CHARACTERIZATION OF ATRIAL NATRIURETIC FACTOR STORAGE POOLS IN HL-1 ATRIAL CARDIOMYOCYTES

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Abstract

Atrial natriuretic factor (ANF) is a cardiac hormone that helps maintain cardiovascular homeostasis. ANF secretion is linked to the constitutive, regulated and constitutive-like pathways. Presence of a monensin-sensitive pool that may follow constitutive-like secretion has previously been identified in an isolated atrial perfusion study. The intracellular ANF storage pools linked to each secretory pathway have not been identified. In this study, ANF storage and secretion was characterized in HL-1 atrial cardiomyocytes through the use of pharmacological agents, density gradient and RP-HPLC analysis. Treatment of HL-1 cells with monensin followed by cell fractionation was unsuccessful in identifying the monensin-sensitive pool. RP-HPLC analysis identified presence of low molecular weight ANF in low density gradient fractions that were defined by the presence of organelle markers of Golgi, early endosome, clathrin and corin. Since the monensin-sensitive pool was thought to be of a constitutive-like nature, targeting this pathway with pharmacological inhibitors of clathrin coat vesicle (CCV) formation and endosomal trafficking failed to prevent stimuli-independent secretion. Based on an inability to prevent ANF secretion by targeting the constitutive-like pathway and the presence of low molecular weight ANF in low density gradient fractions, stimuli-independent ANF secretion seems to be through a constitutive pathway.

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List of Abbreviations

A5	membrane traffic inhibitor A5
ACN	acetonitrile
AF	atrial fibrillation
ANF	atrial natriuretic factor
AP-1	adaptor protein 1
ARF-1	ADP ribosylation factor 1
ASGs	atrial specific granules
ATP	adenosine triphosphate
BFA	brefeldin A
BNP	B-type natriuretic peptide
BSA	bovine serum albumin
CCVs	clathrin coated vesicles
cDNA	complementary deoxyribonucleic acid
cGMP	guanosine 3', 5'- monophosphate
Chr	chromogranin
CNP	C-type natriuretic peptide
Dec-RVKR-CMK	decanoyl-Arg-Val-Lys-Arg-chloromethylketone
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F12
DMSO	dimethyl sulfoxide
DNP	dendroaspis natriuretic peptide
EDTA	ethylenediaminetetraacetic acid
EEA1	early endosome antigen 1
ER	endoplasmic reticulum
ET-1	endothelin 1
ET_A	endothelin receptor A
EtOH	ethanol
fsANF	frameshift ANF
Fz2	second cysteine-rich frizzled-like domain
g_{av}	average gravitational acceleration

GC	guanylyl cyclase
GGA	Golgi-localized-gamma ear containing ARF
g_{max}	maximum gravitational acceleration
GTPase	guanosine triphosphate
h	hour(s)
HCl	hydrochloric acid
HEK293	human embryonic kidney 293 cells
HRP	horseradish peroxidase
ISGs	immature secretory granules
ITS	insulin, transferrin, sodium selenite
M6PR	mannose 6-phosphate receptor
min	minute(s)
mL	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
MSG	mature secretory granules
NaCl	sodium chloride
NEP	neutral endopeptidase 24.11
nM	nanomolar
Nppa	natriuretic peptide precursor A
Nppb	natriuretic peptide precursor B
Nppc	natriuretic peptide precursor C
NPR	natriuretic peptide receptor
NPs	natriuretic peptides
PACS1	phosphofurin acidic cluster sorting protein 1
PAM	peptidylglycine α-amidating monooxygenase
pg	picogram
PI3K	phosphatidylinositol 3-kinase
PK	protein kinase
PM	plasma membrane
PtdIns	phosphatidylinositols

PtdIns(4,5)P2	phosphatidylinositol 4,5-biphosphate
RI	refractive index
RIA	radioimmunoassay
RP-HPLC	reverse-phase high performance liquid chromatography
rpm	revolutions per minute
RRP17	ras-related protein 17
RT-PCR	reverse transcription polymerase chain reaction
SCAMPs	secretory carrier membrane proteins
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGs	secretory granules
SNAP-25	synaptosomal-associated protein-25
TBS	tris-buffered saline
TFA	trifluoroacetic acid
TGN	trans Golgi network
Tris	tris(hydroxymethyl)aminomethane
°C	degree celsius
ρ	density
η	refractive index
µL	microlitre
µM	micromolar

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Introduction

Cardiovascular Endocrinology

Electron microscopy-based, morphological evidence exhibited presence of dense granules in the atrial myocytes (Kisch, 1956); Jamieson and Palade named these ultrastructures "specific granules" (Jamieson and Palade, 1964). Subsequent histochemical studies determined the composition of these electron dense granules to be of a protein or polypeptide nature (de Bold et al., 1978). A relationship between atrial granularity and fluid-electrolyte balance was identified (de Bold, 1979) and rats were injected with crude atrial extracts, which resulted in a potent natriuretic and diuretic response (de Bold et al., 1981). This natriuretic activity was localized to the atrial specific granules (ASGs) as intravenous infusion of purified rat atrial granules had the same natriuretic effect as seen in the crude atrial extract; the component responsible for these natriuretic and diuretic properties came to be known as atrial natriuretic factor (ANF) and thus started the field of cardiovascular endocrinology revealing the heart as an endocrine organ in additional to being a mechanical pump (de Bold, 1982; Garcia et al., 1982). Today, the field of cardiovascular endocrinology includes opioid peptides (Barron, 1999), cardiac adrenomedullin (Nishikimi and Matsuoka, 2005), cardiotrophin-1 (Calabro et al., 2009) and mineralocorticoids (White, 2003).

Natriuretic Peptide System

The natriuretic peptide system includes the natriuretic peptides (NPs) and the NP receptors (NPRs) in vertebrates and invertebrates, thus highlighting the importance of these peptides in water and electrolyte homeostasis (Takei, 2001). After the discovery of ANF, additional members of the NP family with physiological activities similar to ANF were discovered. The second NP member was isolated from porcine brain, thus named brain natriuretic peptide (BNP) and later also known as B-type natriuretic peptide (Sudoh et al., 1988). The third member was also isolated from porcine brain extract and due to its analogous natriuretic and diuretic activity was named C-type natriuretic peptide (CNP) (Sudoh et al., 1990). Two additional NPs homologous to ANF have been identified as urodilatin and dendroaspis natriuretic peptide (DNP). Urodilatin is produced by renal cells after alternative processing of proANF with 4 amino acid extension at the N-terminus to yield $proANF_{95\text{-}126}$ (Schulz-Knappe et al., 1988). DNP was isolated from the green mamba snake venom as a 38 residue peptide with functional and structural properties similar to the other NPs (Schweitz et al., 1992). An additional NP named ventricular natriuretic peptide was isolated from eel cardiac ventricles (Takei et al., 1991).

The second component of the NP system are the two guanylyl cyclase (GC) receptor subtypes NPR-A (GC-A or NPR-1) and NPR-B (GC-B) through which the NPs exert their physiological effects, and the peptides are cleared from circulation via the clearance receptor NPR-C (Tremblay et al., 2002). NPR-A and –B are characterized by a N-terminus extracellular NP binding domain, a transmembrane domain, a C-terminus intracellular kinase homology domain and a GC catalytic domain, which upon binding of

NP results in an increased intracellular cyclic guanosine 3',5'-monophosphate (cGMP) (Chinkers et al., 1989; Lowe et al., 1989). The two receptors have different affinities for the NPs as the extracellular domains in rats are only 43% identical at the amino acid level (Schulz et al., 1989). The clearance receptor or NPR-C lacks the intracellular kinase and GC catalytic domain (Fuller et al., 1988), and functions to remove NPs from circulation via receptor-ligand endocytic internalization and lysosomal hydrolysis of NPs (Nussenzveig et al., 1990). ANF is also removed from circulation by neutral endopeptidase (NEP) 24.11, a zinc metalloprotease, that inactivates ANF by proteolytic cleavage (Erdos and Skidgel, 1989) . NPR-A binds to ANF and BNP with greater affinity than CNP and NPR-B has greater affinity for CNP than ANF or BNP, while NPR-C has greater affinity for ANF than either BNP or CNP (Bennett et al., 1991). The differential binding affinities of the three receptors to their NP ligands mediates the varied biological effects during physiological and pathological states (Potter et al., 2006).

Structure and Function

Natriuretic peptide precursor A (Nppa) and B (Nppb) genes for ANF and BNP, respectively, are found in tandem in the human genome on chromosome 1 at 1p36 (Arden et al., 1995; Yang-Feng et al., 1985) and on chromosome 4 in mouse (Steinhelper, 1993; Yang-Feng et al., 1985). CNP gene, Nppc, is located on chromosome 2 at q24 in human and chromosome 1 in the mouse genome (Ogawa et al., 1994b). Evolutionary linkage studies have indicated that CNP is the ancestral gene with ANF and BNP being evolved through gene duplication events (Inoue et al., 2003). Both ANF and BNP are mainly

expressed in the atria, thus considered cardiac NPs, while CNP is distributed throughout the central nervous system (Ueda et al., 1991) as well as synthesized and secreted by the vascular endothelium (Suga et al., 1992). A distinct structural feature of NP family is the presence of a disulfide bond linking 17 amino acids with varying C- and N- terminus tail lengths, in addition to amino acid and nucleic acid homology (Rosenzweig and Seidman, 1991). Comparative analysis of NPs reveals that ANF and CNP prohormone sequences are conserved within mammals but highly variable at the N-terminus across species, and the proBNP sequence is highly variable even within mammals (Takei, 2001). DNP immunoreactivity has been identified in human plasma and atrial myocardium (Schirger et al., 1999), and in rabbit atria, ventricle, kidney, liver and brain (Kim et al., 2010). Authenticity of DNP as part of the NP family has been questioned as the gene for DNP has not been identified and functional studies in mammals have been performed using peptide and antibody specific for Green Mamba (Richards et al., 2002).

NPs gained clinical importance as cardiac markers when congestive heart failure patients showed elevated levels of circulating ANF (Burnett et al., 1986) and BNP (Mukoyama et al., 1990). NPs have been linked to various physiological and pathological roles in terms of blood pressure and volume homeostasis, cardiac remodelling, vascular remodelling, metabolism, fibrosis and inflammation (Rubattu et al., 2008). During a pathological state, ANF and BNP gene expression increases in atria and ventricles, but circulating BNP levels show a higher fold change as compared to ANF, a quality attributed to a longer plasma half life of BNP (Mukoyama et al., 1991). Cytokine-mediated selective upregulation of BNP and not ANF has shown a distinct role of BNP during inflammation (Ma et al., 2004; Meirovich et al., 2008). This discoordinate

increase in BNP was first observed in acute allograft rejection patients (Masters et al., 1999), and *in vitro* studies have shown an immunomodulatory role for BNP as it reduces the number of monocytes, B cells and natural killer cells (Shaw et al., 2009). BNP and and its receptor NPR-A are expressed in undifferentiated, self-renewing embryonic stem cells to maintain a proliferative phenotype (Abdelalim and Tooyama, 2009). A cardioprotective role of BNP as a locally acting antifibrotic factor has also been established in Nppb knockout mice (Ogawa et al., 2001; Tamura et al., 2000). Initial studies established CNP as new member of the NP family as it displayed similar pharmacological diuretic and natriuretic activities (Sudoh et al., 1990); however, additional reports showed that plasma CNP concentrations reflective of a diseased state fails to exert any changes in systemic hemodynamics or renal function (Barletta et al., 1998). An unexpected phenotype of Nppc knockout mice is dwarfism due to impaired regulation of endochondral ossification (Chusho et al., 2001).

Atrial Natriuretic Factor

The content of ASGs was purified and sequenced to reveal that a 28 amino acid peptide, with an internal disulfide bond indicative of secretory hormones is accountable for the diuretic and natriuretic activity observed in *in vivo* experiments (Flynn et al., 1983). This peptide came to be known as ANF among a multitude of names such as atrial natriuretic peptide, atriopeptin, A-type natriuretic peptide, auriculin, cardionatrin, or atrin; to improve communication and reduce ambiguity, a nomenclature and standardization committee established "atrial natriuretic factor" as the trivial name and amino acid numbering from N-terminus (excluding signal peptide) (Dzau et al., 1987).

Synthesis

Human ANF is synthesized as a 151 amino acid preprohormone, which undergoes cleavage of the 25 amino acid N-terminus leader segment to form $proANF_{1\text{-}126}$ (Oikawa et al., 1984). Human Nppa gene codes for three exons and two introns; the first exon contains the signal peptide and 16 amino acids of proANF, the second exon contains the remaining codons for propeptide except for the last residue, which is found on the last exon in addition to the stop codon (Nemer et al., 1984). Mouse preproANF is composed of 152 residues and terminates with Tyr-Arg-Arg tripeptide sequence instead of Tyr as in the human sequence; however, this tripeptide sequence is not found in circulation (Bovy, 1990; Vlasuk et al., 1986) and as such leaves the mature C-terminus ANF sequence highly conserved between mouse and human with the only difference of residue 110 being isoleucine in mouse, and methionine in humans (Seidman et al., 1984).

Processing and secretion

After cleavage of the signal peptide of preproANF in endoplasmic reticulum (ER), proANF undergoes vectorial transport through the Golgi complex and specific atrial granules have been shown to contain the propeptide $proANF_{1-126}$ (Flynn et al., 1985; Nemer et al., 1984; Thibault et al., 1987) (Figure 1). The prohormone is further processed to yield ANF_{1-98} and ANF_{99-126} through co-secretional maturation that has been associated with corin (Yan et al., 2000), and ANF secretion has been linked to the classical constitutive, regulated and constitutive-like pathway (McGrath and de Bold, 2005) (Figure 1). Furthermore, RP-HPLC profile of atrial extracts showed the proANF form being predominant with approximately 5-10% intracellular peptide of the processed nature corresponding to the ANF_{99-126} (Vuolteenaho et al., 1985). In contrast, BNP is stored mainly as the mature form of 32 amino acids as human BNP_{77-108} (Hino et al., 1990) and 45 amino acids as mouse BNP $_{77-121}$ (Ogawa et al., 1994a). ANF structure is defined by a disulfide bond between cysteine 105 and 121 (Flynn et al., 1983), which has been demonstrated to be critical for the natriuretic and diuretic properties of the peptide (Chartier et al., 1984; Misono et al., 1984). Furthermore, a linear analogue of ANF lacking the disulfide bond elicits a slight cGMP response as compared to native ANF in rat aortic smooth muscle derived cell line (Napier et al., 1986). Additional studies with linear ANF have concluded that the disulfide bond is necessary for the active conformation of the peptide when bound to its receptor but it is not a prerequisite for its biological activity as the linear peptide showed smooth muscle relaxant activity and inhibited aldosterone release from bovine adrenal zona glomerulosa cells; however, the potency of the linear peptide is much lower than the disulfide bond containing ANF

peptide (Schiller et al., 1985). To date, the only post-translational modification is shown to be N-terminal phosphorylation of proANF with approximately 15-25% of SG of the phosphorylated form (Wildey et al., 1990).

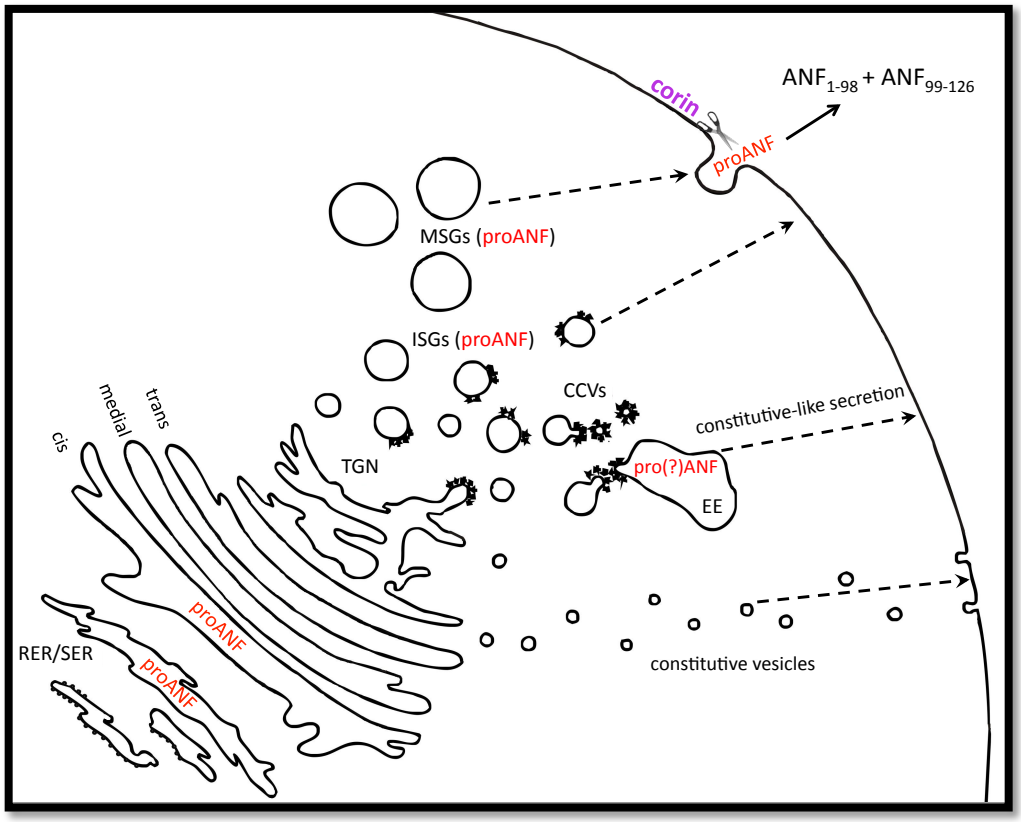


Figure 1: ANF processing and secretion in atrial cardiocytes.

The current model of ANF processing and secretion in atrial cardiocytes describes secretion through a regulated, constitutive, and constitutive-like pathway. PreproANF is processed by removal of signal peptide and proANF is translocated to the Golgi apparatus followed by storage in ASGs. In the current view of literature, $proANF_{1-126}$ is processed via corin at the plasma membrane to yield ANF_{1-98} and ANF_{99-126}. Immature granules are defined by the presence of clathrin. Early endosomes represent the intermediate compartment for constitutive-like secretion. The organelles of the ANF secretory pathway are labelled. MSGs, mature secretory granules; ISGs, immature secretory granules; CCVs, clathrin coated vesicles; TGN, trans-Golgi network; RER/SER, Rough endoplasmic reticulum/smooth endoplasmic reticulum; EE, early endosome.

Corin, a type II transmembrane serine protease with a cytoplasmic N-terminus and an extracellular C-terminus trypsin-like protease domain was identified from a human heart cDNA library (Yan et al., 1999). RT-PCR analysis in rats have shown notable expression of corin in atria, ventricle, septum and aorta (Langenickel et al., 2004). Corin resides on the plasma membrane (PM) of cardiomyocytes as an inactive enzyme precursor and in a catalytically active form (Gladysheva et al., 2008), although a soluble corin form has been detected in human plasma (Peleg et al., 2009). In transfected HEK293 cells, corin has been shown to process proANF as well as proBNP due to sequence similarity at the cleavage site (Yan et al., 2000). Recombinant proANF has been shown to be processed in a sequence specific manner at Arg^{98} by the endogenous corin in the HL-5 cardiac cell line (Wu et al., 2002). Site-directed mutagenesis studies have demonstrated that proANF interacts with frizzled 1 domain and low density lipoprotein receptor repeats 1-4 of corin thus allowing efficient processing of proANF by the protease domain (Knappe et al., 2004).

Increased corin expression has been observed in phenylephrine-induced hypertrophy in primary cultures of neonatal rat cardiomyocytes and in an *in vivo* model of heart failure (Tran et al., 2004). A corin gene allele with two missense mutations (Q568P and T555I) in the second cysteine-rich frizzled-like domain (Fz2) has been identified in United States black population and linked with higher blood pressure and an increased risk for hypertension (Dries et al., 2005). Mutant corin lacking Fz2 retains only 30% of proANF processing activity and variants Q568P and T555I individually have no effect on proANF processing, but T555I/Q568P variants have about 38% of activity as compared to wildtype (Wang et al., 2008). In corin knockout mice, the authors reported

2.6 fold higher content of intracellular proANF and undetectable ANF levels as compared to wildtype (Chan et al., 2005).

Physiological Actions

ANF acts as a potent vasorelaxant to decrease blood pressure (Bolli et al., 1987; Breuhaus et al., 1985; Granger et al., 1986). ANF has a suppressive effect on the sympathetic baroreceptors by activation of vagal afferents (Thoren et al., 1986). ANF causes an increase in glomerular filtration rate and filtration fraction while inhibiting the reabsorption of Na^+ from the collecting duct (Camargo et al., 1984; Cogan, 1985; Huang et al., 1985). ANF inhibits aldosterone synthesis and secretion as well as decreasing renin secretion, which has an impact in decreasing angiotensin II and aldosterone (Atarashi et al., 1985; Atarashi et al., 1984; Burnett et al., 1984; Henrich et al., 1987). ANF inhibits endothelin-1 (ET-1) synthesis and secretion from aortic endothelial cells (Hu et al., 1992); furthermore, ANF also inhibits thrombin-induced ET-1 synthesis in a cGMP dependent manner (Kohno et al., 1992). ANF infusions have been shown to increase hematocrit either by an increase in capillary permeability or capillary pressure gradients (Almeida et al., 1986; Fluckiger et al., 1986). In addition to the well known cardiovascular and renal functions of ANF, recent research shows involvement of ANF in innate and adaptive immune system (Vollmar, 2005), cancer (Saba and Vesely, 2006), and lipolysis (Lafontan et al., 2008).

Genetic Variants

A 2009 review of literature analyzed 29 studies relating to 10 Nppa gene polymorphisms and their role in cardiovascular pathology; even though several studies linked the allelic variants to hypertension and cardiovascular disease, the overall linkage of the variants to a diseased state was found to be inconsistent (Lynch et al., 2009). Among the various polymorphisms that have been identified, only the T2238C (also called ScaI and T1766C) polymorphism results in modification of the ANF peptide at the C-terminus as Arg^{127}-Arg^{128} (Kato et al., 2000; Masharani et al., 1988). A heterozygous frame-shift mutation in the Nppa gene, which results in the extension of ANF at the C-terminus by 12 residues, has been linked with familial atrial fibrillation (AF), and an isolated rat heart model showed that frameshift ANF (fsANF) shortens monophasic action potentials as compared to the wildtype (Hodgson-Zingman et al., 2008). This fsANF has greater affinity for NPR-B and it is resistant to proteolytic degradation by NEP thus elevating its plasma levels (Dickey et al., 2009). Single nucleotide polymorphism rs5063 a non-synonymous mutation Val32Met in Nppa, has been evaluated in two different populations and found to be linked to AF in the Chinese Han population (Ren et al., 2010); however, no correlation has been found in the North American population of European ancestry (Roberts et al., 2010).

Atrial Granule Biogenesis

Atrial Specific Granules

ASGs are the storage site of proANF in cardiac myocytes (Thibault et al., 1987). Characteristics of these granules are similar to secretory granules (SGs) found in endocrine cells (Cantin et al., 1979). ASGs have a high calcium content, low pH and an ability to sustain anion gradients (Somlyo et al., 1988). RP-HPLC analysis of atrial granule content also shows the presence of mature BNP and minor quantity of proBNP (Thibault et al., 1992). The ASGs are between 250 to 500 nm in diameter and their size and number in cardiocytes and natriuretic activity is inversely proportional to the size of the animal (Chang and Bencosme, 1969; de Bold and Salerno, 1983; Jamieson and Palade, 1964; Tomisawa, 1969). Proteomic analysis of isolated ASGs identified 61 distinct proteins related to vesicular trafficking, signal transduction, scaffolding, calcium association and peptide processing in addition to highly abundant proANF and peptidylglycine α-amidating monooxygenase (PAM) (Muth et al., 2004). In the proteomic study, proteins identified in vesicular trafficking included members of the rab guanosine triphosphatase (GTPase) family (rab2, rab3b, rab6a, rab7, rab14) and membrane fusion (annexin II, N-ethylmaleimide sensitive factor, rabaptin5, synaptotagmin VII, syntaxin 6, Syntaxin-binding protein 1); however, the proANF protease corin was not identified in the ASGs.

Granule Biogenesis: Sorting and Maturation

Appropriate signals that segregate proANF from constitutively secreted proteins are required to target the propeptide towards the regulated secretory pathway as this is a prerequisite for the formation of dense core SGs in endocrine cells (Dikeakos and Reudelhuber, 2007). Two models based on the location of protein segregation destined for secretory granules have been proposed. The "sorting by entry" model describes the use of a sorting receptor to segregate cargo at the trans Golgi network (TGN) towards the constitutive, regulated or endosomal/lysosomal pathway (Bauerfeind and Huttner, 1993; Kuliawat and Arvan, 1994). In the "sorting by retention" model, immature secretory granules (ISGs) are formed at the TGN containing unsorted cargo, and the granule matures by the removal of lysosomal or constitutively secreted proteins while regulated secretory proteins are retained (Kuliawat and Arvan, 1992, 1994). Different protein categories that lead to the formation of SGs have been described; these include proteins tethered to TGN/granule membrane, soluble proteins that associate and interact with these membrane-tethered proteins and high molecular weight proteins that lead to the formation of aggregates (Dikeakos and Reudelhuber, 2007).

In ASGs, proANF and PAM constitute more than 95% of total granular membrane proteins and they are present in a molar ratio of 30 (proANF):1 (PAM) (O'Donnell et al., 2003). Importance of proANF sequence as a requirement for ASG biogenesis is highlighted as targeted disruption of Nppa gene in mice leads to loss of ASGs (John et al., 1995). Mutations or deletion of N-terminal proANF changes the size and shape of granules and prevents their docking at the PM (Baertschi et al., 2001). PAM α-amidates C-terminal residues of many bioactive peptides of neuronal or endocrine

origin (Eipper and Mains, 1988); however, in ASGs PAM is suggested to provide a structural instead of an enzymatic role in the packaging of proANF due to lack of its substrate in the granules and tight association of proANF and PAM with the SG membrane (O'Donnell et al., 2003). Presence of both PAM and proANF is required to create the round shape of secretory vesicles; however PAM is not involved in vesicular docking (Labrador et al., 2004). A novel function for the cytosolic domain of PAM has been established as a granule to nuclear signalling molecule to upregulate gene expression upon secretagogue dependent granule exocytosis (Francone et al., 2010).

Sorting towards the regulated secretory pathway is an intrinsic property of α-helices as shown by the routing of synthetic α-helical peptides in the absence of any other sorting signals; this requires presence of an hydrophobic face segregated from charged amino acids (Dikeakos et al., 2007). The N-terminus of proANF contains a leucine zipper-like coiled-coil motif of α-helices (amino acids 12 to 26) capable of oligomerisation (Seidler et al., 1999). The region corresponding to α-helices promotes proANF aggregation in the presence of calcium (Thibault and Doubell, 1992); aggregation is also promoted by members of the chromogranin (Chr) and secretogranin family (Taupenot et al., 2003), among which ChrA and ChrB have been localized with proANF in ASGs (Steiner et al., 1990).

Granule maturation is defined by retention of proteins destined for the regulated secretory pathway to allow quantal release of products in excess of biosynthesis upon a stimuli (Arvan and Castle, 1998). As granule maturation progresses, the core becomes electron dense with simultaneous loss of volume, surface area, and membrane proteins as vesicles are pinched off (Sesso et al., 1980). ISGs are defined by the presence of clathrin

coats that are removed in the form of clathrin coated vesicles (CCVs) to transport cargo towards the endosomal/lysosomal compartments (Fishman and Fine, 1987; Kuliawat et al., 1997). ANF and proANF containing CCVs have been isolated from atrial cardiocytes (Klein et al., 1993), indicating involvement of clathrin in the ANF secretory pathway. The atrial granule proteome also identified components of clathrin coat formation (dynamin 1 & 2), and uncoating machinery (Hsc70, Hop/p60 protein, mortalin); however no clathrin was identified (Muth et al., 2004), and this is in agreement with observations that mature granules are devoid of clathrin (Tooze, 1991).

Heterotrimeric and monomeric G proteins and their effector proteins are important regulators of SG biogenesis and secretion (Kowluru, 2010; Williams et al., 2009). The Rho, Ras and Rab family of small GTPases have been implicated in insulin granule exocytosis (Wang and Thurmond, 2009). Ras-related protein 17 (RRP17), a member of the Ras family of G proteins has been shown to interact with calcium-activated protein for secretion-1 and regulate ANF storage and secretion; RRP17 increases ANF secretion independent of an increase in mRNA expression, suggesting it functions at the level of stored granules (Rybkin et al., 2007). Through immunoblot and immunogold labelling, rab12p (Iida et al., 1996) and rab6p (Iida et al., 1997) have been found to be associated with ASGs at the periphery. $G_{o\alpha}$ is found to be localized to about 60% of SGs in cardiomyocytes (Wolf et al., 1998). Stretch stimulated ANF secretion is mediated via a $G_{i/o}$ pathway, while ET-1 stimulated ANF secretion follows a G_q pathway (Bensimon et al., 2004).

Constitutive Secretion

Newly synthesized proteins follow vectorial transport from ER to PM through multiple and parallel pathways in a stimuli-independent and constitutive manner (Ponnambalam and Baldwin, 2003). Various models for TGN to PM trafficking exist for passive protein transport; the vesicle shuttle model describes movement of transport vesicles through the biosynthetic pathway in a formation- and fusion-dependent mode (Rothman and Wieland, 1996). The vesicular transport is mediated by 60-100 nm vesicles destined for the PM (Buccione et al., 1996). Constitutive vesicle formation at the TGN requires phospholipid-dependent, serine/threonine protein kinases (PKs), namely PKD (Bossard et al., 2007), PKC (Westermann et al., 1996) and PKA (Muniz et al., 1997). Presence of phospholipase C β3 (Diaz Anel, 2007) and interaction of four phosphate adaptor protein 1/2 with small GTPase ADP-ribosylation factor (ARF) and phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) is also necessary for vesicle formation (Godi et al., 2004).

Basal secretion is represented by the turnover of mature secretory granules (MSGs) in the absence of stimuli and contributes towards the passive secretion of proteins alongside constitutive secretion (Matsuuchi and Kelly, 1991; Varro et al., 1996). In the literature it is more common to find the term "basal" as representing the non regulated secretion, which includes the constitutive (vesicle formation at TGN), constitutive-like (vesicle formation at ISGs) and exocytosis of MSGs (Halban and Irminger, 2003). However, sorting of secretory products towards the regulated pathway has been suggested to be a pre-requisite for basal secretion (Giblin et al., 2008). True basal exocytosis has been shown to have a half life of approximately 50 hours with the

peak period of unstimulated granule exocytosis occurring around 9 to 10 hours after chase in pancreatic lobules (Arvan and Castle, 1987).

In the absence of stimuli, the main circulating form of ANF in plasma is the 28-residue peptide; however, presence of minute quantities of proANF has also been observed in human plasma (Macaulay Hunter et al., 1998; Yamaji et al., 1985), rat plasma (Miyata et al., 1985), dog plasma (Cernacek et al., 1988) and isolated rat heart perfusate (de Bold and de Bold, 1989; Thibault et al., 1986), possibly corresponding to basal release from mature granules. Plasma ANF levels in healthy young adults are found to be dependent on gender with circulating ANF almost two-fold higher in premenopausal women as compared with men, while in the elderly the values are higher with no significant difference between men and women (Clark et al., 1990). Plasma half-life of ANF_{99-126} has been determined to be 2.5 minutes for 100 μg of peptide (Yandle et al., 1986) or 5.65 minutes for 32 μg of peptide (Juppner et al., 1986) in humans.

Regulated Secretion

Regulated secretion is a distinctive feature of endocrine cells containing SGs that undergo synthesis-independent and energy-dependent exocytosis upon an external stimuli (Burgess and Kelly, 1987). Granule biogenesis and sorting of proteins destined for secretory granules is a major component of the regulated secretory pathway (discussed in the preceding sections). Exocytosis of ANF under stimulated conditions via secretory granules makes this peptide's secretion fall in the category of regulated secretion (Bloch et al., 1986). Both mechanical and neuroendocrine stimuli regulate ANF secretion to maintain water and electrolyte homeostasis (de Bold et al., 1996).

Neuroendocrine Stimuli

Endocrine- or neurohumoral dependent regulated ANF secretion is mediated by several factors of which ET-1 is the most potent (Schiebinger and Gomez-Sanchez, 1990). Additional neuroendocrine stimuli for ANF secretion include glucocorticoids (Shields et al., 1988), acetylcholine (Hayashi et al., 1988; Inoue et al., 1988), α- and β-adrenergic agonists (Schiebinger et al., 1987; Shields and Glembotski, 1989), prostaglandins (Gardner and Schultz, 1990), thyroid hormone (Mori et al., 1990), angiotensin II (Focaccio et al., 1993), and vasopressin (Zongazo et al., 1991). ANF secretory kinetics vary in terms of stimuli and concentrations, therein illustrating the presence of distinct intracellular ANF pools; in an *ex vivo* rat atria model Schiebinger and colleagues showed that phenylephrine, an α-adrenergic agonist results in ANF secretion in a monophasic mode, while isoproterenol a β-adrenergic agonist increases ANF secretion in a biphasic mode (Schiebinger et al., 1987). ET-1 also shows concentration-

dependent kinetics on ANF secretion; 10 nM ET-1 results in monophasic ANF increase while 100 nM ET-1 shows a biphasic response (Uusimaa et al., 1992). Pathway-specific ANF release is further supported by stretch secretion being augmented by ET-1 and not by norepinephrine or vasopressin (Schiebinger and Greening, 1992).

ET-1, a 21-residue peptide, was first isolated from conditioned medium of porcine aortic endothelial cells and shown to be a potent vasoconstrictor (Yanagisawa et al., 1988). ET-1 functions via G protein coupled receptors ET_A and ET_B (Arai et al., 1990; Sakurai et al., 1990) to activate at least three different signalling pathways (Bouallegue et al., 2007). ET-1 transduces its signal via G_q, which initiates the phosphoinositide cascade to activate PKC (Resink et al., 1988; Smrcka et al., 1991). Mitogen-activated protein kinase and Ras/c-Raf-1 pathway is also activated by ET-1 to activate further downstream effectors ERK1/2, p38, p44/42, JNK to regulate transcription (Wheeler-Jones, 2005; Yue et al., 2000). ET-1 has also been shown to activate phosphatidylinositol-3 kinases (PI3Ks) through $p21^{ras}$ (Foschi et al., 1997). PKC, another downstream product of ET-1 stimulation has also been shown to be a regulator of SG exocytosis; PKC phosphorylates Ser^{187} of Synaptosomal-associated protein-25 (SNAP-25) and this phosphorylated form as well as presence of PKC itself is required for vesicle pool refilling for subsequent granule exocytosis (Nagy et al., 2002).

Mechanical Stimuli

ANF secretion in response to a mechanical stimuli has been termed "stretch-secretion coupling" (Kuroski-de Bold and de Bold, 1991) as it occurs in response to volume-induced atrial stretch (Edwards et al., 1988) to release newly synthesized ANF from ISGs (Mangat and de Bold, 1993). Stretch-stimulated ANF pool consists of a rapidly depleting pool since in the presence of continuous stimuli, peptide secretion returns to basal levels within three hours without upregulation of ANF gene expression (Bruneau and de Bold, 1994). The distinct nature of stretch stimuli has been demonstrated by pertusis toxin, a $G_{i/o}$ protein inhibitor, which abolishes stretch-stimulated but not ET-1-stimulated secretion, which signals through the G_q pathway; furthermore, immunocytochemistry showed that $G_{o\alpha}$ was partially colocalized with ANF (Bensimon et al., 2004). $G_{o\alpha}$ is found to be abundantly expressed in the atria as compared to the ventricles and it is localized to about 60% of SGs in atrial cardiomyocytes (Wolf et al., 1998).

Monensin Sensitive Secretory Pathway

Monensin is a monovalent, sodium ionophore capable of collapsing intracellular proton gradients; thus increasing the pH of intracellular compartments such as TGN, SGs, lysosomes and endosomes in a concentration and time dependent manner (Mollenhauer et al., 1990). Monensin concentrations in the micromolar range inhibit protein processing and transport in the Golgi in addition to neutralizing intracellular acidic compartments including the SGs (Devault et al., 1984), while nanomolar concentrations prevent formation of ISGs at the TGN (Orci et al., 1984). In the myeloid leukemia HL-60 cell

line, 1 μM monensin resulted in vacuolization of the trans Golgi within 30 minutes and after 3 hours the vacuolization disseminated to the cis cisternae; additional monensin induced effects included expanded granules with a lucent periphery and residual electron dense core (Parmley et al., 1988). Monensin inhibited corticotropin-releasing hormone stimulated adrenocorticotropic hormone secretion from anterior pituitary glands in a dose and time dependent manner indicating that monensin has a inhibitory effect on stimulated storage granule exocytosis (Sobel and Shakir, 1988). In pancreatic β cells, Orci and colleagues observed that clathrin coated vesicle formation was inhibited at the TGN with low nanomolar concentrations of monensin (Orci et al., 1984).

Primary cultures of rat atrial myocytes when treated with 0.5 to 5 μM monensin showed redistribution of ASGs from the perinuclear region to the cell periphery within 30 minutes; this study further reported that incubation with 5 μM monensin for more than 3 hours changes granule morphology and kinetic studies on ANF secretion showed a decrease in rate of ANF secretion during a 4.5 hour incubation with 0.5 μM monensin from 0.15 to 0.11 fmol/(hr.myocyte) (Iida et al., 1988). In an isolated perfused rat atrial treatment with 5 μM monensin, secretagogue stimulated ANF secretion was inhibited and basal ANF secretion was decreased suggesting that the monensin-sensitive pathway to be derived from a constitutive-like pool (Ogawa et al., 1999).

Constitutive-like Secretion

Constitutive-like pathway was first observed in parotid acinar cells as a distinct secretory pathway that initiates from ISGs with dissimilar composition than the regulated secretory pathway; hence termed early-phase secretion as opposed to late-phase basal secretion from mature granules (von Zastrow and Castle, 1987). ISGs are distinguished based on the presence of partial clathrin coats (Orci et al., 1985; Tooze and Tooze, 1986), and the only vesicles that have been shown to bud off from ISGs are CCVs (Tooze and Tooze, 1986). This novel pathway was termed constitutive-like pathway to distinguish it from the regulated pathway and constitutive pathway (Arvan et al., 1991). Constitutive-like pathway has been observed in isolated pancreatic islets (C-peptide, insulin, proinsulin, p80), rat pheochromocytoma PC12 cell line (proteoglycans), mouse anterior pituitary cell line AtT20 (soluble PAM, calnuc), parotid acinar cells (salivary protein 1 and α-amylase), and ANF in atrial cardiocytes (Castle and Castle, 1996; De Lisle and Ziemer, 2000; Grimes and Kelly, 1992a; Kuliawat and Arvan, 1992; Lavoie et al., 2002; Milgram et al., 1994; Ogawa et al., 1999).

The newly synthesized products in the ISGs compartment are the first to be secreted in response to stimuli (Castle et al., 1997); however, the constitutive-like pathway corresponds to unstimulated secretion (Castle, 1998). In parotid acinar cells the resting secretion has been attributed to the constitutive-like and the minor regulated pathway (Huang et al., 2001). The sorting mechanism for this pathway is attributed to the inability of polypeptides to be condensed and retained in the mature granules, as a result the products are removed via vesicular budding from the ISGs (Arvan and Castle, 1987). Evidence of this "negative selection" has been illustrated by interrupting the

condensation process in maturing granules by ammonium chloride that enhances the secretion via constitutive-like pathway with secretory content reflecting that of stored granules (Castle, 1998; von Zastrow et al., 1989).

Constitutive-like vesicles proceed to the endosome due to the presence of lysosomal hydrolases in the ISGs and follow a prolonged and indirect route to the PM from TGN as compared to constitutive secretion (Castle, 1998). The route of secretory products in the constitutive-like pathway is segregated into the "first limb" from TGN to endosome mediated by adaptor protein-1 (AP-1) and CCVs, while the "second limb" is the endosome to the PM trafficking, which is enhanced by Brefeldin A (BFA) as shown for procathepsin B (ProB), a marker for AP-1/CCV trafficking (Kuliawat et al., 1997; Turner and Arvan, 2000). In perfused isolated right atria, BFA treatment caused enhanced ANF secretion with the effect being independent of stimulation; furthermore, monensin treatment inhibited stretch- and ET-1-stimulated ANF release without a decrease in basal secretion levels suggesting that the monensin-sensitive pathway is derived from the constitutive-like pool (Ogawa et al., 1999).

Wortmannin Sensitive Secretory Pathway

Wortmannin, a fungal metabolite, is an irreversible and potent inhibitor of PI3K that acts by binding to its p110α catalytic subunit and covalently modifying Lys^{802} residue, which is located in close proximity to the substrate and adenosine triphosphate (ATP) binding site (Powis et al., 1994; Wymann et al., 1996; Yano et al., 1993). PI3K is categorized into three classes based on structural domains and it phosphorylates the 3' position of the inositol ring in phosphatidylinositols (PtdIns), PtdIns(4)P, and

PtdIns(4,5)P_2 (Domin and Waterfield, 1997). Both class I and III enzymes are sensitive to wortmannin in low nanomolar range, while a CCV associated class II enzyme PtdIns3KIIC2α is sensitive at low micromolar range (Domin et al., 1997; Gaidarov et al., 2001).

Wortmannin has been implicated in missorting and hypersecretion of procathepsin D in the TGN to lysosomal pathway with the possible site of mistargeting at TGN to an early endosome compartment (Davidson, 1995). This enhanced secretion effect has also been observed in a pancreatic β cell line where wortmannin potentiates glucose-stimulated insulin secretion (Eto et al., 2002; Hagiwara et al., 1995) . A possible explanation for the augmented secretion of insulin in the presence of the inhibitor is described as being due to the inhibition of phosphodiesterase and subsequent increase in cAMP content (Nunoi et al., 2000). Wortmannin prevents the recruitment of mannose 6-phosphate receptor (M6PR) receptors into CCVs and as a result cathepsin-D, a ligand for M6PR receptor does not get sorted into CCVs and gets secreted in its pro-enzyme form (Gaffet et al., 1997; Karlsson and Carlsson, 1998). Similar effects have been observed for the processing and maturation of procathepsin B in the presence of wortmannin (Turner and Arvan, 2000).

Mis-routing of cargo from TGN to endosomal/lysosomal compartment could also be due to lack of early endosome fusion, which is inhibited in the presence of wortmannin and requires an activated p110 subunit of PI3-K (Jones et al., 1998; Li et al., 1995). Additionally a role for wortmannin in inhibiting trafficking in the late endocytic pathway has also been proposed (Reaves et al., 1996). Wortmannin induces endosomal-specific morphological changes by transforming a vesicular phenotype to the one where

endosomes are enlarged with a tubular network (Shpetner et al., 1996). Wortmannin prevents early endosome antigen 1 (EEA1) from binding to endosomal membranes, an activity that requires functional PI3-K and Rab5 for early endosome fusion (Kjeken et al., 2001; Simonsen et al., 1998).

Role of PI3Ks in granule exocytosis has been implicated in insulin secretion, where the p110γ catalytic subunit of type 1B PI3Ks acts to regulate SG translocation and localization to the PM by decreasing cortical F-actin polymerization (Pigeau et al., 2009). Recently another isoform of PI3Ks, C2α, has been shown to regulate insulin secretion by functioning at the level of granule fusion by negatively regulating proteolysis of SNAP-25 (Dominguez et al., 2010). In chromaffin cells, PI3K-C2α was found to be associated with SGs and partially colocalized with CCVs suggesting a dual role in granule biogenesis and ATP-dependent priming for granule exocytosis (Meunier et al., 2005).

Clathrin-Dependent Secretory Pathway

The first limb of constitutive-like secretion is mediated via CCVs budding off from ISGs to carry cargo destined for endosomes or lysosomes (Dittie et al., 1996; Turner and Arvan, 2000). Cellular trafficking utilizes CCVs during TGN to endosome and PM to endosome transport; CCVs formed at TGN or PM can be distinguished based on the regulatory proteins associated with each region (Brodsky et al., 2001). In primary rat pancreatic beta-cells, use of a dominant-negative clathrin heavy chain mutant showed that clathrin was not involved in regulated secretion of proinsulin or insulin; however, presence of clathrin was implicated in the removal of proteases from the ISGs (Molinete

et al., 2001). Presence of clathrin-coated vesicles predominantly containing proANF has been reported in rat atrial cardiomyocytes; transmission electron microscope studies have shown presence of a partial clathrin coat on dense core granules (Klein et al., 1993).

Clathrin, the major coat protein of CCVs, is a triskelion that forms a polyhedral lattice backbone (Crowther and Pearse, 1981; Pearse, 1975). Clathrin coat self assembles in the presence of factors such as AP-1, ARF-1, secretory carrier membrane proteins (SCAMPs), γ-synergin, and Golgi-localized-gamma-ear-containing ARF-binding proteins (GGA) to form a polyhedral lattice (Brodsky et al., 2001). AP-1, an adaptor or assembly protein (AP) at the TGN triggers clathrin lattice formation and integrates transmembrane molecules such as cargo or receptors into the lattice (Lee et al., 2008; Traub et al., 1993). Cargo recognition is achieved through sorting motifs or AP-1 binding partners; for example, μ1 subunit of AP-1 recognizes tyrosine based YXXΦ motifs where X is any amino acid and Φ is a hydrophobic residue (Marks et al., 1997). Additionally, AP-1 binding partners such as phosphofurin acidic cluster sorting protein 1 (PACS1) recruit furin and M6PRs at the TGN (Wan et al., 1998). During the CCV budding process, dynamin interacts with the bilayer membrane to induce scission of the elongated vesicle neck (Hinshaw and Schmid, 1995). Dynamin II has been localized to TGN, where it interacts with G protein βγ subunit to regulate vesicle formation (Yang et al., 2001). Targeting removal of CCVs from ISGs is expected to prevent initiation of constitutive-like secretory pathway (Kuliawat et al., 1997). Two recently identified inhibitors of clathrin-mediated vesicle trafficking include membrane traffic inhibitor A5 (A5) that targets a TGN to the endosome-specific pathway (Duncan et al., 2007), and dynasore that inhibits the GTPase activity of dynamin (Macia et al., 2006).

Rationale for the Study

ANF plays an important role in maintaining cardiovascular homeostasis (de Bold et al., 2001). In the current view of the literature, $proANF_{1-126}$ is the intracellular storage form in atrial cardiocytes, which is processed co-secretionally at the plasma membrane through the extracellular protease activity of corin (Sei et al., 1992; Yan et al., 2000). However, atrial extracts also show presence of intracellularly processed ANF_{99-126} (Vuolteenaho et al., 1985). Monensin, an ionophore that disrupts protein sorting and transport in the TGN, inhibits stretch- and ET-1-stimulated ANF release without a decrease in basal secretion levels; this demonstrates that this pathway is distinct from both constitutive and regulated pathways with the monensin-sensitive pool to be derived from the constitutive-like pool (Ogawa et al., 1999). The objective of this study is to determine the cell compartments involved in monensin-sensitive storage and secretion in HL-1 atrial cardiomyocytes using cell fractionation techniques based on differential and density gradient ultracentrifugation. An additional aim of this study is to identify the molecular forms of ANF (ANF_{99-126} or $proANF_{1-126}$) in the different cellular pools. Ancillary approaches will make use of pharmacological agents that target endosomes (wortmannin) and clathrin vesicle formation (dynasore and membrane traffic inhibitor A5) to further define the role of constitutive-like pathway in ANF secretion.

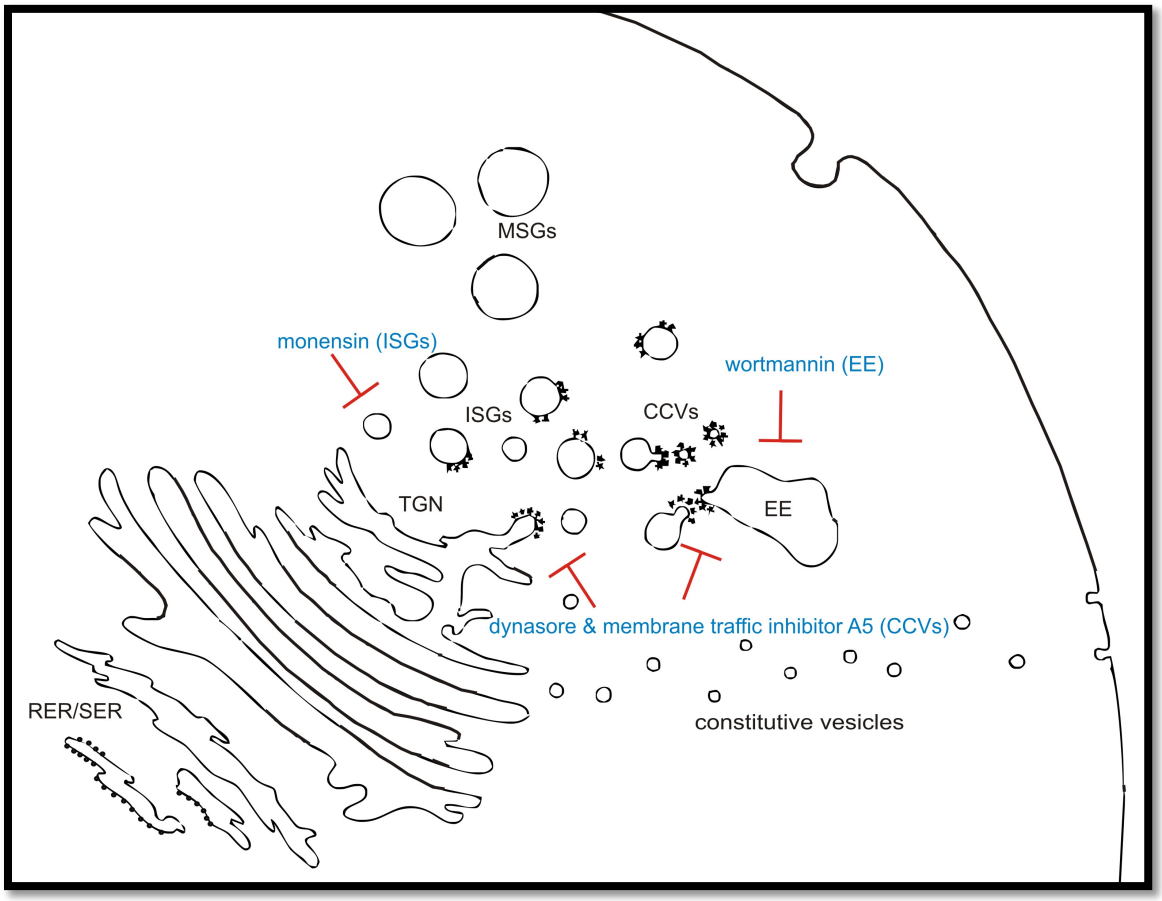


Figure 2: Targeting of secretory pathways with pharmacological agents.

The site of action of monensin (ISGs), wortmannin (endosomes), dynasore and membrane traffic inhibitor A5 (CCVs) are shown. The organelles of the ANF secretory pathway are labelled. MSGs, mature secretory granules; ISGs, immature secretory granules; CCVs, clathrin coated vesicles; TGN, trans-Golgi network; RER/SER, Rough endoplasmic reticulum/smooth endoplasmic reticulum; EE, early endosome.

Hypothesis

There is a monensin-sensitive storage pool of ANF in atrial cardiocytes, which maybe through a constitutive-like pathway.

Materials and Methods

Cell Culture

HL-1 cells (obtained from Dr. William Claycomb, Louisiana State University Medical Center, New Orleans, LA, U.S.A.) were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air in Claycomb medium (51800C, SAFC Biosciences) according to the published protocol (Claycomb et al., 1998) supplemented with 10% fetal bovine serum (F2442, Sigma Aldrich), 100 μg/mL penicillin/streptomycin (15140, Gibco), 100 μM norepinephrine [10 mM norepinephrine stock solution (A0937, Sigma-Aldrich) dissolved in 30 mmol/liter L-ascorbic acid (A7631, Sigma-Aldrich)] and 2 mM L-glutamine (25030, Gibco). HL-1 cells were seeded onto pre-coated 0.02% gelatin (214340, BD Difco Gelatin) and 5 μg/mL fibronectin (F1141, Sigma Aldrich) T-75 culture flasks. HL-1 cells at passage 63 were kept as a frozen stock and once thawed, the cells were cultured in supplemented Claycomb media in the absence of norepinephrine. All the experiments were performed on passage 64.

Pharmacological Agents

Endothelin-1

Endothelin-1 (H-6995, Bachem) was reconstituted in 5% acetic acid and 95% degassed Milli-Q water at a concentration of 1 mg/mL. ET-1 was stored as 5 µL aliquots at -20 °C.

Monensin

Monensin (M5273, Sigma) was dissolved in absolute ethanol as a 50 mM stock solution and stored at 4 °C.

Wortmannin

Wortmannin (W1628, Sigma) was dissolved in DMSO as a 1mg/ml stock solution and stored as 10 µL aliquots at -20 °C.

Dynasore, Dynamin Inhibitor I

Dynasore, Dynamin Inhibitor I (324410, Calbiochem) was dissolved in DMSO as a 100 mM stock solution and stored as 5 µL aliquots at -20 °C.

Membrane Traffic Inhibitor A5

Membrane Inhibitor A5 (444805, Calbiochem) was dissolved in degassed Milli-Q water as a 30 mM stock stolution and stored as 15 µL aliquots at -20 °C.

Dec-RVKR-CMK

Dec-RVKR-CMK (Enzo Life Sciences, ALX-260-022) was dissolved in degassed Milli-Q water as a 10 mM stock stolution and stored as 5 µL aliquots at -20 °C.

Treatment of HL-1 cells

ANF secretion assays

HL-1 cells were plated at a density of 1.25 x 10^4 cells/cm^2 in a 24 well pre-coated with gelatin/fibronectin and allowed to culture for 24 h in norepinephrine-free, fully supplemented Claycomb media. Cells were rinsed twice with DMEM/F12 (D6421, Sigma) and incubated for another 24 h in DMEM/F12 supplemented with 100 μg/mL penicillin/streptomycin, 5 mM L-glutamine and [1X] ITS (insulin, transferrin, sodium selenite) supplement (I1884, Sigma). After a 24 h serum starvation period, cells were rinsed three times with DMEM/F12 and incubated with 500 μL of supplemented DMEM/F12 containing the required agent. All experiments were performed after a 24 h period of serum starvation, unless otherwise stated. After the required incubation time, media was collected and immediately placed on ice. Media was concentrated two fold by freeze drying 450 μL of media and reconstituting in 225 μL of RIA buffer, unless otherwise stated.

Ultracentrifugation

Cell Fractionation

The methodology for cell fractionation performed on HL-1 cells was adapted from a previous study (Bensimon et al., 2004). HL-1 cells were plated at a density of 1.25 x 10^4 cells/cm^2 in a T-75 flask pre-coated with gelatin/fibronectin and allowed to culture for 24 h in norepinephrine-free, fully supplemented Claycomb media. Cells were rinsed twice with DMEM/F12 and incubated for another 24 hours in supplemented DMEM/F12. After a 24 h serum starvation period the flasks were rinsed three times with ice cold homogenization medium (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4). HL-1 cells were homogenized in 10 mL of homogenization medium containing 50 µL of protease inhibitor cocktail (1:200 dilution, P8340, Sigma-Aldrich) with four strokes in a Potter Elvehjem homogenizer. The cell homogenates were centrifuged at 1,900 x g_{max} for 10 min at 4 ^oC using a J20.1 rotor to obtain a nuclear and postnuclear fraction. To 4 mL of working density gradient solution consisting of 2 mL diluent solution (0.25 M sucrose, 10 mM EDTA, 60 mM Tris-HCl, pH 7.4), 60 µL of protease inhibitor cocktail (1:200 dilution, P8340, Sigma-Aldrich) and 10 mL Optiprep (D1556, Sigma), 7.4 mL of the postnuclear fraction were added (final OptiPrep concentration of 17.5%). This mixture was then transferred to tubes fitting the NVT-65 rotor (Beckman Coulter, Fullerton, CA) and centrifuged for 12 h at 341,650 g_{av}. At the completion of ultracentrifugation, 25 fractions of approximately 0.44 mL each were collected by upward displacement using fluorinert FC-40 (F9755, Sigma-Aldrich) as the displacement medium and a fraction recovery system (Beckman Fraction Recovery System) connected to a syringe pump (975 Harvard Apparatus Compact Infusion Pump) set at a flow rate of

0.59 mL/min and a fraction collector (Bio-Rad, Model 2110 fraction collector) set at 0.75 min./fraction.

For ANF quantification, 125 μL of each fraction was mixed with an equal volume of 1M acetic acid, placed in boiling water bath for 10 min, frozen at -80 °C, and freeze dried followed by reconstitution in 250 μL of RIA buffer. An additional 27.5 μL of each fraction was taken for refractive index (RI) measurement followed by protein quantification. RI was measured using a digital handheld refractometer (Kruss, DR201-95). The RI (η) was converted to density (ρ) using the following equation (OptiPrep application sheet S01, Axis-Shield): $\rho = 3.4713\eta - 3.6393$. Protein concentration was determined using BCA protein assay kit (23225, Pierce). The absorbance was read at 562 nm on a BioTek microplate reader and data analysed using Gen5 software.

Tissue Fractionation

Mouse atria ultracentrifugation was performed according to the cell fractionation protocol above except for the following changes. Atria were obtained from male CD-1 mice (Average weight 30 g) after decapitation. One atria was used for one ultracentrifugation gradient. The tissue was placed in 10 mL of ice-cold homogenization medium and homogenized first with a PT30-35 homogenizer fitted with a 7 mm probe for 15 seconds at 70% power and then with four strokes in a Potter Elvehjem homogenizer. The protease inhibitor cocktail was used at a dilution of 1:100 (P8340, Sigma Aldrich).

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

HL-1 extract

HL-1 cells were plated at a density of 1.25 x 10^4 cells/cm^2 in a T-75 flask pre-coated with gelatin/fibronectin and allowed to culture for 24 h in norepinephrine-free, fully supplemented Claycomb media. Cells were rinsed twice with DMEM/F12 and incubated for another 24 h in supplemented DMEM/F12, unless otherwise stated. After a 24 h serum starvation period the flasks were rinsed three times with ice cold PBS and scraped from the cell culture flask in the presence of 10 mL ice cold 1X extractant (1% NaCl, 0.1N HCl, 1.0 M acetic acid). The cells were homogenized with a PT30-35 homogenizer fitted with a 7 mm probe for 15 seconds at 70% power. The cell homogenate was centrifuged at 1,900 x g_{max} for 10 min at 4 ^oC using a J20.1 rotor. The supernatant was immediately passed through a pre-wet Sep-Pak, frozen at -80 ^oC and later freeze dried.

HL-1 media extract

The T-75 cell culture flask was incubated for 1 h in fresh media. Incubation media was removed and immediately centrifuged at 1,900 x g_{max} for 10 min at 4 ^oC using a J20.1 rotor to pellet any cellular debris and dead cells. Equivalent volume of ice cold 2X extractant (2% NaCl, 0.2N HCl, 2.0 M acetic acid) was added to the media and immediately passed through a pre-wet Sep-Pak, frozen at -80 ^oC and later freeze dried.

Tissue extract

Two atria and three ventricle tissue were obtained from male, CD-1 mice and extracted in 10 mL of 1X extractant (1% NaCl, 0.1 N HCl, 1.0 M Acetic acid). The tissue was homogenized using a Polytron fitted with a PT 30-35 probe. The atrial and ventricular homogenates were centrifuged at 1,900 g_{max} for 10 min at 4 ^{o}C using a J20.1 rotor. The supernatant was immediately passed through a pre-wet Sep-Pak, frozen at -80^{o}C and later freeze dried.

Density gradient fraction extract

Density gradient fractions 18 to 23 were pooled from nine gradient runs. To 3.15 mL of pooled density gradient fraction, 3.15 mL of ice-cold 2X extractant (2% NaCl, 0.2N HCl, 2.0 M acetic acid) was added and incubated on ice for 30 min. To this solution 10 mL of 0.1% of TFA was added, and the sample was centrifuged at 1,900 g_{av} for 5 min at 4 °C using a JE-6B rotor to pellet any debris. The sample was loaded onto the column through a three way valve connected to pump A, which was set at a flow rate of 0.85 ml/min and pump B with 80% ACN, 0.1% TFA set at a flow rate of 0.15 ml/min.

RP-HPLC

The RP-HPLC system consisted of Waters 1525 binary HPLC pumps, 2489 UV/Visible detector, and fraction collector III controlled by Breeze 2 software (Waters, Milford, MA). The freeze-dried sample was reconstituted with 1 mL of 0.1% TFA. Separation was achieved on a Jupiter C_{18} column (300 x 7.8 mm, 5 µm, 300 Å) using a linear gradient of 15-55% ACN in 0.1% TFA at a flow rate of 1.5 mL/min over an 80 min

period. Three-milliliter fractions were collected and 100 μL of 1mg/mL BSA was added to each fraction. The fractions were frozen at -80^{o}C and later freeze dried for ANF RIA.

Radioimmunoassay

ANF concentration was measured by RIA using a double antibody method as previously described (Sarda et al., 1989). ANF standard curve was generated using rat ANF_{99-126} peptides (Advanced ChemTech, PX8895) with concentration ranging from 31.25, 62.50, 125.0, 250.0, 500.0 and 1000.0 pg/mL in RIA buffer (0.1 M sodium phosphate; 0.05 M NaCl; 0.01% sodium azide; 0.1% Triton X-100; 0.1% heat treated BSA). All reagents, standards and samples were diluted in RIA buffer. Reactions were carried out in 12 x 75 mm polystyrene tubes (Sarstedt). In each tube, 100 μL of ANF standard or sample was mixed with 100 μL of ANF antiserum at a dilution of 1:12,000 (RAB 005-24, Phoenix Pharmaceuticals) and incubated in the dark at 4 ^{o}C for 4 hours. Following the incubation, 100 μL of iodinated ANF_{99-126} (10,000 counts/minute) was added to the standards or samples and incubated in the dark at 4 ^{o}C for 24 hours. Following this second incubation, 100 μL each of goat anti-rabbit gamma globulin (GAR-500, Phoenix Pharmaceuticals) and normal rabbit serum (16120-107, Gibco) were added to the tubes and incubated at room temperature for 2 h. 1.5 mL of 6.25% polyethylene glycol (Sigma-Aldrich, P2139) was added and the tubes were centrifuged at 865 g_{av} (2700 rpm) on Beckman J-6 M centrifuge at 4 °C for 45 min. The supernatant was discarded and the pellets were counted using a gamma-counter (1272 CliniGamma, LKB Wallac). Quality control was carried out using the 125.0 pg/mL and 250.0 pg/mL concentration of ANF_{99-126}.

Immunoblotting

To 40 μL of gradient fraction, 40 μL of 2X SDS loading buffer (Cell Signaling Technology) containing 2X extractant (2% Triton X-100, 2% sodium deoxycholate, 300 mM NaCl, 2 mM EDTA) and protease inhibitor cocktail (1:200 dilution, P8340, Sigma) was added and incubated for 30 minutes at 4 °C in a tube rotator. Samples were boiled at 95 °C for five minutes and 40 μL of each fraction was loaded onto 6% and 12% SDS-PAGE. Proteins were electrotransferred onto polyvinylidene difluoride membrane (Immun-Blot 0.2 μm, Bio-Rad) for 30 min at 100 V using Criterion Blotter (Bio-Rad) in transfer buffer containing 25 mM Tris, 192 mM Glycine and 10% methanol. Membranes were blocked for 1 h in blocking buffer (5% low fat milk in TBS, 0.1% Tween-20) and then incubated in primary antibody diluted in blocking buffer overnight at 4 °C. All antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. To characterize the gradient fractions antibodies for organelle proteins calnexin (1:500, sc-6465-R), GS28 (1:500, sc-30096), EEA1 (1:250, sc-6414), clathrin (1:500, sc-6579), corin (1:500, sc-67179), ChrA (1:500, sc-13090) and ChrB (1:500, sc-20135) were used. Membrane was washed in TBS containing 0.1% Tween-20 followed by incubation for 1 h in either goat anti-rabbit IgG HRP (1:5000, sc-2004) or donkey anti-goat IgG HRP (1:5000, sc-2020) diluted in blocking buffer. Proteins were visualized by enhanced chemiluminescence (ECL, Roche) and exposure to X-ray film (Santa Cruz Biotechnology).

Statistical Analysis

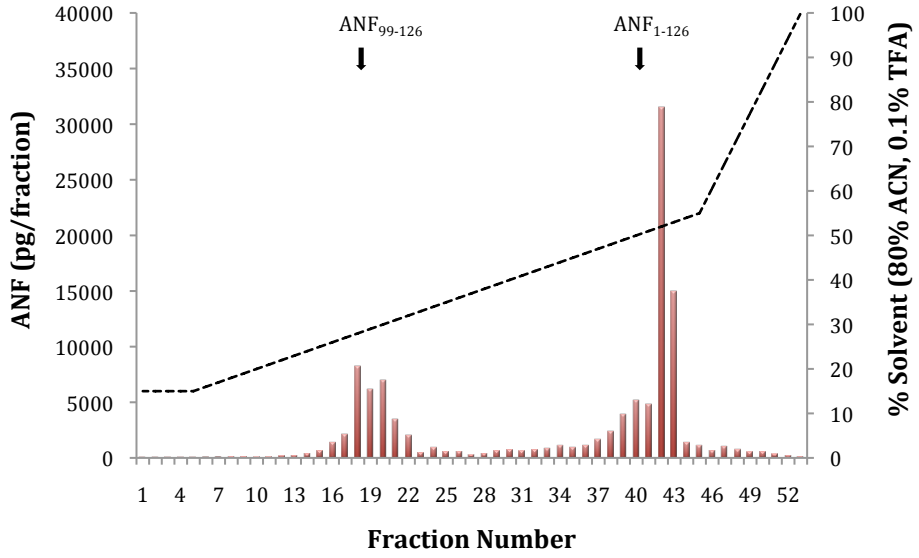
Data is reported as mean ± standard error of the mean (SEM). An unpaired Student's t-test, with two tailed distribution was performed to determine statistical significance between mean pairs. A value of $p \leq 0.05$ was considered significant.

Results

Molecular forms of Intracellular and secreted ANF

As part of establishing the validity of HL-1 atrial cardiomyocyte cell line to study ANF processing and secretion, we compared the intracellular molecular forms of the HL-1 cell line with the mouse atrial and ventricle extract. RP-HPLC profile of mouse atria (Figure 3A) and mouse ventricle (Figure 3B) extract show presence of both processed low molecular weight ANF and high molecular weight proANF, with the latter being in much higher quantities. As a comparison, RP-HPLC profile of HL-1 atrial cardiomyocytes also shows the presence of both the processed and the pro form of ANF. HL-1 cells cultured in fully supplemented Claycomb media show both molecular forms in almost an equivalent ratio due to a decrease in intracellular quantities of proANF (Figure 4A). The RP-HPLC profile of HL-1 cells that had been serum starved for 24 hours had a similar profile as compared to mouse atria, with the predominant intracellular molecular form of proANF (Figure 4B). The RP-HPLC profile of media from cells cultured in fully supplemented Claycomb media shows presence of small quantities of proANF in addition to the main secretory form of low molecular weight ANF (Figure 5A). However, HL-1 cells that had been serum starved for 24 hours show that the only secretory form of ANF is the processed form of low molecular weight ANF (Figure 5B). In summary, RP-HPLC analysis on HL-1 atrial extracts identified two RP-HPLC peaks, one of low molecular weight ANF and another of high molecular weight proANF. HL-1 atrial cardiomyocytes secreted both the processed form of low molecular weight ANF and high molecular weight proANF depending on the cell culture conditions.

A



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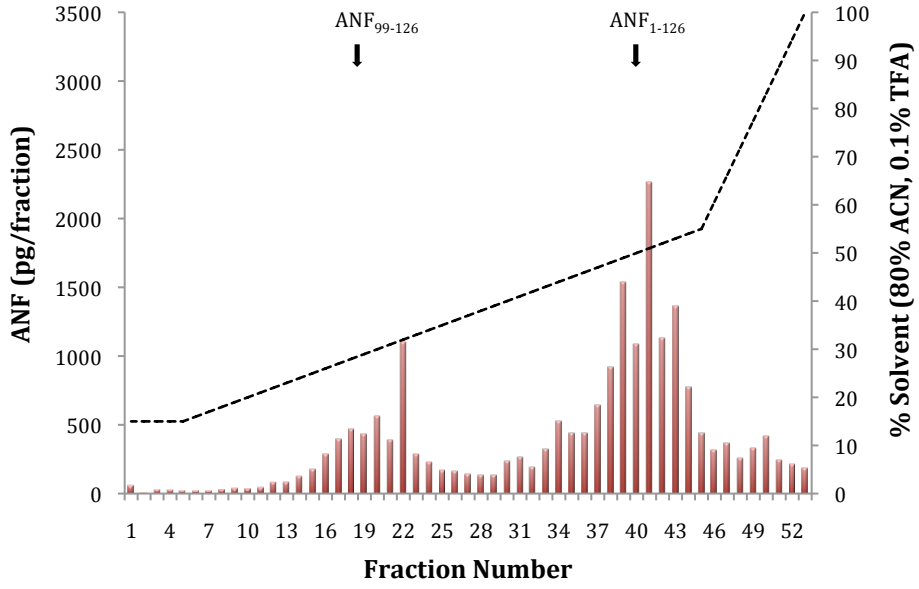
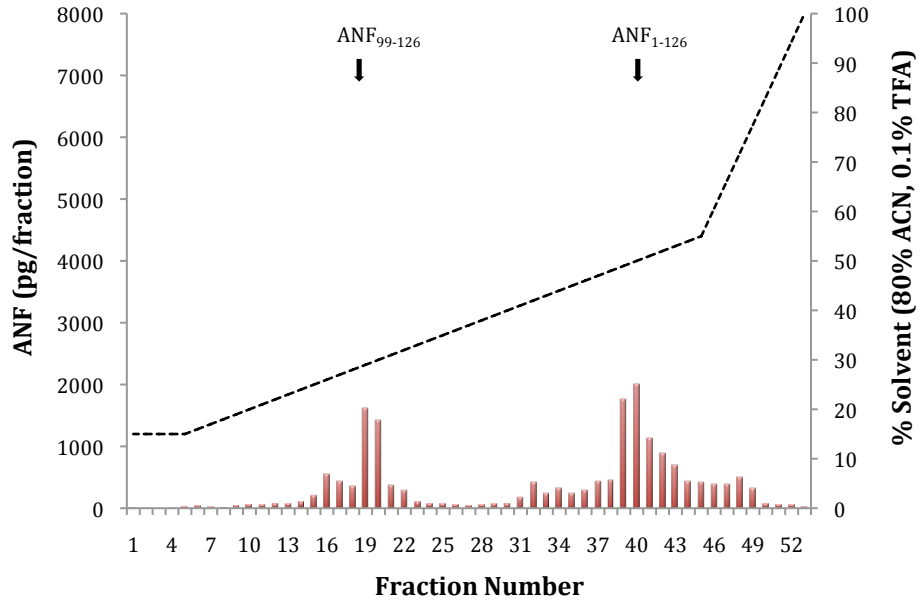


Figure 3: Molecular forms of intracellular ANF in mouse atria and ventricle.

RP-HPLC profiles of intracellular ANF in (**A**) mouse atrial and (**B**) ventricle extract. Arrows indicate the elution position for ANF_{99-126} and $proANF_{1-126}$.

A



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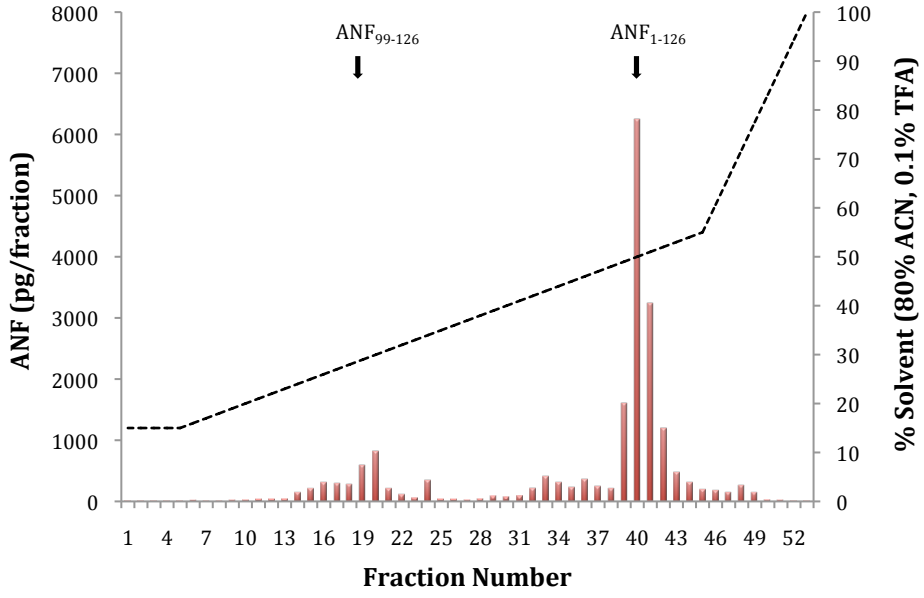
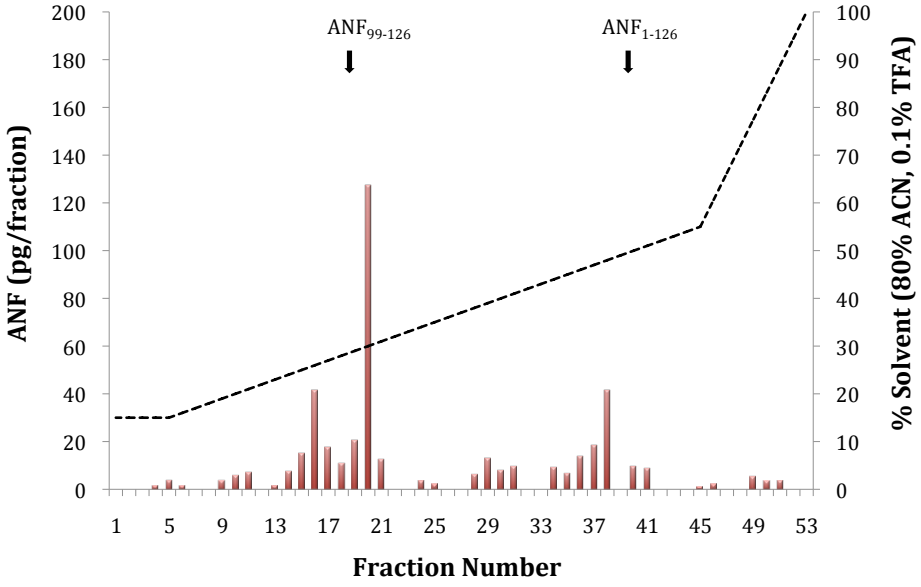


Figure 4: Molecular forms of intracellular ANF in HL-1 atrial cardiomyocytes.

RP-HPLC profiles of intracellular ANF in (**A**) HL-1 cardiomyocytes cultured in fully supplemented Claycomb media (**B**) HL-1 cardiomyocytes serum starved for 24 h in supplemented DMEM/F12. Arrows indicate the elution position for $ANF_{99\text{-}126}$ and $proANF_{1\text{-}126}$. Data is representative of two independent experiments.

A



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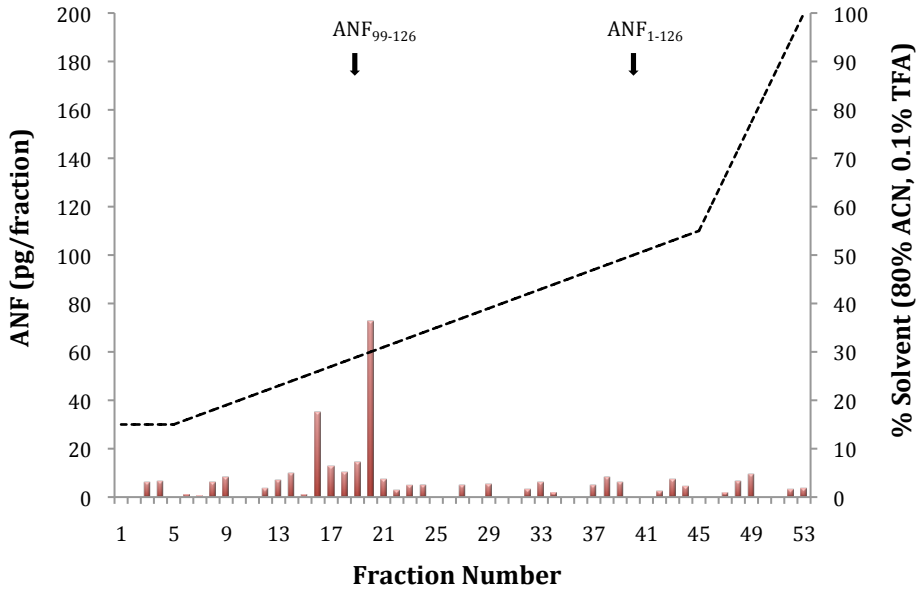


Figure 5: Molecular forms of secreted ANF in HL-1 atrial cardiomyocytes.

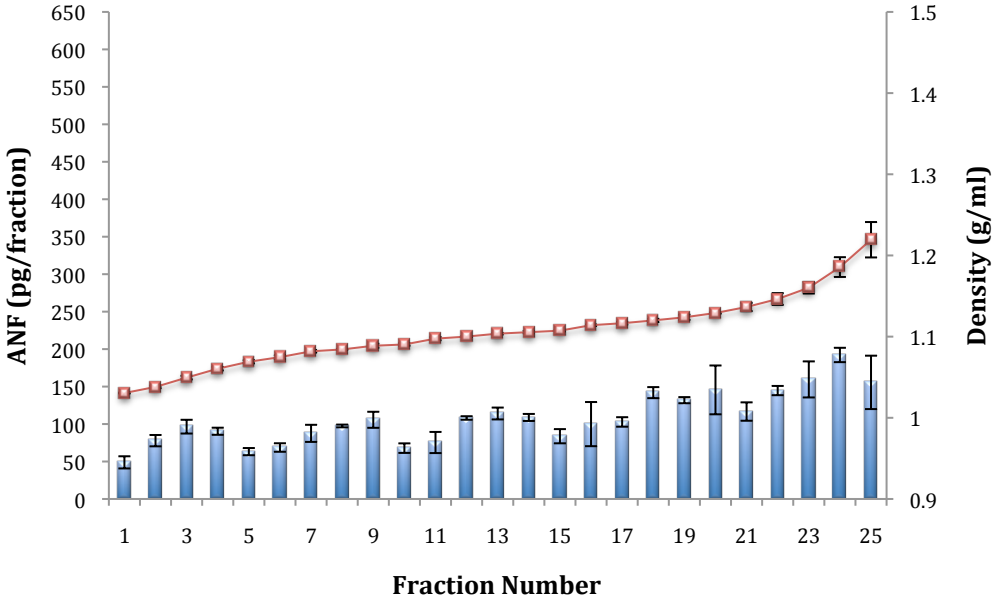
RP-HPLC profiles of secreted ANF in HL-1 cardiomyocytes. (A) 1 h incubation media from cells cultured in fully supplemented Claycomb media. (B) 1 h incubation media from cells serum starved in DMEM/F12 media for 24 h. Arrows indicate the elution position for $ANF_{99\text{-}126}$ and $proANF_{1\text{-}126.}$ Data is representative two independent experiments.

Cell fractionation of HL-1 atrial cardiomyocytes

Gradient Optimization

In order to isolate the different pools of ANF in HL-1 atrial cardiomyocytes, these cells were serum starved for 24 h and subjected to self generated density gradient ultracentrifugation to separate subcellular organelles. Initial runs at 290,000 g_{av} for 5 h showed ANF being distributed throughout the gradient fractions (Figure 6A), while the total protein distribution profile showed protein predominantly in the higher density fractions (Figure 6B). As the centrifugal force and time were increased to 340,000 g_{av} and 7 h, both the ANF and protein distribution shifted towards the higher density fractions corresponding to the steep density curve from fraction 22 to 25 (Figure 7 A&B). Even though a slight shift in ANF quantities towards the higher density fractions was seen, the gradient profile did not show distinct localization of intracellular ANF pools. Ultracentrifugation conditions were further intensified to 12 h at 340,000 g_{av} and as a result the gradient profile shifted towards higher density fractions, with majority of ANF being accumulated at the higher density fractions (Figure 8A). Under these conditions there was a steep change in density between fractions 19 to 25. The total protein profile also shifted towards higher density fractions (Figure 8B). Ultracentrifugation performed on cells cultured in fully supplemented Claycomb media showed a decrease in ANF in the higher density fractions 22 to 25 (Figure 9). To confirm whether the ANF gradient profile seen in HL-1 cells was reproducible in tissue, ultracentrifugation on mouse atria showed similar results to cell fractionation on HL-1 cells that had been serum starved (Figure 10). In conclusion, self-generated density gradient conditions were established at 340,000 g_{av} for 12 h and used in future experiments to isolate intracellular ANF pools.

A



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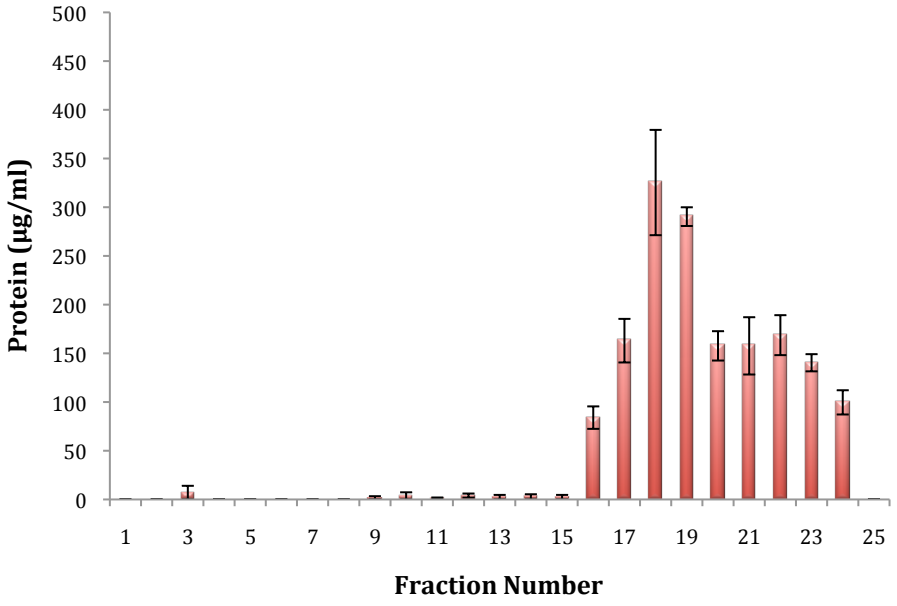
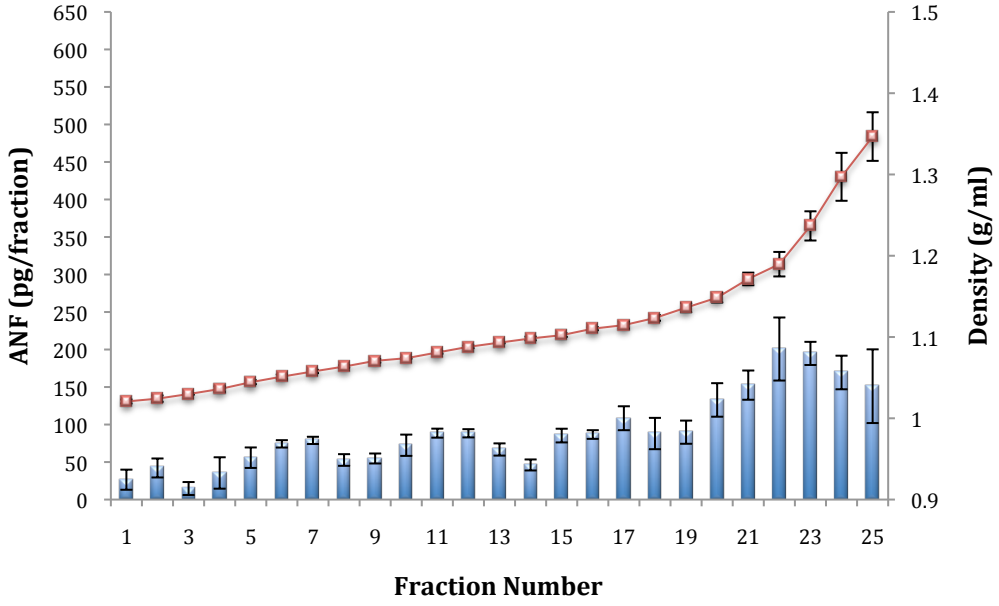


Figure 6: HL-1 cell fractionation at 290,000 g_{av} for 5 h.

HL-1 cell fractionation was performed at 290,000 $_{gav}$ for 5 h. (A) ANF distribution and density of each gradient fraction is shown and (B) shows protein distribution in gradient fractions. Data is represented as mean ± SEM from an experiment done in triplicate.

A



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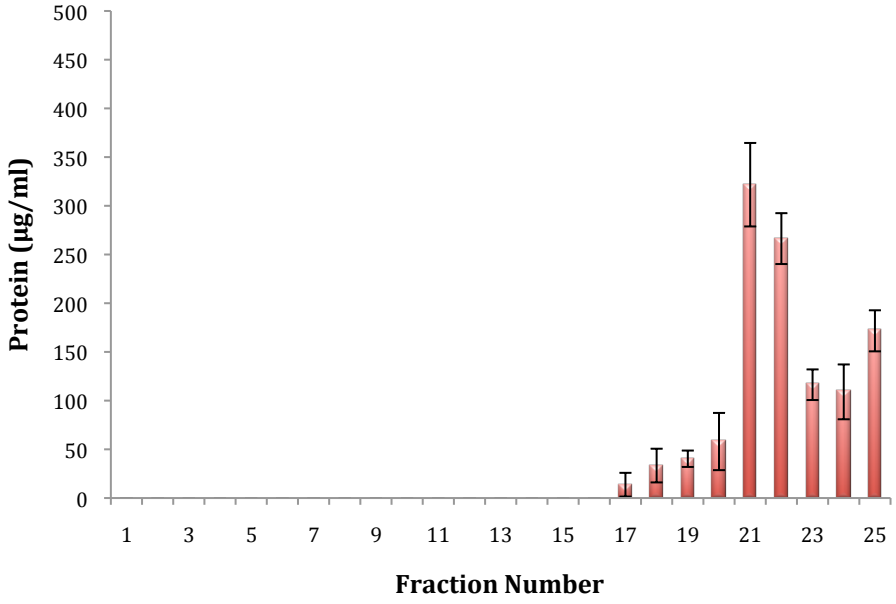
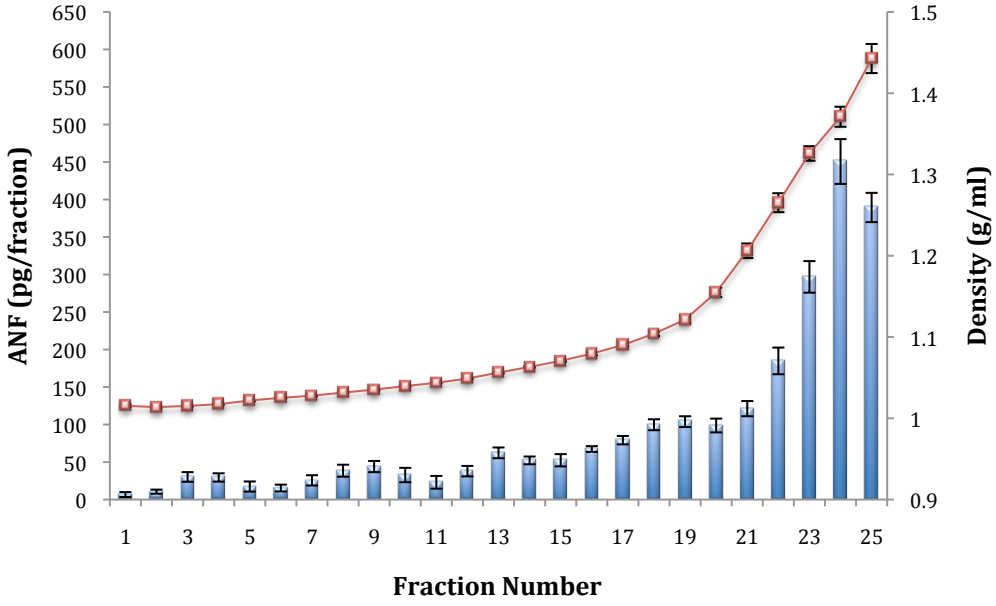


Figure 7: HL-1 cell fractionation at 340,000 g_{av} for 7 h.

HL-1 cell fractionation was performed at 340,000 $_{gav}$ for 7 h. (A) ANF distribution and density of each gradient fraction is shown and (B) shows protein distribution in gradient fractions. Data is represented as mean ± SEM from an experiment done in triplicate.

A



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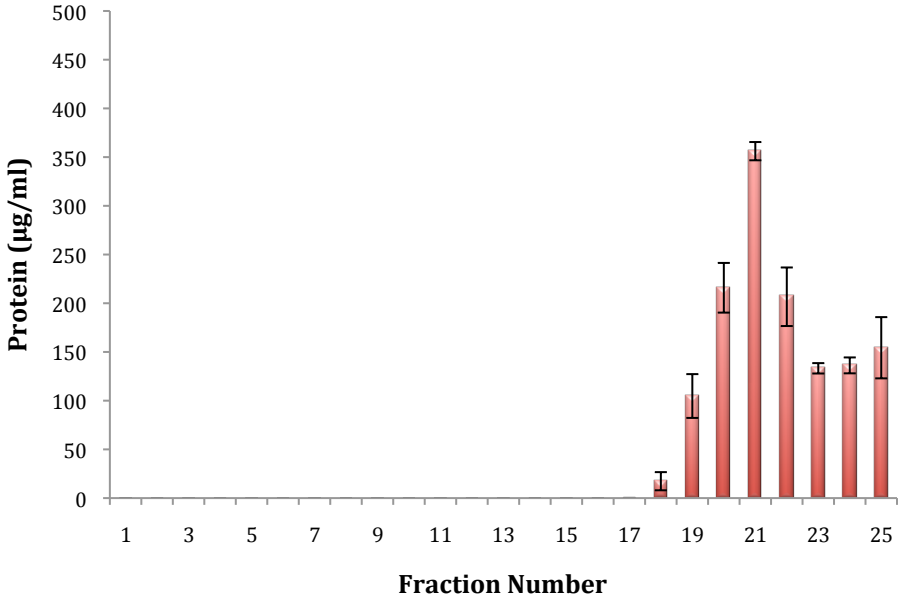


Figure 8: HL-1 cell fractionation at 340,000 g_{av} for 12 h.

HL-1 cell fractionation was performed at 340,000 $_{gav}$ for 12 h. (A) ANF distribution and density of each gradient fraction is shown and (B) shows protein distribution in gradient fractions. Data is represented as mean ± SEM from three independent experiments done in triplicate.

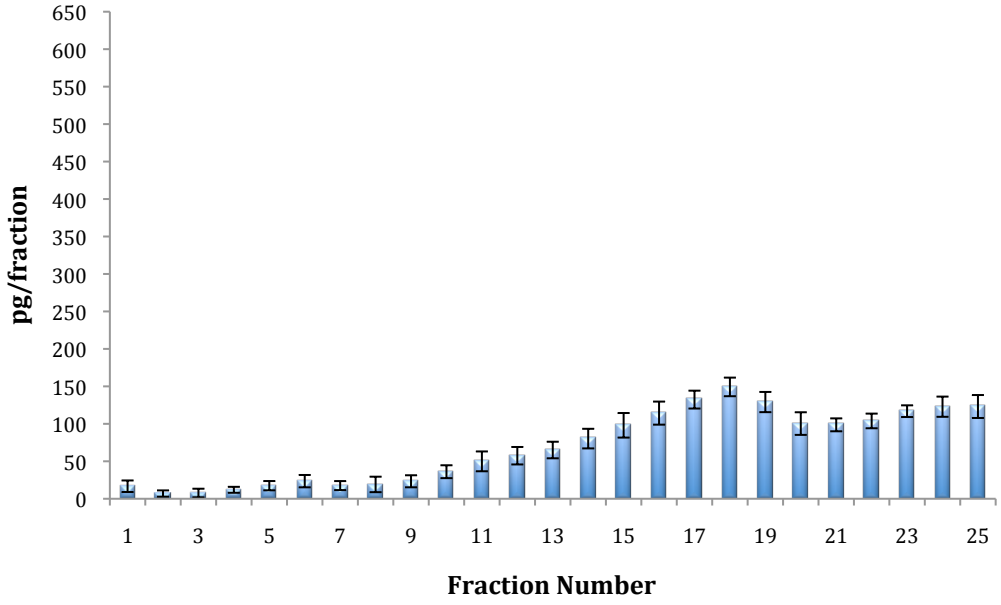


Figure 9: HL-1 cell fractionation for cells cultured in Claycomb media.

HL-1 atrial cardiomyocytes were cultured in fully supplemented Claycomb media. Cell fractionation was performed at 340,000 $_{gav}$ for 12 h. ANF distribution in each gradient fraction is shown. Data is represented as mean ± SEM from three independent experiments done in duplicate.

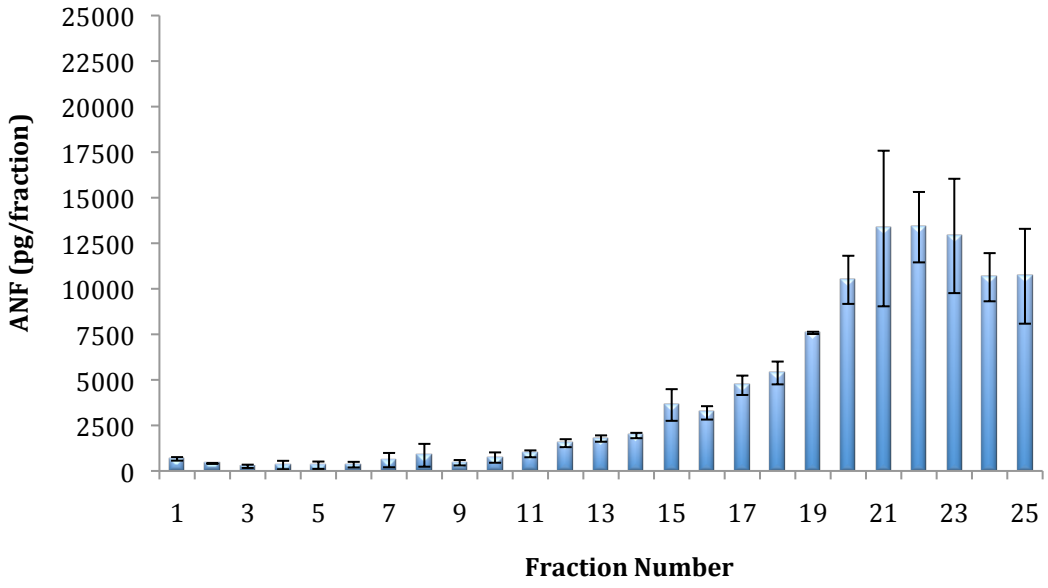


Figure 10: Tissue fractionation on mouse atria.

Tissue fractionation of mouse atria was performed at 340,000 $_{gav}$ for 12 h. ANF distribution in each gradient fraction is shown. Data is represented as mean ± SEM from an experiment done in duplicate.

Gradient Characterization

Density gradient fractions were characterized with organelle markers for ER (calnexin), Golgi apparatus (GS28), early endosomes (EEA1), ISGs (clathrin), PM localized proANF protease (corin), and SGs (ChrA and ChrB) (Figure 11). Calnexin was localized in fractions 18 and 19 with GS28 being also present in fraction 19. EEA1 was identified in fraction 20, which also showed presence of corin and minute quantities of clathrin; however, clathrin was predominantly present in fraction 21. Both ChrA and ChrB, markers for SGs, were localized in low density and high density fractions. Based on the profile of organelle markers and the presence of ANF in each fraction as determined by RIA (Figure 8), fractions 18 to 23 were pooled individually from nine different gradients and taken for RP-HPLC analysis to determine the molecular forms of $ANF_{99\text{-}126}$ and $proANF_{1\text{-}126}$ in each of these fractions (Figure 12). Low molecular weight ANF was found predominantly in fractions 18, 19 and 20. High molecular weight proANF was identified in all the fractions taken for RP-HPLC analysis, except for fraction 19, which showed the presence of only the low molecular weight ANF. Furthermore, fractions 22 and 23 contained proANF in high quantities as compared with the other fractions that were analyzed. In summary, low molecular weight ANF was identified in fractions 18, 19 and 20, which are characterized by the presence of Golgi, early endosome and clathrin, while high molecular weight proANF was identified in fractions 22 and 23, that also showed the presence of ChrA and ChrB, corresponding to mature granules.

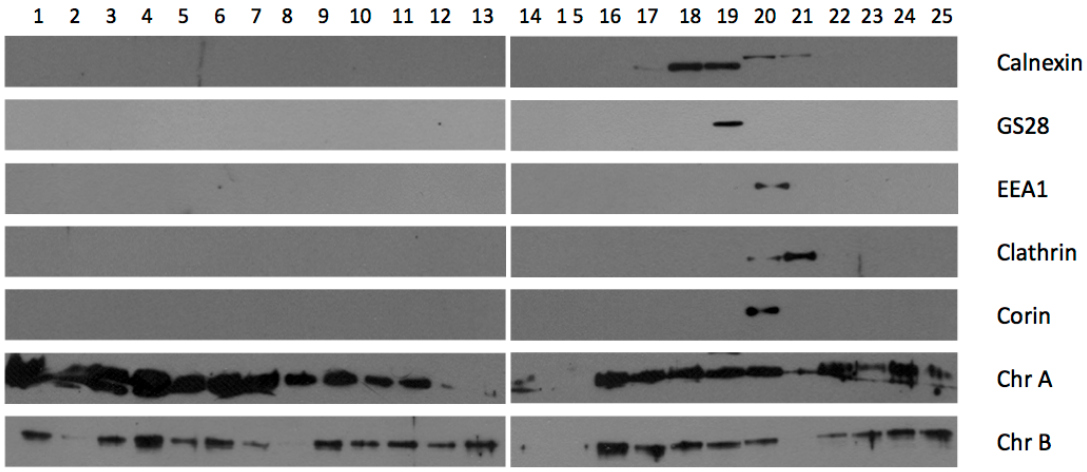


Figure 11: Distribution profile of organelle markers in HL-1 density gradient.

HL-1 cells were fractioned at 340,000 $_{gav}$ for 12 h and equal volume aliquots of each fraction was taken for SDS-PAGE and immunoblotting with antibodies against calnexin (ER), GS28 (Golgi apparatus), EEA1 (early endosomes), clathrin (ISGs), corin (proANF protease), ChrA and ChrB (SGs). Fractions 1 to 13 and 14 to 25 were run on different gels but transferred and probed on the same membrane.

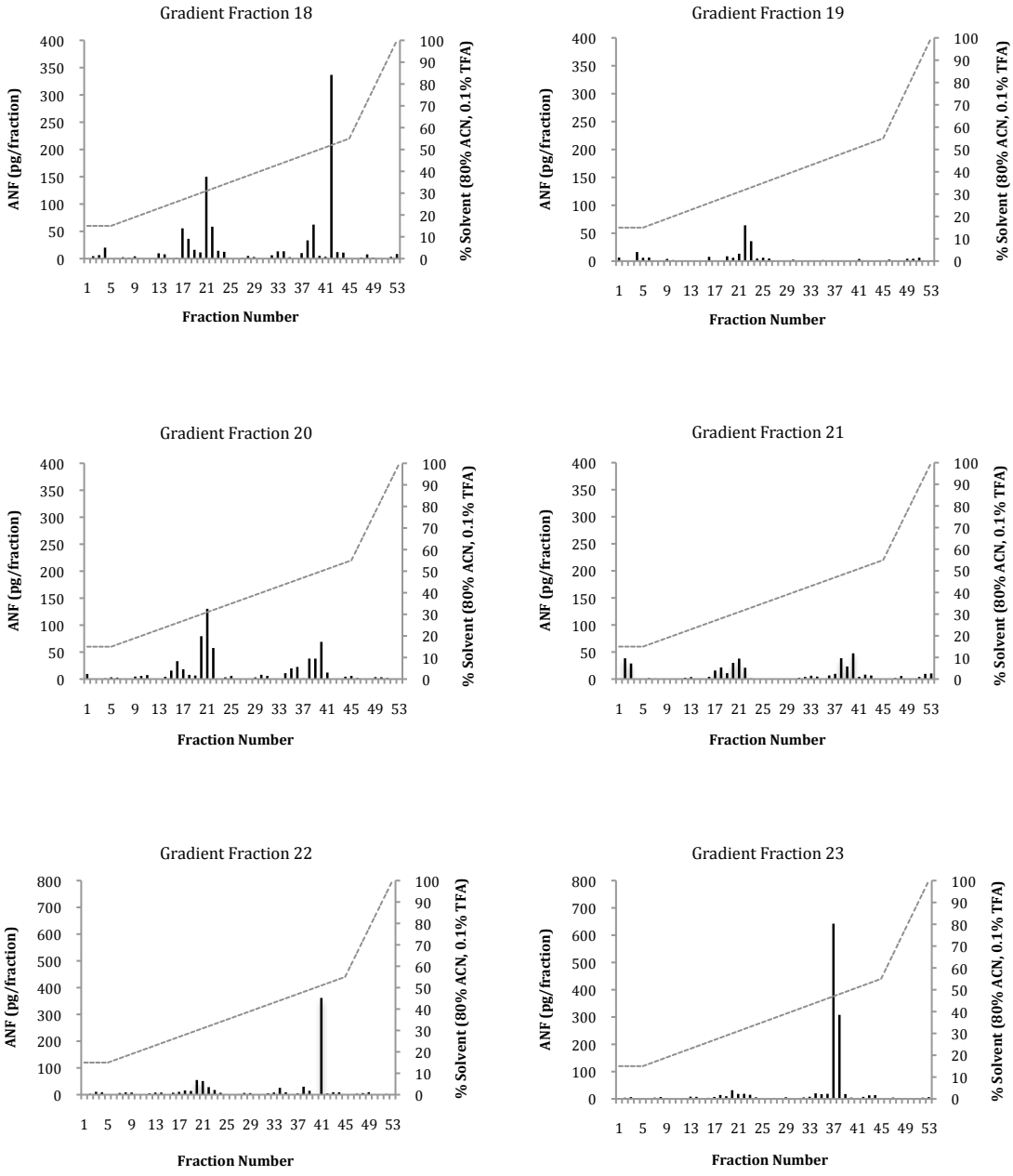


Figure 12: RP-HPLC profile of gradient fractions.

HL-1 cells were fractioned at 340,000 $_{gav}$ for 12 h and 350 µL of each gradient fraction from nine gradients was pooled and taken for RP-HPLC analysis. Low molecular weight ANF was found predominantly in fractions 18, 19 and 20.

ET-1 induced ANF secretion

To distinguish regulated secretion from secretagogue independent secretion, HL-1 cells were stimulated with ET-1 to observe stimuli-dependent increase of ANF in cell culture media. Among the various secretagogues known to stimulate ANF secretion, ET-1 is the most potent (Schiebinger and Gomez-Sanchez, 1990). Prior to stimulation, cells were serum-starved for 24 h in DMEM/F12 supplemented with L-Glut and ITS (insulin, transferring, sodium selenite) and incubated for 1 h with ET-1 in the same media. The established protocol for culturing HL-1 cells in Claycomb media calls for cells to be cultured in highly stimulating conditions. One of the constituents of fully supplemented Claycomb media is 100 μM norepinephrine, which is included to maintain the differentiated and beating phenotype of HL-1 cells (Claycomb et al., 1998); however, norepinephrine is a stimulant of ANF secretion (Schiebinger et al., 1987). As a result HL-1 cells were cultured in norepinephrine-free Claycomb media starting from one passage prior to ET-1 stimulation. ET-1-stimulated ANF secretion in a dose-dependent manner (Figure 13). The most potent concentration was determined to be 20 nM. ANF secretion increased from 38.86 ± 2.50 pg/ml in control to 69.68 ± 6.22 pg/ml in 20 nM ET-1-stimulated secretion, an approximate increase of 1.8 fold. ET-1 concentration as high as 100 nM was also evaluated, however the ANF secretory response was lower than 10 nM (data not shown). All further experiments used 20 nM ET-1 to stimulate ANF secretion. These results demonstrate that HL-1 cells can be stimulated by ET-1 after a serum-starvation period, and 20 nM ET-1 results in the most potent response for ANF secretion.

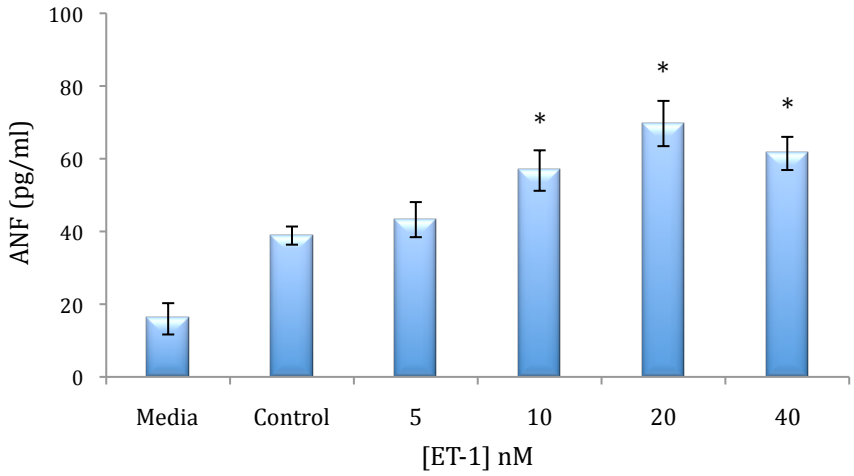


Figure 13: Effect of ET-1 on ANF secretion

Concentration-dependent effect of ET-1 on ANF secretion in HL-1 atrial cardiomyocytes. Cells were incubated for 1 h with the specified concentration of ET-1. Data is represented as mean ± SEM from three independent experiments done in triplicate.*indicates $p<0.01$ for control versus ET-1.

Monensin sensitive secretory pathway

In an *ex vivo* study of rat atrial perfusion with monensin, a decrease in basal ANF secretion was observed in addition to a loss of secretgogue-dependent ANF secretion suggesting that the monensin sensitive pathway is derived from a constitutive-like pool (Ogawa et al., 1999). Initial trial studies on the effect of monensin on ANF secretion in HL-1 cells used the same concentration of monensin that was previously used in the perfusion study. Incubation of non-serum starved HL-1 cells with 5 μM monensin showed a time-dependent increase in ANF secretion (Figure 14). Thereafter, nanomolar concentrations of monensin were used to treat HL-1 cells that were cultured in fully supplemented Claycomb media or that had been serum starved for 24 h. Cells cultured in fully supplemented Claycomb showed an increase in ANF secretion for concentration as low as 5 nM (approximately 1.18-fold) (Figure 15A). Low nanomolar concentrations of monensin showed a dose-dependent effect on increasing ANF secretion, with ANF secretion rising to ~1.24 for 50 nM and ~1.21 nM for 100 nM monensin. However, in cells that had been serum-starved for 24 h, no change in ANF secretion was observed (Figure 15B).

To determine whether monensin prevents secretagogue-dependent ANF release, HL-1 cells were pretreated for 2 h with 10 nM monensin, followed by a 1 h incubation with ET-1 (Figure 16). Monensin prevented ET-1-dependent ANF secretion; there was a slight increase in ANF secretion (~1.21 fold), however not statistically significant. Furthermore, to determine the monensin-sensitive secretagogue compartment in intracellular ANF pools, monensin-treated cells were subjected to cell fractionation (Figure 17); however, no significant changes in the gradient fractions were observed. In

conclusion, a monensin-sensitive, secretagogue dependent ANF secretory pathway was identified in HL-1 cells; however, the monensin-sensitive pool could not be identified through a cell fractionation technique.

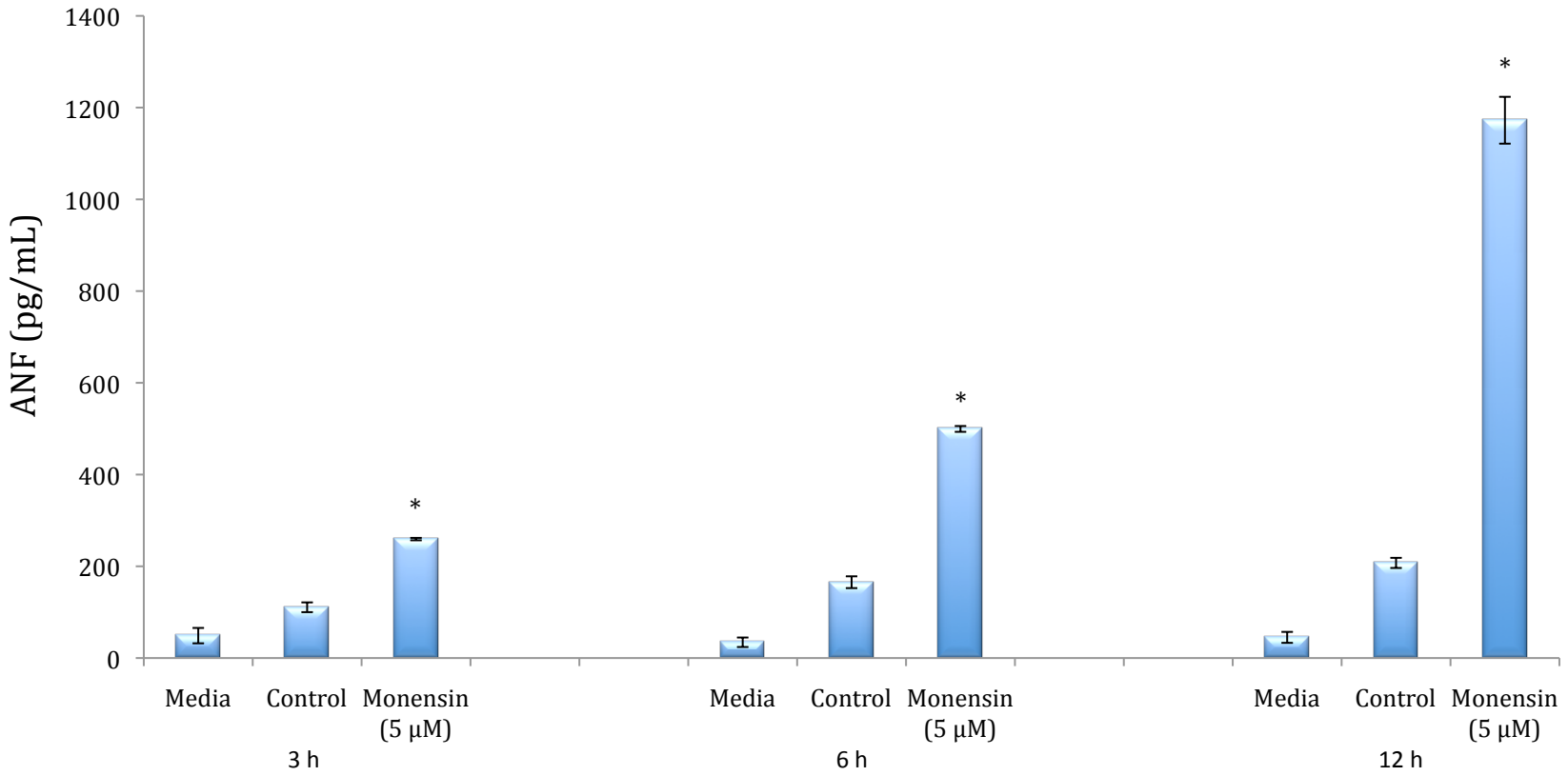
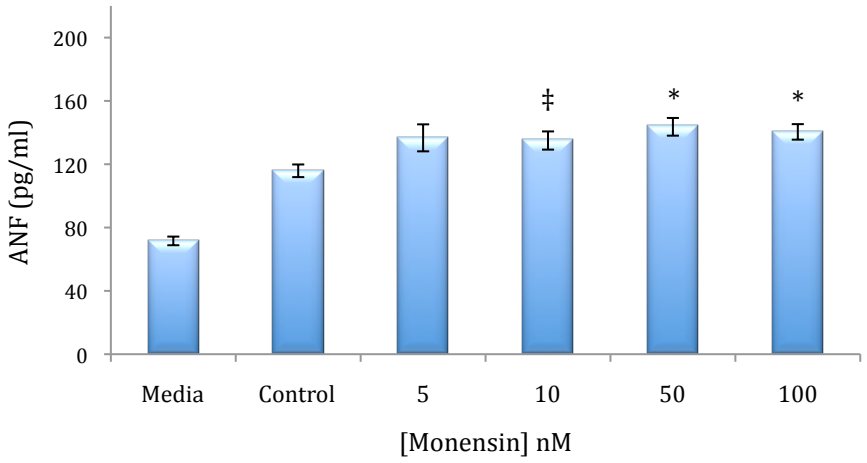


Figure 14: Time-dependent response of 5 µM monensin

HL-1 atrial cardiomyocytes were cultured in fully supplemented Claycomb media. Cells were incubated for 3 h, 6 h and 12 h with 500 µL of supplemented Claycomb containing 5 µM monensin and controls containing 0.01% EtOH. Media was collected at specified time points and immediately placed on ice, and 100 µL of media in duplicate was taken for RIA. Data is represented as mean ± SEM from an experiment done in triplicate. * indicates $p<0.01$ for control versus monensin.

A



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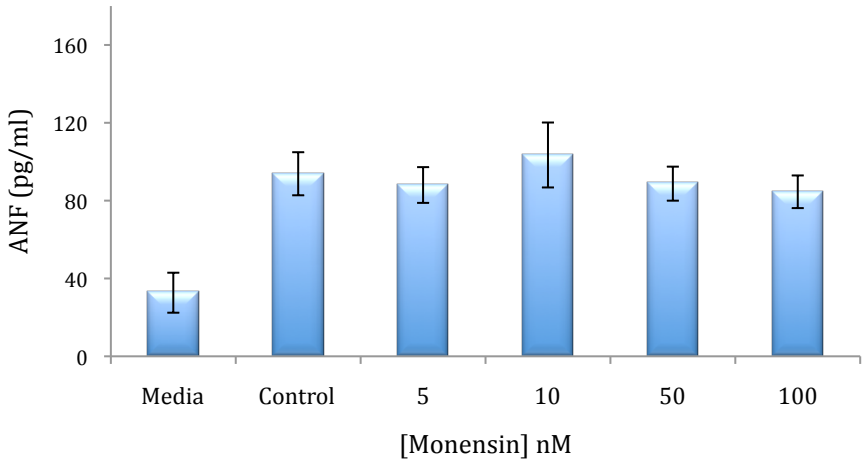


Figure 15: Concentration dependent effect of monensin on ANF secretion.

HL-1 atrial cardiomyocytes were treated with monensin for 3 h with the specified concentrations. The concentration of EtOH in the assay was ≤ 0.0002%. (A) Cells were cultured in fully supplemented Claycomb media and treated with the specified concentration of monensin diluted in fully supplemented Claycomb media. (B) Cells were serum starved 24 h prior to monensin treatment and treated with monensin diluted in supplemented DMEM/F12. Data is represented as mean ± SEM from three independent experiments done in triplicate. * indicates $p<0.01$ for control versus monensin; ‡ indicates $p<0.05$ for control versus monensin.

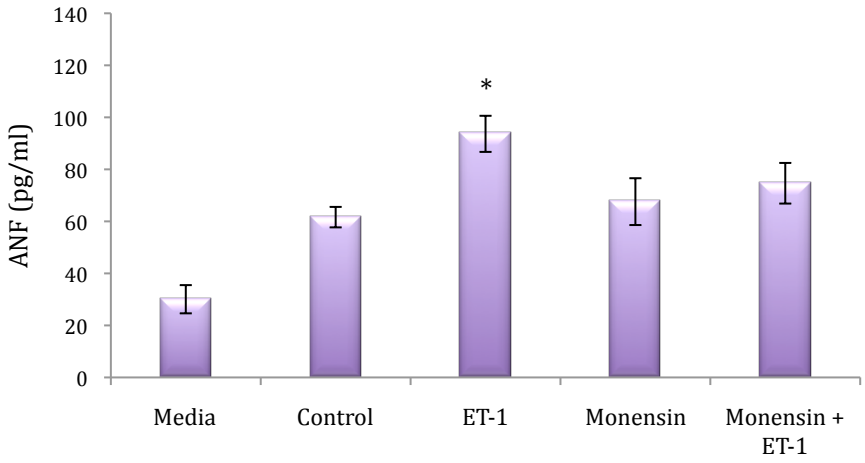


Figure 16: Monensin sensitive secretory pathway

HL-1 atrial cardiomyocytes were pretreated for 2 h with monensin (10 nM) only for the monensin and monensin + ET-1 set. After 2 h, the media was removed and cells were incubated for 1 h in media containing ET-1 (20 nM), monensin (10 nM) and monensin (10 nM) + ET-1 (20 nM). The control and ET-1 set received 0.0002% EtOH during pretreatment and the final 1 h incubation. Data is represented as mean ± SEM from three independent experiments done in triplicate.* indicates $p<0.01$ for control versus ET-1.

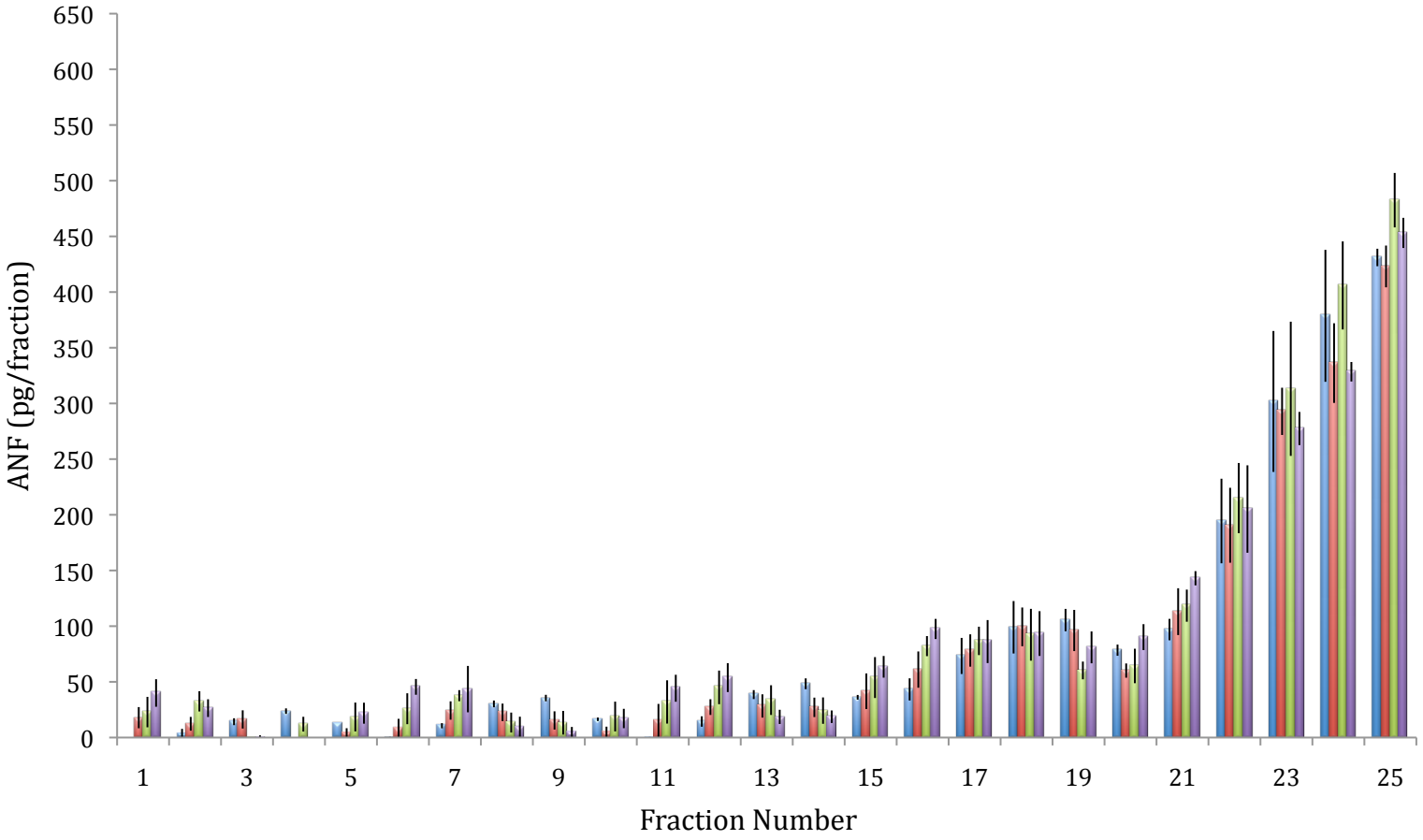


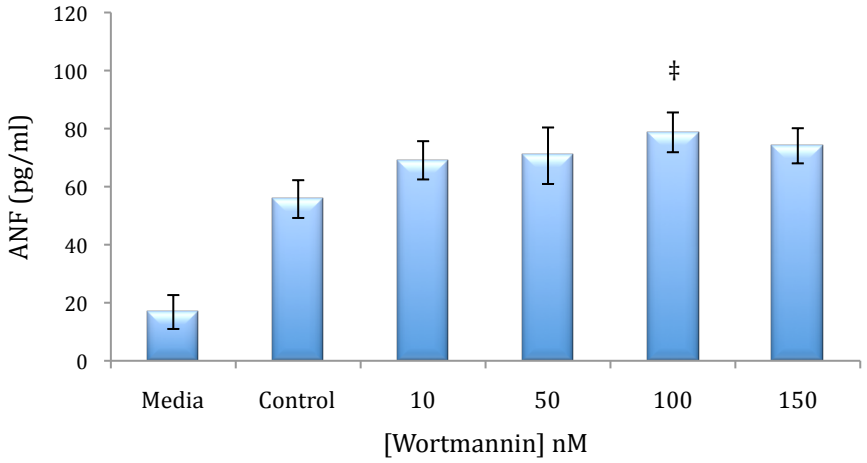
Figure 17: Ultracentrifugation gradient profile of HL-1 cells in the presence of monensin and ET-1

HL-1 atrial cardiomyocytes were pretreated for 2 h with monensin (10 nM) only for the monensin and monensin + ET-1 set. After 2 h, the media was removed and cells were incubated for 1 h in media containing 20 nM ET-1 (■), 10 nM monensin (■) and 10 nM monensin + 20 nM ET-1 (■). The control (■) and ET-1 set received 0.0002% EtOH during pretreatment and the final 1 h incubation. HL-1 cell fractionation was performed at 340,000 $_{gav}$ for 12 h. Data is represented as mean ± SEM from three independent experiments. No significant changes were observed among the treatments in the gradient fractions.

Effect of wortmannin on ANF secretion

Since no evident results were obtained from the treatment of HL-1 cells with monensin in determining whether the constitutive-like pathway is involved in ANF secretion, HL-1 cells were treated with wortmannin, an inhibitor of endosomal traffic (Davidson, 1995). Wortmannin caused an increase in ANF secretion; the highest increase being observed with 100 nM wortmannin (~ 1.41 fold) (Figure 18A). Wortmannin had an effect on ANF secretion in the nanomolar range. Concentration as high as 1 μM was tested; however, no additional increase in ANF secretion was observed as compared to 100 nM (data not shown). Furthermore, the effect of wortmannin on secretagogue-dependent ANF secretion was also evaluated. ET-1 stimulation in the presence of wortmannin was inhibited as no further increase in ANF secretion was observed (Figure 18B). In summary, wortmannin had an effect on both stimuli- dependent and independent ANF secretory pathways in nanomolar concentration.

A



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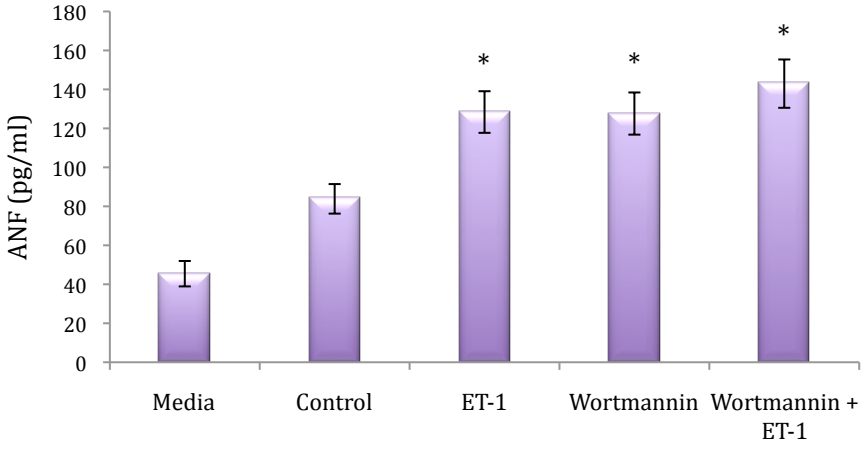


Figure 18: Effect of Wortmannin on ANF secretion.

(A) Dose dependent effect of wortmannin on ANF secretion. HL-1 atrial cardiomyocytes were incubated for 2 h in the presence of wortmannin with the specified concentrations. The concentration of DMSO in the assay was ≤ 0.006%. (B) HL-1 atrial cardiomyocytes were pretreated for 1 h with wortmannin (100 nM) only for the wortmannin and wortmannin + ET-1 set. After 1 h, the media was removed and cells were incubated for 1 h in media containing ET-1 (20 nM), wortmannin (100 nM) and wortmannin (100 nM) + ET-1 (20 nM). The control and ET-1 set received 0.004% DMSO during pretreatment and the final 1 h incubation. Data is represented as mean ± SEM from three independent experiments done in triplicate. ‡ indicates $p<0.05$ for control versus treatment. * indicates $p<0.005$ for control versus treatment.

Effect of dynasore on ANF secretion

Dynasore an inhibitor of the GTPase activity of dynamin, that is required for scission of CCVs (Hinshaw and Schmid, 1995). The constitutive-like pathway is initiated by CCVs budding off from ISGs; therefore, targeting CCV formation would prevent transport of cargo towards the endosomes and the initiation of constitutive-like secretion (Kuliawat et al., 1997). Dynasore had a dose-dependent effect on ANF secretion (Figure 19A). Dynasore at 80 μM caused the highest increase in ANF secretion at approximately 1.52 fold. Effect of dynasore on ANF secretion was also examined for 1 h of incubation with HL-1 cells and during this period an increase in ANF secretion was observed (data not shown), suggesting dynasore had an immediate effect on ANF secretion. Furthermore, HL-1 cells were pretreated with dynasore for 1 h followed by 1 h incubation with ET-1 in the presence of dynasore (Figure 19B). No additional ET-1-induced ANF secretion was observed in the presence of dynasore. In summary, dynasore had an immediate effect on both stimuli- dependent and independent ANF secretory pathways in HL-1 atrial cardiomyocytes.

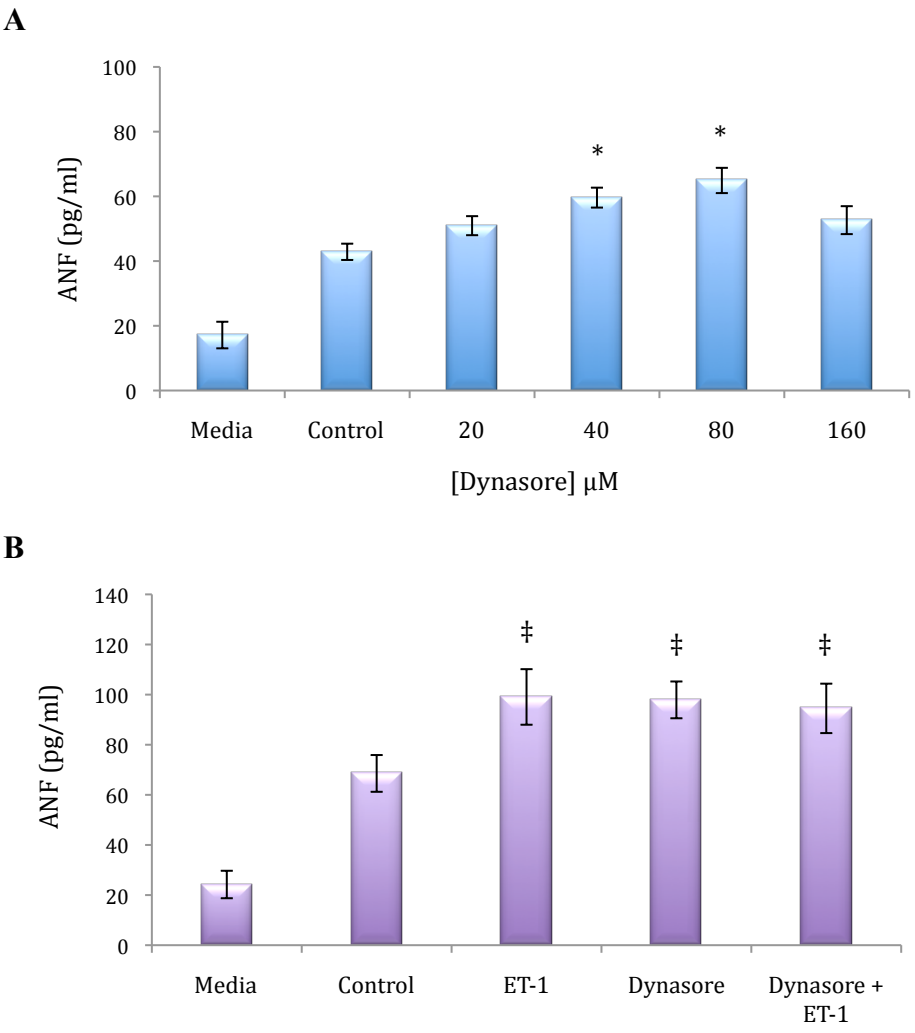


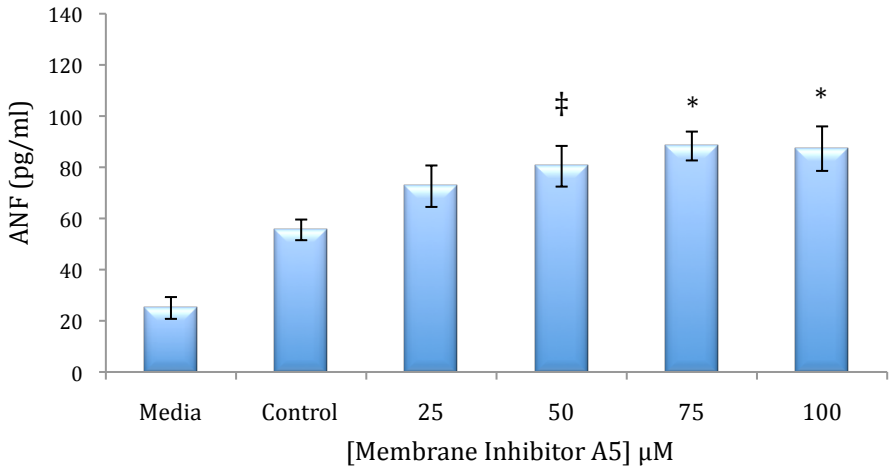
Figure 19: Effect of Dynasore on ANF secretion

(A) Dose dependent effect of dynasore on ANF secretion. HL-1 atrial cardiomyocytes were incubated for 2 h in the presence of dynasore with the specified concentrations. The concentration of DMSO in the assay was ≤ 0.16%. (B) HL-1 atrial cardiomyocytes were pretreated for 1 h with dynasore (80 µM) only for the dynasore and dynasore + ET-1 set. After 1 h, the media was removed and cells were incubated for 1 h in media containing ET-1 (20 nM), dynasore (80 µM) and dynasore (80 µM) + ET-1 (20 nM). The control and ET-1 set received 0.08% DMSO during pretreatment and the final 1 h incubation. Data is represented as mean ± SEM from three independent experiments done in triplicate. ‡ indicates $p \leq 0.05$ for control versus treatment. * indicates $p < 0.005$ for control versus treatment.

Effect of membrane traffic inhibitor A5 on ANF secretion

AP-1 is an adaptor protein localized at TGN, that initiates clathrin lattice formation and integrates cargo and receptors into the newly forming CCV (Lee et al., 2008; Traub et al., 1993). A recently synthesized small molecule called A5 has been shown to prevent translocation of AP-1 from the TGN to specifically target TGN to endosome trafficking (Duncan et al., 2007). Incubation of HL-1 cells with A5 for 1 h caused no change in ANF secretion (data not shown). A5 resulted in an increase in ANF secretion after 2 h of incubation with HL-1 cells in a dose dependent manner (Figure 20A). Pretreatment of HL-1 cells followed by stimulation with ET-1 did not result in additional secretion as compared to ET-1 or A5 alone (Figure 20B). In summary, membrane traffic inhibitor A5 had an effect on both stimuli- dependent and independent ANF secretory pathways in HL-1 atrial cardiomyocytes.

A



B

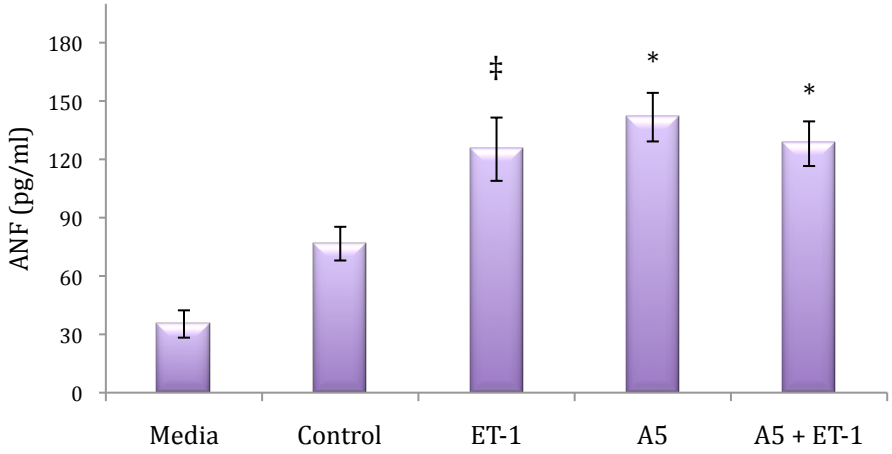


Figure 20: Effect of membrane traffic inhibitor A5 on ANF secretion

(A) Dose dependent effect of membrane traffic inhibitor A5 on ANF secretion. HL-1 atrial cardiomyocytes were incubated for 2 h in the presence of membrane traffic inhibitor A5 with the specified concentrations. (B) HL-1 atrial cardiomyocytes were pretreated for 1 h with A5 (75 µM) only for the A5 and A5 + ET-1 set. After 1 h, the media was removed and cells were incubated for 1 h in media containing ET-1 (20 nM), A5 (75 µM) and A5 (75 µM) + ET-1 (20 nM). Data is represented as mean ± SEM from three independent experiments done in triplicate.‡ indicates $p<0.05$ for control versus treatment. * indicates $p<0.01$ for control versus treatment.

Effect of proprotein convertase inhibitor on ANF secretion

Under serum-starved conditions, HL-1 cells secreted only the processed form of low molecular weight ANF as compared to the secretion of both low and high molecular weight ANF in cells that had been cultured in fully supplemented Claycomb media (Figure 5). The currently known protease for proANF processing is corin, which is localized to the PM with an extracellular catalytic domain (Gladysheva et al., 2008). To determine whether the secreted form of low molecular weight ANF in serum-starved cells was processed intracellularly before being secreted as opposed to the currently accepted model of cosecretional maturation (Sei et al., 1992), HL-1 cells were treated with a non-specific proprotein convertase inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-cmk), commonly known as furin convertase inhibitor. The proprotein convertase inhibitor abolished ANF secretion in a time- and dose- dependent manner (Figure 21). For all the concentrations tested (50 nM to 500 nM), no ANF secretion was observed during the fourth hour as the RIA ANF values were in the same range as non specific binding of the media itself. Inhibitor concentration at 500 nM prevented any further ANF secretion after the first hour and subsequent hourly incubations showed low RIA ANF values corresponding to non-specific binding of the media. In summary, dec-RVKR-cmk, a non-specific proprotein convertase inhibitor, abolished ANF secretion in HL-1 cells in a dose and time dependent manner.

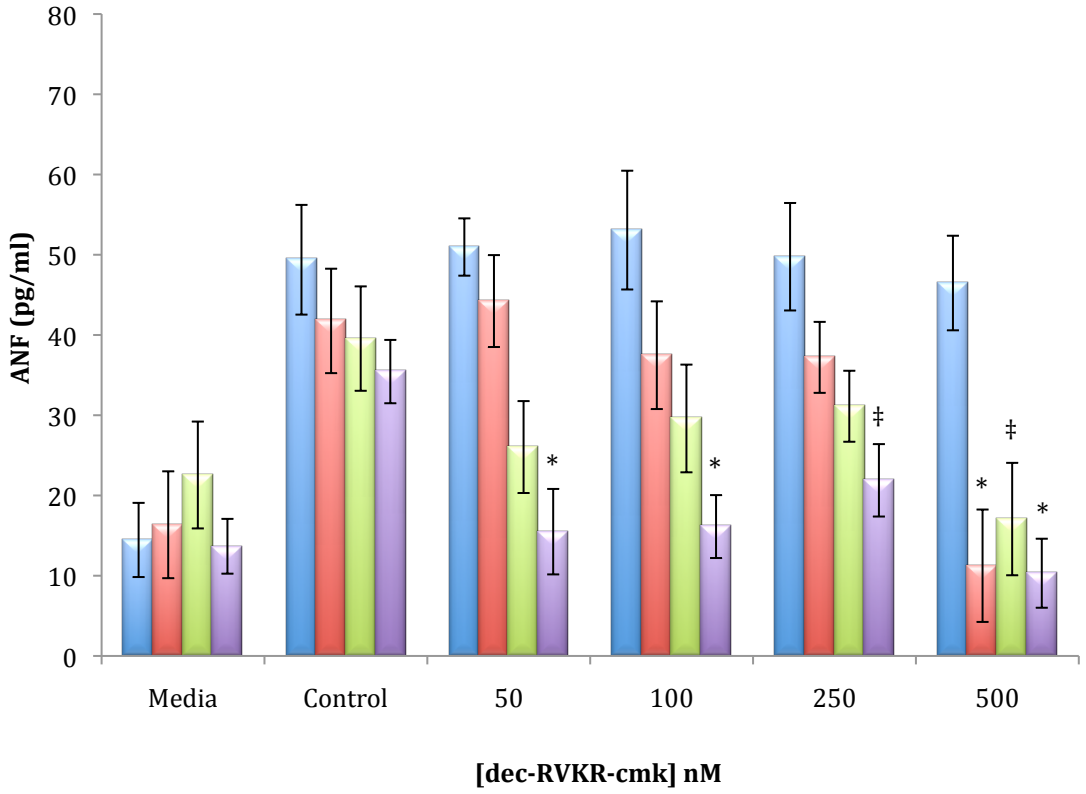


Figure 21: Effect of proprotein convertase inhibitor on ANF secretion

Dose and time dependent effect of proprotein convertase inhibitor dec-RVKR-cmk on ANF secretion. HL-1 atrial cardiomyocytes were incubated with the specified concentrations of the inhibitor for 1 h. Media was removed every hour and replaced with fresh media containing the inhibitor for upto 4 h. The time points are represented as 1 h (■), 2 h (■), 3 h(■), 4 h (■). Data is represented as mean ± SEM from three independent experiments done in triplicate. * indicates $p<0.01$ for control versus inhibitor; ‡ indicates $p<0.05$ for control versus inhibitor.

Discussion

Intracellular and secreted forms of ANF

ANF storage pools

In the current view of the literature, $proANF_{1-126}$ is the intracellular storage form that is processed co-secretionally at the PM through the extracellular protease activity of corin (Sei et al., 1992; Thibault et al., 1987; Yan et al., 2000). RP-HPLC profile of atrial extracts showed that proANF was the predominant form stored in the atria, while approximately 5-10% of the intracellular peptide was processed, corresponding to low molecular weight ANF. (Vuolteenaho et al., 1985). In this study, the presence of processed ANF in both mouse atrial extract and in the HL-1 cells was identified. HL-1 cells that were cultured in fully supplemented Claycomb media showed diminished quantities of proANF as assessed by RP-HPLC. In contrast, serum-starved HL-1 cells and mouse atrial extract had comparable RP-HPLC profiles due to the predominant presence of proANF. Fully supplemented Claycomb media contains norepinephrine, retinoic acid, insulin-like growth factor and epidermal growth factor in addition to 10% fetal bovine serum (White et al., 2004); all these factors contribute to highly stimulating cell culture conditions, which would result in enhanced secretion. Rat pituitary GH3 cells cultured in serum-free conditions, have been shown to have a higher presence of SGs as compared to cells cultured in serum-supplemented media (Brunet-de Carvalho et al., 1989). Pulse labelling studies on primary cultures of rat atrial myocytes have shown that approximately 40% of newly synthesized ANF is immediately secreted while

approximately 60% is retained as an intracellular storage pool (Iida and Shibata, 1994). Stretch-secretion coupling has been shown to use a newly synthesized ANF pool that is readily releasable and rapidly depleted (Mangat and de Bold, 1993), while ET-1 dependent ANF secretion is through newly synthesized as well an older storage pool; therefore, these intracellular pools determine ANF secretory kinetics (Ogawa et al., 1999). In pancreatic β-cells, secretagogue-dependent insulin secretion uses a readily releasable pool in the first phase of secretion, and a reserve pool contributes to the sustained second phase secretion (Bratanova-Tochkova et al., 2002). It would appear that several storage pools exist in endocrine cells, hence RP-HPLC analysis on HL-1 atrial extracts identified two HPLC peaks, one of low molecular weight ANF and another of high molecular weight proANF.

HL-1 cells were fractioned on a self-generated density gradient to separate intracellular ANF pools. As the HL-1 cell fractionation conditions were intensified by increasing centrifugal force and time, more ANF was found in higher density fractions (Figures 4, 5, 6). Cell fractionation of HL-1 cells that were cultured in fully supplemented Claycomb media shows decreased quantities of ANF in higher density fractions; since HL-1 cells cultured under these conditions have depleted quantities of proANF, the loss of ANF in higher density fractions would represent loss of proANF. Tissue fractionation of the mouse atria was also performed, and the gradient profile was similar to cell fractionation of HL-1 cells that had been serum-starved for 24 h. Gradient fractions were characterized with organelle markers for ER (calnexin), Golgi (GS28), early endosome (EEA1), ISGs (clathrin), proANF protease (corin) and SG markers (ChrA and ChrB). In the constitutive-like pathway, endosomes represent the intermediate compartment for the

trafficking between TGN and PM (Castle, 1998); therefore, the gradient fractions were also probed for EEA1, which was located in fraction 20, with some traces of clathrin. Clathrin was predominantly present in fraction 21, which was absent for SG marker ChrB and showed minor quantities of ChrA. In addition to the presence of ChrA and ChrB in higher density fractions, these marker were also found in low density fractions. Both ChrA and ChrB have been localized in ASGs (Steiner et al., 1990). Gradient fractions were also probed for corin, the proANF processing protease (Wu et al., 2002; Yan et al., 2000), which was localized in fraction 20 with EEA1. Organelle marker analysis of density gradients identified EEA1, clathrin, and corin in low density fractions in addition to the ER and Golgi markers.

Gradient fractions 18 to 23 were taken for RP-HPLC analysis to determine the molecular forms of ANF found in these fractions based on the ANF RP-HPLC elution profile. Low molecular weight ANF was found predominantly in fractions 18, 19 and 20, while high molecular weight proANF was also present in fractions 18 and 20. Golgi marker GS28 was found in fraction 19; therefore, the processed ANF found in this region could represent processed ANF found in the Golgi. Additionally, since the gradient fractions do not represent homogenous population of subcellular organelles, low molecular weight ANF could also be localized in low density vesicles in this fraction. The density of fraction 19 is ~ 1.12 g/ml and the density rises to ~ 1.16 g/ml for fraction 20. In human keratinocyte HaCaT cells, ErbB1 has been shown to be secreted via the constitutive pathway in exosomes with density of 1.06-1.11 g/ml (Sanderson et al., 2008). Heparan sulfate proteoglycan, a constitutively secreted protein in hepatocytes, is secreted via low density vesicles with density of 1.05-1.06 g/ml (Nickel et al., 1994). In CHO

cells, glycosaminoglycan chains were found in post-Golgi low density vesicles (~ 1.12 g/ml), that are secreted via the constitutive pathway (Chavez et al., 1996). Fraction 20 showed the presence of EEA1, clathrin and corin in addition to the presence of low molecular weight ANF, as identified by RP-HPLC analysis. In density gradients endosomes have been identified at various densities such as in *S. cerevisiae* at ~1.12-1.14 g/ml (Singer-Kruger et al., 1993), in mouse liver cells at 1.12 g/ml (Chen A., 2010), in rat liver at 1.05 – 1.09 g/ml (Sako et al., 1990), and in HeLa cells at 1.064-1.04 g/ml (Proikas-Cezanne et al., 2006). Additionally, since corin is a transmembrane protease localized at the PM (Gladysheva et al., 2008), it would be expected to find corin in a plasma membrane fraction; plasma membranes have been identified in sucrose gradients at a density of 1.16-1.17 g/ml (Atkinson, 1978) and at 1.03-1.08 g/ml in an iodixanol gradient (Neves et al., 2009). In HL-1 cell fractionation, corin was identified at a density of ~ 1.16 g/ml in fraction 20; therefore, fraction 20 could also contain PM. Furthermore, presence of corin in fraction 20 with the early endosomal marker can also represent the possibility that corin is recycled through early endosomes. Based on the RP-HPLC elution profile of gradient fractions, proANF was identified predominantly in fractions 22 and 23 in addition to minor quantities in fractions 18, 20 and 21. Fraction 22 has a density of ~ 1.27 g/ml and the density rises to 1.33 g/ml for fraction 23. These density fractions demonstrate that proANF is located in mature granules. In fact, MSGs have been identified in density gradients at densities of 1.207 g/ml (Grimes and Kelly, 1992b) and 1.178 g/ml in PC12 cells (Tooze et al., 1991). In parotid acinar cells, SGs have been isolated as low density (1.115-1.125 g/ml), medium density (1.125-1.14 g/ml), and high density (1.14-1.16 g/ml) SGs (Fujita-Yoshigaki et al., 2006; von Zastrow and Castle,

1987). Thibault and colleagues isolated ASGs from rat atria at a density range of 1.11-1.15 g/ml (Thibault et al., 1987). ISGs contain partial clathrin coats (Tooze and Tooze, 1986) and clathrin was identified in fraction 20 and 21; therefore, clathrin was localized at a density range of 1.16 g/ml – 1.21 g/ml. CCVs isolated from bovine brain and rabbit lactating mammary gland have density of ~ 1.23 g/ml (Pauloin et al., 1999). CCVs have also been identified at densities of 1.196 g/ml (Gilbert et al., 1997), and at a density range of 1.20-1.25 g/ml (Daiss and Roth, 1983).The differences in density for organelle markers among the various studies could be due to varying density profiles that are dependent on centrifugal force, time and density material (Arora et al., 1973; Rickwood et al., 1982). In summary, low molecular weight ANF was identified in fractions 18, 19 and 20, which are characterized by the presence of Golgi, early endosome and clathrin, while high molecular weight proANF was identified in fractions 22 and 23, that also showed the presence of ChrA and ChrB, corresponding to mature granules.

ANF processing and secretion revisited

HL-1 atrial cardiomyocytes secreted both the processed form of low molecular weight ANF and high molecular weight proANF depending on the cell culture conditions. Under stimulating conditions high molecular weight proANF was found to be secreted, in addition to the mature and processed form of the peptide. Studies on ANF processing and secretion in primary cultures have given a conflicting view of ANF processing and secretion as compared to *in vivo* studies (Dube et al., 1993). Rat atrial cardiocyte primary cultures have been found to store and secrete proANF (Bloch et al., 1985; Zisfein et al., 1987). Rat atrial primary cultures that had been cultured in serum-free media were also

found to secrete proANF (Glembotski and Gibson, 1985); however, the serum-free media was supplemented with thyroxine, a stimulant of ANF synthesis and secretion (Mori et al., 1990), which can cause proANF secretion. In contrast, freshly isolated atrial primary cultures show predominant secretion of low molecular weight ANF; however, with time the ratio of proANF to mature ANF being secreted increases (Dube et al., 1993). In isolated atrial perfusates and plasma, ANF is predominantly found as the mature hormone $ANF_{99\text{-}126}$ (de Bold and de Bold, 1989; Miyata et al., 1985; Thibault et al., 1986).

The proposed model for proANF processing suggests that proANF is processed extracellularly as the granules are being secreted since the catalytic domain of corin is extracellular (Knappe et al., 2004). A soluble corin has also been identified in human plasma (Peleg et al., 2009), indicating importance of corin's proteolytic activity beyond atrial cardiocytes. In a recent study of corin knockout mice, the authors reported 2.6 fold higher content of intracellular proANF and undetectable low molecular weight ANF levels as compared to wildtype (Chan et al., 2005); the lack of intracellular low molecular weight ANF is questionable since corin has been localized to the PM with an extracellular protease domain as described above. Furthermore, this study showed that the natriuretic activity of ANF in these corin knockout mice was recovered by infusion of a recombinant soluble corin and as a result there was an increase in plasma ANF, which was able to induce cGMP activity in baby hamster kidney cells. However, the authors concluded that corin converted proANF to ANF, which produced an increase in cGMP activity. The initial properties of atrial extracts producing natriuretic and diuretic activity was determined by it's intravenous injection into rats (de Bold et al., 1981). These atrial extracts contain the predominant molecular form of proANF (Vuolteenaho et al., 1985);

therefore, the presence of proANF in plasma seems to be sufficient to induce the same biological effects that are known to be produced by ANF.

While characterization the ANF density gradient fractions, corin was localized to fraction 20 with clathrin and the early endosome marker EEA1. This suggests a possible role for corin in recycling between TGN, endosomes and PM, a quality attributed to the endoprotease furin (Teuchert et al., 1999). Sei and colleagues proposed that proANF processing occurs cosecretionally based on the observation that the intracellular pool was composed of $proANF_{1-126}$, while ANF_{99-126} was released; however, the secretion studies were carried out in primary cultures that had been serum-starved (Sei et al., 1992). Since corin is thought to process proANF at the PM, incubation of exogenous proANF with cardiocyte cultures did not produce processed ANF in the media (Sei et al., 1992). This raises the possibility whether an intracellularly processed ANF pool is being secreted in secretagogue-independent conditions. Under serum-starved conditions, HL-1 cells secreted only the low molecular weight ANF as compared to the secretion of both the low molecular weight ANF and the high molecular weight proANF in cells that had been cultured in fully supplemented Claycomb media. To determine whether the intracellularly processed ANF was responsible for secretion during serum-starved conditions, HL-1 cells were treated with an irreversible, non-specific and potent proprotein convertase called dec-RVKR-cmk (Basak et al., 2008). The proprotein convertase inhibitor abolished ANF secretion in HL-1 cells in a dose and time dependent manner. The highest concentration of the inhibitor used was 500 nM, which is far below what has been used to inhibit proBNP (25-50 μM) (Sawada et al., 1997) and proCNP (5-20 μM) (Wu et al., 2003) processing. Even though this inhibitor is also known as furin convertase inhibitor,

its additional targets include PC6B, PC3, PC2, PACE-4, PC7 and PC4, all of which are inhibited in the nanomolar range (Basak et al., 2008; Jean et al., 1998); therefore, the identity of proprotein convertase responsible for abolishing ANF secretion could not be determined. However, it still needs to be determined through RP-HPLC analysis whether dec-RVKR-cmk prevented intracellular proANF processing, or whether the loss of secretion was due to the inhibition of secretory pathway machinery.

ET-1-induced ANF secretion in HL-1 atrial cardiomyocytes

Among the neurohumoral secretagogues known to stimulate regulated ANF secretion, ET-1, which binds to ET_A receptor and signals through the G_q pathway has been determined to be the most potent (Schiebinger and Gomez-Sanchez, 1990). In AT-1 atrial cardiomyocytes, the precursor for the HL-1 cell line, 10 nM ET-1 induced a 5.77-fold increase in ANF secretion (Lanson et al., 1992). As demonstrated in Figure 13, 20 nM ET-1 increased ANF secretion by approximately 1.8 fold in HL-1 cells. Concentration as high as 100 nM was tested in HL-1 cells, however the ANF secretory response was lower than stimulation with 10 nM ET-1. This dose-dependent discrepancy could be due to ANF secretory kinetics and distinct intracellular storage pools. Uusimaa and colleagues demonstrated that ANF secretory kinetics in rat atrial cardiomyocytes is biphasic in the presence of 100 nM ET-1, where an almost 2-fold rise in ANF secretion within the first ten minutes is followed by decline to 1.4-fold in the next 30 min; in the same study 10 nM ET-1 caused a gradual increase in ANF secretion that was sustained at the same level for the next 30 min (Uusimaa et al., 1992). ET_A receptor desensitization has also been shown to prevent any subsequent ANF secretion post 60 min ET-1

stimulation (Leite et al., 1994). In summary, HL-1 cells were stimulated by ET-1 after a serum-starvation period, and 20 nM ET-1 resulted in the most potent response for ANF secretion.

Monensin-sensitive ANF secretory pathway

Treatment of HL-1 cells with monensin under serum-starved conditions did not inhibit ANF secretion, while monensin treatment of HL-1 cells cultured under stimulating conditions increased ANF secretion by approximately 1.2 fold. Monensin disrupts intracellular proton gradients to increase the pH of intracellular compartments such as TGN, SGs, lysosomes and endosomes in a concentration- and time-dependent manner (Mollenhauer et al., 1990). Various monensin-induced effects in the secretory pathway have been reported depending on the concentration and incubation period used in the study; some of the studies have been summarised in Table 1. A general trend that can be observed from these reports is that monensin concentration in the micromolar range inhibit protein processing and transport at the Golgi in addition to neutralizing intracellular acidic compartments including the SGs (Devault et al., 1984), while nanomolar concentrations prevents formation of clathrin coated ISGs at the TGN (Orci et al., 1984). In primary cultures of rat atrial cardiomyocytes monensin decreased ANF secretion, even though translocation of SGs towards the PM was increased (Iida et al., 1988). Isolated perfused rat atrial treatment with 5 μM monensin inhibited secretagogue stimulated secretion and decreased basal ANF secretion (Ogawa et al., 1999). Treatment of HL-1 cells with 5 μM monensin resulted in a time-dependent increase in ANF secretion; these cells were cultured in fully supplemented Claycomb media and the

observed increase in ANF secretion could be due to nonexocytic release of ANF from vacuolization or swelling of SGs. Monensin induced secretion has been observed for β-endorphin secretion in melanotropes (Back et al., 2000), catecholamine secretion in adrenal medulla cells and pheochromocytoma cells (Izumi et al., 1986; Takahashi et al., 1986), and acetylcholine release from rat cerebrocortical synaptosomes (Satoh and Nakazato, 1991). Schiebinger and colleagues found that monensin increased ANF secretion in isolated rat atrial perfusion, with 5 μM monensin causing an increase of 2.1 fold (Schiebinger et al., 1994). In HL-1 secretion assays, monensin inhibited ET-1 stimulated ANF secretion, as previously observed in an isolated rat atrial preparation (Ogawa et al., 1999). The monensin-sensitive pool was deemed to be of a constitutive-like nature (Ogawa et al., 1999); therefore, to determine the monensin-sensitive intracellular pool, HL-1 cells were treated with monensin and ET-1 followed by cell fractionation. Neither monensin nor ET-1 were able to induce observable changes in density gradient ANF profile; therefore, the monensin-sensitive pool could not be found through a cell fractionation technique. In conclusion, a monensin-sensitive, secretagogue dependent ANF secretory pathway was identified in HL-1 cells; however, monensin-sensitive pool could not be identified as density gradient centrifugation may not have the necessary resolution to identify changes in sedimentation profile with and without treatment.

Table 1: Concentration, time and cell type effects of monensin.

Concentration	Time	Cell Line/Origin	Monensin Induced Effect	References
10 µM	2 h	Baby hamster kidney cells	Golgi: • inhibition of trafficking from medial to trans Golgi	(Griffiths et al., 1983)
1 µM	various	Rat prolactin cell line (GH3 cells)	Golgi: • 1 h – disorganized Golgi with dilated vacuoles Secretion: • 30 min – inhibition of basal prolactin (PRL) secretion • thyrotropin-releasing hormone stimulated PRL secretion is not inhibited; suggested to be due to Golgi-derived vesicles and not granules	(Tougard et al., 1983)
50 nM	1 – 2.5 h	Pancreatic B cells	Golgi: • dilated Golgi cisternae ISGs: • inhibition of clathrin coated vesicle formation at the TGN Secretion: • inhibition of proinsulin processing and release	(Orci et al., 1984)
100 µM	3 h	Neurointermediate lobe cells	Golgi: • pro-opiomelanocortin processing and transport halted at RER/Golgi compartments	(Devault et al., 1984)
various	0.5 h	PC12	≤ 25 nM • Low monensin concentration did not prevent intracellular transport of proteins ≥100 nM • Dilated Golgi cisternae and appearance of cytoplasmic vacuoles and swollen SGs	(Kuhn et al., 1986)
0.15-15 µM	1 - 3.5 h	Anterior pituitary glands	Secretion: • time and dose dependent inhibition of secretagogue stimulated secretion	(Sobel and Shakir, 1988)

Role of constitutive-like pathway in ANF secretion

To determine the presence of constitutive-like pathway in ANF secretion, pharmacological inhibitors of endosomal pathway and clathrin coat vesicle formation were used. Wortmannin, dynasore and membrane traffic inhibitor A5 caused an increase in ANF secretion under secretagogue-independent conditions, while in the presence of ET-1 no further ANF increase was observed. Increase in ANF secretion with these trafficking inhibitors could be due to missorting of proANF from the regulated secretory pathway or missorting of ANF from TGN towards PM instead of endosomes. In the presence of wortmannin, mistargeting of cathepsin B and D towards the PM instead of the lysosomal/endosomal pathway resulted in augmented secretion of these lysosomal enzymes (Davidson, 1995). Membrane traffic inhibitor A5 has been shown to prevent translocation of AP-1 from TGN (Duncan et al., 2007); AP-1 is required for CCV formation at TGN and formation of ISGs (Burgess et al., 2011) (Lee et al., 2008; Traub et al., 1993). Treatment of HL-1 cells with dynasore and membrane inhibitor A5 also prevented ET-1-dependent ANF secretion, suggesting ET-1 secretion is dependent on newly synthesized ANF found in ISGs. Loss of AP-1 or its effectors has been shown to induce increased constitutive secretion and loss of the regulated phenotype in the secretion of von Willebrand factor (Lui-Roberts et al., 2005; Lui-Roberts et al., 2008). Targeting of CCV formation by dynasore and membrane inhibitor A5 or trafficking through endosome with wortmannin did not prevent secretagogue independent ANF secretion; therefore, stimuli independent ANF secretion does not seem to be dependent on clathrin or endosome mediated pathways.

Conclusion

In this study, ANF storage and secretion was characterized in HL-1 atrial cardiomyocytes through the use of pharmacological agents, density gradient and RP-HPLC analysis. Treatment of HL-1 cells with monensin followed by cell fractionation to identify the monensin-sensitive pool was unsuccessful as no significant changes in ANF gradient profile were observed. Since the monensin-sensitive pool was thought to be of a constitutive-like nature, targeting this pathway with pharmacological inhibitors of CCV formation and endosomal trafficking failed to prevent stimuli independent secretion; however, in the presence of ET-1 no further increase in ANF secretion was observed. This suggests a possible role of these inhibitors in the formation of ISGs, as ET-1 has been previously shown to be a stimuli for newly synthesized ANF (Ogawa et al., 1999).

ANF secretion studies and RP-HPLC analysis identified the presence of proANF in the media from HL-1 cells cultured in fully supplemented Claycomb media. The secretion of proANF from primary atrial cultures gave a conflicting view of ANF processing and secretion (Dube et al., 1993). Lack of processing of this high molecular weight proANF questions the ability and efficiency of corin's proteolytic activity. Dec-RVKR-cmk, a non-specific proprotein convertase inhibitor, abolished stimuli-independent ANF secretion in a dose- and time- dependent manner, indicating the importance of an intracellular proprotein convertase in the ANF secretory pathway.

Organelle marker analysis of density gradients identified EEA1, clathrin, and corin in low density fractions in addition to the ER and Golgi markers. RP-HPLC analysis identified the presence of low molecular weight ANF in low density gradient fractions defined by the presence of Golgi, early endosome, clathrin and corin. Since endosomes are the intermediate compartment for constitutive-like secretion, presence of processed ANF with the EEA1 marker is of interest. Furthermore, the density of low molecular weight ANF containing gradient fractions is comparable to the density of constitutive vesicles. Based on an inability to prevent ANF secretion by targeting the constitutive-like pathway and the presence of low molecular weight ANF in low density gradient fractions, stimuli-independent ANF secretion seems to be through a constitutive pathway.

Future Directions

A distinctive feature that segregates constitutive secretion from constitutive-like secretion is the half-time of secretory release of 2.5 h for constitutive-like pathway and 1 h for constitutive pathway (Arvan and Castle, 1987). Pulse-chase labelling of HL-1 atrial cardiomyocytes with tritiated leucine, followed by cell fractionation immediately post-pulse, at 5 min, 10 min and 20 min of chase would determine whether the intracellularly processed ANF pool found in low density fractions turns over rapidly and follows the kinetics of the constitutive pathway. The low density fractions can also be taken for electron microscope autoradiographic analysis to determine the size and morphology of compartment(s) that contain low molecular weight ANF. Furthermore, the low molecular weight ANF found in these fractions can be taken for mass spectrometry analysis to confirm the presence of $ANF_{99\text{-}126}$ in this pool.

Presence of an intracellularly processed ANF pool and secretion of proANF under stimulating cell culture conditions requires further investigation of corin's function and activity through siRNA knockdown of corin in HL-1 cells. Identification of corin as a soluble protein in plasma (Ab et al., 2010; Peleg et al., 2009) and the presence of corin activity in the agouti pathway, which is involved in hair follicle pigmentation (Enshell-Seijffers et al., 2008) indicates that corin may have a greater biological role than just being a proANF protease. Dec-RVKR-cmk inhibited ANF secretion under unstimulated conditions; however, it still needs to be determined through RP-HPLC analysis whether loss of ANF secretion was due to lack of proANF processing that was eventually targeted towards the storage pools as proANF or the proprotein convertase inhibitor was acting

upon machinery required for the secretory pathway. To identify intracellular proANF protease, the proANF amino acids involved in propeptide processing can be synthesized as a fluorogenic substrate, which can be taken for a high throughput protease screen to identify proprotein convertase candidates, that can be further evaluated through siRNA knockdown (Gosalia et al., 2005; Harris et al., 2000). Therefore, identification of intracellular proANF protease would be of interest to determine its role in cardiovascular endocrinology.

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