INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Université d'Ottawa · University of Ottawa

Heterotrimeric $G_{i/o}$ Proteins Regulate Stretch-Stimulated ANF Secretion in Isolated Rat Atria

Michael Bensimon
#1014173

This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular and Molecular Medicine

July 31, 2002
University of Ottawa,
Ottawa, Canada

Department of Cellular and Molecular Medicine

ABSTRACT

Atrial wall stretch plays an important role in regulating the secretion of ANF, a cardiac peptide hormone that regulates water and salt balance, as well as blood pressure. Yet the precise cellular mechanism that couples mechanical stretch to ANF secretion is unknown. In order to elucidate this mechanism, we investigated the role of heterotrimeric $G_{i/o}$ proteins in mechanically-stimulated ANF secretion. G proteins act as molecular switches that have been implicated in the control of intracellular protein transport, and stretch-secretion coupling. We utilized a pharmalogical agent, pertussis toxin (PTX), to inhibit $G_{i/o}$ proteins in male Sprague Dawley rats. Experiments using an isolated atria preparation from PTX-treated animals (25 μg/kg injected ip 48hr prior to atrial isolation) showed that the stretch-induced ANF secretory peak, obtained by increasing intra-atrial pressure from 0.5 to 8 mmHg, was significantly decreased compared to untreated animals. PTX treatment at 40 μg/kg eliminated the stretch-stimulated ANF secretory response. Moreover, basal ANF release was not affected by PTX treatment. Plasma ANF concentrations in PTX-treated animals were significantly decreased compared to controls. An ADP ribosylation assay confirmed that $G_{i/o}$ proteins from PTX-treated animals were inhibited *in vivo* by the toxin. The effect of stimulating $G_{i/o}$ proteins was also investigated using Mastoparan-7 (MAS-7; 10^{-5} M). Infusion with MAS-7 for 30 minutes potently stimulated ANF secretion; a response attenuated by

PTX treatment. Double immunofluorescence confocal microscopy showed that $G_{o\alpha}$ protein colocalized with ANF in atrial secretory granules. We also demonstrated that hormone secretion stimulated by ET-1, a potent ANF secretagogue, was insensitive to $G_{i/o}$ protein inhibition; in line with the hypothesis that PTX would not affect ET-1 because it signals through $G\alpha_q$. Hence, the PTX effect was specific. These results suggest that $G_{i/o}$ proteins couple atrial muscle stretch to ANF secretion in an acute setting, and that there exist two mechanisms, which control natriuretic peptide secretion. The first mechanism controls stretch-stimulated ANF secretion and is PTX-sensitive, while the second regulates basal ANF release and is PTX-insensitive.

TABLE OF CONTENTS

Abstract.....ii

List of tables.....viii

List of figures.....ix

Abbreviations.....x

Acknowledgments.....xii

1. **INTRODUCTION & BACKGROUND** 1

    1. **What are natriuretic peptides?**.....1
        a. Dr. de Bolds landmark discovery.....1
        b. Processing of ANF.....4
        c. ANF receptors.....5
        d. Effects of ANF on the cardiovascular system.....7
        e. Pathophysiological significance of ANF.....8

    2. **Regulated versus constitutive secretion pathways of ANF**.....10

    3. **Formation of secretory granules**.....12

        a. Biogenesis of secretory granules.....12
        b. Properties of secretory granules.....13
        c. Exocytosis of secretory granules.....14
        d. Comparison of immature versus mature secretory granules.16

    4. **Stretch-stimulated ANF secretion**.....17

        a. The role of the cytoskeleton.....18
        b. The role of potassium channels.....19

    5. **G protein intracellular signalling**.....21

        a. Receptor-G protein-effector interactions.....21
        b. Classes of mammalian G_α subunits.....24
        c. GTP-binding proteins in secretory granules of endocrine cells.....26
        d. The role of G proteins in secretory granule formation.....27
        e. GTP-binding proteins and regulated exocytosis.....28

f. G proteins and potassium channels.....29

6. Pharmalogical tools used to investigate GTP binding proteins.....31

a. $G_{i/o}$ binding protein antagonist: Pertussis toxin.....31
b. Entry of bacterial toxins into cells.....33
c. Entry of PTX into cells.....33
d. $G_{i/o}$ binding protein agonist: Mastoparan-7.....35

7. Endothelin-1 (ET-1): an agonist of ANF secretion.....36

2. AIMS AND HYPOTHESIS.....39

3. MATERIALS & METHODS.....40

1. Isolated right atria model.....40
2. Drugs.....42
3. Perfusion protocols.....43

a. Pertussis toxin (PTX) and stretch.....43
b. Mastoparan-7.....44
c. Endothelin-1.....44

4. Preparation of atrial homogenate.....44
5. PTX-induced ADP ribosylation.....45
6. Radioimmunoassay.....46
7. Antibodies used for immunocytochemistry.....48
8. Immunofluorescence labelling.....49
9. Statistical Analysis.....50

4. RESULTS.....51

1. Stretch significantly increases irANF secretion.....51
2. PTX eliminates stretch stimulated irANF secretion in vitro.....51

3. PTX blocks ANF secretion in vivo.....54

4. Degree of ADP-ribosylation by PTX.....57

5. Endothelin-1-stimulated ANF secretion is not affected by PTX.....60

6. Mastoparan-7 significantly increases irANF secretion.....63

7. Immunofluorescence labelling shows colocalization of $G_{\alpha o}$ and ANF in atrial tissue.....66

8. Immunofluorescence labelling of inhibitory G proteins in rat ventricular tissue.....72

5. **DISCUSSION**.....78

1. Association of stretch and ANF secretion.....78

a. Two pools of secretory granules hypothesis: stretch vs. stretch-insensitive.....79
b. Effect of stretch on secretory granules.....80

2. The cellular effect of PTX.....83

a. Entry of PTX into cells.....83

3. Evidence for the association of G proteins and hormone secretion.....85

a. Trans Golgi network.....86
a. Secretory granules.....87
b. Plasma membrane.....89

4. $G_{i/o}$ proteins and the regulation of ANF secretion.....90

a. K_{ATP} channels and the regulation of ANF secretion.....91
b. cAMP and the regulation of ANF secretion.....92

5. Endothelin-1 stimulated ANF secretion.....95

a. Effect of ET-1.....95
b. ET-1 receptor blockers.....95
c. ET-1 and stretch.....96
d. Endothelial cells and mechano-transduction.....97

6. Mastoparan–7 stimulated ANF secretion.....99

6. CONCLUSION.....102

7. FUTURE DIRECTIONS.....103

8. PERSPECTIVE.....107

9. REFERENCES.....109

LIST OF TABLES

Table 1: Published effects of ANF in humans.....3

Table 2: ANF and disease. 10

Table 3: Classes of mammalian G_α subunits. 25

LIST OF FIGURES

Figure 1: Physiologic effects of natriuretic peptides released from the heart following stretch.....2

Figure 2: The regulatory cycle of heterotrimeric proteins.....23

Figure 3: The heterotrimeric GTP-binding protein coupled receptor.....24

Figure 4: Schematic representation of pertussis toxin.....32

Figure 5: Isolated whole atria perfusion model.....42

Figure 6: Competitive radioimmunoassay (RIA).....47

Figure 7: Effect of PTX on stretch-stimulated ANF secretion.....52

Figure 8: In vivo effect of PTX on plasma ANF concentration.....55

Figure 9: PTX blocks the $G_{i/o}$ proteins in vivo.....58

Figure 10: Effect of PTX on ET-1 stimulated ANF secretion.....61

Figure 11: Effect of PTX on MAS-7 stimulated ANF secretion.....64

Figure 12: Immunohistochemistry of rat atria.....67

Figure 13: Immunohistochemistry of rat ventricular tissue.....73

Figure 14: Constitutive vs. regulated ANF secretion.....81

ABBREVIATIONS

1. **AC**: Adenylyl Cyclase
2. **ADP**: Adenosine Di-Phosphate
3. **ATP**: Adenosine Tri-Phosphate
4. **ANF**: Atrial Natriuretic Factor
5. **ARF**: ADP-Ribosylation Factor
6. **AVP**: Arginine-Vasopressin
7. **BNP**: Brain Natriuretic Peptide
8. **cAMP**: Cyclic Adenosine Mono-Phosphate
9. **CCV**: Clathrin-Coated Vesicles
10. **cGMP**: Cyclic Guanosine Mono-Phosphate
11. **CNP**: C-type Natriuretic Peptide
12. **CSVs**: Constitutive Secretory Vesicles
13. **ET-1**: Endothelin-1
14. **ET_A**: Endothelin-1 Receptor A
15. **EM**: Electron Microscopy
16. **GC**: Guanylyl Cyclase
17. **GC-A**: Guanylyl Cyclase A-coupled Receptor
18. **GFR**: Glomerular Filtration Rate
19. **G protein**: GTP-binding protein
20. **GPCR**: GTP-binding Protein Coupled Receptor
21. **GTP**: Guanosine Tri-Phosphate

22. **IL-1β**: Interleukin-1β

23. **HEPES**: *N*-2-Hydroxyethylpirazine-*N'*-2-ethanesulfornic acid

24. **ip**: Intraperitoneal

25. **ir**: Immunoreactive

26. **ISGs**: Immature Secretory Granules

27. **KRBB**: Krebs-Ringer Bicarbonate Buffer

28. **MAS**: Mastoparan

29. **MAS-7**: Mastoparan-7

30. **MSGs**: Mature Secretory Granules

31. **PKA**: Protein Kinase

32. **PTX**: Pertussis Toxin

33. **RIA**: Radioimmunoassay

34. **RSPs**: Regulated Secretory Proteins

35. **SGs**: Secretory Granules

36. **SNARES**: Soluble N-ethyl-maleimide-sensitive fusion protein (NSF) Attachment Protein REceptors

37. **TGN**: Trans Golgi Network

38. **TNF**: Tumor Necrosis Factor

39. **UV**: Urinary Volume

40. **VAMPs**: Vesicle-Associated Membrane Proteins

ACKNOWLEDGEMENTS

My work in Dr. de Bold's laboratory has deepened both my knowledge of the intricacies of cardiovascular research, and my appreciation of the scientific method. I would like to thank him for conveying to me, through his perseverance and scientific inquisitiveness, the importance of hard work, independent thought, and determination with respect to achieving one's goals. Ultimately, the laboratory skills that I developed throughout my graduate studies will serve me well in my future endeavours, and could set the stage for a career in the field of cardiology.

I am also indebted to my friend and colleague, Ken Ma, who shared with me his scientific knowledge and experience without reservation, and who offered constant moral support when it was most needed. Our daily conversations were always insightful and often helped me work through new problems. My interactions with Ken taught me about the crucial role that team work plays in ensuring the success of any scientific research project.

I must also acknowledge the contributions of Dr. Kuroski-de Bold and Amalia Ponce, two individuals who helped create an environment conducive to learning by encouraging me to avail myself of the myriad resources available in the laboratory, and by always lending a helping hand.

My wife Alexandra has been my pilar of strength throughout this research project. Her dedication and understanding have immensely contributed to the completion of this thesis. She has single-handedly taken care of our sons Jacob and Jonathan. I dedicate the work at hand to my entire family, for they have been committed supporters of all the activities I have undertaken in my life.

1. INTRODUCTION & BACKGROUND

1. *What are natriuretic peptides?*

a. Dr. de Bold's landmark discovery

First observed in atrial cardiocytes by electron microscopy (EM), storage granules, or "specific atrial granules", remained a complete mystery for many years. Though their presence was known, no one had been able to decipher the functional or physiological roles of these structures. It took several years of laborious work to isolate and purify these granules in an effort to study their role.

It was Dr. de Bold's seminal discovery in 1981 (de Bold *et al*, 1981), that the infusion of extracts of atrial tissue into rats caused potent natriuresis and reduced systemic blood pressure, that led to the understanding of the physiological role of this unknown factor within atrial cardiac granules. This groundbreaking observation changed the way we look at the heart and broke the dogma that the heart is merely a muscle responsible for mechanically pumping the blood through the body. Dr. de Bold's discovery brought about the belief that the heart has an endocrine function that regulates vasculature and other tissues via the release of two members of a family of natriuretic peptides (Figure 1). This family includes atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (de Bold *et al*, 1996). CNP is primarily found in the central nervous system and vascular endothelial cells, whereas ANF and BNP are cardiac hormones (de Bold *et al*, 1981; Lang *et al*, 1992; Ruskoaho, 1992; de Bold and Bruneau, 2000). The mechanically-induced

release of ANF and the proposed signaling pathways regulating this process, are the central focus of this investigation. ANF is a peptide hormone possessing potent diuretic and vasorelaxant properties (de Bold *et al*, 1981), and is predominantly produced by the atria (Figure 1). The reported effects of ANF in humans are summarized in table 1 (Adapted from: de Zeeuw *et al*, 1992; de Bold and Bruneau, 2000; Kiemer and Vollmar, 2001).

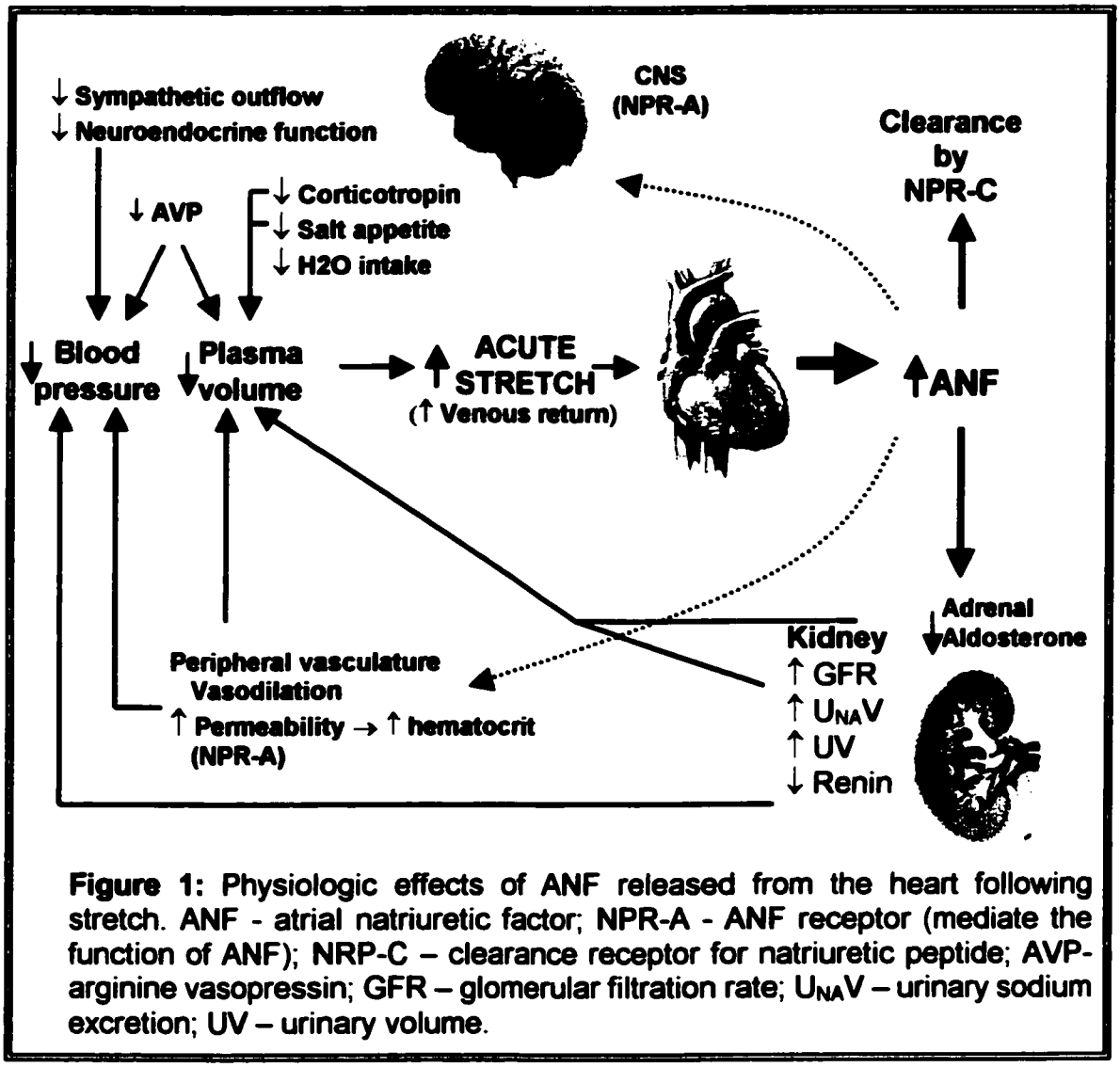


Figure 1: Physiologic effects of ANF released from the heart following stretch. ANF - atrial natriuretic factor; NPR-A - ANF receptor (mediate the function of ANF); NRP-C – clearance receptor for natriuretic peptide; AVP-arginine vasopressin; GFR – glomerular filtration rate; $U_{NA}V$ – urinary sodium excretion; UV – urinary volume.

Table 1*. Main effects of ANF in humans	
Effects	**References**
1. Systemic effects • Hemoconcentration • Decrease in blood pressure	Weidmann *et al*, 1989; Singer *et al*, 1989; Richards *et al*, 1985; Weidmann *et al*, 1986a
2. Effects on hormonal transport • Decrease in plasma renin activity • Decrease in aldosterone, and cortisol • Inhibition of arginine vasopressin secretion and/or effects • Induction of pancreatic juice secretion	Weidmann *et al*, 1986a; Weidmann *et al*, 1989; Singer *et al*, 1989; Richards, 1989; Richards *et al*, 1988; Solomon *et al*, 1988; Moses *et al*, 1990; Wittert *et al*, 1993; Uehlinger *et al*, 1986
3. Renal effects • Increase in glomerular filtration rate • Decrease in effective renal plasma flow • Increase in renovascular resistance • Increase in urinary volume and electrolytes	de Zeeuw *et al*, 1992; Wolfram *et al*, 1996; Richards *et al*, 1988; Weidmann *et al*, 1986b; Solomon *et al*; 1988
4. Central nervous system effects • Modulation of sympathetic activity • Prevents tachycardia in the face of decreased blood volume • Increase in lipolysis • Effects on blood pressure-/volume-regulatory regions in the brain	Floras, 1995; Wiedemann *et al*, 1995; Clerico *et al*, 1999; Sengenes *et al*, 2000; Abramson *et al*, 1999
5. Cardiac effects • Increases coronary vasodilation • Improves left ventricular performance • Improves cardiac output by unloading the heart	de Bold and Bruneau *et al*, 2000; Ruskoaho, 1992; Serizawa *et al*, 1988
6. Pulmonary effects • Relaxation of vascular smooth muscle cells • Relaxation of the trachea	Cargill and Lipworth, 1995; Mikawa *et al*, 1998; Candenas *et al*, 1991
7. Growth and Proliferation effects • Inhibits hypertrophy in cultured vascular smooth muscle cells • Inhibits growth of endothelial cells, and cardiac fibroblasts	Seko *et al*, 1999; Redondo *et al*, 1998; Pedram *et al*, 2001
8. Immunological effects • Reduces the release of tumor necrosis factor α (TNFα) • Attenuates the release of interleukin 1β (IL1β)	Kiemer and Vollmar, 2001; de Bold *et al*, 2001

b. *Processing of ANF*

Secreted proteins reach their destinations by a common biosynthetic pathway proceeding via the endoplasmic reticulum (ER), the Golgi and the trans-Golgi apparatus. Several postranslational events occur in the formation of natriuretic peptides and some aspects of ANF processing distinguish it from other peptide hormone systems. ANF is initially synthesized as a large protein precursor, called preproANF, which undergoes proteolytic cleavage to remove the hydrophobic 25-amino acid signal peptide sequence at its amino terminus during the transport process (Nakayama et, 1984). This produces the 126 amino acid storage form of ANF in the heart, termed $proANF_{1\text{-}126}$ (Glembotski and Gibson, 1985). Further modifications of $proANF_{1\text{-}126}$ involve the formation of an intrachain disulfide bridge at the cysteine residues (Cys^{105}-Cys^{121}) creating a 17-member ring structure that is crucial for its biological activity (Iervasi *et al*, 1993). Unlike conventional endocrine peptides, which are stored in their processed form, $proANF_{1\text{-}126}$ is predominantly packaged in its uncleaved precursor structure as it travels from the Golgi to immature secretory granules (ISGs) and then to mature granules (MSGs) (Thibault *et al*, 1989). It is noteworthy, that BNP is stored as a mixture of processed peptide ($BNP_{51\text{-}95}$) and the precursor pro peptide ($BNP_{1\text{-}95}$) (Kambayashi *et al*, 1989). EM studies utilizing double immunogold labelling show that, ANF and BNP may be often found in the same storage granule, and are both secreted in their processed form into the circulation (Ruskoaho, 1992). In addition, ANF and BNP are co-regulated in the atria, but not in the ventricles (Bianciotti and de Bold, 2000).

proANF$_{1-126}$ is cleaved during secretion at its proximal monobasic site, into its major biologically active form, the COOH-terminal peptide ANF$_{99-126}$ (also called ANF$_{1-28}$) (Flynn *et al*, 1983). This occurs both in humans (Forssmann, 1986) as well as in rats (Schwartz *et al*, 1985; Thibault *et al*, 1985). This cleavage also results in the formation of an NH_2-terminal fragment, proANF$_{1-98}$ (Ruskoaho, 1992). ANF$_{99-126}$ is released from isolated atria (Vuolteenaho *et al*, 1985) and from isolated hearts (Lang *et al*, 1985; Ruskoaho *et al*, 1986; Thibault *et al*, 1986). It has been proposed that many enzymes found in atrial granules or the sarcolemma, cleave the prohormone to its bioactive mature form (de Bold *et al*, 1996; Ruskoaho, 1992). However, their physiological significance is unclear (Ruskoaho, 1992). Three candidate serine protease enzymes exist and are possibly involved in this final processing step: 1) atrioactivase, found in bovine atrial microsomal fractions (Imada *et al*, 1988), 2) IRCMSP1, found in rat hearts (Seidah *et al*, 1986), and 3) a serine proteinase, found in bovine granules (Wypij and Harris, 1992). Recently, Wu *et al* (2002) demonstrated that a type II transmembrane serine protease called 'corin', is the physiological pro-ANF convertase in cardiocytes.

c. *ANF receptors*

Binding sites for ANF are found in target cells of vascular tissues, the brain, the intestine, the eye, the testis, the olfactory mucosa, several renal sites, adrenal glomerulosa cells, the thymus, the spleen, the lymph nodes, the tonsils, and in macrophages (Drewett and Garbers, 1994; Kiemer and Vollmar, 2001).

ANF exerts its physiological actions by binding to the high affinity A type receptor (called ANF-A in humans, Chang *et al*, 1989), a transmembrane guanylyl cyclase-A coupled receptor (called GC-A in rats, Schulz *et al*, 1989). Guanylyl cyclases are cytoplasmic (nitrous oxide synthase) or membrane-associated enzymes, that catalyze the conversion of GTP to cyclic GMP, an intracellular signaling molecule (Schulz *et al*, 1999). The intracellular targets of cGMP encompass cGMP-gated ion channels, cGMP-dependent protein kinases, and cGMP-regulated cyclic nucleotide phosphodiesterases (Bryan, 1991; Lincoln and Cornwell, 1993). Physiologically, the proposed signaling functions of GC-A comprise the induction of diuresis and natriuresis in the kidney, the relaxation of smooth muscle, hypothalamic inhibition of water intake, decreased cardiac output, and inhibition of aldosterone production in the adrenal gland (Samson and Levin, 1997). Cardiorenal regulation of homeostasis is inhibited by blocking guanylate cyclase-coupled natriuretic peptide receptors (Sano *et al*, 1992; Yokota *et al*, 1994). ANF can also bind to other receptors with lower affinity, such as the B type receptor and the C type clearance receptor (Chang *et al*, 1989). ANF and BNP exert their physiological effects by binding to the A type receptor (Koller and Goeddel, 1992). CNP binds to the B-type receptor (Koeller *et al*, 1991). Clearance of natriuretic peptides occurs via the C type receptor (Suga *et al*, 1992), and by an enzymatic process mediated by neutral endopeptidase 24-11 (abundant in the proximal convoluted tubules of the nephron). The binding affinities of the natriuretic peptide ligands vary for each receptor. ANF has the highest binding affinity for the A and C type

receptors, while CNP has the highest binding affinity for the B type receptor (Koller *et al*, 1991; Suga *et al*, 1992).

c. *Effects of ANF on the cardiovascular system*

When administered in low-dose infusions, ANF is a vasorelaxant that lowers blood pressure and decreases peripheral vascular resistance (Fenoy *et al*, 1989; Garcia *et al*, 1985; Granger *et al*, 1986). The blood pressure lowering and decreased cardiac output properties of ANF are attributable to decreased preload (Thibault *et al*, 1999). Reduced cardiac preload is partly caused by an ANF-induced increase in capillary permeability or capillary pressure gradients and thus, by the promotion of extravasation of intravascular fluid into the extravascular compartment (Wijeyaratne and Moult, 1993). There are several mechanisms that promote natriuresis. The main one is the interaction of ANF with its receptor (NPRA) in renal collecting ducts. ANF inhibits Na^+ reabsorption by the inner medullary collecting duct epithelial cells, and promotes Na^+ secretion by these cells (Knepper *et al*, 1991). In the renal vasculature, ANF stimulates increased venous capacitance. This increase promotes natriuresis, which in turn lowers extracellular-fluid volume (Dunn *et al*, 1986). In the peripheral vasculature, ANF lowers sympathetic tone by dampening the baroreceptors, presumably by the suppression of sympathetic outflow from the central nervous system and the inhibition of catecholamine release at the autonomic nerve endings (Levin *et al*, 1998). In addition, ANF probably acts via vagal afferents to suppress the sympathetic baroreceptors sensing the reduced

preload, thereby suppressing the vasoconstriction and tachycardia that would normally accompany loss of blood volume (Thören *et al*, 1986).

d. *Pathophysiological significance of ANF*

Plasma cardiac natriuretic peptides are changed in numerous pathologies (Table 2, adapted from Clerico *et al*, 1999). The pathophysiological significance of ANF is perhaps best defined in patients with congestive heart failure. Myocardial failure is accompanied by increased production of ANF and BNP, both released into the blood plasma (Burnett *et al*, 1986). High concentrations of natriuretic peptides are correlated with the degree of hemodynamic compromise, the development of cardiac arrhythmias, and are predictors of poor long-term survival (Gottlieb *et al*, 1989). In animals with congestive heart failure, the release of ANF inhibits the production of catecholamines, angiotensin II, aldlosterone, and endolthelin-1 (Wada *et al*, 1994). Infusion of HS-142-1 (a microbial polysaccharide that competitively and selectively inhibits ANF binding to its guanylyl cyclase-containing receptor) in these animals leads to a significant increase in the plasma concentrations of these hormones (Wada *et al*, 1994). Moreover, administration of HS-142-1 to rats, markedly impairs water as well as urinary salt excretion, and amplifies the increased blood-pressure response following the administration of exogenous mineralocorticoid (Hirata *et al*, 1993; Yokota *et al*, 1994). Furthermore, rats immunized against their own ANF are unable to excrete a water load normally (Greenwald *et al*, 1988).

The vasodilatory and natriuretic properties of ANF prevent excess water and salt retention, which are clinically relevant in diseases exhibiting vasoconstriction and renal sodium retention such as essential hypertension (De Zeeuw *et al*, 1992). In patients with severe hypertension, high plasma immunoreactive ANF levels have also been measured (Montorsi *et al*, 1987; Genest *et al*, 1988). Chronically hypertensive subjects perfused on a long-term basis with ANF exhibited lowered systolic blood pressure and cardiac output (Franco-Saenz *et al*, 1992).

Genetic manipulations of the ANF gene provide fundamental insights into the pathophysiology of this hormone. In transgenic mice overexpressing the ANF gene, plasma ANF concentrations are 10 times higher and blood pressure was 20 to 30 mmHg lower (Steinhelper *et al*, 1990). The homozygous inactivation of the proANF gene in mice that are fed low and intermediate salt diets, lead to elevated basal blood pressures (John *et al*, 1995). Mice with a homozygous knockout of the proANF gene develop chronic hypertension (Melo *et al*, 1996) and display cardiac enlargement (Levin *et al*, 1998).

Table 2: ANF and disease

Diseases	ANF levels	References
1. Cardiac diseases		
• Heart failure	↑↑↑	Dickstein *et al*, 1995; Cowie *et al*, 1997 Stein & Levin, 1998
• Acute myocardial infarction (first 2-3 d)	↑↑↑	Omland *et al*, 1993; Kettunen *et al*, 1994
• Essential Hypertension with with left ventricular hypertrophy	↑	Sugawara *et al*, 1985
2. Pulmonary diseases		
• Acute dyspnoea	↑	Davis *et al*, 1994
• Obstructive pulmonary disease	↑	Lang *et al*, 1992
3. Endocrine and metabolic diseases		
• Hypothyroidism	↓	Parlapiano *et al*, 1998
• Primary aldosteronism	↑	Lapinski *et al*, 1991
• Cushing's disease	↑	Opocher *et al*, 1990
• Addison's disease	norm. or ↑	Cappuccio *et al*, 1989
• Diabetes mellitus	norm. or ↑	Zietse *et al*, 1997; Straub *et al*, 1996; Pedersen *et al*, 1992
4. Liver cirrhosis with ascitis	↑	Salo *et al*, 1996
5. Renal failure (acute or chronic)	↑↑↑	Ruskoaho, 1992; Wolfram *et al*, 1996
6. Paraneoplastic syndrome	norm. or ↑	Marchioli and Graziano, 1997

2. Regulated versus constitutive release pathways of ANF

ANF secretion from atrial cardiocytes occurs via two mechanisms: the regulated secretory pathway, in response to specific stimuli (i.e.: endothelin-1, atrial muscle stretch, phenylephrine), and the continuous release of hormone, without stimuli (constitutive pathway) (Ogawa *et al*, 1999). Thus, cardiac endocrine cells utilize regulated pathways to release stored peptide hormones in dense–core secretory granules, upon appropriate stimulation (de Bold and Bruneau, 2000). This allows the cardiocytes to release ANF acutely and at a rate that exceeds the endocrine cell's synthesizing capacity. In the absence of secretion stimuli, these secretory granules are stored in the cardiocyte awaiting

subsequent triggers for exocytosis. Mangat and de Bold (1993), have previously demonstrated that exocytosis of ANF under basal and stimulated conditions utilize a pool of newly synthesized hormone acutely released from immature secretory granules. Since ANF secretion is mostly independent of protein synthesis, stimulated release of ANF cannot take place via the constitutive pathway (Doubell and Thibault, 1994; Page *et al*, 1991).

In a previous study, Ogawa *et al* (1999) reported that the regulated and constitutive pathways of ANF release were specifically affected by targeting organelles that regulate hormone secretion. In that experiment, transport from the ER to the TGN was inhibited with brefeldin A; formation of mature secretory granules was disrupted with monensin; protein synthesis was inhibited with cycloheximide. A 'constitutive-like' hormone release pathway was proposed, where the exocytosis of ANF occurs when vesicles budded off from immature secretory granules (Ogawa *et al*, 1999). This type of "constitutive-like" secretion has been observed in the pancreas (Arvan *et al*, 1991; Kuliawat *et al*, 1992). The experiments by Konrad *et al* (1995) point to the involvement of G proteins in the regulation of hormone release from secretory granules (SGs). In addition, pharmacological interventions blocking or stimulating inhibitory GTP-binding proteins, found within storage granules of the pancreas, affected insulin release (Konrad *et al*, 1995).

3. Formation of secretory granules

a. *Biogenesis of secretory granules*

Cardiocytes concentrate and store ANF in specialized organelles, called secretory granules (SGs), which are involved in the regulated secretory pathway. Formation of the nascent storage granule starts as an outpouching of the TGN cisternae generating a condensing vacuole (Palade, 1975; Novikoff et al, 1977), which is at first continuous with the TGN's tubular network (Clermont et al, 1995). Initially, immature secretory granules (ISGs) are formed by the envelopment of a dense core of secretory proteins (Tooze, 1998). ISGs are converted to mature secretory granules (MSGs) containing regulated secretory proteins (RSPs) via several subsequent changes. These events include changes in size as well as modification of the composition of the membrane and content of the SGs (Tooze *et al*, 2001). Proteins destined for SGs are processed by four distinct steps: i) the selective aggregation of the RSPs that form the dense cores and sorting of the RSPs to the membrane in the TGN followed by ii) budding from the TGN, iii) fusion of the membrane bilayers of the immature secretory granule (homotypic fusion), and iv) remodeling of the ISG membrane and content (Tooze *et al*, 2001). Many aspects of these events as well as the mechanisms involved in the formation of both regulated and constitutive secretory granules from the TGN are largely unknown.

Targeting of the secretory proteins to granules occurs at the level of the Golgi apparatus. The trans-Golgi network (TGN) represents the central sorting

station of the cells. It is at this location that proteins are sorted for delivery to the cell surface, or targeted for storage in granules (Tooze, 1991). Thus, constitutively secreted proteins and proteins targeted for storage granules are separated within the late Golgi (Arvan and Castle, 1998). Two non-mutually exclusive theories have evolved to describe the formation of regulated secretory granules: the 'sorting for entry' and 'sorting by retention' models (Kuliawat and Arvan, 1994). The most popular model is the 'sorting for entry', in which selection of protein cargo and membrane occurs in the TGN before ISG formation (Kuliawat and Arvan, 1994). The 'sorting by retention' model argues that little selection occurs in the TGN prior to ISG formation (Tooze *et al*, 1998). Rather, the specific components of SGs are retained throughout granule maturation, and missorted molecules (those not retained) are removed later from a post-TGN compartment, most likely by vesicles budding from the ISG (Urbé *et al*, 1997).

b. *Properties of secretory granules*

Biochemical studies have demonstrated that atrial SGs (Aardal and Helle, 1991) contain many of the proteins characteristic of endocrine and neuronal SGs, including caboxypeptidase E/H (Fricker, 1988; Lynch *et al*, 1988), peptidyl-glycine-α-amidating monooxygenase (PAM) (Eipper and Mains, 1988), chromogranin A and B (Steiner *et al*, 1990), and cytochrome b_{561} (Pruss and Shepard, 1987). Moreover, the SG membrane also contains proteins responsible for the acidification of the secretory proteins such as H^+-ATPase

(Apps and Percy, 1987). Finally, the SGs must be associated with one or more G-proteins, to allow these granules to fuse with the plasma membrane and release their contents (Burgoyne, 1990).

c. *Exocytosis of secretory granules*

Before reaching their final destination, SGs must be transported through the cytoplasm to the plasma membrane for fusion of the granule membrane with the plasma membrane. Translocation of SGs through the cytoplasm occurs via interactions with microtubules, in neurons (Grafstein and Forman, 1980), in endocrine cells (Tooze and Burke, 1987), and in atrial cardiocytes (Iida *et al*, 1988). Thus, the cytoplasmic microtubules provide the tracks along which atrial granules travel in cardiocytes. The association between SGs and microtubules was shown by EM by Orci *et al* (1973).

Rab proteins have been implicated in vesicular transport between the subcellular compartments along the exocytotic pathway (Zerial and Stenmark, 1993; Iida *et al*, 1997). Rab proteins belong to a subfamily of a ras-related GTPase superfamily (known as smgs: small monomeric GTP-binding proteins), which also include the rho/rac, the ras, and the ARF (ADP-ribosylation factor) subfamilies (Watson, 1999). Rab proteins are genetic homologues of Sec4p and Ypt1p proteins, which have been identified as potent regulators of vesicle transport from the Golgi to the plasma membrane in yeast (Salminen and Novick, 1987; Segev *et al*, 1988). Moreover, Rab6p (Iida *et al*, 1997) and

Rab12p (Iida *et al*, 1996) have been identified in atrial SGs, and at least two small GTPases found in purified atrial granules remain unidentified (Iida *et al*, 1997).

The next step in exocytosis requires that SGs fuse with the plasma membrane. Conserved sets of proteins, called SNARES (Soluble N-ethyl-maleimide-sensitive fusion protein (NSF) Attachment Protein REceptors), have recently been implicated in the docking and/or fusion of vesicular structures with their target membranes (Tooze *et al*, 2001). Cytosolic components that promote membrane fusions include NSF and α-SNAP (Tooze et al, 2001). SNARES are classified based on their location on the vesicle (v-SNARE) or target (t-SNARE) membrane (Jahn and Sudhof, 1999). The v-SNAREs in neuronal cells are called synaptobrevin, and their analogs in non-neuronal cells are called VAMPs (vesicle-associated membrane proteins) (Watson, 1999). VAMP 1 and 2 have been shown to be expressed in numerous rat tissues, including kidney adrenal gland, liver, pancreas, thyroid, heart, and smooth muscle (Rossetto et al, 1996). The t-SNARES comprise members of the syntaxin family (Bennett and Scheller, 1993) and the SNAP-25 family (Oyler et al, 1989). While SNAP-25 expression has primarily been identified in neuronal tissues, homologs to SNAP-25 and other members of the SNARE family have been found in adipocytes (Tellam et al, 1997), pancreatic beta cells (Wheeler et al, 1996), and pancreatic acinar cells (Gaisano et al, 1996). In addition, syntaxins 1-4 have also been detected in rat pancreatic and acinar cell fractions, specifically within the plasma membrane

and the SGs (Gaisano et al, 1996). In general, SNARES seem to regulate membrane fusion events throughout the secretory pathway, from the ER to the plasma membrane, independent of membrane fusion of secretory organelles with the plasma membrane (Vitale *et al*, 2000). Furthermore, selective cleavage of membrane-bound SNARES by clostridial toxins inhibits exocytosis in endocrine cells (Blasi *et al*, 1994).

SNARES have also been implicated in the event involving MSGs (mature secretory granules) formation via fusion of the ISGs (termed homotypic fusion); an event that has been observed by electron microscopy in numerous endocrine cells (Tooze, 1991). Reconstitution of homotypic fusion in cell-free extracts (Urbé *et al*, 1998) showed that the same components required for membrane fusion, NSF and α-SNAP, were also needed for ISG-ISG fusion (Tooze et al, 2001).

d. *Comparison of immature vs. mature secretory granules*

Utilizing EM, ISGs have been observed to contain core aggregate localized in close proximity to the TGN (Farquhar et al, 1978). It has also been shown that: i) ISGs are acidic (Orci et al, 1986); ii) processing of pro-hormones occurs in ISGs (Tooze et al, 1987); iii) portions of the ISG membrane have a clathrin coat (Orci et al, 1985; Tooze and Tooze, 1986; Kuliawat et al, 1997). Interestingly, the membrane of both the constitutive secretory vesicle and the MSG do not have a clathrin coat (Tooze, 1991). Other molecules that have been

identified in ISGs and are missing in MSGs, include carboxypeptidase D (Varlamov et al, 1999), SNARES such as syntaxin 6 (Klumperman et al, 1998), and VAMP 4 (Eaton et al, 2000).

4. Stretch-stimulated ANF secretion

While numerous factors regulate ANF release (de Bold *et al*, 2001), atrial wall stretch is the predominant acute stimulus that leads to the exocytosis of ANF from secretory granules (Lang *et al*, 1985; Edwards *et al*, 1988; Bruneau *et al*, 1997). Various experimental systems have been utilized to study hormone release in cardiocytes, including stretch of perfused atria/heart (Mangat and de Bold, 1993; Bruneau and de Bold, 1994; Bruneau et al, 1996, 1997; Ogawa et al, 1999; de Bold et al, 2001), hypotonic swelling (Jiao et al, 2000), and distension of cells grown on elastic membranes (Sadoshima and Izumo, 1997). These mechanical stimuli induced the release of different secretory or exocytotic products such as ANF, endothelin-1, angiotensin II, basic fibroblast growth factor, and vascular endothelial growth factor (Apodaca, 2002; de Bold et al, 1996; Ruskoaho, 1992; Sadoshima et al, 1993; Seko et al, 1999). Mäntymaa *et al* (1990) showed that distension of the right atria in a perfused heart preparation, increased the rate of immunoreactive ANF secretion into the perfusate. Other studies, have confirmed that stretch alone is a major factor regulating ANF release from the heart. A small increase in atrial-stretch elicited a short-lasting potent release of ANF *in vitro* (Bruneau and de Bold, 1994).

Repeated blood volume expansion or increase in preload resulted in a reproducible increase atrial pressure and ANF release (Ruskoaho, 1992).

The mechanisms by which mechanical forces are sensed by the cell and transduced into downstream intracellular effectors that promote exocytosis, have not been fully elucidated. Several mechanosensors have been identified, some of which may be involved in stretch-secretion coupling (Hamill and Martinac, 2001; Sadoshima and Izumo, 1997). The initial step in this process involves some sort of mechanosensor that is able to sense changes in membrane tension or alterations in the underlying cytoskeleton (Apodaca, 2002). Moreover, a single mechanical event may activate several mechanosensors, and each extracellular stimulus may be regulated downstream of these mechanosensors (Hamill and Martinac, 2001; Sadoshima and Izumo, 1997, 1993). Conversely, a number of mechanosensors may selectively control a subpopulation of downstream effectors (Hamill and Martinac, 2001; Sadoshima and Izumo, 1997; Sadoshima *et al*, 1992).

a. *The role of the cytoskeleton*

The cytoskeleton, composed of actin, microtubules, and intermediate filaments, has been shown to be an important player in mechanotransduction (Hamill and Martinac, 2001; Ingber, 1997; Morris and Homann, 2001). Deformation of the plasma membrane leads to the reorganization of the actin cytoskeleton, which affects the mechanotransduction governed by stretch

activated ion channels and by integrins (Hamill and Martinac, 2001; Wilson *et al*, 1995; Hamill and McBride, 1992). In addition, the cytoskeleton acts as a scaffold that coordinates the efficient transport of membranous cargo destined for exocytosis, within the cell (Apodaca, 2002). Agents that alter the integrity of the cytoskeleton significantly affect exocytosis and endocytosis (Apodaca, 2001; Valentijn *et al*, 1999). Moreover, microbutubules (composed of a helical array of repeating α- and β-tubulin subunits) have been involved in the intracellular transportation of various membrane-bound organelles such as SGs (Schliwa, 1984). A close spatial relationship between microtubules and atrial SGs has been shown by EM (Iida *et al*, 1988). This association was supported by double-label immunofluorescence experiments of ANF-containing granules and β-tubulin in cultured atrial cardiocytes (Larsen *et al*, 1993). Depolymerization of the microtubules with the antimitotic agent, nocodazole, caused a dispersal of ANF immunostaining, which was recovered when the microtubules were allowed to repolymerize (Larsen *et al*, 1993).

b. *The role of potassium channels*

Ion channels are macromolecular protein tunnels that span the cell membrane's lipid bilayer. The conformation of a channel protein interchanges between open (activated) and closed states in a process described as gating (Dascal, 2001). Moreover, stretch-activated ion channels have been proposed as potential mediators of cardiac hormone release (Sadoshima and Izumo, 1997). Van Wagoner (1993) described a stretch activated ATP-sensitive

potassium (K^+_{ATP}) channel (Noma, 1983) in rat atrial cardiocytes, and suggested that the stretch-induced potassium efflux somehow lead to ANF release. These K^+_{ATP} channels are normally inhibited by resting concentrations of adenosine tri-phosphate (ATP) and activated when intracellular ATP levels drop (Ashcroft, 1988). K^+_{ATP} channels play a protective role throughout metabolic stress, modulate action potential duration and excitability in the heart, and control insulin release in pancreatic β-cells (Ashcroft, 1988). They are composed by the association of the following two types of protein subunits: 1) a sulphonylurea receptor (SUR) regulatory subunits and 2) a member of the inward rectifier K^+ channel family called Kir6.2, constituting the pore of the channel and containing the major ATP binding site (Sakura *et al*, 1995; Baron *et al*, 1999).

The involvement of K^+_{ATP} channels in ANF secretion has been shown in various experiments. First, hypoxia opens cardiac K^+_{ATP} channels (Deutsch *et al*, 1991) and stimulates ANF release in isolated hearts (Lew and Baertschi, 1989). Second, stretch opened K^+_{ATP} channels within minutes in whole cell patch-clamped neonatal rat atrial cardiocytes (Jiao *et al*, 2000), and adult atrial cardiocytes (Van Wagoner, 1993). Third, the ATP-sensitive potassium channel opener, pinacidil, abolished stretch-induced ANF secretion in isolated rat hearts (Xu *et* al, 1996), yet had no effect in isolated rat atria (Kim SH *et al*, 1997). Alternatively, pretreatment with tolbutamide (K^+_{ATP} channel blocker) potentiated the stretch-induced ANF release and prolonged the response to stretch in isolated hearts (Xu *et al*, 1996), while glibenclamide (K^+_{ATP} channel blocker)

inhibited mechanically stimulated ANF release from isolated atria (Kim SH *et al*, 1997). Recently, Jiao *et al* (2000) showed that K^+_{ATP} channel blockade (tolbutamide) increased ANF release from hypotonically stretched cultures of neonatal atrial cardiocytes (Jiao *et al*, 2000). In that experiment, K^+_{ATP} channel openers (pinacidil and diazoxide) inhibited the stretch alone and the stretch-plus tolbutamide-stimulated ANF release (Jiao *et al*, 2000). Furthermore, in an acute pacing-induced heart failure canine *in vivo* model, investigators showed that glyburide (a K^+_{ATP} channel blocker) impaired stretch-activated ANF release (Chen *et al*, 2000). It is noteworthy that none of these pharmacological interventions affected basal release of ANF (Xu *et al*, 1996; Kim SH *et* al, 1997; Jiao *et al*, 2000; Chen *et al*, 2000).

5. G protein intracellular signaling

a. *Receptor-G protein-effector interactions:*

G proteins (called G proteins because they bind GTP) are a family of proteins that function as switches in signal transduction pathways across cell membranes in eukaryotic cells (Holmer and Homcy, 1991). They are heterotrimers consisting of α-, β-, γ- subunits; the α-subunit binds guanine nucleotide, and the βγ-subunits form a non-covalently associated dimer. G proteins act as fundamental links in signal transduction by coupling receptors (called G protein-coupled receptors, GPCRs) for hormones, neurotransmitters, physical signals (*i.e.*: light, odorants), and growth factors, to various effector molecules, such as ion channels and enzymes that produce second

messengers (Wess, 1997). From sequence and structure analyses, it is known that GPCRs share common structural motifs consisting of an extracellular N-terminal domain, a seven membrane-spanning domain, and a cytoplasmic C-terminal tail (Figure 3) (Wess, 1997). The interaction between GPCRs and the numerous G proteins can be rather diverse; a receptor has been shown to interact with more than one G protein, resulting in multifunctional signaling (Milligan, 1993). Functionally, G-proteins are governed by the GTPase cycle (Figure 2). When GDP is bound, the α and $\beta\gamma$ subunits form an inactive heterotrimer bound to the receptor ("off" conformation). Stimulation of receptors by agonists or physical signals results in the stabilization of its intracellular domain conformation, which promotes the dissociation of GDP from the α-subunit and allows GTP to bind. The replacement of GDP by GTP promotes the dissociation of the α subunit from the $\beta\gamma$ subunit. The "on" conformation allows the α subunit to interact with, and modulate, downstream effectors. This activated state is maintained until the GTP is hydrolysed to GDP by the intrinsic GTPase activity of the α-subunit, converting it back to the "off" conformation. Hence, the heterotrimer is formed again and returns to the receptor.

For many years, G protein signaling pathways were thought to be mainly mediated by the α-subunit. However, it is now recognized that both α and $\beta\gamma$ subunits regulate effectors (Neer, 1995). The rate of GTP hydrolysis represents the timing mechanism that modulates the duration of interactions between the α subunit and its effectors. Inactivation of G protein occurs by blocking G protein

activation or by GTP hydrolysis. Two classes of intracellular proteins can act as inhibitors of G protein activation: GTPase activating proteins (GAPs), which stimulate GTP hydrolysis, and guanine dissociation inhibitors (GDIs), which inhibit GDP dissociation (Geyer & Wittinghofer, 1997). GAPs mostly belong to a recently discovered gene family, the regulator of G-protein signaling (RGS) family, which has been shown to directly stimulate the intrinsic GTPase activity of the α subunit (Berman *et al*, 1996). In rat atrial and ventricular cardiocytes, ten different RGS proteins have been found (Kardestuncer *et al*, 1998).

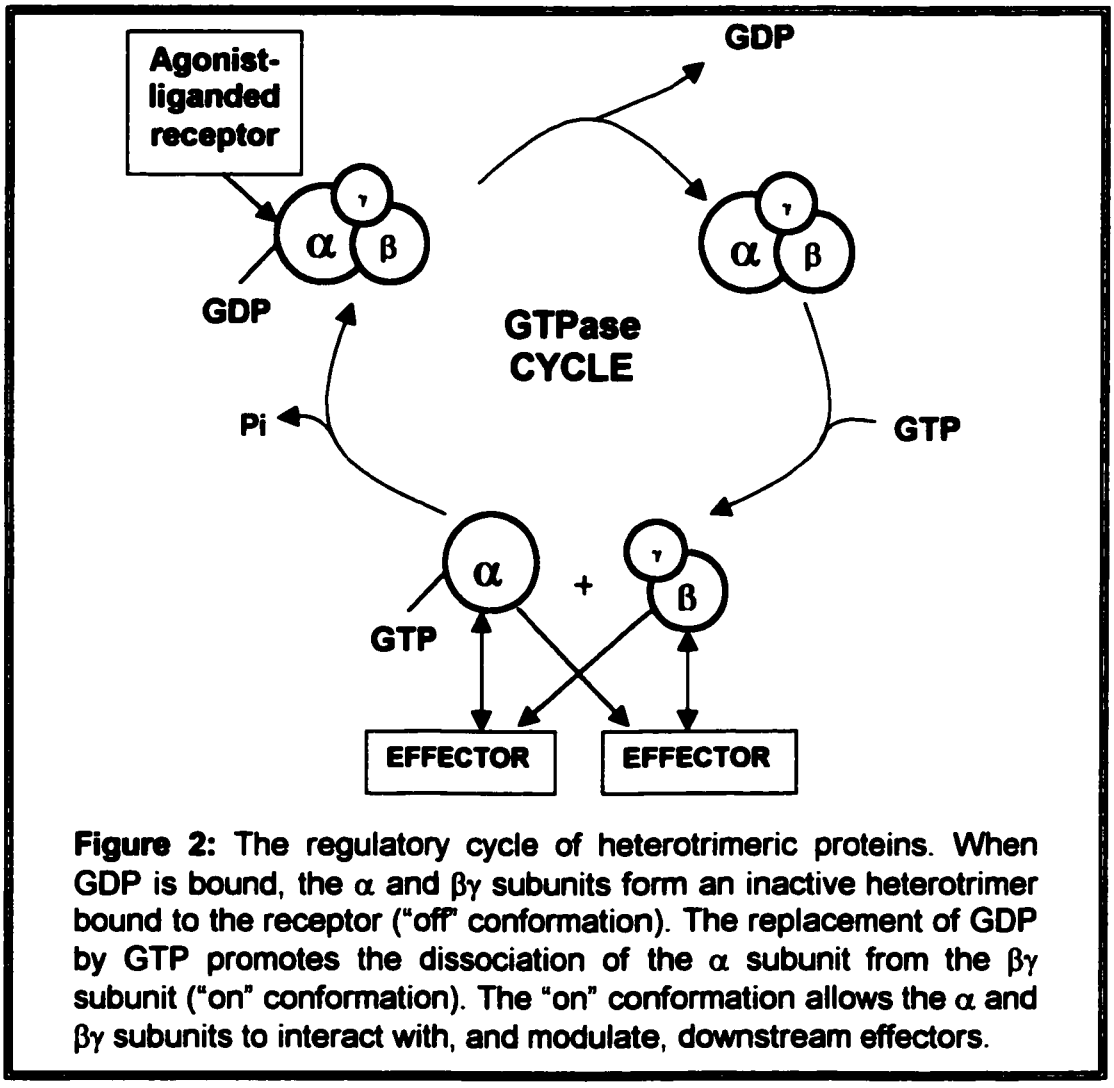


Figure 2: The regulatory cycle of heterotrimeric proteins. When GDP is bound, the α and βγ subunits form an inactive heterotrimer bound to the receptor ("off" conformation). The replacement of GDP by GTP promotes the dissociation of the α subunit from the βγ subunit ("on" conformation). The "on" conformation allows the α and βγ subunits to interact with, and modulate, downstream effectors.

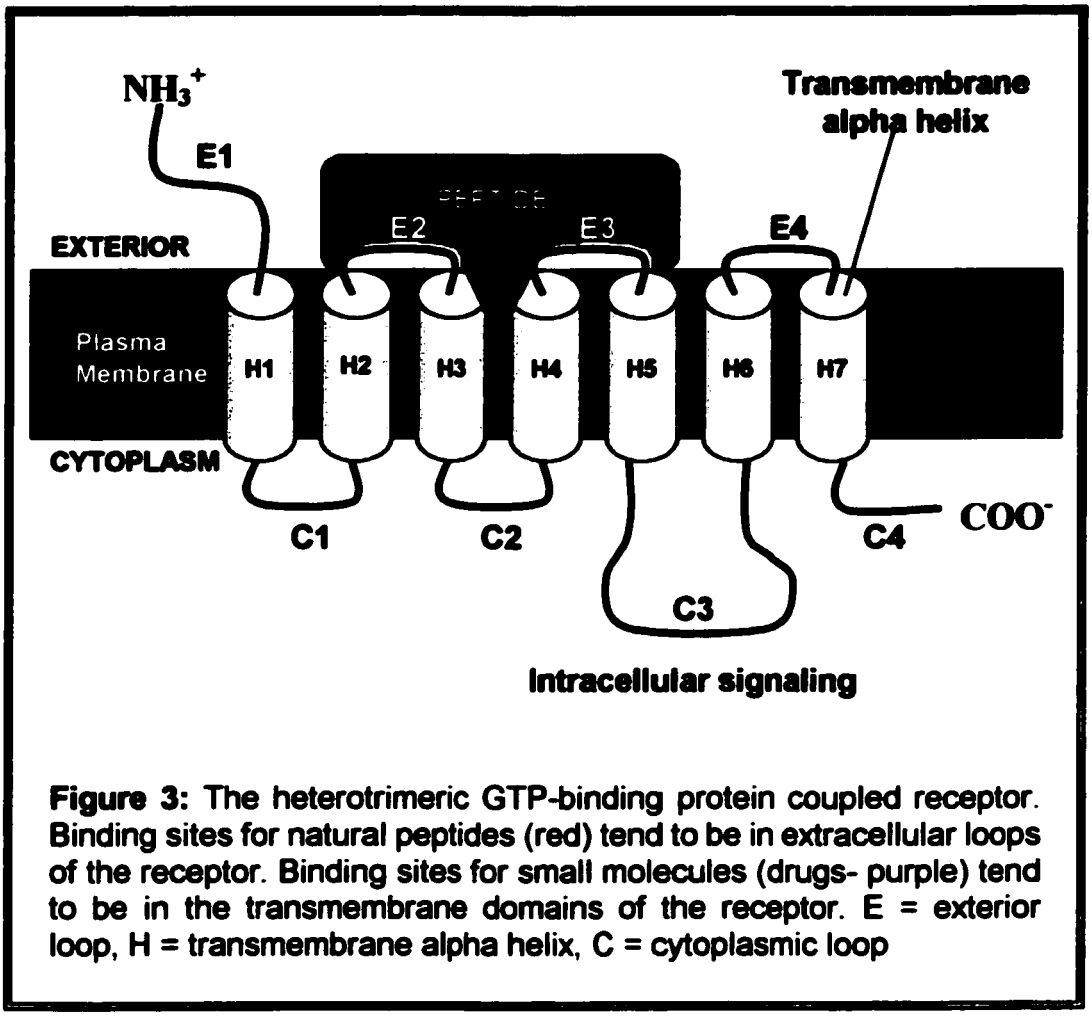


Figure 3: The heterotrimeric GTP-binding protein coupled receptor. Binding sites for natural peptides (red) tend to be in extracellular loops of the receptor. Binding sites for small molecules (drugs- purple) tend to be in the transmembrane domains of the receptor. E = exterior loop, H = transmembrane alpha helix, C = cytoplasmic loop

b. *Classes of mammalian Gα subunits*

Over twenty different G protein α subunits have been found in mammals, and can be subdivided into four major classes (Neer, 1995). The G protein classes (G_s, G_i, G_q, G_{12}) are organized according to the similarity of their amino acid sequences. (Refer to Table 3)

Table 3 Classes of mammalian G_α subunits					
α-subunit	**Inhibitor**	**Expression**	**Receptors for and examples**	**Effector**	**Signaling pathways**
G_s class					
1. G_s	CTX (Cholera Toxin)	Ubiquitous	β-Adrenergic, adrenaline, noradrenaline, histamine, glucagons, ACTH, luteinizing hormone, follicle stimulating hormone, thyroid-stimulating hormone, and others	↑ Adenylyl cyclase, regulate Ca^{2+} channel	↑ cAMP ↑ Ca^{2+} channel
2. G_{olf}	CTX	Olfactory	Odorants	↑ Adenylyl cyclase	↑ cAMP
G_i class					
1. G_{t1}	PTX/CTX (Pertussis toxin)	Rod Photoreceptors	Rhodopsin	↑ cGMP phosphodiesterase	↓ cGMP (vision)
2. G_{t2}	PTX/CTX	Cone Photoreceptors	Cone opsins	↑ cGMP-phosphodiesterase	↓ cGMP (colour vision)
3. G_{gust}	PTX/CTX ?	Taste cells	Taste	unknown	
4. G_{i1}	PTX	Neural > other	α_2-Adrenergic, M2-muscarinic	↓ Adenylyl cyclase ↑ K^+ channel	↓ cAMP
5. G_{i2}	PTX	Ubiquitous		↓ Adenylyl cyclase ↑ K^+ channel	↓ cAMP
6. G_{i3}	PTX	Other tissues> neural		↓ Adenylyl cyclase ↑ K^+ channel	↓ cAMP
7. G_o	PTX	Neural, endocrine	Somatostatin, not well defined	Phospholipase C ↓ Ca^{2+} channels	↑ IP3 ↑ diacylglycerol ↓ Ca^{2+} influx
8. G_z	-	Neural, platelets	unknown	unknown	
G_q class					
1. G_q		Ubiquitous	α1-Adrenergic, M1-muscarinic		
2. G_{11}		Ubiquitous	TRH, V1-vasopressin	↑ Phospholipase C-β1	
3. G_{14}		Liver, lung, kidney	Interleukin-8	↑ Phospholipase C-β1	
4. $G_{15/16}$		Blood cells		↑ Phospholipase C-β1	
G_{12} class					
1. G_{12}		Ubiquitous	Unknown	Unknown	
2. G_{13}		Ubiquitous	Unknown	Unknown	

* Adapted from Spiegel, 1997; Neer, 1995; Bourne *et al*, 1990

c. *GTP-binding proteins in secretory granules of endocrine cells*

Recently, it has been suggested that G_q or $G_{i/o}$ proteins may regulate the exocytotic pathways in a cell-type specific manner (Vitale *et al*, 2000). Complete heterotrimeric G proteins have been found within SGs in different endocrine tissues using immunohistochemistry. Ahnert-Hilger (1994) provided the first evidence that heterotrimeric G_o proteins were found on endocrine bovine chromaffin granules by immunofluorescence microscopy and immunoreplica analysis (Ahnert-Hilger *et al*, 1994). Vitale *et al* (1997) also showed that $G_{\alpha o}$ was associated with the membrane of SGs in chromaffin cells. $G_{o\alpha1}$, $G_{o\alpha2}$, $G_{i\alpha1}$ and $G_{i\alpha2}$ were found in small synaptic vesicles from rodent and bovine brain (Ahnert-Hilger *et al*, 1994). In addition, prolactin SGs in rat anterior pituitary cells contain multiple G proteins ($G_{i\alpha3}$, $G_{\alpha s}$, $G_{o\alpha1}$, $G_{o\alpha2}$) (Muller *et al*, 1994). In the pancreas, the G_i protein appears to be localized to insulin SGs of β-cells (Konrad *et al*, 1995). The heterotrimeric $G_{q/11}$ protein (Ohnishi *et al*, 1997; Padfield and Panesar, 1997) and the $G_{o\alpha}$ protein (Padfield and Panesar, 1997) have been identified within pancreatic acinar zymogen granules. Interestingly, $G_{olf\alpha}$ and $G_{s\alpha}$ (mostly found in immature granules) were localized in β-cell SGs and $G_{11\alpha}$ strongly labeled in the glucagon SGs of pancreatic A-cells (Astesano *et al*, 1999). In the atrial cardiocytes, $G_{o\alpha}$ has been associated with ANF SGs (Figure 12; Wolf *et al*, 1998). Moreover, accessory "downstream" molecules such as the small GTP binding proteins rac, ras, and rho have been linked to atrial SGs (Wildey and Matyas,1993; Iida *et al*, 1996).

d. *The role of G proteins in secretory granule formation*

The formation of SGs from the TGN requires GTP and is inhibited by non-hydrolysable GTP analogues (Xu and Shields, 1993; Tooze *et al*, 1990). Several lines of evidence have implicated heterotrimeric GTP-binding proteins in the regulation of post-Golgi secretory granule formation (Leyte *et al*, 1992; Burgoyne, 1992). A trimeric G protein has been shown to block the process of secretory vesicle formation (Barr *et al*, 1991) in a cell-free system that reconstituted the formation of constitutive secretory vesicles (CSVs) and ISGs from the TGN (Tooze and Huttner, 1990). The $G_{i\alpha/o\alpha}$ subunits have been shown to inhibit vesicle formation when activated with mastoparan-7 ($G_{i/o}$ agonist) in PC12 cells (Leyte *et al*, 1992).

The first step of granule formation involves attachment of ADP-rybosylation factor (ARF) (small GTP-binding protein), and coat proteins to the cytoplasmic phase of the donor membrane (Orci *et al*, 1993). In the second step, the granules are pinched off from the TGN. Trimeric G protein dependence is seen in both steps: binding of ARF is enhanced by aluminum fluoride, an activator of G protein (Donaldson *et al*, 1991), and the activation of $G_{i\alpha/o\alpha}$ proteins inhibits budding of granules (Stow *et al*, 1991; Barr *et al*, 1991; Pimplikar and Simons, 1993; Leyte *et al*, 1992). Furthermore, the inhibition of these proteins by pertussis toxin ($G_{i\alpha/o\alpha}$ proteins antagonist) promotes granule formation (Stow *et al*, 1991; Barr *et al*, 1991; Leyte *et al*, 1992).

e. *GTP-binding proteins and regulated exocytosis*

Low molecular weight (20-29 kDa) GTP-binding proteins of the ras superfamily, are divided into several families (ras, rap, rab, rho, rac, ran and arf), based on sequence homology (Kahn *et al*, 1992). The rab family (for rat brain, from where the original DNAs were cloned) have been found to play key regulatory roles in vesicular transport of proteins from one intracellular compartment to another (Zerial and Stenmark, 1993). Over 30 different rabs have been identified, and each has been localized to specific membrane-bound cell compartments (Watson, 1999). Using transgenic mice, Ohnishi *et al* (1997) showed that rab3D on zymogen granules play a stimulatory role in regulated amylase release. Other rabs that have been identified on SGs include rab6, 8 and 11. In pancreatic acinar zymogen granules, rab6 (Iida *et al*, 1997) and rab11 (Hori *et al*, 1996) have been detected. Moreover, rab6 and rab8 have been detected in α granules of human platelets (Karniguian *et al*, 1993). The involvement of rab 6,8, and 11 proteins in the exocytotic fusion event however, have not been clearly elucidated.

In chromaffin cells, it appears that rab3C regulates exocytosis by controlling the formation and dissociation of a complex of proteins that block secretion (Holz et al, 1994). This complex included VAMPs (identified on SGs membranes), which attaches to syntaxin and SNAP-25 on the plasma membrane (Watson, 1999). Activation of G_o proteins by mastoparan selectively

inhibited priming and abolished the dissolution of the SNAP-25/VAMP-2 complex (Misonou et al, 1997).

The presence of G proteins in the cytosol, on intracellular compartments (ER, Golgi, TGN), and in SGs of numerous endocrine tissues, suggests that these proteins are important regulators of the exocytotic pathway (Watson, 1999). The evidence supporting this conclusion stems from studies in chromaffin and pancreatic cells. In chromaffin cells, ATP-dependent priming of exocytosis was inhibited by activation of G_o proteins in SGs, and G_{i3} proteins played a role in the late calcium-dependent fusion event (Vitale *et al*, 1996). In the pancreas, inhibition of $G_{\alpha q/11}$ localized on SGs with GPAnt-2a (substance-P-related peptide) tonically blocked calcium-regulated amylase secretion by affecting the docking and fusion steps (Ohnishi *et al*, 1997). In another study, stimulation of the granule-associated G_o protein with a synthetic peptide inhibited the exocytotic priming step of secretion in chromaffin cells (Vitale *et al*, 1996).

f. *G proteins and potassium channels*

Many mammalian ion channels are regulated by neurotransmitters and hormones, via G-protein coupled receptors (Dascal, 2001). In the heart muscle, the recognized example is the inward rectifying channels that are opened by the activation of muscarinic receptors via G proteins (Mark and Herlitze, 2000). The ATP-sensitive (K^+_{ATP}) channels are a second type of inwardly rectifying K^+ channel. They are also modulated by heterotrimeric G proteins. Their regulatory

mechanism, however, is poorly understood (Dascal, 2001). These channels are activated by numerous GPCRs (G-protein coupled receptors) by way of pertussis-toxin sensitive G proteins (Kirsh, 1990; Ribalet and Edlestone, 1995). In addition, the ATP sensitivity of insulin secreting cells, is known to be regulated by Gi proteins, stimulated by somatostatin and galanin receptors (Fosset *et al*, 1988; Ribalet and Edlestone, 1995; Dunne *et al*, 1989). Many groups have demonstrated that a PTX sensitive G protein couples purinergic and muscarinic acetylcholine receptors M2 (M_2 AchR) to an inwardly rectifying K^+ channel in atrial cardiocytes (Breitwieser and Szabo, 1985; Pfaffinger *et al*, 1985). Kirsch *et al* (1990) showed that K^+_{ATP} channels are linked to adenosine A1 via inhibitory G proteins in ventricular cardiocytes. Moreover, Kim E *et* al (1997) showed via patch clamp technique, that the ability of adenosine to activate the K^+_{ATP} channel was inhibited by pretreatment of isolated rabbit ventricular cardiocytes with PTX. It has also been shown that the mechanism of G protein action is membrane delimited (Kirsch *et al*, 1990; Terzic *et al*, 1994) and accomplished by antagonizing the ATP-dependent inhibitory gating (Terzic *et al*, 1994). A functional link between PTX sensitive G proteins and K^+_{ATP} channels was also demonstrated in canine vascular tissue (Komaru *et al*, 1997). Furthermore, the affinity of K^+_{ATP} channels for $G_{i\alpha}$ subunit has been shown to be three orders of magnitude (30-50 pM) higher (Ribalet and Edlestone, 1995) than for the most-studied effector of $G_{i\alpha}$, adenylyl cyclase (Sunahara *et al*, 1996). A recent publication (Wada *et al*, 2000), however, has challenged the role of G_α

subunits and shows that the $G_{\beta\gamma}$ subunit directly regulates the reconstituted K^+_{ATP} channel in the COS-7 mammalian cell line.

6. Pharmacological tools used to investigate GTP binding proteins:

a. *$G_{i/o}$ binding protein antagonist:* ***Pertussis toxin***

Bacterial toxins have proven to be extremely useful tools for identifying and studying the role of G proteins. *Bordetella pertussis* is a small, gram negative, non-motile coccobacillus, which produces a 105-kDa toxin called pertussis toxin (PTX). In light of its varied effects, multiple names have been assigned to PTX in the past, including lymphocytosis promoting factor (LPF), histamine-sensitizing factor (HSF), and islet activating protein (IAP) (Kaslow and Burns, 1992). On entry into cells, PTX covalently modifies the α subunits of inhibitory G proteins by ADP-ribosylating a cysteine amino acid residue near its carboxyl terminal (Ui *et al*, 1985; Tanuma *et al*, 1987). PTX consists of six polypeptides (Figure 4) bound together by noncovalent interactions and arranged in the A-B conformation archetypal of many bacterial toxins (Sekura *et al*, 1983; Tamura *et al*, 1982). The A-B architecture comprises two enzymatically active subunits. The A protomer consists of a single S1 subunit, which possesses the toxins catalytic NAD+ glycohydrolase and ADP-ribosyltransferase activities (Tamura *et al*, 1982; Katada *et al*, 1983). NAD is the other substrate for the ADP-ribosyltransferase activity of PTX (Kaslow and Burns, 1992). PTX catalyzes the transfer of the ADP-ribose portion of NAD to inhibitory G proteins in eukaryotic cells (Tamura *et al*, 1982). The B oligomer containing 5 subunits

(S2, S3, 2 x S4, and S5) is responsible for the attachment of the native toxin to eukaryotic target cell surfaces (Tamura *et al*, 1982). The subunits of the B-oligomer are assembled as a "donut shaped" structure to which the S1 subunit is anchored by means of its C-terminal extension (Tamura *et al*, 1982). Following binding of the B-oligomer to the target membrane, the S1 subunit translocates across the membrane, and the toxin works to ADP-ribosylate the alpha subunit of $G_{i/o}$ proteins (Kaslow and Burns, 1992). Thus, PTX inhibits G protein signaling via two ways: 1) PTX inhibits receptor G protein interactions (Wise and Milligan, 1997), 2) ADP-ribosylation by PTX inhibits the dissociation of the G_{α} and $G_{\beta\gamma}$ subunits and consequently blocks effector function (Kaslow and Burns, 1992).

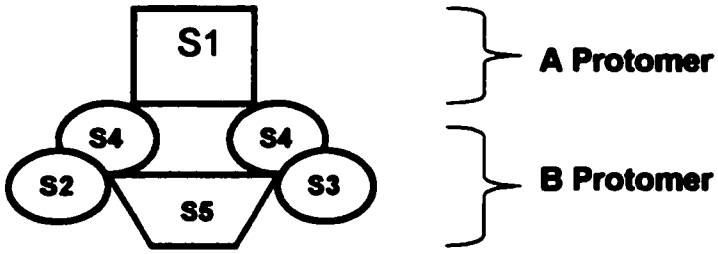


Figure 4: Schematic representation of pertussis toxin. The A-B architecture comprises two enzymatically active subunits. The A protomer consists of a single S1 subunit, which possesses the toxins catalytic NAD+ glycohydrolase and ADP-ribosyltransferase activities. The B oligomer containing 5 subunits (S2, S3, 2 x S4, and S5) is responsible for the attachment of the native toxin to eukaryotic target cell surfaces. (S= subunit)

b. *Entry of bacterial toxins into cells*

In general, bacterial toxins are thought to penetrate eukaryotic cells by endocytosis or by direct penetration of the plasma membrane. Studying the way toxins infect cells has provided important information about their mechanism of action. Diphtheria toxin (ADP-ribosylates a cytoplasmic protein critical for protein synthesis termed EF-2) is internalized after receptor-mediated endocytosis in an acidic compartment where it translocates to the cytosol (Morris *et al*, 1985). This toxin exhibits sensitivity to acidic environments, and exposure to endosomes generates conformational changes believed to enable diphteria toxin to cross the vesicle membrane facilitating the release of the enzymatically active component of the toxin to its target (Durmont and Richards, 1988). Agents that block endosome acidification, such as NH_4Cl, prevented the cytotoxic effect of diphtheria toxin (Krueger and Barbieri, 1995). In addition, Shiga toxin (causes severe dysentery) is endocytosed, and subjected to retrograde transport through the Golgi apparatus to the ER (Sandvig, 2001). Alternatively, cholera toxin (ADP-ribosylates the heterotrimeric Gs protein) may enter some cells by direct penetration of the plasma membrane (Fishman, 1990; Ribi *et al*, 1988), while it may penetrate other cells by means of endocytosis (Janicot *et al*, 1991).

c. *Entry of PTX into cells*

In CHO cells, the B oligomer of PTX was shown to attach to cells containing glycoproteins with N-linked oligosaccharides (Brennan *et al*, 1988;

Witvliet *et al*, 1989). PTX was shown to have a putative lectin-binding site located on the B oligomer that interacted with the carbohydrate sequence NeuAcGalβ4GlcNAc on CHO cells (Brennan *et al*, 1988). In the pancreatic insulin-secreting beta cell derived HIT-T15 line, PTX was able to bind to receptors with sialoglycoproteins (el Baya *et al*, 1995). PTX has properties unlike other toxins, such as its ability to interact with detergents and phospholipids (Kaslow and Lesikar, 1987; Moss *et al*, 1986; Montecucco *et al*, 1986). This suggests that it can cross the plasma membrane directly without the need for endocytosis. In LLC-PK1 (pig kidney) epithelial cells, Stow *et al* (1991) showed that PTX ADP ribosylated $G_{\alpha i}$ proteins located on the cytoplasmic face of Golgi stacks. The involvement of the Golgi complex in the process of ADP-ribosylation by PTX was verified in Chinese hamster ovary (CHO) cells treated with Brefeldin A (BFA) (Xu and Barbieri, 1995). BFA is a compound known to cause disassembly of the Golgi complex, and was shown to inhibit the ADP ribosylation of target proteins by PTX. Further studies in intact CHO cells by Xu and Barbieri (1996) showed that the internalization of PTX was consistent with a model of receptor-mediated endocytosis. In this experiment, chloroquine (inhibits the entry of ligands that act via a endosomal pH-sensitive step), monensin (inhibitor of vesicle formation and sorting at the TGN), and nocodazole (microtubule disrupting drug) inhibited the *in vitro* ADP-ribosylation of CHO cells by PTX.

d. *$G_{i/o}$ binding protein agonist:* ***Mastoparan-7***

Mastoparan (MAS) is an amphiphilic tetradecapeptide from wasp venom with the structure Ile[1]-Asn[2]-Leu[3]-Lys[4]-Ala[5]-Leu[6]-Ala[7]-Ala[8]-Leu[9]-Ala[10]-Lys[11]-Lys[12]-Ile[13]-Leu[14]-NH_2 (Higashijima *et al*, 1988). It is thought that it stimulates G-proteins by mimicking the normal interaction that occurs between G-protein coupled receptors and their respective G-proteins (Mousli *et al*, 1990). Thus, the α-subunit assumes an active configuration, when binding of MAS to the carboxyl terminus of the G-protein, induces the dissociation of the α-subunit from the $\beta\gamma$-subunit, resulting in the release of GDP (Weingarten *et al*, 1990; Tomita *et al*, 1991). MAS increases the rate of nucleotide binding and GTPase activity of purified G_o and G_i binding proteins (Higashijima *et al*, 1988). This toxin also affects the exocytosis of several hormones and neurotransmitters by acting at PTX sensitive sites (Vitale *et al*, 1993; Consolo *et al*, 1997). It stimulates secretion of histamine from mast cells, catecholamines from chromaffin cells, prolactin from the anterior pituitary, serotonin from platelets, and insulin from β-cells (Jones *et al*, 1993; Komatsu *et al*, 1993; Straub *et al*, 1998). Hormone secretion by MAS is blocked by PTX (Yokokawa *et al*, 1989; Higashijima *et al*, 1988). Moreover, Higashijima *et al* (1988) suggested that because MAS is an amphiphilic peptide, it could cross the plasma membrane in response to membrane potential and consequently interact directly with intracellular G proteins. At least seven analogues of MAS are produced in different species (Higashijima and Ross, 1994). The most active analogue, MAS-7, is made by a slight change in sequence: Lys[12]-Ile[13]$\rightarrow$ Ala[12]-Leu[13] (Higashijima *et al*, 1990).

MAS-7 is 5 times more potent and more effective at its optimal concentration than MAS (Higashijima *et al*, 1990).

7. Endothelin-1 (ET-1): an agonist of ANF secretion

In addition to stretch, endothelin-1 (ET-1) was used in the present work as an agonist of ANF secretion and is reviewed in the following paragraphs. The vascular endothelium regulates the function of smooth muscle cells by releasing a range of vasoactive molecules (Ruskoaho *et al*, 1997). The secretion of these factors has been shown to result in acute and chronic changes in local vascular tone, which alters local blood flow (Meininger and Davis, 1992). Chief among these factors, is the most potent mammalian vasoconstrictor discovered to date: endothelin-1 (ET-1) (Yanagisawa *et al*, 1988). Elevated levels of plasma and tissue ET-1 have been documented in many diseases, such as atherosclerosis (Dashwood and Tsui, 2002), essential and pulmonary hypertension (Goldie, 1999; Schiffrin, 2001), myocardial arrhythmias and ischemia (Duru *et al*, 2001), renal failure (Pollock, 2001), and asthma (Hay, 1999; Goldie and Fernandes, 2000).

ET-1 is a member of a family of 21 amino acid peptides, which are expressed in various tissues (Rubanyi and Botelho, 1991; Simonson and Dunn, 1990). ET-1 mRNA has been found in endothelial cells (Yanagisawa *et al*, 1988), in atrial tissue (Elton *et al*, 1992), and in endocardial endothelium (Mebazaa *et al*, 1993). Endothelial cells line the inner surface of blood vessels,

the atrial blood vessels and the trabeculated cavities of the heart chambers (Brutsaert and Andries, 1992; Inagami *et al*, 1995). Substantial evidence acquired during years of research, have clearly shown that ET-1 acts on cardiac cells to stimulate ANF release (de Bold *et al*, 1991; Hu *et al*, 1988; Schiebinger and Gomez-Sanchez, 1990; Stasch *et al*, 1989; Winquist *et al*, 1989; Mäntymaa *et al*, 1990; Fukuda *et al*, 1988; Gardner *et al*, 1991; Horio *et al*, 1993; Leite *et al*, 1994; Suzuki *et al*, 1992). ET-1 was shown to be among the most potent humoral secretagogues of ANF in cultured rat atrial myocytes (Fukuda *et al*, 1988, 1989; Sei and Glembotski, 1990; Gardner *et al*, 1991; Uusimaa *et al*, 1992; Lew and Baertschi, 1992; Muir *et al*, 1993), in isolated atria (Hu *et al*, 1988; Stasch *et al*, 1989; Winquist *et al*, 1989; de Bold *et al*, 1991; Bruneau and de Bold, 1994), and in isolated perfused atria (Mäntymaa *et al*, 1990; Bruneau *et al*, 1997; Ogawa *et al*, 1999). In contrast to hormone release stimulated by stretch, however, ET-1-induced a gradual increase in ANF secretion that reached a plateau and eventually returns to basal levels (Bruneau *et al*, 1994,1997). In addition, while endothelial cells have little SGs to store ET-1 (Yanagisawa *et al*, 1988), they have been shown to store ET-1 and to secrete it acutely (Macarthur *et al*, 1994; Ramaciotti *et al*, 1993).

ET receptors (ET-R) have been divided into two subtypes, ET_A-R and ET_B-R (Hori *et al*, 1992), which share homology with the G-protein coupled receptor family (Arai *et al*, 1990; Sakurai *et al*, 1990). Multiple signaling pathways are stimulated by ET receptors, including production of cAMP

(Sokolovsky *et al*, 1994), production of cGMP (Shraga-Levine *et al*, 1994; Shraga-Levine and Sokolovsky, 1996), activation of phospholipase C (Sokolovsky, 1993), activation of phospholipase D (Ambar and Sokolovsky, 1993), activation of phospholipase A_2 (Aramori and Nakanishi, 1992), an increase in cytosolic Ca^{2+} (Koizumi *et al*, 1994), regulation of Na^+/H^+ exchange (Vigne *et al*, 1991), and activation of the mitogen-activated protein kinase cascade (Kasuya *et al*, 1994). The ET_A-R was shown to be the predominant receptor subtype found in the heart, and was shown to have the greatest affinity for ET-1 and ET-2 (Ihara *et al*, 1992). Quantitive autoradiography has shown that ET_A receptors represent about 91% of the total population of ET receptors in human right atrial cardiocytes (Molenaar *et al*, 1993). The ET_A receptor antagonist, BQ-123, has been shown to inhibit ET-1 stimulated ANF release in a dose-dependent manner in atrial cardiocyte cultures (Irons *et al*, 1993; Thibault *et al*, 1994). Similarly, Skvorak *et al* (1995) showed BQ-123 inhibited the stretch-induced ANF release in an isolated perfused rat atria model. The involvement of G protein signaling in endothelin receptor activation has been confirmed by indirect and direct methods. ET-induced dose-dependent stimulation of high-affinity GTPase was demonstrated by measurement of GTPase activity in rat cardiocytes (Sokolovsky, 1993). Recently, ET-1 was shown to induce coupling of the ET_A receptor to $G_{q/11}$ proteins (Shraga-Levine and Sokolovsky, 2000). Thus, pharmacological treatment with a specific $G_{i/o}$ protein antagonist (PTX) should not affect ET-1 stimulated ANF secretion, since the receptor for ET-1 signals via $G_{\alpha q}$ proteins.

2. AIMS AND HYPOTHESIS:

In order to better understand the pathways involved in stretch-secretion coupling, the role of heterotrimeric GTP binding proteins is investigated. The interest in G proteins stemmed from stretch experiments performed in avian skeletal muscle (Vanderburgh et al, 1995), and cardiac fibroblasts (Gudi et al, 1998), showing that $G_{i/o}$ proteins were involved in stretch-secretion coupling. Ultimately, this project was undertaken to improve our understanding of the pathways involved in converting mechanical stimuli into intracellular signals promoting/inhibiting hormone secretion.

It was hypothesized that stimulation of G protein signaling would promote ANF release and that the inhibition of $G_{i/o}$ protein signaling would impair stretch stimulated ANF release. Utilizing an isolated rat atria model (Bruneau *et al*, 1997), the effect of inhibiting G proteins with the specific $G_{i/o}$ protein antagonist, pertussis toxin, on mechanically-induced ANF secretion was studied. In addition, activation of $G_{i/o}$ proteins with MAS-7 was utilized to further test the present hypothesis. We also wanted to confirm that endothelin-1 (ET-1) stimulated ANF secretion was not affected by PTX, since ET-1 signals through $G\alpha_q$.

3. MATERIALS & METHODS

1. *Isolated right atria*

The isolated atrial preparation was developed in de Bold's laboratory; a model which preserves the morphological and physiological properties of the atria (Bruneau *et al*, 1997). First, the hearts were removed from decapitated male Sprague-Dawley rats (275-350g, Charles River Laboratories) and placed in a Krebs-Ringer bicarbonate buffer (KRBB) solution. The KRBB solution contained 78 mM NaCl, 4.7 mM KCl, 2.54 mM $CaCl_2 \bullet H_2O$, 1.36 MM $NaH_2PO_4 \bullet H_2O$, 1.16 mM $MgCl_2 \bullet H_2O$, 25.0 mM $NaHCO_3$, 20 mM *N*-2-Hydroxyethylpirazine-*N'*-2-ethanesulfonic acid (HEPES), 2.0 Mm sodium glutamate, 4.0 mM sodium fumarate, 2.0 mM sodium lactate, 11.6 mM glucose, 2.46 mU/ml zinc insulin (Humulin R, Eli Lilly, Minneapolis, MN), amino acids and vitamins as in Eagle's Medium (Life Technologies), 0.001% (wt/vol) bovine serum albumin (BSA), 0.01% (wt/vol) gelatin, 100 U/ml penicillin G (Sigma-Aldrich Chemicals), and 0.25 μg/ml fungizone (Life Technologies). The pH was adjusted with 0.1 M NaOH to 7.40 at 30 °C. The KRBB was filtered through a 0.45 μm filter prior to use. The dissection procedure was as follows. A PE-160 cannula (3 cm in length) with a 3 mm opening at the center of the cannula, was inserted in the inferior vena cava and out the superior vena cava. The cannula was positioned such that the opening faced the inside of the right atrial chamber. Each vena cava was secured to the cannula with a double knot of silk suture. Then, the coronary sinus was ligated, the left atria was removed, and the aorta and pulmonary vein were cut off immediately above the ventricles.

Connective tissue and fat were removed from the atrium. The lower 5/6 of the ventricles were removed, and a rigid heat-curved cannula (PE-10), the end of which was heated to form a lip, was inserted in the right atrium via the tricuspid valve; this cannula was held in place by tying a silk suture around the atrioventricular junction.

As illustrated in Figure 5, the right atrium dissected in this manner was fixed to a plastic disc by inserting the inferior vena cava and outflow cannulas onto tubing-covered syringe needle ends that passed through the disc. The disk was then tightly inserted in the opening of an inverted heated (30 °C) organ chamber. The superior vena cava was attached to a cannula from which KRBB was perfused at a rate of 3 ml/min using a programmable solvent delivery module. The inferior vena cava was connected to a pressure transducer (Narco Bio-Systems, Houston, TX) that was linked to a physiograph. The flow of KRBB was from the superior vena cava, into the right atrium, and out the tricuspid valve. The heated (30°C) organ chamber contained 50 ml of KRBB, which was replenished at a rate of 2.5 ml/min, and which was gassed with 95% O_2-5% CO_2 throughout the experiment. To accurately measure intra-atrial pressure, the tips of the pressure transducer and outflow cannulas were positioned at the same height. To adjust the basal atrial pressure to ~0.5 mmHg, the tip of the outflow cannula was raised in relationship with the level of the atrium and pressure transducer. In the stretch experiments, the outflow cannula was raised to generate an intra-atrial pressure of 8 mmHg. ANF hormone secretion was

quantified in the perfusion medium collected for 5-min periods in siliconized glass tubes using a fraction collector. A 50 µl aliquot was transferred to another tube for use in the ANF RIA; this and the remainder of the perfusate were immediately stored at -20 °C.

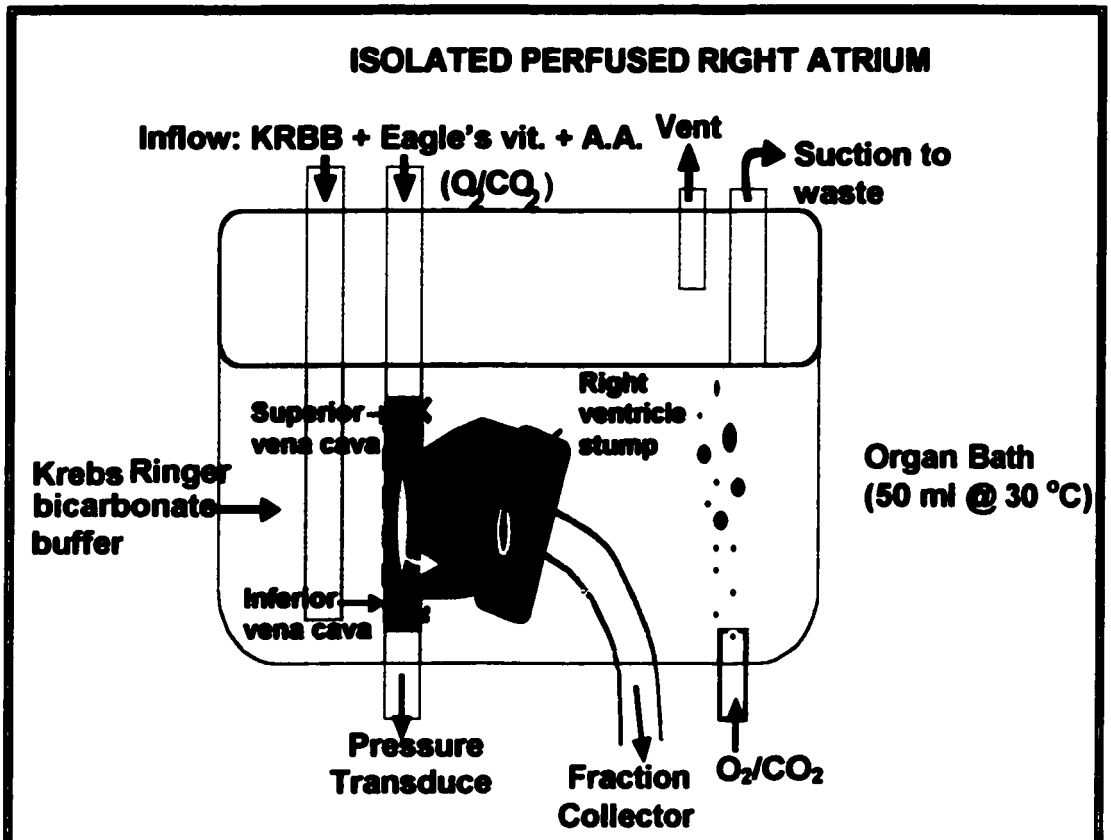


Figure 5: Isolated whole atria perfusion model. Described in text. Adapted from Bruneau *et al*, 1997.

2. Drugs

Each vial containing pertussis toxin (Sigma-Aldrich, Oakville, ON) was reconstituted with 500µl of 0.1 M sodium phosphate buffer (pH = 7.4). Mastoparan-7 (Biosource International, Camarillo, CA) (final concentration

10^{-5}M) was reconstituted with 2 ml of distilled water. The doses of PTX and MAS-7, as well as the time of sacrifice, were based on numerous in vivo and in vitro studies in rats demonstrating that these doses effectively inhibited or stimulated Gi/o proteins (Lasley and Mentzer, 1993; Piacentini *et al*, 1993; Hare *et al*, 1998; Konrad *et al*, 1995; Yokokawa *et al*, 1989; Komatsu *et al*, 1992). Endothelin-1 (Peninsula Laboratories Incorporated, San Carlos, CA) (final concentration 10^{-8}M) was dissolved in 10% acetic acid and diluted with KRBBs solution. Chemicals were purchased from Sigma-Aldrich unless otherwise mentioned.

3. Perfusion Protocols

a. *Pertussis toxin (*PTX*) and stretch*

Two groups of rats were pretreated with pertussis toxin (25 μg/kg ip or 40 μg/kg ip; n=5 respectively) 48 hours prior to the isolated atria experiment. A second group of rats was not pretreated with PTX (baseline group, stretch only group). Following dissection, atria were placed in the organ chamber, and allowed to equilibrate for 1 h. During the basal collection period, atria were left at basal intra-atrial pressure (0.5 mmHg; baseline n=5). Increasing the intra-atrial pressure from 0.5 to 8 mmHg imposed stretch. All atria (with the exception of the control group) were stretched at the 120 min time point for 4 hours. Rats injected with pertussis toxin and vehicle showed no ill effects and exhibited normal physical behavior. At the end of the perfusion period, the tissue was

rapidly removed and placed in a dissecting dish, where the right atrium was dissected and was flash frozen in liquid nitrogen.

b. *Mastoparan-7* (MAS-7)

Drugs (MAS-7, ET-1) were perfused at 9 times their final concentration into the inflow cannula, using a syringe pump (Sage Instruments, Cambridge, MA) set at 0.34 ml/min. The mechanical KRBB delivery module was adjusted to maintain a flow rate of 3 ml/min. In this experiment, MAS-7 (Biosource International) was directly perfused into the cannula perfusing the isolated atria (10^{-5} M, n=5) for 30 minutes (starting at the 120 minutes time point). A second group of rats (n=5) were pretreated with PTX (40 μg/kg ip) 48hr prior to the MAS-7 perfusion experiment.

c. *Endothelin-1* (ET-1)

Atria from untreated (n=4) or PTX-treated (n=4) rats were perfused with ET-1 (Peninsula Laboratories) for a period of 2hr (starting at the 120 min time point; final concentration = 10^{-8}M), as described for MAS-7.

4. Preparation of atria homogenate

Following the perfusion experiments, the atria were flash frozen in liquid nitrogen. The atria were weighed, and sucrose buffer (20 mM HEPES, 1 mM EDTA, 255 mM sucrose, 100 μM phenylmethylsulfonyl fluoride) was added in a 1 (weight of atria) to 4 (vol of sucrose buffer) ratio. The tissue in sucrose buffer

was homogenized using a chilled glass homogenizer. The homogenate was centrifuged at 13,000 rpm for 30 min at 4°C (Model J2-MI centrifuge and model JA-20.1 rotor, Beckman). Supernatant was collected and kept for determination of protein concentrations using a spectrophotometer (Beckman, DU-40). The protocol was as follows: diluted BSA (bovine serum albumin) standards were prepared by diluting 2.0 mg/ml BSA stock standard (Pierce, Rockford, IL) in sucrose buffer. The absorbance of each standard was measured at 280 nm and a standard curve for each BSA standard vs. its concentration in μg/ml was plotted. Using the standard curve, the protein concentrations for the unknown experimental samples were interpolated.

5. PTX-induced ADP ribosylation

To confirm the level of PTX-induced ADP-ribosylation of $G_{i/o}$ protein *in vivo*, a PTX assay was utilized using [^{32}P] NAD (Amersham Pharmacia Biotech Inc., Oakville, ON). The PTX-induced ADP ribosylation reaction was initiated by adding 20 μg of protein from rat atria homogenate to 0.1M Tris-HCl (pH = 7.6), 20 mM dithiothreitol, 1.1 μM [^{32}P] NAD, 0.1 mM ATP, and 1.5 μl of PTX. Following incubation for 1 hour, the reaction was stopped by the addition of 12.5 μl SDS sample buffer (62.5 mM Tris HCl, H 6.8, 25% Glycerol, 2% SDS, 0.01% Bromophenol Blue; Bio-Rad, Oakville, ON) with β-mecaptoethanol (710 mM) and boiled for 5 min at 100 °C. Equal amounts of sample were loaded into 4-15% gradient SDS-Polyacrylamide gel electrophoresis gels (electrophoresed at 80 V for 20 min and 200 V for 50 min) using a Mini-PROTEAN 3 Cell system at

room temperature (Bio-Rad). Broad Range prestained SDS-PAGE standards (Bio-Rad) were also run on each gel. The gel was fixed for 30 min in a solution of 7% glacial acetic acid in 10% methanol, stained with brilliant blue G-colloidal solution (Bio-Rad) for 2 hours, and destained with 10% acetic acid in 25% methanol for 60 seconds. The gel was air-dried overnight. Autoradiography was performed subsequently for 30 min on a PhosphorImager cassette. Radioactivity bound to the blot was quantified using a Bio-Rad personal molecular imager FX and Bio-Rad Quantity One software.

6. Radioimmunoassay

Radioimmuoassay (RIA; Figure 6) was performed using the double antibody technique as previously described in Sarda *et al* (1989). A 1(50 μl perfusate) to 1 (50 μl RIA buffer) dilution of perfusate was used for the ANF RIA. The buffer used for RIA was 0.1 M potassium phosphate (pH 7.4) (VWR International, Mississauga, ON) containing 0.9 % NaCl, 0.01% sodium azide, 0.1 % triton-X, and 0.1% heat-treated BSA. RIA buffer was used to dilute all reagents and samples. All reactions were carried out in polystyrene tubes (12 X 75 mm). The standard curves were calculated by using ANF_{99-126} (Peninsula Laboratories, Belmont, CA) at the following concentrations (pg/ml): 31.25, 62.50, 125.0, 250.0, 500.0, and 1000.0, prepared on ice and kept out of direct sunlight. Quality control (QC) tubes were prepared by using low (125.0 pg/ml) or medium (250.0 pg/ml) concentrations of ANF_{99-126}. The protocol is as follows: 100 μl of antiserum raised against rabbit ANF_{99-126} (1:30,000 dilution; Peninsula

Laboratories) was added to 100 µl of sample, standard, or QC and left at 4°C for 4 hours. Following the incubation period, 100 µl (10,000 cpm) of iodinated ANF_{99-126} was added to all the tubes, which were left at 4°C for approximately 20 hours. Free and bound fractions of ANF_{99-126}, were separated by adding 100 µl of 5% normal rabbit serum (Invitrogen, Burlington, ON) and 100 µl of goat anti-rabbit gamma globulin (Advanced Chemtech, Louiville, KY) to the tubes. Following a 2hr incubation at room temperature, 1.5 ml of 6.25 % polyethylene glycol (PEG) dissolved in distilled water (Sigma-Aldrich) was added to each tube. The tubes were then centrifuged at 2000Xg (2700 rpm on Beckman J-6 M centrifuge) at 4°C for 45 minutes. The supernatant was then carefully discarded, and the radioactivity in each pellet was counted in a gamma counter.

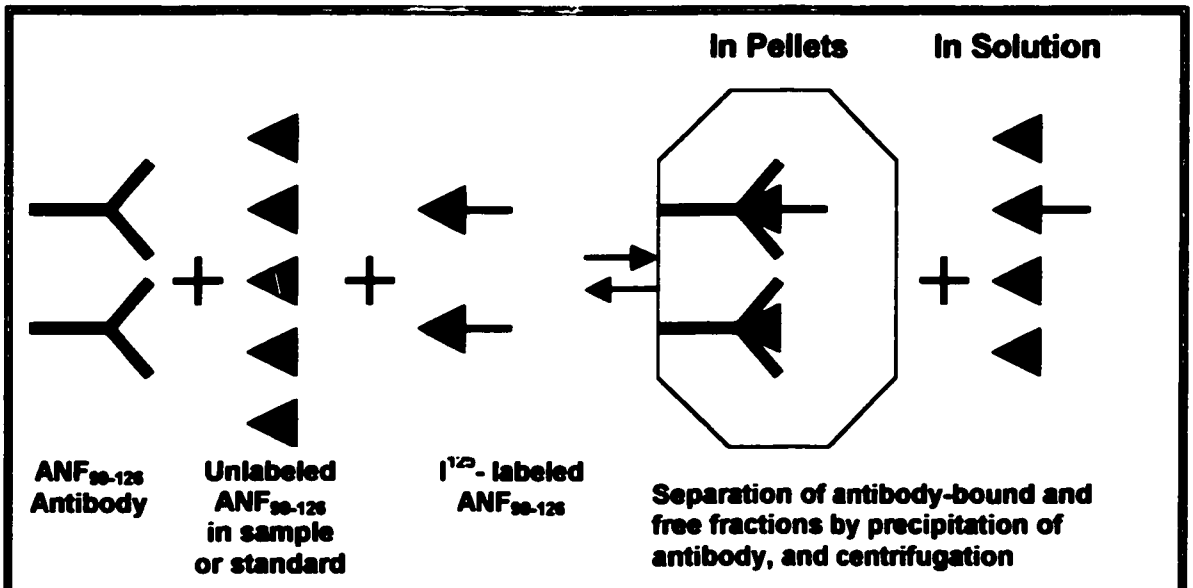


Figure 6: Competitive radioimmunoassay (RIA). RIA is described in terms of competition between a radiolabeled antigen and its unlabeled counterpart for binding to a limited quantity of specific antibody (Ab). Thus, the amount of unlabeled ANF_{99-126} present, limits the amount of radiolabled ANF_{99-126} that can bind to the Ab. Upon suitable calibration, the amount of unlabeled species can be accurately measured via the amount of radioactivity associated with the antigen-antibody complex.

For ANF determination in plasma, trunk blood samples were collected from PTX pretreated (n = 6) and untreated (injected with 0.9 % NaCl; n=6) male Sprague Dawley rats in chilled EDTA-containing vacutainer tubes. Plasma was immediately separated by centrifugation at 2000Xg for 20 min at 4°C and stored in cryovials at 80°C until assayed. Plasma samples were acidified by adding 1.0 N hydrochloric acid (HCl) (ratio of 100µl to 1 ml of plasma) and extracted with Sep-Pak C-18 cartridges (Millipore-Waters). The cartridge had been previously moistened by passing 5 ml of 80% acetonitrile containing 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich) followed by 5 ml of 0.1% TFA. The C-18 cartridge containing absorbed ANF peptide was washed with 20 ml of 0.1% TFA, and subsequently eluted with 3 ml of 80% acetonitrile containing 0.1% TFA. Then, the eluate was lyophilized via rapid freeze drying, dissolved in 100 µl of 20 mM HCl and 900 µl of RIA buffer, and centrifuged for 15 min at 1000 g. 100 µl of the supernatant was then subjected to RIA.

7. Antibodies used for immunocytochemistry

Affinity purified rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. The $G_{\alpha i1}$ antibody (sc-391) was raised against a peptide mapping within a highly divergent domain of $G_{\alpha i1}$ of rat origin. sc-391 was described as reacting with $G_{\alpha i-1}$ and to a lesser degree, $G_{\alpha i-2}$ and $G_{\alpha i-3}$ of mouse, rat, and human origins; non cross-reactive with other G_{α} subunit proteins. The $G_{\alpha i2}$ antibody (sc-7276) mapped within a highly divergent domain of $G_{\alpha i2}$ of human origin. sc-7276 was described as reacting with $G_{\alpha i-2}$ of mouse, rat and

human origin; non cross-reactive with $G_{\alpha i1}$ or $G_{\alpha i3}$. The $G_{\alpha i3}$ antibody (sc-262) was raised against a peptide mapping at the carboxy terminus of $G_{\alpha i-3}$ of rat origin. sc-262 was described as reacting with $G_{\alpha i1}$, $G_{\alpha i2}$ and $G_{\alpha i3}$ of mouse, rat and human origin; non cross-reactive with other G_{α} subunit proteins. A polyclonal rabbit antibody (551) against purified $G_{o\alpha}$ from bovine brain was purchased from MBL International Corporation. This antibody showed cross-reactivity with rat and human $G_{o\alpha}$ and did not react with $G_{\alpha i2}$ and $G_{\alpha i3}$ as stated by MBL. Double labelling with ANF antibody (Chemtech) was performed.

8. Immunofluorescence labelling

All the secondary antibodies, immunological reagents, and fluorescent conjugates used in the labelling procedure were purchased from Vector Laboratories (Burmingham, CA). Atrial and ventricular tissue sections (4μm) from rat hearts fixed in 4% paraformaldehyde tissue buffer and embedded in paraffin, were mounted on positively charged glass slides. The tissue sections were deparaffinized and hydrated through toluene and graded ethanol series immediately before staining, by double labelling procedures. Sections were washed for 5 minutes in tap water, and incubated in 3% hydrogen peroxide to quench endogenous peroxidase activity. Then, sections were incubated for 20 minutes with diluted normal blocking serum prepared from goat. The excess serum was blotted off and each section was incubated with one of the $G_{i\alpha 1-3}$ antibodies (1:50) or the $G_{o\alpha}$ antibody (1:200) for 30 minutes. The immunostaining was performed using the avidin-biotin technique (Vector

Laboratories, Burlingame, CA). Sections were washed in phosphate buffer (10mM, pH = 7.4) between each step of the staining procedure and incubated for 30 minutes with diluted biotinylated goat anti-rabbit antibody solution. Then, sections were incubated with Fluoreiscein Avidin DCS (1:200) to visualize the GTP binding proteins. The sections were then incubated for 30 minutes with biotin from an avidin blocking kit to block the unbound avidin sites. Subsequently, double labeling was performed with the ANF antibody (1:1000) in accordance with the same protocol described above but using Texas Red Avidin DCS (1:200). After undergoing rehydration in a graded alcohol series and toluene, the sections were mounted in VECTASHIELD mounting medium. Finally, the mounted sections were examined using a Bio-Rad 1024 confocal microscope. The green channel had an excitation of 488nm and an emission of 525 nm. The red channel had an excitation of 594 nm and an emission 620 nm. Lack of cross-talk between the channels was established. The controls for the immunohistochemical reaction included the omission of the primary antibody, and the use of blocking peptides for primary antibody competition studies.

9. Statistical analysis

All data are reported as mean ± standard error of the means (SEM). The Unpaired Student's t test was performed to determine statistical significance between mean pairs. Two-way analysis of variance (ANOVA) was performed to determine statistical significance among multiple groups. The statistical package used was Graph Pad PRISM 3.0.

4. RESULTS

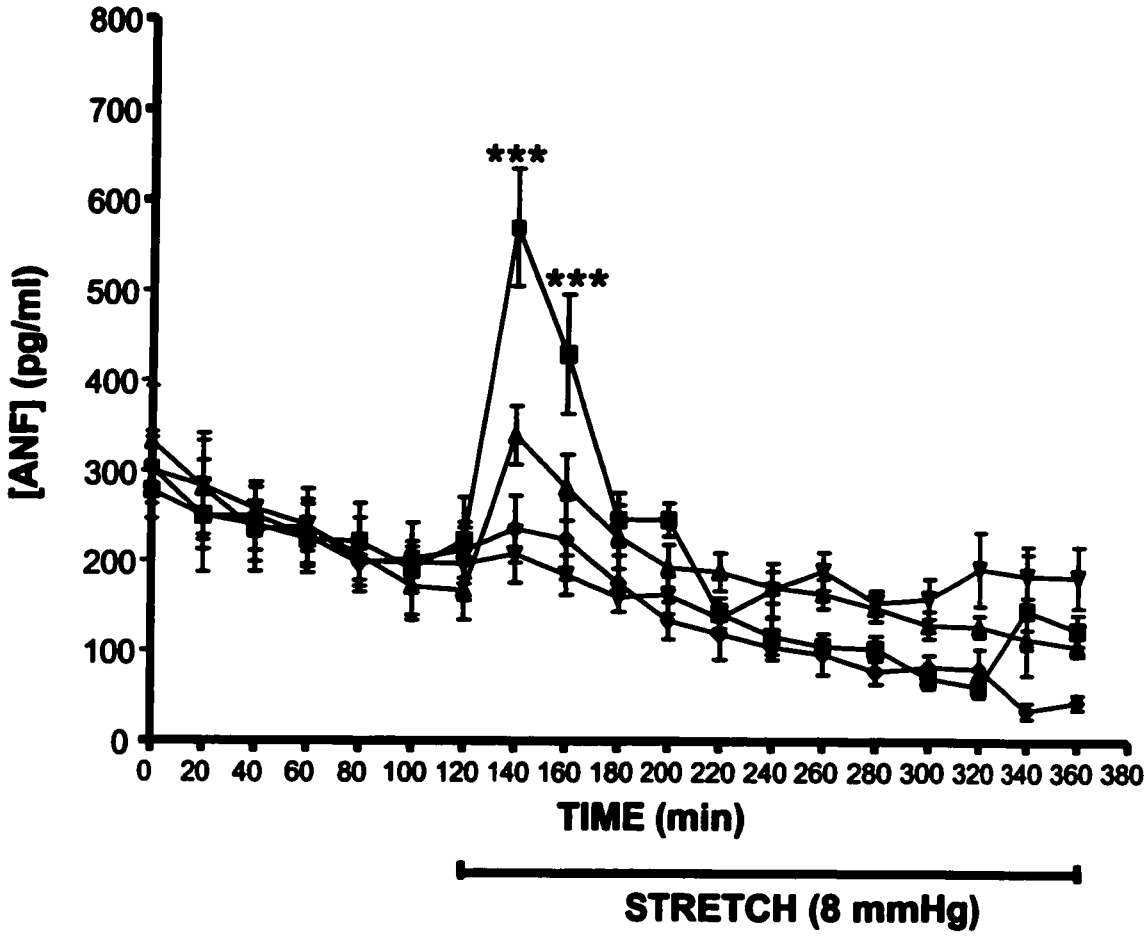
1. *Stretch significantly increases irANF secretion*

Atrial stretch (induced by increasing intra-atrial pressure from 0.5 to 8 mmHg) induced an immediate increase in ANF secretion, and started to decrease within 20 min (Figure 7). Stretch-induced ANF secretion returned to basal levels 1 h following induction of stretch, although mechanical stimulation continued until the end of the experiment. Two-way ANOVA analysis showed a statistically significant difference between the means (at 140 min) of the stretch (n = 5) experiment versus the baseline (N = 5) at 140 min. (570.9±65.2 pg/ml vs. 208±32 pg/ml respectively; $P<0.001$) and 160 min. (430.9±66.1 vs. 184.9±21.9 pg/ml respectively; $P<0.001$).

2. *Pertussis Toxin eliminates stretch stimulated irANF secretion in vitro*

Pretreatment of animals with PTX (25 μg/kg intra-peritoneal (ip), n = 5) significantly decreased the stretch-stimulated ANF secretory peak (at 140 min; from 570.9 ± 65.2 pg/ml to 340.5±32.5 pg/ml; $P<0.001$) (Figure 7). Pretreatment with 40 μg/kg ip (n=5) completely eliminated the stretch-stimulated ANF secretion (Figure 7).

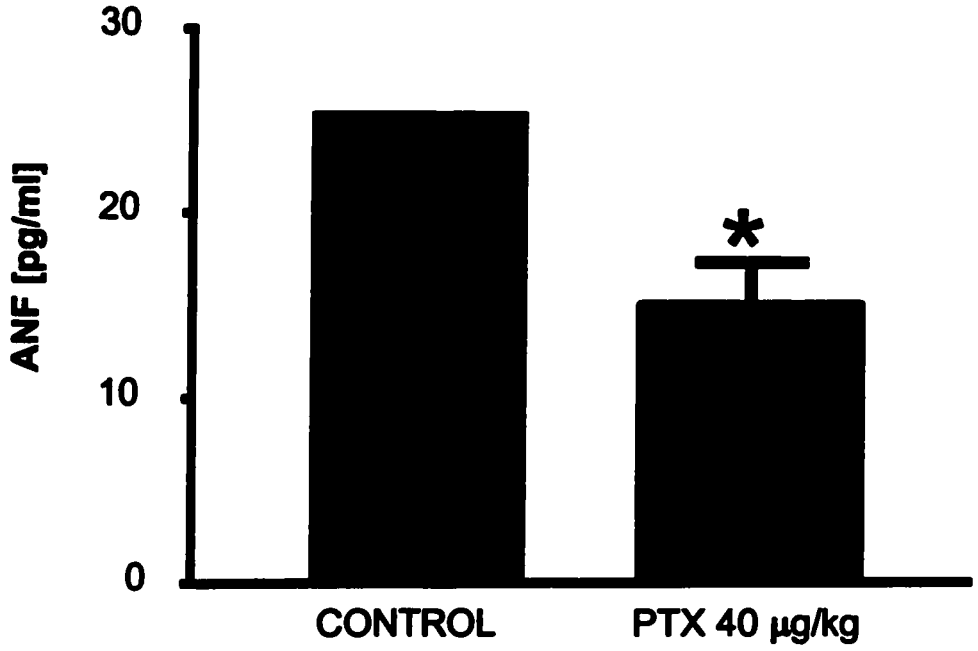
Figure 7: Effect of PTX on stretch-stimulated ANF secretion. The isolated atria were perfused with Krebb's solution in four experimental groups: 1) baseline (▼) 2) stretch (■) 3) stretch + PTX (▲; 25 μg/kg ip) 4) stretch + PTX (♦; 40 μg/kg ip). Intra-atrial pressure was changed from 0.5 mmHg to 8 mmHg (stretch) at 120 min as indicated by arrow. Values are means ± SEM; n=5 respectively. *** $P<0.001$ (stretch vs. stretch + PTX by ANOVA)


800
700
600
500
400
300
200
100
0
[ANF] (pg/ml)
***
***
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380
TIME (min)
STRETCH (8 mmHg)

3. *Pertussis Toxin blocks ANF secretion in vivo*

Plasma ANF concentration was significantly decreased (25.7 ± 1.6 to 15.2 ± 2.6 pg/ml; $P<0.05$) in PTX (40 μg/kg ip, n = 6) treated animals compared to basal ANF levels in untreated animals injected with saline (n = 6) (Figure 8). The animals displayed no adverse effects during the 48 h prior to sacrifice.

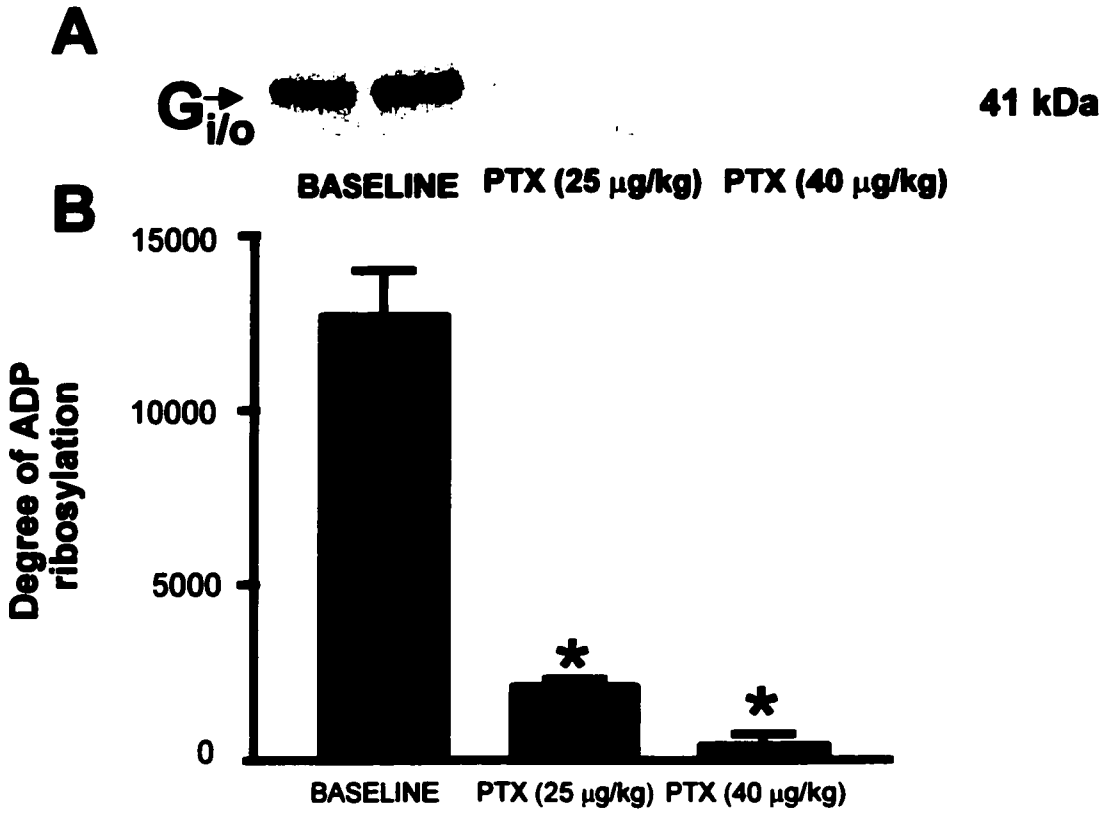
Figure 8: *In vivo* effect of PTX on plasma ANF concentration. Blood was collected from two groups of Sprague-Dawley rats: 1) Control animals injected with 0.9% saline 2) treated animals injected with PTX (40 μg/kg ip). Injections were given 48 h prior to sacrifice. Values are means ± SEM; n=6 per group. * $P<0.05$ (PTX vs. Control)


30
20
10
0
ANF [pg/ml]
*
CONTROL
PTX 40 µg/kg

4. Degree of ADP-ribosylation by PTX

To establish the extent of $G_{i/o}$-binding protein within rat atrial tissue that was inactivated via ADP-ribosylation by PTX *in vivo*, an ADP ribosylation assay was utilized. The autoradiogram of the pretreated hearts showed weak bands for the 25 μg/kg treated samples and no bands for the 40 μg/kg treated samples (Figure 9A) indicating that no further ribosylation could take place *in vitro* and that the degree of $G_{i/o}$ binding protein subunit ribosylation was dose dependent (Figure 9B). Two-way ANOVA analysis of the degree of ribosylation showed a statistically significant difference between the untreated and pretreated animals ($P<0.05$). Broad Range prestained SDS-PAGE standards confirmed that the $G_{i/o}$ proteins electrophoresed to their known molecular weight of approximately 39-41kDa.

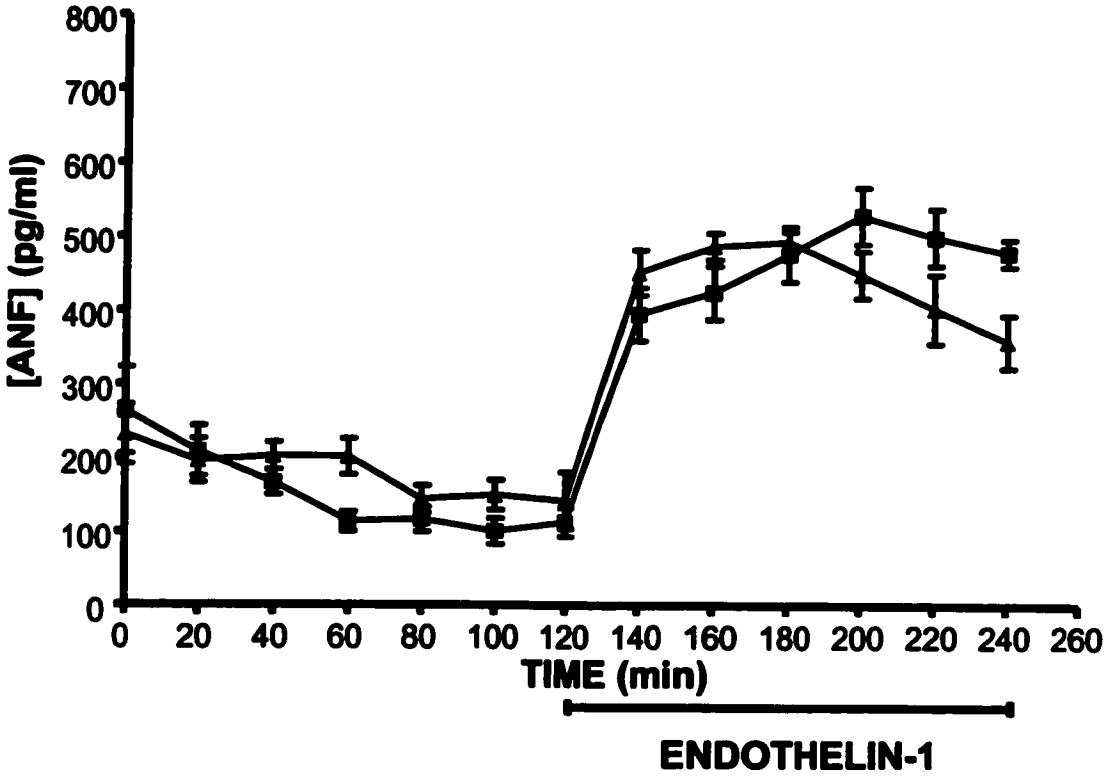
Figure 9: PTX blocks the $G_{i/o}$ proteins in vivo. A) The autoradiogram displays the PTX-catalysed incorporation of [^{32}P] ADP-ribose from [^{32}P] NAD into atria homogenate from un- and PTX- treated (48 h before assay) Sprague Dawley rats. PTX ADP-ribosylated $G_{i/o}$ proteins had a relative molecular weight of 39-41 kDa. B) The intensity of the radioactivity in each lane was quantified and averaged for each group. Values are means ± SEM. * $P<0.05$ (PTX vs. Baseline)


A
$G_{i/o}$
41 kDa
B
BASELINE
PTX (25 μg/kg)
PTX (40 μg/kg)
15000
10000
5000
0
Degree of ADP ribosylation
*
*
BASELINE
PTX (25 μg/kg)
PTX (40 μg/kg)

5. *Endothelin-1- stimulated ANF secretion is not affected by PTX*

PTX (40 μg/kg ip 48 hours prior to perfusion) treated right atrial appendages were perfused with endothelin-1 (ET-1 = 10^{-8} M) for 2 h. The release of ANF was stimulated throughout the infusion of ET-1. ET-1 stimulated ANF release similarly in PTX treated or untreated rats (Figure 10).

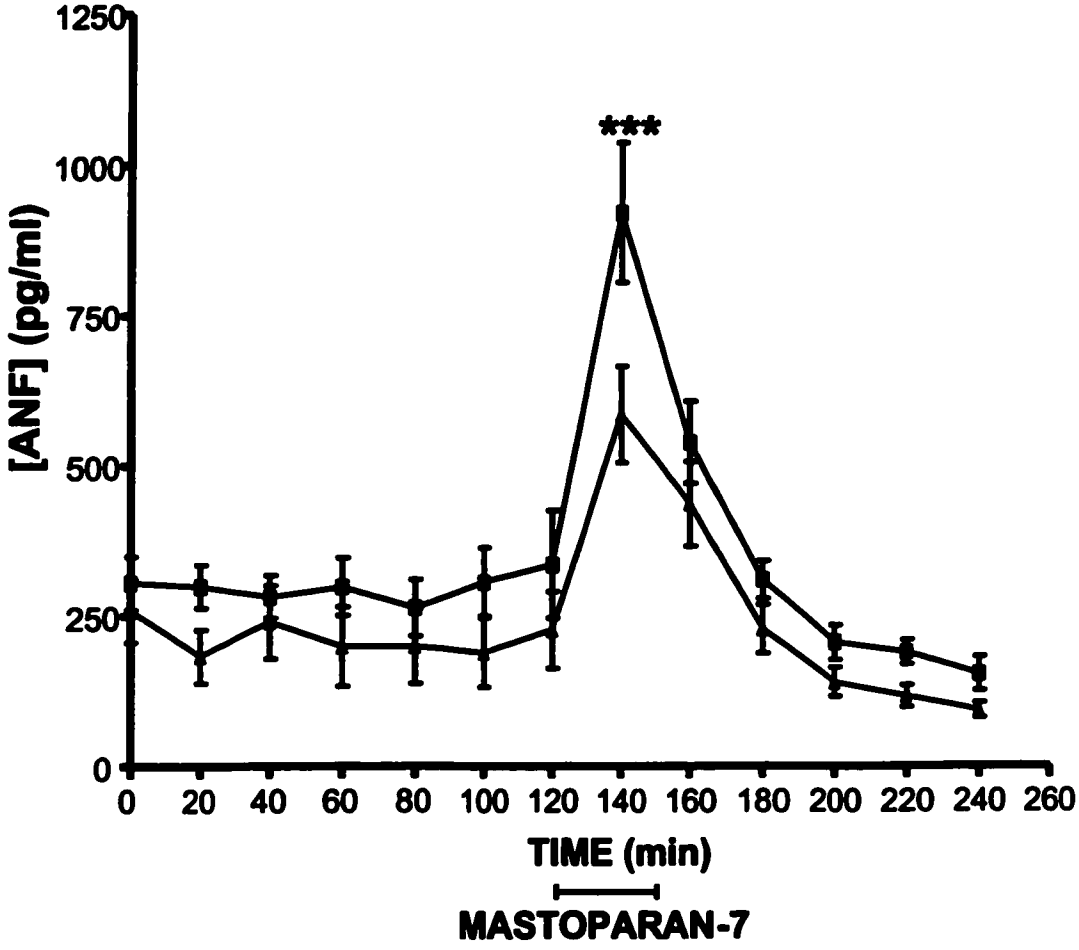
Figure 10: Effect of PTX on ET-1 stimulated ANF secretion. ET-1 (10^{-8}M) was perfused (0.34 ml/min) for 2 h starting at 120 min in 2 groups of rats: 1) untreated (■) 2) PTX-treated (▲; 40 μg/kg ip 48 h before perfusion). Values are means ± SEM; n = 4 per group; no statistical difference between each time point.


800
700
600
500
400
300
200
100
0
[ANF] (pg/ml)
0 20 40 60 80 100 120 140 160 180 200 220 240 260
TIME (min)
ENDOTHELIN-1

6. *Mastoparan-7 Toxin significantly increases irANF secretion*

MAS-7 (10^{-5} M) perfused for 30 min potently stimulated ANF secretion (Figure 11). ANF secretion returned to basal levels after MAS-7 infusion was stopped. PTX pretreatment (40 μg/kg ip) attenuated the MAS-7 stimulated ANF secretion ($P<0.001$; $n = 5$) (Figure 11). Lower doses of MAS-7 (10^{-6} M, 10^{-8}M; $n = 5$ respectively) did not stimulate ANF secretion.

Figure 11: Effect of PTX on MAS-7 stimulated ANF secretion. MAS-7 (10^{-5}M) was perfused (0.34 ml/min) for 30 min starting at 120 min in 2 groups of rats: 1) untreated (■) 2) PTX-treated (▲; 40 μg/kg ip 48 h before perfusion). Values are means ± SEM; n = 5 per group. *** P<0.001 (MAS-7 vs. MAS-7 + PTX by T-test)

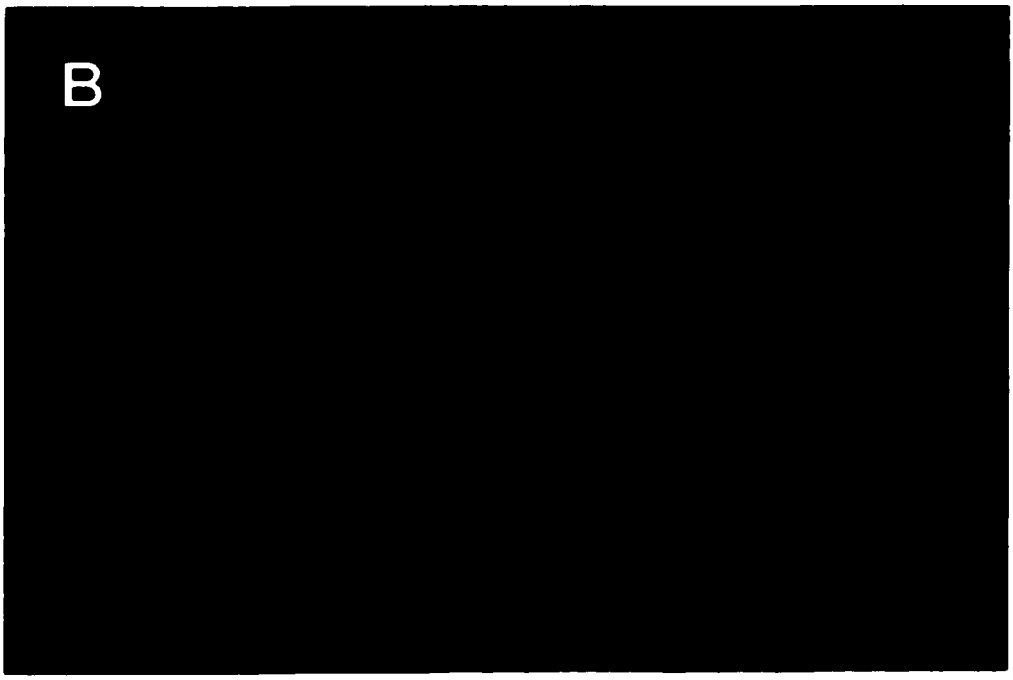

1250
1000
750
500
250
0
[ANF] (pg/ml)
***
0
20
40
60
80
100
120
140
160
180
200
220
240
260
TIME (min)
MASTOPARAN-7

7. *Immunofluorescence labelling shows colocalization of $G_{o\alpha}$ and ANF in atrial tissue*

By immunofluorescence labelling and confocal microscopy, it was demonstrated that $G_{o\alpha}$ colocalized with ANF (Figure 12D, 12E). The atrial sections were labeled with anti-$G_{o\alpha}$ and ANF antibodies, which were then complexed with biotinylated secondary antibodies. This complex was visualized by treatment with Fluorescein Avidin DCS (G proteins) or Texas Red Avidin DCS (ANF). $G_{o\alpha}$-immunoreactivity appears to have a concentrated staining pattern consistent with the sarcolemma and ANF granules within the perinuclear region (Figure 12F, 12G). At higher magnification (100X), a distinct colocalization pattern was observed for ANF and $G_{o\alpha}$ proteins (Figure 12 G, H, I). Colocalization was established by the appearance of a yellow color (resulting from the overlap of the green and red channels). Not all $G_{o\alpha}$ proteins, however, colocalized with ANF visualized by the remaining red staining (12 E, F). Specificity of the $G_{o\alpha}$ -antibody was confirmed with control paraffin sections of Langerhans' islet (Figure 12D), staining with the same pattern as Asano *et al* (1988). Other members of the $G_{i/o}$ family were tested, such as G_{i1} (Figure 12A), G_{i2} (Figure 12B), and G_{i3} (Figure 12C); however, no colocalization with ANF was found.

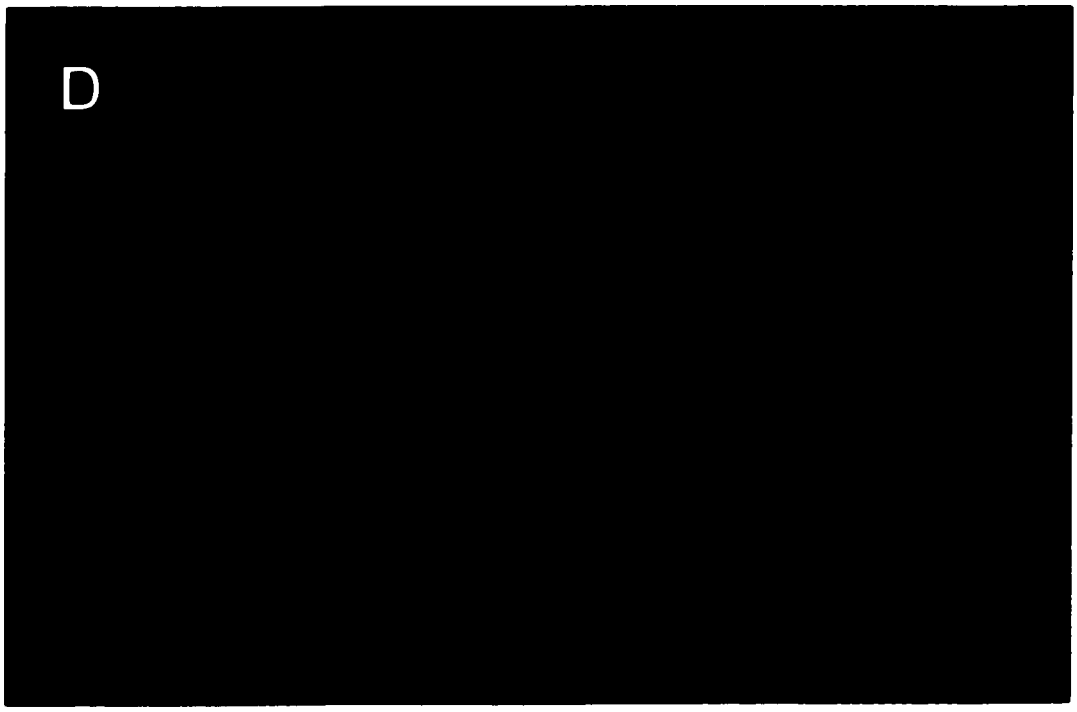
Figure 12: Immunohistochemistry of rat atria. Sections of atria were fixed with 4% paraformaldehyde tissue buffer and incubated with $G_{i/o}$ protein (green) and ANF (red) antibodies. Tissue-bound antibodies were visualized by fluorochrome-conjugated secondary antibodies using confocal microscopy. Intense labelling was seen for all the $G_{i/o}$ proteins of the sectioned cardiocytes of the atria. ANF labelling had a perinuclear distribution. No colocalization with ANF was observed for (A) G_{i1} (B) G_{i2} and (C) G_{i3}. (E, F, I) G_o proteins colocalized with ANF (yellow colour). (D) Control paraffin sections of Langerhans' islet stained with anti-$G_{o\alpha}$ antibody. Bars: A-E,I = 50 μm.

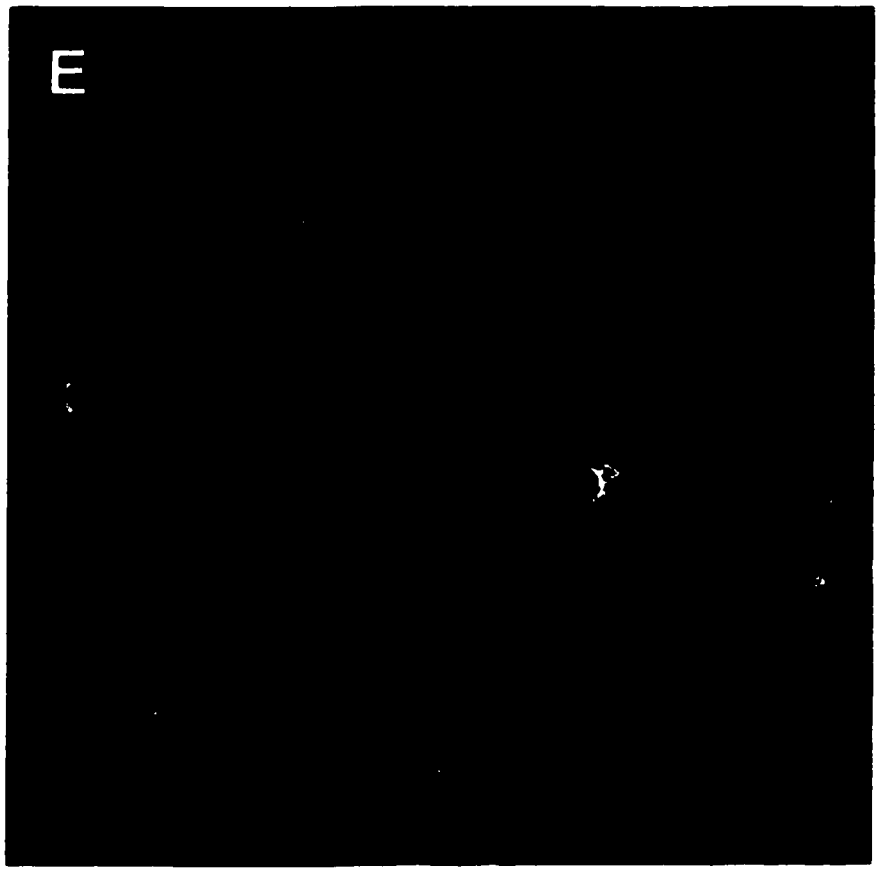
G_{i1}



G_{i2}

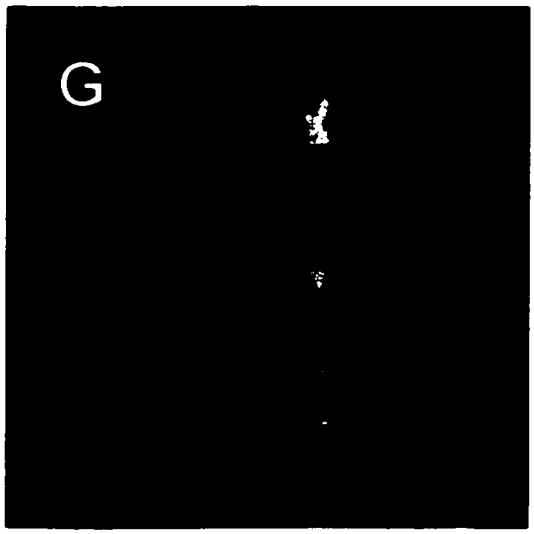

C
G_{i3}


D
G_o

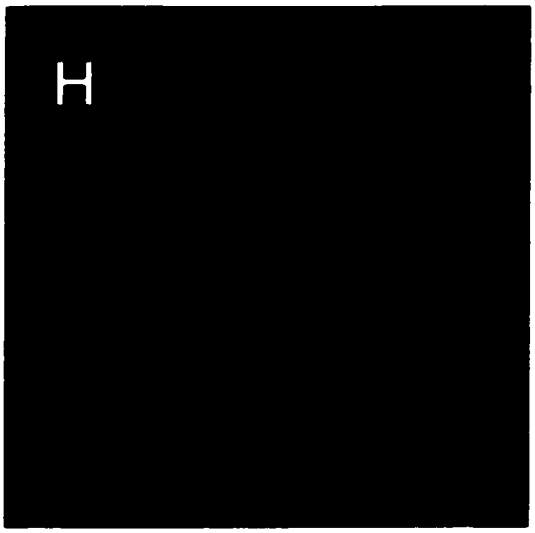


G_o

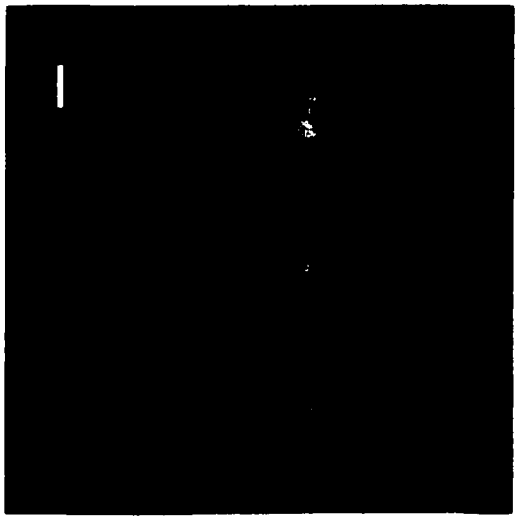
G_o



ANF



$G_{o\alpha}$

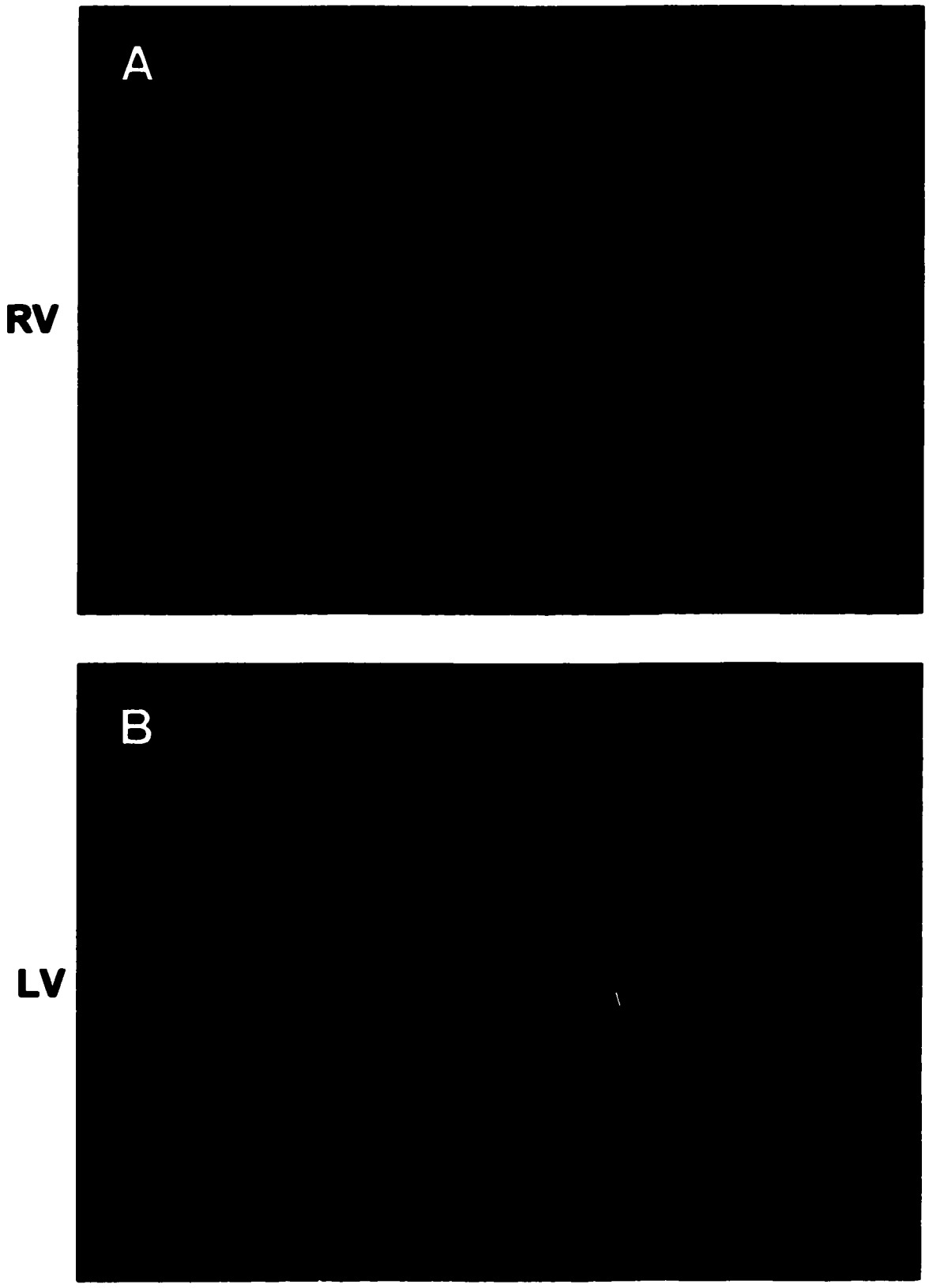


ANF + $G_{o\alpha}$

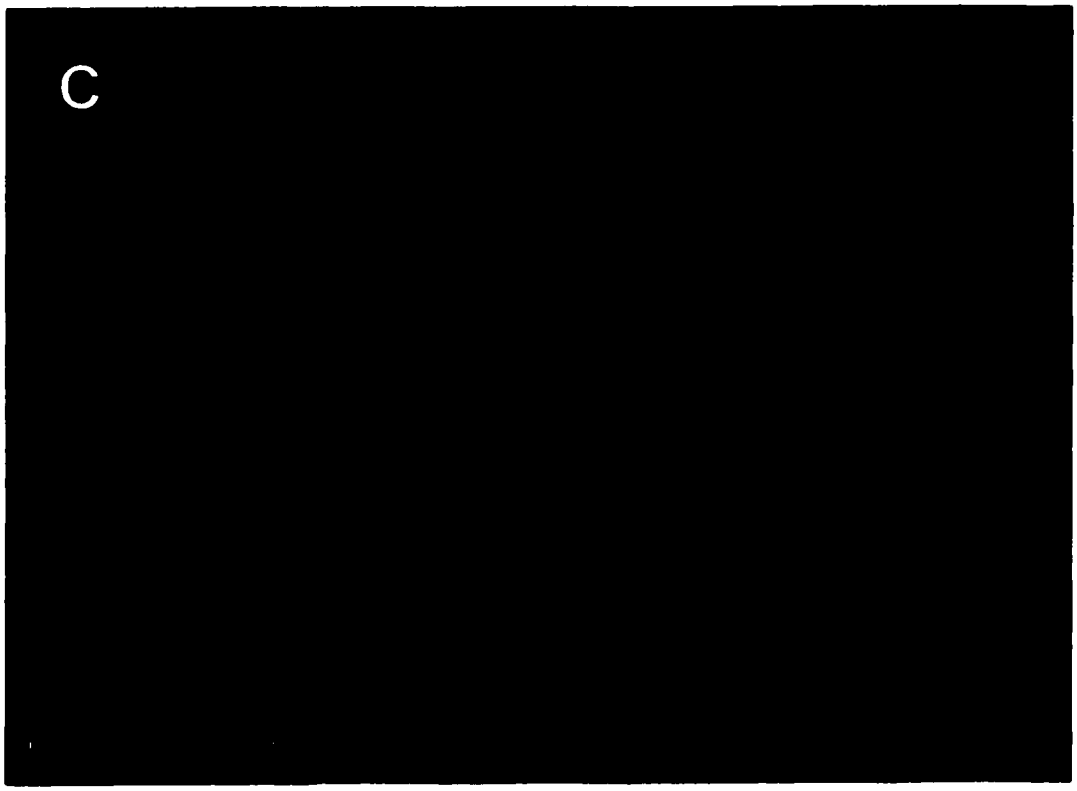
8. *Immunofluorescence labelling of inhibitory G proteins in rat ventricular tissue*

Ventricular tissue was treated with the same G protein and ANF antibodies as the atrial tissue. There was a lack of ANF immunoreativity in the ventricles (Figure 13). The ventricular cardiocytes displayed different G protein staining patterns in the right ventricle compared to the left ventricle. The right ventricle had a striated pattern of labelling, whereas the left ventricle had a staining pattern consistent with that of intercalated discs. The $G_{o\alpha}$-immunoreactivity was concentrated along the sarcolemma, though the labelling in both ventricles was weak compared to that of the atrial cardiocytes (Figure 13A,B). The ventricular cardiocytes showed stronger labelling for the $G_{i\alpha1}$ (Figure 13C,D), $G_{i\alpha2}$ (Figure 13E,F), and $G_{i\alpha3}$ (Figure 13G,H). RV=right ventricle, LV=left ventricle.

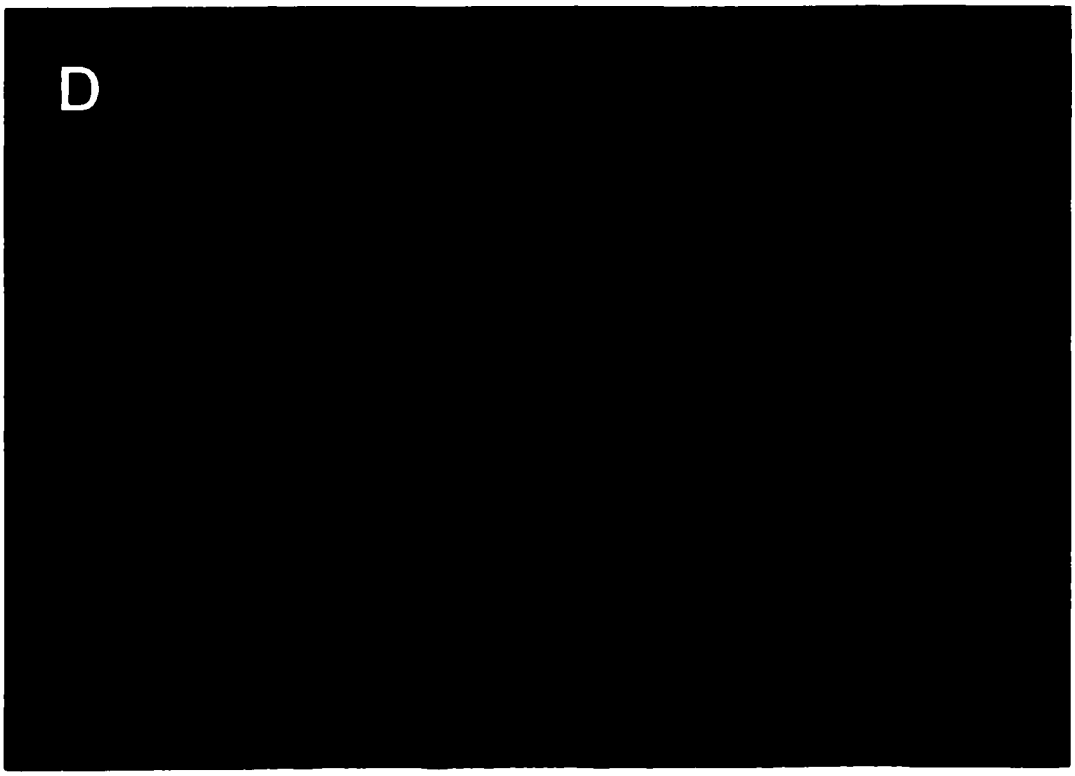
Figure 13: Immunohistochemistry of rat ventricular tissue. Sections of ventricles were fixed with 4% paraformaldehyde tissue buffer and incubated with $G_{i/o}$ protein (green) and ANF antibodies. Tissue-bound antibodies were visualized by fluorochrome-conjugated secondary antibodies using confocal microscopy. Weak labelling was observed for the $G_{\alpha o}$ proteins of the right (A) and left (B) ventricular sections. Ventricular cardiocytes (RV, LV) labeled intensively for $G_{i\alpha 1}$ (C, D), $G_{i\alpha 2}$ (E, F), and $G_{i\alpha 3}$ (G, H). Bars: A-H = 50 μm.


G_o
A
RV
B
LV

G_{i1}



RV

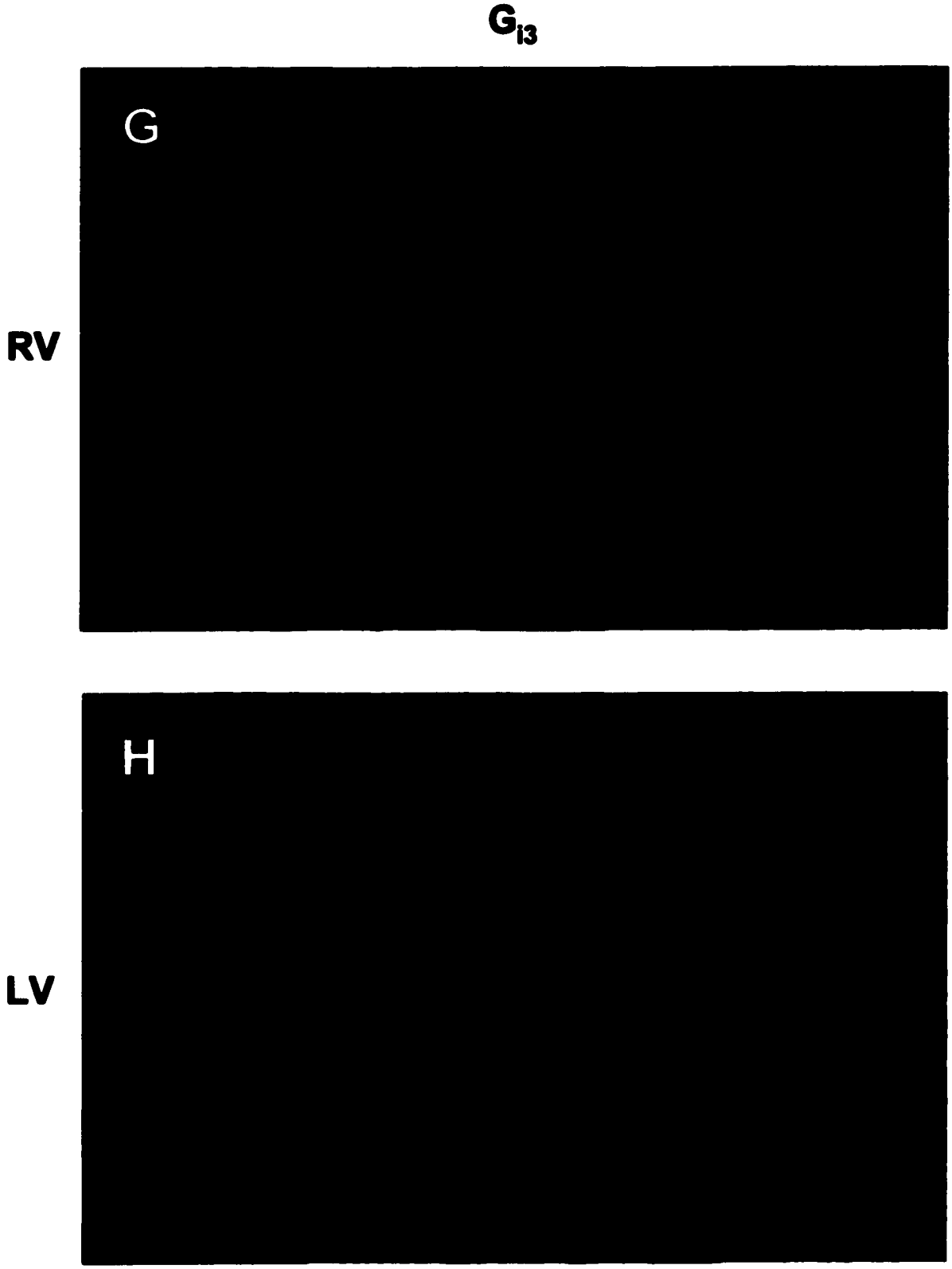


LV

G_{i2}

RV

LV


G_{i3}
G
RV
H
LV

5. DISCUSSION

This study provided important insight into the mechanisms that control ANF release in the adult rat atria. Such mechanisms were observed by directly activating/inhibiting heterotrimeric G proteins in adult atria stimulated by mechanical stretch. The involvement of $G_{i/o}$ proteins was established in stretch-stimulated ANF secretion by utilizing PTX, a pharmacological agent recognized for its ability to inhibit these proteins (Ui et al, 1985). Conversely, the activation of $G_{i/o}$ proteins by MAS-7, potently stimulated ANF release which was attenuated by PTX. Immunofluorescent labelling confirmed the colocalization of $G_{\alpha o}$ proteins with ANF, providing insight into the interactions of G proteins and the hormone they regulate. Furthermore, the present experiments showed that ANF secretion stimulated by ET-1 was insensitive to $G_{i/o}$ protein inhibition.

1. Association of stretch and ANF secretion

Stretch-secretion coupling was coined to describe the process whereby mechanical stretch is coupled to ANF release via an unknown signaling mechanism (Kuroski-de Bold and de Bold, 1991). Using double pulse chase analysis, Mangat and de Bold (1993) showed that stretch-stimulated ANF release was associated with the rapid depletion of an immediately releasable pool of newly formed granules. In the present experiments, inhibiting $G_{i/o}$ proteins exclusively effected stretch-stimulated ANF secretion and not basal

release. This observation suggests that $G_{i/o}$ proteins regulate the secretion of newly synthesized ANF granules that are acutely sensitive to stretch.

Numerous hypotheses have evolved addressing how mechanical stretch is converted into biochemical signals (Sadoshima and Izumo, 1997; Dostal and Baker, 1998). For example, stretch-activated ion channels have been proposed as potential mediators of cardiac hormone release (Sadoshima et al, 1997). In addition, mechanical stress-induced signal transduction has been shown to simultaneously trigger several second messenger systems (Sadoshima and Izumo, 1997). Stretch experiments performed in avian skeletal muscle (Vanderburgh et al, 1995), and cardiac fibroblasts (Gudi et al, 1998), have shown $G_{i/o}$ proteins are involved in the stretch signaling pathways. In the present experiments, the inhibition of stretch-stimulated ANF secretion by PTX provided strong evidence for the involvement of $G_{i/o}$ heterotrimeric proteins in regulated hormone secretion (Figure 7). The role of $G_{i/o}$ proteins in hormone secretion was strengthened by immunohistochemical studies demonstrating the relationship between G proteins and ANF secretory granules (Figure 12).

a. *Two pools of secretory granules hypothesis: stretch vs. stretch-insensitive*

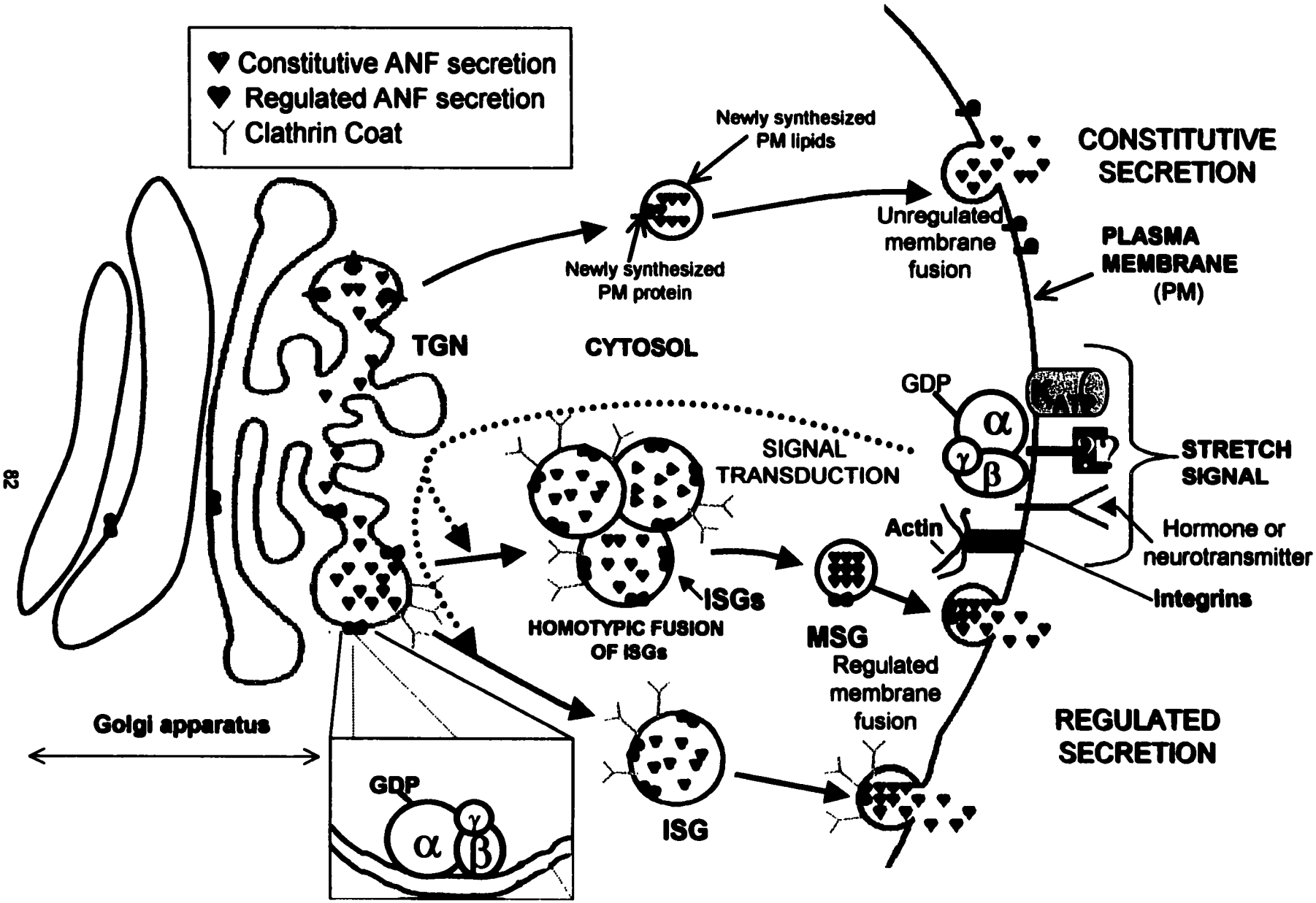
The association of G proteins with secretory granules is well established (Ahnert-Hilger, 1994; Vitale et al, 1997; Konrad et al, 1995; Ohnishi et al, 1997; Padfield and Panesar, 1997). Wolf et al (1998) provided immunohistochemical and immunoelectron microscopy evidence suggesting that $G_{o\alpha}$ regulate ANF

secretion from atrial granules; such was confirmed in this study (Figure 12H-J) using the $G_{o\alpha}$ antibody created by Asano et al (1988). Colocalization of $G_{o\alpha}$ proteins and ANF at the TGN further supported the hypothesis that ISGs are regulated by the inhibitory G proteins. PTX treatment did not eliminate ANF secretion, suggesting that different subpopulations of secretory granules exist. The two pools of secretory hypothesis suggests: 1) stretch activated a population of secretory granules sensitive to PTX; 2) a second class of PTX-insensitive secretory granules mediate the basal release of ANF. (Figure 14).

b. *Effect of stretch on secretory granules*

In vitro experiments by Agnoletti *et al* (1989) studied the effect of stretch on SGs in isolated superfused rat atria followed by morphometric assessment via electron microscopy. It was found that the overall number of SGs did not change significantly with stretch. However, a substantial change in granule distribution was observed (Agnoletti *et al*, 1989). Stretching of the atrial cardiocytes led to the movement of what the author described as 'atrial specific granules' from the interior to the periphery of the cell. The location of these SGs was refined with an *in situ* tannic acid perfusion technique that arrested exocytosis in anaesthetized rats, and showed atrial SGs fusing with the plasma membrane (Newman and Severs, 1990, 1993). Caution must be taken in interpreting these results because the movement of SGs, seen from the center of the cell to the periphery in the Agnoletti study, describe a cell where the Golgi network is

Figure 14: Constitutive vs. regulated ANF secretion. A schematic representation of the formation SGs. Formation of the nascent storage granule starts as an outpouching of the TGN generating a condensing vacuole, which is at first continuous with the TGN's tubular network. Initially, immature secretory granules (ISGs) are formed by the envelopment of a dense core of secretory proteins. ISGs are converted to mature secretory granules (MSGs) via many subsequent changes. Alternatively, clathrin-coated ISGs may directly bind with the plasma membrane (constitutive-like secretion). The final step of ANF exocytosis, involves fusion of the secretory granules with the plasma membrane (PM). Newly synthesized PM proteins are delivered to the PM via the constitutive pathway. Distinct features of ISGs are the presence of a clathrin coat (orange). Inhibitory G proteins are present in the golgi, the regulated SGs, and the plasma membrane. PTX (yellow) may inhibit G proteins within all these compartments. Mastoparan-7 (MAS-7, pink) is an agonist of $G_{i/o}$ proteins. Stretch may be activating: 1) an unknown receptor (navy blue); 2) receptor coupled to K^{+}_{ATP} channels (purple); 3) a known cardiac receptor stimulated by hormones or neurotransmitters (baby blue) 4) integrins coupled to the actin (brown) cytoskeleton. Signal transduction occurs via the molecular switches: GTP-binding proteins. Red hearts in the regulated pathway, and blue hearts in the constitutive pathway represent ANF.


Constitutive ANF secretion
Regulated ANF secretion
Clathrin Coat
Newly synthesized PM lipids
Newly synthesized PM protein
CONSTITUTIVE SECRETION
Unregulated membrane fusion
PLASMA MEMBRANE (PM)
TGN
CYTOSOL
GDP
SIGNAL TRANSDUCTION
STRETCH SIGNAL
Actin
Hormone or neurotransmitter
Integrins
ISGs
HOMOTYPIC FUSION OF ISGs
MSG
Regulated membrane fusion
REGULATED SECRETION
Golgi apparatus
GDP
ISG
82

centrally located within the cell. In cardiocytes, the Golgi network is distributed throughout the cytosol.

2. The cellular effect of PTX

PTX has been characterized as a toxin that inhibits G proteins localized on the inner surface of the plasma membrane (Krueger and Barbieri, 1995; Ui *et al*, 1985). The second PTX substrate in eukaryotic cells is NAD, which has also been found in the cytoplasm. Therefore, Kaslow and Burns (1992) have suggested that the S1 subunit of PTX likely crosses the sarcolemma of the cell, in order to gain access to its substrates. This raises the intriguing possibility that PTX is directly acting on the G proteins found in SGs in the cytoplasm. Conversely, PTX may be interacting with SGs fused to the plasma membrane prior to exocytosis.

a. *Entry of PTX into cells*

The endocytotic uptake of PTX in CHO cells has been characterized by EM, fractionation, and inhibition studies. el Bayâ *et al* (1997) showed that PTX was routed to the Golgi apparatus in an enzymatically active form following receptor-mediated endocytosis via a retrograde transport mechanism. In that experiment, the involvement of the Golgi network in PTX-induced G protein ADP-ribosylation was demonstrated by various means. Subcellular fractionation of CHO cells incubated with PTX, showed that the active form of PTX was detected in Golgi fractions (el Bayâ *et al*, 1997). As shown by Xu and Barbieri

(1995), pretreatment of HIT-T15 (insulin-secreting β cells derived from hamster pancreas) and CHO cells with BFA, inhibited ADP-ribosylation by PTX (el Bayâ *et al*, 1997). Since BFA has been reported to have other effects such as influencing endocytotic processes (Hunziker *et al*, 1991; Stoorvogel *et al*, 1996), the investigators wanted to show that the protective effect of BFA against PTX intoxication, depended on disorganization of the Golgi network. Hence, the same experiments were repeated with Madin Darby Canine Kidney epithelial (MDCK) cells, which have a Golgi network insensitive to BFA (Hunzike *et al*, 1991). The inability to block the ADP-ribosylation of the G-proteins in MDCK cells with BFA confirmed the involvement of the Golgi network in this process. Additional support for this conclusion was demonstrated with a mutant CHO cell line (V24.1) exhibiting a temperature-sensitive Golgi complex (Kao and Draper, 1992). In the V24.1 cell line, elevated temperatures (e.g. 41 °C) disrupted the Golgi apparatus and abolished the ability of PTX to inhibit G proteins (el Bayâ *et al*, 1997).

Taken together, these results demonstrated that PTX 1) is transported into the cell; and 2) require a functional Golgi apparatus to mediate its inhibitory effects. They also suggested that the G proteins affected by PTX treatment in the present study were the G proteins localized in the Golgi network. This further supports the possibility that the inhibitory G proteins localized in the TGN (Leyte *et al*, 1992; Maier *et al*, 1995; Barr *et al*, 1991) and SGs (Figure 12, Wolf *et al*, 1998; Toutant *et al*, 1987; Ahnert-Hilger *et al*, 1994; Muller *et al*, 1994; Vitale *et*

al, 1997; Padfield and Panesar, 1997) regulate granule formation, maturation, and ultimately the exocytosis of ANF. However, the endocytotic model proposed by Xu and Barbieri (1995) also suggested that the internalized S1 subunit was retransported to the G proteins in the plasma membrane following Golgi passage; a hypothesis which could not be confirmed or refuted by the el Bayâ *et al* (1997) study.

3. Evidence for the association of G proteins and hormone secretion

The release of ANF results from the contribution of many players in the process of exocytosis, some of which are regulated by G proteins. $G_{\alpha o}$ proteins have been located on intracellular membranes and are most likely implicated in secretion and vesicular protein transport (Helms, 1995; Numberg & Ahnert-Hilger, 1996). While $G_{\alpha o}$ protein is not present in the human ventricular myocardium, small amounts of $G_{\alpha o}$ have been detected in the human left atrium. Two isoforms $G_{o1\alpha}$ & $G_{o2\alpha}$ have been identified in rat atria, with $G_{o2\alpha}$ being the primary isoform expressed in the ventricles (Asano et al, 1992). Other studies have demonstrated that $G_{\alpha o}$ is expressed in the heart and other peripheral tissues (Mumby et al, 2002; Asano et al, 1988). This protein seems to be specifically concentrated within the atria (Luetje et al, 1988; Asano et al, 1988). $G_{\alpha o}$ appears to generate a direct inhibitory effect on regulated exocytosis (Ohara-imaizumi et al, 1992; Vitale et al, 1996). Though the signaling pathways governing hormone release are complex, the implications for the regulation of

ANF secretion by G proteins at the various intracellular compartments of the cell, are discussed in the following sections.

a. *TGN*

The presence of heterotrimeric G proteins within the TGN provided insight into the possible sorting mechanisms that regulate the packaging of regulated secretory proteins. Leyte *et al* (1992) show that in a cell-free system, stimulated inhibitory G proteins within the TGN, inhibit the formation of CSVs and ISGs. Moreover, the 'sorting-for-entry' hypothesis (Arvan and Castle, 1998; Tooze, 1998) argues that the TGN acts as the principal operator for protein sorting in the biosynthetic transport pathway. This model proposes that the formation of regulated SGs within the TGN involves the specific binding of regulated secretory proteins either to a receptor in the nascent granule membrane or other regulated secretory proteins that are already bound (Arvan and Castle, 1998; Tooze, 1998). It is conceivable that the receptor that serves as an anchor for regulated protein aggregation interacts with $G_{\alpha i/\alpha o}$ subunits.

The formation of SGs involves budding from the TGN. Hence, the ISG membrane contains the same proteins as its donor TGN membrane, including heterotrimeric G proteins. The heterotrimeric G proteins found in numerous endocrine cells presumably are from the TGN membrane from which the regulated SGs were formed. Maier *et al* (1995) showed that $G_{\alpha o}$ was found at the Golgi of adrenal medulla. Barr *et al* (1991) have previously demonstrated

that pertussis toxin-sensitive α subunits cofractionate with the TGN of PC12 cells. Fractionation followed by analysis by SDS-PAGE confirmed that $G_{\alpha i2}$, $G_{\alpha o2}$ and $G_{\alpha i3}$ were associated with the TGN in PC12 cells (Leyte *et al*, 1992). The PTX-induced ADP-ribosylated αi/αo subunits found in the TGN-containing fractions of the velocity gradient comigrated with the TGN, and were identified by [^{35}S] sulfate pulse-labeled hsPG (heparan sulfate proteoglycan) and SgII (secretogranin II), following sucrose gradient centrifugation (Leyte *et al*, 1992). The sulfated markers were used by Leyte *et al* (1992) because Tooze and Huttner (1990) showed that, hsPG and SgII, were localized in the TGN, and were then found after chase in constitutive secretory vesicles (CSVs) and ISGs respectively. PTX treatment led to the stimulation of cell-free ISG formation. Thus, these experiments confirmed that inhibitory G proteins on the TGN are involved in the regulation of secretory granules (Leyte *et al*, 1992). In the experiments presented here, the presence of these G proteins would confer their sensitivity to PTX treatment, observed under physical stimulation with stretch. Perhaps the secretory granules that participate in the basal release of ANF are not packaged with these $G_{i/o}$ proteins, which explain why basal release of ANF is insensitive to PTX treatment.

b. *Secretory granules*

$G_{\alpha o}$ has been found in neuronal tissues (Asano *et al*, 1990, 1992), testis (Asano *et al*, 1992), and adipocytes (Asano *et al*, 1992). Subcellular immunofluorecence analysis has localized this G protein specifically within SGs

in the heart (Figure 12; Wolf *et al*, 1998), the brain (Toutant *et al*, 1987; Ahnert-Hilger *et al*, 1994; Muller *et al*, 1994; Vitale *et al*, 1997), and the pancreas (Padfield and Panesar, 1997). Maier *et al* (1995) showed that antibodies against $G_{\alpha o}$ stimulated regulated secretion.

Following the formation of ISGs, they undergo maturation and become MSGs (Arvan and Castle, 1998; Tooze, 1998). Tooze *et al* (1991) has suggested that this process involves the fusion of ISGs to form an MSG, and that this process is regulated by G proteins. Moreover, it has long been suggested that the patches of clathrin identified on the surface of ISGs could be involved in the formation of clathrin-coated vesicles released from the ISG (Urbé *et al*, 1997). In such a model, homotypic fusion of the ISG as seen by EM (Tooze and Stinchcombe, 1992) would produce additional membrane surface area, allowing the formation of clathrin-coated vesicles to bud-off from the ISG (Urbé *et al*, 1997).

In all neuroendocrine and endocrine cells examined to date, a key characteristic of maturation involves the removal of soluble proteins, peptides and membrane proteins from the ISG (Tooze et al, 2001). The 'constitutive-like secretion' describes the process that occurs when soluble peptides and unprocessed hormones are removed from the ISGs and secreted from the cell (Arvan, 1991). Hence, ISG-derived clathrin-coated vesicles (CCVs) have been shown to remove membrane proteins such as mannose-6-phosphate receptors,

furin, and bound lysosomal enzymes from ISGs (Figure 14) (Dittié et al, 1996; Kuliawat et al, 1997). Taken together, it has been suggested that inhibitory G protein may participate in the pathways regulating the formation and maturation of SGs or their fusion with the cell membrane (Tooze, 2001).

c. *Plasma membrane*

Support for the regulation of hormone release by inhibitory G proteins at the plasma membrane, rather than at the Golgi, stems from the interpretation of various studies. Stimulation of inhibitory G proteins was shown to inhibit granule formation at the TGN (Leyte *et al*, 1992), while PTX was able to counteract these effects. Hence, assuming mechanically stimulated ANF release depended on the formation of ISGs, PTX pretreatment would have conceivably promoted stretch stimulated ANF secretion. In the experiments presented here, however, the inhibition of stretch-stimulated ANF secretion by PTX suggests that regulation for stretch secretion coupling occurs at a post TGN stage in exocytosis. However, it does not eliminate the possibility that the G proteins packaged with SGs are participating in this process. For example, regulation may occur at the level of ISGs containing newly synthesized hormone.

During exocytosis, the membranes of secretory granules fuse with the plasma membrane to release their contents (Tooze *et al*, 2001). Apodaca (2002) has suggested that SG membranes are recycled into the plasma membrane. Thus, fusion of their membranes provides another site of action for PTX. The G

proteins found in the granule membrane would avail themselves to ADP ribosylation via PTX. Furthermore, the model proposed by Xu and Barbieri (1995) suggested that the target for the S1 subunit was the membrane-bound cytoplasmic G protein, following passage through the Golgi. These plasma membrane-bound heterotrimeric $G_{i/o}$ proteins interact with ion channels, various cardiac receptors, integrins, and the cytoskeleton. While no specific receptor has been found to exclusively regulate stretch-stimulated ANF secretion in the heart, it is probable that more than one mechanosensor participates in the signaling cascade that operates via the G proteins. In addition, PTX has been shown to interact with a carbohydrate sequence on the plasma membrane of CHO cells (Brennan *et al*, 1988). All in all, these results suggest that the G proteins affected by PTX in the present experiments were located at the plasma membrane.

4. $G_{i/o}$ proteins and the regulation of ANF secretion

The results presented here suggest that stretch activates inhibitory G proteins, which promotes the release of ANF, and that inhibition of these G proteins abolishes the stretch-stimulated ANF response. Thus, a component of the pathway(s) regulating stretch-secretion coupling involves inhibitory G proteins. Integration of the data presented in this thesis and that of studies by others allows for the development of different hypothetical models for the regulation of ANF release following stretch.

a. *K^+_{ATP} channels and the regulation of ANF secretion*

In the heart, discrepancies exist with regards to the pharmacological properties of K^+_{ATP} channels in various experimental models. These channels have been; 1) implicated in the regulation of stimulated-ANF secretion, 2) shown to act as mechanosensors, and 3) shown to interact with GPCR. Both hypoxia and stretch have been shown to stimulate ANF release (de Bold *et al*, 1991; Lew and Baertschi, 1989), and to open cardiac K^+_{ATP} channels (Deutsch *et al*, 1991; Van Wagoner, 1993). Blocking the K^+_{ATP} channels impaired stretch-activated ANF release in isolated atria (Kim SH *et al*, 1997), and in an *in vivo* canine model (Chen *et al*, 2000). Taken as a whole, these results suggest that stretch stimulated opening of the K^+_{ATP} channel, results in ANF secretion. On the other hand, it has been shown that K^+_{ATP} channel blockers: 1) promoted mechanically stimulated ANF release from atrial cell cultures (Jiao *et al*, 2000); and 2) potentiated stretch-induced ANF release in isolated hearts (Xu *et al*, 1996). Since stretch opens these channels and is accompanied by ANF release, it follows that ANF secretion would be inhibited by blocking these channels. Moreover, it is conceivable that opening of the K^+_{ATP} channels by stretch activates the $G_{i/o}$ proteins or that stretch may be activating some unknown receptor coupled to $G_{i/o}$ proteins, stimulating the K^+_{ATP} channel to open. PTX treatment in the present experiments inhibits the ability for the heterotrimeric subunits to dissociate.

b. *cAMP and the regulation of ANF secretion*

cAMP was first identified as an intracellular effector of hormone action in 1959, and has since been implicated in intracellular signaling pathways in prokaryotic and eukaryotic cells. It is produced when a plasma membrane bound adenylyl cyclase (AC) synthesizes cAMP from ATP. Many extracellular signaling molecules control cAMP levels by binding to heterotrimeric GTP-binding coupled receptors which, in turn, alter the activity of AC (Ishikawa and Homcy, 1997). The G protein that participates in AC activation, is referred to as stimulatory G protein (Gs). The best–studied example of receptors coupled to the activation of AC, are the cardiac β-adrenergic receptors, which mediate some of the effects of adrenaline. AC is inhibited when adrenaline binds α_2-adrenergic receptors, which are coupled to inhibitory G proteins.

G_i proteins also regulate AC, which directly affects the formation of cAMP. cAMP exerts its effects in eukaryotic cells, primarily by activating the enzyme cAMP-dependent protein kinase (PKA), which catalyzes the transfer of terminal phosphate group from ATP to specific serines or threonines of various effector proteins. Therefore, the central postulate of cAMP-mediated hormone action is that hormones regulate cAMP production, cAMP stimulates PKA, and PKA regulates cellular substrates by phosphorylation (Steinberg and Brunton, 2001). Taking into consideration the experimental results that support the involvement of $G_{i/o}$ proteins in stretch mediated ANF release, and the regulatory role of $G_{i/o}$ proteins for the inhibition of PKA production, it is conceivable that

PKA is involved in stretch-secretion coupling of ANF. Indeed, compounds that increase the intracellular concentrations of cAMP have been shown to affect the secretion rate of endocrine cells by stimulating PKA (Blackshear *et al*, 1988). Forskolin, a direct activator of AC, has been shown to decrease ANF release from cultured atrial cardiocytes (Iida and Page, 1988; Muir *et al*, 1993; Shields and Glembotski, 1989). In an isolated heart preparation, Ruskoaho *et al* (1990) showed that forskolin decreased stretch-stimulated ANF secretion. Recently, similar effects were found in isolated rabbit atrial preparations; AC activation with forskolin was shown to inhibit the release of ANF (Cui *et al*, 2002). The role of cAMP is controversial though; others showed that cAMP-elevating agents stimulated ANF release from cultured cardiac myocytes (Church *et al*, 1994), and sliced atria (Azizi *et al*, 1995). Nonetheless, it is conceivable that stretch activates $G_{i/o}$ proteins, which in turn inhibit AC; the net result being a permissive effect on the release of ANF. This hypothesis is supported by two recent publications. Stretch of cultured neonatal cardiac fibroblasts was found to stimulate G protein ($G_{\alpha q}$ and $G_{\alpha i1}$) activation within one minute of mechanical stress, the response being regulated by the rate and the degree of stretch (Gudi *et al*, 1998). Furthermore, Tavi *et al* (2000) showed that the concentration of cAMP did not change in stretched rat atria (Tavi *et al*, 2000). Taken together, these studies suggest that G proteins might potentiate ANF release during stretch, by inhibiting cAMP formation.

The study by Tavi *et al* (2000) also highlighted the difference of intracellular signaling in the atria compared to the ventricles. In contrast to the results by Tavi *et al* (2000) in the atria, stretch was shown to change cAMP levels in ventricular tissue such as blood perfused canine heart (Todaka *et al*, 1998), ferret papillary muscle (Calaghan *et al*, 1999), and frog ventricle (Singh, 1982). This suggests that the cAMP in the atrial tissue responds to stretch differently than ventricular tissue.

The G protein-mediated inhibition of cAMP following stretch, suggested in this thesis, provides a mechanistic explanation for the results published by de Bold and de Bold in 1989. They showed that the stretch-secretion coupling of ANF was negatively modulated by calcium (Ca^{2+}). Thus, a decrease in calcium concentration, resulted in an increase in ANF secretion. The involvement of Ca^{2+} in stretch-mediated ANF release has been controversial. In one study, stretch-secretion coupling of ANF secretion in isolated superfused rat atria was dependent on extracellular Ca^{2+} (Laine *et al*, 1996). In contrast, Ca^{2+} was shown to negatively regulate ANF stretch-secretion coupling in perfused hearts (Ito *et al*, 1988; Ruskoaho *et al*, 1990), and in isolated atria (Cui *et al*, 2002). Thus, inhibiting cAMP formation (via Gi/o protein inhibition) benefits ANF secretion by decreasing the production of PKA, which in turn indirectly decreases Ca^{2+} influx. This further suggests that Ca^{2+} is a negative regulator of the intracellular signaling pathways regulating ANF stretch-secretion coupling.

5. Endothelin-1 stimulated ANF secretion

a. *Effect of ET-1*

While stretch is the predominant stimulus for ANF release, Bruneau and de Bold (1994) demonstrated that ET-1 stimulation resulted in increased ANF secretion. In fact it has been shown that ET-1 is among the most potent neuroendocrine factors that stimulate ANF secretion (Bruneau and de Bold, 1994; Ogawa et al, 1999). In contrast to the ANF secretory response of cardiac muscle to stretch, Ogawa et al (1999) utilizing double-label pulse-chase experiments, showed that the secretory response to ET-1 was based on a peptide that was not newly synthesized or, newly stored ANF. This further supports the hypothesis that newly synthesized immature granules participates in the stretch response.

b. *ET-1 receptor blockers*

ET-1 stimulated ANF secretion occurs via binding to a single class of endothelin receptors (ET_A) in neonatal and adult atrial myocytes (Irons et al, 1993;Thibault et al, 1994; Leite et al, 1994), and neonatal rat ventricular myocytes (Hilal-Dandan et al, 1997). ET_A receptor blockade with the antagonist BQ-123 resulted in a decrease in IP_3 formation paralleled with an inhibition of ANF secretion (Irons et al, 1993). In view of the fact that ET_A receptor blockade prevented ANF release, it can be suggested that ET-1 stimulated ANF secretion was regulated at the level of the plasma membrane. Recently, Bianciotti and de Bold (2000) showed that chronic blockade of DOCA-salt-treated rats with the

selective ET-1 type-A receptor antagonist ABT-627, prevented the increase of natriuretic peptide (NP) gene expression in ventricles, but that the atria NP gene expression was unaffected. These results demonstrated two important concepts: 1) that regulation of ANF is tissue specific in the rat heart; and 2) that receptor blockade at the level of the plasma membrane did not inhibit ANF release, suggesting that an alternate regulatory mechanism exists for the release of ANF at the level of the atria.

The cardiac signaling receptors, mediating the effects of endothelin have been reported to couple with both G_q- and G_i-linked pathways (Hilal-Dandan et al, 1992, 1994). In a study conducted by Hilal-Dandan (1997), the partial inhibition of ANF-luciferase construct expression caused by PTX treatment in neonatal ventricular myocytes, stimulated by ET-1, suggested $G_{i/o}$ proteins were components of the signaling pathway activated by ET-1 to stimulate ANF secretion. These experiments, however, were conducted in cultured ventricular myocytes. Furthermore, different signaling pathways may exist in different cell types within a given tissue (Meszaros et al, 2000).

c. *ET-1 and stretch*

Skvorak *et al* (1997) has suggested that ET and nitrous oxide (NO) interact to regulate stretch-induced ANF secretion. Cultured endothelial cells released ET-1 immediately in response to mechanical stretch (Macarthur et al, 1994). In rat left atria preparations, endothelin significantly increased the ANF

secretion in response to stretch (Schiebinger and Greening, 1992). Perhaps, two pools of ANF granules were activated in these experiments, suggesting that $G_{i/o}$ and G_q mediated pathways can simultaneously be turned on depending on the stimulus. It was shown that cross talk between $G_{\alpha i}$- and $G_{\alpha q}$-coupled receptors was mediated by $G_{\beta\gamma}$ exchange (Quitterer and Lohse, 1999). It is possible that this was the mechanism by which ET enhanced stretch-stimulated secretion in the Shiebinger and Greening (1992) study. Activation of one pathway can stimulate the other. Stimulation of two $G_{\alpha i}$-coupled receptors (the α_{2c}-adrenergic & the A1 adenosine receptors), enhanced the potency and efficacy of the signal generated by two $G_{\alpha q}$-coupled receptors (UTP-preferring P2Y AND B_2 receptor) (Quitterer and Lohse, 1999). Cross talk between $G_{q/11}$ protein- and $G_{i/o}$ protein-coupled receptors has also been demonstrated in mouse atria stimulated with angiotensin II and bradykinin (Cox et al, 2000).

d. *Endothelial cells and mechanotransduction*

Stimulation of ANF release by ET-1 and stretch has been shown to be mostly independent of protein synthesis (Doubell and Thibault, 1994; Page *et al*, 1991). The ANF secretory response to stretch and ET-1 was kinetically different; while atrial muscle stretch caused an immediate increase in ANF secretion followed shortly by a return to basal levels (Ogawa *et al*, 1999; de Bold *et al*, 2001), stimulation by ET-1 resulted in a slower and more sustained hormonal release (Bruneau and de Bold, 1994; Bruneau *et al*, 1996, 1997). Following mechanical stretch, endothelial cells have been shown to sense the change in

transmural pressure, stimulating the cells to release vasoconstrictors and relaxant factors (Rubanyi *et al*, 1990; Ruskuaho *et al*, 1997). Macarthur *et al* (1994) showed that stretching of bovine aortic endothelial cells resulted in the immediate and sustained release of ET-1. In an isolated rat heart preparation, infusion of ET stimulated both basal and stretch-stimulated ANF release (Mäntymaa *et al*, 1990). Thus, it is reasonable to suggest that atrial endothelial cells respond to mechanical stimulation by releasing endothelial factors that act via paracrine/autocrine signaling on ANF secretion in cardiocytes. However, the kinetics of release by these factors, where known, is different from that of stretch-secretion coupling.

In another isolated atria model, locally produced ET-1 acted through paracrine mechanisms to regulate the endocrine response of ANF to stretch and anoxia (Skvorak et al, 1995, 1996). However, two caveats must be considered: 1) different hormone secretion pathways may exist in the ventricles; and 2) the atrial cell culture experiments may not preserve the signaling interactions between the different types of cells in whole tissue. A number of studies have showed differences in the transmembrane signaling processes in whole ventricles compared to isolated ventricular myocytes (Chesley et al, 2000; Buxton et al, 1985; Hilal-Dandan et al, 1992; Ishihata and Endoh, 1995). Moreover, the interactions between different cell types have physiological significance, since paracrine signaling plays a significant endocrine role in the heart.

6. Mastoparan-7 stimulated ANF secretion

Since inhibiting the $G_{i/o}$ proteins eliminate the stretch-stimulated ANF response, it can be hypothesized that stimulating the $G_{i/o}$ proteins might increase ANF secretion. In the pancreas, a class of compounds known as mastoparans has been shown to stimulate insulin secretion by signaling through heterotrimeric G proteins (Robertson et al, 1991). It is thought that MAS exerts its effects by acting through heterotrimeric G proteins localized in the membrane (Higashijima *et al*, 1990; Sukumar and Higashijima, 1992). A cross-linking study found a binding site for MAS on the amino terminus of the α-subunits of G-proteins (Higashijima and Ross, 1991). Moreover, MAS was shown to selectively activate $G_{i/o}$ proteins (Higashijima et al, 1990).

MAS-7 was selected because it was an analogue with 5-fold greater potency than MAS (obtained by substituting Ala for Lys in position 12) ((Higashijima et al, 1990). Secondly, PTX had been shown to block MAS stimulated insulin secretion (Konrad *et al*, 1995). Thirdly, Ross et al (1994) had showed that MAS-7 accelerated the GTP/GDP nucleotide exchange of the $G_{\alpha o}$ subunit more than 30-fold. G_o proteins were of interest because the immunofluorescent staining experiments suggested that $G_{o\alpha}$ was the inhibitory G protein colocalizing with ANF. As a result, MAS-7 was deemed the appropriate compound to test the hypothesis that stimulating $G_{i/o}$ proteins would promote ANF release. As discussed earlier, it was suggested that the regulatory mechanism of stretch-secretion coupling involved the activation of $G_{i/o}$ proteins.

Stimulation of ANF secretion with MAS-7 and attenuation of this secretion with PTX support the involvement of $G_{i/o}$ proteins in ANF secretion (Figure 11). However, PTX only attenuated ANF secretion stimulated by MAS-7 and did not completely inhibit hormone release. This suggests MAS-7 is also acting on a PTX-insensitive hormone-releasing pathway.

A recent publication suggested that MAS-7 possessed membrane-perturbing properties that affected the lipid composition of L1210 cells (Park et al, 2000). Increases in free fatty acids and phosphatidylethanol as well as a decrease of phosphatidylcholine were among the effects measured in these cells following MAS-7 treatment (Park et al, 2000). Others have reported that MAS-7 acted by the induction of membrane pore formation (Suh et al, 1996, 1998). In insulin secreting β-TC3 cells, MAS had also been shown to cause lysis of the plasma membrane at high concentrations (Lorenzo *et al*, 1994). Nevertheless, Konrad *et al* (1995) showed that MAS acted selectively via G_i proteins to stimulate insulin release from β-TC3 cells, and that release was not due to lytic toxicity.

In the present experiments, two observations suggest that the cardiocytes were not lysed. First, MAS-7 stimulated ANF secretion was attenuated by PTX. Secondly, ANF secretion returned to basal levels upon removal of MAS-7 from the perfusate. A nonspecific destruction of the cell membrane, would prevent return to basal ANF levels. The current data,

however, cannot rule out the possibility that MAS-7 caused pore formation in the plasma membrane of the cardiocytes. The partial inhibition of MAS-7-induced ANF release by PTX, suggests that there maybe a specific effect on $G_{i/o}$ proteins, and a non-specific effect due to perturbation of the membrane and muscle.

6. CONCLUSION

In summary, this study demonstrates that pertussis toxin ($G_{i/o}$ antagonist) eliminated the stretch-coupled ANF secretion and that mastoparan-7 ($G_{i/o}$ agonist) potently stimulated ANF secretion. These results support the hypothesis that interference with G protein signaling impairs stretch-mediated ANF release. They further suggest that $G_{i/o}$ proteins are involved in the secretory pathways regulating ANF release. The co-localization of $G_{\alpha o}$ with ANF storage granules provides a possible source of hormone regulation within the cardiocyte. In addition, the inability to inhibit ET-1 stimulated ANF secretion suggests multiple signaling pathways regulate ANF secretion. Mechanical (stretch) and autocrine/paracrine (ET-1) stimuli of ANF secretion do not seem to share a common $G_{i/o}$ protein regulatory mechanism. Taken together, both the hypothesis that stretch-stimulated secretion involves SGs regulated by heterotrimeric G proteins, and the localization of this control (SGs vs. plasma membrane), require further investigation.

7. FUTURE DIRECTIONS

A number of experiments would clarify some of the issues raised in this thesis. To test the hypothesis that different pools of SGs exist, immunofluorescent microscopy could be utilized. In such an experiment, antibodies to various marker proteins that have been located on the immature SGs could be used to distinguish immature SGs from mature SGs. For example, a clathrin coat has only been found on ISGs and not on MSGs (Urbé *et al*, 1997), which provides a way of distinguishing ISGs from MSGs. Furthermore, the predominant variant of clathrin light chains (LCa and LCb) found in cells with a regulated pathway was LCb (Acton and Brodsky, 1990). These findings indicate that regulated, as opposed to constitutively released, SGs could be separated based on the LCb proteins found in their membranes.

The experimental strategy would involve a combination of Western blot analysis, two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis/isoelectric focusing, and light and electron microscopic immunocytochemistry to study tissue sections of rat atria. A clathrin light LCb polyclonal antibody combined with a G protein antibody could help determine: 1) which SGs are ISGs, 2) which SGs contain the inhibitory G proteins, and 3) which pathway is utilized by the ISGs. Double immunofluorescence confocal microscopy and double label electron microscopic immunogold localizations using ANF, $G_{i/o}$ protein, and clathrin coat antibodies would provide further insight

into the perinuclear location of these ISGs. Unlike other clathrin-coated vesicles, ISGs possess the unique property that their clathrin coat appears discontinuous when observed by electron microscopy (Orci, 1982; Orci *et al*, 1984).

In the present experiments, PTX was used to inhibit the $G_{i/o}$ proteins in atrial cardiocytes. A key issue that needs to be addressed is which G proteins were ADP-ribosylated by pretreatment with this pharmacological agent. While the ADP-ribosylation results clearly show that the $G_{i/o}$ proteins were inhibited with the selected dose of PTX (40 μg/kg), pinpointing the location of the inhibitory G proteins affected by this treatment would provide crucial information about the intracellular pathways regulating ANF secretion. It has been suggested that PTX is transported to the Golgi apparatus, and back to the plasma membrane (Xu and Barbieri, 1995). This raises the interesting possibility that G proteins in the Golgi, the SGs, as well as the plasma membrane were targets for this toxin. At the plasma membrane, PTX has been shown to interact with a specific carbohydrate sequence (Brennan *et al*, 1988). Hence, determining which receptor(s) were acting as the point of attachment for PTX may yield information about the receptor(s) participating in stretch-secretion coupling. Searching for the presence of this glycoprotein sequence in plasma membrane cardiac receptors could implicate receptors that have not been considered. These types of protein-protein interaction studies could conceivably yield which $G_{i/o}$ proteins (at the plasma membrane, SGs, or Golgi) are primarily involved in ANF stretch-secretion coupling.

Another concern in the experiments was the reported lytic properties of MAS-7. This issue raised the possibility that the ANF secretion following MAS-7 infusion may have been the result of ANF SGs leaking out into the perfusate. Evaluating this possibility could be accomplished in two ways. First, the physiological responsiveness of the atria to known stimuli could be assessed following MAS-7 treatment. If the integrity of the cell membranes were compromised, the cardiocytes would be unable to exhibit the well-documented ANF secretory response to stretch. Thus, the atria could be stretched after MAS-7 infusion had been stopped. Maintenance of the stretch response would suggest that the cardiocytes were not damaged. Second, leakage could also be examined by determining if leakage of marker enzymes of myocardial damage had occurred.

In the discussion, ATP-sensitive potassium channels and cAMP were suggested as possible players in the mechanism of stretch-secretion coupling. Measuring cAMP, and PKA in the experiments at hand could have helped in accepting or rejecting this hypothesis. Furthermore, it could be interesting to evaluate the effect of PTX and MAS-7 on cAMP concentrations; utilizing a cAMP radioimmunoassay, cAMP levels could be measured in the perfusate of pharmacologically treated atria, which were stretched or not stretched. Moreover, K^{+}_{ATP} channel blockers and activators have been tested in numerous models to evaluate their involvement in stretch-secretion coupling. It would be

interesting to test the involvement of these ion channels in the isolated atria preparation, in conjunction with the treatments shown in the present experiments thus far.

8. PERSPECTIVES

To date, mechanism(s) for stretch-secretion coupling have not been unraveled for atrial cardiocytes. This task has become increasingly challenging as it has become clear that there is not an all-encompassing intracellular model regulating exocytosis in endocrine cells. Differences are apparent even within the same organ, such as the differences seen in natriuretic peptide secretion in the atria compared to that in the cardiac ventricles. Evidently, every cell is specialized to perform different functions. Nonetheless, the presence of $G_{i/o}$ proteins in numerous endocrine and exocrine cells, supports the possibility that hormone-secreting cells share a common $G_{i/o}$ protein regulatory mechanism.

Cells have evolved with numerous redundant pathways. The essential feature of heterotrimeric G proteins is that, they serve as molecular switches, and act as a point of regulatory convergence for many pathways in the cells. Pharmacological inhibition of $G_{i/o}$ proteins has demonstrated that they are involved in the intracellular pathways regulating stretch-stimulated ANF release. As research in the area of G protein signaling carries on, the list of systemic functions associated with these proteins continues to grow (Neves *et al*, 2002). The next step involves establishing which effectors are regulated by $G_{i/o}$ proteins in ANF stretch-secretion coupling. Many researchers have outlined candidate effectors involved in stretch-secretion coupling. Perhaps no individual effector

functions alone in this process; rather these effectors may be operating in unison, a possibility, which will be solved with further research.

9. REFERENCES:

Aardal, S., Helle, K.B. (1991) Comparative aspects of the endocrine myocardium. *Acta. Physiol. Scand. Suppl.* **599**, 31-46.

Abramson, B.L., Ando, S., Notarius, C.F., Rongen, G.A., and Floras, J.S. (1999) Effect of atrial natriuretic peptide on muscle sympathetic activity and its reflex control in human heart failure. *Circulation.* **99**, 1810-5.

Acton, S. L. and Brodsky, F. M. (1990). Predominance of clathrin light chain LCb correlates with the presence of a regulated secretory pathway. *J. Cell Biol.* **111**,1419-1426.

Agnoletti, G., Ferrari, R., Slade, A.M., Severs, N.J., and Harris, P. (1989) Stretch-induced centrifugal movement of atrial specific granules- a preparatory step in atrial natriuretic peptide secretion. *J. Mol. Cell. Cardiol.* **21**, 235-239.

Ahnert-Hilger, G., Schäfer, T., Spicher, K., Grund, C., Schultz, G., and Wiedenmann, B. (1994) Detection of G-protein heterotrimers on large dense core and small synaptic vesicles of neuroendocrine and neuronal cells. *Eur. J. Cell Biol.* **65**, 26-38.

Ambar, I., Sokolovsky, M. (1993) Endothelin receptors stimulate both phospholipase C and phospholipase D activities in different cell lines. *Eur. J. Pharmacol.* **245**, 31-41.

Apodaca, G. (2001) Endocytic traffic in polarized epithelial cells: role of the actin and microtubule cytoskeleton. *Traffic* **2**, 149-159.

Apodaca, G. (2002) Modulation of membrane traffic by mechanical stimuli. *Am. J. Physiol. Renal Physiol.* **282**, F179-F190.

Apps, D.K., and Percy, J.M. (1987) The H+-translocating ATPase of chromaffin granule membranes. *Ann. N. Y. Acad. Sci.* **493**, 178-88.

Arai, H., Hori, S., Aramori, I., Ohkubo, H., and Nakanishi, S. (1990) Cloning and expression of a cDNA encoding an endothelin receptor. *Nature.* **348**, 730-2.

Aramori, I., and Nakanishi, S. (1992) Coupling of two endothelin receptor subtypes to differing signal transduction in transfected Chinese hamster ovary cells. *J. Biol. Chem.* **267**, 12468-74.

Arvan, P. and Castle, D. (1998). Sorting and storage during secretory granule biogenesis: looking backward and looking forward. *Biochem. J.* **332**,593 -610.

Arvan, P., Kuliawat, R., Prabakharan, D., Zavacki, A. -M., Elahi, D., Wang, S. and Pilkey, D. (1991). Protein discharge from immature secretory granules displays both regulated and constitutive characteristics. *J. Biol. Chem.* **266**,14171-14174.

Asano, T., Morishita, R., Kato, K. (1992) Two forms of G(o) type G proteins: identification and distribution in various rat tissues and cloned cells. *J. Neurochem.* **58**, 2176-81.

Asano, T., Nagahama, M., and Kato, K. (1990) Subcellular distribution of GTP-binding proteins, Go and Gi2, in rat cerebral cortex. *J. Biochem (Tokyo).* **107**, 694-8.

Asano, T., Semba, R., Kamiya, N., Ogasawara, N., and Kato, K. (1988) Go, a GTP-binding protein: Immunochemical and immunohistochemical localization in rat. *J. Neurochem.* **50**, 1164-1169.

Ashcroft, F.M. (1988) Adenosine 5'-triphosphate-sensitive potassium channels. *Annu. Rev. Neurosci.* **11**, 97-118.

Astesano, A., Regnauld, K., Ferrand, N., Gingras, D., Bendayan, M., Rosselin, G., and Emami, S. (1999) Cellular and subcellular expression of Golf/Gs and Gq/G11 α-subunits in rat pancreatic endocrine cells. *J. Histochem. & Cytochem.* **47**, 289-302, 1999.

Azizi, C., Barthelemy, C., Masson, F., Maistre, G., Eurin, J., and Carayon, A. (1995) Myocardial production of prostaglandins: its role in atrial natriuretic peptide release. *Eur J Endocrinol* **133**, 255-259.

Baron, A., van Bever, L., Monnier, D., Roatti, A., and Baertschi, A.J. (1999) A novel K(ATP) current in cultured neonatal rat atrial appendage cardiomyocytes. *Circ Res.* **85**, 707-15.

Barr, F.A., Leyte, A., Mollner, S., Pfeuffer, T., Tooze, S.A., and Huttner, W.B. (1991) Trimeric G-proteins of the trans-Golgi network are involved in the formation of constitutive secretory vesicles and immature secretory granules. *FEBS Lett.* **294**, 239-43.

Bennett, M.K., and Scheller, R.H. (1993) The molecular machinery for secretion is conserved from yeast to neurons. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2559-63.

Berman, D.M., Wilkie, T.M., and Gilman, A.G. (1996) GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits. *Cell.* **86**, 445-52.

Bianciotti, L.G., and de Bold, A.J. (2000) Effect of selective ET(A) receptor blockade on natriuretic peptide gene expression in DOCA-salt hypertension. *Am. J. Physiol.* **279**, H93-H101.

Blackshear, P.J., Naim, A.C., Kuo, J.F. (1988) Protein kinases 1988: a current perspective. *FASEB J.* **2**, 2957-2969.

Blasi, J., Binz, T., Yamasaki, S., Link, E., Niemann, H., and Jahn, R. (1994) Inhibition of neurotransmitter release by clostridial neurotoxins correlates with specific proteolysis of synaptosomal proteins. *J. Physiol. Paris.* **88**, 235-41.

Bourne, H.R., Sanders, D.A., and McCormick, F. (1990) The GTPase superfamily: a conserved switch for diverse cell functions. *Nature.* **348**, 125-32.

Breitwieser, G.E., and Szabo, G. (1985) Uncoupling of cardiac muscarinic and beta-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature.* **317**, 538-40.

Brennan, M.J., David, J.L., Kenimer, J.G., and Manclark, C.R. (1988) Lectin-like binding of pertussis toxin to a 165-kilodalton Chinese hamster ovary cell glycoprotein. *J. Biol. Chem.* **263**, 4895-4899.

Bruneau, B.G., and de Bold, A.J. (1994) Selective changes in natriuretic peptide and early response gene expression in isolated rat atria following stimulation by stretch and endothelin-1. *Cardiovascular Res.* **28**, 1519-1525.

Bruneau, B.G., Piazza, L.A., and de Bold, A.J. (1996) α1-Adrenergic stimulation of isolated rat atria results in discoordinate increases in natriuretic peptide secretion and gene expression and enhances *Egr-1* and c-*Myc* expression. *Endocrinology.* **137**(1), 137-143.

Bruneau, B.G., Piazza, L.A., and de Bold, A.J. (1997) BNP gene expression is specifically modulated by stretch and ET-1 in a new model of isolated rat atria. *Am. J. Physiol.* **273**, H2678-H2686.

Brutsaert, D.L., and Andries, L.J. (1992) The endocardial endothelium. *Am. J. Physiol.* **263**, H985–H1002.

Bryan, W.M. (1991) Design of minimum active fragments of biologically active peptides. *Methods Enzymol.* **202**, 436-48.

Burgoyne, R.D. (1990) Secretory vesicle-associated proteins and their role in exocytosis. *Annu. Rev. Physiol.* **52**, 647-59

Burgoyne, R.D. (1992) Trimeric G proteins in Golgi transport. *Trends Biochem. Sci.* **17**, 87-8.

Burnett, J.C. Jr., Kao, P.C., Hu, D.C., Heser, D.W., Heublein, D., Granger, J.P., Opgenorth, T.J., and Reeder, G.S.(1986) Atrial natriuretic peptide elevation in congestive heart failure in the human. *Science.* **231**,1145-7.

Buxton, I.L.O., and Brunton, L.L. (1985) Direct analysis of β-Adrenergic receptor subtypes on intact adult ventricular myocytes of the rat. *Circ. Res.* **56**, 126-132.

Calaghan, S.C., Colyer, J., and White, E. (1999) Cyclic AMP but not phosphorylation of phospholamban contributes to the slow inotropic response to stretch in ferret papillary muscle. *Pflügers Arch.* **437**, 780-782.

Candenas, M.L., Naline, E., Puybasset, L., Devillier, P., and Advenier, C. (1991) Effect of atrial natriuretic peptide and on atriopeptins on the human isolated bronchus. Comparison with the reactivity of the guinea-pig isolated trachea. *Pulm. Pharmacol.* **4**, 120-5.

Cappuccio, F.P., Markandu, N.D., Buckley, M.G., Sugden, A.L., Sagnella, G.A., and MacGregor, G.A. (1989) Raised plasma levels of atrial natriuretic peptides in Addison's disease. *J. Endocrinol. Invest.* **12**, 205-207.

Cargill, R.I., and Lipworth, B.J. (1995) Acute effects of ANP and BNP on hypoxic pulmonary vasoconstriction in humans. *Br. J. Clin. Pharmacol.* **40**, 585-90.

Chang, M.S., Lowe, D.G., Lewis, M., Hellmiss, R., Chen, E., and Goeddel, D.V. (1989) Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature.* **341**, 68-72.

Chen, Z.J., Miao, Z.H., Vetter, M., Dulin, N., Liu, S., Che, D., Hughes, B., Murad, F., Douglas, J., Chang, C.H. (2000) Molecular cloning of a regulatory protein for membrane-bound guanylate cyclase GC-A. *Biochem. Biophys. Res. Commun.* **278**, 106-11.

Chesley, A., Lundberg, M.S., Asai, T., Xiao, R-P., Ohtani, S., Lakatta, E.G., and Crow, M.T. (2000) The β_2-Adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through Gi-dependent coupling to phosphatidylinositol 3'-Kinase. *Circ. Res.* **87**, 1172-1179.

Church, D.J., Van der Bent, V., Vallotton, M.B., Capponi, A.M., and Lang, U. (1994) Calcium influx in platelet activating factor-induced atrial natriuretic peptide release in rat cardiomyocytes. *Am. J. Physiol. Endocrinol. Metab.* **266**, E403-E409.

Clerico, A., Iervasi, G., and Mariani, G. (1999) Pathophysiogic relevance of measuring the plasma levels of cardiac natriuretic peptide hormones in humans. *Horm. Metab. Res.* **31**, 487-498.

Clermont, Y., Rambourg, A., and Hermo, L. (1995) Trans-Golgi network (TGN) of different cell types: three-dimensional structural characteristics and variability. *Anat. Rec.* **242**, 289-301.

Consolo, S., Baldi, G., Nannini, L., Uboldi, M.C., Pooga, M., Langel, U., and Bartfai, T. (1997) Galparan induces in vivo acetylcholine release in the frontal cortex. *Brain Res.* **756**, 174-8.

Cowie, M.R., Struthers, A.D., Wood, D.A., Coats A.J.S., Thompson, S.G., Poole-Wilson, P.A., and Sutton, G.C. (1997) Value of natriuretic peptides in assessment of patients with possible new heart failure in primary care. *Lancet* **350**, 1347-1351.

Cox, S.L., Schelb, V., Trendelenburg, A.U., and Starke, K. (2000) Enhancement of noardrenaline release by angiotensin II and bradykinin in mouse atria : evidence for cross-talk between $G_{q/11}$ protein- and $G_{i/o}$ protein-coupled receptors. *Br. J. Pharmacol.* **129**, 1095-1102.

Cui, X., Wen, J.F., Jin, J.Y., Xu, W.X., Kim, S.Z., Kim, S.H., Lee, H.S., and Cho, K.W. (2002) Protein kinase-dependent and Ca(2+)-independent cAMP inhibition of ANF release in beating rabbit atria. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **282**, R1477-R1489.

Dascal, N. (2001) Ion-channel regulation by G proteins. *Trends Endocrinol. Metab.* **12**, 391-8.

Dashwood, M.R., and Tsui, J.C. (2002) Endothelin-1 and atherosclerosis: potential complications associated with endothelin-receptor blockade. *Atherosclerosis.* **160**, 297-304.

Davis, M., Espiner, E., Richards, G., Billings, J., Town, I., Neill, A., Drennan, C., Richards, M., Turner, J., and Yandle, T. (1994) Plasma brain natriuretic peptide in assessment of acute dyspnoea. *Lancet* **343**, 440-444.

de Bold, A.J., Borenstein, H.B., Veress, A.T., and Sonnenberg, H. (2001) A rapid and potent natriuretic response to intravenous injections of atrial myocardial extract in rats. Reprint *J. Am. Soc. Nephrol.* **13**, 403-409.

de Bold, A.J., Borenstein, H.B., Veress, A.T., and Sonnenberg, H. (1981) A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.* **28**, 89-94.

de Bold, A.J., Bruneau, B.G. (2000) Natriuretic peptides. In Fray JCS, Goodman MH, eds. Handbook of Physiology. Section 7. The Endocrine System. Vol III. Endocrine Regulation of Water and Electrolyte Balance. New York, American Physiological Society by Oxford University Press, 377-409.

de Bold, A.J., Bruneau, B.G., and Kuroski de Bold, M.L. (1996) Mechanical and neuroendocrine regulation of the endocrine heart. *Cardiovasc. Res.* **31**, 7-18.

de Bold, A.J., Bruneau, B.G., Kuroski de Bold, M.L. (1996) Mechanical and neuroendocrine regulation of the endocrine heart. *Cardiovascular Res.* **31**, 7-18.

de Bold, A.J., Kuroski-de Bold, M.L., Boer, P.H., Dube, G., Mangat, H., and Johnson F. (1991) A decade of atrial natriuretic factor research. Can J Physiol Pharmacol. **69**, 1480-5.

de Bold, M.L., and de Bold, A.J. (1989) Effect of manipulations of Ca2+ environment on atrial natriuretic factor release. *Am. J. Physiol.* **256**, H1588-94.

de Zeeuw, D., Janssen, W.M., and de Jong, P.E. (1992) Atrial natriuretic factor, its (patho)physiogical significance in humans. *Kidney Int.* **41**, 1115-1133.

Deutsch, N., Klitzner, T.S., Lamp, S.T., and Weiss, J.N. (1991) Activation of cardiac ATP-sensitive K+ current during hypoxia: correlation with tissue ATP levels. *Am. J. Physiol.* **261**, H671-6.

Dickstein, K., Larsen, A.L., Bonarjee, V., Thoresen, M., Aarsland T., and Hall, C. (1995) Plasma proatrial natriuretic factor is predictive of clinical status in patients with congestive heart failure. *Am. J. Cardiol.* **76**, 679-683.

Dittie, A. S., Hajibagheri, N. and Tooze, S. A. (1996). The AP-1 adaptor complex binds to immature secretory granules from PC12 cells, and is regulated by ADP-ribosylation factor. *J. Cell Biol.* **132**,523 -536.

Donaldson, J.G., Kahn, R.A., Lippincott-Schwartz, J., and Klausner, R.D. (1991) Binding of ARF and beta-COP to Golgi membranes: possible regulation by a trimeric G protein. *Science.* **254**, 1197-9.

Dostal, D.E., and Baker, K.M. (1998) Angiotensin and Endothelin. Messengers that couple ventricular stretch to the Na^+/H^+ exchanger and cardiac hypertrophy. *Circ. Res.* **83**, 870-873.

Doubell, A.F., and Thibault, G. (1994) Calcium is involved in both positive and negative modulation of the secretory system for ANP. *Am. J. Physiol.* **266**, H1854-63.

Drewett JG, Garbers DL. (1994) The family of guanylyl cyclase receptors and their ligands. *Endocr. Rev.* **15**, 135-62.

Dumont, M.E., and Richards, F.M. (1988) The pH-dependent conformational change of diphtheria toxin. *J. Biol. Chem.* **263**, 2087-97.

Dunn, B.R., Ichikawa, I., Pfeffer, J.M., Troy, J.L., and Brenner, B.M. (1986) Renal and systemic effects of synthetic atrial natriuretic peptide in the anesthesized rat. *Circ. Res.* **59**, 237-246.

Dunne, M.J., Bullett, M.J., Li, G.D., Wollheim, C.B., and Petersen, O.H. (1989) Galanin activates nucleotide-dependent K+ channels in insulin-secreting cells via a pertussis toxin-sensitive G-protein. *EMBO J.* **8**, 413-20.

Duru, F., Barton, M., Luscher, T.F., Candinas, R. (2001) Endothelin and cardiac arrhythmias: do endothelin antagonists have a therapeutic potential as antiarrhythmic drugs? *Cardiovasc. Res.* **49**, 272-80.

Eaton, B.A., Haugwitz, M., Lau, D., and Moore, H.P. (2000) Biogenesis of regulated exocytotic carriers in neuroendocrine cells. *J. Neurosci.* **20**, 7334-44.

Edwards, B.S., Zimmerman, R.S., Schwab, T.R., Heublein, D.M., and Burnett, J.C. Jr. (1988) Atrial stretch, not pressure, is the principal determinant controlling the acute release of atrial natriuretic factor. *Circ. Res.* **62**, 191-5.

Eipper, B.A., and Mains, R.E. (1988) Peptide alpha-amidation. *Annu. Rev. Physiol.* **50**, 333-44.

el Baya, A., Linnemann, R., von Olleschik-Elbheim, L., and Schmidt, M.A. (1995) Identification of binding proteins for pertussis toxin on pancreatic beta cell-derived insulin-secreting cells. *Microb. Pathog.* **18**, 173-85.

el Baya, A., Linnemann, R., von Olleschik-Elbheim, L., Robenek, H., and Schmidt, M.A. (1997) Endocytosis and retrograde transport of pertussis toxin to the Golgi complex as a prerequisite for cellular intoxication. *Eur. J. Cell. Biol.* **73**, 40-8.

el Baya, A., Linnemann, R., von Olleschik-Elbheim, L., and Schmidt, M.A. (1997) Pertussis toxin. Entry into cells and enzymatic activity. *Adv. Exp. Med. Biol.* **419**, 83-6.

Elton, T.S., Oparil, S., Taylor, G.R., Hicks, P.H., Yang, R.H., Jin, H., and Chen, Y.F. (1992) Normobaric hypoxia stimulates endothelin-1 gene expression in the rat. *Am. J. Physiol.* **263**, R1260-4.

Farquhar, M.G., Reid, J.J., Daniell, L.W. (1978) Intracellular transport and packaging of prolactin: a quantitative electron microscope autoradiographic study of mammotrophs dissociated from rat pituitaries. *Endocrinology*. **102**, 296-311.

Fenoy, F.J., Quesada, T., Garcia-Salom, M., Romero, J.C., and Salazar, F.J. (1989) Hemodynamic effects of chronic infusion of rANP in renal hypertensive rats. *Am J Physiol.* **256**, H1393-8.

Fishman, P.H. (1990) Mechanism of action of cholera toxin. In *ADP-Ribosylating Toxins and G Proteins* (Moss. J., and Vaughan, M., eds) pp. 127-140, American Society for Microbiology, Washington, DC.

Floras, J.S. (1995) Inhibitory effect of atrial natriuretic factor on sympathetic ganglionic neurotransmission in humans. *Am. J. Physiol.* **269**, R406-12.

Flynn, T.G., de Bold, M.L., and de Bold, A.J. (1983) The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties. *Biochem. Biophys. Res. Commun.***117**, 859-65.

Forssmann, W.G. (1986) Cardiac hormones. I. Review on the morphology, biochemistry, and molecular biology of the endocrine heart. Eur J Clin Invest. **16**, 439-51.

Fosset, M., Schmid-Antomarchi, H., de Weille, J.R., and Lazdunski, M. (1988) Somatostatin activates glibenclamide-sensitive and ATP-regulated K+ channels in insulinoma cells via a G-protein. *FEBS Lett.* **242**, 94-6.

Franco-Saenz, R., Somani, P., and Mulrow, P.J. (1992) Effect of atrial natriuretic peptide (8-33-Met ANP) in patients with hypertension. *Am. J. Hyperten.* **5**, 266-75.

Fricker, L.D. (1988) Activation and membrane binding of carboxypeptidase E. *J. Cell Biochem.* **38**, 279-89.

Fukuda, Y., Hirata, Y., Taketani, S., Kojima, T., Oikawa, S., Nakazato, H., and Kobayashi, Y. (1989) Endothelin stimulates accumulations of cellular atrial natriuretic peptide and its messenger RNA in rat cardiocytes. *Biochem. Biophys. Res. Comm.* **164**, 1431–1436.

Fukuda, Y., Hirata, Y., Yoshimi, H., Kojima, T., Kobayashi, Y., Yanagisawa, M., and Masaki, T. (1988) Endothelin is a potent secretagogue for atrial natriuretic peptide in cultured rat atrial myocytes. *Biochem. Biophys. Res. Comm.* **155**, 167–172.

Gaisano, H.Y., Ghai, M., Malkus, P.N., Sheu, L., Bouquillon, A., Bennett, M.K., and Trimble, W.S. (1996) Distinct cellular locations of the syntaxin family of proteins in rat pancreatic acinar cells. Mol Biol Cell. **7**, 2019-27.

Gaisano, H.Y., Sheu, L., Grondin, G., Ghai, M., Bouquillon, A., Lowe, A., Beaudoin, A., and Trimble, W.S. (1996) The vesicle-associated membrane protein family of proteins in rat pancreatic and parotid acinar cells. *Gastroenterology*. **111**, 1661-9.

Garcia, R., Thibault, G., Gutkowska, J., Hamet, P., Cantin, M., and Genest, J. (1985) Effect of chronic infusion of synthetic atrial natriuretic factor (ANF 8-33) in conscious two-kidney, one-clip hypertensive rats. *Proc. Soc. Exp. Biol. Med.* **178**, 155-9.

Gardner, D.G., Newman, E.D., Nakamura, K.K., and Nguyen. K.P. (1991) Endothelin increases the synthesis and secretion of atrial natriuretic peptide in neonatal rat cardiocytes. *Am. J. Physiol.* **271**, E177-E182.

Gardner, D.G., Wirtz, H., and Dobbs, L.G. (1992) Stretch-dependent regulation of atrial peptide synthesis and secretion in cultured atrial cardiocytes. *Am. J. Physiol.* **263**, E239-E244.

Genest, J., Larochelle, P., Cusson, J.R., Garcia, R., Gutkowska, J., and Cantin, M. (1988) The atrial natriuretic factor in hypertension. *Am. J. Med. Sci.* **295**, 299-304.

Geyer, M., and Wittinghofer, A. (1997) GEFs, GAPs, GDIs and effectors: taking a closer (3D) look at the regulation of Ras-related GTP-binding proteins. *Curr. Opin. Struct. Biol.* **7**, 786-92.

Glembotski, C.C., and Gibson, T.R. (1985) Molecular forms of immunoactive atrial natriuretic peptide released from cultured rat atrial myocytes. *Biochem. Biophys. Res. Commun.* **132**, 1008-17.

Goldie, R.G. (1999) Endothelins in health and disease: an overview. *Clin. Exp. Pharmaco. Physiol.* **26**,145-8.

Goldie, R.G., and Fernandes, L.B. (2000) A possible mediator role for endothelin-1 in respiratory disease. *Monaldi. Arch. Chest. Dis.* **55**, 162-7.

Gottlieb, S.S., Kukin, M.L., Ahern, D., and Packer, M. (1989) Prognostic importance of atrial natriuretic peptide in patients with chronic heart failure. *J. Am. Coll. Cardiol.* **13**, 1534-9.

Grafstein, B., and Forman, D.S. (1980) Intracellular transport in neurons. *Physiol. Rev.* **60**, 1167-283.

Granger, J.P., Opgenorth, T.J., Salazar, J., Romero, J.C., and Burnett, J.C. Jr. (1986) Long-term hypotensive and renal effects of atrial natriuretic peptide. *Hypertension.* **8**, II112-6.

Greenwald, J.E., Sakata, M., Michener, M.L., Sides, S.D., and Needleman, P. (1988) Is atriopeptin a physiological or pathophysiological substance? Studies in the autoimmune rat. *J. Clin. Invest.* **81**, 1036-41.

Gudi, S.R.P., Lee, A.A., Clark, C.B., and Frangos, J.A. (1998) Equibiaxial strain and strain rate stimulate early activation of G proteins in cardiac fibroblasts. *Am. J. Physiol.* **274**, C1424-C1428.

Hamill, O.P., and Martinac, B. (2001) Molecular basis of mechanotransduction in living cells. *Physiol. Rev.* **81**, 685-740.

Hamill, O.P., and McBride, D.W. (1992) Rapid adaptation of single mechanosensitive channels in Xenopus oocytes. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7462-7466.

Hare, J.M., Kim, B., Flavahan, N.A., Ricker, K.M., Peng, X., Colman, L., Weiss, R.G., and Kass, D.A. (1998) Pertussis toxin-sensitive G proteins influence nitric oxide synthase III activity and protein levels in rat heart. *J. Clin. Invest.* **101**, 1424-31.

Hay, D.W. (1999) Putative mediator role of endothelin-1 in asthma and other lung diseases. *Clin. Exp. Pharmacol. Physiol.* **26**, 168-71.

Helms, J.B. (1995) Role of heterotrimeric GTP binding proteins in vesicular protein transport: indications for both classical and alternative G protein cycles. *FEBS Lett.* **369**, 84-8.

Higashijima, T., Burnier, J., and Ross, E.M. (1990) Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. *J. Biol. Chem.* **265**(24), 14716-14186.

Higashijima, T., Ross, E.M. (1991) Mapping of the mastoparan-binding site on G proteins. Cross-linking of [^{125}I-Tyr3,Cys11]mastoparan to Go. *J. Biol. Chem.* **266**, 12655-12661.

Higashijima, T., Ross, E.M. (1994) Regulation of G-protein activation by mastoparans and other cationic peptides. *Methods Enzymol.* **237**, 26-37.

Higashijima, T., Uzu, S., Nakajima, T., and Ross, E. (1988) Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). *J. Biol. Chem.* **263**, 6491-6494.

Hilal-Dandan, R., Ramirez, M.T., Villegas, S., Gonzalez, A., Endo-Mochizuki, Y., Brown, J.H., and Brunton, L.L. (1997) Endothelin ET_A receptor regulates signaling and ANF gene expression via multiple G protein-linked pathways. *Am. J. Physiol.* **272**, H130-H137.

Hilal-Dandan, R., Urasawa, K., and Brunton, L.L. (1992) Endothelin inhibits adenylate cyclase and stimulates phosphoinositide hydrolysis in adult cardiac myocytes. *J. Biol. Chem.* **267**, 10620-10624.

Hirai, Y., Kuwada, M., Yasuhara, T., Yoshida, H., and Nakajima, T. (1979) A new mast cell degranulating peptide homologous to mastoparan in the venom of Japanese hornet (Vespa xanthoptera). *Chem. Pharm. Bull. (Tokyo)* **27**, 1945-1946.

Hirai, Y., Yasuhara, T., Yoshida, H., Nakajima, T., Fujino, M., and Kitada, C. (1979) A new mast cell degranulating peptide "mastoparan" in the venom of Vespula lewisii. *Chem. Pharm. Bull. Tokyo.* **27**,1942-4.

Hirata, Y., Matsuoka, H., Suzuki, E., Hayakawa, H., Sugimoto, T., Matsuda, Y., Morishita, Y., Kangawa, K., Minamino, N., Matsuo, H., and Sugimoto, T. (1993) Role of endogenous atrial natriuretic peptide in DOCA-salt hypertensive rats. Effects of a novel nonpeptide antagonist for atrial natriuretic peptide receptor. *Circulation.* **87**, 554-61.

Holmer, S.R., and Homcy, C.J. (1991) G proteins in the heart. A redundant and diverse transmembrane signaling network. *Circulation.* **84**, 1891-902.

Holz, R.W., Brondyk, W.H., Senter, R.A., Kuizon, L., and Macara, I.G. (1994) Evidence for the involvement of Rab3A in Ca (2+)-dependent exocytosis from adrenal chromaffin cells. *J. Biol. Chem.* **269**, 10229-34.

Hori, S., Komatsu, Y., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1992) Distinct tissue distribution and cellular localization of two messenger ribonucleic acids encoding different subtypes of rat endothelin receptors. *Endocrinology.* **130**, 1885-95.

Hori, Y., Takeyama, Y., Hiroyoshi, M., Ueda, T., Maeda, A., Ohyanagi, H., Saitoh, Y., Kaibuchi, K., and Takai, Y. (1996) Possible involvement of Rab11 p24, a Ras-like small GTP-binding protein, in intracellular vesicular transport of isolated pancreatic acini. *Dig. Dis. Sci.* **41**, 133-8.

Horio, T., Kohno, M., Takeda, T. (1993) Cosecretion of atrial and brain natriuretic peptides stimulated by endothelin-1 from cultured rat atrial and ventricular cardiocytes. *Metabolism.* **42**, 94-6.

Hu, J.R., Berninger, U.G., Lang, R.E. (1988) Endothelin stimulates atrial natriuretic peptide (ANP) release from rat atria. *Eur. J. Pharmacol.* **158**, 177-8.

Hunziker, W., Whitney, J.A., and Mellman, I. (1991) Selective inhibition of transcytosis by brefeldin A in MDCK cells. *Cell.* **67**, 617-27.

Iervasi, G., Clerico, A., Berti, S., Pilo, A., Vitek, F, Biagini, A., Baratto, M.T., Bianchi, R., and Donato, L. (1993) ANP kinetics in normal men: *in vivo* measurement by a tracer method and correlation with sodium intake. *Am. J. Physiol.* **264**, F480-F489.

Ihara, M., Noguchi, K., Saeki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M., and Yano, M. (1992) Biological profiles of highly potent novel endothelin antagonists selective for the ETA receptor. *Life Sci.* **50**, 247-55.

Iida, H., and Page, E. (1988) Inhibition of atrial natriuretic peptide secretion by forskolin in noncontracting cultured atrial myocytes. *Biochem. Biophys. Res. Commun.*, **157**, 330-336.

Iida, H., Barron, W.M., and Page, E. (1988) Monensin turns on microtubule-associated translocation of secretory granules in cultured rat atrial myocytes. *Circ. Res.* **62**, 1159-70.

Iida, H., Tanaka, S., and Shibata, Y. (1997) Small GTP-binding protein, Rab6, is associated with secretory granules in atrial myocytes. *Am. J. Physiol.* **272**, C1594-601.

Iida, H., Wang, L., Nishii, K., Ookuma, A., and Shibata Y. (1996) Identification of rab12 as a secretory granule-associated small GTP-binding protein in atrial myocytes. *Circ. Res.* **78**, 343-7.

Inagami, T., Naruse, M., and Hoover R (1995) Endothelium as an endocrine organ. *Ann. Rev. Physiol.* **57**, 171–189.

Ingber, D.E. (1997) Tensegrity: the architectural basis of cellular mechanotransduction. *Annu. Rev. Physiol.* **59**, 575-599.

Irons, C.E., Murray, S.F., and Glembotski, C.C. (1993) Identification of the receptor subtype responsible for endothelin-mediated protein kinase C activation and atrial natriuretic factor secretion from atrial myocytes. *J. Biol. Chem.* **31**, 23417-23421.

Ishihata, A., and Endoh, M. (1995) Species-related differences in inotropic effects of angiotensin II in mammalian ventricular muscle: receptors, subtypes and phosphoinositide hydrolysis. *Br. J. Pharmacol.* **114**, 447-453.

Ishikawa, Y., Homcy, C.J. (1997) The adenylyl cyclases as integrators of transmembrane signal transduction. Circ Res. **80**, 297-304.

Ito, T., Toki, Y., Siegel, N., Gierse, J.K., and Needleman, P. (1988) Manipulation of stretch-induced atriopeptin prohormone release and processing in the perfused rat heart. *Proc. Natl. Acad. Sci. USA* **85**, 8365-8369.

Jahn, R., and Sudhof, T.C. (1999) Membrane fusion and exocytosis. *Annu. Rev. Biochem.* **68**, 863-911.

Janicot, M., Fouque, F., and Desbuquois, B. (1991) Activation of rat liver adenylate cyclase by pertussis toxin by cholera toxin requires toxin internalization and processing in endosomes. *J. Biol. Chem.* **266**, 12858-12865.

Jiao, J-H., Baumann, P., Baron, A., Roath, A., Pence, R.A., and Baertschi, A.J. (2000) Sulfonylurea receptor ligands modulate stretch-induced ANF secretion in rat atrial myocyte culture. *Am. J. Physiol. Heart. Circ. Physiol.* **278**, H2028-H2038.

John, S.W., Krege, J.H., Oliver, P.M., Hagaman, J.R., Hodgin, J.B., Pang, S.C., Flynn, T.G., and Smithies, O.(1995) Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *Science*. **267**, 679-81

Jones, P.M., Mann, F.M., Persaud, S.J., and Wheeler-Jones, C.P. (1993) Mastoparan stimulates insulin secretion from pancreatic beta-cells by effects at a late stage in the secretory pathway. *Mol. Cell. Endocrinol.* **94**, 97-103.

Joyce-Brady, M., Rubins, J.B., Panchenko, M.P., Bernardo, J., Steele, M.P., Kolm, L., Simons, E.R., and Dickey, B.F. (1991) Mechanisms of mastoparan-stimulated surfactant secretion from isolated pulmonary alveolar type 2 cells. *J. Biol. Chem.* **266**, 6859-6865.

Kahn, R.A., Der, C.J., Bokoch, G.M. (1992) The ras superfamily of GTP-binding proteins: guidelines on nomenclature. *FASEB J.* **6**, 2512-3.

Kambayashi, Y., Nakao, K., Itoh, H., Hosoda, K., Saito, Y., Yamada, T., Mukoyama, M., Arai, H., Shirakami, G., Suga, S. (1989) Isolation and sequence determination of rat cardiac natriuretic peptide. *Biochem. Biophys. Res. Commun.* **163**, 233-40.

Kardestuncer, T., Wu, H., Lim, A.L., and Neer, E.J. (1998) Cardiac myocytes express mRNA for ten RGS proteins: changes in RGS mRNA expression in ventricular myocytes and cultured atria. *FEBS Lett.* **438**, 285-8.

Kamiguian, A., Zahraoui, A., and Tavitian, A. (1993) Identification of small GTP-binding rab proteins in human platelets: thrombin-induced phosphorylation of rab3B, rab6, and rab8 proteins. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 7647-51.

Kaslow, H.R., and Burns, D.L. (1992) Pertussis toxin and target eukaryotic cells: binding, entry, and activation. *FASEB J.* **6**, 2684-90.

Kaslow, H.R., and Lesikar, D.D. (1987) Sulfhydryl-alkylating reagents inactivate the NAD glycohydrolase activity of pertussis toxin. *Biochemistry.* **26**, 4397-402.

Kasuya, Y., Abe, Y., Hama, H., Sakurai, T., Asada, S., Masaki, T., and Goto, K. (1994) Endothelin-1 activates mitogen-activated protein kinases through two independent signalling pathways in rat astrocytes. *Biochem. Biophys. Res. Commun.* **204**,1325-33.

Katada, T., Tamura, M., and Ui, M. (1983) The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. *Arch Biochem Biophys.* **224**, 290-8.

Kawai, Y., and Arinze, I.J. (1996) Differential localization and development-dependent expression of G-protein subunits, Goα and Gβ, in rabbit Heart. *J. Mol. Cell. Cardiol.* **28**, 1555-1564.

Kettunen, R.V.J., Leppaluoto, J., Jounela, A., and Vuolteenaho, O. (1994) Plasma N-terminal atrial natriuretic peptide in acute myocardial infarction. *Am. Heart J.* **127**, 1449-1455.

Kiemer, A.K., and Vollmar, A.M. (2001) The atrial natriuretic peptide regulates the production of inflammatory mediators in macrophages. *Ann. Rheum. Dis.* **60**, Suppl 3:iii68-70.

Kim, E., Han, J., Ho, W., and Earm, Y.E. (1997) Modulation of ATP-sensitive K+ channels in rabbit ventricular myocytes by adenosine A1 receptor activation. *Am. J. Physiol.* **272**, H325-33.

Kim, S.H., Cho, K.W., Chang, S.H., Kim, S.Z., and Chae, S.W. (1997) Glibenclamide suppresses stretch-activated ANP secretion: involvements of K^{+}_{ATP} channel and L-type Ca^{2+} channel modulation. *Pflügers Arch.* **434**, 362-372.

Kinnunen, P., Vuolteenaho, O., and Ruskoaho, H. (1993) Mechanisms of atrial and brain natriuretic peptide release from rat ventricular myocardium : Effect of stretching. *Endocrinology.* **132**, 1961-1970.

Kirsch, G.E., Codina, J., Birnbaumer, L., and Brown, A.M. (1990) Coupling of ATP-sensitive K+ channels to A1 receptors by G proteins in rat ventricular myocytes. *Am. J. Physiol.* **28**, H820-H826.

Klumperman, J., Kuliawat, R., Griffith, J.M., Geuze, H.J., Arvan, P. (1998) Mannose 6-phosphate receptors are sorted from immature secretory granules via adaptor protein AP-1, clathrin, and syntaxin 6-positive vesicles. *J. Cell. Biol.* **141**, 359-71.

Knepper, M.A., Lankford, S.P., and Terada, Y. (1991) Renal tubular actions of ANF. *Can. J. Physiol. Pharmacol.* **69**, 1537-45.

Koizumi, S., Kataoka, Y., Niwa, M., Yamashita, K., Taniyama, K., and Kudo, Y. (1994) Endothelin increased $[Ca^{2+}]_i$ in cultured neurones and slices of rat hippocampus. *Neuroreport.* **5**, 1077-80.

Koller, K.J., Lowe, D.G., Bennett, G.L., Minamino, N., Kangawa, K., Matsuo, H., Goeddel, D.V. (1991) Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science.* **252**, 120-3.

Koller KJ, Goeddel DV. (1992) Molecular biology of the natriuretic peptides and their receptors. *Circulation.* **86**, 1081-8.

Komaru, T., Tanikawa, T., Sugimura, A., Kumagai, T., Sato, K., Kanatsuka, H., and Shirato, K. (1997) Mechanisms of coronary microvascular dilation induced by the activation of pertussis toxin-sensitive G proteins are vessel-size dependent. Heterogeneous involvement of nitric oxide pathway and ATP-sensitive K+ channels. *Circ. Res.* **80**, 1-10.

Komatsu, M., Aizawa, T., Yokokawa, N., Sato, Y., Okada, N., Takasu, N., and Yamada, T. (1992) Mastoparan-induced hormone release from rat pancreatic islets. *Endocrinology.* **130**, 221-228.

Komatsu, M., McDermott, A.M., Gillison, S.L., and Sharp, G.W.G. (1993) Mastoparan stimulation exocytosis at a Ca^{2+}-independent late site in stimulus-secretion coupling. *J. Biol. Chem.* **268**, 23297-23306.

Konrad, R.J., Young, R.A., Record, R.D., Smith, R.M., Butkerait, P., Manning, D., Jarett, L., and Wolf, B.A. (1995) The heterotrimeric G-protein G_i is localized to the insulin secretory granules of β-cells and is involved in insulin secretion. *J. Biol. Chem.* **270**, 12869-12876.

Krueger, K.M., and Barbieri, J.T. (1995) The family of bacterial ADP-ribosyalting exotoxins. *Clin. Microbiol. Rev.* **8**, 34-47.

Kuliawat, R. and Arvan, P. (1992). Protein targeting via the 'constitutive-like' secretory pathway in isolated pancreatic islets: passive sorting in the immature granule compartment. *J. Cell Biol.* **118**,521 -529.

Kuliawat, R. and Arvan, P. (1994). Distinct molecular mechanisms for protein sorting within immature secretory granules of pancreatic beta-cells. *J. Cell Biol.* **126**, 77-86.

Kuliawat, R., Klumperman, J., Ludwig, T. and Arvan, P. (1997). Differential sorting of lysosomal enzymes out of the regulated secretory pathway in pancreatic beta-cells. *J. Cell Biol.* **137**,595 -608.

Kurihara, H., Kitajima, K., Senda, T., Fujita, H., and Nakajima, T. (1986) Multigranular exocytosis induced by phospholipase A2-activators, melittin and mastoparan, in rat anterior pituitary cells. *Cell Tissue Res.* **243**, 311-316.

Kuroski-de Bold, M.L., and de Bold, A.J. (1991) Stretch-secretion coupling in atrial cardiocytes. Dissociation between atrial natriuretic factor release and mechanical activity. *Hypertension.* **18**, III169-178.

Laine, M., Id, L., Vuolteenaho, O., Weckstrom, M., and Ruskoaho, H. (1996) Role of calcium in stretch-induced release and mRNA synthesis of natriuretic peptides in isolated rat atrium. *Pflugers Arch.* **432**, 953-960.

Lang, C.C., Coutie, W.J., Struthers, A.D., Dhillon, D.P., Winter, J.H., and Lipworth, B.J. (1992) Elevated levels of brain natriuretic peptide in acute hypoxaemic chronic obstructive pulmonary disease. *Clin. Sci. (Lond.)* **83**, 529-33.

Lang, R.E., Tholken, H., Ganten, D., Luft, F.C., Ruskoaho, H., and Unger, T. (1985) Atrial natriuretic factor--a circulating hormone stimulated by volume loading. *Nature.* **314**, 264-6.

Lapinski, M., Stepniakowski, K., Januszewicz, A., Wocial, B., Chodakowska, J., Feltynowski, T., and Januszewicz, W. (1991) Plasma atrial natriuretic peptide concentration in patients with primary aldosteronism. *J. Hyperten.* **9**, S260-261.

Larsen, T.H., Huitfeldt, H.S., Myking, O., Saetersdal, T. (1993) Microtubule-associated distribution of specific granules and secretion of atrial natriuretic factor in primary cultures of rat cardiomyocytes. *Cell. Tissue Res.* **272**, 201-10.

Lasley, R.D., and Mentzer, R.M. (1993) Pertussis toxin blocks adenosine A1 receptor mediated protection of the ischemic rat heart. *J. Mol. Cell. Cardiol.* **25**, 815-21.

Leite, M.F., Page, E., and Ambler, S.K. (1994) Regulation of ANP secretion by endothelin-1 in cultured atrial myocytes: desensitization and receptor subtype. *Am. J. Physiol.* **267**, H2193-203.

Levin, E.R., Gardner, D.G., and Samson, W.K. (1998) Natriuretic peptides. *N. Engl. J. Med.* **339**, 321-8.

Lew, R.A., and Baertschi, A.J. (1992) Endothelium-dependent ANF secretion in vitro. *Am. J. Physiol.* **263**, H1071-7.

Leyte, A., Barr, F.A., Kehlenbach, R. H., and Huttner, W.B. (1992) Multiple trimeric G-proteins on the trans-Golgi network exert stimulatory and inhibitory effects on secretory vesicle formation. *EMBO J.* **11**, 4795-4804.

Lincoln, T.M, and Cornwell, T.L. (1993) Intracellular cyclic GMP receptor proteins. *FASEB J.* **7**, 328-38.

Lorenzo, A., Razzaboni, B., Weir, G.C., and Yankner, B.A. (1994) Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. *Nature.* **368**, 756-60.

Luetje, C.W., Tietje, K.M., Christian, J.L., and Nathanson, N.M. (1988) Differential tissue expression and developmental regulation of guanine nucleotide binding regulatory proteins and their messenger RNAs in rat heart. *J. Biol. Chem.* **263**,13357-65.

Lynch, D.R., Venable, J.C., and Snyder, S.H. (1988) Enkephalin convertase in the heart: similar disposition to atrial natriuretic factor. Endocrinology. **122**, 2683-91.

Macarthur, H., Warner, T.D., Wood, E.G., Corder, R., and Vane, J.R. (1994) Endothelin-1 release from endothelial cells in culture is elevated both acutely and chronically by short periods of mechanical stretch. *Biochem. Biophys. Res. Commun.* **200**, 395-400.

Maier, O., Ehmsen, E., and Westermann, P. (1995) Trimeric G protein alpha subunits of the Gs and Gi families localized at the Golgi membrane. *Biochem. Biophys. Res. Commun.* **208**, 135-43.

Mangat, H., and de Bold, A.J. (1993) Stretch-induced atrial natriuretic factor release utilizes a rapidly depleting pool of newly synthesized hormone. *Endocrinology.* **133**, 1398-1403.

Mäntymaa, P., Leppaluoto, J., and Ruskoaho, H. (1990) Endothelin stimulates basal and stretch-induced atrial natriuretic peptide secretion from the perfused rat heart. *Endocrinology.* **126**, 587-95.
Marchioli, C.C., and Graziano, S.L. (1997) Paraneoplastic syndromes associated with small lung cancer. *Chest Surg. Clin. N. Am.* **7**, 65-80.

Mark, M.D., and Herlitz, S. (2000) G-protein mediated gating of inward-rectifier K+ channels. *Eur. J. Biochem.* **267**, 5830-6.

Mebazaa, A., Mayoux, E., Maeda, K., Martin, L.D., Lakatta. E.G., Robotham, J.L., and Shah, A.M. (1993) Paracrine effects of endocardial endothelial cells on myocyte contraction mediated via endothelin. *Am. J. Physiol.* **265**, H1841-6.

Meininger, G.A., and Davis, M.J. (1992) Cellular mechanisms involved in the vascular myogenic response. *Am. J. Physiol.* **263**, H647-59.

Melo, L.G., Veress, A.T., Ackermann, U., Pang, S.C., Flynn, T.G., and Sonnenberg, H. (1999) Chronic hypertension in ANP knockout mice: contribution of peripheral resistance. *Regul. Pept.* **79**, 109-15.

Meszaros, J.G., Gonzalez, A.M., Endo-Mochizuki, Y., Villegas, S., Villarreal, F., and Brunton, L. (2000) Identification of G protein-coupled signalling pathways in cardiac fibroblasts: cross talk between Gq and Gs. *Am. J. Physiol. Cell. Physiol.* **278**, C154-C162.

Mikawa, K., Kume, H., and Takagi, K. (1998) Effects of atrial natriuretic peptide and 8-brom cyclic guanosine monophosphate on human tracheal smooth muscle. *Arzneimittelforschung.* **48**, 914-8.

Milligan, G. (1993) Agonist regulation of cellular G protein levels and distribution: mechanisms and functional implications. *Trends Pharmacol.Sci.* **14**, 413-8.

Misonou, H., Ohara-Imaizumi, M., and Kumakura, K. (1997) Regulation of the priming of exocytosis and the dissociation of SNAP-25 and VAMP-2 in adrenal chromaffin cells. *Neurosci. Lett.* **232**, 182-4.

Molenaar, P., O'Reilly, G., Sharkey, A., Kuc, R.E., Harding, D.P., Plumpton, C., Gresham, G.A., and Davenport, A.P. (1993) Characterization and localization of endothelin receptor subtypes in the human atrioventricular conducting system and myocardium. *Circ. Res.* **72**, 526–538.

Montecucco, C., Tomasi, M., Schiavo, G., and Rappuoli, R. (1986) Hydrophobic photolabelling of pertussis toxin subunits interacting with lipids. *FEBS Lett.***194**, 301-4.

Montorsi, P., Tonolo, G., Polonia, J., Hepburn, D., and Richards, A.M. (1987) Correlates of plasma atrial natriuretic factor in health and hypertension. *Hypertension.* **10**, 570-6.

Morris, C.E., and Homann, U. (2001) Cell surface area regulation and membrane tension. *J. Membr. Biol.* **179**, 79-102.

Morris, R.E., Gerstein, A.S., Bonventre, P.F., and Saelinger, C.B. (1985) Receptor-mediated entry of diphtheria toxin into monkey kidney (Vero) cells: electron microscopic evaluation. *Infect. Immun.* **50**, 721-7.

Moses, A.M., Jones, C., and Yucha, C.B. (1990) Effects of sodium intake, furosemide, and infusion of atrial natriuretic peptide on the urinary and metabolic clearances of arginine vasopressin in normal subjects. *J. Clin. Endocrinol. Metab.* **70**, 222-9.

Moss, J., Stanley, S.J., Watkins, P.A., Burns, D.L., Manclark, C.R., Kaslow, H.R., and Hewlett, E.L. (1986) Stimulation of the thiol-dependent ADP-ribosyltransferase and NAD glycohydrolase activities of Bordetella pertussis toxin by adenine nucleotides, phospholipids, and detergents. *Biochemistry.* **25**, 2720-5.

Mousli, M., Bronner, C., Landry, Y., Bockaert, J., and Rouot, B. (1990) Direct activation of GTP-binding regulatory proteins (G-proteins) by substance P and compound 48/80. *Febs letters.* **259**, 260-262.

Mousli, M., Bueb, J-L., Bronner, C., Rouot, B., and Landry, Y. (1990) G protein activation: a receptor-independent mode of action for cationic amphiphilic neuropeptides and venom peptides. *TiPS.* **11**, 358-362.

Muir, T.M., Hair, J., Inglis, G.C., Dow, J.W., Lindop, G.B., and Leckie, B.J. (1993) Hormonal control of atrial natriuretic peptide synthesis and secretion from cultured atrial myocytes. *J. Mol. Cell. Cardiol.* **25**, 509-518.

Muller, L., Picart, R., Barret, A., Bockaert, J., Homburger, V., and Tougard, C. (1994) Identification of multiple subunits of heterotrimeric G proteins on the membrane of secretory granules of rat prolactin anterior pituitary cells. *Mol. & Cell. Neuro.* **5**, 556-566.

Mumby, S.M. (2002) Subcellular localization of G protein subunits. *Methods Enzymol.* **344**, 383-403.

Nakayama, K., Ohkubo, H., Hirose, T., Inayama, S., and Nakanish, S. (1984) mRNA sequence for human cardiodilatin-atrial natriuretic factor precursor and regulation of precursor mRNA in rat atria. *Nature.* **310**, 699-701.

Neer, E.J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell.* **80**, 249-257.

Neubig, R.R. (1994) Membrane organization in G-protein mechanisms. *FASEB J.* **8**, 939-946.

Neves, S.R., Prahlad, T., and Iyengar, R. (2002) G protein pathways. *Science.* **296**, 1636-1639.

Newman, T.M., and Severs, N.J. (1990) Arrested exocytosis of atrial secretory granules. *J. Mol. Cell. Cardiol.* **22**, 771-86.

Newman, T.M., and Severs, N.J. (1993) Stretch and anesthetic dependency of atrial natriuretic peptide release demonstrated by an ultrastructural assay. *J. Cell. Physiol.* **155**, 240-247.

Noma, A. (1983) ATP-regulated K+ channels in cardiac muscle. *Nature.* **314**, 147-148.

Novikoff, A.B., Mori, M., Quintana, N., and Yam, A. (1977) Studies of the secretory process in the mammalian exocrine pancreas. I. The condensing vacuoles. *J. Cell Biol.* **75**, 148-65.

Numberg, B., and Ahnert-Hilger, G. (1996) Potential roles of heterotrimeric G proteins of the endomembrane system. *FEBS Lett.* **389**, 61-5.

Ogawa, T., Vatta, M., Bruneau, B.G., and de Bold, A.J. (1999) Characterization of natriuretic peptide production by adult heart atria. *Am. J. Physiol.* **276**, H1977-86.

Ohara-Imaizumi, M., Kameyama, K., Kawae, N., Takeda, K., Muramatsu, S., and Kumakura, K. (1992) Regulatory role of the GTP-binding protein, G(o), in the mechanism of exocytosis in adrenal chromaffin cells. *J Neurochem.* **58**, 2275-84.

Ohnishi, H., Ernst, S.A., Yule, D.I., Baker, C.W., and Williams, J.A. (1997) Heterotrimeric G-protein Gq/11 localized on pancreatic zymogen granules is involved in calcium-regulated amylase secretion. *J. Biol. Chem.* **272**, 16056-16061.

Omland, T., Bonarjee, W.S., Nilsen, D.W.T, Sundsfjord, J.A., Lie, R.T., Thibault, G., and Dickstein, K. (1993) Prognostic significance of N-terminal proatrial natriuretic factor (1-98) in acute myocardial infarction: comparison with atrial natriuretic factor (99-126) and clinical evaluation. *Brit. Heart J.* **70**, 409-414.

Opocher, G., Rocco, S., Carpene, G., Pedini, F., Scarante, M., Milani, R., Boscaro, M., and Mantero, F. (1990) Atrial natriuretic peptide in Cushing's disease. *J. Endocrinol. Invest.* **13**, 133-137.

Orci, L. (1982). Macro and micro-domains in the endocrine pancreas. *Diabetes.* **31**, 538 -565.

Orci, L. Ravazzola, M., Amherdt, M., Louvard, D. and Perrelet, A. (1985). Clathrin-immunoreactive sites in the Golgi apparatus are concentrated at the trans pole in polypeptide hormone-secreting cells. *Proc. Natl. Acad. Sci. USA.* **82**,5385-5389.

Orci, L., Halban, P., Amherdt, M., Ravazzola, M., Vassalli, J.-D. and Perrelet, A. (1984). A clathrin-coated, Golgi-related compartment of the insulin secreting cell accumulates proinsulin in the presence of monensin. *Cell.* **39**,39-47.

Orci, L., Le Marchand, Y., Singh, A., Assimacopoulos-Jeannet, F., Rouiller, C., and Jeanrenaud, B. (1973) Letter: Role of microtubules in lipoprotein secretion by the liver. *Nature.* **244**, 30-2.

Orci, L., Palmer, D.J., Ravazzola, M., Perrelet, A., Amherdt, M., and Rothman, J.E. (1993) Budding from Golgi membranes requires the coatomer complex of non-clathrin coat proteins. *Nature.* **362**, 648-52.

Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J.D., and Anderson, R.G. (1986) Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles. *J. Cell. Biol.* **103**, 2273-81.

Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E., and Wilson, M.C. (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. *J. Cell. Biol.* **109**, 3039-52.

Ozaki, Y., Matsumoto, Y., Yatomi, Y., Higashihara, M., Kariya, T., and Kume, S. (1990) Mastoparan, a wasp venom, activates platelets via pertussis toxin-sensitive GTP-binding proteins. *Biochem. Biophys. Res. Commun.* **1990**,779-785.

Padfield, P.J., and Panesar, N. (1997) Identification of Goα, Gqα, and Gsα immunoreactivity associated with the rat pancreatic zymogen granule membrane. *Biochem. Biophys. Res. Comm.* **237**, 235-238.

Page, E., Upshaw-Earley, J., Goings, G.E., and Hanck, D.A. (1991) Inhibition of atrial peptide secretion at different stages of the secretory process: Ca^{2+} dependence. *Am. J. Physiol.* **261**, C1162-72.

Palade G. (1975) Intracellular aspects of the process of protein synthesis. *Science.***189**, 347-58.

Park, H.S., Lee, S.Y., Kim, Y.H., Kim, J.Y., Lee, S.J., Choi, M-U. (2000) Membrane perturbation by mastoparan 7 elicits a broad alteration in lipid composition of L1210 cells. *Biochimica et Biophysica Acta.* **1484**, 151-162.

Parlapiano, C., Campana, E., Alessandri, N., Rota, C., Sellini, M., Vecci, E., Tonnarini, G., Borgia, M.C., and Negri, M. (1998) Plasma atrial natriuretic hormone in hyperthyroidism. *Endocri. Res.* **24**, 105-112.

Pedersen, M.M., Christiansen, J.S., Pedersen, E.B., and Mogensen, C.E. (1992) Determinations of intra-individual variation in kidney function in normoalbuminuric insulin-dependent diabetic patients: importance of atrial natriuretic peptide and glycaemic control. *Clin. Sci.* **83**, 445-451.

Pedram, A., Razandi, M., Kehrl, J., and Levin, E.R. (2000) Natriuretic peptides inhibit G protein activation. *J. Biol. Chem.* **275**, 7365-7372.

Pedram, A., Razandi, M., and Levin, E.R. (2001) Natriuretic peptides suppress vascular endothelial cell growth factor signaling to angiogenesis. *Endocrinology.* **142**, 1578-86.

Pfaffinger, P.J., Martin, J.M., Hunter, D.D., Nathanson, N.M., and Hille, B. (1985) GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature.* **317**, 536-8.

Piacentini, L., Wainwright, C.L., and Parratt, J.R. (1993) The antiarrhythmic effect of ischaemic preconditioning in isolated rat heart involves a pertussis toxin sensitive mechanism. *Cardiovasc. Res.* **27**, 674-80.

Pimplikar, S.W., and Simons, K. (1993) Regulation of apical transport in epithelial cells by a Gs class of heterotrimeric G protein. *Nature.* **362**, 456-8.

Pollock, D.M. (2001) Endothelin antagonists in the treatment of renal failure. *Curr. Opin. Investig. Drugs.* **2**, 513-20.

Pruss, R.M., and Shepard, E.A. (1987) Cytochrome b_{561} can be detected in many neuroendocrine tissues using a specific monoclonal antibody. *Neuroscience.* **22**, 149-57.

Quitterer, U., and Lohse, M. (1999) Crosstalk between $G\alpha_i$- and $G\alpha_q$- coupled receptors is mediated by $G_{\beta\gamma}$ exchange. *Proc. Natl. Acad. Sci. USA.* **96**, 10626-10631.

Ramaciotti, C., McClellan, G., Sharkey, A., Rose, D., Weisberg, A., and Winegrad, S. (1993) Cardiac endothelial cells modulate contractility of rat heart in response to oxygen tension and coronary flow. *Circ. Res.* **72**, 1044-64.

Redondo, J., Bishop, J.E., and Wilkins, M.R. (1998) Effect of atrial natriuretic peptide and cyclic GMP phosphodiesterase inhibition on collagen synthesis by adult cardiac fibroblasts. *Br. J. Pharmacol.* **124**, 1455-62.

Ribalet, B., and Eddlestone, G.T. (1995) Characterization of the G protein coupling of a somatostatin receptor to the K^+ATP channel in insulin-secreting mammalian HIT and RIN cell lines. *J. Physiol.* **485**, 73-86.

Ribi, H.O., Ludwig, D.S., Mercer, K.L, Schoolnik, G.K., and Kornberg, R.D. (1988) Three-dimensional structure of cholera toxin penetrating a lipid membrane. Science. **239**, 1272-6.

Richards, A.M. (1989) Atrial natriuretic factor administered to humans: 1984-1988. *J. Cardiovasc. Pharmacol.* **13**, S69-74.

Richards AM, McDonald D, Fitzpatrick MA, Nicholls MG, Espiner EA, Ikram H, Jans S, Grant S, Yandle T. (1988) Atrial natriuretic hormone has biological effects in man at physiological plasma concentrations. *J. Clin. Endocrinol. Metab.* **67**, 1134-9.

Richards AM, Nicholls MG, Ikram H, Webster MW, Yandle TG, Espiner EA. (1985) Renal, haemodynamic, and hormonal effects of human alpha atrial natriuretic peptide in healthy volunteers. *Lancet.* **1**, 545-9.

Robertson, R.P., Seaquist, E.R., and Walseth, T.F. (1991) G proteins and modulation of insulin secretion. *Diabetes.* **40**, 1-6.

Ross, E.M., and Higashijima, T. (1994) Regulation of G-protein activation by mastoparans and other cationic peptides. *Methods in Enzymology.* **237**, 26-37.

Rossetto, O., Gorza, L., Schiavo, G., Schiavo, N., Scheller, R.H., and Montecucco, C. (1996) VAMP/synaptobrevin isoforms 1 and 2 are widely and differentially expressed in non neuronal tissues. J Cell Biol. **132**, 167-79.

Rubanyi, G.M., and Botelho, L.H. (1991) Endothelins. *FASEB J.* **5**, 2713-20.

Rubanyi, G.M., Freay, A.D., Kauser, K., Johns, A., and Harder, D.R. (1990) Mechanoreception by the endothelium: mediators and mechanisms of pressure- and flow-induced vascular responses. *Blood Vessels.* **27**, 246-57.

Ruskoaho, H. (1992) Atrial natriuretic peptide: synthesis, release, and metabolism. *Pharmacol. Rev.* **44**, 479-601.

Ruskoaho, H., Leskinen, H., Magga, J., Taskinen, P., Mantymaa, P., Vuolteenaho, O., and Leppaluoto, J. (1997) Mechanisms of mechanical load-induced atrial natriuretic peptide secretion: role of endothelin, nitric oxide, and angiotensin II. J Mol Med. **75**, 876-85.

Ruskoaho, H., Toth, M., Ganten, D., Unger, T.H., and Lang, R.E. (1986) The phorbol ester induced atrial natriuretic peptide secretion is stimulated by forskolin and BAY K 8644 and inhibited by 8-bromo-cyclic GMP. *Biochem. Biophys. Res. Commun.* **139**, 266-274.

Ruskoaho, H., Vuolteenaho, O., and Leppaluoto, J. (1990) Phorbol esters enhance stretch-induced atrial natriuretic peptide secretion. *Endocrinology.* **127**, 2445-2455.

Sadoshima, J., and Izumo, S. (1993) Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J.* **12**, 1681-1692.

Sadoshima, J., and Izumo, S. (1997) The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu. Rev. Physiol.* **59**, 551-571.

Sadoshima, J., Takahashi, N., Jahn, L., and Izumo, S. (1992) Roles of mechano-sensitive ion channels, cytoskeleton, and contractile activity in stretch-induced immediate-early gene expression and hypertrophy of cardiac myocytes. *Proc. Natl. Acad. Sci. USA* **89**, 9905-9909.

Sakura, H., Ammala, C., Smith, P.A., Gribble, F.M., and Ashcroft, F.M. (1995) Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic beta-cells, brain, heart and skeletal muscle. *FEBS Lett.* **377**, 338-44.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., and Masaki, T. (1990) Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature.* **348**, 732-5.

Salminen, A., and Novick, P.J. (1987) A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell.* **49**, 527-38.

Salo, J., Jemenez, W., Kuhn, M., Gines, A., Gines, P., Fernandez-Esparrach, G., Angeli, P., Claria, J., Bataller, R., Arroyo, V., Forssmann, W.G., and Rodes, J. (1996) Urinary excretion of urodilatin in patients with cirrhosis. *Hepatology.* **24**, 1428-1432.

Samson, W.R., and Levin, E.R. (1997) Natriuretic peptides in health and disease. In Humana Press. Totowa, New Jersey.

Sandvig, K. (2001) Shiga toxins. *Toxicon.* **39**, 1629-35.

Sano, T., Morishita, Y., Matsuda, Y., and Yamada, K. (1992) Pharmacological profile of HS-142-1, a novel nonpeptide atrial natriuretic peptide antagonist of microbial origin. I. Selective inhibition of the actions of natriuretic peptides in anesthetized rats. *J. Pharmacol. Exp. Ther.* **260**, 825-31.

Sano, T., Morishita, Y., Yamada, K., and Matsuda, Y. (1992) Effects of HS-142-1, a novel non-peptide ANP antagonist, on diuresis and natriuresis induced by acute volume expansion in anesthetized rats. *Biochem. Biophys. Res. Commun.* **182**, 824-9.

Sarda, I.R., de Bold, M.L., and de Bold, A.J. (1989) Optimization of atrial natriuretic factor radioimmunoassay. *Clin. Biochem.* **22**, 11-15.

Schiebinger, R.J., and Gomez-Sanchez, C.E. (1990) Endothelin: a potent stimulus of atrial natriuretic peptide secretion by super-fused rat atria and its dependency on calcium. *Endocrinology.* **127**, 119–125.

Schiebinger, R.J., and Greening, K.M. (1992) Interaction between stretch and hormonally stimulated atrial natriuretic peptide secretion. *Am. J. Physiol.* **262**, H78-H83.

Schiffrin, E.L. (2001) A critical review of the role of endothelial factors in the pathogenesis of hypertension. *J. Cardiovasc. Pharmacol.* **38**, S3-6.

Schliwa, M. (1984) Mechanisms of intracellular organelle transport. *Cell. Muscle. Motil.* **5**, 403-6.

Schultz, J. E. J., Hsu, A. K., Barbieri, J.T., Li, P-L., and Gross, G. (1998) Pertussis toxin abolishes the cardioprotective effect of ischemic preconditioning in intact rat heart. *Am. J. Physiol.* **275**, H495-H500.

Schulz, S., and Waldman, S.A. (1999) The guanylyl cyclase family of natriuretic peptide receptors. *Vitam. Horm.* **57**, 123-51.

Schulz, S., Singh, S., Bellet, R.A., Singh, G., Tubb, D.J., Chin, H., and Garbers, D.L. (1989) The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell.* **58**, 1155-62.

Schwartz, D., Geller, D.M., Manning, P.T., Siegel, N.R., Fok, K.F., Smith, C.E., and Needleman, P. (1985) Ser-Leu-Arg-Arg-atriopeptin III: the major circulating form of atrial peptide. *Science.* **229**, 397-400.

Segev, N., Mulholland, J., and Botstein, D. (1988) The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell.* **52**, 915-24.

Sei, C.A., and Glembotski, C.C. (1990) Calcium dependence of phenylephrine-, endothelin-, and potassium chloride-stimulated atrial natriuretic factor secretion from long term primary neonatal rat atrial cardiocytes. *J. Biol. Chem.* **265**, 7166–7172.

Seidah, N.G., Cromlish, J.A., Hamelin, J., Thibault, G., and Chretien, M. (1986) Homologous IRCM-serine protease 1 from pituitary, heart atrium and ventricle: a common pro-hormone maturation enzyme? *Biosci. Rep.* **6**, 835-44.

Seko, Y., Seko, Y., Takahashi, N., Shibuya, M., and Yazaki, Y. (1999) Pulsatile stretch stimulates vascular endothelial growth factor (VEGF) secretion by cultured rat cardiac myoctyes. *Biochem. Biophys. Res. Commun.* **254**, 462-465.

Sekura, R.D., Fish, F., Manclark, C.R., Meade, B., Zhang, Y.L. (1983) Pertussis toxin. Affinity purification of a new ADP-ribosyltransferase. *J. Biol. Chem.* **258**, 14647-51.

Sengenes, C., Berlan, M., De Glisezinski, I., Lafontan, M., and Galitzky, J. (2000) Natriuretic peptides: a new lipolytic pathway in human adipocytes. *FASEB J.* **14**, 1345-51.

Serizawa, T., Hirata, Y., Kohmoto, O., Iizuka, M., Matsuoka, H., Sato, H., Takahashi, T., Mochizuki, T., Ishii, M., Sugimoto, T. (1988) Acute hemodynamic effects of alpha human atrial natriuretic polypeptide in patients with congestive heart failure. *Jpn. Heart J.* **29**, 143-9.

Shields, P.P., and Glembotski, C.C. (1989) Regulation of atrial natriuretic factor-(99-126) secretion from neonatal rat primary atrial cultures by activators of protein kinases A and C. *J. Biol. Chem.* **264**, 9322-9328.

Shraga-Levine, Z., and Sokolovsky, M. (1996) cGMP formation in rat atrial slices is ligand and endothelin receptor subtype specific. *Circ. Res.* **78**, 424-30.

Shraga-Levine, Z., and Sokolovsky, M. (2000) Functional coupling of G proteins of endothelin receptors is ligand and receptor subtype specific. *Cell. & Mol. Neurobiol.* **20**, 305-317.

Shraga-Levine, Z., Galron, R., and Sokolovsky, M. (1994) Cyclic GMP formation in rat cerebellar slices is stimulated by endothelins via nitric oxide formation and by sarafotoxins via formation of carbon monoxide. *Biochemistry.* **33**, 14656-9.

Simonson, M.S., and Dunn, M.J. (1990) Cellular signaling by peptides of the endothelin gene family. *FASEB J.* **4**, 2989-3000.

Singer DR, Markandu ND, Buckley MG, Miller MA, Sugden AL, Sagnella GA, MacGregor GA. (1989) Prolonged decrease in blood pressure after atrial natriuretic peptide infusion in essential hypertension: a new anti-pressor mechanism? *Clin. Sci.* **77**, 253-8

Singh, J. (1982) Stretch stimulates cyclic nucleotide metabolism in the isolated frog ventricle. *Pflügers Arch.* **395**,162-164.

Skoglund, G., Basmaciogullari, A., Rouot, B., Marie, J-C., and Rosselin, G. (1999) Cell-specific localization of G protein α-subunits in the islets of Langerhans. *J. Endocrinol.* **162**, 31-37.

Skvorak, J.P., and Dietz, J.R. (1997) Endothelin and nitric oxide interact to regulate stretch-induced ANP secretion. *Am. J. Physiol.* **273**, R301-R306.

Skvorak, J.P., Nazian, S.J., and Dietz, J.R. (1995) Endothelin acts as paracrine regulator of stretch-induced atrial natriuretic peptide release. *Am. J. Physiol.* **269**, R1093-R1098.

Skvorak, J.P., Sutton, E.T., Rao, P.S., and Dietz, J.R. (1996) Mechanism of anoxia-induced atrial natriuretic peptide release in the isolated rat atria. *Am. J. Physiol.* **271**, R237-R243.

Sokolovsky, M. (1993) Endothelin receptors in rat cerebellum: activation of phosphoinositide hydrolysis is transduced by multiple G-proteins. *Cell. Signal.* **5**, 473-83.

Sokolovsky, M.(1993) Functional coupling between endothelin receptors and multiple G-proteins in rat heart myocytes. *Receptors Channels.* **1**, 295-304.

Sokolovsky, M., Shraga-Levine, Z., Galron, R. (1994) Ligand-specific stimulation/inhibition of cAMP formation by a novel endothelin receptor subtype. *Biochemistry.* **33**, 11417-9.

Solomon, L.R., Atherton, J.C., Bobinski, H., Hillier, V., and Green, R. (1988) Effect of low dose infusion of atrial natriuretic peptide on renal function in man. *Clin. Sci.* **75**, 403-10.

Spiegel, A.M. (1997) The molecular basis of disorders caused by defects in G proteins. *Horm. Res.* **47**, 89-96.

Stasch, J.P., Hirth-Dietrich, C., Kazda, S., and Neuser, D. (1989) Endothelin stimulates release of atrial natriuretic peptides in vitro and in vivo. *Life Sci.* **45**, 869–875.

Stein, B.C., and Levin, R.I. (1998) Natriuretic peptides: physiology, therapeutic potential, and risk stratification in ischemic heart disease. *Am. Heart. J.* **135**: 914-923.

Steinberg, S.F., and Brunton, L.L. (2001) Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. *Annu. Rev. Pharmacol. Toxicol.* **41**, 751-773.

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

Steinhelper, M.E., Cochrane, K.L., and Field, L.J. (1990) Hypotension in transgenic mice expressing atrial natriuretic factor fusion genes. *Hypertension.* **16**, 301-7.

Stoorvogel, W., Oorschot, V., Geuze, H.J. (1996) A novel class of clathrin-coated vesicles budding from endosomes. *J. Cell Biol.* **132**, 21-33.

Stow, J.L., de Almeida, J.B., Narula, N., Holtzman, E.J., Ercolani, L., and Ausiello, D.A. (1991) A heterotrimeric G protein, G alpha i-3, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK1 epithelial cells. *J Cell Biol.* **114**, 1113-24.

Straub, R.H., Hall, C., Kramer, B.K., Elbracht, R., Palitzsch, K.D., Lang, B., and Scholmerich, J. (1996) Atrial natriuretic factor and digoxin-like immunoreactive factor in diabetic patients: their interrelation and the influence of the autonomic nervous system. *J. Clin. Endocrinol. Metab.* **81**, 3385-3389.

Straub, S.G., James, R.F., Dunne, M.J., and Sharp, G.W. (1998) Glucose augmentation of mastoparan-stimulated insulin secretion in rat and human pancreatic islets. *Diabetes.* **47**, 1053-7.

Suga, S., Nakao, K., Hosoda, K., Mukoyama, M., Ogawa, Y., Shirakami, G., Arai, H., Saito, Y., Kambayashi, Y., Inouye, K., and Imura, H. (1992) Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology.* **130**, 229-39.

Sugawara, A., Nakao, K., Sakamoto, M., Morii, N., Yamada, T., Itoh, H., Shiono, S., Imura, H. (1985) Plasma concentration of atrial natriuretic polypeptide in essential hypertension. *Lancet* **2**, 1426-1427.

Suh, B.C., Lee, I.S., Chae, H.D., Han, S., and Kim, K.T. (1998) Characterization of Mas-7-induced pore formation in SK-N-BE(2)C human neuroblastoma cells. *Mol. Cells.* **8**,162-168.

Suh, B-C., Song, S-K., Kim, Y-K., and Kim, K-T. (1996) Induction of cytosolic Ca^{2+} elevation mediated by Mas-7 occurs through membrane pore formation. *J. Biol. Chem.* **271**, 32753-32759.

Sukumar, M., and Higashijima, T. (1992) G protein-bound conformation of mastoparan-X, a receptor-mimetic peptide. *J. Biol. Chem.* **267**, 21421-4.

Sunahara, R.K., Dessauer, C.W., and Gilman, A.G. (1996) Complexity and diversity of mammalian adenylyl cyclases. *Annu. Rev. Pharmacol. Toxicol.* **36**, 461-80.

Suzuki, E., Hirata, Y., Kohmoto, O., Sugimoto, T., Hayakawa, H., Matsuoka, H., Sugimoto, T., Kojima, M., Kangawa, K., Minamino, N., and Matsuo, H. (1992) Cellular mechanisms for synthesis and secretion of atrial natriuretic peptide and brain natriuretic peptide in cultured rat atrial cells. *Circ Res.* **71**, 1039-48.

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M., and Ishii, S. (1982) Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry.* **21**, 5516-22.

Tanuma, S., Kawashima, K., and Endo, H. (1987) An NAD: cysteine ADP-ribosyltransferase is present in human erythrocytes. *J Biochem (Tokyo).* **101**, 821-4.

Tavi, P., Laine, M., Voutilainen, S., Lehenkari, P., Vuolteenaho, O., Ruskoaho, H., and Weckström, M. (1999) Potentiation of stretch-induced atrial natriuretic peptide secretion by intracellular acidosis. *Am. J. Physiol.* **277**, H405-H412.

Tavi, P., Weckstrom, M., and Ruskoaho, H. (2000) cAMP- and cGMP-independent stretch-induced changes in the contraction of rat atrium. *Pflugers Arch.* **441**, 65-68.

Tellam, J.T., Macaulay, S.L., McIntosh, S., Hewish, D.R., Ward, C.W., and James, D.E. (1997) Characterization of Munc-18c and syntaxin-4 in 3T3-L1 adipocytes. Putative role in insulin-dependent movement of GLUT-4. *J. Biol. Chem.* **272**, 6179-86.

Terzic, A., Tung, R.T., Inanobe, A., Katada, T., Kurachi, Y. (1994) G proteins activate ATP-sensitive K^+ channels by antagonizing ATP-dependent gating. *Neuron.* **12**, 885-93.

Thibault, G., Amiri, F., and Garcia, R. (1999) Regulation of natriuretic peptide secretion by the heart. *Annu. Rev. Physiol.* **61**, 193-217.

Thibault, G., Doubell, A.F., Garcia, R., Lariviere, R., and Schiffrin, E.L. (1994) Endothelin-stimulated secretion of natriuretic peptides by rat atrial myocytes is mediated by endothelin A receptors. *Circ Res.* **74**, 460-70.

Thibault, G., Garcia, R., Gutkowska, J., Lazure, C., Seidah, N.G., Chretien, M., Genest, J., and Cantin, M. (1986) Identification of the released form of atrial natriuretic factor by the perfused rat heart. *Proc. Soc. Exp. Biol. Med.* **182**, 137-41.

Thibault, G., Haile-Meskel, H., Wrobel-Konrad, E., Ballak, M., Garcia, R., Genest, J., Cantin, M. (1989) Processing of the atrial natriuretic factor propeptide by atrial cardiocytes as revealed by immunocryoultramicrotomy. *Endocrinology.* **124**, 3109-16.

Thibault, G., Lazure, C., Schiffrin, E.L., Gutkowska, J., Chartier, L., Garcia, R., Seidah, N.G., Chretien, M., Genest, J., and Cantin, M. (1985) Identification of a biologically active circulating form of rat atrial natriuretic factor. *Biochem. Biophys. Res. Commun.* **130**, 981-6.

Thören, P., Mark, A.L., Morgan, D.A., O'Neill, T.P., Needleman, P., and Brody, M.J. (1986) Activation of vagal depressor reflexes by atriopeptins inhibits renal sympathetic nerve activity. *Am. J. Physiol.* **251**, H1252-9.

Todaka, K., Ogino, K., Gu, A., and Burkhoff, D. (1998) Effects of ventricular stretch on the contractile strength, calcium transient, and cAMP in intact canine hearts. *Am. J. Physiol.* **274**, H990-H1000.

Tomita, U., Inanobe, A., Kobayashi, I., Takahashi, K., Ui, M., and Katada, T. (1991) Direct interactions of mastoparan and compound 48/80 with GTP-binding proteins. *J. Biochem (Tokyo).* **109**, 184-9.

Tooze J, Tooze SA. (1986) Clathrin-coated vesicular transport of secretory proteins during the formation of ACTH-containing secretory granules in AtT20 cells. *J. Cell. Biol.* **103**, 839-50.

Tooze, J., and Burke, B. (1987) Accumulation of adrenocorticotropin secretory granules in the midbody of telophase AtT20 cells: evidence that secretory granules move anterogradely along microtubules. *J. Cell. Biol.* **104**, 1047-57.

Tooze, J., Hollinshead, M., Frank, R., and Burke, B. (1987) An antibody specific for an endoproteolytic cleavage site provides evidence that pro-opiomelanocortin is packaged into secretory granules in AtT20 cells before its cleavage. *J Cell Biol.* **105**, 155-62.

Tooze, S.A. (1991) Biogenesis of secretory granules. Implications arising from the immature secretory granule in the regulated pathway of secretion. *FEBS Lett.* **285**, 220-4.

Tooze, S.A. (1998) Biogenesis of secretory granules in the trans-Golgi network of neuroendocrine and endocrine cells. *Biochim. Biophys. Acta.* **1404**, 231-44.

Tooze, S.A., and Huttner, W.B. (1990) Cell-free protein sorting to the regulated and constitutive secretory pathways. *Cell.* **60**, 837-47.

Tooze, S.A., and Stinchcombe, J.C. (1992) Biogenesis of secretory granules. *Semin. Cell. Biol.* **3**, 357-66.

Tooze, S.A., Martens, G.J., and Huttner, W.B. (2001) Secretory granule biogenesis: rafting to the SNARE. *Trends Cell. Biol.* **11**, 116-22.

Tooze, S.A., Weiss, U., and Huttner, W.B. (1990) Requirement for GTP hydrolysis in the formation of secretory vesicles. *Nature.* **347**, 207-8.

Toutant, M., Aunis, D., Bockaert, J., Homburger, V., and Rouot, B. (1987) Presence of three pertussis toxin substrates and Go alpha immunoreactivity in both plasma and granule membranes of chromaffin cells. *FEBS Lett.* **215**, 339-44.

Uehlinger, D.E., Weidmann, P., Gnadinger, M.P., Hasle,r L., Bachmann, C., Shaw, S., Hellmuller, B., and Lang, R.E. (1986) Increase in circulating insulin induced by atrial natriuretic peptide in normal humans. *J. Cardiovasc. Pharmacol.* **8**, 1122-9.

Ui, M., Katada, T., and Tamura, M. (1985) ADP-ribosylation of a membrane protein catalyzed by islet-activating protein, pertussis toxin. In: E. Althaus, H. Hilz, S. Shall (eds.): ADP-ribosylation of proteins. pp. 551-561. Springer-Verlag, Berlin 1985.

Urbé S, Page LJ, and Tooze SA. (1998) Homotypic fusion of immature secretory granules during maturation in a cell-free assay. *J. Cell. Biol.* **143**, 1831-44.

Urbé, S., Tooze, S.A., and Barr FA. (1997) Formation of secretory vesicles in the biosynthetic pathway. *Biochim. Biophys. Acta.* **1358**, 6-22.

Uusimaa, P., Ruskoaho, H., Vuolteenaho, O., and Hassinen, I.E. (1992) Endothelin-induced atrial natriuretic peptide release from cultured neonatal cardiac myocytes: the role of calcium and protein kinase C. *Endocrinology* **130**, 2455–2464.

Valentijn, K.M., Gumkowski, F.D., and Jamieson, J.D. (1999) The subapical actin cytoskeleton regulates secretion and membrane retrieval in pancreatic acinar cells. *J Cell Sci.* **112**, 81-96.

Van Wagoner, D.R. (1993) Mechanosensitive gating of atrial ATP-sensitive potassium channels. *Circ Res.* **72**, 973-83.

Vanderburgh, H.H., Shansky, J., Solerssi, R., and Chromiak, J. (1995) Mechanical stimulation of skeletal muscle increases prostaglandin F2α production, cyclooxygenase activity, and cell growth by a pertussis toxin sensitive mechanism. *J. Cell. Physiol.* **163**, 285-294.

Varlamov, O., Eng, F.J., Novikova, E.G., and Fricker, L.D. (1999) Localization of metallocarboxypeptidase D in AtT-20 cells. Potential role in prohormone processing. *J. Biol. Chem.* **274**, 14759-67.

Vigne, P., Ladoux, A., and Frelin, C. (1991) Endothelins activate Na+/H+ exchange in brain capillary endothelial cells via a high affinity endothelin-3 receptor that is not coupled to phospholipase C. *J. Biol. Chem.* **266**, 5925-8.

Vitale, N., Gasman, S., Caumont, A-S., Gensse, M., Galas, M-C., Chasserot-Golaz, S., and Bader, M-F. (2000) Insight in the exocytotic process in chromaffin cells: Regulation by trimeric and monomeric G proteins. *Biochimie.* **82**, 365-373.

Vitale, N., Gensse, M., Chasserot-Golaz, S., Aunis, D., and Bader, M-F. (1996) Trimeric G proteins control regulated exocytosis in bovine chromaffin cells: sequential involvement of Go associated with secretory granules and Gi3 to the plasma membrane. *Eur. J. Neurosci.* **8**, 1275-1285.

Vitale, N., Gonon, F., Thiersé, D., Aunis, D., Bader, M-F. (1997) Exocytosis in single chromaffin cells : regulation by a secretory granule-associated Go protein. *Cell & Mol. Neurobiol.* **17**, 71-87.

Vitale, N., Mukai, H., Rouot, B., Thiersé, D., Aunis, D., and Bader, M-F. (1993) Exocytosis in chromaffin cells. Possible involvement of the heterotrimeric GTP-binding protein Go. *J. Biol.Chem.* **268**, 14715-14723.

Vuolteenaho, O., Arjamaa, O., and Ling, N. (1985) Atrial natriuretic polypeptides (ANP): rat atrial store high molecular weight precursor but secrete processed peptides of 25 amino acids. *Biochem. Biophys. Res. Commun.* **129**, 82-88.

Wada, A., Tsutamoto, T., Matsuda, Y., and Kinoshita, M. (1994) Cardiorenal and neurohumoral effects of endogenous atrial natriuretic peptide in dogs with severe congestive heart failure using a specific antagonist for guanylate cyclase-coupled receptors. *Circulation.* **89**, 2232-40.

Wada, Y., Yamashita, T., Imai, K., Miura, R., Takao, K., Nishi, M., Takeshima, H., Asano, T., Morishita, R., Nishizawa, K., Kokubun, S., and Nukada, T. (2000) A region of the sulfonylurea receptor critical for a modulation of ATP-sensitive K(+) channels by G-protein betagamma-subunits. *EMBO J.* **19**, 4915-25.

Watson, E.L. (1999) GTP-binding proteins and regulated exocytosis. *Crit. Rev. Oral. Biol. Med.* **10**, 284-306.

Weidmann P, Saxenhofer H, Shaw SG, Ferrier C. (1989) Atrial natriuretic peptide in man. *J. Steroid. Biochem.* **32**, 229-41.

Weidmann P, Hellmueller B, Uehlinger DE, Lang RE, Gnaedinger MP, Hasler L, Shaw S, Bachmann C. (1986a) Plasma levels and cardiovascular, endocrine, and excretory effects of atrial natriuretic peptide during different sodium intakes in man. *J. Clin. Endocrinol. Metab.* **62**, 1027-36.

Weidmann P, Hasler L, Gnadinger MP, Lang RE, Uehlinger DE, Shaw S, Rascher W, Reubi FC. (1986b) Blood levels and renal effects of atrial natriuretic peptide in normal man. *J. Clin. Invest.* **77**, 734-42.

Weingarten, R., Ransnas, L., Mueller, H., Sklar, L.A., and Bokoch, G.M. (1990) Mastoparan interacts with the carboxyl terminus of the alpha subunit G_i. *J. Biol. Chem.* **265**, 11044-11049.

Wess, J. (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J.* **11**, 346-54.

Wheeler, M.B., Sheu, L.., Ghai, M., Bouquillon, A., Grondin, G., Weller, U., Beaudoin, A.R., Bennett, M.K., Trimble, W.S., and Gaisano, H.Y. (1996) Characterization of SNARE protein expression in beta cell lines and pancreatic islets. *Endocrinology.* **137**, 1340-8.

Wiedemann, K., Herzog, L., and Kellner, M. (1995) Atrial natriuretic hormone inhibits corticotropin-releasing hormone-induced prolactin release in man. *J. Psychiatr. Res.* **29**, 51-8.

Wijeyratne, C.N., and Moult, P.J. (1993) The effect of alpha human atrial natriuretic peptide on plasma volume and vascular permeability in normotensive subjects. *J. Clin. Endocrinol. Metab.* **76**, 343-346.

Wildey, G.M., and Matyas, A.E. (1993) Detection of low molecular weight GTP-binding proteins associated with rat atrial secretory granules. *J. Mol. Cell. Cardiol.* **25**, 459-68.

Wildey, G.M., Fischman, A.J., Margolies, M.N., Graham, R.M., and Homcy, C.J. (1990) Phosphorylation state of pro-atrial natriuretic factor in rat atrial secretory granules. *Endocrinology* **127**, 2839-2848.

Wilson, E., Sudhir, K., and Ives, H.E. (1995) Mechanical strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrin interactions. *J. Clin . Invest.* **96**, 2364-72.

Wilson, S.P. (1989) Effects of mastoparan on catecholamine release from chromaffin cells. *FEBS Lett.* **247**, 239-241.

Winquist, R.J., Scott, A.L., and Vlasuk, G.P. (1989) Enhanced release of atrial natriuretic factor by endothelin in atria from hypertensive rats. *Hypertension.* **14**, 111–114.

Wise, A., and Milligan, G. (1997) Rescue of functional interactions between the alpha2A-adrenoreceptor and acylation-resistant forms of Gi1alpha by expressing the proteins from chimeric open reading frames. *J. Biol. Chem.* **272**, 24673-8.

Wittert, G.A., Espiner, E.A., Richards, A.M., Donald, R.A., Livesey, J.H., and Yandle, T.G. (1993) Atrial natriuretic factor reduces vasopressin and angiotensin II but not the ACTH response to acute hypoglycaemic stress in normal men. *Clin. Endocrinol.* **38**, 183-9.

Witvliet, M.H., Burns, D.L., Brennan, M.J., Poolman, J.T., and Manclark, C.R. (1989) Binding of pertussis toxin to eucaryotic cells and glycoproteins. *Infect. Immun.* **57**, 3324-30.

Wolf, W-P., Spicher, K., Hannelore, H., and Schulze, W. (1998) Immunocytochemical localization of the G-protein sub-unit, Goα, in rat heart. Implications for a role of Goα in secretion of cardiac hormones. *J. Mol. Cell. Cardiol.* **30**, 1149-1162.

Wolfram, G., Sitter, T., Gottsman, M., Gerzer, R., and Schiffl, H. (1996) Assessment of dry weight in haemodialysis patients by the volume markers ANP and cGMP. *Nephol. Dial. Transplant.* **11 Suppl 2**, 28-30.

Wu F, Yan W, Pan J, Morser J, Wu Q. (2002) Processing of pro-atrial natriuretic peptide by corin in cardiac myocytes. *J. Biol. Chem.* **277**, 16900-5.

Wypij, D.M., and Harris, R.B. (1992) Characterization of homogeneous atrial granule serine proteinase, a candidate processing enzyme of pro-atrial natriuretic factor. *Life Sci.* **50**, 523-31.

Xu H, Shields D. (1993) Prohormone processing in the trans-Golgi network: endoproteolytic cleavage of prosomatostatin and formation of nascent secretory vesicles in permeabilized cells. *J. Cell. Biol.* **122**, 1169-84.

Xu, T., Jiao, J.H., Pence, R.A., and Baertschi, A.J. (1996) ATP-sensitive potassium channels regulate stimulated ANF secretion in isolated rat heart. *Am. J. Physiol.* **271**, H2339-45.

Xu, Y., and Barbieri, J.T. (1995) Pertussis toxin-mediated ADP-ribosylation of target proteins in Chinese hamster ovary cells involves a vesicle trafficking mechanism. *Infection and Immunity* **63**, 825-232.

Xu, Y., and Barbieri, J.T. (1996) Pertussis toxin-catalyzed ADP-ribosylation of G_{i-2} and G_{i-3} in CHO cells is modulated by inhibitors of intracellular trafficking. *Infection Immunity.* **64**, 593-9.

Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Nagai, R., Aikawa, R., Uozumi, H., Yazaki, Y. (1998) Role of ion channels and exchangers in mechanical stretch-induced cardiomyocytes hypertrophy. *Circ. Res.* **82**, 430-437.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* **332**, 411-5.

Yokokawa, N., Komatsu, M., Takeda, T., Aizawa, T., and Yamada, T. (1989) Mastoparan, a wasp venom, stimulates insulin release by pancreatic islets through pertussis toxin sensitive GPT-binding protein. *Biochem. Biophys. Res. Commun.* **158**, 712-716.

Yokota N, Bruneau BG, Kuroski de Bold ML, de Bold AJ. (1994) Atrial natriuretic factor significantly contributes to the mineralocorticoid escape phenomenon. Evidence for a guanylate cyclase-mediated pathway. *J Clin Invest.* **94**, 1938-46.

Zerial, M., and Stenmark, H. (1993) Rab GTPases in vesicular transport. *Curr. Opin. Cell. Biol.* **5**, 613-20.

Zietse, R., Weimar, W., and Schalekamp, M.A. (1997) Atrial natriuretic peptide in diabetic nephropathy. *Kidney. Int.* **60**, S33-41.