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SNARE – Mediated Exocytosis of Atrial Natriuretic Peptide from Atrial Cardiac Myocytes

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SNARE-Mediated Exocytosis of Atrial Natriuretic Peptide from Atrial Cardiac Myocytes

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DEDICATION

I would like to dedicate this work to my wife, Julie, for whom I am eternally grateful.

Her love, support, and sacrifice throughout these years allowed for this to happen.
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CHAPTER I - INTRODUCTION

A PubMed search (March 2007) reveals that since their discovery in 1981 almost 19,000 publications have referred to natriuretic peptides, indicating high levels of interest in this area. There are three main family members in the natriuretic peptide family. They consist of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide. These natriuretic peptides are natural antagonists to the renin-angiotensin-aldosterone system, and their role in regulating fluid balance appears to be particularly important in patients with hemodynamic stress such as in heart failure. Whereas excitation-contraction coupling in the heart has been a subject of intense investigation, the endocrine function of the heart by comparison has been far less studied.

Studies involving the contraction of heart date back to 1883. In these studies, Sydney Ringer began to understand the role of Ca$^{2+}$ in the contracting beating heart, where solutions lacking Na$^+$, K$^+$ and Ca$^{2+}$ were unable to maintain a normal heart beat (Ringer, 1883). It was not until nearly 100 hundred years later when the discovery of ANP by de Bold in 1981 when the heart became recognized as an organ of both mechanical and endocrine function (de Bold et al., 1981). Since then, ANP has been shown to regulate blood volume and pressure, smooth muscle/fibroblast proliferation and cardiac myocyte growth. In addition, work from many laboratories have established natriuretic peptides as an important physiological and pathophysiological counterbalance to the renin-angiotensin-aldosterone system (Rosenkranz et al., 2003). Moreover, based on physiological characteristics, ANP may be a useful marker of
acute overload or rapid cardiovascular hemodynamic changes. When cardiac performance decreases, neurohormonal systems are stimulated to adjust for the loss of cardiac output. To adapt and possibly protect the heart, ANP exocytosis is stimulated as an adaptive and protective response to heart failure, renal failure, diabetes mellitus and liver cirrhosis (Woods, 2004). Accordingly, natriuretic peptides, in particular ANP and BNP, have emerged as valuable clinical tools that serve as diagnostic and prognostic markers for heart failure.

Atrial cardiac myocytes secrete ANP by both constitutive and regulated exocytotic fusion of ANP-containing secretory granules with the sarcolemma. Amazingly, detailed information regarding the identity and function of specific membrane fusion proteins involved in exocytosis in the endocrine heart is lacking (Lang et al., 1985; Ryu et al., 2002). SNARE proteins (derived from soluble N-ethylmaleimide sensitive fusion protein attachment receptor) are a large protein superfamily consisting of more than 60 members in yeast and mammalian cells. In endocrine cells, the primary function of SNARE proteins is to mediate fusion of secretory granules with the plasma membrane (or other intracellular target compartments). Generally, SNAREs can be divided into two categories; vesicle or v-SNAREs, which are associated with the membranes of the secretory granules, and target or t-SNAREs, which are located on the target membrane (Sudhof et al., 1993). Recent classification however takes account for the structural features of the SNARE proteins and divides them into R-SNAREs and Q-SNAREs (Fasshauer et al., 1998). The best-studied SNAREs are those that mediate docking of secretory granules with the presynaptic membrane. These proteins include VAMP-2 (vesicle associated
membrane protein – 2), syntaxin 1 and SNAP-25 (synaptosomal-associated protein of 25 kDa). Interestingly, skeletal muscle cells have been shown to contain SNARE proteins involved in the trafficking of the glucose transporter-4 (GLUT-4) to the plasma membrane in response to insulin. This set of SNARE proteins consists of VAMP-2, SNAP-23 and syntaxin 4 (Slot et al., 1997; Foster et al., 1998).

In this dissertation, I report evidence that multiple SNARE protein isoforms, including those implicated in the late stages of Ca\textsuperscript{2+}-dependent exocytosis in neurons and endocrine cells, are expressed in neonatal and adult atrial cardiac myocytes. Atrial cardiac myocytes were screened for mRNA transcripts by RT-PCR to determine which SNAREs were present. Identified SNARE proteins known to be involved in regulated exocytosis in other cell types were further characterized in myocytes by Western blot. Functional interaction was demonstrated using immunoprecipitation techniques. VAMP-2 was shown to interact with its putative binding partners, syntaxin 4 and SNAP-23, in neonatal cardiac myocytes, and syntaxin 1 and SNAP-25 in adult cardiac myocytes. Using cell fractionation and immunocytochemical methods, it was revealed that VAMP-1, VAMP-2 and synaptotagmin-1 localized to subpopulations of ANP-containing secretory granules in atrial myocytes. This demonstrated that multiple SNARE proteins are present in neonatal and adult cardiac myocytes, revealed an age-dependent reconfiguration of SNARE proteins, and suggests the importance of SNARE proteins in exocytosis of ANP from the endocrine heart.

Although mechanical stretch of the atrial wall is thought to be the predominant stimulus for ANP exocytosis, it has been shown that treatment with
ouabain can also efficiently evoke ANP exocytosis. However, the Ca\(^{2+}\)-dependence and molecular mechanisms that mediate ANP exocytosis remain unclear. Presently, there are conflicting reports on the role of intracellular Ca\(^{2+}\) (De Bold and De Bold, 1989; Uusimaa et al., 1990; Ryu et al., 2002). However, using patch clamp methods, I have demonstrated that controlled rises in intracellular Ca\(^{2+}\) efficiently evokes exocytosis in single neonatal myocytes (Peters et al., 2006). In addition, I identified the integral membrane protein of secretory vesicles containing two Ca\(^{2+}\) binding C2 domains, synaptotagmin 1. Synaptotagmin 1 is a low affinity Ca\(^{2+}\) sensor believed to trigger exocytosis via increased interaction between SNARE proteins upon binding Ca\(^{2+}\), thus connecting a rise in intracellular Ca\(^{2+}\) to exocytosis (Brose et al., 1992; Augustine, 2001). Also in this study, I report evidence that tyrosine phosphorylation of synaptotagmin 1 by ouabain enhances the calcium sensitivity of exocytosis by activation of the Na\(^{+}/K^{+}\)-ATPase / Src signaling complex. This mechanism may provide a Ca\(^{2+}\)-dependent signal for exocytosis in myocytes.

In addition to a cellular ion pump, Na\(^{+}/K^{+}\)-ATPase can assemble into a functional signaling complex (Haas et al., 2002; Xie and Cai, 2003; Tian et al., 2006). Thus, Na\(^{+}/K^{+}\)-ATPase can function as a receptor for cardiotonic steroids and depending on concentration, can activate or inhibit Na\(^{+}/K^{+}\)-ATPase mediated signals. The binding of ouabain activates the bound non-receptor tyrosine kinase Src, resulting in activation of Src and tyrosine phosphorylation of various effectors (Haas et al., 2000; Haas et al., 2002; Tian et al., 2006). This results in a variety of signals ranging from activation of the Ras/Raf/Erk cascade and hypertrophic growth in myocytes and Ca\(^{2+}\) release from intracellular stores in LLC-PK1 cells (Huang et al.,
Interestingly, cultured neonatal rat myocytes have been shown to express two isoforms of Na\(^+/\)K\(^+\)-ATPase, α1 and α3, which differ in their affinities for the binding of ouabain (Orlowski and Lingrel, 1990). These differences may provide activation or inhibition of the Na\(^+/\)K\(^+\)-ATPase isoforms that provides regulatory control of exocytosis in neonatal atrial myocytes.

Also in this study, utilizing co-immunoprecipitation methods also reveals that synaptotagmin 1 is regulated by tyrosine phosphorylation. Increased tyrosine phosphorylation of synaptotagmin 1 can dissociate it from a complex with the α3 subunit of Na\(^+/\)K\(^+\)-ATPase. Treatment with the tyrosine dephosphorylation inhibitor, pervanadate or sub-toxic concentrations of ouabain in a dose dependent manner was found to increase tyrosine phosphorylation of synaptotagmin 1. Additionally a decreased interaction between synaptotagmin 1 and an immune complex of Na\(^+/\)K\(^+\)-ATPase α3 / Src / syntaxin 4 was observed. To further define the role of tyrosine phosphorylation, the broad inhibitor genistein, which inhibits tyrosine kinases, was utilized. Atrial tissue incubated in 100 µM genistein had less tyrosine phosphorylation than treatment with 100 nM ouabain. Interestingly, it was revealed that synaptotagmin 1 has six candidate tyrosine phosphorylation sites that are located within the highly conserved C\(_2\) (Ca\(^{2+}\) binding) domains.

The observation that tyrosine phosphorylation of synaptotagmin 1 occurs may lead to the increased sensitivity of Ca\(^{2+}\) to synaptotagmin 1. This was revealed using C\(_m\) to follow exocytotic activity of quiescent neonatal atrial myocytes. Application of 100 nM ouabain specifically targeting the Na\(^+/\)K\(^+\)-ATPase α3 significantly enhanced the average C\(_m\) change to the first stimulus and diminished the response of the second
stimulus with no effect on intracellular Ca\(^{2+}\) levels. This suggested ouabain and subsequent tyrosine phosphorylation enhanced the Ca\(^{2+}\)-sensitivity of exocytosis. These new findings propose SNARE proteins are assembled with Na\(^+\)/K\(^+\)-ATPase \(\alpha_3\) in a signal complex that upon application of ouabain induces tyrosine phosphorylation of synaptotagmin 1, therefore enhancing the Ca\(^{2+}\) sensitivity of exocytosis.
CHAPTER II: LITERATURE REVIEW

In 1956, a distinct myocyte from the atrium of a guinea pig heart which contained unique dense inclusions was discovered by Kisch (Kisch, 1956). It was not till eight years later, in 1964, when Jamieson and Palade demonstrated these dense inclusions were secretory granules (Jamieson and Palade, 1964). Initially, these granules were suggested to contain catecholamines (Shore et al., 1958). However, it was later shown by fluorescent microscopy that the bulk of catecholamines were in the cardiac nerves (Dahlstrom et al., 1965). The next significant finding would not be made till 1976 by Marie et al., who discovered that sodium electrolyte balance influenced the distribution index of the atrial granules (Marie et al., 1976). It was not till 1981 when de Bold et al. discovered the biological effects of the contents of the granules by focusing on atrial extracts. His laboratory found that injection of extracts from the atria, but not the ventricles, elicited a rapid decrease in blood pressure (de Bold et al., 1981). Soon after, the same atrial extracts were found to have a relaxant effect on smooth muscle (Flynn et al., 1983). Two years later, atrial natriuretic factor (ANF), also know as atrial natriuretic peptide (ANP) was purified from atrial extracts, allowing for its structure and sequence to be identified. Since these discoveries, the heart is no longer considered only as an efficient pump but also as an important endocrine organ, secreting a family of related peptide hormones known as natriuretic peptides.
**Natriuretic Peptides**

Natriuretic peptides are a group of peptide hormones characterized by a conserved ring structure of 17 amino acids sharing the conserved sequence CFGXXXDRIXXXXGLGX (where X is any amino acid) (Misono et al., 1984). As seen in Figure 1, the structures of natriuretic peptides consists of N-terminal and C-terminal extensions in addition to containing a cysteine bridge (Clerico et al., 2006). This structure is highly conserved allowing specificity of the hormones to their receptors. In general, natriuretic peptides function as natural antagonists to the renin-angiotensin-aldosterone system and play a role in regulating fluid balance. In addition, natriuretic peptides have several other physiological actions, including but not limited to: vasodilation, natriuresis, diuresis, endothelin release, inhibition of the sympathetic nervous system, and inhibition of the mechanisms responsible for vascular hypertrophy and remodeling. Most importantly, natriuretic peptides are considered to be protective due to their ability to compensate for heart failure.

The natriuretic peptide family currently consists of four family members. The first member, ANP, is mainly synthesized in the atrium under normal conditions, but low concentrations can be found in the ventricles and kidneys. Second, brain natriuretic peptide (BNP), was initially discovered in porcine brain, although it was later found to be synthesized and secreted from atrial and ventricular myocytes. Structurally related to ANP and BNP is that of C-type natriuretic peptide (CNP). CNP is mainly produced by vascular endothelial cells and acts primarily in the peripheral vasculature. The newest member of the natriuretic peptide family is a recently identified peptide, dendroaspis natriuretic peptide (DNP). DNP was
identified from the venom of the Green Mamba snake and has similar physiological functions to ANP (Schweitz et al., 1992), however a DNP homolog in humans has not been identified (Singh et al., 2006).

**Figure 1. Chemical Structures of Natriuretic Peptide Family (Clerico et al. 2006)**

Fig. 1. Amino acid chains demonstrating the peptide ring and cysteine bridge structure of atrial natriuretic peptide (ANP), Brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and Dendroaspis natriuretic peptide (DNP).
**Atrial Natriuretic Peptide**

The synthesis of ANP begins with specific genes encoding a product consisting of a 151-amino acid preproANP precursor molecule. The preprohormone contains a 25 amino acid signal sequence that undergoes proteolytic cleavage into a prohormone (Michener et al., 1986). The importance of the signal peptide allows for the translocation of the preprohormone from the ribosome into the sarcoplasmic reticulum. The prohormone (proANP\textsubscript{1-126}) is transported through the Golgi complex to secretory granules for storage (Vuolteenaho et al., 1985). These secretory granules are morphologically similar to that of large dense core granules that are an average diameter of about 350 nm (Jamieson and Palade, 1964), with an estimated capacitance of 3.8 fF / granule given the specific capacitance of membranes of 1 μF / cm\textsuperscript{2} (Peters et al., 2006). The final cleavage event occurs upon secretion which proANP\textsubscript{1-126} is cleaved by the cardiac protease, corin (Yan et al., 1999). Corin, a membrane-bound type II serine protease, converts proANP\textsubscript{1-126} into the biologically active C-terminal peptide ANP\textsubscript{99-126} by cleaving proANP following arginine at position 98 (Yan et al., 2000). Interestingly, mice lacking corin are found to be hypertensive as well as having undetectable levels of mature ANP (Chan et al., 2005).

Once secreted, ANP diffuses into the blood stream where it is distributed to various target organs. ANP exerts its effects by binding to one of three functional natriuretic peptide receptors (NPR) (Pandey, 2005). NPR-A and NPR-B are linked to guanylyl cyclases through which the production of cyclic guanosine monophosphate (cGMP) and downstream signaling through the catalytic domain occurs (Potter, 2005). ANP and BNP bind with high affinity and mediate their effects through the
NPR-A, whereas NPR-B appears to have a high affinity for CNP. NPR-A receptors are expressed in kidneys, adrenal, adipose, aortic and lung tissues (Nagase et al., 1997). NPR-B is found in similar tissues as NPR-A, however, it is the predominant receptor in the brain (Nagase et al., 1997). Moreover, mice lacking NPR-A were severely hypertensive (30-40 mm Hg) and volume expanded by 30 % (John et al., 1995). The third receptor type, NPR-C, is referred to as the clearance receptor. This receptor is responsible for removing natriuretic peptides from circulation through receptor mediated internalization and degradation (Berg et al., 1988).

The overall effect of ANP is to counter the blood pressure raising effects on the renin-angiotensin-aldosterone system and vasopressin/antidiuretic hormone response. More specifically, ANP elicits its physiologic effect through the synthesis of cGMP, a classic intracellular second messenger, that regulates responses through a family of nonselective cation channel called cyclic nucleotide gated ion channels and cGMP-dependent protein kinases (Jiang et al., 1992).

The effects that ANP place on blood pressure are through the inhibition of the renin-angiotensin-aldosterone system, in particular inhibiting renin secretion. Renin is a protease secreted from renal juxtaglomerular cells that cleaves angiotensinogen to angiotensin I (Sielecki et al., 1989). An angiotensin converting enzyme converts angiotensin I to angiotensin II, in the pulmonary vascular endothelium. The major hormone responsible for regulating sodium absorption, angiotensin II, stimulates vasoconstriction resulting in increased blood pressure (Ruskoaho et al., 1991; Ruskoaho, 1992; Yandle, 1994). In addition to inhibiting renin secretion, ANP inhibits aldosterone production in the adrenal gland. The mechanisms for reduction
in aldosterone involve inhibition of synthesis and phosphorylation of regulatory proteins responsible for aldosterone synthesis (Potter and Hunter, 2000; Potthast et al., 2004).

In the kidney, ANP increases the glomerular filtration rate, reduces renin secretion and inhibits sodium and water reabsorption. Glomerular filtration rate is elevated by afferent arteriolar dilation and efferent arteriolar constriction (Marin-Grez et al., 1986). Reabsorption of water and sodium is blocked through ANP inhibition of angiotensin II stimulated sodium and water transport (Light et al., 1990). In the collecting ducts, ANP reduces sodium absorption by directly inhibiting an amiloride-sensitive cation channel (Light et al., 1990). Interestingly, ANP and natriuretic peptides have been found in the brain, although CNP and NPR-B are the most abundant. ANP has been shown to suppress salt appetite (Itoh et al., 1986) and inhibit the release of vasopressin from the hypothalamus (Samson et al., 1987).
CHAPTER III: METHODS AND MATERIALS

Materials

Chemicals of the highest purity were purchased from Sigma and Fisher. Cell culture media, fetal bovine serum, horse serum, newborn calf serum, material for mRNA isolation, cDNA synthesis and PCR were obtained from Invitrogen. Collagenase type II was attained from Worthington Biochemical. Oregon green bapta-5N, NP-EGTA, phalloidin, SP-A Alexa-488, SP-A Alexa-555 and DAPI were obtained from Invitrogen. Halt protease inhibitor, protein A/G agarose beads and enhanced chemiluminescence kit were purchased from Pierce. Electrophoresis reagents and molecular weight markers were obtained from BioRad.

Antibodies

All antibodies were purchased from commercial sources. Polyclonal anti-SNAP-23, SNAP-25, VAMP-2, VAMP-1, synaptotagmin-1, and syntaxin 4 were obtained from Synaptic Systems. Monoclonal anti-VAMP-2, synaptotagmin-1, and syntaxin-1 were purchased from Synaptic Systems. Monoclonal anti-Na⁺/K⁺-ATPase α1 and polyclonal anti-Na⁺/K⁺-ATPase α3 were purchased from Upstate Biotechnology. Monoclonal anti-phosphotyrosine (PY99) and c-Src antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-MAP-2 and alpha-actinin (sarcomeric), polyclonal anti-proANP and proBNP were purchased from Chemicon International. Donkey anti-mouse IgG Alexa Fluor 488, donkey anti-mouse IgG Alexa Fluor 555, donkey anti-rabbit IgG Alexa Fluor 488 and donkey
anti-rabbit IgG Alexa Fluor 555 was purchased from Invitrogen. Anti-mouse IgG (H + L) horseradish peroxidase conjugate and anti-rabbit IgG (H + L) horseradish peroxidase conjugate were obtained from Bio-Rad Laboratories.

**Cell Preparation and Culture**

Neonatal rat atrial cardiac myocytes were prepared from Sprague-Dawley rats as previous described with minor modifications (Chlopcikova et al., 2001; Peters et al., 2006). Briefly, the atria were dissected from whole hearts of one to three day old rats (~25 rats) and washed in ADS buffer contained (in mM): 116 NaCl, 20 HEPES, 1.0 NaH2PO4, 5.5 glucose, 5 KCl, and 0.8 MgSO4, pH 7.35. The dissociation of atrial myocytes occurred in three consecutive incubations with ADS containing 0.1% collagenase type 2 (Worthington) in a shaking water bath at 37 °C for 20 mins. Following dissociation, the enzymatic solution was removed by centrifugation at 340 x g for three mins. The pellet was resuspended in 1 ml newborn calf serum and kept on ice during the subsequent incubations. Cells were then combined and washed with ADS and resuspended in plating medium for 1 to 1.25 hs. Plating medium consisted of Dulbecco’s modified essential medium (DMEM) supplemented with 10% horse serum and 5% newborn calf serum. The non-adhesive cells (atrial myocytes) were then transferred, washed and centrifuged for one min at 340 x g. The pelleted myocytes were resuspended in plating medium and allowed to adhere on coverslips pre-coated with collagen (Sigma) and maintained at 37 °C in 95%/5% O2/CO2.

Atria from male Sprague-Dawley rats (200-250g) were used to prepare adult atrial myocytes. Rats were injected with heparin (1.3 μl /g of body weight) and
pentobarbital (1.3 μl/g of body weight) 25 mins before surgery. Using a modified Langendorf perfusion apparatus, removed whole hearts were perfused with Jolik modified MEM (Sigma). Perfusion continued in three 15 mins intervals, with each increasing the levels of Ca$^{2+}$ in the medium. Following the changes in Ca$^{2+}$, the media was supplemented with 0.1% collagenase type 2 and perfused for 15 mins. Atria were dissected following enzymatic disassociation and placed in a shaking water bath at 37°C for three mins and centrifuged for four mins at 340 x g. The myocytes were resuspended in Medium-199 (Invitrogen) and plated on laminin coated coverslips.

**mRNA Extraction and cDNA Synthesis**

mRNA from atrial neonatal myocytes, adult atrial myocytes and brain tissue and mouse parotid gland were prepared using TRIzol (Invitrogen) as described by the manufacturer. Briefly, tissue and cells were quickly washed with PBS and lysed with TRIzol and incubated for five mins at room temperature. Total RNA was isolated by the addition of chloroform, forming a colorless upper aqueous phase. Centrifugation at 12,000 x g at 4 °C for 10 mins isolated the RNA following the addition of isopropyl alcohol. The pellet was washed once with 75% ethanol, dried briefly for the removal of ethanol and resuspended in 20 μl RNase-free water. Purification of the sample was accomplished by digestion of double-stranded DNA to oligonucleotides with the addition of 1 μl DNase I (1 U/μl) and 1 μl 10x DNase I reaction buffer (proportional to 1 μg of RNA sample) for 15 mins at room temperature to remove DNA. EDTA was added for inactivation of the reaction. First
strand cDNA synthesis was accomplished by the addition of SuperScript II RT, 1 μl Oligo (dt)_{12-18} and 1 μl 10 mM dNTP mix. Following incubation and deactivation of reaction, samples were stored at -20°C until further use.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

PCR primers were designed using Primer 3 as previously described (Rozen and Skaletsky, 2000). Standard PCR reactions were performed using 2 U of Taq DNA polymerase under the following standard conditions: denaturing at 94 °C for 45 secs, annealing at 55 °C for 30 secs and primer extension at 72 °C for 1 min and 30 secs. Due to various melting temperatures, the annealing temperature varied between experiments under the premise of 5°C lower than the melting temperature of primer-template.

PCR products were separated by electrophoresis (90 V for 1 – 1.5 hs) in running buffer (in mM): 890 Tris, 20 EDTA, and 890 Boric acid on a 2% agarose gel stained and visualized with ethidium bromide. Products were identified by using a 50 bp DNA ladder (Invitrogen) and imaged with a Kodak digital imaging system.

**Immunoblot Analysis**

Protein concentrations were determined by using a shift in absorbance in Coomassie brilliant blue as determined by the Bio-Rad Bradford assay (Bio-Rad Laboratories). Subsequent to sample preparations, samples were denatured by boiling in 2X sample buffer (15% glycerol, 1% sodium dodecyl sulfate detergent (SDS), 0.625 M Tris-base, 1% bromophenol blue, 0.015 M DTT). Using a BioRad Mini-
PROTEAN 3 system, proteins were resolved on 10 or 12% SDS-Polyacrylamide gels in running buffer (in mM; 236 Tris-base, 80 glycine, 0.1% SDS) for 45 mins at 200 V. Following separation of proteins, proteins were transferred to nitrocellulose membranes (90V for 2.5 hs in mM; 25 Tris-base, 192 glycine, 20% methanol). Membranes were then blocked in 4% non-fat milk in Tris-buffered saline (TBS-T) (in mM: 20 Tris-base, 137 NaCl, 3.8 HCL, 1% Tween 20, pH 7.6) overnight at 4°C. Membranes were washed and probed with specific antisera for VAMP-1 (1:1000), VAMP-2 (1:10,000), synaptotagmin 1 (1:1000), syntaxin 4 (1:4000), SNAP-25 (1:1000), GLUT-4 (1:1200), MAP-2 (1:1000), SNAP-23 (1:1000), syntaxin 1A (1:1000), munc 18 (1:5000), pro-atrial natriuretic peptide (1:1000), Na⁺/K⁺-ATPase α1 (1:1000), Na⁺/K⁺-ATPase α3 (1:1000), phosphotyrosine (1:2500) and c-Src (1:1000). Visualization by enhanced chemiluminescence (GE Biosciences) was completed following incubation with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:3000. Immunoreactive bands were detected on CL-XPosure film (Pierce) and protein signal was quantified using ImageJ software as described (Rasband, 1997-2004).

Subcellular Fractionation and Sucrose Density Gradients

For cell fractionation experiments, myocytes were removed from dishes by a cell scraper and collected by centrifugation at 340 x g for two mins as previously described (Peters et al., 2006). Pellets were resuspended in fractionation buffer in mM: 250 sucrose, 20 HEPES, 1 EDTA, pH 7.2. In order to keep secretory granules intact, a working clearance of 15.3 μm for neonatal myocytes and 25.5 μm for adult
cells was achieved using the Cell Cracker, a ball bearing based homogenizer (H and Y Enterprise, Redwood City, CA). Cells were passed through the Cell Cracker six times, resulting in lysed cells with intact secretory granules. Working clearances for intact secretory granules were attained by varying clearances in prior experiments. Lysates were centrifuged at 900 x g for five mins to remove remaining intact cells and nuclei.

Sucrose density gradient fractionation was completed by stepwise centrifugation using a Beckman Coulter Ultracentrifuge equipped with a Beckman TLA 120.2 rotor. Lysates were cleared of nuclei, mitochondria, and cellular debris by sequential centrifugations of 1000 x g for five mins followed by 3500 x g for 15 mins at 4ºC. The resulting pellet was resuspended in fractionation buffer (200 µL). The sample was then placed onto a 0.4 M to 2.2 M sucrose gradient. Using a Beckman TLA 55 swing rotor, the sample was centrifuged for two hs at 200,000 x g at 4ºC. Following centrifugation, the sample was removed from the top of the centrifuge tube in 15 equal fractions of 147 µL. Aliquots were diluted in 2X sample buffer and proteins were separated by SDS-PAGE as described above.

**Immunocytochemistry**

**Myocyte Cell Cultures**

Cultured cardiac myocytes were washed two times in phosphate buffered saline (PBS) supplemented with (in mM): 0.1 CaCl$_2$ and 1 MgCl$_2$ (PBS-Ca-Mg). Cells were fixed to the coverslips by methanol or 4% paraformaldehyde in PBS. Following fixation, cells were rinsed three times in PBS. For cells that were fixed
using paraformaldehyde, incubation in cell permeabilization buffer (PBS-Ca-Mg, 0.3% Triton X-100, and 0.1% bovine serum albumin) for five mins provided sufficient permeabilization. Cells were incubated in blocking buffer (PBS-Ca-Mg, 0.2% Triton X-100, 5% goat serum) for 60 mins to block nonspecific IgG binding sites. Myocytes were then incubated at room temperature for one h or 4 °C overnight with anti-ANP polyclonal antibody (1:100), anti-VAMP-1 polyclonal antibody (1:1000), anti-synaptotagmin 1 monoclonal antibody (1:100), anti-VAMP-2 monoclonal antibody (1:1000), anti-VAMP-2 polyclonal antibody (1:1000), anti-actinin monoclonal antibody (1:200), anti-Na⁺/K⁺-ATPase α1 monoclonal antibody (1:100) and polyclonal anti-Na⁺/K⁺-ATPase α3 (1:100) in antibody dilution buffer (PBS-Ca-Mg, 5% goat serum, 0.1% Triton X-100). Following incubation with primary antibody, myocytes were washed twice and incubated at room temperature with Alexa Fluor 488-conjugated anti-mouse IgG (H+L) and Alexa Fluor 546-conjugated anti-rabbit IgG (H+L) antibodies for 1 h. After several washes, coverslips were mounted onto slides with the anti-bleaching mounting medium Vectorshield (Vector).

Dual labeling of cells with two polyclonal antibodies (ANP and VAMP-1) were completed as previously described (Morris and Stanley, 2003). Briefly, original antibody storage buffer was replaced with PBS using a Micron centrifugal filter (Millipore) with a 30 kDa cut off. In order to form an antibody-protein A complex, SP-A Alexa (488 or 555) was mixed with the antibody (ANP or VAMP-1) at equal proportions (1 mg/ml IgG to 1 mg/ml SP-A Alexa dye). The antibody mixture was allowed to incubate at 4°C overnight. Purification of the antibody complex was
completed by dilution with PBS and re-concentrated using a 100 kDa Microcon centrifugal concentrator (Millipore). Staining of samples were completed following fixation and permeabilization by methanol followed by a 30 min incubation in blocking solution (0.1% BSA, fragmented rabbit immunoglobulin (1:20 of 10 mg/ml stock solution) in PBS). The SP-A-Alexa tagged antibodies were directly added to the blocking solution at a 2X concentration. Samples were incubated overnight at 4°C and rinsed three times in 10 min intervals and mounted with Vectashield mounting medium. Slides were stored at 4°C until imaged.

**Heart Tissue Slices**

For tissue preparation, atria or ventricles were removed, washed and fixed overnight by 4% paraformaldehyde dissolved in PBS prepared freshly. Tissue was then cut on a freezing microtome in 25 µM sections. Following two washes in PBS, sections were incubated overnight with polyclonal anti-ANP antibody (1:300) in PBS-Triton (PBS-Tx) containing 5% goat serum at 4°C. Sections were rinsed in PBS-Tx and incubated for 1 h with 5 µg/mL of Alexa Fluor 488-conjugated anti-rabbit IgG at room temperature. F-actin was visualized by staining with 6.6 µM Alexa Fluor 546-conjugated phalloidin or Alexa Fluor 633-conjugated phalloidin (Invitrogen) for 20 mins at room temperature. Following several washes, stained sections were mounted on glass slides using Vectashield mounting medium and stored at 4°C.
**Confocal Microscopy**

Confocal micrographs were captured utilizing one of three available confocal microscopes. Images were acquired using a confocal laser scanning microscope (BioRad Radiance 2000) equipped with Ar and HeNe laser sources and an Olympus BX51 WI upright microscope coupled to a 60x Uplan-Apo water-immersion objective. Images were acquired, stored, and visualized with LaserSharp 2000 (BioRad).

Additional images were acquired with a Leica TCS SP2 confocal laser scanning microscope equipped with an AOBS spectral scanner system and multiple laser sources (633 nm, 543 nm and 458 nm lines of red HeNe, green HeNe and blue argon) (Leica, Mannheim, Germany). Further images were captured using a Leica TCS SP5 broadband confocal microscope (Leica, Mannheim, Germany) equipped with Argon-488 and diode pumped solid state-561 and 633 laser sources and 63.0x 1.40 N.A. oil immersion objective. Sections were collected and visualized as projection images using Leica LAS software. Colocalization of labeled proteins were analyzed using ImageJ software as previously described (Rasband, 1997-2004).

**Co-Immunoprecipitation**

Tissue or cells were washed and lysed in immunoprecipitation buffer (in mM): 50 Tris-HCl, pH 7.4, 150 NaCl, 0.25% deoxycholic acid, 1% NP-40 supplemented with Halt Protease inhibitor. Lysates were pre-cleared and combined with anti-Na⁺/K⁺-ATPase alpha 3, anti-synaptotagmin 1, anti-VAMP 2 or anti-syntaxin 4 antibody overnight at 4 °C, followed by a second incubation with
immobilized protein A/G agarose beads for 2 hs. The ensuing immunoprecipitates were resolved on 12% SDS-PAGE, followed by transfer to nitrocellulose membranes for immunoblotting.

An additional method was utilized to eliminate detection of heavy and light chains of the IP antibody with ExactaCruz (Santa Cruz Biotechnology) as described by the manufacturer. Briefly, cell or tissue lysate were mixed with a preformed immunoprecipitation antibody – immunoprecipitation matrix complex and allowed to incubate overnight at 4ºC. Following incubation, the sample was microcentrifuged at maximum speed for one minute to pellet the matrix. The pelleted matrix was washed twice in immunoprecipitation buffer and re-suspended in 2X sample buffer and boiled for three minutes. Samples were resolved on 12% SDS-PAGE, followed by transfer to nitrocellulose membranes for immunoblotting.

**Digital Fluorescence Imaging and Whole Cell Patch Clamp**

Changes in cytosolic Ca\(^{2+}\) and C\(_m\) were measured as previously described (Peters et al., 2006). Briefly, cytosolic Ca\(^{2+}\) changes were monitored by a monochromometer-based illumination system, high speed CCD camera (T.I.L.L. Photonics) and Oregon Green Bapta-5N (100 μM). A pulsed Xenon arc lamp (T.I.L.L. Photonics) was used to induce exocytotic activity by the controlled photolysis of NP-EGTA. A high intensity flash (0.1 - 5 ms) of UV light was reflected onto the plane of focus with a DM400 dichroic mirror. Axopatch 200A patch clamp amplifier (Axon Instruments), ITC-16 digital interface (Instrutech), IGOR Pro (Wavemetrics) and Pulse Control XOP software-based phase sensitive detector were
Changes in membrane capacitance ($\Delta C_m$) of single neonatal atrial cardiac myocytes were measured by monitoring the current response to a 30 mV rms sine wave at 1201 Hz applied at a holding potential of -90 mV. The intracellular recording solution for the photolytic release of caged Ca$^{2+}$ contained (in mM): 130 CsCl, 10 HEPES-tris, 10 NP-EGTA, 5 CaCl$_2$, 2 Mg$^{2+}$-ATP, 1.2 MgCl$_2$, 100 µM OGB-5N, pH 7.2. The intracellular recording solution for the photolytic release of caged InsP$_3$ (D-myo-inositol 1,4,5-triphosphate, P4(5)-(1-(2-nitrophenyl)ethyl) ester, tris(triethylammonium) salt (Invitrogen) contained (in mM): 140 CsCl, 10 HEPES-Tris, 1.5 MgCl$_2$, 3 Mg-ATP, 1 n-hydroxyethylethylediaminetriacetic acid (HEDTA), 0.075 OGB-2 or OGB-5N, and 0.2 caged-InsP$_3$, pH 7.3. The intracellular recording solution for activation of voltage-dependent Ca$^{2+}$ channels by step depolarization contained (in mM): 110 KCl, 1 MgCl$_2$, 0.1 CaCl$_2$, 1 N-methyl-D-glucamine (NMDG)-EGTA, 10 HEPES. Millisecond time resolution of $C_m$ was achievable with this method. For kinetic analysis, individual capacitance traces were fitted with an exponential function: $f(t) = A_0 + A\exp\left[-(t-t_0)/\tau\right]$, where $A$ and $\tau$ are the amplitude and time constant of secretory granule fusion.

**Data Analysis**

Data are given as mean ± S.E. Statistical analysis was performed using the Student’s $t$-test, and significance was accepted at $p < 0.05$. Presented immunoblots are representatives of similar results from at least three separate experiments.
CHAPTER IV – IDENTIFICATION OF SNAREs

Hypothesis I: SNARE and SNARE-associated Proteins are Present in Atrial Myocytes and Capable of Forming Functional Interactions.

SNARE Proteins

The term exocytosis describes the process in which membranes of intracellular vesicles fuse with the plasma membrane. Exocytosis is mediated by a complex series of protein-protein interactions. This process governs the exocytotic release of hormones or neuropeptides from exocrine or neuronal cells. In 1984, two soluble proteins were purified that efficiently mediated protein movement along the vesicle trafficking pathway, N-ethylmaleimide sensitive factor (NSF) and an adaptor protein called NSF attachment protein (SNAP) (Balch et al., 1984; Block et al., 1988). These proteins were found to act in many intracellular pathways except that of fusion events with mitochondria and peroxisomes which appear to utilize a set of unrelated proteins. Using affinity purification of NSF and SNAP from brain samples, a set of additional proteins necessary for vesicle fusion were discovered (Sollner et al., 1993). The identification and purification led to the group of proteins that function as receptors to the SNAP receptor or SNARE (N-ethylmaleimide-sensitive factor attachment protein receptor) proteins. Work by many groups have shown that they form a protein superfamily consisting of more than 100 members in *Saccharomyces cerevisiae*, *Arabidopsis thaliana* and *Homo sapiens* acting not only
during release at the synapse, but in most intracellular trafficking pathways (Rizo and Sudhof, 2002; Jahn et al., 2003).

The discoveries of the SNARE proteins lead to the proposal of a SNARE hypothesis. This hypothesis proposed that these proteins provided specificity for targeting of vesicles and provide the molecular machinery necessary for fusion between vesicle and target membranes (Sollner et al., 1993). Figure 2 illustrates the formation of a core SNARE complex based on the above hypothesis. Due to the localization of these proteins to synaptic vesicles or presynaptic membranes, it was suggested that two classes of SNARE proteins existed. SNARE proteins were then classified as t- (target) or v- (vesicle) SNAREs depending on the membrane which they are located (Sollner et al., 1993). However, this hypothesis has since undergone many revisions, one of which is the nomenclature of SNARE proteins. Structurally, SNAREs are now categorized based on the residues that are located in the center of the core SNARE complex. This residue is highly conserved and consists of either arginine (R-SNARE) or glutamine (Q-SNARE) (Fasshauer et al., 1998).

It has now been shown that three Q-SNAREs and one R-SNARE is required for fusion events (McNew et al., 2000). The basic structure of the SNARE proteins consists of heptad repeat domains in the amino acid sequences (Hardwick and Pelham, 1992). This allows the coiled-coil sequences of SNAREs to form into a α-helix bundle. The interacting amino acid residues at the core hydrophilic ionic layer are flanked by hydrophobic leucine-zipper layers (Sutton et al., 1998). It is thought that clamping of the membranes and initiation of the fusion of the membrane proceeds from the N-terminal to the C-terminal end (Hanson et al., 1997). Figure 3
Figure 2. Model of Proposed Core SNARE Complex Formation (Blatt et al. 1999)

Figure 2. Illustration of the formation of the core SNARE complex, synaptobrevin (v-SNARE) will interact with the t-SNAREs, SNAP-25 and syntaxin. These interactions allow for the membranes to fuse and subsequent release of the cargo contained within the vesicle.
Figure 3. Structure of Neuronal SNARE Core Complex (Jahn and Scheller, 2006)

(A) Demonstrates the coiled-coil motifs of the neuronal SNARE complex which contains 4-α-helixes consisting of VAMP-2 (blue), syntaxin 1 (red) and SNAP-25 (green). (B) Skeleton drawing of the layers in which the core complex is formed. Layer ‘0’ is shown in red, at this layer of the core is hydrophilic. (C) Illustrates the layers approaching the ‘0’ layer that are highly conserved.
illustrates the 16 layers of the core SNARE complex as well the contribution of each SNARE to the core SNARE complex. The zero ionic layer as shown in Figure 3 is composed of R56 from VAMP-2, Q226 from syntaxin-1, Q53 and Q174 from two individual SNAP 25 proteins (Fasshauer et al., 1998). This interaction of the SNARE complex allows for it to be very stable. The SNARE complex is capable of resisting denaturation by SDS, botulinum and tetanus neurotoxins while having a melting temperature greater than 90ºC (Hayashi et al., 1994; Poirier et al., 1998).

One of the first SNARE elements to be identified, syntaxin 1, was found in the plasma membrane of neuronal cells (Bennett et al., 1993). Since then, at least 20 syntaxin isoforms have been described across the animal and plant kingdoms. Typically, syntaxins have molecular weights of approximately 35 kDa and fall into Q- or t- SNARE classification. Syntaxins are type II integral membrane proteins containing a single C-terminal transmembrane domain, a SNARE domain (referred to as H3) and a N-terminal regulatory domain (Habc) (Hirai et al., 1993). The H3 domain is highly conserved among all syntaxin isoforms and is required for binding SNAP-25 and VAMP (Calakos et al., 1994). Syntaxin, in particular syntaxin 1, is believed to be regulated by the SNARE-associated protein, Munc18. Munc18 binds to syntaxin 1 and holds the protein in a closed conformation by collapsing the H3 helix (Hata et al., 1993). The dissociation of syntaxin from Munc18 allows for the conformation of syntaxin opening and interacting with other SNARE proteins (ex. VAMP-2 and SNAP-25). The distribution of syntaxin varies upon isoforms, for example, syntaxin 1A is primary localized to the neuronal plasma membrane and is concentrated in synapses (Bennett et al., 1993). Syntaxin 2, 3 and 4 have also been
shown to be localized at the plasma membranes of cells, however, they have a much wider tissue distribution (Bennett et al., 1993; Jahn and Sudhof, 1999). Syntaxin 5 and 6 have been found to be localized to the Golgi membrane as well as endosomal structures (Banfield et al., 1994). Moreover, syntaxin 7 is involved in the fusion of late endosomes and lysosomes. In endosomal membranes, it has been shown to form complexes with VAMP-8 and syntaxin 8 (Advani et al., 1998).

The synaptobrevin family, also known as vesicle associated membrane protein (VAMP), are small integral membrane proteins of secretory granules with molecular weights on average of 11 – 13 kDa (Baumert et al., 1989). They are classified as members of v- or R- SNAREs. They have two domains, a N-terminal region exposed to the cytoplasm and a C-terminal transmembrane domain including a conserved 60 amino acid central sequence that enables binding to syntaxin and SNAP-25. Two isoforms have been identified in the central nervous system, VAMP-1 and VAMP-2. They are distributed within different regions of the brain and are highly homologous with approximately 80% amino acid identity (Sudhof, 1995). These proteins have been shown to be the major constituents of synaptic vesicles and other secretory granules as well as an essential component of the exocytotic fusion machinery.

VAMP-3, also known as cellubrevin, is a non-neuronal isoform that has been identified and localized to the endosomal membrane pool and ubiquitously expressed. Interestingly, VAMP-5 has been reported to be expressed in skeletal muscle and heart and localized to the plasma membrane in these tissues (Zeng et al., 1998).

Joining syntaxin and VAMP as a key components of the minimum machinery required for exocytosis is that of a synaptosome-associated protein of 25,000 daltons.
(SNAP-25). SNAP-25 is a membrane bound protein anchored to the cytosolic face of membranes by palmitoyl side chains (Lin and Scheller, 2000). SNAP-25 is classified as a Q- SNARE contributing two α-helices to the formation of the fusion complex. In neurons, SNAP-25 assembles with syntaxin 1 and VAMP-2. Interestingly, homozygous deletions of the SNAP-25 genes are lethal compared to heterozygous deletions which results in a hyperactive phenotype (Hess et al., 1994; Heyser et al., 1995). A ubiquitously expressed isoforms and functional homologue of SNAP-25 is the synaptosome-associated protein of 23,000 daltons, SNAP-23. Similar to SNAP-25, SNAP-23 is able to function in regulated exocytosis. In addition to SNAP-23 and SNAP-25, there are two other members of the family. SNAP-29 and SNAP-47, expressed in a variety of tissues, lack a membrane anchor and may be localized to a vesicle pool which is not confined to a specific organelle (Wong et al., 1999; Holt et al., 2006).

The core of the membrane fusion complex is comprised around the synaptic vesicle protein (VAMP) and plasma membrane proteins syntaxin 1 and SNAP-25. However, the exocytotic process is not as straightforward as the coming together of three proteins. This was touched upon earlier in regards to the regulation of syntaxin 1 by Munc18. In vitro, purified core SNARE proteins undergo fusion on the time scale of minutes and is not regulated by Ca\textsuperscript{2+} (Weber et al., 1998). However, it is well known that regulated exocytosis found in neurons where membrane trafficking is finely tuned to intracellular signals such as Ca\textsuperscript{2+}. These findings suggested those Ca\textsuperscript{2+} sensors, as well as other proteins, likely function with the core SNARE proteins to drive fusion in a regulated timely manner.
The search for a Ca\textsuperscript{2+} sensor lead to the discovery of a 65-kDa protein localized to the synaptic vesicle and large dense core vesicles of the rat brain (Walch-Solimena et al., 1993). The protein structure consists of a variable N-terminal domain that is exposed to the lumen of the vesicle and a conserved cytoplasmic tail that contains two Ca\textsuperscript{2+}-binding domains (Perin et al., 1990). Since the discovery of this protein, synaptotagmin, 13 isoforms have been identified. Each of which contains a conserved C2 domain, which consists of a 130 amino acid motif capable of binding Ca\textsuperscript{2+}. Interestingly, this domain is not limited to the synaptotagmin family, suggesting a unique structural specialization may be required.

Synaptotagmin, in particular synaptotagmin 1, is capable of binding five Ca\textsuperscript{2+} ions (Brose et al., 1992). This binding is achieved by three Ca\textsuperscript{2+} ions to the C2A and two to C2B domains (Ubach et al., 1998). Crystal structures suggest that Ca\textsuperscript{2+} binds to the C2 domain by electrostatic potentials formed by domains created by the folding of eight stranded \( \beta \) sandwiches (Sutton et al., 1995). It has been suggested that the binding of Ca\textsuperscript{2+} to synaptotagmin 1 acts on primed secretory vesicles. Evidence suggests synaptotagmin can penetrate both membranes and bind to the core of the SNARE complex (Davis et al., 1999). Synaptotagmin binding to the core complex may allow for further pulling of the membranes allowing fusion to occur (Chapman, 2002). Mice that are heterozygotes for the synaptotagmin 1 gene were phenotypically normal. However, those that were homozygotes died within 48 hours of birth. Cultured neurons from these mice revealed that the fast component of neurotransmitter release was abolished (Geppert et al., 1994). In summary, Ca\textsuperscript{2+}
entry and subsequent binding to synaptotagmin, allows synaptotagmin to facilitate
SNARE complex assembly and fusion of membranes in a rapid timely event.

**Known SNAREs Involved in GLUT-4 Translocation**

Adipose tissues and striated muscle, both skeletal and cardiac, contain the
insulin responsive glucose transporter 4 (GLUT-4). GLUT-4 is responsible for the
insulin regulated glucose disposal located in secretory granules of atrial cardiac
myocytes. GLUT-4 has been shown to enter ANP-containing granules in the trans-
Golgi reticulum (Slot et al., 1997). Interestingly, targeting of GLUT-4 into secretory
granules appears to be unique to atrial cardiac myocytes. GLUT-4 is expressed in
renal arteriolar cells, however, GLUT-4 is not located to renin-containing secretory
granules (Slot et al., 1997). In insulin responsive cells the core SNARE complex
consists of VAMP-2, SNAP-23 and syntaxin 4. Initially, GLUT-4 was localized to a
population of secretory granules that contained VAMP-2 in rat skeletal muscle (Cain
et al., 1992). The presence of VAMP-2 has since been confirmed in rat skeletal
muscle and the rat L6 muscle cell line demonstrating that no contaminates were
present (Ralston et al., 1994; Volchuk et al., 1994). In addition, VAMP-1 and
VAMP-5 have been identified (Jagadish et al., 1996; Zeng et al., 1998). VAMP-5 is
preferentially expressed in the heart and does not appear in the brain (Zeng et al.,
1998).

The syntaxin isoforms 2, 4, and 5 were also detected in membranes from rat
skeletal muscle (Volchuk et al., 1996; Sevilla et al., 1997). The dominant syntaxin
isoforms present in rat skeletal muscle is syntaxin 4, which has been localized in
membrane fractions (Wang et al., 1997). Finally, SNAP-23 and SNAP-25 have also
been identified in skeletal muscle (Jagadish et al., 1996; Ravichandran et al., 1996).
The presence of these SNARE proteins suggests their involvement in the
translocation of GLUT-4 to the plasma membrane.

**Identification of SNARE and SNARE-associated Proteins**

Physical stretch of the atrial wall is the canonical stimulus for exocytotic
secretion of ANP. However, detailed information regarding the function and identity
of specific SNAREs involved in exocytosis of ANP from atrial myocytes has been
lacking. Specifically, whereas the proteins that mediate exocytotic events in neurons
and neuroendocrine cells have been well characterized, those that mediate ANP
release are not known. To advance our understanding and to test the hypothesis that
SNARE and SNARE-associated proteins are present in atrial cardiac myocytes, I
utilized RT-PCR and Western blot analysis.

To begin, neonatal and adult primary cultures of atrial cardiac myocytes from
Sprague-Dawley rats were used to ask whether SNARE and SNARE-associated
proteins were present in neonatal and adult myocyte primary cultures. RT-PCR was
used to screen for the presence of SNARE and SNARE-associated transcripts.
mRNA isolated from neonatal and adult atrial cardiac myocytes were used to created
cDNA libraries which were probed using specific PCR primers encoding R- and Q-
SNAREs and the SNARE-associated families of proteins. The primers sets used are
given in Table I, from which a select set of primers have been previously described to
probe for SNAREs in the rat salivary gland and atrial cardiac myocytes (Imai et al.,
# Table I. PCR Primers Encoding SNARE and SNARE-associated Proteins
(Peters et al., 2006)

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*Accession numbers from GenBank/EMBL data bank

Table I. PCR primers designed as described in Methods section.
As a positive control in the current set of experiments, rat brain was utilized. These data are summarized in Table II, showing the results for the SNARE and SNARE-associated protein families.

As indicated, multiple SNARE products were identified including those that are thought to form core SNARE complexes in neuroendocrine cells (Sollner et al., 1993). Identification of six VAMP isoforms was noted. Expression of several isoforms were expected due to the involvement of VAMP isoforms in protein trafficking within the Golgi network, late endosomal fusion and maturation of the secretory granules (Advani et al., 1998; Zeng et al., 1998; Ward et al., 2000). Of particular interest was the identification of VAMP-1 and VAMP-2 as seen in Figure 3. These proteins have been identified as core SNARE proteins in the regulated secretion from endocrine cells. The SNARE transcripts of VAMP binding partners were also probed. As shown in Table II and Figure 3, SNAP-23 and SNAP-25 were identified in adult myocytes but was limited to SNAP-23 in neonatal myocytes.

Multiple syntaxin isoforms were also identified including syntaxin 4, which was previously identified and localized to the plasma membrane of skeletal muscle (Volchuk et al., 1996). Although syntaxin 4 has been previously described, the presence of syntaxin 1 in adult myocytes was a novel finding. An additional SNARE family was probed, the synaptotagmin family of proteins. Products encoding synaptotagmin 1 (Fig. 3) and synaptotagmin 3-6 were identified as shown in Table II. This finding was of particular interest, due to the role of synaptotagmin in neuronal cells. Moreover, the presence of Munc18 was identified in both neonatal and adult cultures (data not shown).
Table II. Identification of SNARE and SNARE-associated Transcripts by RT-PCR (Peters et al., 2006)

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Table II. No data available (nd). (+) indicates the identification of the specific isoforms, (-) indicates that the isoforms was not detected. Results are representative of three independent experiments.

I next focused my investigation on the SNARE proteins which are known to function in the late stages of the secretory pathway and that I identified as associated with ANP-containing secretory granules. As previously discussed, ANP has been shown to be stored in secretory granules that are morphologically similar to large dense core granules (Vuolteenaho et al., 1985). Figure 4A demonstrates that proANP is identified in lysates of primary cultures from neonatal and adult myocytes as well as the positive control, the whole heart. Further studies using western blot analysis confirmed the RT-PCR findings, identifying core SNARE complexes in the neonatal...
and adult myocytes. As shown in Figure 4B, these proteins consisted of syntaxin 1, syntaxin 4, SNAP-23, SNAP-25, VAMP-1, VAMP-2, and synaptotagmin 1. The expression of many of these SNARE proteins suggests that they may be able to form a functional SNARE complex. In addition, the SNARE-associated proteins of Munc18, synaptogyrin and syntaxin 6 (adult only) were identified using western blot.

Figure 3. Identification of SNARE Proteins by RT-PCR (Peters et al., 2006)

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Figure 3. RT-PCR products encoding the neuronal marker MAP-2 and core SNARE proteins. Brain tissue (Lane 1), neonatal atrial myocytes (Lane 2) and adult atrial myocytes (Lane 3) were visualized by ethidium bromide separated on a 2% agarose gel following standard RT-PCR conditions. Representative images from at least 3 independent experiments.
Although many SNARE and SNARE-associated proteins involved in regulated exocytosis were identified, the question of neuronal contamination was an issue. Using immunofluorescence with antisera for cardiac muscle specific α-actinin, greater than 90% of cultured cells were myocytes. To further examine the purity of the cultures and to ensure that no neuronal components were in the cultures due to the innervations of the atria by sympathetic and parasympathetic nerve fibers, lysates were probed with the neuronal marker, microtubule associated protein-2 (MAP-2). As shown in Figure 3, MAP-2 was undetectable by RT-PCR in the cultures; however a strong signal was detected in the control brain sample. These results were confirmed in Fig. 4B by Western blot analysis which was unable to detect MAP-2. These data suggests that the identified SNARE proteins were endogenous to the atrial cardiac myocytes and not originated from neuronal contaminations of the primary cultures.
Figure 4. Identification of ANP and SNARE Proteins by Western blot (Peters et al. 2006)

A

Pro-ANP

60 kDa

B

Brain  Neonatal  Adult

MAP-2

200 kDa

Syntaxin 4

35 kDa

Syntaxin 1A

35 kDa

SNAP-23

23 kDa

SNAP-25

25 kDa

VAMP-2

17 kDa

VAMP-1

17 kDa

Figure 4. (A) Western blot analysis demonstrating expression of proANP in adult and neonatal atrial myocytes. Whole adult rat heart was used as positive control. (B) Western blot analysis for SNARE proteins in neonatal and adult atrial myocytes and the positive control adult rat brain. 50 µg of total protein was added to each lane, similar results were obtained in three independent experiments.
Localization of SNARE Proteins to ANP-containing Secretory Granules

Neonatal Atrial Myocytes

The distribution and localization of the R-SNAREs, VAMP-1 and VAMP-2 to ANP-containing secretory granules were examined using cultured neonatal cardiac myocytes. Atrial myocytes were lysed using a ball-bearing cell homogenizer. The benefits of this homogenizer were that intracellular organelles, including secretory granules, remained intact. The total cell lysate was subjected to cell fractionation analysis by step-wise sucrose density gradients. Figure 5A shows Western blot analysis of 15 equal aliquots collected from the top of the centrifuged gradient (0.4 to 2.2 M sucrose). Each fraction was separated by 12% SDS-PAGE and immunoblotted for proANP, SNAP-23, VAMP-1 and VAMP-2. Each protein was identified in low density fractions (fractions 1-4), likely due to a portion of these proteins located in the cytosol or plasma membrane or lysis of secretory granules during preparation. The presence of the t-SNARE, SNAP-23 was expected to be contained in these fractions. Of particular interest, synaptotagmin 1, VAMP-1 and VAMP-2 were localized to the densities which ANP was found. These data suggest these proteins may be localized to secretory granules containing ANP.

To further investigate the localization, differential centrifugation was utilized to purify a secretory granule fraction prior to sucrose gradient fractionation. Figure 5B represents fractions 9-15, in which ANP was found to migrate to fractions 12 and 13 where the SNARE proteins VAMP-1, VAMP-2 and synaptotagmin 1 were found to migrate. The distribution of these proteins suggests that VAMP-1, VAMP-2 and synaptotagmin are present on a population of secretory granules in neonatal atrial
Figure 5. Migration of ANP and SNARE Proteins on Sucrose Density Gradients

(Peters et al., 2006)

(A) Neonatal atrial myocyte cell lysate migration on a sucrose gradient. Fractions one through 15 represent equal aliquots removed from the top of the gradient following cell fractionation. (B) An enriched fraction of secretory granules subjected to sucrose density fractionation. Not shown are fractions one through eight which contained no signal. Fractions were separated on 12% SDS-Page gels and subjected to immunoblot analysis with anti-proANP, anti-VAMP-1, anti-VAMP-2, anti-SNAP-23 and anti-synaptotagmin 1. Similar results were obtained in three independent experiments.
myocytes. The distribution of VAMP-1, VAMP-2 and synaptotagmin 1 to secretory granules in neonatal atrial myocytes is consistent to the known distribution of these proteins in other cell types. However, the above result may be due to similar densities migrating with a granule population of equal densities. To rule this possibility out, the subcellular distribution of SNARE proteins were examined by immunocytochemistry and confocal microscopy.

These experiments were completed using cultured neonatal atrial myocytes or tissue sections of the heart labeled with specific antisera and visualized using Alexa-488 and Alexa-555 conjugated secondary antibodies. Colocalization was quantified using ImageJ fluorescent correlation algorithm and micrographs expressed as full frame averages obtained from each 0.25 µm or 0.5 µm optical slices from designated Z-series. In Figure 6A, tissue slices of the whole heart were labeled for ANP and stained with phalloidin. Phalloidin is a high affinity probe for F-actin that is made from a mushroom toxin conjugated to Alexa-555 for visualization. ANP (green) displayed largely punctuate labeling in the atria with little to no staining in the ventricle. Staining with the nuclear dye DAPI demonstrated that not all cells within the atria contain ANP as seen in Figure 6B. In Figure 6C, single neonatal myocytes were stained for ANP and α-actinin. Punctate signal representing the ANP-containing granules were found to be located in a perinuclear distribution.

Following the identification of ANP, neonatal atrial myocytes were labeled for specific SNARE proteins. In Fig. 7A, ANP (green) exhibited the characteristic perinuclear labeling as seen above and colocalized in part with VAMP-2 (red). The Pearson’s correlation value resulting from the colocalization analysis was 0.52 ± 0.08.
Figure 6. Localization of ANP

(A) Confocal micrographs of whole heart tissue stained for ANP (green) and F-actin (red). F-actin was labeled with phalloidin-conjugated Alexa 555. The atria portion is clearly labeled with ANP and the ventricle showed F-actin staining only. (B) Confocal micrographs of whole heart tissue labeled for ANP (green), F-actin (red) and the nuclear dye DAPI (blue). Images illustrate that not all atrial cardiac myocytes contain ANP. (C) Single neonatal atrial myocyte labeled for ANP (red) and α-actinin (green), demonstrating the perinuclear distribution of ANP.
The VAMP-2 signal appeared to be more distributed than ANP, possibly suggesting VAMP-2 may also be localized to a subpopulation of granules not containing ANP. Due to this finding, it was then logical to examine the localization of VAMP-1 and ANP. The presence of VAMP-1 was previously identified in RT-PCR and confirmed by Western blot. However, the antibodies against these proteins are both polyclonal. To circumvent this issue, I utilized a technique referred to as ‘pretty poly’ which enables dual-labeling of cells with two polyclonal antibodies (Morris and Stanley, 2003). To accomplish the labeling, anti-proANP and anti-VAMP-1 were pre-conjugated to protein A conjugate to Alexa 488 or Alexa 555. In Figure 7B, using widefield fluorescence microscopy, ANP (green) was distributed in a perinuclear fashion, while VAMP-1 appeared to be widely distributed. The corresponding Pearson’s value was $0.74 \pm 0.08$ (n = 4). The calculated Pearson value was higher than VAMP-2 and ANP; however, given the differences in resolution that was achieved it would be difficult to compare directly.

To examine the possibility that two populations of granules may be present, VAMP-1 and VAMP-2 were directly compared within cells. In Figure 7C, VAMP-2 (green) appeared to be generally in the perinuclear region with small quantities localized to the cell surface. VAMP-1 appeared to have a much wider distribution, however, was generally excluded from regions containing VAMP-2. The signals appeared to have only a minor overlap, with a resulting Pearson’s correlation value of $0.21 \pm 0.13$. In Figures 7D – F, the relationship of synaptotagmin 1 with ANP, VAMP-1, and VAMP-2 were determined. In each panel, synaptotagmin 1 (green) exhibited a broadly punctate distribution. The relationship of synaptotagmin 1 with
Figure 7. Confocal Micrographs of Neonatal Atrial Myocytes (Peters et al., 2006)

Figure 7. Multiple confocal micrographs and widefield fluorescence of neonatal atrial myocytes demonstrating colocalization (yellow) of SNARE proteins and ANP-containing secretory granules. (A) Confocal micrograph demonstrating the colocalization of ANP (green) with a portion of VAMP-2 (red). (B) The colocalization of ANP (green) with VAMP-1 (red) as seen by using widefield fluorescence microscopy and a specialized labeling technique using two polyclonal antibodies. (C) Confocal micrograph of VAMP-1 (red) and VAMP-2 (green) demonstrating little overlap of the vesicle SNARE proteins. Panels D-F illustrates confocal micrographs of synaptotagmin 1 (green) and ANP (D), VAMP-2 (E) or VAMP-1 (F) (red).

ANP (red) yielded a Pearson’s correlation value of $0.51 \pm 0.05$ in Figure 7D.

Synaptotagmin 1 (green) and VAMP-2 (red) resulted in a similar relationship, with a Pearson’s value of $0.69 \pm 0.07$. This differed from the Pearson’s value of $0.90 \pm 0.06$ when comparing synaptotagmin 1 (green) and VAMP-1 (red). These data suggest the
majority of ANP-containing granules contain synaptotagmin 1 and either VAMP-1 or VAMP-2. The wide distribution of VAMP-1 suggest that it may be involved in other trafficking pathways in the cell, possibly involved in secretion of BNP containing granules or translocation of granules carrying GLUT-4 alone. The involvement of other pathways may also explain why a subpopulation of granules contained VAMP-2 and were negative for ANP.

In an additional set of immunocytochemistry experiments, the localization of syntaxin 4 and SNAP-23 were examined. As seen in Fig. 8A, syntaxin 4 appears to have a very punctate labeling pattern. The bulk of the signal appears to be located on or near the plasma membrane, but it does appear that a small quantity has been internalized. Of particular interest, the majority of internalized signal appears to be punctate and measuring near the size of a secretory granule as determined by using Leica Application software (LAS). The labeling of SNAP-23 appeared to exhibit a similar pattern. In Fig. 8B, the staining patterns were consistent to distinct labeling of the plasma membrane with a small quantity internalized. The localization of syntaxin 4 and SNAP-23 to distinct points in the plasma may represent pre-designated points of exocytosis.
Figure 8. Localization of Q-SNAREs in Neonatal Atrial Myocytes

(A) Confocal micrograph demonstrating the punctate labeling of the plasma membrane or close proximity of the plasma membrane. Cells were labeled with antisera specific for syntaxin 4 and visualized with Alexa Fluor 555 conjugated secondary antibody.

(B) Micrograph illustrating the punctate labeling of SNAP-23. Cells were stained with anti-SNAP-23 antibody and visualized with Alexa Fluor 555 conjugated secondary antibody.

Adult Cardiac Myocytes

Unlike the neonatal myocyte, the adult cardiac myocyte is a binucleated non-dividing cell. As the myocyte matures into the adult stage, multiple morphological changes occur. One significant change is the rearrangement of actin and myocyte into specific striations including junctions in the form of intercalated disks. With this and other morphological changes, it would be difficult to extrapolate results from neonatal myocytes to describe that of the adult myocyte. Because of these changes, the distribution of VAMP-2 to ANP-containing granules was examined using freshly prepared adult atrial myocytes. Following differential plating to eliminate non-
myocytes from the primary cultures, cells were lysed using the cell cracker as previously described. Cell lysate was subjected to differential centrifugation and the purified secretory granule fraction was further separated by sucrose gradient fractionation. Figure 9 shows the migration of VAMP-2 and ANP on sucrose gradients (0.4 to 2.2 M). The vesicle SNARE protein, VAMP-2, largely colocalized with ANP, suggesting VAMP-2 is located on ANP-containing granules similar to that of neonatal myocytes.

Figure 9. Migration of ANP and VAMP-2 in Adult Atrial Myocytes

In Figure 10, a set of immunocytochemistry experiments was completed using freshly prepared adult atrial myocytes. In Fig. 10A, VAMP-1 was localized in a distinct banding pattern within the myocyte. VAMP-1 appears to extend from the plasma membrane along the transverse axial tubule system (TATS), allowing for the
appearance of light striations. Interestingly, very little of the labeling appeared to be punctate or localized to the plasma membrane. This staining is very diverse from what is seen by VAMP-2, as shown in Fig. 10B. VAMP-2 is clearly found in the plasma membrane as well as diverse distribution within the cell. Interestingly, VAMP-2 also has punctate signal approximately the size of secretory granules near the TATS as measured by LAS. To confirm that VAMP-1 or VAMP-2 was localized to the transverse tubule system and not that of the myofibril Z-bands, VAMP-1 was co-labeled with α-actinin. In Fig. 10C-E, the co-labeled myocyte clearly illustrates that VAMP-1 did not colocalize with α-actinin which is a component of the myofibril Z-band. Therefore demonstration that VAMP-1 localized to the invaginations of the plasma membrane or the transverse t-tubules.

To explore the localization of Q- SNAREs, syntaxin 1 and syntaxin 4 were labeled. In Fig. 10F, similar to the distribution of VAMP-1, syntaxin 1 appeared to follow TATS with essentially no signal in the plasma membrane. Figure 10G illustrates the labeling of syntaxin 4. Syntaxin 4 was found to be punctate in the perinuclear region as well as select regions near the transverse tubules. A similar staining pattern was demonstrated in Fig. 10H. Synaptotagmin 1 was also located in the perinuclear region as well as containing punctate signal. Interestingly, synaptotagmin 1 also localized to the plasma membrane.
Figure 10. Localization of SNAREs in Adult Atrial Myocytes

(A) VAMP-1 was stained with antisera for VAMP-1 and visualized with Alexa-488 conjugated secondary antibody. (B) VAMP-2 was labeled with anti-VAMP-2 and Alexa-488 conjugated antibody. Distinct labeling of the plasma membrane and punctate signal was observed. Diameter of measured punctate signal ranged from 250 – 450 nm in length. Panels C-E represents a dual-labeled cell with anti-VAMP-1 (C) and α-actinin (D). (E) Merged image of C and D demonstrating that VAMP-1 and the myofibril Z-bands do not colocalize. (F) Syntaxin 1 labeled with anti-syntaxin 1. (G) Syntaxin 4 labeling at the intercalated disks. (H) The SNARE-associated synaptotagmin 1 stained with antisera for synaptotagmin.
Interactions of SNAREs in vitro (Neonatal and Adult Myocytes)

The presence of the identified SNAREs suggests that SNAREs may form complexes within the atrial cardiac myocytes. To determine if this is the case, I used immunoprecipitation methods to determine if the SNAREs were capable of interactions by using VAMP-2 specific antibody to probe cell lysates for associated immune complexes. Within neonatal myocytes, VAMP-2 pulled down an immune complex containing syntaxin 4, SNAP-23 and synaptotagmin 1 (Fig. 11A). Conversely, an additional immune complex to that of the neonatal complex was uncovered in adult atrial myocytes. As shown in Fig. 11B, VAMP-2 immunoprecipitated syntaxin 1 and SNAP-25 as well as syntaxin 4 and SNAP-23. Although nonspecific interactions can be detected using immunoprecipitation techniques they nevertheless demonstrate that the SNARE proteins in atrial myocytes assemble in a manner consistent with their hypothesized function.

Discussion

Findings from this set of experiments demonstrate that multiple SNAREs are present and capable of interacting in atrial myocytes. Furthermore, these proteins are crucial for vesicle fusion mediated neurotransmitter release, and therefore may play an essential role in regulating the controlled secretion of ANP from neonatal and adult atrial cardiac myocytes. As seen in Fig. 12, neonatal myocytes contain the SNARE proteins responsible for GLUT-4 transporter translocation. This complex consists of VAMP-1/VAMP-2, SNAP-23 and syntaxin 4, all of which have been previously identified in skeletal muscle (Slot et al., 1997; Foster et al., 1998).
Figure 12. Proposed SNARE Hypothesis in Neonatal and Adult Cardiac Myocytes

Figure 12. SNARE hypothesis in neonatal and adult atrial cardiac myocytes. The left side of the illustration demonstrates the SNARE proteins present in neonatal atrial myocytes. These proteins consist of syntaxin 4, VAMP-1, VAMP-2, SNAP-23 and synaptotagmin 1. The right side of the illustration representing adult atrial myocytes contains those found on the left in addition to SNAP-25 and syntaxin 1.
Although these proteins were also identified in adult cardiac myocytes, an additional complex consisting of VAMP-1/VAMP-2, SNAP-25 and syntaxin 1 was identified (Fig. 12). This combination of SNARE proteins is believed to mediate synaptic transmitter release in neurons (Lin and Scheller, 2000). These findings have been previously unknown in adult atrial cardiac myocytes. Interestingly, this may suggest an age dependent reconfiguration of SNARE proteins and may have relevance for the Ca\(^{2+}\)-dependent exocytosis in adult atrial myocytes.

Alternatively, the appearance of the adult complex may coincide with the appearance and formation of the TATS system in atrial cardiac myocytes. The tubule system, comprised of invaginations of the plasma membrane, is not as developed as that of ventricular cardiac myocytes which are highly organized and run transversely at Z-lines. The morphology of TATS may allow the cardiac myocyte to increase docking sites for exocytosis of ANP-containing granules which are mostly perinuclear. As shown in Fig. 13, the invaginations of the membranes to the docking sites of ANP-containing granules may allow for specificity of Ca\(^{2+}\) mediated ANP release amongst the background influxes of Ca\(^{2+}\) with each heart beat. These interpretations may not be relevant to neonatal cardiac myocytes, where a TATS system is not observed. For example, the neonatal myocyte does not appear to as structured or as large as that of the adult myocyte. By comparing the α-actinin staining, it is clear that in the adult cell the internal structures are highly organized and contain more filaments when compared to that of the neonatal myocyte.
Figure 13. Proposed Role of TATS System in Exocytosis in Adult Atrial Myocytes

Figure 13. Model of proposed role of TATS system in adult atrial cardiac myocyte. Invaginations of the membranes (T-tubules or TATS) are shown approaching the docking sites of ANP-containing secretory granules. This may allow for the secretory granules to dock at this membrane system allowing for secretion to occur.
CHAPTER 5 – REGULATORY CONTROL OF EXOCYTOSIS

Hypothesis II: Secretory Activity in Neonatal Atrial Myocytes can be Evoked by Rises in Intracellular Ca\textsuperscript{2+}

Regulation of ANP Exocytosis

The atrium of the heart is capable of secreting ANP that functions in the regulation of body fluid and electrolyte balance. In addition, it has been shown to secrete BNP, cardiac adrenomedullin and cardiotrophin-1 (Asai et al., 2000; Eto et al., 2003). The first stimuli studied and predominant signal for ANP release is stretch of the atrial wall (Lang et al., 1985). A more detail explanation for ANP release was later described as the increased pressure on the wall of the atria (Ruskoaho et al., 1986). It was subsequently shown that the ratio of atrial wall tension to wall stress was key for secretion (Thibault et al., 1999). In perfused hearts, increased atrial contraction rates had enhancement effects on ANP release as well as increased atrial pressure (Ruskoaho, 1992). In addition, cultured neonatal atrial myocytes stimulated by electrical pacing induced a rapid increase in ANP secretion (McDonough et al., 1994). Although this evidence suggests that volume expansion is responsible for secretion it is not well understood if atrial wall stretch acts directly or through local factors such as hormones, endothelial factors (endothelin-1), oxytocin, ouabain and Ca\textsuperscript{2+}.

Endothelin (ET-1), a hormone synthesized by vascular endothelium, has been shown to be a potent ANP secretagogue in rat atrial myocytes (Fukuda et al., 1988; Sei and Glembotski, 1990) as well as in isolated atria (Hu et al., 1988; Stasch et al., 1989). In paced rat atria, ET-1 (1 to 100 nM) increased ANP secretion in a dose-
dependent manner from 1.6 to 6.7 fold above the baseline (Schiebinger and Gomez-Sanchez, 1990). ET-1 functions through a heterotrimeric guanine nucleotide binding protein, G-protein. The dissociation of the heterotrimer leads to the activation of phospholipase C. Phospholipase C then hydrolyzes phosphatidylinositol 4’, 5’-bisphosphate into two second messengers, diacylglycerol and IP₃ (inositol 1’, 4’, 5’-trisphosphate) (Rhee, 2001). IP₃ then induces a Ca²⁺ release from the sarcoplasmic reticulum resulting in a release of ANP. This has been shown to occur within seconds following the application of ET-1 to atrial cardiac myocytes (Clerk and Sugden, 1997).

**Ca²⁺ in Atrial Cardiac Myocytes**

Although the canonical stimulus for ANP release is stretch of the atrial myocyte, no clear role for intracellular Ca²⁺ has been defined. In contrast, exocytosis in most endocrine or neuronal cells is regulated by an increase in intracellular Ca²⁺ concentrations (Knight et al., 1989). However, a major difference between these cells and atrial myocytes is that the myocytes undergo rapid internal Ca²⁺ fluctuations during each contraction via Ca²⁺ entry through voltage-gated Ca²⁺ channels. The voltage-gated Ca²⁺ channels mediate Ca²⁺ entry in atrial myocytes in response to membrane depolarization. Electrophysiological studies have revealed that multiple types of Ca²⁺ channels designated L-, N-, P-, Q-, R-, and T-type exist. In cardiac tissue, the two main classified Ca²⁺ channels are L-type and T-type.

L-type Ca²⁺ channels, also referred to high-voltage activated channel (HVA), was designated by their ability to have long lasting currents in the presence of Ba²⁺
These channels are responsible for the major Ca\(^{2+}\) current in cardiac, smooth and skeletal muscle. In addition, these channels are also present in endocrine cells where they trigger the release of hormones (Milani et al., 1990). The major subtypes of HVA channels expressed in the heart are Ca\(_v\)1.2 and Ca\(_v\)1.3. Knockout studies of Ca\(_v\)1.2 in cardiac tissue was demonstrated to be embryonic lethal in mice, stressing the importance of the HVA Ca\(^{2+}\) channels (Seisenberger et al., 2000). Moreover, one other HVA channel has been described in cardiac myocytes the R-type, or Ca\(_v\)2.3 (Lu et al., 2004). Once these channels are activated, they remain open long enough to drive a Ca\(^{2+}\) tail current (Metz et al., 2005).

The other main type of Ca\(^{2+}\) channels described in cardiac tissue is that of the T-type Ca\(^{2+}\) channels or low voltage activated (LVA) channels. T-type currents activate and inactivate at more negative voltages (~30 mV). There are three T-type Ca\(^{2+}\) channel subtypes, two of which are expressed in the heart (Ca\(_v\)3.1 and Ca\(_v\)3.2) (Perez-Reyes et al., 1998). The likely role for LVA channels is to provide an inward depolarizing current during the slow diastolic depolarization which contributes to regulating the pace of the heart (Nilius, 1986; Hagiwara et al., 1988). In addition to the discussed voltage-dependent Ca\(^{2+}\) channels, Ca\(^{2+}\) can enter the myocyte through stretch activated channels. These channels are mechanically opened by non-osmotic stretch and may raise Ca\(^{2+}\) in localized areas (Sigurdson et al., 1992; Lipp and Niggli, 1996).

In order to restore Ca\(^{2+}\) homeostasis following influx and for muscle relaxation, Ca\(^{2+}\) removal from the cytosol is necessary. This is primarily completed through the Ca\(^{2+}\)-ATPase in the sarcoplasmic reticulum (SERCA) or the Ca\(^{2+}\)-ATPase
and Na⁺/Ca²⁺ exchanger in the plasma membrane. The major Ca²⁺ store in cardiac myocytes is that of the sarcoplasmic reticulum. Interestingly, the sarcoplasmic reticulum has dual roles. In addition to removal of Ca²⁺ from the cytosol following Ca²⁺ influx, Ca²⁺ is subsequently released from the sarcoplasmic reticulum by the opening of the ryanodine receptors in a Ca²⁺ induced Ca²⁺ release. This release of Ca²⁺ is an essential mechanism leading to the contraction of myocytes and the migration of the Ca²⁺ wave involving the activation of phospholipase C by Ca²⁺ leading to IP₃ formation and subsequent release of Ca²⁺ by IP₃ mediated channels (Ehrlich et al., 1994; Laver, 2006).

Other mechanisms for removal of Ca²⁺ from the cytosol include the Ca²⁺-ATPase. The Ca²⁺-ATPase is a form of P-ATPase, which transfers Ca²⁺ out of from the myocyte following a contraction with the hydrolysis of ATP. This intracellular process contributes to the relaxation of cardiac myocytes following contraction. In addition, the Na⁺/Ca²⁺ exchanger transports Ca²⁺ across the membrane by utilizing the electrochemical gradient for Na⁺. In the cardiac myocyte, this transporter is essential for homeostasis of intracellular Ca²⁺ and relaxation following contraction by exchanging one Ca²⁺ ion for three Na⁺ ions. Ca²⁺ can also be stored in mitochondria, which may contribute to Ca²⁺ sparks in cardiac myocytes (Kirichok et al., 2004). Ca²⁺ uptake occurs through the mitochondrial Ca²⁺ uniporter located in the inner membrane of the organelle. Finally, Ca²⁺ can be sequestered with a mobile buffer or by a calcium-binding protein.
Role of Ca$^{2+}$ in Secretory Control

In most secretory cells, Ca$^{2+}$ has been demonstrated to evoke exocytosis; however, there is no clear consensus in regards to Ca$^{2+}$ evoked ANP secretion. Numerous studies support an inhibitory role for Ca$^{2+}$ in the system while others clearly support a positive role. In 1989, De Bold and De Bold demonstrated that ANP secretion was independent of extracellular Ca$^{2+}$ in spontaneous beating rat atria and concluded that a reduction in cytosolic Ca$^{2+}$ actually stimulated ANP release (De Bold and De Bold, 1989). It was later demonstrated in perfused rat heart that BAY K8644, a Ca$^{2+}$ channel agonist that increases Ca$^{2+}$ entry, increased the concentration of intracellular Ca$^{2+}$, however inhibited ANP release (Ruskoaho, 1990). Similar results were reported in beating rabbit atria where BAY K8644 increased atrial stroke volume and suppressed ANP secretion (Wen et al., 2000). In addition, release of Ca$^{2+}$ from intracellular stores by application of ryanodine did not have a positive effect on ANP release (Kuroski-de Bold and de Bold, 1991). These results suggest that rises in intracellular Ca$^{2+}$ through voltage-dependent channels or release of Ca$^{2+}$ from intracellular stores have no effect on the secretion of ANP.

In contrast, BAY K8644 in cultured neonatal myocytes evoked a Ca$^{2+}$ influx and subsequent release of ANP in a dose dependent manner (Saito et al., 1986). Similar findings were observed in paced rat atria (Schiebinger et al., 1994). Further studies demonstrated Ca$^{2+}$ influx from BAY K8644 functions through a PKC independent mechanism, similar to the mechanism ET-1 induces ANP secretion (Irons et al., 1992). These effects of BAY K8644 were blocked by the Ca$^{2+}$ channel antagonist nifedipine, suggesting that intracellular Ca$^{2+}$ has a direct role in ANP.
secretion (Matsubara et al., 1988). Further conformation for the positive role of Ca\(^{2+}\) was demonstrated in superfused rat atria where extracellular Ca\(^{2+}\) concentrations were dependent for ANP release (Laine et al., 1996). To judge whether secretory activity could be evoked in single neonatal atrial myocytes, time-resolved membrane capacitance measurements were used in combination with the flash photolysis of caged compounds.

**Ca\(^{2+}\)-evoked Exocytosis**

Previously, I identified the Ca\(^{2+}\) sensing protein, synaptotagmin 1, in neonatal atrial cardiac myocytes and demonstrated that this protein was associated with ANP-containing secretory granules. The presence of synaptotagmin 1 suggests that a Ca\(^{2+}\)-dependent component of exocytosis in neonatal cardiac myocytes may be present. Therefore, I sought to establish whether a controlled rise in cytosolic Ca\(^{2+}\) was sufficient to evoke exocytosis and to test the hypothesis that a rise in intracellular Ca\(^{2+}\) sufficiently evokes exocytosis in neonatal atrial myocytes. To accomplish this, I used time-resolved membrane capacitance measurements in combination with the flash photolysis of caged Ca\(^{2+}\), caged IP\(_3\) or voltage step depolarizations to follow the exocytotic activity of single neonatal atrial myocytes.

The neonatal myocytes used in these experiments were plated on collagen coated coverslips where caged Ca\(^{2+}\) and the low affinity Ca\(^{2+}\) sensitive dye OGB-5N was introduced via the patch clamp pipette. Isolated, quiescent myocytes were chosen for recordings. Moreover, these non-beating myocytes were able to remain patch clamped for longer periods of time. An additional benefit of the quiescent cells
was that I had full control over the rise of intracellular Ca$^{2+}$ to evoke secretory activity without significant background changes in Ca$^{2+}$ as observed in spontaneous contracting myocytes.

To evoke a consistent rise in intracellular Ca$^{2+}$, a 1 msec 300 V UV flash was applied to single neonatal myocytes following a period of three minutes for diffusion of dye and caged Ca$^{2+}$ solution. Photorelease of Ca$^{2+}$ induced a rapid consistent controlled rise in intracellular Ca$^{2+}$ in parallel to an exponential rise in C$_m$.

Previously, it was shown that rat atrial myocytes contain secretory granules with an average diameter of 350 nm (Jamieson and Palade, 1964). Given the specific capacity of membranes (1 $\mu$F/ cm$^2$) and known diameter of granules it was calculated that each individual secretory granule would have a given capacitance of 3.8 fF (Peters et al., 2006). Although single secretory granule fusions were below the resolution of our system, changes in C$_m$ can be correlated to the number of secretory granules undergoing secretion upon evoked rises in intracellular Ca$^{2+}$.

As shown in Fig. 14A, a single UV flash induced an exponential change in C$_m$ measurements. Also given are the corresponding changes in membrane conductance by the isolation of the capacitative current, demonstrating that little change of ion flow across the plasma membrane occurs during the capacitance measurements. The average evoked change in C$_m$ for the first UV flash was 298.1 ± 21.4 (n = 18). In Fig. 14B, a second sequential flash evoked an average change of 232.9 ± 22.5 (n = 16). These changes in C$_m$ correspond to the fusion of 78 and 61 granules for the first and second flash, respectively for all untreated neonatal myocytes (Fig. 14C). Corresponding changes in intracellular Ca$^{2+}$ was recorded simultaneously with C$_m$.
Figure 14.  Flash Photorelease of Ca$^{2+}$ in Single Neonatal Atrial Myocytes

(Peters et al., 2006)

(A) Representative traces from recordings of myocytes loaded with 10 mM NP-EGTA. The individual traces show exponential increases in $C_m$ (upper trace) evoked by a 1 ms UV flash. The lower trace illustrates the corresponding changes in resistance. (B) Representative recordings of the second flash given to a myocyte three minutes following the first UV flash. Each trace begins with a series of calibration pulses. (C) Summation of evoked $C_m$ changes by first ($n = 18$) or second ($n = 16$) UV flash in fF. Data is cumulative over all experiments. These data have a $p$ value of ($p = 0.04$).
Figure 15. Changes in Ca\textsuperscript{2+} and Kinetics of C\textsubscript{m} Rise (Peters et al., 2006)

Figure 15. Changes in Ca\textsuperscript{2+} evoked exocytosis by UV flash photorelease of caged Ca\textsuperscript{2+} and kinetics of response. (A) The top set of traces represent an overlay of change in C\textsubscript{m} of first flash (black trace) and second flash (blue trace). The lower overlay represents the changes in cytosolic Ca\textsuperscript{2+} as monitored by loading individual cells with 100 µM OGB-5N. The second flash had nearly identical rises in Ca\textsuperscript{2+}. (B) Representative trace of the change in C\textsubscript{m} (upper trace) where the rising phase was fit to a single exponential line. Rate constant for the evoked rise was 1.79 ± 0.18 s\textsuperscript{-1} or 1.63 ± 0.21 s\textsuperscript{-1} for the first and second flash, respectively.

measurements by monitoring the changes in OGB-5N. As shown in Fig. 15A, sequential flashes evoked nearly identical Ca\textsuperscript{2+} elevations despite a reduced C\textsubscript{m} response to the second flash application.

The analyses of the kinetics of the exocytotic bursts lead to a direct comparison of neonatal atrial myocytes to neuroendocrine cells. The kinetics of atrial myocytes was best fit to an exponential line with a τ = 476 msecs (Fig. 15B). The initial rate of exocytosis was calculated at 490 fF sec\textsuperscript{-1}, corresponding to more than 100 secretory granule fusion events a second. This finding is unique to neonatal atrial myocytes. Similar measurements in neuroendocrine cells exhibit complex kinetics.
existing of two or three exponential rate components, unlike that of neonatal atrial myocytes (Sudhof, 2002).

These experiments demonstrated that a controlled rise in intracellular Ca\textsuperscript{2+} can evoke a secretory response in neonatal atrial myocytes. I next determined if other secretagogues were capable of inducing similar changes in C\textsubscript{m}. As previously discussed, ET-1 is an established secretagogue of ANP. The cellular mechanism in which ET-1 induces a rise is thought to function through the activation of phospholipase C and subsequent production of IP\textsubscript{3}. To test this directly, the controlled photolysis of 200 µM caged IP\textsubscript{3} in combination with C\textsubscript{m} was used to monitor exocytosis in neonatal atrial myocytes. In Fig. 16A, the flash photolysis of caged IP\textsubscript{3} evoked a small rise in C\textsubscript{m} (12 ± 12 fF; n = 9); however, this response was inconsistent. Interestingly in Fig. 16B, simultaneous measurements of intracellular Ca\textsuperscript{2+} displayed that the photorelease of caged IP\textsubscript{3} did induce a rise in intracellular Ca\textsuperscript{2+} levels. In addition, the application of ET-1 to single neonatal atrial myocytes induced a rise in intracellular Ca\textsuperscript{2+} as determined by 1 µM Fura-2 loaded myocytes (Fig 16C). This may indicate that IP\textsubscript{3} mediated release of Ca\textsuperscript{2+} alone is unable to induce a consistent exocytotic response in neonatal atrial myocytes and may require an additional regulatory signal.

Following the set of experiments investigating IP\textsubscript{3} mediated Ca\textsuperscript{2+} release effects on exocytosis, Ca\textsuperscript{2+} influx through voltage-dependent Ca\textsuperscript{2+} channels were examined. In these experiments, membrane step depolarizations were utilized for activation of Ca\textsuperscript{2+} channels were used in combination with C\textsubscript{m}. Single step depolarizations were made from a holding potential of -90 to 10 mV for 100 msecs or
Figure 16. IP$_3$ Mediated Rise in C$_m$ and Intracellular Ca$^{2+}$

(A) Representative recordings of increases in C$_m$ evoked by a 1 msec UV flash in neonatal atrial myocytes loaded with 200 µM caged IP$_3$ and 100 µM OGB-5N. The trace shown indicates a small rise in C$_m$, a response that was not consistent in all recorded myocytes. (B) The flash photolysis of caged IP$_3$ resulted in robust changes in intracellular Ca$^{2+}$ levels. (C) The rise in intracellular Ca$^{2+}$ following the application of 100 nM ET-1 in neonatal myocytes loaded with 1 µM Fura-2, confirming the presence of functional IP$_3$ receptors in neonatal atrial myocytes.
Figure 17. Voltage Dependent Influx of Ca$^{2+}$

(A) Changes in $C_m$ induced by single step or trains of 100 msec or 500 msec depolarizing pulses applied at 5 Hz or photocleavage of caged IP$_3$ or caged Ca$^{2+}$. (B) Representative trace of $C_m$ changes induced by 500 msec step depolarizations from a holding potential of -90 to 10 mV at 5 Hz. The initial rise and cumulative changes in $C_m$ are marked in blue. $C_m$ data is not accurate during activation of voltage dependent channels; therefore the breaks in the trace represent application of the individual voltage pulses.
500 msecs. As shown in Fig. 17A, single step depolarization did not result in a change in C\textsubscript{m}. In order to increase the influx of Ca\textsuperscript{2+}, a train of 10 or 12 pulses at a frequency of 5 Hz were applied. At 100 msecs, a positive rise in C\textsubscript{m} was not observed (Fig. 17A). In contrast, a train of pulses at 500 msecs induced a modest rise in C\textsubscript{m} (Fig. 17B). The average change in C\textsubscript{m} from this stimulation was 233 ± 191 fF (n = 4), corresponding to approximately 61 secretory granule fusions. Interestingly, application of a second train resulted in a reduced exocytotic response.

**Discussion**

In these experiments, direct elevation of Ca\textsuperscript{2+} by the photolytic release of caged Ca\textsuperscript{2+} induced exocytosis in quiescent neonatal atrial myocytes. Similarly, others have implicated Ca\textsuperscript{2+} has a positive role in regulating exocytosis (Matsubara et al., 1988; Suzuki et al., 1992; Rebsamen et al., 1997). In contrast to these findings are to those that demonstrated Ca\textsuperscript{2+} was ineffective at inducing ANP release (Gibbs, 1987; Iida and Page, 1988; Uusimaa et al., 1990). These differences may reflect the role Ca\textsuperscript{2+} plays at select points in the secretory pathway including granule biogenesis, mobilization, recruitment, docking, priming and fusion (Dietz, 2005).

In order to focus on the final stages of exocytosis, the controlled elevation of intracellular Ca\textsuperscript{2+} at room temperature (~22 °C) was used to limit the recruitment of a pool of granules available for release. In neonatal myocytes, the evoked exocytotic burst was described by a single exponential line. In contrast, evoked exocytosis in chromaffin cells exhibited multiple components (Henkel and Almers, 1996). Each kinetic component has been interpreted to reflect multiple pools of secretory granules.
at different stages of readiness to undergo release. Therefore, at room temperature, neonatal atrial myocytes may lack the multiple pools as has been described in neuroendocrine cells (Schneggenburger and Neher, 2000). In addition, sequential flashes had the ability to deplete the readily releasable pool over the temperature and time frame of these experiments. These findings for a readily releasable pool of ANP is supported by evidence that ANP exocytosis declines over time in isolated rat atria, however, the decline is not due to significant depletion of total ANP stores (Dowsley et al., 1995). Moreover, although the amplitude of each successive response was diminished, the time constant of each burst was not significantly altered, further suggesting that depletion was occurring from a finite granule pool. This suggests that a readily releasable pool of secretory granules is present and rises in intracellular Ca^{2+} can efficiently evoke exocytosis in neonatal atrial myocytes.
Hypothesis III: Ouabain Treatment Modulates the Exocytotic Response by Enhancing the Ca\(^{2+}\) Sensitivity of Exocytosis

Changes in intracellular Ca\(^{2+}\) play a key role in the excitation-contraction mechanism within the cardiac myocyte. With every beat of the heart, the concentration of Ca\(^{2+}\) rises from a resting level of approximately 100 nM to 1 µM (Marks, 2003). Therefore it is difficult to imagine how a Ca\(^{2+}\)-dependent mechanism can play a key role in regulating exocytosis where global Ca\(^{2+}\) levels raise 10-fold with every heart beat. Despite this background change in Ca\(^{2+}\), a regulatory role for Ca\(^{2+}\)-dependent exocytosis remains a possibility. Perhaps an additional signal in combination with the rise in intracellular Ca\(^{2+}\) is required for the controlled release of ANP in a Ca\(^{2+}\)-dependent manner. For example, this signal may result in an increase in the amplitude of the Ca\(^{2+}\) signal, an enhancement of the spatial overlap of the exocytotic machinery to Ca\(^{2+}\) or an alteration to Ca\(^{2+}\) sensitivity of synaptotagmin-1. One possible regulatory signal may result from the application of ouabain which binds to a signal complex consisting of the Na\(^+\)/K\(^+\)-ATPase and Src. Binding of ouabain to the Na\(^+\)/K\(^+\)-ATPase / Src signaling complex may provide the additional regulatory signal required for Ca\(^{2+}\)-dependent exocytosis in cardiac myocytes.

The Na\(^+\)/K\(^+\)-ATPase

In 1997, Jens Christian Skou was awarded the one half of the Nobel Prize for the discovery of an ion-transporting enzyme, Na\(^+\)/K\(^+\)-ATPase. His work was first published in 1957. Using a crab nerve membrane preparation, he identified that the
sodium pump was most active in the presence of Na⁺ and K⁺ (Skou, 1957). One year later, Skou demonstrated that Na⁺/K⁺-ATPase was inhibited by ouabain. Interestingly, the term sodium pump did not originate with Skou. The term was first used by Dean in 1941, which was further used in 1954 by Gardos investigating red blood cells (Scheiner-Bobis, 2002). The Na⁺/K⁺-ATPase also known as the Na⁺/K⁺ pump, is a highly conserved enzyme found in the plasma membrane of higher eukaryotes and virtually every human cell. The Na⁺/K⁺-ATPase is important for establishing the cell resting potential and cell volume.

The Na⁺/K⁺-ATPase belongs to the P-type ATPases, the ATPase family that becomes phosphorylated during ATP hydrolysis at a conserved aspartic acid (Lutsenko and Kaplan, 1995). This family consists of more than 50 members (Scheiner-Bobis, 2002). The mechanism by which Na⁺ and K⁺ is transported utilizes ATP hydrolysis. Upon the binding of ATP to the Na⁺/K⁺-ATPase, three intracellular Na⁺ ions are bound with very high affinity. ATP is subsequently hydrolyzed, leading to the phosphorylation of the pump at a highly conserved aspartate residue (Skou, 1988). Adenosine diphosphate (ADP) is released, resulting in a conformational change, exposing the Na⁺ ions to the extracellular region (Glynn, 1993). The affinity of Na⁺ to the phosphorylated Na⁺/K⁺-ATPase is very low, allowing Na⁺ to be released to the outside (Glynn, 1993). At this state, Na⁺/K⁺-ATPase affinity for K⁺ is increased, therefore binding K⁺ to the pump which subsequently leads to dephosphorylation of the pump and returning Na⁺/K⁺-ATPase to its original conformation where K⁺ is released inside the cell (Skou, 1988; Glynn, 1993). Upon release of K⁺, ATP binds and process begins again.
The structure of the Na\(^+\)/K\(^+\)-ATPase consists of a catalytic \(\alpha\) subunit and regulatory \(\beta\) and \(\gamma\) subunits. Unlike the \(\alpha\) and \(\beta\) subunits, \(\gamma\) is not expressed as widely nor is it essential for Na\(^+\)/K\(^+\)-ATPase activity (Scheiner-Bobis and Farley, 1994). The \(\alpha\) subunit is the subunit that contains binding sites for ATP and the cardiac glycosides. This subunit crosses the membrane 10 times, forming 10 membrane domains with both N- and C- terminal ends on the cytosolic side with a molecular mass of 100-113 kDa (Antolovic et al., 1991). There are four isoforms of the \(\alpha\) subunit each of which have different affinities for ouabain (Sweadner, 1989). Within the rat, \(\alpha_2\) and \(\alpha_3\) have a 1000-fold higher affinity for ouabain than that of \(\alpha_1\) (Blanco and Mercer, 1998). In neonatal rat atrial myocytes, \(\alpha_1\) and \(\alpha_3\) are the only isoforms expressed (Orlowski and Lingrel, 1988). Therefore, at low concentrations of ouabain, the activity of the \(\alpha\) isoforms can be functionally separated in neonatal myocytes.

**Ligands of the Na\(^+\)/K\(^+\)-ATPase**

The Na\(^+\)/K\(^+\)-ATPase contains a binding site for cardiac glycosides and steroids such as ouabain, digoxin, palytoxin and marinobufagenin. Ouabain is a cardiac glycoside found as a secondary metabolite of the foxglove plant (*Digitalis purpurea*), the source of digitalis and digitonin used to improve heart performance by increasing the force of contractions. Ouabain and digoxin block the Na\(^+\)/K\(^+\)-ATPase at high concentrations used in treatment for atrial fibrillation and congestive heart failure. At low concentrations ouabain appears to have a much different effect, stimulating a signal cascade.
In 1991, an endogenous isoform of ouabain was identified (Hamlyn et al., 1991; Hamlyn et al., 2003). This endogenous ouabain is synthesized in the adrenal gland and may also be produced in the hypothalamus. In normal human plasma, circulating levels of endogenous ouabain ranging from 9 – 600 pM have been identified (Hamlyn et al., 1991). These levels tend to be much higher in patients with congestive heart failure, hypertension, pregnancy and nephrectomy (Yamada et al., 1994; Vakkuri et al., 2000). Another endogenous ligand found to inhibit the Na+/K+-ATPase is that of marinobufagenin (MBG) (Bagrov et al., 1995). Marinobufagenin acts as a vasoconstrictor by inhibition of Na+/K+-ATPase in cardiovascular tissues (Fedorova and Bagrov, 1997). Interestingly, MBG exhibits greater affinity to rat α1 subunit than that of α3 (Fedorova and Bagrov, 1997; Fedorova et al., 2001). Most recently, it has been demonstrated that MBG may play an important role in cardiac disease and oxidant stress state observed with renal failure (Kennedy et al., 2006).

**Ouabain and Na⁺/K⁺-ATPase in Signal Transduction**

The function of the Na⁺/K⁺-ATPase is not limited to only a Na⁺ pump, but may also function as a signal transducer. Work by numerous groups have shown that Na⁺/K⁺-ATPase can assemble with multiple protein complexes and transmit intracellular signals. Interestingly, high or toxic concentrations of ouabain inhibit the ion transporting functions of the Na⁺/K⁺-ATPase. In contrast, low or non-toxic levels (100 nM to 100 µM) appear to activate the signal transduction pathways of the Na⁺/K⁺-ATPase. Recent evidence in LLC-PK1 cells demonstrate that two pools of Na⁺/K⁺-ATPase exists (Liang et al., 2007). In these cells, evidence points to a large
pool of a non-pumping $\text{Na}^+/\text{K}^+$-ATPase. This pool of $\text{Na}^+/\text{K}^+$-ATPase may function as receptors capable of binding ouabain and performing cellular functions other than ion pumping (Liang et al., 2007).

The signaling $\text{Na}^+/\text{K}^+$-ATPase is localized in caveolae, small invaginations of the plasma membrane consisting of caveolins (Liu et al., 2003). Binding of ouabain to the $\text{Na}^+/\text{K}^+$-ATPase activates Src, the nonreceptor tyrosine kinase, resulting in the activation of two different select pathways (Haas et al., 2000; Haas et al., 2002). These include the activation of Ras/Raf/ERK cascade or the mitochondrial reactive oxygen species (ROS) pathway. In addition, the binding of ouabain stimulates tyrosine phosphorylation of the proteins that are associated with the $\text{Na}^+/\text{K}^+$-ATPase (Tian et al., 2006). Subsequently, this also leads to the activation of MAP kinase, PKC, rises in intracellular $\text{Ca}^{2+}$ and myocyte contraction (Xie and Askari, 2002).

Src, a non-receptor tyrosine kinase, was discovered in Rous avian sarcoma virus. This tyrosine kinase plays critical role in signaling in cells in responses to extracellular ligands (Brown and Cooper, 1996). Src has a structure composed of two peptide binding domains, in addition to a catalytic kinase domain. One of the peptide binding domains is the SH2 domain, and the other is an SH3 domain. The binding of ligands to these domains are capable of activating Src with the kinase activity regulated by tyrosine phosphorylation (Ma et al., 2000; Tatosyan and Mizenina, 2000). In particular, the binding of ouabain to $\text{Na}^+/\text{K}^+$-ATPase activates Src kinase and stimulates subsequent tyrosine phosphorylation (Tian et al., 2006).
Regulation of SNARE proteins

As discussed earlier, SNARE proteins play crucial roles in the regulated process of membrane fusion events. These events include the interaction of R- and Q- SNAREs to induce membrane fusion. In neuroendocrine cells, these fusion events are regulated by increases in intracellular \(Ca^{2+}\) levels. In addition to changes in \(Ca^{2+}\), there are many SNARE-associated proteins involved in the regulation of membrane fusion. These include NSF, \(\alpha\)-SNAP, synaptophysin, munc18 and synaptotagmin (Snyder et al., 2006). These associated SNARE proteins function as regulators in membrane fusion. In addition to SNARE-associated proteins, phosphorylation of the core SNARE proteins or by SNARE-associated proteins may regulate membrane fusion.

Serine and threonine phosphorylation of SNARE and SNARE-associated proteins are common for regulation, including preventing the SNARE complex from forming while away from the active fusion sites (Gerst, 2003). For example, VAMP has three phosphorylation sites, all of which are in the conserved SNARE motif (Snyder et al., 2006). It has been suggested that these phosphorylation sites affect the interactions with other SNARE proteins, however they are not essential for the interaction with the SNARE core complex (Nielander et al., 1995; Regazzi et al., 1996)

A recent convincing example of tyrosine phosphorylation regulating a SNARE protein is that of the SNARE–associated protein, Munc18c. In 3T3L1 adipocytes, Munc18c undergoes rapid tyrosine phosphorylation upon response to insulin treatment (Oh and Thurmond, 2006). Tyrosine phosphorylation of Munc18c
appears to result in dissociation from its inhibitory relationship with syntaxin 4. Similar results were reported in MIN6, pancreatic beta cells, upon treatment with glucose (Oh and Thurmond, 2006). Tyrosine phosphorylation of Munc18c and others may provide a conserved mechanism that may be required for vesicle fusion to occur or allow for a regulatory signal to enhance secretory responses.

*Localization of Na\(^+\)/K\(^+\)-ATPase Subunits*

The cellular mechanisms by which ouabain induce secretion is not very well understood in cardiac myocytes. However, it has been shown that ouabain stimulation is associated with a rise in intracellular Ca\(^{2+}\). Others have demonstrated that Na\(^+\)/K\(^+\)-ATPase can assemble into a functional signaling complex (Haas et al., 2002; Xie and Cai, 2003). Therefore, Na\(^+\)/K\(^+\)-ATPase can function as a receptor for ouabain and depending on concentration can activate or inhibit Na\(^+\)/K\(^+\)-ATPase mediated signals. With this understanding, Na\(^+\)/K\(^+\)-ATPase may function as a receptor that provides a regulatory signal for the control of exocytosis in cardiac myocytes on a background of continuous oscillations of intracellular Ca\(^{2+}\) critical for contractile function.

The localization of α1 and α3 subunits was completed using immunocytochemical methods in cultured neonatal atrial myocytes. In Fig. 18A, a single neonatal atrial myocyte was labeled with specific antisera for α1 and α3 subunits of the Na\(^+\)/K\(^+\)-ATPase. The α1 (green) and α3 (red) was generally limited to the plasma membrane. A portion of signal colocalized with α1 at the plasma membrane, however the majority of the signal did not colocalize. A similar pattern of
non-colocalized signals occurred in adult atrial cardiac myocytes (Fig. 18B), where the α3 signal was excluded from the plasma membrane and appears to be associated in T-tubules.

Figure 18. Localization of Na\(^+\)/K\(^+\)-ATPase Subunits

![Image of confocal micrographs showing localization of Na+/K+ ATPase subunits in neonatal and adult atrial cardiac myocytes.](image-url)

Figure 18. Localization of α1 and α3 subunits of the Na\(^+\)/K\(^+\) ATPase in neonatal and adult atrial cardiac myocytes. (A) Confocal micrographs of neonatal atrial myocytes stained with specific antisera for α1 (green) and α3 (red). The merge image demonstrates very little colocalization of signals. Nuclear staining was completed with DAPI. (B) Confocal micrographs of adult atrial myocytes. α1 (green) and α3 (red) exhibit very little colocalization of signals (yellow).
Ouabain Enhancement of Ca<sup>2+</sup> Evoked Exocytosis

Previously, I demonstrated that a controlled rise in cytosolic Ca<sup>2+</sup> by the photorelease of caged Ca<sup>2+</sup> was sufficient to evoke exocytosis as monitored by C<sub>m</sub> in neonatal atrial myocytes (see Fig. 14). To test the hypothesis that ouabain will enhance the secretory response, the controlled photolysis of caged Ca<sup>2+</sup> was utilized as the primary method to elevate intracellular Ca<sup>2+</sup> and evoke exocytosis. This controlled and nearly uniform Ca<sup>2+</sup> elevation allowed for direct investigation of the effects of Ca<sup>2+</sup> without any effects on Ca<sup>2+</sup> influx or release machinery. Similar to previous experiments, patch clamped neonatal atrial myocytes were introduced to 10 mM NP-EGTA and 1μM OGB-5N via the patch pipette. The photolysis of caged Ca<sup>2+</sup> occurred following a three minute equilibration period by a UV flash.

In Fig. 19A, I show representative traces of the response for the first UV flash application for control (black trace) and following treatment with 100 nM ouabain (blue trace). The ouabain treated myocyte responded with a greater C<sub>m</sub> amplitude then that of the untreated myocyte. Interestingly, an enhancement of secretory activity was not observed with the second sequential flash. This augmented response to the first UV flash was also observed in 100 μM ouabain treated myocytes. These data are summarized in Fig. 19B. In the untreated neonatal myocyte, the photolytic release of Ca<sup>2+</sup> induced a secretory response of 286.5 ± 22.4 (n = 11) for the first UV flash. This response corresponds to approximately 78 secretory granule fusion events. The average change in C<sub>m</sub> of the second sequential flash was 203.9 ± 12.7 (n = 11), a modest decrease from the first UV flash. The average changes in C<sub>m</sub> for 100 nM ouabain treated neonatal myocytes was 526.9 ± 59.5 (n = 5) and 131.5 ± 16.3 (n =
5) for the first and second flash, respectively. The augmented response of the first UV flash was significantly different from the control myocytes (p < 0.01). In 100 μM ouabain treated myocytes, average change in C_m was 361.1 ± 33.8 (n = 16) for the first UV flash and for the second UV flash was 131.8 ± 17.7 (n = 14). Similar to 100 nM, 100 μM ouabain significantly augmented the response to the photolytic release

Figure 19. Ouabain Enhancement of Exocytosis

A

B

Figure 19. Summation of changes in C_m and representative trace of treated neonatal atrial myocyte with ouabain (100 nM). (A) Summation of collected data for untreated and treated myocytes of first and second sequential flash. 100 nM and 100 μM ouabain treated myocytes exhibited an increase in evoked exocytosis to the first UV flash and diminished responses to the second UV flash. (B) Representative trace of untreated (black trace) and 100 nM ouabain (blue trace) treated neonatal atrial myocytes in response to the first UV flash stimulus.
of Ca\textsuperscript{2+} (p < 0.01). Interestingly, the differences between 100 nM and 100 µM were not significantly different (p = 0.51). These data are consistent with the hypothesis that ouabain enhances Ca\textsuperscript{2+} sensitivity and efficacy of membrane fusion events without changing the total number of secretory granules available in the readily releasable pool of granules. Following the augmented response at these concentrations, a range of ouabain concentrations were examined (0.1, 50, 100, 150, 250 µM). In Fig. 20, the summated normalized data for each was plotted. As indicated by asterisks, only the concentrations of 100 nM and 100 µM ouabain evoked significant augmented responses.

Figure 20. Dose Dependent Response of Evoked Exocytosis

![Figure 20](image.png)

Figure 20. Cultured neonatal atrial myocytes treated with a range of ouabain concentrations prior to the photolytic release of caged Ca\textsuperscript{2+}. As illustrated with asterisks, only 100 nM and 100 µM evoked significant augmented exocytotic responses. The data has been normalized to the response of the untreated myocyte of the experimental day. The significance values were p < 0.01 and p < 0.01 for 100 nM and 100 µM, respectively.
Depletion of Readily Releasable Pool of Secretory Granules

These data are consistent with the idea that the readily releasable pool of secretory granules can be exhausted or depleted in the time and temperature of my experiment. This was observed in the consistent diminished response to the second sequential flash. If the pool can be depleted, multiple sequential Ca\(^{2+}\) challenges by the photolytic release of Ca\(^{2+}\) would diminish any response. To test this, I applied four sequential UV flashes to each neonatal atrial myocyte. Each individual flash delivered essentially identical Ca\(^{2+}\) rises with diminished responses in C\(_m\). As shown in Fig. 21A, the exocytotic response was nearly exhausted following the fourth flash in a series of flashes (three minute intervals between flashes were given to maintain consistent Ca\(^{2+}\) challenges over the depletion paradigm). In Fig. 21B, the diminished response of normalized C\(_m\) illustrates near complete depletion of the readily releasable pool by the fourth UV flash. The corresponding decrease in secretory granules is reported in Fig. 21C. These studies agree with others, suggesting ANP-containing granules undergo rapid exocytosis without significant depletion of total stores of ANP-containing granules (Mangat and de Bold, 1993; Dowsley et al., 1995).

Interactions of Src and SNARE Proteins with Na\(^+\)/K\(^+\)-ATPase

Work by others have previously demonstrated that the Na\(^+\)/K\(^+\)-ATPase \(\alpha_1\) interacts with the non-receptor kinase Src (Tian et al., 2006). To investigate if the \(\alpha_3\) subunit of the Na\(^+\)/K\(^+\)-ATPase interacts with Src in a similar manner, co-immunoprecipitation methods were utilized. In Fig. 22A, co-immunoprecipitations using specific antisera for the \(\alpha_3\) subunit was demonstrated to interact with Src. This
Figure 21. Depletion of Releasable Secretory Granules

(A) Representative traces from an untreated myocyte demonstrating sequential diminishment of response to rises in intracellular Ca\(^{2+}\) by the photorelease of caged Ca\(^{2+}\) following four sequential flashes. (B) Summated data of untreated and treated myocytes following four sequential flashes. Very little change in C\(_m\) is observed by the fourth sequential flash. (C) Data were transformed into the number of secretory granules undergoing exocytosis upon a given treatment and stimuli using a specific capacitance of 1\(\mu\)F/cm\(^2\).
Figure 22. Co-Immunoprecipitation Using the α3 subunit of the Na⁺/K⁺-ATPase

(A) A representative blot showing the interaction of Na⁺/K⁺-ATPase with Src (lane 1). Lane 2, control experiment using agarose beads only. (B) Representative blots showing the interaction of the α3 subunit with synaptotagmin 1, syntaxin 4 and SNAP-23. The control lane represents the basal interactions. A similar experiment was repeated to investigate the effects of 100 μM ouabain (Oua) on these interactions.
finding suggests that the \( \alpha_3 \) subunit may form a signal complex similar to the reported complex involving the \( \alpha_1 \) subunit (Haas et al., 2002; Tian et al., 2006).

Furthermore, co-immunoprecipitation methods were also utilized to test specific interactions between SNARE and SNARE-associated proteins with the \( \text{Na}^+\text{K}^-\text{ATPase} \). In Fig. 22B, \( \alpha_3 \) was used to immunoprecipitate the membrane SNARE proteins, syntaxin 4 and SNAP-23, in untreated or treated (100 \( \mu \text{M} \) ouabain) neonatal atrial tissue. In addition to these t-SNAREs, the vesicle associated protein synaptotagmin 1 was also found to interact with the \( \alpha_3 \) subunit of \( \text{Na}^+/\text{K}^-\text{-ATPase}. \) Of particular interest, these interactions appear to be specific to the \( \alpha_3 \) subunit. A duplicate set of experiments was repeated using \( \alpha_1 \) as the immunoprecipitating antisera. In these experiments no specific interactions between \( \alpha_1 \) and the probed SNARE proteins were observed (data not shown).

Based on these observations and previous reports, I hypothesized that the observed increased sensitivity upon ouabain treatment may be a result of tyrosine phosphorylation events involving the associated SNARE proteins. To test whether the interacting SNARE proteins could be tyrosine phosphorylated, I treated neonatal atrial tissue with the irreversible protein tyrosine phosphatase inhibitor, pervanadate. In Fig. 23A, the representative immunoblots of a co-immunoprecipitation experiment following treatment with a range of pervanadate concentrations (0.01, 0.1 and 1 mM) and 100 \( \mu \text{M} \) ouabain indicate tyrosine phosphorylation of synaptotagmin 1 and syntaxin 4 with no effects on SNAP-23. Figure 23B contains the averaged data from two duplicate experiments. Upon treatment with pervanadate, synaptotagmin 1 underwent on average a five-fold increase in tyrosine phosphorylation. To a lesser
Figure 23. Tyrosine Phosphorylation of SNARE Proteins

A  IP: Phosphotyrosine

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B  IP: Phosphotyrosine

Figure 23. Stimulation of tyrosine phosphorylation by pervanadate and ouabain. (A) Neonatal atrial tissue was treated with a range of pervanadate (0.01 to 1 mM) and ouabain (100 μM) concentrations to induce tyrosine phosphorylation. (B) Immunoblot analysis of tyrosine phosphorylation of associated SNAREs. As indicated, ouabain and pervanadate were capable of inducing tyrosine phosphorylation of synaptotagmin 1 and to a lesser extent, syntaxin 4.
extent, syntaxin 4 demonstrated on average a 2.6 fold increase. The interaction with SNAP-23 appeared to have no change. Similar increases in the interaction of tyrosine phosphorylation with synaptotagmin 1 were observed in Fig. 23C. Following 100 μM ouabain treatment, synaptotagmin 1 had an average seven fold increase in tyrosine phosphorylation. As noted with pervanadate, syntaxin 4 underwent a modest change while no change was observed in SNAP-23.

The tyrosine phosphorylation of synaptotagmin was very interesting because this observation has to our knowledge not been previously reported and tyrosine phosphorylation in exocytosis in general is not well documented. Utilizing the online software program NetPhos 2.0, revealed rat synaptotagmin 1 has six predicted tyrosine phosphorylation sites (Fig. 24). This program closely examined the amino acid sequences for known tyrosine phosphorylation motifs ([R/K]xx[D/E]xxxY or [R/K]xxx[D/E]xxY) (Blom et al., 1999). Of particular interest, all the predicted sites occur within the highly conserved C2 binding domains (Ca\textsuperscript{2+} binding domains). Although not studied in further detail here, it was interesting to note that syntaxin 4 was shown to interact with the signal complex and may also undergo tyrosine phosphorylation and has 2 predicted tyrosine phosphorylation sites.

To further examine the effects of ouabain induced tyrosine phosphorylation of synaptotagmin 1, reciprocal co-immunoprecipitation experiments using synaptotagmin 1 and phosphotyrosine following a range of ouabain treatments showed that synaptotagmin 1 underwent tyrosine phosphorylation in a ouabain dose dependent manner (Fig. 25).
Figure 24. Predicted Tyrosine Phosphorylation of Synaptotagmin 1

*Homo sapiens*

*Pan troglodytes*

*Rattus norvegicus*

*Drosophila melanogaster*

*Caenorhabditis elegans*

Figure 24. Predicted tyrosine phosphorylation sites in synaptotagmin 1. The predicted sites (red) were determined using NetPhos 2.0 following identification of amino acid sequences from the NCBI database. Interestingly, all the tyrosine phosphorylation sites occur within the highly conserved C2 domains.
Figure 25. Tyrosine Phosphorylation of Synaptotagmin 1

Discussion

Given the continuous oscillations of Ca^{2+} during extraction-contraction coupling, Ca^{2+} alone may not be sufficient to regulate exocytosis. Therefore an addition regulatory signal may be required to trigger exocytosis that is superimposed over the background changes in Ca^{2+}. Based on these data, ouabain activation of Na^{+}/K^{+}-ATPase may provide one regulatory signal.

As demonstrated, the SNARE proteins (synaptotagmin 1, syntaxin 4 and SNAP-23) are assembled with the α3 subunit of Na^{+}/K^{+}-ATPase in a signal complex. The binding of low concentrations of ouabain to the Na^{+}/K^{+}-ATPase induce subsequent tyrosine phosphorylation of the associated SNARE proteins, including the Ca^{2+}
sensor synaptotagmin 1. The tyrosine phosphorylation of synaptotagmin 1 may enhance the sensitivity of Ca\(^{2+}\)-dependent exocytosis in neonatal atrial cardiac myocytes. This may occur through a direct interaction with the secretory granules or an increased sensitivity to Ca\(^{2+}\) through synaptotagmin 1.

The physiological importance of ouabain signaling through the \(\alpha_3\) subunit of Na\(^{+}/K^{+}\)-ATPase and subsequent tyrosine phosphorylation of synaptotagmin 1 may have clinically relevance. Endogenous ouabain synthesis by the adrenal cortex can cause increased secretion of ANP. Low levels of digitalis have been demonstrated to induce ANP secretion. Although these hormones have been demonstrated to induce secretion, the cellular mechanisms that mediate the exocytotic release of ANP are unknown. Therefore, endogenous ouabain may provide a natural regulatory signal for Ca\(^{2+}\)-dependent exocytosis in atrial myocytes. The regulatory signal induced by low levels endogenous ouabain may enhance the Ca\(^{2+}\) sensitivity of exocytosis similar to the observations with ouabain. This enhancement may allow a Ca\(^{2+}\)-dependent signal \textit{in vivo}. In disease states were Ca\(^{2+}\) within the cells is increased, this signal may provide the explanation for increased ANP plasma levels.

Figure 26 represents a model hypothesis of the Na\(^{+}/K^{+}\)-ATPase signal complex with SNARE proteins. SNARE proteins are assembled with the Na\(^{+}/K^{+}\)-ATPase signal complex in the plasma membrane that upon ouabain binding to the Na\(^{+}/K^{+}\)-ATPase induces tyrosine phosphorylation of synaptotagmin 1 and ultimately enhances the Ca\(^{2+}\) sensitivity of exocytosis. This signal may direct secretory granules to the complex and provide the regulatory control for release or increase the
interaction of the SNARE proteins with the membranes undergoing fusion. An alternative explanation for increased sensitivity may lie with tyrosine phosphorylation of synaptotagmin 1. To speculate, the phosphorylation of synaptotagmin 1 may provide a negative charge to the protein, which would attract Ca\(^{2+}\) ions with a higher affinity. Moreover, a conformational change following the tyrosine phosphorylation may simply enhance its interaction with the other core SNARE proteins, therefore enhancing exocytosis at the final stages of membrane fusion. This observation of tyrosine phosphorylation of synaptotagmin 1 may underlie a novel signaling pathway.
whereby the Ca$^{2+}$ sensitivity of exocytosis is enhanced in neonatal atrial cardiac myocytes. Interestingly, the tyrosine phosphorylation of synaptotagmin 1 may be relevant in neuroendocrine pathways which may be overlooked.

The combination of ouabain induced tyrosine phosphorylation and Ca$^{2+}$ may provide the regulatory signals required for Ca$^{2+}$-dependent exocytosis. Future studies will test this hypothesis directly, using a pharmacological approach in combination with capacitance measurements. The use of genistein or a Src family specific inhibitor (PP2) may elucidate the role of tyrosine phosphorylation mediated by ouabain. Furthermore, to demonstrate that tyrosine phosphorylation of synaptotagmin 1 is directly involved in the enhancement of exocytosis a loss of function study should be completed.
CONCLUSIONS

1. Multiple SNARE proteins were identified in rat neonatal and adult cardiac myocytes by RT-PCR and Western blot analysis.

2. VAMP-1, VAMP-2 and synaptotagmin-1 localized to subpopulations of ANP-containing secretory in atrial cardiac myocytes.

3. VAMP-2 was shown to interact with its putative binding partners syntaxin 4 and SNAP-23 in neonatal cardiac myocytes and syntaxin 1 and SNAP-25 in adult cardiac myocytes.

4. The controlled rise in intracellular Ca^{2+} was effective at inducing exocytosis in neonatal atrial myocytes.

5. Application of non-toxic levels of ouabain enhances the average change in C_m for the first sequential flash while decreasing subsequent flashes.

6. The pool of readily releasable granules can be depleted by sequential rises in intracellular Ca^{2+}; application of non-toxic levels of ouabain augments this response.

7. Synaptotagmin 1, SNAP-23 and syntaxin 4 are capable of interacting with the α_3 subunit of Na^+/K^+ -ATPase.

8. Applications of 100 µM ouabain lead to subsequent tyrosine phosphorylation of synaptotagmin 1.
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cross-activation of cGMP-dependent protein kinase by cAMP in pig coronary

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The heart, often overlooked as an endocrine gland, is responsible for secreting the vasoactive hormone atrial natriuretic peptide (ANP). Physical stretch of the atrial wall is the conical stimulus; however, ouabain has been shown to efficiently evoke exocytosis. Nevertheless, the Ca^{2+}-dependence and molecular mechanisms that mediate ANP exocytosis remain unclear. In the current study, I identified SNARE proteins and determined their association with ANP-containing secretory granules using primary cultures of neonatal and adult rat atrial cardiac myocytes. Myocytes were screened for mRNA transcripts by RT-PCR and further characterized by Western blot analysis. Functional interactions of identified core SNARE proteins were demonstrated using co-immunoprecipitation methods. Localization of core SNARE proteins were completed using cell fractionation and immunocytochemical methods, revealing that VAMP-1, VAMP-2 and synaptotagmin-1 were localized to subpopulations of ANP-containing secretory granules, suggesting an importance for SNARE proteins. To judge whether secretory activity could be evoked by intracellular Ca^{2+} elevation and the role of ouabain in this process, we used time-resolved membrane capacitance measurements (C_m) in combination with the flash photolysis of caged Ca^{2+} to follow the exocytotic activity of individual myocytes. Two sequential flashes at room temperature evoked nearly identical Ca^{2+} changes that induced exponential C_m rises (78 and 66 granules, respectively). The application of 100 nM ouabain to target the α_3-subunit of the Na^+/K^+-ATPase significantly enhanced the average C_m change to the first stimulus and diminished the response to the second stimulus (138 and 58 granules, respectively) with no effect on Ca^{2+} levels or size of
the readily-releasable pool, suggesting ouabain enhanced the Ca$^{2+}$-sensitivity of exocytosis. Others have shown binding of ouabain to the Na$^{+}$/K$^{+}$-ATPase activates Src and subsequent downstream tyrosine phosphorylation. Using co-immunoprecipitation methods, I demonstrated that synaptotagmin-1 associates with an immune complex comprised of Na$^{+}$/K$^{+}$-ATPase α3/Src/syntaxin-4. Moreover, treatment with pervanadate (inhibitor of protein tyrosine phosphatases) or ouabain induced tyrosine phosphorylation of synaptotagmin-1 in a dose dependent manner. Based on these findings, I propose SNARE proteins are required for exocytosis and are assembled with Na$^{+}$/K$^{+}$-ATPase α3/Src/syntaxin-4 in a signal complex that upon activation by ouabain induces tyrosine phosphorylation of synaptotagmin-1 to enhance the Ca$^{2+}$-sensitivity of exocytosis.