHCNP-mediated negative inotropism (i.e. LVP and RPP) at all concentrations tested, while slightly decreasing HR at 10^{-9} and 10^{-8} M, and increasing CP (Fig. 25).

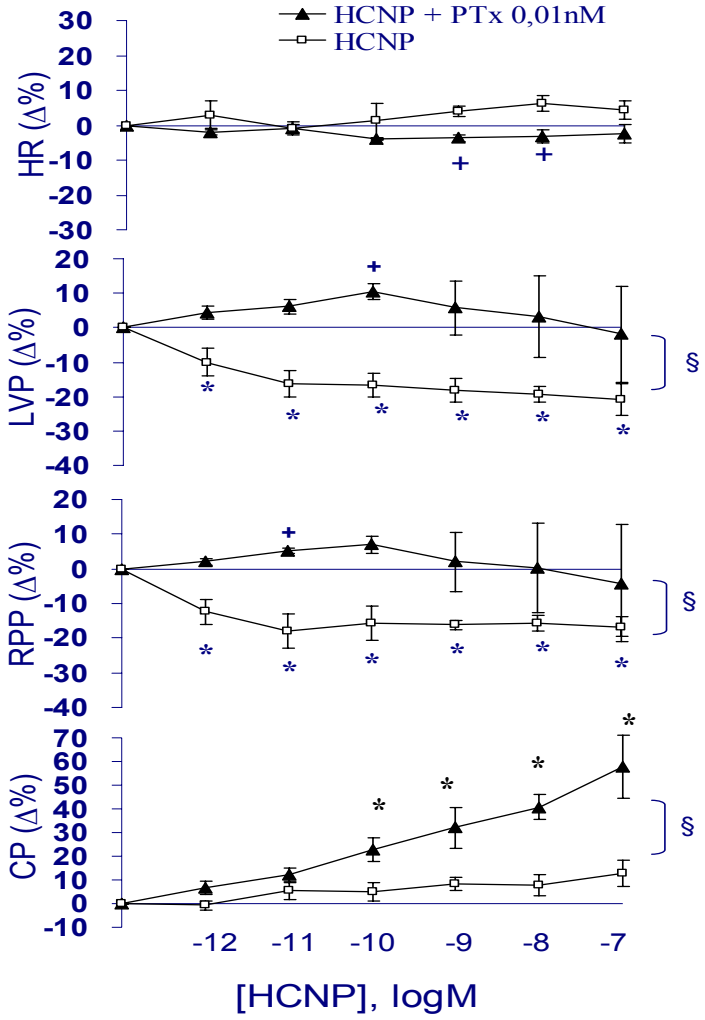

Figure 25. Concentration-dependent responses of rHCNP alone (from 10^{-12} to 10^{-7} M) and of rHCNP (from 10^{-12} to 10^{-7} M) plus PTx (0,01 nM) on HR, LVP, RPP and CP on the isolated and perfused *Langendorff* rat heart preparation. Percentage changes were evaluated as means ± SEM of 4 experiments for each group. Significance of difference from control values (t-test); *=p<0.05. Comparison between groups (ANOVA, Duncan's test); §=p<0.05.

4.3 Adrenergic-stimulated preparations

To test whether rHCNP is able to counteract the ββ-adrenergic-mediated positive chronotropism, inotropism and coronary dilation, dose-response curves were generated by perfusing heart preparations with increasing concentrations of ISO (from 10^{-10} to 10^{-6} M) alone and together with a single concentration of rHCNP (0.01 nM). rHCNP (0.01 nM) abolished the ISO (from $5x10^{-9}$ M to 10^{-7} M)-mediated positive increase of LVP and RPP. At all ISO concentrations, rHCNP significantly reduced HR without modifying CP (Fig. 26).

The antagonistic effect of rHCNP against the ISO-dependent stimulation, evaluated by fitting the sigmoid concentration–response curves of LVP and RPP in presence of ISO alone (from 10^{-10} to 10^{-6} M) and ISO (from 10^{-10} to 10^{-6} M) plus rHCNP (0.01 nM), provided the corresponding percentage of LVP variations in terms of EC_{50} values. These data are consistent with a competitive type of antagonism of rHCNP (Fig. 27A and B).

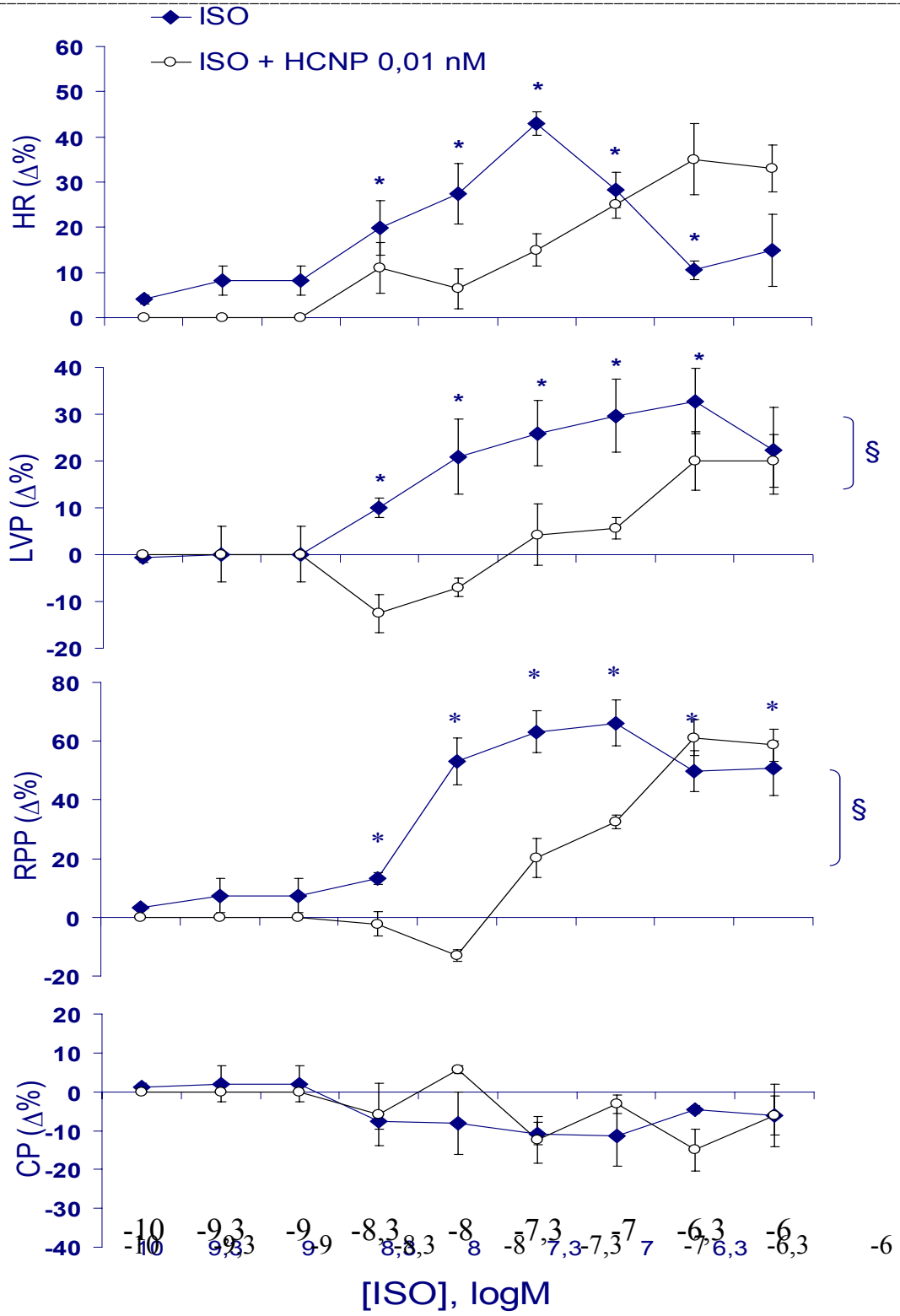

Figure 26. Concentration-dependent responses of ISO alone (from 10^{-10} to 10^{-6} M) alone and of ISO (from 10^{-10} to 10^{-6} M) plus of rHCNP (0.01 nM) on HR, LVP, RPP and CP on the isolated and perfused *Langendorff* rat heart preparation. Percentage changes were evaluated as means ± SEM of 7 experiments for each group. Significance of difference from control values (t-test); *=p<0.05. Comparison between groups (ANOVA, Duncan's test); §=p<0.05.

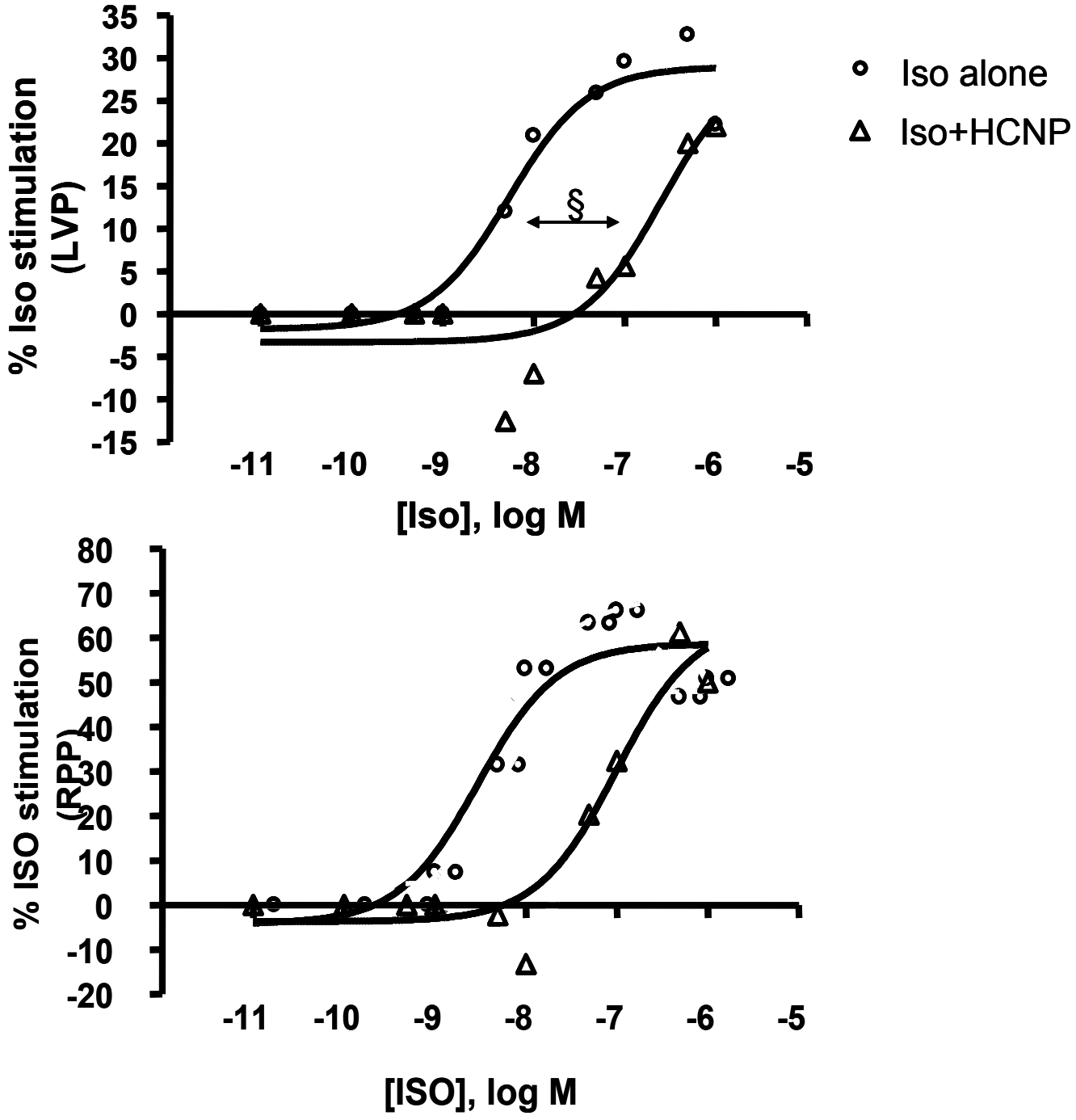

Figure 27 The sigmoid concentration-response curves of ISO-mediated stimulation on LVP (A) and RPP (B) of ISO (from 10^{-10} to 10^{-6} M) alone and of ISO (from 10^{-10} to 10^{-6} M) plus a single concentration of rHCNP at 0.01 nM on the isolated and perfused *Langendorff* rat heart preparation. Contraction is expressed as a percentage of LVP and RPP [baseline=0%, peak constriction by ISO and ISO plus VS1=100%].
The EC_{50} values (in logM) of ISO alone was -8.5 ± 0.3 (r^2= 0.90), of ISO plus HCNP (0.01 nM) was -6.9 ± 0.36 (r^2= 0.88). Comparison between groups (ANOVA, Duncan's test); §=$p<0.05$.

Discussion

1. Vasostatins on Isolated heart preparation

This study tested the hypothesis that vasostatins may act as inotropic modulators of cardiac performance in mammals with an "anti-adrenergic" potential. We have demonstrated that human recombinant VS1and VS2 exert a cardiotropic action on the rat heart (Cerra et al., 2005). These results represent the first indication that CGA-derived N-terminal peptides containing the vasostatin 1 (CGA 1-76) and vasostatin 2 (CGA 1-113) sequences can affect the cardiac performance of mammals, with potentially important physiological implications.

On the Langendorff-perfused rat heart under basal conditions VS1 caused a dose-dependent reduction of LVP and RPP, thus inducing a negative inotropism. Significant effects were elicited by VS1at concentrations of CGA in the plasma of patients with severe heart failure (5-20 nM, (Ceconi et al., 2002)). Since the negative inotropy is attained without affecting neither HR nor CP, the reduction of contractile performance, and consequently of cardiac work, is consistent with a direct myocardial influence of the peptide which is independent from coronary reactivity. This result is in agreement with the direct negative inotropism induced by VS1on the frog heart which lacks the vascular supply and therefore is a useful natural bioassay for uncovering specific myocardial effects elicited by cardioactive agents (Corti et al. 1997). Moreover, the VS1 inotropic potency detected on the rat heart is within the same range of concentrations as in frog and eel hearts (Corti et al. 1997, Curry et al. 1991). This finding is of evolutionary interest in view of the high conservation (83%) between frog and human peptide (Turquier et al. 1999). In particular, homology reaches 100% in both the CGA_{14-29} (frog, cattle, man, pig, horse and ostrich), and the

CGA_{51-62} sequence (former species plus rat and mouse) (Aardal and Helle, 1992 and references therein). Notably, the phylogenetically conserved domains CGA_{17-38} and CGA_{7-57} appear crucial for eliciting the negative inotropism and counteracting theβ-adrenergic stimulation (Corti et al. 2002, Tota et al. 2003).

The lack of species-specificity of human VS1 on the isolated and Langendorff perfused rat heart suggests that this model may be exploited for studying the effect of CGA N-terminal fragments on mammalian heart, thus overcoming the difficulty of using human hearts. However, the rat heart model could have some limitations that must be taken into consideration. The N-terminal sequence of rat CGA, in contrast to the human sequence, does not contain the $Lys_{77}Lys_{78}$ dibasic site, while it contains an insertion of 15 glutamine residues (Fig. 1, Mandalà et al 2005 and references therein).

Thus, proteolytic processing in humans and in rats is likely different. In previous work Stainer et al., 1990 showed by immunoblotting that CGA is extensively processed in the rat atrial tissue, but the presence of vasostatin-like fragments was not demonstrated. It is difficult to speculate whether lack of detection was due to lack of production of vasostatin-like fragments or to lack of vasostatin-specific antibodies in the polyclonal antiserum. Further studies are therefore necessary to clarify whether vasostatin-like fragments are produced in the rat heart.

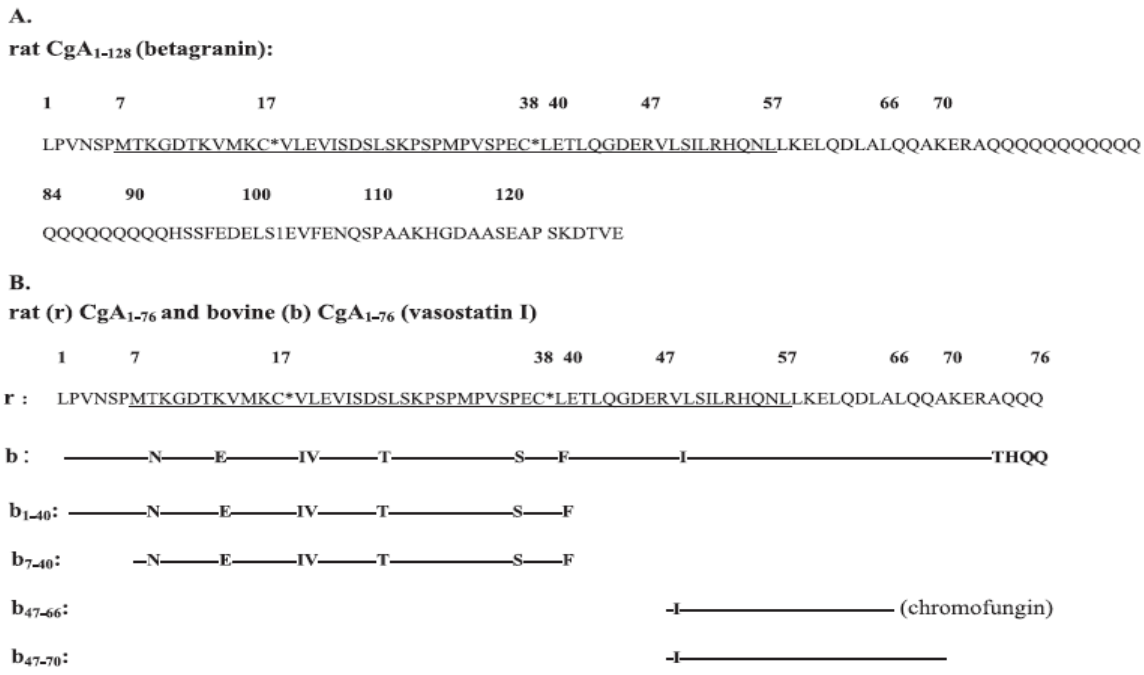

Fig. 1 Primary structure of (A) rat betagranin [Iacangelo et al., 1988] and (B) a structural comparison of the rat (r) and bovine [Metz-Buotigue et al., 1993] (b) CgA1–76, including the synthetic bovine peptides used in the present study. In (A), the sequence of the synthetic rat peptide (CgA7–57) is underlined. In (B), the substituted amino acid residues in the bovine peptides relative to the sequence of rat CgA1–76 are given in bold.

Nevertheless, the high homology of structural domains (i.e. 82% homology of CGA 17-38) between human and rat sequences (Table 1), together with the negative inotropism exerted by human recombinant VS1 on the rat heart, well comparable to that documented in eel and frog hearts (see for references Imbrogno et al, 2004), strongly support the view that the inotropic activity of CGA N-terminal fragments is highly conserved. Knowledge on the biologically active determinants of CGA provides important information regarding structure-function relationship of this

peptide. The alignment of the reported sequences of CGA_{1-76} indicates that VS1 is a highly conserved segment of the protein.

Peptide	Minimum concentration for intrinsic negative inotropy (nM)	*n*	Minimum concentration for counteracting the ISO-evoked inotropy	*n*
CGA_{1-40SS}	68*	3	45*	4
CGA_{1-40SH}	>148	3	>68	3
$fCGA_{4-16}$	>695	4	206*	4
$bCGA_{4-16}$	68*	5	206*	4
$fCGA_{47-66}$	125*	4	125*	4
$bCGA_{47-66}$	125*	5	272*	4

Table I. Minimum effective concentrations of VS1-derived peptides for intrinsic negative inotropy and for counteracting the isoproterenol (ISO)-evoked inotropy in paced frog hearts. *P<0.05 for difference from control (without peptide) for *n* hearts at that concentration.

Shorter VS1 sequences have been shown to mimic some of the VS1 actions, while other regions such as the disulfide-bridge loop**,** formed by the two cysteine residues, appear important for the biological actions of VS1 in some but not all bioassays. We have attempted to identify specific sequences within VS1 with which the intrinsic negative inotropy and the counteracting action against the β-adrenergic-mediated positive inotropism may be associated. We have used as bioassay the electrically paced, isolated and perfused working heart of the frog, a preparation very suitable for assessing the direct effects of cardiotropic substances on myocardial performance (Tota et al., 2003). The bovine sequences CGA_{4-16} and CGA_{47-66} have been compared with the frog counterparts and the importance of the disulfide bridge has been analysed by means of the bovine CGA_{1-40} with (CGA_{1-40SS}) and without (CGA_{1-40SH})

an intact loop. Their minimally effective concentrations for intrinsic negative inotropism and for counteracting the ISO-mediated positive inotropism are reported in Table 1. The results show that structurally different sequences of frog and bovine VS1 are both able to modulate inotropy in frog myocardium, emphasizing the high phylogenetic conservation of the sequence CGA_{1-76}. The intact VS1, the fragment CGA_{7-57} and the shorter peptide CGA_{1-40} with the intact disulfide-bridge loop appear the most potent in exerting intrinsic negative inotropy and counteraction against ISO-mediated positive inotropism.

The amphipatic sequences of frog and bovine CGA_{4-16} and CGA_{47-66} induce negative inotropic modulation of cardiac performance both under basal and ISO-stimulated conditions, although with significantly lower apparent potencies (Table 1) than reported for the human recombinant VS1 and CGA_{7-57} and for the natural configuration of the bovine CGA_{1-40}, i.e. CGA_{1-40SS}. The minimally effective concentrations (Table 1) show that the least effective sequence is the frog CGA_{4-16}. Of note, this peptide carries a net negative charge and, compared to the bovine counterpart, lacks of amphipathy.

By comparison, while the N- and C-terminal sequences of frog and bovine VS1, CGA_{4-16} and CGA_{47-66} counteract the ISO-stimulated inotropy at higher threshold concentrations of peptide, the bovine CGA_{1-40SS} but not CGA_{1-40SH} counteracts the ISO-evoked positive inotropy at threshold concentrations of 45nM, similar to that of human VS1 and CGA_{7-57}. These data indicate that peptides containing the bovine CGA_{17-38} domain might substitute for the intact human VS1 in counteracting the β-adrenergic-mediated positive inotropy. This indicates that the disulfide-bridge region

of VS1 may be relevant for the marked negative inotropism elicited by VS1 and CGA_{7-57}, both under basal or adrenergically stimulated cardiac performance. The fact that the intact CGA_{1-40SS} sequence is more potent than its reduced counterpart could reflect a steric requirement exposed in the loop configuration.

This suggests that our data are relevant in the context of mammalian cardiac physiology.

The recombinant VS1 contains the human vasostatin 1 sequence plus two residues (KK) at the C-terminus and three additional residues (STA) at the N-terminus, due to the cloning procedures. The biological activity observed with VS1 and the results of previous work showing that a synthetic peptide CGA7-57 is sufficient to induce negative inotropic effects in the eel and frog hearts (Corti et al. 2002, Imbrogno et al. 2004, Tota et al. 2003), indicate that the N-terminal amino-group and the C-terminal carboxylic group of vasostatin 1 are likely not critical for the activity.

We have also found that STA-CGA1-115 induces positive chronotropism and coronary constriction without modifications of contractile performance. Therefore, the two peptides appear to differently affect the non-stimulated rat heart. On the frog heart, both VS1 and VS2 elicited a negative inotropism, VS2 showing lower inotropic potency than STA-CGA1-78. However, in 40% of the frog heart preparations VS2 induced positive inotropism (Corti et al. 2002, Tota et al. 2003). The present work does not clarify whether the different activities shown by the two vasostatin peptides on the rat heart depend on different mechanisms activated by specific domains of the two fragments. Structure-function analyses carried out on the

frog heart showed that 1-40, 47-66 domains, and particularly the 7-57 region, represent structural determinants of the cardiosuppressive action of vasostatins (Corti et al., 2002; Tota et al., 2003). Furthermore, cleavage at Lys_{77}-Lys_{78} of VS2 with plasmin and removal of the C-terminal domain was associated with an increase of pro-adhesive effects in fibroblast adhesion assays (Colombo et al. 2002). It is conceivable that the C-terminal domain of VS2, lacking in VS1, can either mask or modify the structural determinants of inotropic and pro-adhesive activities located within the VS1 domain.

In our beating heart preparation VS1 did not exert any coronary activity. In contrast, vasodilation was observed on segments of bovine coronary resistance arteries exposed to the VS1 active domain 1-40 (Brekke et al., 2002), as well as on segments of intrathoracic artery and saphenous vein exposed to vasostatin-1 (Aardal et al., 1993). The possibility exists that differences in either the experimental conditions (i.e. *in situ* perfused whole coronary bed *vs* isolated vascular segments), or species-specific sensitivities towards vasostatin peptides may account for these different results.

As a second step of our research we aimed to explore if VS1 cardiotropic action involves membrane receptors or it requires the interaction with inter- and/or intra-cellular proteins. On the rat heart, VS1-dependent negative inotropism is abolished in the presence of alfa and beta adrenoceptor antagonists, while it is unaffected by the cholinergic inhibition (Angelone et al., 2005). Rather being directly related to a specific adrenergic receptor antagonism, VS-induced inotropism could be due to secondary interactions between VSs and inter- or intra-cellular

regulatory proteins affecting, for example, allosteric sites of the adrenergic receptors. Indeed, as postulated for the VS1 antifungal and antibacterial activities (Maget-Dana et al., 2002), it is possible that VSs through their lipophilic region may interact with some plasma membrane lipid domains (caveolae, scavenger receptors, etc…), thereby activating inter- and/or intracellular signal-transduction pathways, including Gi/0 protein and the NO-cGMP-PKG systemMoreover, in agreement with our work on the ell heart, On the ß-adrenergic stimulated rat heart, both VS1 and VS2 showed similar inhibitory activity towards the typical ISO-dependent increase of cardiac contractility and work. This result is consistent with the negative inotropism exerted on ISO-stimulated cardiac preparations of eel and frog (Corti et al. 2002, Imbrogno et al. 2004). To test the selectivity of this counteracting activity we tested VS1against the positive inotropic agent ouabain (Muller-Ehmsen et al. 2003; Schiffmann et al., 2002) and we found that the peptide was unable to modify the glycoside-induced positive inotropism.

The effects of VS1on cardiac work analysed by means of competitive experiments indicated a functional noncompetitive antagonism between the peptide and the ISO-elicited increase of RPP. A similar noncompetitive behavior was also demonstrated for catestatin, another active CGA fragment corresponding to residues 344-364, which inhibits nicotine cholinergic receptor-mediated catecholamine release from chromaffin cells (Mahata et al., 1997). At present, it is not known if the biological effects of vasostatins on their cellular targets (e.g. endothelium, smooth muscle, fibroblasts (Helle et al., 2001) occur via a classic receptor-ligand interaction. In fact, although binding sites for vasostatins and their fragments have been

suggested in calf aorta vascular smooth muscle and bovine parathyroid cells (Maget-Dane et al. 2002), the demonstration of a classic receptor remains elusive. Alternatively, it has been proposed that vasostatin-derived fragments (i.e. CGA_{1-40} and CGA_{47-66}) exert their effects by interacting with, or penetrating into, the cell membrane by means of hydrophobic interactions between specific domains of the peptides and spatially localized regions of the lipid bilayer with consequent modulation of cellular effectors (Lugardon et al. 2000; Maget-Dane et al. 2002). In line with this hypothesis, at least two possible mechanisms underlying the inhibition of the ISO-mediated positive inotropy stimulation may be suggested. One mechanism could be via an allosteric modulation of the ß-adrenergic receptor independent from the ligand binding site; the other could be via modulation of the downstream intracellular signalling triggered by the activation of the ß-adrenoceptor itself. An involvement of the adrenergic receptor system in the vasostatin-dependent cardiosuppression has been reported in eel but not in frog Corti et al 2004, Imbrogno et al 2004), indicating the existence of different species-specific mechanisms. Further analyses (e.g. radioligand competitive binding studies) can clarify this aspect. Of note, the dose-response curve of VS1 shows a bell-shaped behavior, maximal inhibition of isoproterenol activity being achieved with 65 nM peptide concentration. This is not unprecedented. In fact, previous studies on cell adhesion showed that also the pro-adhesive activity of VS1on murine fibroblasts is bell-shaped (Koeslag et al., 1999). We do not know the reason for this behaviour. Possibly, considering that VS1can exist in a monomer-dimer equilibrium (Corti et al., 1997) the bell-shaped

curve could reflect structural changes occurring as a function of peptide concentration.

On the Langendorff-perfused rat heart both VS1and VS2 did not modify the ISO-induced coronary dilation. Of note, vasostatins maintain the vasodilation evoked by moderate extracellular potassium on segments of bovine coronary arteries, leading to speculate that synergism between vasostatins and coronary dilators facilitates the cardiac blood supply in face of stress-induced increased activity (Brekke et al. 2002). In conclusion, we demonstrated that VS1and VS2 exert an inhibitory tone on the performance of the isolated and perfused rat heart both under basal conditions and under ß-adrenergic stimulation. This action is mainly attained by reducing contractility and cardiac work. The "anti-adrenergic" action of vasostatins points to a putative counter-regulatory role of these CGA fragments in cardiovascular homeostasis, which may protect the heart against overstimulation at several levels (i.e. circulating catecholamines or/and local adrenergic stimuli via intra-cardiac nerves, enterochromaffin cells or cardiomyocytes themselves (Steiner et al. 1990). This may have important pathophysiological implications for example in relation to the multiple cardiovascular functions exerted by N-terminal CGA peptides

Interestingly, we report for the first time a VS-induced negative lusitropic effect . Moreover, it is of note that, in parallel with the negative inotropism, VS1 counteracted the positive lusitropic action of the β-adrenergic stimulation. Thus, VS1 appears to act in an opposite manner with respect to the β-adrenergic agonists. In fact, the peptide is able to counteract not only the positive inotropism elicited by ISO, but also its well-known enhancement of the rate of relaxation (McIvor et al.,

1988). These data are relevant when considering the high levels of CGA observed in patients with systolic and/or diastolic dysfunction.

Ischemic preconditioning is a phenomenon in which brief periods (2-20 min) of ischemia can evoke mechanisms leading to protection against myocardial cellular damage in the event of a subsequent prolonged period of ischemia. In ischemic preconditioning, nitric oxide (NO) limits the extension of a subsequent infarct and protects against ischemia/reperfusion-induced endothelial dysfunction, arrhythmias and myocardial stunning (Gattullo et al., 1999). The protective activity concerns both the first and the second window of protection. The antiarrhythmic effect is attributed to microvessel dilation and to the production of cyclic guanosine monophosphate in the myocardium. The limitation of the infarct size is likely to depend on the opening of the mitochondrial adenosine triphosphate-sensitive potassium channels, to which NO participates via the activation of a protein kinase C (PKC) (Gattullo et al., 1999 and references therein).

On the basis of the above feature, a possible protective role of VS1 in limiting the extent of the necrosis induced by ischaemia and reperfusion (I/R) was studied. At present, the results of preliminary experiments show that VS1 is able to limit the damage caused by I/R and that the protective activity is mediated by NO signalling.

Thus, experiments were performed to assess whether rat cardiac tissues may produce vasostatins. This may help to clarify the molecular components and the mechanisms involved in the vasostatin-mediated cross-talk between the adrenergic system and the heart and, at the same time, its putative significance in cardiac protection.

2. VS1 on myocardiocyte Culture

Studies on the proadhesive activities of VS1, suggest their involvement in modulating adhesion mechanisms in the extracellular space, influencing ligand capacity or affinity of the integrins (Gasparri et al., 1997). Recently, studies on frog and eel hearts on influence of the cytoskeleton on the VSs-mediated inotropism response showed that the VSs-mediated negative inotropy is abolished by different inhibitor of actin polymerization, suggesting the inotropic influence of vasostatins is cytoskeleton-dependent (Mazza et al., 2005). There is convincing evidence in the myocardiocytes that cytoskeleton interaction with adrenergic receptor modulation of ionic current may influence contractility (Malan et al., 2003). Using fluorescently tagged phalloidin, we showed that VS1 induced remarkably actin polymerization at all concentration tested. In order to verify that the action of peptide CGA-derived is not secondary to osmosis effects, we tested an inert peptide, Chromogranin B (CGB614-626), and ISO. In this case the effect induced by VS1 was undetectable after treatment with both CGB and ISO. These data strongly suggest a role of VS1 in modulating actin microfilaments network. The exact mechanism by which VS1 may exert this effect on actin microfilaments is to be defined.

3. Characterization of Vasostatin containing peptides in Rat Heart

We investigate for the first time the N-terminal processing of CGA in rat heart. Our results indicate that in rat heart extract, a major board immunoreactivity for CGA at an apparent molecular weight of 75-50 kDa is present. This high molecular weight immunoreactivity is due to the whole CGA protein (with and without posttranslational modifications), as well as C-terminal truncated CGA fragments, and suggests that the maturation process is incomplete in this organ. By comparison with N-terminal CGA fragments present in chromaffin cell intragranular matrix (Metz-Boutigue,et al. 1993, Strub et al. 1997, Lugardon, et al. 2000), it appears that only few low molecular major fragments are present (27 and 37kDa, respectively *d* and *g* labels), whereas a ladder of N-terminal fragments appears to be present in the bovine chromaffin granule matrix. In addition to the previously described N-terminal CGA fragments, a proteomic approach using RP-HPLC and mass spectrometry techniques allowed us to characterize three peptides starting at the first residue and one at the fourth residue of the protein. All these peptides ended at a monobasic potential cleavage site that could be recognized by prohormone convertases and carboxypeptidases that are present in rat heart (Zheng et al., 1994). Interestingly, the difference of molecular mass obtained by the comparison of the theoretical and the experimental masses allowed us to determine that phosphorylations, oxidations and/or glycosylations were present on some of these

fragments. Thus, the presence of a phosphorylation previously described on residues Ser_{81} of bovine CGA was extrapolated to the Ser_{96}, since this site is conserved (Bauer et al. 1999, Zhang et al. 1997) and predicted to be phosphorylated. In addition, we observed mass differences that could be attributed to a O-glycosylation (NAcGal-Gal-NeuAc) that might be present on the Thr_{126} according to O-glycosylation site predictions (NetPhos 2.0 software) and the fact that this modification is only observed on CGA_{1-135} (preventing proteolytic degradations) and not for shorter fragments (*i.e.,* CGA_{1-124} and CGA_{4-113}). One of the characterized fragments, the CGA_{1-135}, is of particular interest because it is found under various forms: non modified, oxidized, phosphorylated and O-glycosylated (NAcGal-Gal-NeuAc).

CGA phosphorylations have been extensively studied in bovine and human, and were described on serine located on residues 81, 124, 297, 307, 372 and 376, as well as on Tyr_{173} of the bovine CGA (Bauer et al. 1999, Strub,et al. 1997). In human, phosphorylations were described on serine residues 200, 252 and 315 of the CGA sequence (Gadroy,et al. 1998). That kind of posttranslational modifications could be related to the peptide activities. Thus, phosphorylations represent a modulator of the antimicrobial activities of the chromacin (Strub et al., 1996; Goumon et al., 1996). The results of our research also indicate the presence of oxidized fragments. In the chromaffin granules, for instance, mass spectrometry analysis have indicated the presence of oxidation on methionine residues that could be related to a redox mechanism taking place inside the granules (Sirimanne et al., 1995).

CGA and its derived fragments are present in secretion organelles and are secreted by various organs and cell types (Helle, 2004) . Interestingly, a co-

localisation of CGA and atrial natriuretic peptides was found in secretion vesicles of rat heart (Steiner et al., 1990) indicating that CGA and CGA-derived peptides could be secreted. Together with our study, these data strongly suggest the secretion of the CGA N-terminal derived fragments by the cardiomyocytes leading to the concept of an implication as a para- or autocrine cardiomodulator according to the cardiosupressive effects of VS1-derived peptides (Tota et al., 2003, Corti et al., 2004, Corti et al., 2002).

4. HCNP on Isolated heart preparation

To date, recent information about PEBP and its derived HCNP implicates an involvement in multiple signalling mechanisms. In the present study we show the involvement of HCNP in the myocardial G protein-coupled receptor pathways acting as negative modulator of the adrenergic signalling. In fact, using the model of isolated and perfused rat *Langendorff* heart, we demonstrate for the first time, in mammals, that HCNP exerts direct inotropic effects, providing also clear evidence that these cardiotropic actions involve interaction with receptor-mediated heterotrimeric G protein signalling.

These data confirm our preliminary study showing that HCNP depresses the mechanical performance of the isolated and perfused working heart of the frog by inducing negative inotropism under basal conditions and counteracting the ISO-dependent positive inotropism.

Under basal conditions (*i.e.* non-stimulated heart), HCNP acts as an efficient negative modulator of myocardial performance, since it depresses relevant contractile parameters such as LVP and RPP, an index of cardiac work, within a nanomolar range of concentrations. Notably, the concentration/response curves show that neither HR nor CP are modified by HCNP, highlighting a selective inotropic characteristic of the peptide. This feature makes it of particular interest for physio-pharmacological studies designed to analyze myocardial contractility separately from rythmogenic or coronary motility.

Cholinergic signalling

The findings that HCNP-dependent negative inotropism is enhanced in presence of cholinergic stimuli (*i.e.* carbachol), demonstrate the undecapeptide involvement in the cardiac cholinergic signal-transduction pathway. Moreover, using selective muscarinic subtype inhibitors, we have provided evidence that HCNP acts as competitive antagonist of ACh at the M_2 subtype receptors level, without any significant interaction with the M_1-M_3 receptor subtypes. The M_2 subtype is preferentially located on the myocardiocytes and in the rat heart ventricle M_2 muscarinic receptors were detected mainly on the sarcolemma and T-tubules of the cardiomyocytes (Hove-Madsen et al. 1996) (Fig. 2).

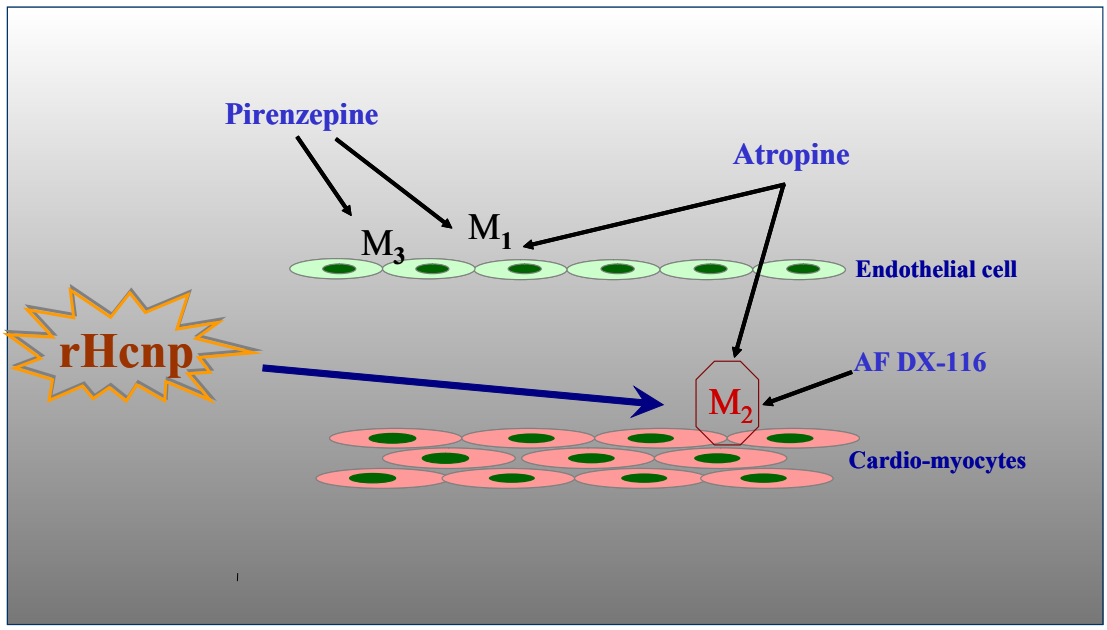

Figure 2. Differential localization of muscarinic receptors in the rat heart.

They are principally coupled to adenylate cyclase inhibition, reduce intracellular cAMP levels and decrease the L-type Ca^{2+} current, thereby eliciting negative cholinergic chronotropic and inotropic effects (for references, see (Hove-Madsen et al. 1996, Gattuso et al. 1999). Cholinergic-mediated negative inotropism can also be elicited by activation of constitutive NOS activity (eNOS) linked to the muscarinic receptor-G protein system detected in the caveolae of ventricular myocytes of several mammalian hearts (for references, see (Dessy et al., 2000). Our results are consistent with an endothelium-independent inotropic action of HCNP, indicating that the peptide targets directly the myocardium. Remarkably, the HCNP-mediated inotropism is G protein dependent. Using the precursor of HCNP, i.e. the

human PEBP it as been shown that hPEBP modulates G protein and G protein-coupled receptor signaling (Kroslak et al. 2001).

Receptor-receptor interactions between G-protein-coupled receptors occurring at the plasma-membrane level have been identified. In particular, it has been shown that clustering of G-protein-coupled receptors in aggregates or receptor mosaics results in the reciprocal modulation of their binding and decoding characteristics, their cooperativity playing an important part in the decoding of signal transduction (Agnati et al. 2005).

The analysis of the interactions between HCNP and the adrenergic system has revealed that the peptide exerts a functional but not receptorial competitive antagonism. Taken together, these data suggest that in the rat heart HCNP may act as a new cholinergic modulator through direct interaction with the G-protein-muscarinic coupled receptor signal-transduction system localized at the myocardiocyte sarcolemmal level. HCNP-induced activation of the cholinergic pathway appears to counteracts functionally the adrenergic pathway, thereby modulating myocardial contractility under basal conditions and under adrenergic stimulations.

Conclusion

The brain and the cardiovascular system are involved in a functionally important cross-talk, whose major function is to maintain homeostasis. Two pathways, known for over a century, link the brain and the cardiovascular system: the autonomic nervous system (ANS) via direct neural actions, and the neuroendocrine humoral outflow via the hypothalamic-pituitary-adrenal (HPA) axis.

Knowledge on their interactions, particularly on the effects of the stress “system” on organ homeostasis, is evolving dramatically, at an accelerating pace. Looking back one can recognize some milestones. About hundred years ago, the functional characterization of how the sympathetic and parasympathetic systems were able to influence effector tissues was paralleled by the discovery of the adrenomedullary "suprarenine", i.e. epinephrine (E) or adrenaline, the first hormone to be isolated from tissue (Benschop et al., 1996 for references), along with the concept of chemical neurotransmission introduced by Sherrington. In the early 1920s Loewi demonstrated that the mechanical effects of electrical stimulation of the innervation of the frog heart were mediated by "Vagus-Stoff", acetylcholine, or by "Sympatikus-Stoff", adrenaline. In the 1930's, the emerging role played by the sympathetic nervous system (SNS) and its end products, catecholamines (CAs), in the stress response was highlighted by Walter Cannon and Hans Selye. Notably, Cannon studied the stress response, named by him as "fight or flight" reaction, and recognized the "wisdom of the body" in the "generalized" sympathetic response acting during stress, emphasizing at the same time the more "discrete" influences exerted by the parasympathetic nervous system (PNS) (Chrousos and Gold, 1992). Soon after, the "general adaptation syndrome" or "stress response" concept,

developed by Selye (1936), revealed the crucial role of the peripheral limbs of the stress system, the SNS and the HPA axis, in the maintenance of stress-related homeostasis through increased peripheral levels of CAs and glucocorticoids acting synergistically.

In the following decades an explosive growth of studies, stemmed from Selye's theory, clearly demonstrated how the stress response itself could threaten the homeostasis of target organs and tissues. In fact, it was shown that such disturbed homeostasis could result from overactivation of the sympathetic system and/or positive reverberatory feedback loop so that activation of one system (SNS) tends to activate another excitatory as well (Chrousos and Gold, 1992). The renin-angiotensin system (RAS) was indeed one of the first peptide system studied in this connection from the 1950s on. The heart and the vascular system then have become a classical paradigm of a stress-threatened organs. In particular, the heart, now recognized as an endocrine organ, is viewed as an integrative interface between the noradrenergic nerve terminals, releasing their major neurotransmitter norepinephrine (NE), and the circulating CAs (E and, to lesser extent, NE) secreted by the adrenal medulla. Moreover, in the heart, as in other organs such as the gastrointestinal tract (GI) and the lymphoid organs, CAs are co-stored and co-released with other humoral principles, including neuropeptides, in the chromaffin cells as well as in the myocardiocytes themselves, the endocardial endothelium, the coronary vessels, and the connective cells of the interstitium.

Elevated CAs levels have long been known to induce necrotic damage in the heart (Raab, 1963; Benjamin et al., 1989; Teerlink et al., 1994; Tan et al., 2003;

Goldspink et al., 2004, and references therein). It is also well known that the initial response of the heart to prolonged and excessive stress is to enlarge morphologically to a state defined as cardiac hypertrophy, i.e. a compensatory adjustment preventing progressive deterioration of cardiac function (Grossman et al., 1975; Chien et al., 1991, and references therein). However, often, as we have learned in the last decades, the stress will overwhelm the system, leading the hypertrophied heart to failing processes. Concomitantly, in an attempt to control cardiac output and systemic blood pressure, there is a sustained heightened activation of the SNS and the RAS (Esler et al., 1997). Recent evidence indicates that, if left uncontrolled, these excitatory cascades may be more important in the deterioration process than the actual stress placed on the heart. Importantly, in human heart failure, chronic heightened activation of the SNS and associated enhancement of adrenergic signalling pathways via the CAs, has adverse prognostic significance and may accelerate the pathological processes (Cohn et al., 1984). Clearly, the outcome of the studies in this area of cardiovascular research has been of great clinical relevance, providing the rationale for anti-adrenergic drug therapy, including the β-blockers, still amongst the world's most used drugs.

The present work showing the inotropic and “antiadrenergic” characteristic of VS1 and HCNP suggests as next breakthrough the search for some basic neuroendocrine mechanisms which may counteract the overactivation of the adrenergic system and which may have an impact on the onset and course of patho-physiological conditions.

These two peptides may represent new functionally important links between the brain and the cardiovascular system. Knowledge on VSs and HCNP and cardiac signalling may provide a new molecular dimension to the "wisdom of the body", uncovering how the sympathetic and parasympathetic systems influence effector tissues homeostasis and, at the same time, how over-activation of the adrenergic system during the onset and course of patho-physiological conditions may be functionally counteracted.

References

- **Aardal, S., Helle, K.B. (1992)** The vasoinhibitory activity of bovine chromogranin A fragment (vasostatin) and its independence of extracellular calcium in isolated segments of human blood vessels. *Reg Pept* **41(1):** 9-18.

- **Aardal, S., Helle, K.B., Elsayed, S., Reed, R.K., Serck-Hanssen, G. (1993)** Vasostatins, comprising the N-terminal domain of chromogranin A, suppress tension in isolated human blood vessel segments. *J Neuroendocrinol* **5(4):** 405-12.

- **Abbott, D., Lodola, A. (1992)** The effects of isoproterenol on the isolated perfused rat heart. *Res Commun Chem Pathol Pharmacol* **76(1):** 125-8.

- **Agnati, L.F., Tarakanov, A.O., Guidolin, D**. **(2005)** A simple mathematical model of cooperativity in receptor mosaics based on the "symmetry rule". *Biosystems* **80(2):** 165-73.

- **Ali, N.N., Xu, X., Brito-Martins, M., Poole-Wilson, P.A., Harding, S.E., Fuller, S.J. (2004)** Beta-adrenoceptor subtype dependence of chronotropy in mouse embryonic stem cell-derived cardiomyocytes. *Basic Res Cardiol* **99(6):** 382-91.

- **Angeletti, R.H., Aardal, S., Serck-Hanssen, G., Gee, P., Helle, K.B. (1994)** Vasoinhibitory activity of synthetic peptides from the amino terminus of chromogranin A. *Acta Physiol Scand* **152(1):** 11-9.

- **Angelone, T., Goumon, Y., Metz-Boutigue, M.H., Glattard, E., Cerra, M.C. (2005)** The Hippocampal Cholinergic Neurostimulating Peptide (HCNP): a novel cardioregulatory molecule? Annual Main Meeting of the Society for Experimental Biology published in Comparative Biochemistry and Physiology. **Vol. 141** p. S155. Barcellona, 11-15 Luglio.

- **Angelone, T., Goumon, Y., Cerra, M.C., Metz-Boutigue, M.H., Aunis, D., Tota, B.(2005)** The emerging cardio-inhibitory role of the Hippocampal Cholinergic Neurostimulating Peptide (HCNP). *Submitted*

- **Angelone, T., Goumon, Y., Corti, A., Cerra, M.C., ,Tota, B., Metz-Boutigue, M.H. (2004)** Role of Vasostatin-1 on Cytoskeletal modulation in rat cardiac myocytes. XI Congresso Nazionale della Società Italiana di Ricerche Cardiovascolari (S.I.R.C). Latina, 23-25 Settembre.

- **Aunis, D., Metz-Boutigue, M.H. (2000)** Chromogranins: current concepts. structural and functional aspects. *Adv Exp Med Biol* **482:** 21-38.

- **Bauer, S.H., Zhang, X.Y., Van Dongen, W., Claeys, M., Przybylski, M. (1999)** Chromogranin A from bovine adrenal medulla: molecular characterization of

glycosylations, phosphorylations, and sequence heterogeneities by mass spectrometry. *Anal Biochem* **274:** 69-80.

- **Benjamin, I.J., Jalil, J.E., Tan, L.B., Cho, K., Weber, K.T., Clark, W.A. (1989)** Isoproterenol-induced myocardial fibrosis in relation to myocyte necrosis. *Circ Res* **65**: 657-70.

- **Benschop, R.J., Rodriguez-Feuerhahn, M., Schedlowski, M. (1996)** Catecholamine-induced leukocytosis: early observations, current research, and future directions. *Brain Behav Immun* **10(2)** :77-91.

- **Brekke, J.F., Osol, G.J., Helle, K.B. (2002)** N-terminal chromogranin-derived peptides as dilators of bovine coronary resistance arteries. *Reg Pept* **105(2):** 93-100

- **Ceconi, C., Ferrari, R., Bachetti, T., Opasich, C., Volterrani, M., Colombo, B., Parrinello, G., Corti, A. (2002)** Chromogranin A in heart failure; a novel neurohumoral factor and a predictor for mortality. *Eur Heart J* **23(12):** 967-74.

- **Cerra, M.C., De Iuri, L., Angelone, T., Corti, A., Tota, B. (2005)** Recombinant N-terminal fragments of chromogranin-A modulate cardiac function of the Langendorff-perfused rat heart. *Basic Res Cardiol* **100:** 1-10.

- **Chien, K.R., Knowlton, K.U., Zhu, H., Chien, S. (1991)** Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiological response. *FASEB J* **5**: 3037-46.

- **Chrousos, G.P., Gold, P.W., (1992)** The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. *JAMA* **267**: 1244-52.

- **Cohn, J.N., Levine, T.B., Olivari, M.T., Gaberg, V., Lura, D., Francis, G.S., Simon, A.B., Rector, T. (1984)** Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. *N Engl J Med* **311**: 819-23.

- **Colombo, B., Longhi, R., Marinzi, C., Magni, F., Cattaneo, A., Yoo, S.H., Curnis, F., Corti, A. (2002)** Cleavage of chromogranin A N-terminal domain by plasmin provides a new mechanism for regulating cell adhesion. *J Biol Chem* **277(48):** 45911-9.

- **Corti, A., Sanchez, L.P., Gasparri, A., Curnis, F., Longhi, R., Brandazza, A., Siccardi, A.G., Sidoli, A. (1997)** Production and structure characterisation of recombinant chromogranin A N-terminal fragments (vasostatins) evidence of dimer-monomer equilibria. *Eur J Biochem* **248(3):** 692-9.

- **Corti, A., Mannarino, C., Mazza, R., Colombo, B., Longhi, R., Tota, B. (2002)** Vasostatins exert negative inotropism in the working heart of the frog. *Ann N Y Acad Sci* **971:** 362-5.

- **Corti, A., Mannarino, C., Mazza, R., Angelone, T., Longhi, R., Tota, B. (2004)** Chromogranin A N-terminal fragments vasostatin-1 and the synthetic CGA 7-57 peptide act as cardiostatins on the isolated working frog heart. *Gen Comp Endocrinol* **136(2):** 217-24

- **Cryer, P.E. (1980)** Physiology and pathophysiology of the human sympathoadrenal neuroendocrine system. *N Engl J Med* **303(8):** 436-44.

- **Curry, W.J., Johnston, C.F., Hutton, J.C., Arden, S.D., Rutherford, N.G., Shaw, C., Buchanan, K.D. (1991)** The tissue distribution of rat chromogranin A-derived peptides: evidence for differential tissue processing from sequence specific antisera. *Histochemistry* **96(6):** 531-8.

- **Dessy C, Kelly RA, Balligand JL, Feron O. (2000)** Dynamin mediates caveolar sequestration of muscarinic cholinergic receptors and alteration in NO signaling. *EMBO J* **19(16):** 4272-80.

- **Doblinger, A., Becker, A., Seidah, N.G., Laslop, A. (2003)** Proteolytic processing of chromogranin A by the prohormone convertase PC2. *Reg Pept* **111**:111-6.

- **Esler, M., Kaye, D., Lambert, G., Esler, D., Jennings, G. (1997)** Adrenergic nervous system in heart failure. *Am J Card* **80**: 7L-14L.

- **Eskeland, N.L., Zhou, A., Dinh, T.Q., Wu, H,, Parmer, R.J., Mains, R.E., O'Connor, D.T. (1996)** Chromogranin A processing and secretion: specific role of endogenous and exogenous prohormone convertases in the regulated secretory pathway. *J Clin Invest* **98(1)**:148-56.

- **Frayne, J., Ingram, C., Love, S., Hall, L. (1999)** Localisation of phosphatidylethanolamine-binding protein in the brain and other tissues of the rat. *Cell Tissue Res* **298(3):** 415-23.

- **Gadroy, P., Stridsberg, M., Capon, C., Michalski, J.C., Strub, J.M., Van Dorsselaer, A., Aunis, D., Metz-Boutigue, M.H. (1998)** Phosphorylation and O-glycosylation sites of human chromogranin A (CGA79-439) from urine of patients with carcinoid tumors. *J Biol Chem* **273:** 34087-97

- **Gasparri, A., Sidoli, A., Sanchez, L.P., Longhi, R., Siccardi, A.G., Marchisio, P.C., Corti, A. (1997)** Chromogranin A fragments modulate cell adhesion. Identification and characterization of a pro-adhesive domain. *J Biol Chem* **272:** 20835-20843.

- **Gattullo, D., Linden, R.J., Losano, G., Pagliaro, P., Westerhof, N. (1999)** Ischaemic preconditioning changes the pattern of coronary reactive hyperaemia in the goat: role of adenosine and nitric oxide. *Cardiovasc Res* **42(1)**: 57-64.

- **Gattuso, A., Mazza, R., Pellegrino, D., Tota, B. (1999)** Endocardial endothelium mediates luminal ACh-NO signaling in isolated frog heart. *Am J Physiol* **276(2)**: H633-41.

- **Georget, M., Mateo, P., Vandecasteele, G., Lipskaia, L., Defer, N., Hanoune, J., Hoerter, J., Lugnier, C., Fischmeister, R. (2003)** Cyclic AMP compartmentation due to increased cAMP-phosphodiesterase activity in transgenic mice with a cardiac-directed expression of the human adenylyl cyclase type 8 (AC8). *FASEB J* **17(11**): 1380-91.

- **Glattard, E., Angelone, T., Strub, J.M., Tota, B., Aunis, D., Metz-Boutigue, M.E., Goumon. Y. (2005)** Identification and characterization of Vasostatin-containing peptides in rat heart. *Submitted.*

- **Goldspink, D.F., Burniston, J.G., Ellison, G.M., Clark, W.A., Tan, L.B., (2004)** Catecholamine-induced apoptosis and necrosis in cardiac and skeletal myocytes of the rat in vivo: the same or separate death pathways? *Exp Physiol* **89(4)**: 407-16.

- **Goumon, Y., Strub, J.M., Moniatte, M., Nullans, G., Poteur, L., Hubert, P., Van Dorsselaer, A., Aunis, D., Metz-Boutigue, M.H. (1996)** The C-terminal bisphosphorylated proenkephalin-A-(209-237)-peptide from adrenal medullary chromaffin granules possesses antibacterial activity. *Eur J Biochem* **235(3):** 516-25.

- **Goumon, Y., Lugardon, K., Kieffer, B., Lefevre, J.F., Van Dorsselaer, A., Aunis, D., Metz-Boutigue, M.H. (1998)** Characterization of antibacterial COOH-terminal proenkephalin-A-derived peptides (PEAP) in infectious fluids. Importance of enkelytin, the antibacterial PEAP209-237 secreted by stimulated chromaffin cells. *J Biol Chem* **273(45)**: 29847-56.

- **Goumon, Y., Angelone, T., Schoentgen, F., Chasserotgolaz, S., Almas, B., Fukami, M.M., Strub, J.M., Tota, B., Aunis, D., Mezt-Boutigue, M.H. (2004)** The hippocampal cholinergic neurostimulating peptide, the N-terminal fragment of the secreted phosphatidylethanolamine-binding protein, possess a new biological activity on cardiac physiology. *J Bio Chem* **279**: 13054-13064.

- **Grossman, L.A., Tiroa, L.O., Page, H.L. (1975)** Left main coronary artery obstruction. Survival and clinical improvement without surgery. *J Tenn Med Assoc* **68**: 903-4.

- **Helle, K.B., Serck-Hanssen, G., Aardal, S. (1993)** Functional aspects of the adrenal medullary chromogranins. *Neurochem Int* **22(4):** 353-60.

- **Helle, K.B., Angeletti, R.H. (1998)** Chromogranins as multifunctional prohormones: a role in protection against excessive responses to stress?. In: Kanno, T., et al (Eds): The Adrenal Chromaffin Cell: Archetype and Exemplar of Cellular Signalling in Secretory Control. Hokkaido University Press, Sapporo, Japan, pp. 273-281.

- **Helle, K.B., Metz-Boutigue, M.H., Aunis, D. (2001)** Chromogranin A as a calcium-binding precursor for multiple regulatory peptides for the immune, endocrine and metabolic systems. *Curr Med Chem* **1**: 119-40.

- **Helle, K.B. (2004**) The granin family of uniquely acidic proteins of the diffuse neuroendocrine system: comparative and functional aspects. *Biol Rev Camb Philos Soc* **79(4)**: 769-94.

- **Hove-Madsen, L., Mery, P.F., Jurevicius, J., Skeberdis, A.V., Fischmeister, R. (1996)** Regulation of myocardial calcium channels by cyclic AMP metabolism. *Basic Res Cardiol* **91**: 1-8.

- **Iacangelo, A., Okayama, H., Eiden, L.E. (1988)** Primary structure of rat chromogranin A and distribution of its mRNA. *FEBS* **227(2):**115-21.

- **Iacangelo, A., Eiden, L.E. (1995)** Chronogranin A: current status as a precursor for bioactive peptides and a granulogenic/sorting factor in the regulated secretory pathway. *Regul Pept* **58:** 65-88.

- **Imbrogno S, Angelone T, Corti A, Adamo C, Helle KB, Tota B. (2004)** Influence of vasostatins, the chromogranin A-derived peptides, on the working heart of the eel (Anguilla anguilla): negative inotropy and mechanism of action. *Gen Comp Endocrinol* **139(1):** 20-8.

- **Javadov, S.A., Lim, K.H., Kerr, P.M., Suleiman, M.S., Angelini, G.D., Halestrap, A.P. (2000)** Protection of hearts from reperfusion injury by propofol is associated with inhibition of the mitochondrial permeability transition. *Cardiovasc Res* **45:** 360-69.

- **Katada, E., Mitake, S., Matsukawa, N., Otsuka, Y., Tsugu, Y., Fujimori, O., Ojika, K. (1996)** Distribution of hippocampal cholinergic neurostimulating peptide (HCNP)-like immunoreactivity in organs and tissues of young Wistar rats. *Histochem Cell Biol* **105(1):** 43-51.

- **Kieffer, A.E., Goumon, Y., Ruh, O., Chasserot-Golaz, S., Nullans, G., Gasnier, C., Aunis, D., Metz-Boutigue, M.H. (2003)** The N- and C-terminal fragments of ubiquitin are important for the antimicrobial activities. *FASEB J* 17: 776-778.

- **Koeslag, J.H., Saunders, P.T., Wessels, J.A. (1997)** Glucose homeostasis with infinite gain: further lessons from the Daisyworld parable? *J Endocrinol* **154(2)**: 187-92.

- **Koeslag, J.H., Saunders, P.T., Wessels, J.A. (1999)** The chromogranins and the counter-regulatory hormones: do they make homeostatic sense? *J Physiol* **517 (3)**: 643-9.

- **Kornfeld, R., Kornfeld, S. (1985)** Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* **54:** 631-64.

- **Kratchmarova, I., Kalume, D.E., Blagoev, B., Scherer, P.E., Podtelejnikov, A.V., Molina, H., Bickel, P.E., Andersen, J.S., Fernandez, M.M., Bunkenborg, J., Roepstorff, P., Kristiansen, K., Lodish, H.F., Mann, M., Pandey, A**. **(2002)** A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics* **1(3):** 213-22.

- **Kroslak, T., Koch, T., Kahl, E., Hollt, V**. **(2001)** Human phosphatidylethanolamine-binding protein facilitates heterotrimeric G protein-dependent signaling. *J Biol Chem* **276(43):** 39772-78.

- **Lugardon, K., Raffner, R., Goumon, Y., Corti, A., Delmas, A., Bulet, P., Aunis, D., Metz-Boutigue, M.H. (2000)** Antibacterial and antifungal activities of vasostatin-1, the N-terminal fragment of chromogranin. *J Biol Chem* **275:** 10745-53.

- **Lugardon, K., Chasserot-Golaz, S., Kieffer, A.E., Maget-Dana, R., Nullans, G., Kieffer, B., Aunis, D., Metz-Boutigue, M.H. (2001)** Structural and biological characterization of chromofungin, the antifungal chromogranin A-(47-66)-derived peptide. *J Biol Chem* **276(38):** 35875-82.

- **Maget-Dana, R., Metz-Boutigue, M.H., Helle, K.B. (2002)** The N-terminal domain of chromogranin A (CgA1-40) interacts with monolayer of membrane lipids of fungal and mammalian compositions. *Ann N Y Acad Sci* **971**: 352-354.

- **Mahata, S.K., O'Connor, D.T., Mahata, M., Yoo, S.H., Taupenot, L., Wu, H., Gill, B.M., Parmer, R.J. (1997)** Novel autocrine feedback control of catecholamine release. A discrete chromogranin a fragment is a noncompetitive nicotinic cholinergic antagonist. *J Clin Invest* **100(6):** 1623-33.

- **Malan, D., Gallo, M.P., Bedendi, I., Biasin, C., Levi, R.C., Alloatti, G. (2003)** Microtubules mobility affects the modulation of L-type I(Ca) by muscarinic and beta-adrenergic agonists in guinea-pig cardiac myocytes. *J Mol Cell Card* **35:** 195-206.

- **Mandala, M., Brekke, J.F., Serck-Hanssen, G., Metz-Boutigue, M.H., Helle, K.B. (2005)** Chromogranin A-derived peptides: interaction with the rat posterior cerebral artery. *Regul Pept* **1241**: 73-80.

- **Mazza, R., Imbrogno, S., Mannarino, C., Tota, B. (2005**) Vasostatins interact with the cytoskeleton in amphibian and teleost hearts. Annual Main Meeting of the Society for

Experimental Biology published in Comparative Biochemistry and Physiology **Vol. 141A** p. S326. Barcelona, 11-15 July.

- **McIvor, M.E,, Orchard, C.H., Lakatta, E.G. (1988)** Dissociation of changes in apparent myofibrillar Ca^{2+} sensitivity and twitch relaxation induced by adrenergic and cholinergic stimulation in isolated ferret cardiac muscle. *J Gen Physiol* **92(4):** 509-29.

- **McVicar, C.M,, Cunningham, R.T., Harriott, P., Johnston, C.F., Buchanan, K.D., Curry,W.J. (2001)** Analysis of the post-translational processing of chromogranin A in rat neuroendocrine tissue employing an N-terminal site-specific antiserum. *J Neuroendocrinol* **13(7):** 588-95.

- **Metz-Boutigue, M.H., Angeletti, R.H., Aunis, D. (1993)** Intracellular and extracellular processing of chromogranin A. Determination of cleavage sites. *Eur J Biochem* **217**: 247-57.

- **Muller-Ehmsen, J., Nickel, J., Zobel, C., Hirsch, I., Bolck, B., Brixius, K., Schwinger, R.H. (2003)** Longer term effects of ouabain on the contractility of rat isolated cardiomyocytes and on the expression of Ca and Na regulating proteins. *Basic Res Cardiol* **98(2):** 90-6.

- **Nobels, F.R., Kwekkeboom, D.J., Bouillon, R., Lamberts, S.W. (1998)** Chromogranin A: its clinical value as marker of neuroendocrine tumours. *Eur J Clin Invest* **28(6):** 431-40.

- **Ojika, K., Appel, S.H. (1984)** Neurotrophic effects of hippocampal extracts on medial septal nucleus in vitro. *Proc Natl Acad Sci U S A* **81(8):** 2567-71.

- **Ojika, K., Mitake, S., Kamiya, N., Kosuge, N., Taiji, M**. **(1994)** Two different molecules, NGF and free-HCNP, stimulate cholinergic activity in septal nuclei in vitro in a different manner. *Dev Brain Res* **79:** 1-9.

- **Ojika, K., Mitake, S., Tohdon, N., Appel, S.H., Otsuka, Y., Katada, E., Matsukawa, N.K. (2000).** Hippocampal cholinergic neurostimulating peptide (HCNP). Pr*og Neurobiol* **60:** 37-83.

- **Pieroni, M., Corti, A., Chimenti, C., Angelone, T., Tota, B., Flavio, C., Colombo, B., Russo, M.A., Maseri, A., Frustaci, A.** (2005) Myocardial Production of Chromogranin A In Human Heart: A New Regulatory Peptide of Myocyte Function. *Submitted*

- **Raab, W. (1963)** Neurogenic multifocal destruction of myocardial tissue (pathogenic mechanism and its prevention). *Rev Can Biol* **22**: 217-239.

- **Ratti, S., Curnis, F., Longhi, R., Colombo, B., Gasparri, A., Magni, F., Manera, E., Metz-Boutigue, M.H., Corti, A. (2000)** Structure-activity relationships of chromogranin

A in cell adhesion. Identification of an adhesion site for fibroblasts and smooth muscle cells. *J Biol Chem* **275(38):**29257-63.

- **Saunders, B.D., Sabbagh, E., Chin,W.W., Kaiser, U.B. (1998)** Differential use of signal transduction pathways in the gonadotropin-releasing hormone-mediated regulation of gonadotropin subunit gene expression. *Endocrinology* **139(4):**1835-43.

- **Schiffmann, H., Flesch, M., Hauseler, C., Pfahlberg, A., Bohm, M., Hellige, G. (2002)** Effects of different inotropic interventions on myocardial function in the developing rabbit heart. *Basic Res Cardiol* **97(1):** 76-87.

- **Schoentgen, F., Jolles, P. (1995)** From structure to function: possible biological roles of a new widespread protein family binding hydrophobic ligands and displaying a nucleotide binding site. *FEBS Lett* **369(1):** 22-26.

- **Sirimanne, S.R., May, S.W. (1995)** Interaction of non-conjugated olefinic substrate analogues with dopamine beta-monooxygenase: catalysis and mechanism-based inhibition. *Biochem J* **306 (Pt 1):** 77-85.

- **Steiner, H.J., Weiler, R., Ludescher, C., Schmid, K.W., Winkler, H. (1990)** Chromogranins A and B are co-localized with atrial natriuretic peptides in secretory granules of rat heart. *J Histochem Cytochem* **38(6):** 845-50.

- **Strub, J.M., Goumon, Y., Lugardon, K., Capon, C., Lopez, M., Moniatte, M., Van Dorsselaer, A., Aunis, D., Metz-Boutigue, M.H. (1996)** Antibacterial activity of glycosylated and phosphorylated chromogranin A-derived peptide 173-194 from bovine adrenal medullary chromaffin granules. *J Biol Chem* **271(45):** 28533-40.

- **Strub, J.M., Sorokine, O., Van Dorsselaer, A., Aunis, D., Metz-Boutigue, M.H. (1997)** Phosphorylation and O-glycosylation sites of bovine chromogranin A from adrenal medullary chromaffin granules and their relationship with biological activities. *J Biol Chem* **272:** 11928-36.

- **Sudhof, T.C., Jahn, R. (1991)** Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron* **6(5):** 665-77.

- **Tan, L.B., Burniston, J.G., Clark, W.A., Ng, Y., Goldspink, D.F. (2003)** Characterization of adrenoceptor involvement in skeletal and cardiac myotoxicity Induced by sympathomimetic agents: toward a new bioassay for beta-blockers. *J Cardiovasc Pharmacol* **41**: 518-25.

- **Taupenot, L., Harper, K.L., O'Connor, DT. (2003)** The chromogranin-secretogranin family. *N Engl J Med* **348(12):** 1134-49.

- **Teerlink, J.R., Pfeffer, J.M., Pfeffer, M.A., (1994)** Progressive ventricular remodeling in response to diffuse isoproterenol-induced myocardial necrosis in rats. *Circ Res* **75(1):**105-13.

- **Tota, B., Mazza, R., Angelone, T., Nullans, G., Metz-Boutigue, M.H., Aunis, D., Helle, K.B. (2003)** Peptides from the N-terminal domain of chromogranin A (vasostatins) exert negative inotropic effects in the isolated frog heart. *Reg Pept* **114(2-3):** 123-30.

- **Trifaro, J.M. (1977)** Common mechanisms of hormone secretion. *Annu Rev Pharmacol Toxicol* **17:** 27-47.

- **Tsugu, Y., Ojika, K., Matsukawa, N., Iwase, T., Otsuka, Y., Katada, E., Mitake, S. (1998)** High levels of hippocampal cholinergic neurostimulating peptide (HCNP) in the CSF of some patients with Alzheimer's disease. *Eur J Neurol* **5:** 561-9.

- **Turquier, V., Vaudry, H., Jegou, S., Anouar, Y. (1999)** Frog chromogranin A messenger ribonucleic acid encodes three highly conserved peptides. Coordinate regulation of proopiomelanocortin and chromogranin A gene expression in the pars intermedia of the pituitary during background color adaptation. *Endocrinology* **140(9):** 4104-12.

- **Vallèe, B.S., Coadou, G., Labbè, H., Sy, D., Novelle, F., Schoentgen, F. (2003)** Peptide corresponding to the N- and C-terminal parts of PEBP are well-structurated in solution: new insights into their possible interaction with partners in vivo. *J Pept Res* **61:** 47-57.

- **Vitale, M.L., Seward, E.P., Trifaro, J.M. (1995)** Chromaffin cell cortical actin network dynamics control the size of the release-ready vesicle pool and the initial rate of exocytosis. *Neuron* **14(2):** 353-63

- **Vittone, L., Mundina-Weilenmann, C., Mattiazzi, A., Cingolani, H. (1994)** Physiologic and pharmacologic factors that affect myocardial relaxation. *J Pharmacol Toxicol Methods* **32(1):** 7-18.

- **Vorm, O., Roepstorff, P. (1994**) Peptide sequence information derived by partial acid hydrolysis and matrix-assisted laser desorption/ionization mass spectrometry. *Biol. Mass Spectrom* **23(12):** 734-40.

- **Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K.D., Rose, D.W., Mischak, H., Sedivy, J.M., Kolch, W. (1999)** Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature* **401(6749):** 173-7.

- **Zhang, X., Dillen, L., Bauer, S.H., Van Dongen, W., Liang, F., Przybylski, M., Esmans, E., De Potter, W.P., Claeys, M. (1997)** Mass spectrometric identification of

phosphorylated vasostatin II, a chromogranin A-derived protein fragment (1-113). *Biochim Biophys Acta* **1343(2):** 287-98.

- **Zheng, M., Streck, R.D., Scott, R.E., Seidah, N.G., Pintar, J.E. (1994)** The developmental expression in rat of proteases furin, PC1, PC2, and carboxypeptidase E: implications for early maturation of proteolytic processing capacity. *J Neurosci* **14(8):** 4656-73.

Curriculum vitae et studiorum

Curriculum Vitae et Studiorum

Dr. Tommaso Angelone

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Curriculm Studiorum

- **1995:** Secondary school diploma in classical studies with full marks at "Liceo Ginnasio Pasquale Candela"-San Marco Argentano (CS) – Italy
- **2001**: Doctor degree in "Pharmaceutic Chemistry and Technology" with full marks at the Faculty of Pharmacy, University of Calabria –Cosenza (Italy). Title of Thesis: "New cardioactive hormones: Vasostatins. Compared analysis of various peptide fragments"
- **2003:** Qualified to the profession of Pharmacist since July 2003.

Main Research Interest

- Isolated and perfused preparations of frog, eel and rat hearts (Working and Langendorff)
- Neurohumoral control of cardiac performance in mammalian, amphibia and fishes
- Cell Culture (cardiomyocytes); Differential Proteomic; Assay of proteins; Immunocytochemestry analysis; HPLC and Mass Spectrometry

Research Activities

- **From 2002:** Participating in the Research Program "Activity of Cardiomodulators agents in the cardiac response to stress" (operative unit at the University of Calabria, coordinator : Prof.ssa M.C.Cerra)

- **From 2003:** Participating in the "Vinci Programme" for the collaboration with "Unitè INSERM Physiopathologie du Systeme Nerveux" University Louis Pasteur-Strasbourg, France

- **From 7th to 15th October 2003**: Carried out research activity on cardiomyocytes cultures at the "Unitè INSERM Physiopathologie du Systeme Nerveux" University Louis Pasteur-Strasbourg, France

- **From 2003:** Participating in the Research Program PRIN "Vasostatins as new cardioregulatory hormones" (operative unit at the University of Calabria, coordinator : Prof. Bruno Tota)

- **From Jenuary 2004 to March 2004**: Carried out research activity on cardiomyocytes cultures at the "Unitè INSERM Physiopathologie du Systeme Nerveux" University Louis Pasteur-Strasbourg, France

- **From 7th to 11th June 2004:** Participated at experiments for a project on the myocardial protection at Dept. of Neuroscience, University of Turin, Italy

- **From 7th to 11th June 2004:** Participated at experiments on cardiomyocytes cell cultures by Confocal Microscopy Analysis and Patch Clamp at Dept. of Animal and Human Biology, University of Turin, Italy

- **From 2th November to 3th Dicember 2004 :** Carried out research activity on cardiomyocytes cultures (Assay of peptides in stardard and ET-1-conditioned medium; Confocal Microscopy Analysis) at the "Unitè INSERM Physiopathologie du Systeme Nerveux" University Louis Pasteur-Strasbourg, France

- **From 2005:** Participating in the "Galileo Programme" for the collaboration with "Unitè INSERM Physiopathologie du Systeme Nerveux" University Louis Pasteur-Strasbourg, France

- **From 2005:** Fellowship into Research Unit of "Istituto Italiano di Ricerche Cardiovascolari" (operative unit of the University of Calabria, coordinator : Prof. Bruno Tota)

- **From 8th to 10th February 2005:** Participated at experiments for a project on the myocardial protection at Dept. of Neuroscience, University of Turin, Italy

- **From 31th August to 4th September 2005**: Carried out research activity on cardiomyocytes cultures at the "Unitè INSERM Physiopathologie du Systeme Nerveux" University Louis Pasteur-Strasbourg, France

Awards and Academic Recognition

- **T.Angelone**, E. Pulerà, A. Quintieri, E. Filice, Y. Goumon, M-H. Metz-Boutigue, B. Tota, M.C. Cerra, "The newly discovered "Hippocampal Cholinergic Neurostimulating Peptide" (HCNP) modulates myocardial function in the rat. XII Congresso Nazionale della Società Italiana di Ricerche Cardiovascolari Imola (BO), 22-24 September 2005 ***This work received an award from Italian National Institute of Cardiovascular Research***

- D. Pellegrino, **T. Angelone**, B.Tota "Inotropic effects of Nitrite in the vertebrate heart" International Meeting on Role of Nitrite in Physiology, Pathophysiology and Therapeutics, September 8-9, 2005 USA ***This work received an award from National Institutes of Health***

- De Iuri L., **Angelone T.,** Corti A., Tota B. and Cerra M.C.. "Anti adrenergic action of vasostatins on *Langendorff* isolated and perfused rat heart". X Congresso Nazionale S.I.R.C. (Sept 24-26,2003, Istituto Superiore di Sanità, Roma) ***This work received an award from "Consorzio Interuniversitario per la Ricerca Cardiovascolare"***

Societies and Scientific Committees

- **From 2003**: Member of "Society for Experimental Biology" (SEB)

- **From 2004**: Member of "Società Italiana di Ricerche Cardiovascolari" (SIRC)

Publications

Proceeding

1. **T.Angelone**, E. Pulerà, A. Quintieri, E. Filice, Y. Goumon, M-H. Metz-Boutigue, B. Tota, M.C. Cerra, "The newly discovered "Hippocampal Cholinergic Neurostimulating Peptide" (HCNP) modulates myocardial function in the rat. XII Congresso Nazionale della Società Italiana di Ricerche Cardiovascolari Imola (BO), 22-24 September 2005

2. **T. Angelone**, A. Corti, B. Tota and M. Cerra,"Vasostatins as modulators of cardiac performance in the isolated perfused rat Langendorff heart" ."International Workshop on Vasostatins and CGA-derived peptides", Capri (Italy), 8 th - 11 th September 2005

3. Y. Goumon, F. Glattard, **T. Angelone**, B. Tota, D. Aunis and M.-H. Metz-Boutigue "Structural and biological characterization of secreted vasostatin-containing peptides as paracrine cardiac mediators". "International Workshop on Vasostatins and CGA-derived peptides", Capri (Italy), 8th-11th September 2005

4. A. Corti, C. Chementi, **T. Angelone**, B. Tota, F. Curnis, B. Colombo, M.A. Russo, A. Maseri, A. Frustaci and M. Pieroni "Chromogranin A in hypertrophic and dilated cardiomyopathies". "International Workshop on Vasostatins and CGA-derived peptides", Capri (Italy), 8th-11th September 2005

5. P. Pagliaro, S. Cappello, R. Rastaldo, C. Penna, **T. Angelone**, A. Corti, B. Tota, G. Losano, "Protection against ischemia/reperfusion injures by Vasostatin 1 in the isolated rat heart" . "International Workshop on Vasostatins and CGA-derived peptides", Capri (Italy), 8th-11th September 2005

6. D. Pellegrino, **T. Angelone**, B.Tota "Inotropic effects of Nitrite in the vertebrate heart" International Meeting on Role of Nitrite in Physiology, Pathophysiology and Therapeutics, September 8-9,

2005 USA

7. P. Pagliaro, S. Cappello, R. Rastaldo, C. Penna, **T. Angelone**, A. Corti, B. Tota, G. Losano, "Protection against ischemia/reperfusion injuries by Vasostatin-1 in the isolated rat heart" **Physiological Society** Focused Meeting, 5-7th September 2005, University of Oxford,UK .

8. S. Cappello, R. Rastaldo, C. Penna, A. Corti, P. Pagliaro, **T. Angelone**, B. Tota, G. Losano "Limitazione della necrosi miocardica da ischemia e riperfusione mediante vasostatina-1" 65° Congresso Nazionale della SOCIETà ITALIANA di CARDIOLOGIA 10-13 December 2005

9. S. Cappello, R. Rastaldo, C. Penna, A. Corti, P. Pagliaro, **T. Angelone**, B. Tota, G. Losano. "Vasostatin-1 limits ischemia/reperfusion induced myocardial necrosis"XII Congresso Nazionale della Società Italiana di Ricerche Cardiovascolari Imola (BO), 22-24 September 2005

10. S. Cappello, R. Rastaldo, **T. Angelone**, A. Folino, C. Penna, P. Pagliaro, B. Tota, G. Losano." Vasostatin-1 Affords Nitric Oxide Dependent Myocardial Protective Effects Akin To Early Preconditioning" 16th European Students' Conference (ESC), 19-23 October Berlin, 2005.

11. **Angelone T. ,** Tota B., Goumon Y., Metz-Boutigue M.-H., Glattard E., Cerra M. C., " The Hippocampal Cholinergic Neurostimulating Peptide (HCNP): A novel cardioregulatory molecule?". "Annual Main Meeting of the Society for Experimental Biology published in Comparative Biochemistry and Physiology", Barcelona (Spain), 11-15 July, 2005, Vol. 141A

12. Cerra M. C. , **Angelone T.** , Corti A. , Tota B. , " Cardiotropic role of Chromogranin A-derived peptides in vertebrates". "Annual Main Meeting of the Society for Experimental Biology published in Comparative Biochemistry and Physiology", Barcelona (Spain), 11-15 July, 2005, Vol. 141A

13. **Angelone T.** , Goumon Y. , Corti A. , Cerra M. C. , Tota B. , Metz-Boutigue M.-H. , " Role of Vasostatin-1 on Cytoskeletal modulation in rat cardiac myocytes". "XI Congresso Nazionale della Società Italiana di

Ricerche Cardiovascolari (S.I.R.C).", Latina, 23-25 September, 2004, 2004

14. **Angelone T.**, Pulera' E., Corti A., Tota B., Cerra M.C., "Cardiosuppressive action of vasostatin-1 on the rat heart: NO-cGMP-mediated mechanism". "XI Congresso Nazionale della Società Italiana di Ricerche Cardiovascolari (S.I.R.C).", Latina, 23-25 September, 2004.

15. Tota B. , Cerra M. C. , **Angelone T.** , Mazza R. , Imbrogno S. , Mannarino C. , Corti A. , " Chromogranin A-derived peptides in "zero steady-state error"homeostatis: vasostatins and the heart.". " Abstract Annual Main Meeting of the Society for Experimental Biology published in Comparative Biochemistry and Physiology", Edimburgo-UK, 29 March-2 April, 2004, Elsevier:2004, Vol. 137/A

16. **Angelone T.**, Pulera' E. , Corti A. , Tota B. , Cerra M. C. , " Cardiosuppressive action of vasostatin-1 on the rat heart: involvement of membrane receptors". "XI Congresso Nazionale della Società Italiana di Ricerche Cardiovascolari (S.I.R.C)", Latina, 23-25 settembre, 2004.

17. Cerra M. C. , De Iuri L. **, Angelone T.** , Corti A. , Tota B. , " Cardioinhibitory role of vasostatins on the rat isolated Langendorff perfused heart". "Abstract for the Annual Meeting of the Society for Experimental Biology published in Comparative Biochemistry and Physiology", Edimburgo, UK, 29 March-2 april, 2004, Elsevier:2004, Vol. 137/A

18. Mazza R. , Mannarino C. , **Angelone T. ,** Corti A. , Imbrogno S. , Tota B. , " Cardiosuppressive action of vasostatins on the frog heart". "12 ISCCB", Isla de La Palma Canary Islands Spain, 21-25 September, 2003

19. Mazza R. , Imbrogno S. , **Angelone T. ,** Corti A. , Helle K. B. , Tota B. , " Cardiosuppressor activity of vasostatins in eel and frog". "Sixth Intenational Congress of Comparative Physiology and Biochemistry", La Trobe, Mt Buller, Australia, 2-7 Febbraio, 2003

20. De Iuri L. , **Angelone T. ,** Corti A. , Tota B. , Cerra M. C. , " Anti-adrenergic action of vasostatins on Langendorff isolated and perfused rat heart"."10 Congresso Nazionale S.I.R.C", Roma, 24/26 September, 2003

21. **Angelone T. ,** De Iuri L. , Corti A. , Tota B. , Cerra M. C. , " Cardiosuppressive action of vasostatins on the Langendorff isolated and perfused rat heart". "12 ISCCB", Isla de La Palma Canary Islands Spain, 21-25 September, 2003.

22. Imbrogno S. , **Angelone T.** , Corti A. , Mazza R. , Mannarino C. , Tota B. , Helle K. B., " The inotropic influence of vasostatins on the working heart of the eel (Anguilla Anguilla): paracrine aspects and subcellular mechanisms". "12 ISCCB", Isla de La Palma Canary Islands Spain, 21-25 September, 2003

Full Paper

1. **Angelone T.,** Goumon Y., Cerra M.C., Metz-Boutigue M.-H., Aunis D., Tota B., " The emerging cardio-inhibitory role of the Hippocampal Cholinergic Neurostimulating Peptide (HCNP)". **2005** Submitted

2. Glattard E., **Angelone T.,** Strub J.-M., Tota B., Aunis D., Metz-Boutigue M.-H., Goumon Y., "Identification and characterization of Vasostatin-containing peptides in rat heart" **2005** Submitted

3. Pieroni M., Corti A., Chimenti C., **Angelone T.,** Tota B., Curnis F., Colombo B., Russo M.A., Maseri A., Frustaci A., "Myocardial production of chromogranin A in human heart a new regulatory peptide of myocyte function". **2005** submitted

4. Cerra M.C., De Iuri L., **Angelone T.,** Corti A., Tota B., "Recombinant N-terminal fragments of chromogranin-A modulate cardiac function of the Langendorff-perfused heart". *Basic Res Cardiol*, **2005**, 100: 1-10.

5. Goumon Y., **Angelone T.**, Schoentgen F., Chasserotgolaz S., Almas B., Fukami M., Strub J., Tota B., Aunis D., Metz-Boutigue M.-H., " The hippocampal cholinergic neurostimulating peptide, the N-terminal fragment of the secreted phosphatidylethanolamine-binding protein, possess a new biological activity on cardiac physiology". *Journal of Biological Chemistry*, **2004**, 279(13): 13054-13064.

6. Imbrogno S., **Angelone T.**, Corti A., Adamo C., Helle K. B., Tota B., " Influence of Vasostatins, the Chromogranin A-derived peptides, on the working heart of the eel (Anguilla Anguilla): negative inotropy and mechanism of action". *General and Comparative Endocrinology*, **2004**, 139(1): 20-28.

7. Corti A., Mannarino C., Mazza R., **Angelone T.**, Longhi R., Tota B., "Chromogranin A N-terminal fragments vasostatin-1 and the synthetic CGA7-57 peptide act as cardiostatins on the isolated working frog heart". *General and Comparative Endocrinology,* **2004**, 136(2): 217-224.

8. Tota B., Mazza R., **Angelone T.**, Nullans G., Metz-Boutigue M.-H., Aunis D., Helle K. B., " Peptides from the N-terminal domain of Chromogranin A (vasostatins) exert negative inotropic effects in the isolated frog heart". *Regulatory Peptides*, **2003**, 114(2-3): 123-130.